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The preparation optimization and immune effect of epimedium polysaccharide-propolis flavone liposome

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ABSTRACT

The preparation conditions of epimedium polysaccharide-propolis flavone liposome (EPL) were optimized by response surface methodology taking entrapment rates of epimedium polysaccharide and propolis flavone as indexes. The immunoenhancement of EPL prepared with optimized condition was determined taking epimedium polysaccharide-propolis flavone suspension (EPS) and epimedium polysaccharide-propolis flavone watery solution (EPW) as control. The results showed that the optimized preparation condition was as follows: the ratio of drug to lipid was 14:1, the ratio of soybean phospholipid to cholesterol was 6:1, and the ultrasonic time was 19 min. EPL could significantly promote the proliferation of T and B lymphocytes singly or synergistically with PHA or LPS, mRNA expression of IL-2 and IL-6 and secretion of IgG and IgM as compared with EPS and EPW. These results indicated that liposome could significantly improve the immunoenhancement of epimedium polysaccharide-propolis flavone immunopotentiator (EPI) and would be as the suitable dosage form of EPI.

1. Introduction

All drugs should be made into suitable form which was called as the dosage form for clinical use (Li, Qiao, & Yan, 2008). Although the efficacy of drug mainly depends on the drug itself, the dosage form also plays an important role under certain conditions (Gu & Peng, 1987). For instance, the suitable dosage forms can ensure the efficacy and stability of the drug, change the property of drug, regulate the release of drug, reduce or eliminate the toxic side effect. Moreover, some dosage forms have the positioning or targeting action (Chen, 2006). The different dosage forms might lead to different efficacy for same medicine (Yao, Fu, & Jiang, 2002).

The liposome is a new material for dosage form. It is artificially prepared membranous vesicles composed of natural phospholipids and cholesterol. Its structure is similar to cell membrane with hydrophilicity and lipophilicity and suitable for use as the carrier of drugs (Li, Braiteh, & Kurzrock, 2005). It has the action of targeting and slow releasing, reducing toxicity, improving bioavailability and so on (Wang, Qiu, & Xu, 2004). Therefore, it is attracting more and more attention in medical domain. Many researches indicated that the actions of some Chinese herbal medicinal ingredients were obviously enhanced after they are encapsulated with liposome (Deng, 1996; Lee, Chung, & Lee, 2008; Liu & Guo, 1999).

Herba epimedii, a commonly used Chinese herbal medicine, could promote the specific and non-specific immune function of human and animal (Tian, 2010). Its active ingredients include polysaccharide, flavonoid glycosides, terpenoids and alkaloid, especially the polysaccharide playing an important role (Nada, Miodrag, Aleksandar, & Zvezdana, 2006). As one kind of new-type immunopotentiator, *propolis* has been applied widely, and obtains marked achievements. The propolis as immunopotentiator has the following advantages, such as higher protection rate, longer immune protection period, producing immunity earlier and so on (Shen, 1989). Its main active component is propolis flavone (PF). Our previous researches demonstrated that EP and PF possessed synergistically immunologic enhancement and antiviral effect, the efficacy of EP–PF prescription (named EP–PF immunopotentiator, EPI) was better than those of other three prescriptions (Fan et al., 2010, 2011), and had been applied for national new

Abbreviations: EP, epimedium polysaccharide; PF, propolis flavone; EPI, epimedium polysaccharide-propolis flavone immunopotentiator; EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PHA, phytohemagglutinin; CC, cell control; PHAC, PHA control; LPS, lipopolysaccharide; LPSC, LPS control; DMSO, dimethyl sulfoxide; IL-2, interleukin-2; IL-6, interleukin-10; Ig, immunoglobulin.

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veterinary drug and invent patent in China as a new immunopotentiator.

In the present research, EPI was made into three dosage forms, EPI liposome (EPL), EPI suspension (EPS) and EPI watery solution (EPW). The preparation conditions of EPL were optimized by response surface method. EPL was prepared with the optimized condition so as to obtain the highest entrapment rates of EP and PF. The effects of EPL on T and B lymphocytes proliferation, IL-2 and IL-6 mRNA expression of T lymphocyte, IgG and IgM secretion of B lymphocyte were determined and compared with EPS and EPW. The purpose of this research was to select the optimum preparation condition of EPL, probe into the probability of liposome on improving the immune enhancement of EPI, and select a suitable dosage form for this immunopotentiator.

2. Materials and methods

2.1. EPS, EPW and reagents

Epimedium polysaccharide (net content of 71.23%) and propolis flavone (net content of 70.99%) were provided in our laboratory. EP–PF suspension (EPS) and EP–PF watery solution (EPW) were also prepared in our laboratory, they were diluted into five working concentrations (15.625–0.977 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum based on the previous determination result of safe concentration (Fan et al., 2011). When the endotoxin amount was up to the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU mL^{-1}) (Veterinary Pharmacopoeia commission of the People's Republic of China, 2006), they were stored at 4 °C for the test.

