PHARMACEUTICAL TECHNOLOGY

PREPARATION, CHARACTERIZATION OF *POGOSTEMON CABLIN* BENTH. ESSENTIAL OIL/ β -CYCLODEXTRIN INCLUSION COMPLEX AND THEIR- ANTIMICROBIAL ACTIVITIES

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Abstract: Essential oil of *Pogostemon cablin* Benth. (PAEO) has a wide range of pharmacological therapeutic properties and biological activities and is widely used in the medicine, food and cosmetics industries. However, poor water-solubility and instability limit its application in the food and pharmaceutical industries. Beta-cyclodextrin (β -CD) has a specific hollow structure that can form inclusion complexes with essential oil and is commonly used to improve its stability, solubility and bioavailability. In this study, the inclusion complexes of PAEO with β -CD were prepared and optimized by orthogonal experimental design. The formation of the inclusion complexes was confirmed with Fourier transform infrared spectroscopy, microscopic imaging, and differential scanning calorimetry. The thermal stability, light stability, and wet stability test results demonstrate that the formation of β -CD inclusion complex has significantly increased the stability of PAEO. By comparing with free PAEO, the PAEO- β -CD inclusion complexes against common pathogenic bacteria were significantly increased.

Keywords: Patchouli essential oil, inclusion complex, antimicrobial activity

Pogostemon cablin Benth. (patchouli, PA), a plant of the Lamiaceae family is a well-known traditional medicine. In China, India, Japan, and Malaysia, PA is presently cultivated extensively and used to remove dampness, relieve fever, relieve diarrhea, stop vomiting, and stimulate the appetite (1). Essential oil (EO), secondary metabolites, and biological activities of some plants are one of the most important medicinal substances of patchouli. Pharmacological studies have suggested that the PA essential oil (PAEO) has a wide spectrum of activity, e.g. anti-inflammatory (2-4), anti-oxidative (5, 6) and antimicrobial activities (7, 8). However, due to its unpleasant odor, poor water solubility, volatility, and instability, these limited the therapeutic effect and application of PAEO (9, 10).

Cyclodextrin (CD) is a cyclic oligosaccharide compound obtained by linking 6 to 12 glucose molecules after enzymatic hydrolysis of starch. The structure is hollow cylindrical with a hydrophobic inside (11). Common CDs are made up of 6, 7, and

8 glucose molecules connected by α -1, 4 glycosidic bonds, which are called α -CD, β -CD, and γ -CD, respectively. CD inclusion technology can encapsulate drug molecules into the hollow structure of CD to improve drug solubility, stability, and bioavailability (12, 13).

Saturated aqueous solution method (14), ultrasonic method (15), and grinding method (16) are commonly used to prepare the inclusion complex. The saturated aqueous solution method has simple operation and low requirements for equipment and has been widely used in laboratory preparation and industrial production.

The aim of this work is to prepare PAEO- β -CD inclusion complex using a saturated aqueous solution method and optimize the preparation conditions by orthogonal test design. The morphology and microstructure were verified by microscopic imaging, Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC). Then, the thermal stability, light stability, and wet stability

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of the inclusion complex were evaluated. Furthermore, the potential use of PAEO-β-CD inclusion complex for the treatment of infectious diseases was evaluated by common pathogenic bacteria.

EXPERIMENTAL

Materials

The dried decoction pieces of PA were purchased from the Anguo herb market (Hebei Province, China). A voucher specimen (No. 2018-09) was deposited in the Department of Pharmacy, Hebei North University.

Extraction of essential oil from PA

The extraction experiment was carried out according to the previous method (17). The decoction pieces were pulverized and submitted to steam distillation using a Clevenger’s apparatus with a round-bottom flask of 1000 mL. The crushed PA sample (100 g) was boiled with 400 mL of deionized water for 4 h. The extracted essential oil was collected by the volatile oil extractor, and the supernatant was carefully separated. The extracted EO was then dried over anhydrous sodium sulfate to remove water.

Preparation procedure

Preparation of PAEO-β-CD inclusion complex

The inclusion complex of PAEO and β-CD was prepared by a saturated aqueous solution

method with entrapment efficiency as an evaluation index. Briefly, an appropriate amount of β-CD and distilled water were put into a 200 mL beaker and stirred for 2 h to obtain a saturated solution. Then, 2 mL of PAEO-ethanol solution (1 : 1) was slowly added into β-CD solution under stirring and heating conditions. After stored overnight in a refrigerator at 4°C, the inclusion complex was filtered and washed with water and ethanol several times to remove residual PAEO. The dried powder was stored in a desiccator at room temperature for subsequent experiments.

