Optimization of Preparation Process of Flavonoid Phospholipid Complex of Guava Leaves by Box-Behnken Response Surface Methodology

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Abstract: In this study, a flavonoid phospholipid complex of guava leaves was prepared to improve its lipid solubility and bioavailability. The flavonoid phospholipid complex of guava leaves was prepared by solvent method. Taking the recombination rate as the evaluation index, single factor investigation and Box-Behnken response surface methodology were used to screen and optimize the preparation process. The results showed that the optimal preparation process of the flavonoid phospholipid complex extract of guava leaves was as follows: total flavonoids of guava leaves: soybean phospholipids (1:2), absolute ethanol as the reaction solvent, the mass concentration of total flavonoids of guava leaves as 5 mg/mL, and the reaction was carried out at 50°C for 2 h. The recombination rate reached 72.84±0.77% (n=3). The research shows that the preparation process is simple and feasible, and the prepared total flavonoid phospholipid complex of guava leaves has good stability and improved lipophilicity.

Keywords: Total Flavonoids of Guava Leaves; Phospholipid Complex; Box-Behnken Response Surface Methodology; Preparation Process

1. Introduction

Guava leaf, belonging to the leaves of psidium guajava of the myrtaceae family, are mainly distributed in tropical and subtropical regions. In China, Guangdong, Guangxi and other places have introduced and cultivated it from Taiwan [1-4]. It contains terpenoids, flavonoids, phenols, polysaccharides, volatile oils, etc. The important active ingredients are flavonoids [5], and guava leaves have a variety of pharmacological activities, such as hypoglycemic, lipid-lowering, antioxidant, hepatoprotective, etc.; among which the hypoglycemic effect may be related to its flavonoids and other phenolics substances it contains [6]. However, flavonoids have high polarity and poor lipid solubility, which are difficult to be absorbed orally, restricting their clinical application.

The phospholipid complex is a new drug-carrying system discovered and proposed by Italian scholar Bambardelli during his researches on liposomes, which is a substance formed by the combination of drugs and phospholipids through charge-transfer., and many studies have confirmed that the phospholipid complex can significantly increase the lipophilicity of active substances. Domestic and foreign researchers have made complexes of poorly-soluble drugs and phospholipids in order to enhance the oral absorption of the drugs, e.g., silybin-phospholipid complex [7] is an early-studied complex which has increased the bioavailability and therapeutic effect of the drug. In China, there have been relevant reports on the phospholipid complexes made of the isoflavones of tempeh [8], the total flavonoids of pueraria lobata [9], the licorice flavonoids [10], the epimedium flavonoids [11] and so on. The results showed that the lipid solubility of the drugs increased and the bioavailability was improved after the preparation of the phospholipid complexes.

At present, there is no relevant research on the total flavonoid phospholipid complex of guava leaves. In this research, the total flavonoids of guava leaves are prepared into phospholipid complexes by solvent method, and the optimal preparation process is screened out, so as to enhance the lipid solubility of drugs, improve oral absorption and increase bioavailability, laying a theoretical foundation for a further

development of new preparations of total flavonoids of guava leaves and a wide application of total flavonoids of guava leaves.

2. Instruments and Reagents

PX124ZH/E electronic analytical balance (Ohaus Instruments Co., Ltd.); RE-2000A rotary evaporator (Shanghai Yarong Biochemical Instrument Factory); DF-101S heat-collecting constant temperature heating magnetic stirrer (Gongyi Yuhua Instrument Co., Ltd.); DZF vacuum drying oven (Shanghai Lichen Bangxi Instrument Technology Co., Ltd.) ; SHZ-88A water-bath thermostatic oscillator (Taicang Experimental Equipment Factory); UV-5500PC ultraviolet-visible spectrophotometer (Shanghai Metash Instruments Co., Ltd.)

Total flavonoid extract of guava leaves (Shaanxi Kanglichuan Biotechnology Co., Ltd); rutin standard (Original Plant Extract Standard Product Distribution Center); soybean phospholipids (Hebei Mersway Bio-Tech Co., Ltd.); reagents such as aluminum nitrate, absolute ethanol, methanol, dichloromethane, trichloromethane, ethyl acetate, acetone, etc. are all analytically pure; water is purified water.

