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Optimization of Conditions of *Zanthoxylum* Alkylamides Liposomes by Response Surface Methodology and the Absorption Characteristics of Liposomes in the Caco-2 Cell Monolayer Model

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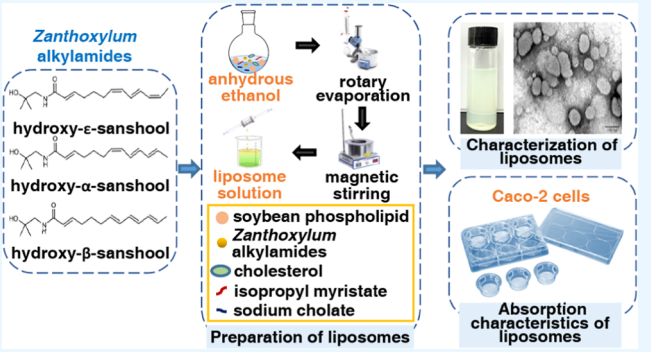
ABSTRACT: *Zanthoxylum* alkylamides, as a numbing substance in *Zanthoxylum bungeanum* has many physiological effects. However, the numbing taste and unstable properties limited its application. This study aimed to optimize the preparation process of *Zanthoxylum* alkylamides liposomes by response surface methodology (RSM) and to investigate the *in vitro* absorption characteristics of the liposomes through the Caco-2 cell monolayer model. The process parameters of liposomes were as follows: *Zanthoxylum* alkylamides was 15 mg, phospholipid−feedstock ratio was 6.14, phospholipid−cholesterol ratio was 8.51, sodium cholate was 33.80 mg, isopropyl myristate was 29.49 mg, and the theoretical encapsulation efficiency of the prepared liposomes could reach 90.23%. Further, the particle size of the liposomes was 155.47 ± 3.16 nm, and the ζ-potential was −34.11 ± 4.34 mV. Meanwhile, the liposomes could be preserved for 14 days under the condition that the content of *Zanthoxylum* alkylamides was less than 2 mg/mL and the preservation temperature was lower than 25 °C. Moreover, the uptake characteristics of the *Zanthoxylum* alkylamides liposomes in the Caco-2 cell monolayer model were also investigated. The results showed that the *Zanthoxylum* alkylamides liposomes could be taken up and absorbed by Caco-2 cells. Also, the *Zanthoxylum* alkylamides liposomes had a better uptake performance than the unembedded *Zanthoxylum* alkylamides and conformed to the passive uptake.



1. INTRODUCTION

Zanthoxylum bungeanum, a spice plant of the genus *Zanthoxylum* L. in the family Rutaceae is mainly distributed in Africa, America, Oceania, and tropical and subtropical regions of Asia.[1] It mainly contains volatile oils, alkaloids, and more than 25 *Zanthoxylum* amides.[2−4] Meanwhile, the numbing taste of *Z. bungeanum* is mainly produced by *Zanthoxylum* alkylamides.[5] Recent studies have shown that *Zanthoxylum* alkylamides has various physiological effects such as anesthetic, analgesic, hypolipidemic, and hypoglycemic and has the potential to be developed into functional foods.[6−8] However, the stability of *Zanthoxylum* alkylamides is easily affected by factors such as ultraviolet light, oxygen, and heating, resulting in changes in its chemical structure and a decrease in its content. Previous studies have also shown the effect of *Zanthoxylum* alkylamides in improving blood glucose levels in Sprague-Dawley rats.[9] However, it is not widely accepted by consumers due to its pungent and numbing taste, making it difficult to consume directly as a functional food. Moreover, directly consuming a large amount of *Zanthoxylum* alkylamides can also damage the intestinal mucosa and cellular structure, leading to intestinal discomfort.[10] Additionally, the poor water solubility of *Zanthoxylum* alkylamides, which is usually sealed with petroleum ether at −20 °C or preserved in airtight brown bottles filled with nitrogen, also limits its wide application. Therefore, the research on the related functional products of *Zanthoxylum* alkylamides is of great significance to improve the stability of *Zanthoxylum* alkylamides, reduce irritation, and expand its application.