Orthogonal experimental design

Based on the single-factor experiment results, three factors and three levels of the orthogonal assay ($L_9(3^4)$) was designed and evaluated the influence of the ratio of PAEO to β-CD (A), inclusion temperature (B) and inclusion time (C) on the entrapment efficiency of inclusion complex. (Factors and levels see Table 1)

Determination of entrapment efficiency

According to the essential oil determination method (Pharmacopoeia of People’s Republic of China, 2015), 1 mL of PAEO and 300 mL of distilled water were added in a 500 mL round-bottomed flask equipped with volatile extractor. After the extraction and dehydration, the volume of volatile was 0.91 mL. And the blank recovery was calculated by equation (1).

Table 1. Orthogonal design and results.

No.	A	B	C	Entrapment efficiency (%)
	Ratio of PAEO to β-CD	Inclusion temperature	Inclusion time	
1	1 (1 : 6)	1 (50°C)	1 (1.5 h)	52.73 ± 1.35
2	1 (1 : 6)	2 (60°C)	2 (2 h)	72.66 ± 0.99
3	1 (1 : 6)	3 (70°C)	3 (2.5 h)	63.45 ± 1.17
4	2 (1 : 8)	1 (50°C)	2 (2 h)	60.18 ± 1.29
5	2 (1 : 8)	2 (60°C)	3 (2.5 h)	67.34 ± 0.73
6	2 (1 : 8)	3 (70°C)	1 (1.5 h)	54.3 ± 1.05
7	3 (1 : 10)	1 (50°C)	2 (2.5 h)	51.28 ± 1.01
8	3 (1 : 10)	2 (60°C)	1 (1.5 h)	86.34 ± 1.48
9	3 (1 : 10)	3 (70°C)	3 (2.5 h)	66.09 ± 0.67
K ₁	62.95	54.73	64.46	
K ₂	60.61	75.45	66.31	
K ₃	67.9	61.28	60.69	
Range	7.29	20.72	5.62	
	A ₃ B ₂ C ₂			

K_i was calculated by the average of the entrapment efficiency under various factors and levels. Range was the difference between the maximum value and the minimum value of K_i.

Blank recovery (%) = $\frac{\text{volume of collected EO (mL)}}{\text{volume of added EO (mL)}}$ (1)

The recovery of EO from the inclusion complex was also determined in this method, and the entrapment efficiency was calculated according to equation (2).

Entrapment efficiency (%) = $\frac{\text{the recovery amount of EO in inclusion complex (mL)}}{\text{addition amount of EO in inclusion complex (mL)} \times \text{blank recovery (\%)}}$ (2)

Stability study of PAEO-β-CD inclusion complex

Thermal stability test

PAEO-β-CD inclusion complex and PAEO were accurately weighed and placed in a weighing bottle with a thickness about 0.3~0.5 cm, then put them in a constant humidity chamber (set the humidity to 45%), while the temperature was set to 40°C, 60°C, and 80°C, respectively. Samples were taken at 0, 5, and 10 days to determine the PAEO content.

Light stability test

PAEO-β-CD inclusion complex and PAEO were accurately weighed and placed in a drug stability test chamber with a luminance of 4500 Lx. Samples were taken at 0, 5, and 10 days to determine the PAEO content.

Wet stability test

PAEO-β-CD inclusion complex and PAEO were accurately weighed and placed in two closed containers with a relative humidity of 75% and 90%, respectively. Samples were taken at 0, 5, and 10 days to determine the PAEO content.

Microscopic imaging analysis

The morphology and crystal form change of the inclusion complex and the mixture of PAEO and β-CD were observed through a microscope to determine whether the inclusion is formed. After high-speed dispersion in water and filtration through a 100 mesh sieve, the β-CD, the physical mixture of PAEO and β-CD, and the PAEO-β-CD inclusion complex were observed under a 100 × microscope.

Infrared spectroscopy analysis

For FT-IR measurements, a Nicolet IS5 Infrared spectrometer (Thermo Scientific, USA) with a resolution of 4 cm⁻¹, was used to record the spectra of β-CD, PAEO, physical mixture of PAEO and β-CD, and PAEO-β-CD inclusion complex from 4000 to 500 cm⁻¹.