3. Methods and Results

3.1 Ultraviolet and Visible Spectrum Methodological Investigation on the Determination of Total Flavonoids Content in Guava Leaves Extracts

3.1.1 Establishment of the standard curve

Rutin reference substance (20.00 mg) was precisely weighed and dissolved in methanol solution to volume, and then made up to a reference substance solution with a concentration of 0.2 mg/mL. Guava leaf extract (0.20 g) was precisely weighed and dissolved in methanol solution and then made up to obtain a test solution. Accurately transfer 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mL of rutin reference substance solution into volumetric flasks respectively. Add 1.0 mL of 10% aluminum nitrate solution, add methanol solution to make up to the mark, measure the absorbance (A) at 413 nm and plot a graph with the rutin concentration (c) [12]. The regression equation obtained is $y = 0.0255x - 0.0118$, and $R^2 = 0.9994$. The results show that the linearity of rutin reference standard is good within the concentration range of 8.0~32.0 μg/mL.

3.1.2 Precision test

Accurately measure 0.2 mL of 5 test solutions samples, add 1.0 mL of 10% aluminum nitrate solution, dilute to volume with methanol solution, measure their absorbance and calculate the RSD (n=5) of 0.20%, indicating that the instrument has good precision.

3.1.3 Stability test

Accurately measure 0.2 mL of 6 test solutions samples. Then add 1.0 mL of 10% aluminum nitrate solution. Make up to the mark with methanol solution. Measure their absorbance, and calculate an RSD (n=6) of 0.51%, indicating that the prepared test solution is color-stable within 2 h.

3.1.4 Repeatability test

Take 6 portions of the same batch of guava leaf extract powder to prepare the test solutions, add 1.0 mL of 10% aluminum nitrate solution, make up to the scale with methanol solution, measure their absorbance, and calculate an RSD (n=5) is 1.57%, which indicates that the method has good repeatability.

3.1.5 Spiked-sample recovery test

Accurately measure 0.05 mL of three groups of test solution samples with determined total flavone content, with three replicates in each group. Add 0.15 mL, 0.20 mL and 0.25 mL of rutin reference substance solution into each group respectively, and then add 1.0 mL of 10% aluminum nitrate solution. Make up to the mark with methanol. Measure their absorbance and calculate the recovery rate and RSD value [13]. The calculated average recovery rate is 103.94% and RSD is 1.79% (n=9), which indicates that the method is accurate.

3.2 Preparation of Total Flavonoid Phospholipid Complex of Guava Leaves

3.2.1 Method of the preparation of total flavonoid phospholipid complex of guava leaves

A certain amount of total flavonoid extract from guava leaves and soybean phospholipids are weighed and dissolved in the reaction solvent. After being mixed for a certain period of time at a certain temperature, the solvent is removed by low-temperature rotary evaporation, and vacuum drying is carried out at 40°C for 12 hours to obtain the total

flavonoid phospholipid complex of guava leaves.

3.2.2 Determination method of the recombination rate of phospholipid complex

Soybean phospholipids and the total flavonoid phospholipid complex of guava leaves are both easily soluble in dichloromethane, but the total flavonoid extract of guava leaves is difficult to be dissolved in dichloromethane. According to the solubility of the three in dichloromethane, the total flavonoid extract of guava leaves (W_1) and soybean lecithin are reacted to obtain the phospholipid complex, the phospholipid and the complex are dissolved in a proper amount of dichloromethane. Centrifuge to collect the precipitate and wash the precipitate multiple times with a small amount of dichloromethane, and the precipitate is dried and weighed (W_2). The compound ratio of the total flavonoid extract of guava leaves and soybean lecithin can be calculated [14, 15], and the formula is as follows:

$$\text{Recombination rate (\%)} = \frac{(W_1 - W_2)}{W_1} \times 100\%$$

3.3 Single Factor Experiment to Investigate the Preparation Process of Phospholipid Complex

3.3.1 Investigation of reaction solvent

Referring to the method in item “3.2.1”, with a feed ratio of 1:1 added, the mass concentration of total flavonoids in guava leaves is 5 mg/mL, and the reaction is carried out at 30°C for 2 h. Investigate the solution of absolute ethanol, methanol, ethyl acetate, acetone and dichloromethane, and calculate the recombination rate. The results are shown in Figure 1 which show that when methanol is used as reaction solvent, the recombination rate is the highest, followed by absolute ethanol. In consideration of the toxicity and safety of methanol, absolute ethanol is selected as the reaction solvent in this design.

