Development of tea tree oil-loaded liposomal formulation using response surface methodology

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Abstract

The aim of this study is to prepare tea tree oil liposome (TTOL) and optimize the preparation condition by single factor experiment and statistical design. TTOL was prepared using a thin-film hydration with the combination of sonication method and the preparation conditions of TTOL were optimized with response surface methodology (RSM). The optimal preparation conditions for TTOL by response surface methodology were as follows: the mass ratio of PC and Cho 5.51, TTO concentration 1.21% (v/v) and Tween 80 concentration 0.79% (v/v). The response surface analysis showed that the significant (p<0.05) second-order polynomial regression equations successfully fitted for all dependent variables with no significant (p>0.05) lack of fit for the reduced models. Furthermore, the interaction of the mass ratio of PC/Cho and TTO concentration had a significant effect. The amounts of Tween 80 required were also reduced with RSM. Under these conditions, the experimental encapsulation efficiency of TTOL was 97.81±0.33%, which was close with the predicted value. Therefore, the optimized preparation condition was very reliable. The increased entrapment efficiency would significantly improve the TTO stability and bioavailability.

Keywords

Encapsulation efficiency, liposomes, response surface methodology, tea tree oil

Introduction

Tea tree oil (TTO), the essential oil of Melaleuca alternifolia exhibits broad-spectrum antibacterial, antifungal, antiviral, and antiprotozoal activity (Carson et al., 2006). It has a long history as a medicinal essential oil and is one of the most widely used medicinal essential oils for the treatment of many pathologies. In recent decades, the misuse of conventional antibiotics has resulted in the emergence of many multidrug-resistant strains (Alanis, 2005; Heymann, 2006). The resistance of the bacteria and fungi to the innumerable antimicrobial agents constitutes one of the great challenges in the treatment of infections. In vitro, TTO has the antimicrobial activity against a variety of microorganisms (Carson et al., 2006). The use of TTO as antimicrobial agents can overcome problems caused by the misuse of conventional antibiotics (Ninomiya et al., 2011; Thomsen et al., 2013). TTO and its components compromise the cytoplasmic membrane. It can induce a loss of cellular electrondense material and coagulation of cytoplasmic constituents, stimulate leakage of cellular potassium ions, and inhibit microbial cell respiration. However, TTO has the proper lipophilicity with a very low solubility in water, which may result in its poor bioavailability (Carson et al., 2006). On the other hand, TTO is unstable and susceptible to degradation in the presence of oxygen, light and temperature (Sherry et al., 2013). Therefore, it is apparently necessary to research new-type preparations or select a highly effective carrier of TTO to promote its clinical application.

A liposome is an artificially-prepared vesicle composed of a lipid bilayer as cell membrane. The liposome can be used as a vehicle for administration of nutrients and pharmaceutical drugs. Liposomes can encapsulate hydrophilic molecules in the aqueous internal space or lipophilic and amphiphilic molecules that become embedded in their concentric bilayers (Vazquez-Gonzalez et al., 2014). Liposomes are promising carriers for TTO by reverse-phase evaporation vesicles method or dispersion method (Biju et al., 2005; Low et al., 2013). They can protect TTO and are stable at 4–5°C for several months. The thin-film hydration technique in addition to sonication or freeze–thaw cycles, the modified rapid expansion of the supercritical solution into surfactant method and the precipitation from gas saturated solution-drying-spraying technique were used to prepare liposomes encapsulating essential oils. Among them, the thin-film hydration technique in addition to sonication is one of the most commonly used method (Sherry et al., 2013). Therefore, we attempted to prepare liposomes encapsulating TTO by the thin-film hydration technique in addition to sonication in this study.

The application of statistical experimental design techniques in liposome process development can result in...
improved entrapment efficiency and particle size, reduced process variability, closer confirmation of the output response to nominal and target requirements and reduced development time and overall costs. Conventional practice of single factor optimization is a time-consuming process and requires a number of experiments to determine optimum levels, which are unreliable (Elibol, 2004). However, response surface methodology (RSM) is an effective synthesized statistical and optimization technique. It uses quantitative data to determine and simultaneously solve multivariate equations in order to optimize processes or products (Tang et al., 2014). RSM has been widely applied in the optimal preparation of liposomal formulation, such as glyceryllyglyceric acid liposome (Zhao et al., 2012), gypenosides liposome (Yu et al., 2014), Rehmannia glutinosa polysaccharide liposome (Huang et al., 2014), and PEGylated estradiol benzoate liposomes (Haeri et al., 2012).