Differential scanning calorimetry analysis

A Mettler differential scanning calorimetry (DSC) was used to detect β-CD, PAEO, physical mixture of PAEO and β-CD, and PAEO-β-CD inclusion complex. Samples were weighted approximately 2 mg. An empty aluminum pan was used as a reference sample. The heating rate was 20°Cmin⁻¹, and the heating range was 30 ~ 500°C.

Chemical composition of PAEO

Gas Chromatography (GC) analyses were performed coupled to mass spectrometry (GC/MS) (Shimadzu QP2010 Ultra, Japan). The capillary column was Rtx-5MS (30 m × 0.25 mm × 0.25 μm), and the helium carrier gas flow rate was 1.2 mL/min. Injector temperature was 250°C, and the injection volume was 2 μL. The oven program temperature was at 60°C for 2 min after injection and then ramp at 3°C/min to 210°C. The mass detector electron ionization was 70 eV. The identification of the components was performed based on mass spectra library search (NIST14) and the comparison of the retention index described in the literature (18, 19).

Antimicrobial activity

The Agar dilution method was carried out for the determination of antibacterial activity. PAEO and its inclusion complex were dissolved and sequentially diluted in a 96 well plate to produce a concentration range of 0.24-48 mg/mL.

Microbial strains (*Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiellapneumonia*, *Typhoid bacillus*) were incubated under sterilized conditions at 37°C for 24 h. Activated strains were distributed on nutrient agar and diluted to reach about 1 × 10⁶ CFU·mL⁻¹. After

Table 2. Results of the thermal stability test of PAEO and PAEO-β-CD inclusion complex (%).

Time/day	40°C		60°C		80°C	
	PAEO	Inclusion complex	PAEO	Inclusion complex	PAEO	Inclusion complex
5	45.67 ± 0.76	99.23 ± 1.32	6.08 ± 0.45	98.93 ± 1.12	0.00	96.34 ± 1.28
10	0.00	98.34 ± 0.56	0.00	97.56 ± 0.76	0.00	95.43 ± 0.45

Figure 1. Effect of different ratios of PAEO to β-CD (A), inclusion temperature (B), and inclusion time (C) on the entrapment efficiency of PAEO-β-CD inclusion complexes.

Table 3. Results of light stability and wet stability test of PAEO and PAEO-β-CD inclusion complex (%).

Time/day	Light (intensity 4500 Lx)		Relative humidity 75%		Relative humidity 90%	
	PAEO	Inclusion complex	PAEO	Inclusion complex	PAEO	Inclusion complex
5	76.85 ± 0.86	99.06 ± 1.02	89.05 ± 1.06	98.74 ± 1.04	92.45 ± 0.65	99.03 ± 0.76
10	38.65 ± 0.65	98.76 ± 0.94	82.64 ± 0.92	98.02 ± 0.98	90.32 ± 0.62	98.03 ± 0.98

Figure 2. Microscopic image of β-CD (A), mixture of PAEO and β-CD (B) and PAEO-β-CD inclusion complexes (C) under magnification 100×.

solidification, 5 μL PAEO-β-CD inclusion complex solution or free essential oil solution at proper concentration were applied on the surface of agar plates previously seeded with standardized test microorganisms. Then, the plates were incubated under 37°C for 24 h, and the diameters of inhibition

growth zones were measured using a caliper rule. To determine the minimum bactericidal concentration (MBC), 40 μL of aliquot broth were taken from each well and plated in agar for 24 h at 37°C for quality control strains, and 37°C for plant bacteria. The MBC represents the concentration required to kill 99.9% or more of the initial inoculum. All assays were performed in triplicate. The data were statistically analyzed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Preparation procedure

Properties of the PAEO

PAEO is a colorless oily liquid with a density of 0.97 g/cm³ and a boiling point of 148°C.

Single-factor experiment results

The single factor experiment was an experiment in which only one factor was changing, and other factors remained unchanged. The specific role and influence factor in the experiment can be determined through a single factor experiment, so as to provide a reasonable level range for orthogonal experiments. In this study, the effects of the ratio of PAEO to β-CD, inclusion temperature, and inclusion time on the entrapment efficiency were performed. As shown in Figure 1A, when the ratio of PAEO to β-CD increases from 1:4 to 1:8, the entrapment efficiency of the inclusion complex gradually increases, and then the entrapment efficiency gradually decreases with the increase of the ratio of PAEO to β-CD. Therefore, the ratio of essential oil to β-CD of 1:8 was chosen as the center point of the orthogonal experiment. As can be seen from Figure 1B, the entrapment efficiency gradually increases with increasing temperature at 30, 40, 50, and 60°C, and decreases at 70, 80°C. The high-temperature condition which is conducive to the inclusion process may lead to the volatility of PAEO. However, the solubility of β-CD decreases at low temperatures also reduces the entrapment efficiency. Therefore, 60°C was chosen as the center point of the orthogonal experiment. Figure 1C describes the relationship between inclusion time and entrapment efficiency.