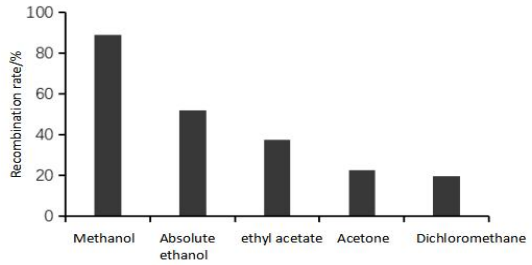

Figure 1. Investigation Results of Reaction Solvent

3.3.2 Investigation on the feeding ratio of drug and soybean lecithin

According to the method in item “3.2.1”, absolute ethanol is used as the reaction solvent and the other conditions are the same as those in “3.3.1”. The influence of the feeding ratio of the total flavonoids from guava leaves and soybean lecithin (5: 1, 4: 1, 3: 1, 2: 1, 1: 1, 1: 2, 1: 3, 1: 4, 1: 5) on the recombination rate is investigated, and the results are shown in Figure 2. It can be seen that the rate of recombination is greatly affected by the ratio of drug to lipid, and shows a tendency of initial increasing and then decreasing. When the ratio of drug to lipid is 1: 3, the rate of recombination is the highest.

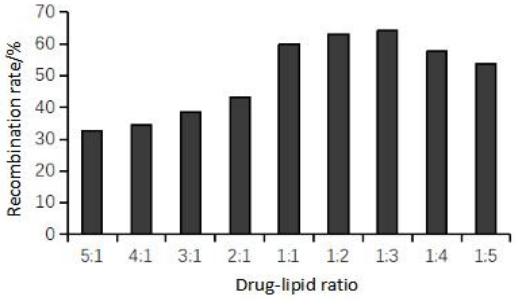

Figure 2. Investigation Results of Feeding Ratio

3.3.3 Investigation of reaction time

Referring to the method in “3.2.1”, using absolute ethanol as the reaction solvent, other conditions are the same as those in “3.3.1”. Investigate the effect of reaction time (1, 2, 3, 5, 10, 12 h) on the recombination rate. The results are shown in Figure 3. With the increase of time, the recombination rate increases, but the increase is relatively small and tends to level off after 2h, so 2h is selected as the reaction time in this design.

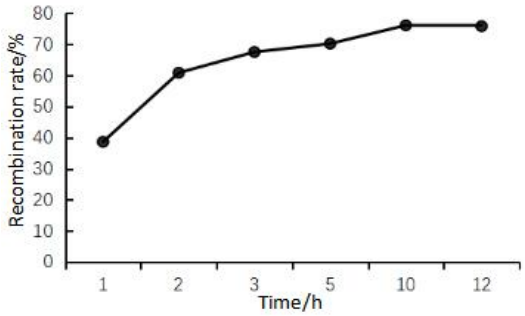

Figure 3. Investigation Results of Reaction Time

3.3.4 Investigation of mass concentration of reactants

Referring to the method in “3.2.1”, use absolute ethanol as the reaction solvent, and

other conditions are the same as those in "3.3.1". Investigate the effect of reactant mass concentration (2, 5, 10, 15, 20 mg/mL) on the recombination rate. The results are shown in Figure 4. The results show that when the mass concentration of the total flavonoids in guava leaves was 5 mg/mL, the recombination rate of total flavonoid phospholipid complex of guava leaves reached the highest.

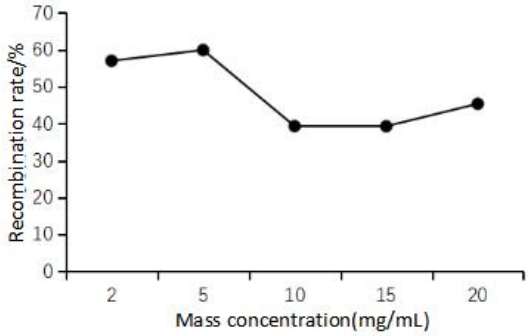

Figure 4. Investigation Results of Reactant Mass Concentration

3.3.5 Investigation of reaction temperature

Referring to the method in term"3.2.1", using absolute ethanol as the reaction solvent, other conditions are the same as those in "3.3.1", investigate the influence of temperature of 30, 40, 50, 60 and 70°C on the recombination rate. The results are shown in Figure 5 which show that the recombination rate increases with the increase of temperature, but decreased above 60°C.