In this study, we encapsulated TTO into liposomes using the thin film hydration method and sonication. Meanwhile, the ultraviolet absorption components of TTO was determined by spectrophotometric method. Moreover, the application of this technique for optimizing preparation conditions of TTOL by single factor experiments of seven factors, a central composite design (CCD) and RSM had not been reported. Plackett–Burman (PB) design was applied for determination of the significant parameters. Path of the steepest ascent method was used to locate the optimum region, finally CCD and RSM were utilized to determine the TTOL optimal preparation conditions for maximum entrapment efficiency of TTOL, which can obtain high bioavailability of TTO.

Methods

Materials

Soybean phosphatidylcholine (PC), cholesterol (Cho) and Tween 80 were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Tea tree oil was obtained from Nanning Innovative Pharmaceutical Technology Co., Ltd (Nanning, China). Concentrations of the TTO components determined by gas chromatographic analysis (and the range specified by the international standard ISO 4730:1996, shown in parentheses) were as follows: 2.75% α-pinene (1–6%); 0.53% sabinene (trace to 3.5%); 9.79% α-terpinene (5–13%); 1.05% limonene (0.5–4%); 2.19% p-cymene (0.5–12%); 2.9% 1,8-cineole (0–15%); 18.91% γ-terpinene (10–28%); 3.11% terpinolene (1.5–5%); 46.96% terpinen-4-ol (>30%); 2.56% α-terpineol (1.5–8%); 0.6% aromadendrene (trace to 7%); 0.44% δ-cadinene (trace to 8%); 0.26% globulol (trace to 3.5%); and 0.29% viridiflorol (trace to 1.5%). Other agents were of analytical grade from Sinopharm Chemical Reagent Co., Ltd.

Basal TTOL preparation

TTOLs were prepared using the thin-membrane hydration method with bath-type sonicator (da Silva Malheiros et al., 2010). Briefly, TTOL was prepared using PC and Cho in the mass ratio of 2:1. The lipid components, 25 mg Cho and the PC corresponding to Cho were dissolved in 10 ml chloroform in a dry 50 ml round bottom flask. The solvents were removed using a rotary evaporator at room temperature, 15 rpm, and a high vacuum, which resulted in a uniform, thin lipid membrane on the flask wall. Traces of organic solvents were removed by storage for 12 hours in a vacuum desiccator. The deposited lipid membrane was hydrated with 10 ml of 12.0 mmol/l phosphate-buffered saline (PBS, containing 1% v/v Tween 80 and 0.2% v/v TTO), pH 5.8, by agitation for 5 minutes at 45 °C. Simultaneously, sonication of the preparation was carried out in a bath-type sonicator (Branson Ultrasonics, Danbury, CT) with 240 W of sonication power to form small unilamellar vesicles (SUVs). Finally, the liposomal dispersions prepared were stored at room temperature for 3 hours in order to anneal any structural defects.

Determination of liposome entrapment efficiency

A known concentration of TTO in PBS solution containing 3% (v/v) Tween 80 was scanned in the range of 200–400 nm using a ultraviolet spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan). The sharp peaks caused by the sequential concentrations of 0.004–0.04 ml/100 ml TTO in 3% (v/v) Tween 80 at 265 nm (λmax) were recorded. It was found that α-terpinene and p-cymene displayed the significant absorption peaks at 265 nm according to UV scanning spectrum (Figure S1). However, TTO components have similar molecular weight and physico-chemical properties, especially octanol–water partition coefficient (Kow) (Carson et al., 2006). Therefore, we used the ultraviolet absorption components in TTO as the index to calculate encapsulation efficiency of TTOLs. Unknown concentration of TTO was determined by extrapolating the absorbance values from the calibration curve prepared by plotting the known TTO concentration versus its corresponding absorbance values.