As an emerging technology, nanotechnology can improve the functionality of products, thus changing the existing primary stage of agricultural processing.[11] Nanoparticles, also known as solid colloidal particles, include nanospheres and nanocapsules.[12] Liposomes are nanocarriers composed of one or more layers of natural or synthetic lipids, which can be safely applied.[13,14] Their structure effectively encapsulates hydrophilic and hydrophobic substances, with hydrophilic molecules being encapsulated in the aqueous core and hydrophobic molecules

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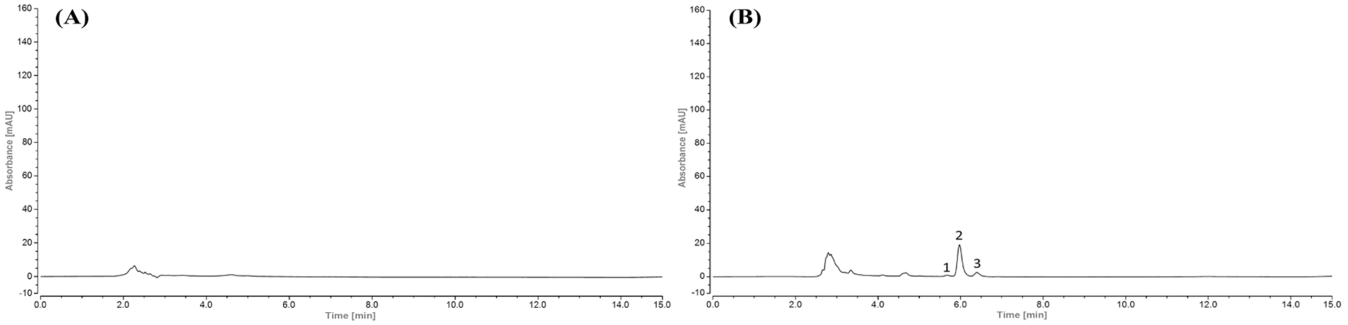

Figure 1. Liquid chromatogram of liposomes of *Zanthoxylum* alkylamides [(A) blank liposome; (B) alkylamides-containing liposome; (1) hydroxy-ε-sanshool; (2) hydroxy-α-sanshool; (3) hydroxy-β-sanshool].

Table 1. Box−Behnken Design with Experimental Values

run	phospholipid−feedstock ratio	phospholipid−cholesterol ratio	sodium cholate (mg)	isopropyl myristate (mg)	EE (%)
1	5	8	50	25	78.3
2	6	8	30	25	90.39
3	6	6	10	25	77.24
4	6	10	30	5	82.03
5	6	8	50	5	81.55
6	6	8	30	25	88.91
7	6	6	30	5	82.72
8	5	10	30	25	76.51
9	6	10	10	25	79.58
10	5	8	10	25	68.19
11	6	8	10	45	79.2
12	6	6	30	45	77.04
13	6	6	50	25	79
14	7	8	30	45	86
15	5	6	30	25	80.15
16	7	6	30	25	76.31
17	6	10	50	25	85
18	7	10	30	25	78.24
19	7	8	50	25	79.2
20	6	8	30	25	91.57
21	6	8	30	25	89.6
22	6	8	10	5	79.2
23	7	8	10	25	76.3
24	5	8	30	45	73.69
25	6	8	50	45	83
26	5	8	30	5	82.09
27	6	8	30	25	88.43
28	6	10	30	45	86
29	7	8	30	5	76.51

being retained in the lipid bilayer. Further, liposomes can increase the stability and cycling time of substances and decrease the toxicity of substances,[15] which is suitable for the development of food products with *Zanthoxylum* alkylamides as a functional ingredient, thus improving the original sensory quality and physical properties and enhancing the value of the application.[16,17] It has been shown that hydroxy-α-sanshool, one of the components of *Zanthoxylum* alkylamides, could be detected in blood and urine of healthy volunteers after oral administration of 15 g of a traditional Japanese Medicine Daikenchuto. Meanwhile, the drug concentration in blood peaked at 30 min after administration, and then the concentration decreased.[18] Moreover, the rat intestinal absorption of *Zanthoxylum* alkylamide followed a passive transport way, Jejunum and ileum were the major absorption sites.[19] These studies provide a theoretical basis for the development of functional foods with hypolipidemic and hypoglycemic effects based on *Zanthoxylum* alkylamides.

This study aimed to improve the unfavorable aspects such as inconvenient preservation, easy degradation, bad taste, and intestinal irritation of *Zanthoxylum* alkylamides by embedding *Zanthoxylum* alkylamides in the form of liposomes; optimize the preparation process by analyzing the relevant indexes of liposomes to improve the stability of liposomes; and explore the *in vitro* absorption of the *Zanthoxylum* alkylamides liposomes by Caco-2 cells, which could be used to expand the application of *Zanthoxylum* alkylamides.

2. RESULTS AND DISCUSSION

2.1. Preparation of *Zanthoxylum* Alkylamides Liposomes. As shown in Figure 1, the reagents in the preparation process of *Zanthoxylum* alkylamides liposomes did not interfere

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with the analysis of the content of *Zanthoxylum* alkylamides in the liposomes.

The results of the Box−Behnken test for the liposome preparation process of *Zanthoxylum* alkylamides are shown in Table 1. Meanwhile, the data in Table 1 were subjected to model fitting to obtain the analysis of variance (ANOVA) results for the liposome preparation process (Table 2).