Soybean phospholipid (No. 20100728) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd. Cholesterol (No. 20100908) was purchased from Anhui Tianqi Chemical Technology Co., Ltd. Protamine (Sigma, P4380) was dissolved by physiological saline to 10 $mg\,mL^{-1}$. Lymphocyte separation medium (No. 100218) was the product of Shanghai Huajing Biology Inc. RPMI-1640 (GIBCO) with the supplement of 100 IU mL^{-1} benzylpenicillin, 100 IU mL^{-1} streptomycin and 10% fetal bovine serum was used for washing and re-suspending cells, diluting mitogen and culturing the cells. Phytohemagglutinin (PHA, Sigma, No. L-8754), as a T-cell mitogen, was dissolved into 0.1 $mg\,mL^{-1}$ with RPMI-1640. Lipopolysaccharide (LPS, Sigma, No. L2880), as a B-cell mitogen, was dissolved into 0.05 $mg\,mL^{-1}$ with RPMI-1640. Hanks' solution was used for diluting blood. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, American Co.) was dissolved into 5 $mg\,mL^{-1}$ with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.2). These reagents were filtered through a 0.22 μm millipore membrane filter. PHA solution was stored at −20 °C, MTT solution, at 4 °C in dark bottles, RPMI-1640 were stored at 4 °C. Dimethyl sulfoxide (DMSO, No. 20090519) was produced by Kemiou Institute of Chemical Engineering in Tianjin. RNAiso Plus, DEPC and SYBR Green Real-time PCR Master Mix were supplied by TakaRa Biochnology Co. Ltd. Other chemicals used in experiments were analytical grade.

2.2. Optimization of EPL preparation conditions by response surface method

2.2.1. Box–Behnken design of the preparation conditions

Base on the single-factor experiments and our previous researches, the ratio of drug to lipid, ratio of soybean phospholipid to cholesterol and ultrasonic time were mainly effective factors on entrapment rate of EPL. Therefore these three factors at three levels were used respectively and seventeen reactive conditions were designed according to Box–Behnken central composite design. These three factors at three levels were as follows: the ratio of drug to lipid (10:1, 15:1 and 20:1, w/w); the ratio of soybean phospholipid to cholesterol (4:1, 6:1 and 8:1, w/w); ultrasonic time (10, 20 and 30 min).

2.2.2. Preparation of EPL

PF, soybean phospholipid, cholesterol and tocopherol were dissolved in ethanol–chloroform solution (1:1, v/v), and poured into round bottom flask. The solution was evaporated to dryness in 40 °C water bath by rotary evaporator (Model RE-52A, Yarong Biochemical Instrument Manufacturer, Shanghai City), finally a dry film was formed in the sidewall. EP and sucrose was dissolved in phosphate buffered saline (PBS, pH 7.2), and then poured into the round bottom flask, joggled until the film was completely dissolved at room temperature (25 °C). The suspension was dealt with ultrasonication in ice bath for 20 min. Then, crude suspension was filtered through 0.8 μm, 0.45 μm and 0.22 μm millipore membrane filters successively (Zhang, Anyarambhatla, & Ma, 2005).

Under the transmission electron microscope, the particles of EPL presented spherical or nearly spherical shape with uniform size whose diameters were less than 200 nm. EPL was diluted into five working concentrations (15.625–0.977 $\mu g\,mL^{-1}$) in two-fold serial dilution with RPMI-1640 containing 10% fetal bovine serum, sterilized and stored at 4 °C for the test.

2.2.3. Entrapment rate assay of EPL

The entrapment rate of EPL was assayed by protamine method according to reference (Yu, Zhang, & Zheng, 2003). The entrapment rate (ER) was calculated according to the formula: $ER = 1\text{-}C_f/(C_e + C_f) \times 100\%$ (C_f: the content of free drug; C_e: the content of encapsulated drug) (Yu et al., 2003). The contents of EP and PF (the content of free drug, C_f) were determined respectively by vitriol–phenol method and rutin method (Gunter, Gunter, Jarkowski, & Rosier, 1982; Meng & Chen, 1990).

2.2.4. Confirmatory test

According to the optimum preparation condition optimized by response surface method, five verification experiments were carried out, the entrapment rates of EP and PF were determined in order to observe whether the experimental results were consistent with regression model or not.