Percentage entrapment efficiency (EE) was determined using petroleum ether extraction method as described by Jacobsohn et al. (1986) and Nakamura et al. (1999). Briefly, samples were extracted three times with 3.0 ml of petroleum ether each time. For each extraction, the organic layers were aspirated and collected into a round-bottom flask. The solvents were removed using a rotary evaporator at room temperature, 60 rpm, and a high vacuum. And then, the PBS solution containing 3% (v/v) Tween 80 was added into the flask and the mixture was vortexed. TTO concentration was determined and calculated by 265 nm absorbance. Finally, percentage entrapment efficiency of TTOLs was determined using the calibration curve and the following formula (Ocak et al., 2011):

\[
\text{EE} = \left( \frac{\text{Total amount of TTO} - \text{amount of free TTO}}{\text{Total amount of free TTO}} \right) \times 100
\]

Determination of tea tree oil components

Concentrations of tea tree oil components were determined by gas chromatography. Gas chromatography appliances used included a Shimadzu GC-2014C System equipped with a fused-silica Stabilwax® column (60 m × 0.32 mm i.d.; film thickness 0.50 μm) and a FID detector. Carrier gas was hydrogen at a constant voltage. The oven temperature programme was 75 °C for 4 min, then 4 °C/min until 200 °C was reached, and maintained 10 min. The detector and the
injector temperature was 250°C. The volume of injected sample was 0.2 μL and the split ratio was 1:50.

Transmission electron microscopy

The size and morphology of TTOLs were studied using a transmission electron microscope (TEM; JEM-2100, Tokyo, Japan). Briefly, the TTOLs suspension was diluted ten-fold in 12.0 mmol/l PBS solution, and the sample was deposited on a sample grid (carbon membrane supported by a copper grid) and negatively stained with phosphotungstic acid solution 2% (w/v). All preparations were allowed to dry for 2 hours and then observed with JEM 2100 transmission electron microscope operating at the voltage of 100 kV. The particle size was reported as the mean diameter of randomly selected structures.

Liposome size determination

Mean particle size diameter of TTOLs was directly measured using Zetasizer Nano ZS90 equipment (Malvern Instruments Ltd., Worcestershire, UK). All samples were diluted in the ratio 1:10 (v/v) with 12.0 mmol/l PBS solution, pH 7.4 and the mean standard deviation of three determinations was reported.

Experimental design and data analysis

Single factor experiments

For determining the scope of variables in the factorial experiment, single factor experiments of seven variables were carried out, including the mass ratio of PC and Cho, TTO concentration, Tween 80 concentration, hydration medium pH, temperature, sonication time and power.

PB design

A PB design can rapidly screen multiple factors and identify influential factors. Seven real and four dummy factors, the mass ratio of PC and Cho, TTO concentration, Tween 80 concentration, hydration medium pH, temperature, sonication time and power, were examined to evaluate key factors significantly affecting TTOL entrapment efficiency. Elements related to the level of choice are very important for the experimental results. Each variable was represented at two levels according to the results of single factor experiments (Table 1), high and low, which were denoted by “+1” and “−1”, respectively. In the experiments, the variables, which were significant at 95% confidence level (p < 0.05), were considered to influence TTOL entrapment efficiency significantly and were further optimized by a CCD. An 11-run PB design (Table 2) was used, and the experimental responses were analyzed by the method of least squares to fit the following first-order model:

\[ Y = \beta_0 + \sum \beta_iX_i \]  

(1)

where Y was the predicted response, \( \beta_0 \) was the intercept, and \( \beta_i \) was the linear regression coefficients, and \( X_i \) was the coded levels of the independent variables.

Path of steepest ascent method

The effects of three significant factors determined by PB design were screened to locate the optimum region with respect to TTOL entrapment efficiency by applying steepest ascent experiments. Experiments were performed along the steepest ascent path until the response showed no further increase. The point obtained was used as the center point for further optimization. The experimental design of the steepest ascent is shown in Table 3.

Central composite design and response surface methodology

The three most significant factors (the mass ratio of PC and Cho, TTO concentration and Tween 80 concentration) led to optimization using a CCD to enhance TTOL entrapment efficiency. The three independent factors were observed at five different levels (−1.68, −1, 0, +1, +1.68) (Table 4). A set of 20 experiments was carried out. All the variables were taken at a central-coded value considered as zero (Table 5). To validate the optimization of TTOL preparation methods, triplicates were used for each experimental design to confirm the results from the response surface analysis. The behavior of the system was explained by the following second-order polynomial equation:

\[ Y = \beta_0 + \sum \beta_iX_i + \sum \beta_{ii}X^2_i + \sum \beta_{ij}X_iX_j \quad i = 1, 2, \ldots, k \]  

(2)

where Y was the predicted response, \( \beta_0 \) was the intercept, \( X_i \) and \( X_j \) were the coded independent factors, \( \beta_i \) was the linear coefficient, \( \beta_{ii} \) was the quadratic coefficient and \( \beta_{ij} \) was the interaction coefficient.