Table 2. Variance Analysis of the Results of the Response Surface Experiment

source	sum of squares	df	mean square	F value	p value
model	776.02	14	55.43	14.39	<0.0001[b] significant
A	15.48	1	15.43	4.02	0.0648
B	18.50	1	18.50	4.80	0.0458[a]
C	57.82	1	57.82	15.01	0.0017[b]
D	0.057	1	0.057	0.015	0.9046
AB	7.76	1	7.76	2.01	0.1778
AC	13.00	1	13.00	3.37	0.0876
AD	80.01	1	80.01	20.77	0.0004[b]
BC	3.35	1	3.35	0.87	0.3670
BD	23.28	1	23.28	6.04	0.0276[a]
CD	0.53	1	0.53	0.14	0.7174
A^2	389.34	1	389.34	101.05	<0.0001[b]
B^2	114.76	1	114.76	29.79	<0.0001[b]
C^2	230.70	1	230.70	59.88	<0.0001[b]
D^2	60.54	1	60.54	15.71	0.0014[b]
residual	53.94	14	3.85		
lack of fit	47.75	10	4.78	3.09	0.1443 not significant
pure error	6.19	4	1.55		
cor total	829.96	28			

[a]$p < 0.05$ indicates a significant difference. [b]$p < 0.01$ indicates a highly significant difference.

The optimized model equation was as follows:

$$EE(\%) = 89.78 + 1.14A + 1.24B + 2.20C + 0.069D + 1.39AB - 1.80AC + 4.47AD + 0.91BC + 2.41BD + 0.36CD - 7.75A^2 - 4.21B^2 - 5.96C^2 - 3.05D^2$$

The optimization model had a misfit term of 0.1443 (not significant), $R^2 = 0.9350$, and $R_{Adj}^2 = 0.8700$, indicating a good fit for the model. C, AD, A^2, B^2, and C^2 had significant influences ($P < 0.001$; Table 2). The effect of B (phospholipid−cholesterol ratio) on the results was highly significant, the effect of C (sodium cholate) was significant, and the effects of A (phospholipid−ingredient ratio) and D (isopropyl myristate) were not significant on the results.

Based on the results of model fitting, the interaction between the four factors affecting the encapsulation efficiency (EE) (phospholipid−feedstock ratio, phospholipid−cholesterol ratio, sodium cholate dosage, and isopropyl myristate dosage) was examined. Two of these were kept unchanged, and a three-dimensional effect surface plot was obtained for the EE in relation to the other two factors, which led to the prediction of the optimal process for the *Zanthoxylum* alkylamides liposomes (Figure 2).

Response surface methodology (RSM), a mathematical and statistical method of fitting an empirical model to experimental data obtained based on an experimental design, typically uses linear or squared polynomial functions to describe the system under study and explore the experimental conditions until they are optimized.[11,20] The EE showed an increasing and then decreasing trend with the increase in the phospholipid−feedstock ratio, phospholipid−cholesterol ratio, amount of sodium cholate, and amount of isopropyl myristate (Figure 2). Phospholipids are amphiphilic lipids with a glycerol molecule bound to a phosphate group (PO_4^{2-}) and two chains of saturated or unsaturated fatty acids.[21] As a key component that can provide specific properties to liposomes, phospholipids can encapsulate compounds and improve functionality.[22] They are also a major component of biological cell membranes, which enables the coexistence of liposomes and cellular membranes in the release mechanism. Cholesterol also plays an essential role in the preparation of liposomes by binding to phospholipid chains, increasing the mechanical strength and decreasing the permeability and fluidity of liposomes.[23−25] It is essential for the structural stability of liposome membranes in the intestinal environment.[26] Therefore, liposomes with a better EE can be prepared using phospholipids and cholesterol as the main raw materials. The phenomena and results of the preliminary pretests revealed that the liposomes prepared when the phospholipid−raw material ratio was around 6:1 had a better encapsulation ability for the *Zanthoxylum* alkylamides. The EE of the liposomes increased first. Then, it decreased with the