2.3. T lymphocyte proliferation assay

The blood samples were collected from non-immune White Roman chickens (male) at 60-day old (provided by Tangquan Poultry Farm) and transferred immediately into aseptic capped tubes containing sodium heparin, then diluted with an equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After centrifugation at $800 \times g$ for 20 min, the lymphocytes was collected and washed twice with RPMI-1640 without fetal bovine serum. The resulting pellet was re-suspended and diluted to 5×10^6 mL^{-1} with RPMI-1640 after the cell viability was assessed by trypan blue exclusion. The solution was divided into two parts, one part was added with PHA, and respectively incubated into 96-well culture plates, 100 μL per well. Then, EPL, EPS and EPW at series of concentrations were added, in cell control group and PHA control group, RPMI-1640 and PHA respectively, 100 μL per well, four wells each concentration. The final concentration of PHA reached to 20 $\mu g\,mL^{-1}$. The plates were incubated in a humid atmosphere with 5% CO_2 (Revco, Co., USA) at 39.5 °C for 48 h. Briefly, 20 μL of MTT (5 $mg\,mL^{-1}$) was added into each well at 4 h before the end of incubation. Then the plates were centrifuged at $1000 \times g$ for 10 min at room temperature. The supernatant was removed carefully and 100 μL of DMSO was added into each well. The plates were shaken for 5 min to dissolve the crystals

completely. The absorbance of cells in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model RT-6000, Leidu Co., Ltd. Shenzhen City) at a wave length of 570 nm (A_{570} value) as the index of lymphocytes proliferation (Wang et al., 2010). Meanwhile the cellular proliferation rate was calculated to compare the strength of lymphocytes proliferation according to the equation (Yu, Jiang, Yan, & Zhu, 2005): proliferation rate (%) = $\bar{A}_{570}$ value of drug group − $\bar{A}_{570}$ value of cell or PHA control group/$\bar{A}_{570}$ value of cell or PHA control group) × 100 (the $\bar{A}$ was the average value of five concentration groups of drug or four wells of control group).

2.4. B lymphocyte proliferation assay

The spleen were aseptically collected from non-immune 60-day-old White Roman chicken (male), washed twice with physiological saline, and immediately transferred into a Petri dish with Hanks' solution. The spleen was minced using a pair of scissors, the tissues were blown fully with pipette which made the cell suspended in Hanks' solution, then filtered through 3-tier gauze. Finally, the Hanks' solution contained cell was collected and carefully layered on the surface of lymphocyte separation medium. Then the treatment and determinations were similar to 2.3. (T lymphocyte proliferation assay) except that PHA (final concentration, 20 μg mL^{-1}) was instead of LPS (final concentration, 10 μg mL^{-1}) (Zhao et al., 1995).

2.5. IL-2 and IL-6 mRNA expression assay

2.5.1. Cell and total RNA extraction

Lymphocyte was prepared according to Section 2.3. The cells were inoculated into a 24-well culture plate, 800 μL per well. One milliliter of EPL, EPS and EPW at three concentrations (15.625–3.906 μg mL^{-1}) and 200 μL of PHA were added into each well. The plates were incubated at 39.5 °C in an atmosphere of 5% CO_2 and each sample seeded four wells. After 12 h incubation, the plates were centrifuged at 1000 × *g* for 10 min, the supernatant was removed carefully and 200 μL of DEPC-treated water was added to suspend the lymphocytes (Wang, Li, & Xu, 2006). Total RNA was isolated according to instruction of Trizol kit (TakaRa). The RNA quality was assessed by both agarose gel (1%) electrophoresis and spectrometry (A_{260}/A_{280}).

2.5.2. Primers for the RT-PCR

The primers were designed according to the sequences of IL-2, IL-6 and β-actin (as a house keeping gene for normalization) of chickens which were published by Genbank (Harvey, Barbara, Chris, & Peter, 2000) and synthesized by Shanghai Gene Core Bio Technologies Co. Ltd. They were used in PCR amplification of cDNA and cytokine quantification in real-time PCR. The primer sequences used were listed as follow: IL-2 (138 bp), forward: 5′-ATGGAAAACTCTTCAAACA-3′, reverse: 5′-ACTTCTCCCAGGTAA CAC-3′; IL-6 (155 bp), forward: 5′-GCTCGCCGGCTTCGA-3′, reverse: 5′-GGTAG GTCTGAAAGGCGAACAG-3′; β-actin (280 bp), forward: 5′-CTGACACCACACT TTCTACAATG-3′, reverse: 5′-GATCTT CATGAG GTAGTCCGTCAG-3′.

2.5.3. Quantifications of cytokine mRNA

The cDNA was synthesized with 1 μL of total RNA (500 ng), 5 μL of 5× PrimeScript buffer, 1 μL of Oligo dT Primer (50 μM), 1 μL Random 6 mers and 12 μL DEPC-treated water at the conditions of 37 °C for 15 min, 85 °C for 5 s and 4 °C for 5 min. Real-time PCR was performed with SYBR Premix Ex Taq Kit (TakaRa) and mini Cycler Detection System (ABI Prism 7300). The reaction system was 25 μL of final volume including 2 μL cDNA, 12.5 μL SYBR Green Real-time PCR Master Mix, 0.5 μL ROX Reference Dye, 1 μL forward primer, 1 μL reverse primer and 8 μL dH_2O. The reaction conditions were as follows: initial 1 cycle of enzyme inactivation for 3 min at 95 °C, followed by 40 cycles of amplification including initial denaturation at 95 °C for 30 s, annealing at 60 °C for 31 s and elongation at 72 °C for 30 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primer specificity and the purity of the products. Relative mRNA levels of IL-2 and IL-6 genes in samples were determined using the Δ cycle threshold (ΔC_t) method using β-actin as the reference gene. For each of the IL-2 and IL-6 genes, the $\Delta\Delta C_t$ values of all the samples were then calculated by subtracting the average ΔC_t of the sample from the ΔC_t of the control. The $\Delta\Delta C_t$ values were converted to fold differences by raising 2 to the power $-\Delta\Delta C_t$ ($2^{-\Delta\Delta C_t}$).