Statistical analysis

Design-Expert V8.0.6 (Stat-Ease, Inc., Minneapolis, MN) was used for the experimental designs and regression analysis of the experimental data. The significance of the data was evaluated by variance analysis (ANOVA). 3D surface plots were drawn to indicate the effects of independent variables on the response. The variance explained by the model was shown by the multiple determination coefficient (R²) value. The statistical significance of the model equation was evaluated by the F-test for ANOVA.

Results and discussion

Single factor experiments of seven variables for TTOL entrapment efficiency

In order to obtain the range of factors used in PB experiment, we firstly did a single-factor experiment of seven factors. The mass ratio of PC and Cho was in the range of 2–6; TTO...
concentration was from 0.2% to 1.6% (v/v); Tween 80 concentration was from 1% to 3.5% (v/v); pH value of hydration medium was 5.8–8.2; temperature was 45–65°C; sonication time was 5–25 min; sonication power was 240–360 W.

The results of the experiment are shown in Figure 1. TTOL entrapment efficiency was gradually increased by single factor experiments. When the mass ratio of PC and Cho, TTO and Tween 80 concentrations, the pH of hydration medium, temperature, sonication time, and sonication power were as follows: 4:1, 0.4% (v/v), 3% (v/v), 7.4, 55°C, 15 min and 300 W, the maximal encapsulation efficiency reached 91.09 ± 0.08% (Figure 1G). Then we designed PB experiments according to the results of single factor experiments.

Table 2. Design matrix (in coded levels) with observed and predicted response (TTOL entrapment efficiency) for the experiments performed using PB design.

| Run | X₁ | X₂ | X₃ | X₄ | X₅ | X₆ | X₇ | X₈ | X₉ | X₁₀ | X₁₁ | Actual response (%) | Predicted response (%) |
|-----|----|----|----|----|----|----|----|----|----|-----|----|--------------------|-----------------------|
| 1   | +1 | +1 | -1 | +1 | +1 | -1 | -1 | -1 | +1 | -1  | -1 | 92.74              | 92.78                 |
| 2   | -1 | +1 | +1 | -1 | +1 | +1 | -1 | -1 | -1 | +1  | -1 | 92.02              | 92.04                 |
| 3   | +1 | -1 | +1 | +1 | -1 | +1 | -1 | -1 | -1 | -1  | +1 | 92.11              | 92.13                 |
| 4   | -1 | -1 | -1 | +1 | -1 | +1 | -1 | +1 | -1 | -1  | +1 | 92.34              | 92.36                 |
| 5   | -1 | -1 | +1 | -1 | +1 | -1 | +1 | -1 | +1 | -1  | +1 | 91.70              | 91.72                 |
| 6   | -1 | -1 | -1 | -1 | +1 | -1 | +1 | -1 | +1 | +1  | -1 | 92.00              | 92.02                 |
| 7   | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | +1 | +1  | +1 | 92.39              | 92.41                 |
| 8   | +1 | +1 | -1 | -1 | -1 | +1 | +1 | +1 | +1 | +1  | +1 | 92.70              | 92.72                 |
| 9   | +1 | +1 | +1 | -1 | -1 | +1 | -1 | +1 | +1 | +1  | +1 | 92.44              | 92.46                 |
| 10  | +1 | +1 | +1 | +1 | -1 | +1 | -1 | +1 | +1 | +1  | +1 | 92.05              | 92.07                 |
| 11  | +1 | +1 | +1 | +1 | +1 | -1 | +1 | -1 | +1 | +1  | +1 | 92.08              | 92.10                 |
| 12  | +1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1  | -1 | 91.96              | 91.98                 |
| 13  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0   | 0  | 92.36              | 92.23                 |
| 14  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0   | 0  | 92.36              | 92.23                 |
| 15  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0   | 0  | 92.36              | 92.23                 |
| 16  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0   | 0  | 92.36              | 92.23                 |