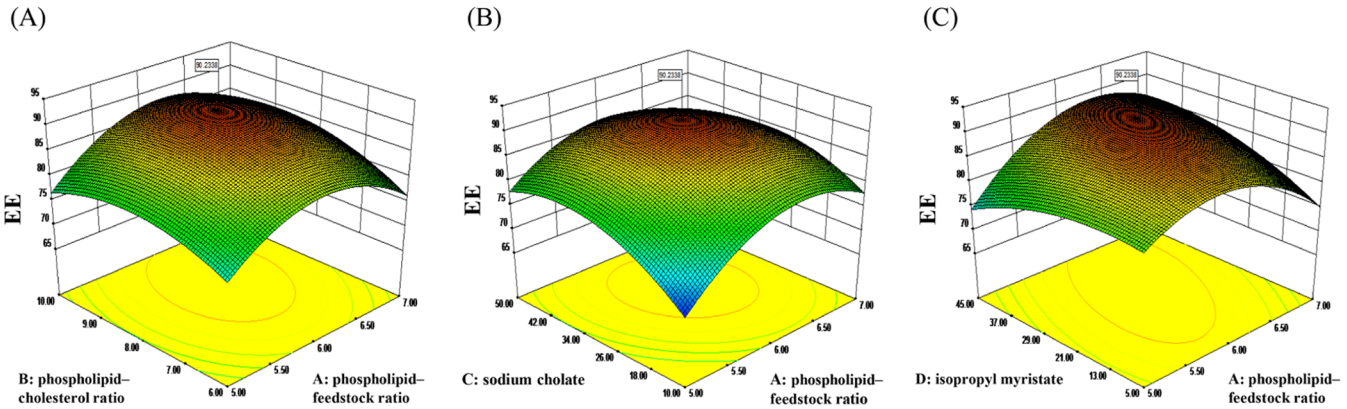

Figure 2. Three-dimensional surface representation of the experiment. Plots (A−C) show the effects of phospholipid−feedstock ratio (A), phospholipid−cholesterol ratio (B), sodium cholate (C), and isopropyl myristate (D) on the EE %.

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Table 3. Content of *Zanthoxylum* Alkylamides Liposomes[a]

content (mg/mL)	temperature (°C)	preservation time (days) 0	7	14	21
1.0	4	0.98 ± 0.05[a]	0.98 ± 0.03[a]	0.97 ± 0.02[a]	0.96 ± 0.01[a]
	25	0.98 ± 0.05[a]	0.97 ± 0.01[a]	0.96 ± 0.01[a]	0.95 ± 0.01[a]
	40	0.98 ± 0.05[a]	0.96 ± 0.01[a]	0.96 ± 0.01[a]	0.93 ± 0.01[a]*
2.0	4	1.99 ± 0.03[a]	1.98 ± 0.01[a]	1.96 ± 0.01[a]	1.94 ± 0.06[a]
	25	1.99 ± 0.03[a]	1.96 ± 0.01[a]	1.94 ± 0.02[a]	1.87 ± 0.01[b]*
	40	1.99 ± 0.03[a]	1.95 ± 0.02[a]*	1.91 ± 0.06[a]	1.82 ± 0.01[b]*
4.0	4	3.95 ± 0.03[a]	3.93 ± 0.02[a]	3.86 ± 0.01[a]	3.70 ± 0.08[b]
	25	3.95 ± 0.03[a]	3.92 ± 0.02[a]	3.81 ± 0.02[b]*	3.60 ± 0.04[c]
	40	3.95 ± 0.03[a]	3.88 ± 0.01[b]*	3.81 ± 0.02[c]*	3.54 ± 0.03[d]*

[a]The experimental data are expressed as mean ± standard error (n = 3). (a–d) Different letters indicate that there is a significant difference between the experimental groups of the same content and preservation temperature for each preservation day ($P < 0.05$), and * indicates that the experimental group of the same content and preservation day for each temperature is significantly different from the experimental group of 4 °C ($P < 0.05$).

Table 4. Particle Size of *Zanthoxylum* Alkylamides Liposomes[a]

content (mg/mL)	temperature (°C)	preservation time (days) 0	7	14	21
1.0	4	153.03 ± 3.15[a]	155.47 ± 3.16[a]	157.66 ± 3.03[a]	158.36 ± 2.00[a]
	25	153.03 ± 3.15[a]	155.97 ± 4.45[a]	161.53 ± 4.80[a]	162.01 ± 1.03[a]
	40	153.03 ± 3.15[a]	156.85 ± 2.40[ab]	162.17 ± 1.90[b]*	162.76 ± 1.67[b]*
2.0	4	161.67 ± 3.21[a]	163.80 ± 2.27[a]	165.78 ± 6.69[a]	167.68 ± 3.26[a]
	25	161.67 ± 3.21[a]	164.27 ± 5.13[a]	166.39 ± 1.74[a]	169.34 ± 6.50[a]
	40	161.67 ± 3.21[a]	166.52 ± 1.74[a]	167.49 ± 4.83[a]	173.69 ± 7.78[a]
4.0	4	165.85 ± 4.14[a]	169.38 ± 3.23[a]	171.18 ± 8.18[a]	205.83 ± 2.19[b]
	25	165.85 ± 4.14[a]	171.68 ± 7.14[a]	174.15 ± 3.33[a]	206.14 ± 4.32[b]
	40	165.85 ± 4.14[a]	172.12 ± 5.23[ab]	177.81 ± 5.63[b]	212.36 ± 2.73[c]