2.6. IgG and IgM contents assay

Lymphocyte was prepared according to Section 2.4. The cells were inoculated into a 96-well culture plate, 80 μL per well. 100 μL of EPL, EPS and EPW at three concentrations (15.625–3.906 μg mL^{-1}) and 20 μL of LPS were added into each well. The plates were incubated at 39.5 °C in an atmosphere of 5% CO_2 and each sample seeded four wells. After 48 h incubation, the plates were centrifuged at 1000 × *g* for 10 min, the supernatant was collected carefully for determination of the contents of immunoglobulin. The contents of IgG and IgM secreted by B lymphocyte were assayed by ELISA kit (R&D Systems Inc.).

2.7. Statistical analysis

Data are expressed as the mean ± S.D. Duncan's multiple range test was used to determine the differences among groups with the software SPSS 16.0. Significant differences were considered at $p < 0.05$.

3. Results

3.1. The optimization of preparation condition of EPL

Seventeen preparation experimental conditions and results are listed in Table 1. The experimental data were dealt with analysis of variance by Design-Expert 8.0 software. The regression equations was as follows: entrapment rate of EP (%) = $12.72125 + 7.14170 \times A + 8.53613 \times B - 0.53298 \times C - 0.014750 \times A \times B + 0.066250 \times A \times C + 0.11625 \times B \times C - 0.29294 \times A^2 - 0.82400 \times B^2 - 0.030510 \times C^2$. Entrapment rate of PF (%) = $12.16875 + 7.33875 \times A + 11.30500 \times B - 0.30713 \times C - 0.016750 \times A \times B + 0.043800 \times A \times C + 0.19587 \times B \times C - 0.28610 \times A^2 - 1.19375 \times B^2 - 0.039325 \times C^2$.

Variance analysis of regression model for the entrapment rate of EP was checked by *F*-test and *p*-value. The *p*-value of regression equation was 0.0008 and extremely significant smaller than 0.01, which showed that the equation was extremely significant. It indicated that the equation about entrapment rate of EP was right, this method was reliable. At the same time, the *p*-value of lack of fit was 0.9408 and extremely significant larger than 0.05, the difference was not significant. It indicated that the reasons of lack of fit were not exist and the fitted regression equation was better. These data showed that this regression equation could be used for explaining and predicting the experimental result. The maximum entrapment rate of EP was presumed by regression model, which was 85.4755%. At this time, the optimized preparation condition of EPL was as follows: the ratio of drug to lipid was 14.16:1, the ratio of soybean phospholipid to cholesterol was 6.38:1, and the ultrasonic time was 18.78 min.

Table 1
Design and response values of RSM test in preparation of EPL.

Test number	*A* (ratio of drug to lipid)	*B* (ratio of soybean phospholipid to cholesterol)	*C* (ultrasonic time/min)	Entrapment rate of EP (%)	Entrapment rate of PF (%)
1	15:1	6:1	20	87.94	75.07
2	20:1	4:1	20	70.54	56.67
3	15:1	8:1	30	81.80	67.41
4	15:1	6:1	20	84.94	70.07
5	15:1	4:1	30	74.97	58.47
6	20:1	6:1	30	75.30	58.33
7	15:1	8:1	10	77.93	59.60
8	10:1	6:1	30	72.49	60.93
9	15:1	6:1	20	86.40	74.00
10	20:1	6:1	10	70.38	55.84
11	15:1	6:1	20	81.80	66.20
12	10:1	6:1	10	80.82	67.20
13	15:1	6:1	20	84.53	72.96
14	10:1	4:1	20	74.63	58.73
15	15:1	4:1	10	80.40	66.33
16	10:1	8:1	20	78.76	63.13
17	20:1	8:1	20	74.08	60.40

Table 2
Changes of T lymphocyte proliferation (A_{570} value).