Table 3. Experimental design of steepest ascent and corresponding results.

| Run | the mass ratio of PC and Cho | the mass ratio of PC and Cho (A) | TTO concentration | Tween 80 concentration | TTOL entrapment efficiency |
|-----|-------------------------------|----------------------------------|-------------------|------------------------|----------------------------|
| 0   | 4.0                           | 9.43                             | 1.00              | 1.12                   | 92.35 ± 0.23               |
| 0 + 1Δ | 4.4                           | 9.43                             | 1.05              | 1.12                   | 92.63 ± 0.17               |
| 0 + 2Δ | 4.8                           | 9.43                             | 1.10              | 1.12                   | 93.41 ± 0.31               |
| 0 + 3Δ | 5.2                           | 9.43                             | 1.15              | 1.12                   | 95.16 ± 0.11               |
| 0 + 4Δ | 5.6                           | 9.43                             | 1.20              | 1.12                   | 97.35 ± 0.26               |
| 0 + 5Δ | 6.0                           | 9.43                             | 1.25              | 1.08                   | 93.98 ± 0.25               |

Table 4. Coded and actual values of independent factors for CCD.

| Factors | Levels of factors | the mass ratio of PC and Cho (A) | TTO concentration (B, %) | Tween 80 concentration (C, %) |
|---------|-------------------|----------------------------------|--------------------------|-----------------------------|
|         | −1.68             | 4.93                             | 1.12                     | 0.72                        |
|         | −1               | 5.20                             | 1.15                     | 0.75                        |
|         | 0                | 5.60                             | 1.20                     | 0.80                        |
|         | 1                | 6.00                             | 1.25                     | 0.85                        |
| A       | +1.68             | 0.00                             | 1.08                     | 0.88                        |

The analysis of variance for PB experiments is shown in Table 5. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB model.

\[ Y = 92.25 + 0.20X_1 + 0.17X_2 - 0.14X_3 + 0.0092X_4 + 0.0008X_5 + 0.0008X_6 + 0.0058X_7 \] (3)

The PB Design

The PB design was employed to identify which variables have important effects on TTOL entrapment efficiency. TTOL preparation conditions contained the mass ratio of PC and Cho, TTO concentration, Tween 80 concentration, hydration medium pH, temperature, sonication time and power. Table 2 shows the PB experimental design for 16 trials with two levels for each variable. Four controls were used to estimate the experimental error and check the adequacy of the first-order model. According to the analysis by PB design, a first-order model can be obtained from the regression results of the PB experiment:

\[ Y = 92.25 + 0.20X_1 + 0.17X_2 - 0.14X_3 + 0.0092X_4 + 0.0008X_5 + 0.0008X_6 + 0.0058X_7 \] (3)

The analysis of variance for PB experiments is shown in Table 6. The model was significant (p<0.05) and the determination coefficient (R²) was 0.9469, indicating that 94.69% of the variability in the response could be explained...
by the model. Based on the statistical analysis, the factors which had the greatest impact on TTOL entrapment efficiency were identified as $X_1$ (the mass ratio of PC and Cho), $X_2$ (TTO concentration) and $X_3$ (Tween 80 concentration). These variables were ranked as follows: $X_1 > X_2 > X_3$. It can be seen from Table 6 that the other variables had confidence levels below 95%, and hence were considered insignificant. Especially, the $p$ values of four dummy factors, $X_8, X_9, X_{10}$, and $X_{12}$ were below 95%.

Table 6. Analysis of variance (ANOVA) for the experiments performed using PB design.