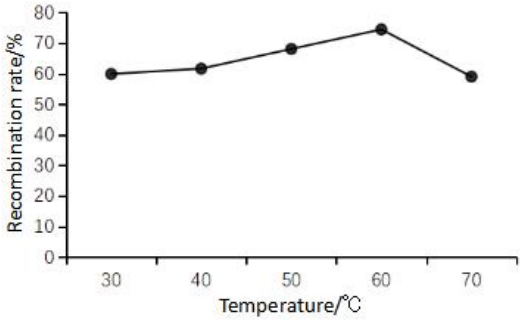

Figure 5. Investigation Results of Reaction Temperature

3.4 Optimization of the Response Surface Methodology

3.4.1 Optimization of the process

On the basis of the results of single factor test, the Box-Behnken response surface experiment was used to further optimize the process. The reaction temperature (factor A), mass concentration of total flavonoids from guava leaves (factor B) and feeding ratio (factor C) were selected as independent variables, and the recombination rate of total flavonoid phospholipid complex of guava leaves was taken as the response value. A response surface analysis of three factors and three levels was designed. The response surface test design and results are shown in Table 1 and Figure 6. Design-Expert 13 software was used to simulate and fit on the data with multiple variables, and the quadratic regression formula using the recombination rate of total flavonoid phospholipid complex of guava leaves as the response value as the objective function was obtained as follows:

$Y = 73.02 + 3.42\times A - 3.65\times B + 3.97\times C - 0.6073\times AB + 0.9725\times AC - 0.2973\times BC - 2.04\times A^2 - 4.22\times B^2 - 6.54\times C^2$. As can be seen from the variance analysis results in Table 2, the model $P < 0.0001$, which indicates that the regression model is significant; the lack-of-fit term $P = 0.0574 > 0.05$, indicating that the model has no obvious deviation. The multiple correlation coefficient R^2 of the model is 0.9936, indicating that the model can explain that 99.36% of the changes in the data come from test factors. Among them, the three first-order terms A, B, and C have extremely significant effects on the model ($P < 0.01$); the three quadratic terms A^2, B^2, and C^2 have extremely significant effects on the model ($P < 0.01$); the interaction terms AB and AC are not significant ($P > 0.05$), and the interaction term BC has a significant effect ($P < 0.05$). The variance analysis results show that the reaction temperature, the mass concentration of total flavonoids from guava leaves, and the feeding ratio have significant effects on the recombination rate of the total flavonoid phospholipid complex of guava leaves, but the effects between the reaction temperature and the mass concentration of total flavonoids from guava leaves and between the reaction temperature and the feeding ratio on the recombination rate are not significant. According to the response surface results, the influence of each factor on the recombination rate of the phospholipid complex of total flavonoid extract from guava leaves is in the order of factor C > factor B > factor A, that is, the feeding ratio > the mass concentration of total flavonoids from guava leaves > the reaction temperature.

As can be seen from the contour curves of the response surface, the interaction between the

mass concentration of total flavonoids from guava leaves and the feeding ratio is significant, manifested as a steep curve. The interaction between temperature and feeding ratio is secondary. The interaction between feeding ratio and reaction temperature is less significant, and the curve is flat and smooth. Judging from the steepness of the response surface, the feeding ratio has the most significant impact on the recombination rate, followed by the mass concentration of total flavonoids from guava leaves and reaction temperature, which is consistent with the variance analysis results.

Table 1. Response Surface Design and Results

No.	Factor			Recombination rate (%)
	A	B	C	
1	40	5	1:1	58.25
2	50	10	1:1	54.77
3	50	5	1:2	73.81
4	50	5	1:2	72.88
5	50	10	1:3	62.64
6	40	10	1:2	60.59
7	50	5	1:2	73.87
8	50	5	1:2	73.88
9	60	10	1:2	65.41
10	50	2	1:1	61.09
11	60	5	1:1	64.53
12	40	5	1:3	63.69
13	50	2	1:3	70.50
14	60	2	1:2	73.86
15	60	5	1:3	73.86
16	50	5	1:2	73.88
17	40	2	1:2	67.15