Modes	Groups	Concentration ($\mu g\,mL^{-1}$)				
		15.625	7.813	3.907	1.954	0.977
Single stimulation	EPL	0.210 ± 0.006^{a}	0.225 ± 0.006^{a}	0.222 ± 0.004^{a}	0.195 ± 0.008^{a}	0.205 ± 0.004^{a}
	EPS	0.153 ± 0.004^{b}	0.175 ± 0.006^{b}	0.187 ± 0.004^{b}	0.181 ± 0.002^{ab}	0.204 ± 0.003^{a}
	EPW	0.146 ± 0.004^{b}	0.165 ± 0.008^{b}	0.179 ± 0.004^{b}	0.175 ± 0.002^{b}	0.169 ± 0.002^{b}
	CC	0.138 ± 0.006^{b}	0.138 ± 0.006^{c}	0.138 ± 0.006^{c}	0.138 ± 0.006^{c}	0.138 ± 0.006^{c}
Synergistical stimulation with LPS	EPL	0.210 ± 0.006^{a}	0.249 ± 0.004^{a}	0.233 ± 0.004^{a}	0.200 ± 0.007^{a}	0.186 ± 0.003^{a}
	EPS	0.158 ± 0.004^{b}	0.214 ± 0.004^{b}	0.194 ± 0.005^{b}	0.173 ± 0.005^{b}	0.173 ± 0.005^{ab}
	EPW	0.163 ± 0.004^{b}	0.216 ± 0.002^{b}	0.190 ± 0.008^{b}	0.161 ± 0.007^{b}	0.164 ± 0.003^{b}
	PHAC	0.169 ± 0.005^{b}	0.169 ± 0.005^{c}	0.169 ± 0.005^{c}	0.169 ± 0.005^{b}	0.169 ± 0.005^{b}

[a–c] Data in same mode within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; CC, cell control; PHAC, phytohemagglutinin control.

Variance analysis of regression model for the entrapment rate of PF was checked by *F*-test and *p*-value. The *p*-value of regression equation was 0.0115 and extremely significant smaller than 0.01. The *p*-value of lack of fit was 0.7408 and extremely significant larger than 0.05. These data showed that this regression equation could be used for explaining and predicting the experimental result. The maximum entrapment rate of PF was presumed by regression model, which was 71.9508%. At this time, the optimized preparation condition of EPL was as follows: the ratio of drug to lipid was 14.14:1, the ratio of soybean phospholipid to cholesterol was 6.23:1, and the ultrasonic time was 19.50 min.

3.2. Confirmatory test

Considering the feasibility and convenience of experiment, the optimized preparation condition of EPL was set that the ratio of drug to lipid was 14:1, the ratio of soybean phospholipid to cholesterol was 6:1, and the ultrasonic time was 19 min. At this time, the entrapment rates of EP and PF presumed by regression equations were 85.23899% and 71.84195% respectively. Five verification experiments were carried out at these optimum conditions, the average entrapment rates of EP and PF were 84.95% and 71.36% respectively. The relative error between actual values and predicted values is 0.3% and 0.7%. It fits well with the predicted values. These results demonstrated that optimizing the preparation condition of EPL by response surface methodology was reasonable and feasible.

3.3. Changes of T lymphocyte proliferation

The A_{570} values in every group are listed in Table 2. During single stimulation, the A_{570} values in EPL, EPS and EPW at 7.813–0.977 $\mu g\,mL^{-1}$ groups were significantly higher than those in cell control (CC) groups ($p < 0.05$). The A_{570} values in EPL at all concentration groups were the highest and significantly higher than those in EPW and CC groups ($p < 0.05$), and significantly higher than those in EPS at 15.625–3.907 $\mu g\,mL^{-1}$ group ($p < 0.05$).

During synergistical stimulation with PHA, the A_{570} values in EPL, EPS and EPW at 7.813 and 3.907 $\mu g\,mL^{-1}$ groups were significantly higher than those in PHA control (PHAC) group ($p < 0.05$). The A_{570} values in EPL at all concentration groups were the highest and significantly higher than those in EPW and PHAC groups ($p < 0.05$), and significantly higher than those in EPS at 15.625–1.954 $\mu g\,mL^{-1}$ groups ($p < 0.05$).

The T lymphocyte proliferation rates in every group are illustrated in Fig. 1. During single stimulation and synergistical stimulation with PHA, the lymphocyte proliferation rates in EPL

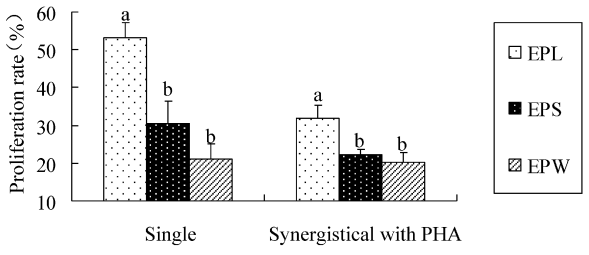

Fig. 1. The T lymphocyte proliferation rate in every group. [a,b] Data in same index within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution.

Table 3
Changes of B lymphocyte proliferation (A_{570} value).