| Factors          | Sum of squares | Degree of freedom | Mean square | F value   | Prob > F |
|------------------|----------------|-------------------|-------------|-----------|----------|
| Model            | 1.07849167     | 11                | 0.098044697 | 6.4840263 | 0.0429*  |
| A-PC:Cho         | 0.47600833     | 1                 | 0.476008333 | 31.4802976| 0.0050*  |
| B-TTO            | 0.35020833     | 1                 | 0.350208333 | 23.16065032| 0.0086*  |
| C-Tween 80       | 0.24940833     | 1                 | 0.249408333 | 16.49435106| 0.0153*  |
| D-Temperature    | 8.3333E-06     | 1                 | 8.3333E-06  | 0.000551116| 0.9824   |
| E-Time           | 8.3333E-06     | 1                 | 8.3333E-06  | 0.000551116| 0.9824   |
| F-Power          | 8.3333E-06     | 1                 | 8.3333E-06  | 0.000551116| 0.9824   |
| G-pH             | 0.00040833     | 1                 | 0.000408333 | 0.027004684| 0.8774   |
| H-H              | 0.00020833     | 1                 | 0.000208333 | 0.0137779  | 0.9122   |
| J-J              | 8.3333E-06     | 1                 | 8.3333E-06  | 0.000551116| 0.9824   |
| K-K              | 0.00100833     | 1                 | 0.00100833  | 0.8090    |
| L-L              | 0.00020833     | 1                 | 0.000208333 | 0.8090    |

$R^2 = 0.9469$; C.V. = 0.13%.

*Statistically significant at 95% probability level.
and $X_1$ and $X_2$ were far higher than 0.05. The result showed that four variables were nearly irrelevant to TTOL entrapment efficiency. Thus, the mass ratio of PC and Cho, TTO concentration and Tween 80 concentration were selected for further optimization to achieve a maximum response.

The path of steepest ascent

Based on the first-order model equation obtained above and regression results, the mass ratio of PC and Cho, TTO concentration and Tween 80 concentration were considered to be significant variables. The coefficients of $X_1$ and $X_2$ were positive, $X_3$ was negative, which meant that increasing $X_1$, $X_2$ and decreasing $X_3$ had a positive effect on TTOL entrapment efficiency. The path of the steepest ascent was determined to identify the correct direction of the changing variables. The corresponding results of the steepest ascent are shown in Table 3. The values of the other factors were fixed at optimal conditions in single factor experiment. The maximum TTOL entrapment efficiency was obtained in run 0 + 4$\Delta$, which meant that the point was near the region of maximum TTOL entrapment efficiency response. Therefore, this point was chosen as the center point of the CCD for further optimization.

Central composite design and response surface methodology

A highly significant quadratic polynomial obtained by CCD was very useful in determining the optimal TTOL preparation
The levels of the factors chosen were set based on the previous data analysis. Each variable was studied at five coded levels (\(C0\), 1.68, 1, 0, +1, +1.68) shown in Table 5. The other variables were taken at a central-coded value of zero as seen in Table 1. Experimental design and results are displayed in Table 5. The screened variables were expressed by the following fitted second-order polynomial equation from the regression results:

\[
Y = 97.10 - 0.31A + 0.34B - 0.20C + 0.46AB - 0.36AC \\
+ 0.11BC - 0.41A^2 - 0.58B^2 - 0.42C^2
\]

where \(Y\) was the predicted response, \(A\), \(B\) and \(C\) were coded values of the mass ratio of PC and Cho, TTO concentration conditions which had a significant effect on TTOL entrapment efficiency. The levels of the factors chosen were set based on the previous data analysis. Each variable was studied at five coded levels (−1.68, −1, 0, +1, +1.68) shown in Table 5. The other variables were taken at a central-coded value of zero as seen in Table 1. Experimental design and results are displayed in Table 5. The screened variables were expressed by the following fitted second-order polynomial equation from the regression results:

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+ 0.11BC - 0.41A^2 - 0.58B^2 - 0.42C^2
\]
and Tween 80 concentration, respectively. Statistical significance of the model equation was checked by the F-test, and the proportion of variance explained by the model was obtained by the multiple coefficient of determination ($R^2$). The $R^2$ value was a measure of the goodness of fit of the model. In this experiment, $R^2$ was calculated to be 86.25%. The inputs of the test data set were presented to the model for prediction, and the model’s predictive results were compared with the actual output values. $R^2$ values depict the percentage of response variability accounted for by the model (Cui et al., 2010). The plot of predicted values versus experimental values, seen in Figure 2, indicated that all the predicted values of the RSM model were close to the experimental values. The regression coefficients and corresponding $p$ values were used to check the significance of each coefficient (Ren et al., 2011). This represented that the smaller the $p$ value was, the bigger the significance of the corresponding coefficient was.