Table 2. Results of Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-value	P-value	
Model	663.80	9	73.76	120.87	<0.0001	significant
A-temperature	90.90	1	90.90	148.98	<0.0001	
B-concentration	106.51	2	106.51	174.55	<0.0001	
C-lipid-drug ratio	122.32	1	122.32	200.47	<0.0001	
AB	1.52	1	1.52	2.49	0.1584	
AC	3.78	1	3.78	6.20	0.0416	
BC	0.3645	1	0.3645	0.5974	0.4649	
A2	17.51	1	17.51	28.70	0.0011	
B2	63.92	1	63.92	104.75	<0.0001	
C2	180.20	1	180.20	295.32	<0.0001	
Residual	4.27	7	0.6102			
Lack of fit	3.50	3	1.17	6.05	0.0574	Not significant
Pure Error	0.7717	4	0.1929			
Cor Total	668.07	16				

3.4.2 Validation experiment

Through result analysis by Design-Expert.13 software, the optimal process conditions for the total flavonoid phospholipid complex of guava leaves can be obtained as follows: the reaction time is 2 hours, the reaction solvent is anhydrous ethanol, the reaction temperature is 50 °C, the feeding ratio (total flavonoid extract of guava leaves: soybean lecithin) is 1:2, and the mass concentration of the total flavonoid extract of guava leaves is 5 mg/mL. Under these conditions, the maximum recombination rate can reach 73.88%. Three verification experiments were carried out under the above conditions. The recombination rates were 72.05%, 73.59%, and 72.87%, respectively. The average recombination rate was 72.84%, and RSD (n = 3) was 0.77%.

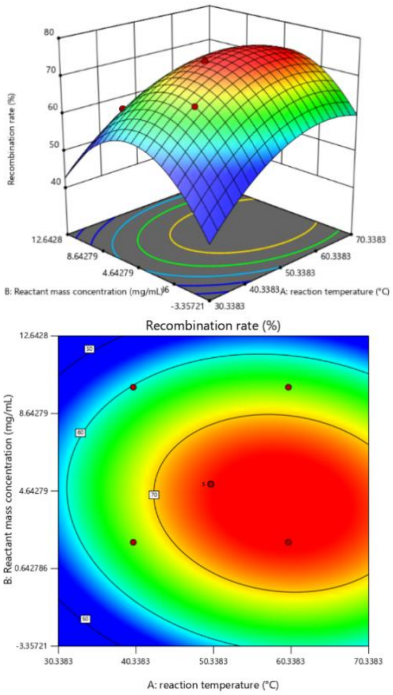

Figure 6-1. Response Surface and Contour Diagram of Reaction Temperature and Mass Concentration of Guava Leaf Total Flavone to the Recombination Rate of The Total Flavonoid Phospholipid Complex of Guava Leaves

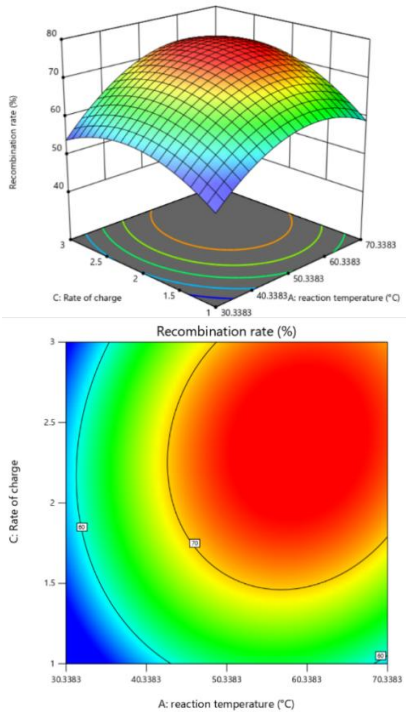

Figure 6-2. Response Surface and Contour Diagram of Reaction Temperature and Feeding Ratio to the Recombination Rate of the Total Flavonoid Phospholipid Complex of Guava Leaves