[a]The experimental data are expressed as mean ± standard error (n = 3). (a–d) Different letters indicate that there is a significant difference between the experimental groups of the same content and preservation temperature for each preservation day ($P < 0.05$), and * indicates that the experimental group of the same content and preservation day for each temperature is significantly different from the experimental group of 4 °C ($P < 0.05$).

increasing phospholipid–cholesterol ratio, which could be attributed to the fact that a moderate amount of cholesterol regulated the membrane fluidity and improved the stability of the liposomes. However, when the addition of cholesterol exceeded the saturation value of liposomes, the liposome membrane ruptured, resulting in a decrease in the EE. The phospholipid–cholesterol ratio should not be more than 10:1. Sodium cholate can improve the deformability of liposomes, thus improving liposome shaping and encapsulation. When the addition of sodium cholate exceeded a certain ratio, it increased the deformability of liposomes so that the stability of liposomes was reduced, decreasing the EE. The amount of sodium cholate should not exceed 50 mg so that the EE does not increase. The addition of isopropyl myristate also has a certain effect on the stability of liposomes, so its addition should not exceed 50 mg.

The optimal parameters obtained from the RSM were optimized as follows: *Zanthoxylum* alkylamides 15 mg, phospholipid–feedstock ratio 6.14, phospholipid–cholesterol ratio 8.51, sodium cholate 33.80 mg, and isopropyl myristate 29.49 mg, which could achieve a theoretical EE of 90.23%. This was further validated, and the EE was 90.12%, which was close to the theoretical value. This finding showed that the model had a good fit and the liposome preparation process through optimization was highly feasible.

2.2. Properties of *Zanthoxylum* Alkylamides Liposomes. *2.2.1. Stability.* When the theoretical content of the liposomes was 1 mg/mL, the content of *Zanthoxylum* alkylamides in the liposomes at different temperatures and different preservation times did not change much (Table 3). However, when the theoretical content was 4 mg/mL, the content of *Zanthoxylum* alkylamides in the liposomes changed significantly at a preservation temperature of more than 25 °C and the number of preservation days as more than 14. The particle sizes of liposomes with different theoretical contents in each group did not show large changes in the preservation temperature of 25 °C and the number of preservation days as 14 (Table 4). The aforementioned results showed that the *Zanthoxylum* alkylamides liposomes could be stably preserved for 14 days at a content of less than 2 mg/mL and a preservation temperature of less than 25 °C. The content in the *Zanthoxylum* alkylamides liposomes and the state of the liposomes were relatively stable during this period. This might be attributed to the fact that liposomes buried *Zanthoxylum* alkylamides in a hydrophobic layer, which effectively avoided the contact of *Zanthoxylum* alkylamides with the water phase and improved the stability.

2.2.2. Morphology, Particle Size, and ζ-Potential. The properties of liposomes are affected by not only their composition but also factors such as liposome size and surface charge.[27] The *Zanthoxylum* alkylamides liposomes were homogeneous milky white suspensions, whereas the *Zanthoxylum* alkylamides fluorescent liposomes were homogeneous yellow–green suspensions (Figure 3). Both liposome solutions

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were free from visible precipitates to the naked eye, indicating that the prepared liposome solutions had good stability.

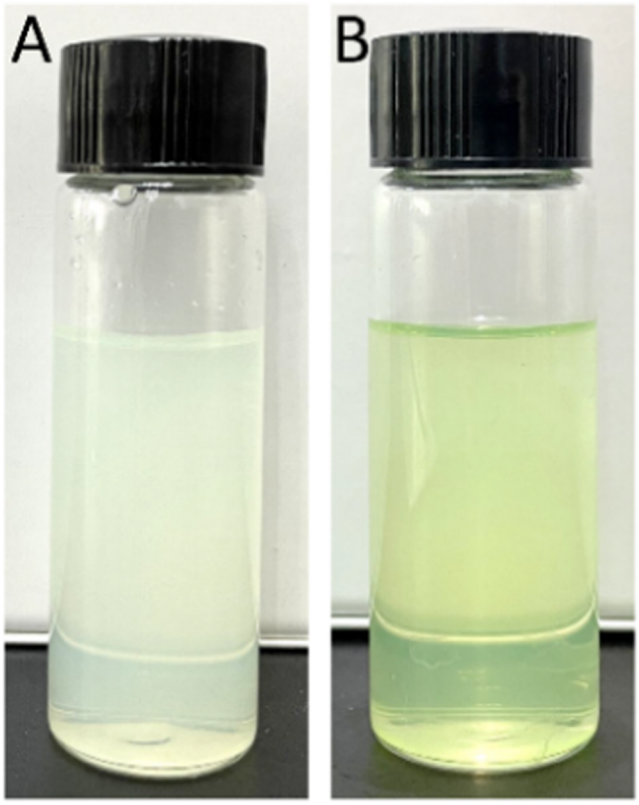

Figure 3. Appearance of liposomes [(A) *Zanthoxylum* alkylamides liposome and (B) *Zanthoxylum* alkylamides fluorescent liposome].