Modes	Groups	Concentration ($\mu g\,mL^{-1}$)				
		15.625	7.813	3.907	1.954	0.977
Single stimulation	EPL	0.186 ± 0.004^{a}	0.163 ± 0.004^{a}	0.154 ± 0.005^{a}	0.127 ± 0.002^{a}	0.133 ± 0.006^{a}
	EPS	0.148 ± 0.004^{b}	0.127 ± 0.004^{b}	0.146 ± 0.006^{a}	0.107 ± 0.002^{b}	0.109 ± 0.003^{b}
	EPW	0.146 ± 0.005^{b}	0.131 ± 0.002^{b}	0.118 ± 0.004^{b}	0.100 ± 0.005^{b}	0.118 ± 0.007^{b}
	CC	0.107 ± 0.003^{c}	0.107 ± 0.003^{c}	0.107 ± 0.003^{b}	0.107 ± 0.003^{b}	0.107 ± 0.003^{b}
Synergistical stimulation with LPS	EPL	0.189 ± 0.003^{a}	0.187 ± 0.005^{a}	0.152 ± 0.001^{a}	0.164 ± 0.004^{a}	0.164 ± 0.002^{a}
	EPS	0.164 ± 0.002^{b}	0.145 ± 0.001^{b}	0.137 ± 0.002^{b}	0.140 ± 0.003^{b}	0.112 ± 0.003^{c}
	EPW	0.166 ± 0.004^{b}	0.153 ± 0.002^{b}	0.130 ± 0.003^{b}	0.123 ± 0.001^{c}	0.108 ± 0.004^{c}
	LPSC	0.136 ± 0.002^{c}	0.136 ± 0.002^{c}	0.136 ± 0.002^{b}	0.136 ± 0.002^{b}	0.136 ± 0.002^{b}

[a–c] Data in same mode within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; CC, cell control; LPSC, lipopolysaccharide control.

group were the highest and significantly higher than those in EPS and EPW groups ($p < 0.05$), EPS group were higher than EPW group but not significant ($p > 0.05$).

3.4. Changes of B lymphocyte proliferation

The A_{570} values in every group are listed in Table 3. During single stimulation, the A_{570} values in EPL, EPS and EPW at 15.625 and 7.813 $\mu g\,mL^{-1}$ groups were significantly higher than those in CC group ($p < 0.05$). The A_{570} values of EPL at all concentration groups were the highest and significantly higher than those in EPW and CC groups ($p < 0.05$), and significantly higher than those in EPS group except at 3.907 $\mu g\,mL^{-1}$ ($p > 0.05$).

During synergistical stimulation with LPS, the A_{570} values of EPL, EPS and EPW at 15.625 and 7.813 $\mu g\,mL^{-1}$ groups were significantly higher than those in LPS control (LPSC) group ($p < 0.05$). The A_{570} values in EPL at all concentration groups were the highest and significantly higher than those in other three groups ($p < 0.05$).

The B lymphocyte proliferation rates in every group are illustrated in Fig. 2. During single stimulation and synergistical stimulation with LPS, the lymphocyte proliferation rates in EPL group were the highest and significantly higher than those in EPS and EPW groups ($p < 0.05$), in EPS group was significantly higher than that in EPW during single stimulation ($p < 0.05$).

3.5. Changes of IL-2 and IL-6 mRNA expression

3.5.1. Changes of IL-2 mRNA expression

The relative expression levels of IL-2 mRNA are illustrated in Fig. 3. The IL-2 mRNA expression, in EPL at 15.625–3.907 $\mu g\,mL^{-1}$ group were the highest and significantly higher than those in EPW, PHAC and CC groups and in EPS except at 3.907 $\mu g\,mL^{-1}$ group ($p < 0.05$), in EPS at 15.625–3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in PHAC and CC groups and in EPW at 15.625 $\mu g\,mL^{-1}$ group ($p < 0.05$), and in EPW at 15.625–3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in CC group and in PHAC at 7.813 $\mu g\,mL^{-1}$ group.

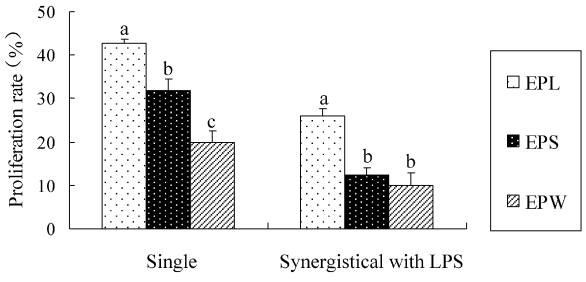

Fig. 2. The B lymphocyte proliferation rate in every group. [a–c] Data in same index within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution.

3.5.2. Changes of IL-6 mRNA expression

The results are illustrated in Fig. 4. The IL-6 mRNA expression, in EPL at 15.625–3.907 $\mu g\,mL^{-1}$ group were the highest and significantly higher than those in other four groups except EPS at 15.625 $\mu g\,mL^{-1}$ group ($p < 0.05$), in EPS at 15.625–3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in PHAC and CC groups, and in EPW at 15.625–3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in CC group and in PHAC except at 3.907 $\mu g\,mL^{-1}$ group ($p < 0.05$).