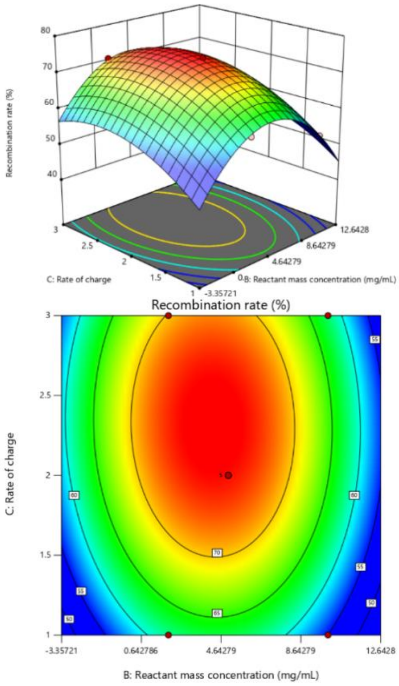

Figure 6-3. Response Surface and Contour Diagram of Mass Concentration and Feeding Ratio of Total Flavonoid in Guava Leaves to the Recombination Rate of the Total Flavonoid Phospholipid Complex of Guava Leaves

Figure 6. Response Surface and Contour Diagram

3.5 Characterization of Phospholipid Complexes

3.5.1 Ultraviolet analysis (UV)

Weigh total flavonoids of guava leaves, soybean phospholipid and their physical mixtures and phospholipid complexes, dissolve them with absolute ethanol, and scan at full wavelength within the wavelength range of 200~600 nm. The results are shown in Figure 7. The results show that both the physical mixture and the phospholipid complex display the absorption characteristics of total flavonoids of guava leaves, with absorption peaks at about 260, 370, and 390 nm, while the absorption peaks of soybean phospholipids are at 269 nm and 244 nm, indicating that no new chromophore was produced during the recombination process, i. e. no new compound was formed.

3.5.2 Infrared analysis (FTIR)

Take total flavonoids of guava leaves, soybean lecithin and their physical mixture and phospholipid complex respectively, add potassium bromide for grinding and tableting, and perform infrared scanning within the range of 4000~400 cm^{-1}. The results are shown in Figure 8. The main absorption peaks of soybean lecithin were at 3401 cm^{-1}(-OH), 2852 cm^{-1} (-CH), 1743 cm^{-1} (C=O), 1224 cm^{-1} (P=O) and 1085 cm^{-1} (P-O-C). The main absorption peaks of total flavonoids from guava leaves are at 3440 cm^{-1} (-OH), 1656 cm^{-1} (C=O), and 1085 cm^{-1} (C-O-C). The physical mixture of total flavonoids from guava leaves and lecithin basically has no superposition of the two, and more shows the infrared absorption of lecithin. Compared with the physical mixture, the position, shape and width of the peaks of the phospholipid complex all change to a certain extent. The intensity of the hydroxyl characteristic peak becomes weaker. The peak shape of the phosphorus-oxygen double bond in lecithin becomes wider and the intensity becomes weaker. It migrates from 1259 cm^{-1} to 1251 cm^{-1}. This indicates that some parts of total flavonoids from guava leaves and soybean lecithin in the total flavonoid

phospholipid complex of guava leaves are combined through interaction to form a complex.

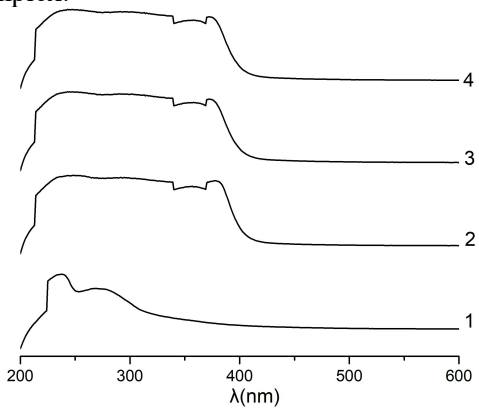

Figure 7 UV Curves of Soybean Phospholipids (1), Total Flavonoids of Guava Leaves (2), Physical Mixture of Total Flavonoids of Guava Leaves and Phospholipids (3) and Their Phospholipid Complexes (4)