60°C, and the inclusion time was 2 h. Three verification experiments were performed under optimized conditions, and the average entrapment efficiency reached $(87.02 \pm 1.06)\%$.

The stability test of PAEO- β -CD inclusion complex

Thermal stability test

As shown in Table 2, PAEO was unstable and volatile under high-temperature conditions. The stability was greatly improved after preparation into PAEO- β -CD inclusion complex, indicating that the inclusion complex can significantly improve the thermal stability of PAEO.

Light stability and wet stability test

As shown in Table 3, PAEO was unstable and easily volatilized under a light. It can also be observed that PAEO has a certain degree of volatility under a high humidity environment. On the contrary, a remarkable improvement of the stability has been exhibited in PAEO- β -CD inclusion complex. In general, the formation of the inclusion complex is beneficial to enhance the stability of volatile to light, heat and humidity.

Microscopic imaging analysis

Figure 2A shows the microstructure of β -CD, which is colorless and transparent, showing plate-like crystals; Figure 2B depicts the physical mixture of PAEO and β -CD. The microstructure of β -CD can be observed, as well as the surrounding oil droplets; Figure 2C is the photograph of the inclusion complex of PAEO- β -CD. It can be seen that the black opaque mass is wrapped in the β -CD cavity, and free oil droplets are not observed. These results indicate that the PAEO was successfully encapsulated in β -CD.

Infrared spectroscopy analysis

FT-IR can be used to identify the formation of the inclusion complex between β -CD and guest molecules by the changes of peak shape, intensity and position. Infrared spectroscopy of PAEO, β -CD, physical mixture, and PAEO- β -CD inclusion complex were shown in Figure 3. β -CD (Fig. 3A) had characteristic absorption peaks at 3300 cm^{-1} for O-H stretching vibration, 2924 cm^{-1} for C-H stretching vibration, 1152 cm^{-1} , 1076 cm^{-1} and 1020 cm^{-1} for asymmetrical stretching vibration and symmetrical stretching vibration of C-O-C. PAEO (Fig. 3C) had characteristic absorption peaks at 3461 cm^{-1} for symmetrical stretching vibration of =CH, 2924 cm^{-1} and 2869 cm^{-1} for asymmetrical stretching vibration and symmetrical stretching vibration of $-\text{CH}_3$, 1743 ,

1718 , 1642 , 1557 , 1464 and 1374 cm^{-1} for stretching vibration of C=C. The physical mixture of PAEO and β -CD (Figure 3D) exhibited a combination of characteristics of PAEO with characteristic absorption peaks at 2924 , 2869 , 1743 , 1642 , 1557 , 1464 , 1374 cm^{-1} and β -CD with characteristic absorption peaks at 3300 , 2924 , 1152 , 1076 , 1020 cm^{-1} . However, the characteristic absorption of PAEO- β -CD inclusion complex (Fig. 3B) is similar to β -CD, the infrared vibration of PAEO in the inclusion complex was limited, and many characteristic absorption peaks such as 2869 , 1743 , 1718 , and 1557 cm^{-1} disappeared in the inclusion complex. These results indicate that the inclusion complex has been successfully prepared.

Differential scanning calorimetry

The thermal curves of PAEO, β -CD, physical mixture, and PAEO- β -CD inclusion complexes are shown in Figure 4. For the β -CD (Fig. 4C), wide endothermic peaks in a range of 95 - 132°C are associated with the release of water from β -CD (20), and the exothermic peak above 360°C can be attributed to melting decomposition of β -CD. As can be seen from Figure 4B, the PAEO shows two sharp endothermic from 130 to 150°C and a broad endothermic event at 300°C , which is possibly corresponding to the volatilization or evaporation process of different ingredients. For the physical mixture of PAEO and β -CD (Fig. 4D), the endothermic peaks of PAEO and β -CD were both observed, establishing the physical property of the physical mixture was similar to the two components, and it was still the simple mixture of PAEO and β -CD (21). In the DSC curve of the PAEO- β -CD inclusion complex (Fig. 4A), the endothermic peak of the PAEO disappeared. While the complexes showed a higher transition temperature than the corresponding β -CD. It indicated that a new phase was produced after the wrapping of essential oil into the β -CD and the formation of the inclusion complex. The phenomena observed in the experiment are consistent with that reported in the literature (22).