The microscopic morphology of the *Zanthoxylum* alkylamides liposomes was dispersed or aggregated and circular or elliptical, and the particle size was small (Figure 4).

The particle size of *Zanthoxylum* alkylamides liposomes was mainly about 100 nm, with an average size of 155.47 ± 3.16 nm, and the ζ-potential of the liposomes was −34.11 ± 4.34 mV (Figure 5). The ζ-potential is an important index for evaluating the stability of liposomes, and a ζ-potential of less than −30 mV or more than 30 mV indicates that the electrostatic force on the surface of the liposomes is relatively stable.[28] The results showed that the particle size distribution of liposomes was uniform, and the quality was relatively stable. Researches have shown that particle sizes of liposomes could affect the effectiveness of liposomes in oral drug delivery systems. Generally, liposomes with smaller particle sizes have better absorption effects than liposomes with larger particle sizes. For example, liposomes loaded with insulin could have better bioavailability with a particle size of 150 nm.[29] Therefore, the prepared *Zanthoxylum* alkylamides liposomes can meet the requirements of the corresponding indexes of liposomes in terms of both the particle size and ζ-potential.

2.3. Uptake Characteristics of *Zanthoxylum* Alkylamides Liposomes in Caco-2 Cells. *2.3.1. High-Performance Liquid Chromatography (HPLC) Chromatograms of Zanthoxylum Alkylamides.* The contents of the three sanshools in the *Zanthoxylum* alkylamides liposomes were determined by the established HPLC method (Figure 6). The three sanshools had a good separation, which could be used for subsequent experimental studies.

2.3.2. Effects of Zanthoxylum Alkylamides Liposomes on the Proliferation Rate of Caco-2 Cells. Under cell culture conditions, Caco-2 cells (human colon adenocarcinoma cells) form cell monolayers and spontaneously undergo epithelioid differentiation, resulting in tight intercellular junctions and microvillous structures, which are similar to the epithelial cells of the intestinal tract in terms of morphology and physiological functions and are widely used to mimic the study of the mechanism of the transport and absorption of substances in the small intestine.[30−32] The effect of liposomes with different concentrations on the proliferation rate of Caco-2 cells varied (Figure 7). The proliferation rate of Caco-2 cells was greater than 80% when the content of *Zanthoxylum* alkylamides in liposomes was 25 and 50 μg/mL. In contrast, the proliferation rate of Caco-2 cells significantly reduced when the content of *Zanthoxylum* alkylamides liposomes was 100 and 200 μg/mL, with both concentrations resulting in rates lower than 50%. Therefore, the content of *Zanthoxylum* alkylamides in liposomes should not exceed 50 μg/mL in the subsequent study to ensure that the cells were in good condition in the experiment.

2.3.3. Effects of Zanthoxylum Alkylamides Liposomes on the Growth Morphology of Caco-2 Cells. Normally growing Caco-2 cells had distinct cell morphology and good growth density (Figure 8). The cell morphology gradually changed with the increase in the content of *Zanthoxylum* alkylamides in liposomes. When the content of *Zanthoxylum* alkylamides in liposomes was 50 μg/mL, the cells still had a certain morphology and growth density. However, when the content of *Zanthoxylum* alkylamides in liposomes reached 100 μg/mL, the cells had more rounded shapes and the cell density decreased accordingly. Therefore, combined with the results of the cell proliferation rate test, the content of *Zanthoxylum* alkylamides in liposomes did not exceed 50 μg/mL in the subsequent test.

2.3.4. Uptake of Zanthoxylum Alkylamides Liposomes in Caco-2 Cells. The normally growing Caco-2 cells in the cell

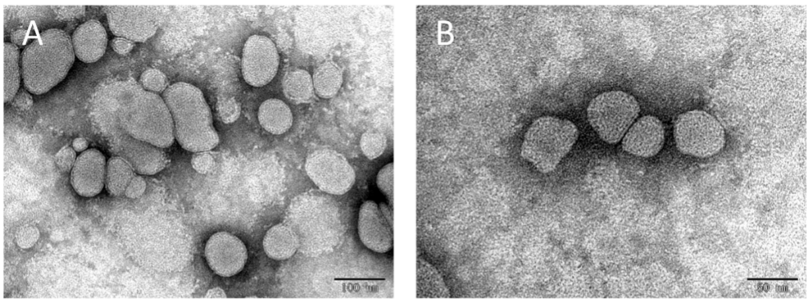

Figure 4. Microscopic morphology of liposomes [(A)100 nm and (B) 50 nm].