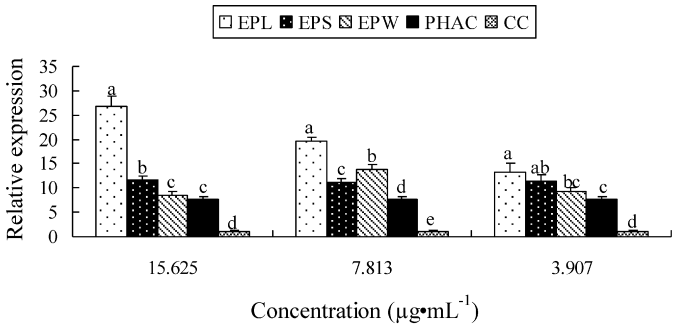

Fig. 3. The relative expression changes of IL-2 mRNA in every group. [a–d] Data in same mode within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; CC, cell control; PHAC, phytohemagglutinin control.

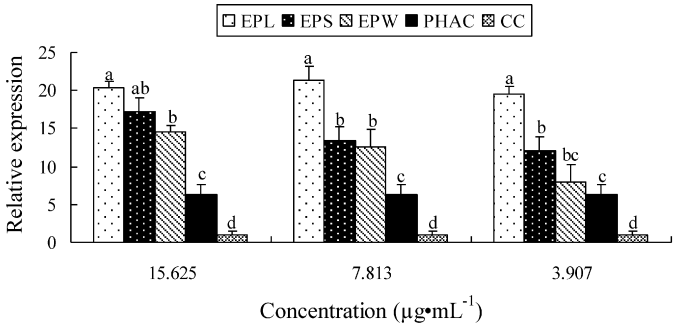

Fig. 4. The relative expression changes of IL-6 mRNA in every group. [a–d] Data in same mode within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; CC, cell control; PHAC, phytohemagglutinin control.

Table 4
The changes of IgG and IgM concentrations ($\mu g\,mL^{-1}$).

Indexes	Groups	Concentration ($\mu g\,mL^{-1}$)		
		15.625	7.813	3.907
IgG	EPL	129.16 ± 1.11^{a}	141.01 ± 3.89^{a}	150.82 ± 0.56^{a}
	EPS	84.21 ± 9.47^{b}	102.77 ± 4.9^{b}	126.33 ± 2.55^{b}
	EPW	67.49 ± 2.87^{c}	97.63 ± 2.14^{b}	99.58 ± 2.18^{c}
	LPSC	57.81 ± 3.84^{c}	57.81 ± 3.84^{c}	57.81 ± 3.84^{d}
IgM	EPL	30.93 ± 0.45^{a}	31.44 ± 0.91^{a}	33.31 ± 1.04^{a}
	EPS	16.22 ± 0.43^{b}	25.76 ± 0.25^{b}	29.17 ± 1.74^{b}
	EPW	13.98 ± 1.32^{bc}	18.44 ± 1.01^{c}	21.35 ± 0.31^{c}
	LPSC	11.94 ± 0.67^{c}	11.94 ± 0.67^{d}	11.94 ± 0.67^{d}

[a–d] Data in same index within a column without the same superscripts differ significantly ($p < 0.05$). EPL, epimedium polysaccharide-propolis flavone liposome; EPS, epimedium polysaccharide-propolis flavone suspension; EPW, epimedium polysaccharide-propolis flavone watery solution; CC, cell control; LPSC, lipopolysaccharide control.

3.6. Changes of IgG and IgM concentrations

The data are listed in Table 4. The IgG concentrations, in EPL at 15.625–3.907 $\mu g\,mL^{-1}$ group were the highest and significantly higher than those in other three groups ($p < 0.05$), in EPS group were significantly higher than those in LPSC group and in EPW except at 7.813 $\mu g\,mL^{-1}$ group ($p < 0.05$), and in EPW at 7.813 and 3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in LPSC group ($p < 0.05$).

The IgM concentrations, in EPL at 15.625–3.907 $\mu g\,mL^{-1}$ group were the highest and significantly higher than those in other three groups ($p < 0.05$), in EPS group were significantly higher than those in LPSC group and in EPW except at 15.625 $\mu g\,mL^{-1}$ group ($p < 0.05$), and in EPW at 7.813 and 3.907 $\mu g\,mL^{-1}$ group were significantly higher than those in LPSC group ($p < 0.05$).

4. Discussion

The quality of drug liposome is affected by encapsulation efficiency. The high entrapment rate is one of the critical factors to the clinical curative effect of drugs (Xu, Zhang, & Wang, 2004). Therefore, the entrapment rate was regard as the index of inspecting the quality of liposome in this experiment. The membrane distribution-micromembrane extruding method was used for preparing the EPL in this experiment. It is a short-cut method of the film dispersion method and the high pressure homogeneous method, which reduces the production cost greatly, and suits for small-scale production in laboratory. The results showed that the EPL liposome prepared by this method possesses the advantages of high entrapment rate and particle size.