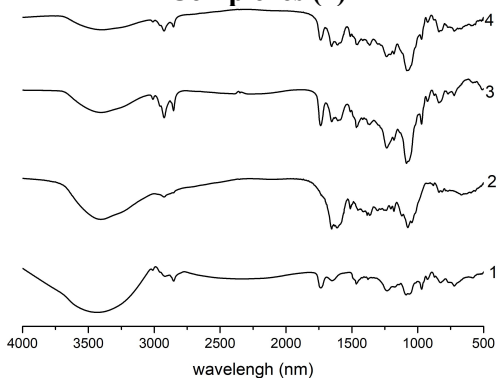

Figure 8 FTIR Curve of Soybean Phospholipid (1), Total Flavonoids of Guava Leaves (2), Physical Mixture of Total Flavonoids of Guava Leaves and Phospholipids (3) and Their Phospholipid Complex (4)

3.6 Comparison of Oil-Water Partition Coefficient

Weighing a proper amount of total flavonoids from guava leaves and its phospholipid complex, add octanol to dissolve them, and prepare the n-octanol solution containing the medicine. Centrifuge at 4000r/min for 10min. Accurately pipette 10 mL of n-octanol solution containing drugs into 5 different conical flasks, respectively add 10 mL of n-octanol saturated buffer solution with pH value of 1.2, 4.5, 6.8, 7.4 and 9.0, shake at constant temperature of 37°C for 24 h, separate the n-octanol solution on the upper layer and the water solution on the bottom layer, measure their absorbance, and calculate the apparent oil-water partition coefficient according to the formula. The results are shown in Table 3.

$$P = \frac{c_0}{c_1} \quad (1)$$

In the above formula, c_0 represents the concentration of total flavonoids in the oil phase after partition equilibrium, and c_1 represents the concentration of total flavonoids in the aqueous phase after partition equilibrium.

The results showed that the oil-water partition coefficients of the total flavonoid phospholipid complex of guava leaves first increased and then decreased within the pH range of 1.2 to 9.0. The oil-water partition coefficients of the total flavonoid phospholipid complex of guava leaves were higher than those of total flavonoids of guava leaves at different pH values, which indicated that the lipophilicity of total flavonoid phospholipid complex of guava leaves was better.

Table 3. Determination Results of Oil-Water Partition Coefficient

pH	Total Flavonoids of Guava Leaves Log P±SD	Total flavonoid phospholipid complex of guava leaves. Log P±SD
1.2	0.0969±0.0331	0.2815±0.0.0383
4.5	0.0956±0.0183	0.2821±0.0303
6.8	0.1167±0.0146	0.2986±0.0325
7.4	0.0974±0.0357	0.3176±0.0209
9.0	0.1054±0.0618	0.2809±0.0274

4. Discussion

Regarding the selection of reaction solvents in the preparation process, phospholipid complexes are substances formed by complexation of polar groups of phospholipids and drugs by intermolecular forces, and non-proton transfer solvents are usually used as reaction solvents in the preparation of phospholipid complexes of the active ingredients of traditional Chinese medicines[16], and the use of proton transfer solvents as reaction solvents will hinder the intermolecular forces[17], but at the same time, they are also affected by the solubility of the solvent of drugs or phospholipids. Solvents

with low polarity such as dichloromethane and ethyl acetate have low solubility for the components with high polarity in total flavonoids from guava leaves. And phospholipids have extremely low solubility in acetone, resulting in a low recombination rate. When methanol and absolute ethanol are used as reaction solvents, the recombination rate is relatively high. However, considering the toxicity and the development of subsequent oral preparations, absolute ethanol is finally selected as the reaction solvent.

The oil-water partition coefficient can be used to evaluate the solubility of the drug, which is important in predicting the absorption of the drug in the body. The total flavonoids of guava leaves contain a variety of flavonoids with high polarity, which causes poor lipid solubility and oral absorption of the drug. The oil-water partition coefficient was experimentally measured to be improved after preparation into phospholipid complexes, i.e., the drug's lipid solubility increased, which may be due to the fact that after interacting with phospholipids, the polar groups are masked to a certain extent [18], the lipophilicity and the lipid solubility of the drug are improved, which is conducive to improving the oral absorption of the drug and increasing the bioavailability.

In this experiment, the phospholipid complex was prepared from the total flavonoids of guava leaves through the phospholipid complex technology, and the preparation process was optimized by Box-Behnken response surface methodology, which was simple, feasible and easy to operate. At the same time, after being prepared into phospholipid complexes, the lipid solubility of the total flavonoids of guava leaves has been improved, and the oral absorption efficiency and bioavailability were expected to be improved, which laid a foundation for further preparation of other formulations of the total flavonoid phospholipid complex of guava leaves.

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