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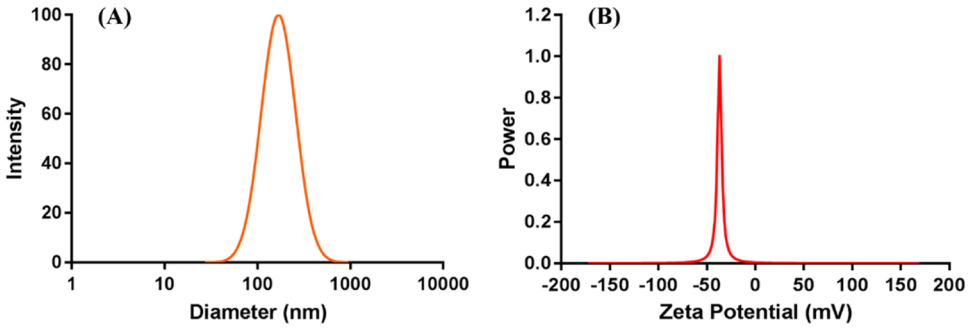

Figure 5. Size distribution and ζ-potential of liposomes [(A) size distribution and (B) ζ-potential].

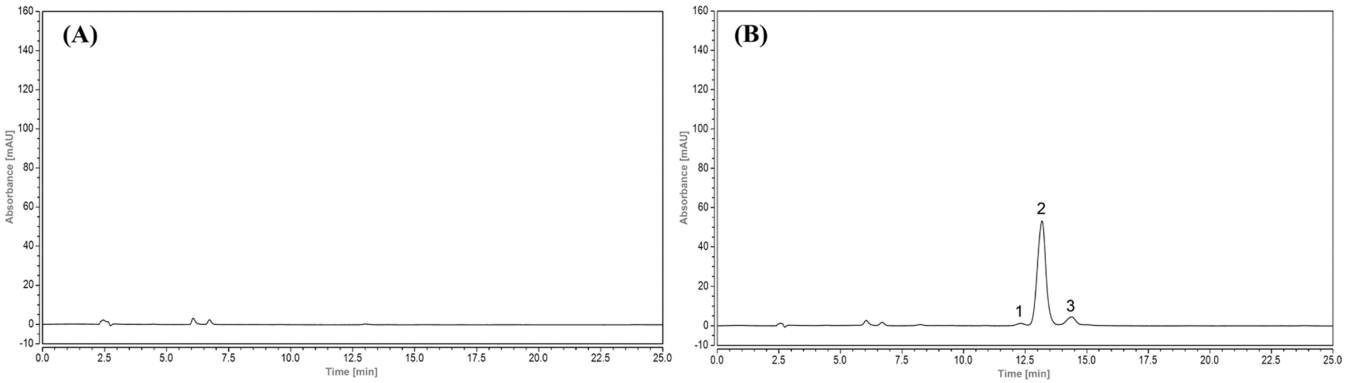

Figure 6. Liquid chromatogram of liposomes of *Zanthoxylum* alkylamides [(A) blank solution; (B) blank standard addition; (1) hydroxy-ε-sanshool; (2) hydroxy-α-sanshool; (3) hydroxy-β-sanshool].

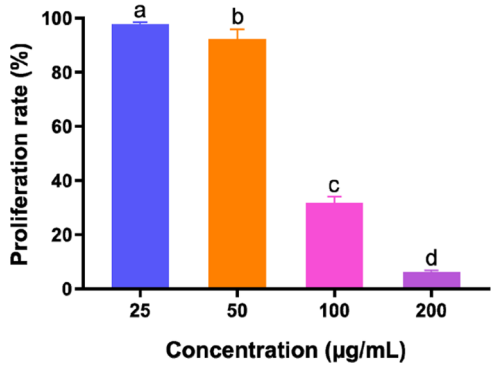

Figure 7. Effect of *Zanthoxylum* alkylamides liposomes on Caco-2 cell viability. 25−200 μg/mL represents the liposome groups of different concentration of *Zanthoxylum* alkylamides, and the letters (a−d) indicate significant differences between the groups ($P < 0.05$).

culture plate on the second day of observation were not covered by the field of view (Figure 9). The density of the cells gradually increased with the increase in the culture time. On the 10th day, the Caco-2 cells were almost completely covered by the field of view and showed the characteristics of the "paving stone" morphology of Caco-2 cells. Caco-2 cells cultured to the 14th day completely filled the bottom of the culture wells. A dense monolayer of the membrane thus formed could be used for the study on the uptake of liposomes.