Table 7 shows that A (the mass ratio of PC and Cho) and B (TTO concentration) had a significant effect on TTOL entrapment efficiency, and C (Tween 80 concentration) was found to have no significance. The interaction of A (the mass ratio of PC and Cho) and B (TTO concentration) also had a significant effect. In addition, the quadratic terms for the three factors were found to be significant effects. Three-dimensional response surface curves were plotted to explain the interaction between two factors for maximum TTOL entrapment efficiency (Figures 3–5). The 3D plots and the shapes of the contour plots show the interaction between two variables. The maximum TTOL entrapment efficiency was obtained when the actual values of the test variables were as follows: the mass ratio of PC and Cho 5.51; TTO concentration 1.21% (v/v); Tween 80 concentration 0.79% (v/v).

Predicted maximum TTOL entrapment efficiency corresponding to these values was 97.18%, which led to significantly improve entrapment efficiency when compared with that (50.16 ± 0.42%) using the basal preparation conditions (Figure 1A).

Lipid bilayer of liposome membrane was composed of PC. The ratio of PC and Cho controlled membrane fluidity and determined liposome stability (Jaafar-Maalej et al., 2010; Ortan et al., 2009); TTO was the encapsulated substance, whose components inserted into the lipid bilayer, and formed TTOLs (Varona et al., 2011); Tween 80 as emulsifier was added into hydration medium in order to enhance the dispersion and solubility of essential oils to form uniform and stable PBS solution involving essential oils. Thus, three factors (the ratio of PC and Cho, TTO and Tween 80) had important influence on the entrapment efficiency of TTOLs. Only when the three parameters above were appropriate, stable TTOLs with high encapsulation efficiency could be obtained.

Moreover, TTO components, lipophilic molecules, competed with cholesterol molecules for the lipophilic space in the lipid bilayer; cholesterol decreased the flexibility of the bilayer and thus the possibility of integration of lipophilic molecules into the lipid membrane (Jaafar-Maalej et al., 2010). Therefore, cholesterol might lower the incorporation of hydrophobic molecules into the bilayer membrane, and PC/Cho and TTO concentration had a significant interaction effect.

**Experimental validation of the optimized conditions**

Validation experiments were performed to verify the accuracy of the models. The results showed that the predicted values were in accordance with the experimental results. The experimental TTOL entrapment efficiency was 97.81 ± 0.33%, which was in excellent agreement with the predicted value (97.18%). Through gas chromatography analysis, the results showed that entrapment efficiencies of main TTO components in liposome suspension were between 96 and 100% (Table 8), which were close to that measured by UV spectrophotometric method. This indicated that the entrapment efficiencies of ultraviolet absorption TTO
components in liposome could indirectly characterize the encapsulation efficiency of TTO in liposome.

The size of liposome was also an important factor for in vivo application. The size and morphology of TTOLs were analyzed by transmission electron microscopy and Zetasizer, and liposomes had a spheroid shape with a homogeneous size (Figure 6). The average particle diameter of TTOLs was about 80 ± 8 nm before optimization and 58 ± 5 nm after optimization, which was in agreement with that obtained by Zetasizer. The thickness of lipid membrane was about 10–20 nm (Figure 6A–F). The results represented that the particle size decreased significantly after optimization, which was caused by the changes of TTO concentration in optimization process (Valenti et al., 2001). Further, the light and dark areas in the lipid bilayer of TTOLs indicated non-uniform distribution of compositions. The average particle diameter of TTOLs were 60 ± 5 nm after 3 months of preservation at 4°C. The result indicated that the formulations were very stable for 3 months in terms of the size. Generally, it was concluded that EOs loaded into liposomes were stable at 4 ± 1°C for at least 6 months (Ortan et al., 2009; Sinico et al., 2005; Valenti et al., 2001).

Conclusion

The application of single factors experiment and statistical design for the screening and optimization of TTOL preparation conditions allowed quick identification of the important factors and the interaction between these factors. The final optimal preparation condition was: the mass ratio of PC and Cho, 5.51; TTO concentration, 1.21% (v/v); Tween 80 concentration, 0.79% (v/v); hydration medium pH, 7.4; temperature, 55°C; sonication time, 15 min; sonication power, 300 W, which led to increase the actual TTOL entrapment efficiency by 95.00% to reach 97.81% when compared with that (50.16%) using the basal preparation conditions. The increased TTOL entrapment efficiency would promote TTO bioavailability and protect TTO from the damage of oxygen, light and temperature.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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