Chemical composition of PAEO

The major components of PAEO were patchouli alcohol (32.02%), α -bulnesene (11.32%), α -guaiene (8.7%), α -Patchoulene (6.0%), Pogostol (5.9%) and Seychellene (5.6%). The minor components were (E)-Caryophyllene (3.2%), Aciphyllene (2.6%), Pogostone (2.5%) and β -Patchoulene (1.8%). Similar phenomena were found by Manoj et al. (23). The essential oil obtained by APEO- β -CD inclusion complexes contained patchouli alcohol (32.63%), α -bulnesene (10.46%), α -guaiene

Table 4. The minimum inhibitory concentration (MIC, mg/mL) and minimum bactericidal concentration(MBC, mg/mL) of free PAEO and PAEO-β-CD inclusion complex against different bacterias.

	MIC (mg/mL)		MBC(mg/mL)	
	Free PAEO Dinclusion complex	PAEO-β-C	Free PAEO Dinclusion complex	PAEO-β-C
<i>Escherichia coli</i>	24	12*	48	24*
<i>Stphylococcus epidermidis</i>	0.6	0.24*	1.2	0.48*
<i>Staphylococcus aureus</i>	0.6	0.48	1.2	1.2
<i>Klebsiella pneumonia</i>	6	1.2*	12	6*
<i>Typhoid bacillus</i>	48	12*	-	48

*Difference between PAEO-β-CD inclusion complex and free PAEO were significant (p<0.05).

(8.91%), α-Patchoulene (6.34%), Pogostol (6.12%), Seychellene (5.4%), (E)-Caryophyllene (3.6%), Aciphyllene (2.8%), Pogostone (2.74%) and β-Patchoulene (1.92%). There was no significant difference in composition and content between free volatile oil and PAEO-β-CD inclusion complex. According to previous reports (24, 25), patchouli alcohol and Pogostone are responsible for the anti-bacterial and antifungal effects.

Antibacterial activity of PAEO- β-CD inclusion complex

In this study, *E. coli*, *S. epidermidis*, *S. aureus*, *K. pneumonia*, *T. bacillus* were selected to investigate the antibacterial activity of PAEO and PAEO-β-CD inclusion complex. The minimum inhibitory concentration (MIC) and MBC values are displayed in Table 4. The antibacterial activity of the inclusion complex was better than that of free PAEO of equal concentration. PAEO-β-CD inclusion complex had the strongest inhibitory activity on *S. epidermidis* with MIC values of 0.24 mg/mL and MBC values of 0.48 mg/mL, then followed by *S. aureus* with MIC value of 0.48 mg/mL and MBC values of 1.2 mg/mL, and *K. pneumonia* with MIC value of 1.2 mg/mL. The PAEO-β-CD inclusion complex showed similar inhibitory activity to *E. coli* and *T. bacillus* (MIC value 12 mg/mL). The effect of PAEO-β-CD inclusion complexes on Gram-positive bacteria was better than against Gram-negative bacteria, this result is consistent with previous research (26). The antibacterial mechanism is related to destroy the structural integrity, affect the permeability, change the conformation of protein of cell membrane and cell wall (27). The *in vitro* antibacterial test illustrates the action mechanism of PAEO as an antibacterial drug in traditional medical treatment.

CONCLUSIONS

In this study, the orthogonal design was successfully used to optimize the preparation conditions of the PAEO-β-CD inclusion complex. The optimum entrapment efficiency was 87.02% with the ratio of PAEO to β-CD of 1:10, the inclusion temperature of 60°C, and the inclusion time of 2 h. The results of FT-IR, DSC and microscopy revealed the formation of the inclusion complex. Furthermore, antibacterial experiments were carried out, and it was found that PAEO and PAEO-β-CD inclusion complex had a good inhibitory effect on Gram-positive bacteria. However, further research is still necessary to fully understand the mechanism. The research provides the basis of further study and therapeutic prospects in bacterial infection of essential oil from *P. cablin* Benth.

Conflicts of interest

The authors declare no conflict of interest.

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