Response surface methodology is a rapid technique which is able to simulate the functional relation between factor and response by applying multivariant quadratic regression equation, seek the optimized technological parameter by analyzing the regression equation, and solve multivariable questions. It is commonly applied in multifactor experimental design and has many advantages, such as convenient, high precision, good predictability and so on (Kalil, Maugeri, & Rodrigues, 2000; Kramar, Turk, & Vrecer, 2003). As compared with orthogonal design and homogeneous design, the response surface methodology could analyze each level of experiment consecutively, and the prediction model is consecutive (Mu, Zhu, & Guo, 2009). Besides, it could work out the optimum parameter and has better experimental design and expression of results. In this experiment, Box–Behnken central composite design was used for optimizing the preparation condition of EPL. The results found that the optimized preparation condition of EPL was that the ratio of drug to lipid was 14:1, the ratio of soybean phospholipid to cholesterol was 6:1, and the ultrasonic time was 19 min.

Lymphocyte proliferation is an important index of evaluating cellular immune function. After stimulated by antigenic properties, T and B lymphocytes could proliferate and differentiate, generate specific immune response, and produce lymphokine and antibody, they are considered as the most important immune effector cell (Minato, Kawakami, Nomura, Tsuchida, & Mizuno, 2004). Therefore, the determination of T and B lymphocytes proliferation is the better method of studying the activity and action mechanism of drugs (He, 2002). The results of this experiment showed that the A_{570} values and lymphocyte proliferation rates in EPL at five concentration groups were the highest in single stimulation and synergistical stimulation with PHA or LPS, and significantly higher than those in EPS and EPW at most concentration groups, which indicated that EPL possessed the strongest effect, and the immune-enhancing activity of EPI was significantly improved after it was encapsulated by liposome. Cai et al. reported that lentinan liposome could promote lymphocyte transformation, and which was significantly better than lentinan injection (Cai, Peng, Chen, & Pang, 2009), Li also proved that cistanche deserticola polysaccharide liposome could significantly promote lymphocyte proliferation (Li, 2008). These experimental results indicated that the liposome could enhance the efficacy of drug, which was consistent with our experimental results.

In order to further investigate the effect of EPL on immune response of T lymphocyte, the effects of EPL on the mRNA expression of IL-2 and IL-6 were studied. IL-2 mediates cellular immunity generally by promoting the proliferation and differentiation of T cell (Yang, 2003; Yang, Qin, & Zhu, 2009), and IL-6 mainly mediates humoral immunity mainly by promoting the proliferation of B cell and the production of immunoglobulin (Salgame et al., 1991; Yang, 2003). The experimental results showed that the relative contents of IL-2 and IL-6 in EPL at three concentration groups were highest and numberly or significantly higher than those in EPS and EPW groups. It indicated that the action of EPL was stronger than those of EPS and EPW in promoting cytokines secretion thus increasing the cellular immune response. It was reported that the dynamic balance and mutual adjustment between Th1 and Th2 played an important role in maintaining normal immunologic function of organism (Lee et al., 2004; Ying, Kikuchi, Meng, Kay, & Kaplan, 2002). The experimental results also revealed that IL-2 contents were increasing along with the IL-6 contents increased, which kept the dynamic balance of the Th1 cell and the Th2 cell to some extent.

In order to further investigate the effect of EPL on immune response of B lymphocyte, the effect of EPL on secretion of IgG and IgM was studied. IgG and IgM mainly mediate humoral immunity (Yang, 2003). As a main antibody produced by organism, IgG is also the essential material for anti-infection immunity, which possesses not only high content, but also long duration. IgM appears earliest when organism contacts with antigenic substance for the first time. Therefore, it plays an important role in early period of anti-infection immunity. IgG and IgM both have many kinds of immunologic competences such as antibacteria, antivirus, antitoxin and the anti-tumor effect and so on (Yang et al., 2011). The experimental results showed that the contents of IgG and IgM in EPL at three concentration groups were highest and significantly higher than those in EPS and EPW groups. It indicated that the action of EPL was stronger than that of EPS and EPW in promoting IgG and IgM secretion thus increasing the humoral immunity.

Based on the comparison and analysis of the immunoenhancement of the three dosage forms, it could be found that the effect of EPL was the best. The mechanism may be that phosphatide and cholesterol, as principal constituents of liposome, are also the main ingredients of cell membrane, and can promote the interactions between liposome and cell, such as absorption, fusion, lipid exchange, endocytosis and so on (Wang, Zhu, & Liao, 2007). So EPI encapsulated by liposome is easy to be absorbed by cell. On the

other hand, the particle diameter of EPL is smaller, less than 200 nm, which is smaller and conducive to penetrating cell membrane. Besides liposome can decelerate the degradation and lengthen the action time of drug. Many other researches also confirmed that the efficacy of drug encapsulated by liposome was significantly better than that of non-encapsulated drug (Cai, Peng, Chen, & Pang, 2009; Lee, Chung, & Lee, 2008; Li, 2008; Liu & Guo, 1999).

In conclusion, liposome could significantly improve the immune enhancement of EPI and would be developed as the suitable dosage form of EPI.

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