The fluorescence intensity in the field of view increased with the increase in incubation time when the content of *Zanthoxylum* alkylamides in the liposomes was 50 μg/mL; the difference in fluorescence intensity between 90 and 120 min was not significant (Figure 10). The aforementioned results indicated that Caco-2 cells could bind to the *Zanthoxylum* alkylamides liposomes, which provided a theoretical basis for the subsequent uptake and transport studies.

After adding liposome solution with the content of *Zanthoxylum* alkylamides of 20 μg/mL, the uptake rate at 30 min of incubation significantly increased compared with the initial uptake rate (Figure 11). With the increase in the incubation time, the highest uptake rate was observed in 90 min, the uptake rate slightly decreased beyond 90 min, and the difference in the uptake rate at the incubation time of 90 and 120 min was not significant. Therefore, the uptake of liposomes by Caco-2 cells was examined after 90 min in subsequent studies.

The uptake of liposomes by Caco-2 cells gradually increased with the increase in the content of *Zanthoxylum* alkylamides liposomes (Figure 12). The uptake rate was significantly higher at 50 μg/mL *Zanthoxylum* alkylamides liposomes than at 30 μg/mL *Zanthoxylum* alkylamides liposomes. In the subsequent uptake and absorption studies, the content of *Zanthoxylum* alkylamides liposomes was 50 μg/mL.

P-Glycoprotein, an efflux protein, is involved in the absorption of orally administered components mainly in the small intestine, where it can reduce substrate uptake by binding to the substrate and excreting it from the cell plasma out of the cell. Verapamil was added as an exocytosis inhibitor to examine the effect of cellular exocytosis on uptake. The difference in the uptake rates of liposomal solutions of *Zanthoxylum* alkylamides with different contents before and after the addition of verapamil was not significant in the uptake study, suggesting that exocytosis had no significant effect on the uptake rate of *Zanthoxylum* alkylamides liposomes (Figure 13).

2.3.5. Absorption of Zanthoxylum Alkylamides Liposomes in Caco-2 Cells. An RE1600 epithelial cell resistivity meter was used to determine the transepithelial electrical resistance (TEER) value of the apical (AP) and basolateral (BL) sides of

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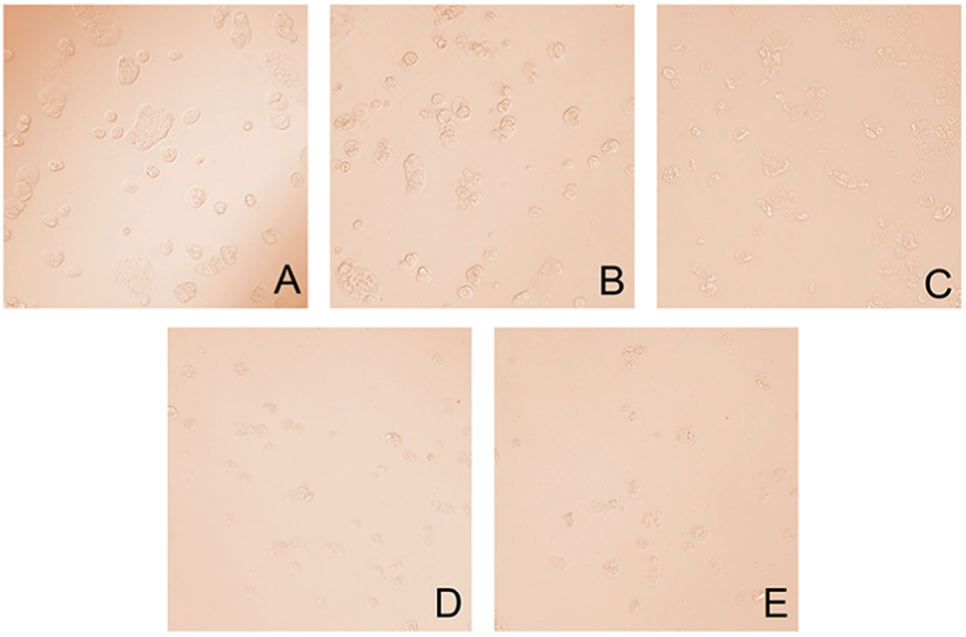

Figure 8. Effect of *Zanthoxylum* alkylamides liposomes on Caco-2 cell morphology [(A) blank group; (B) 25 μg/mL *Zanthoxylum* alkylamides liposome group; (C) 50 μg/mL *Zanthoxylum* alkylamides liposome group; (D) 100 μg/mL *Zanthoxylum* alkylamides liposome group; (E) 200 μg/mL *Zanthoxylum* alkylamides liposome group].

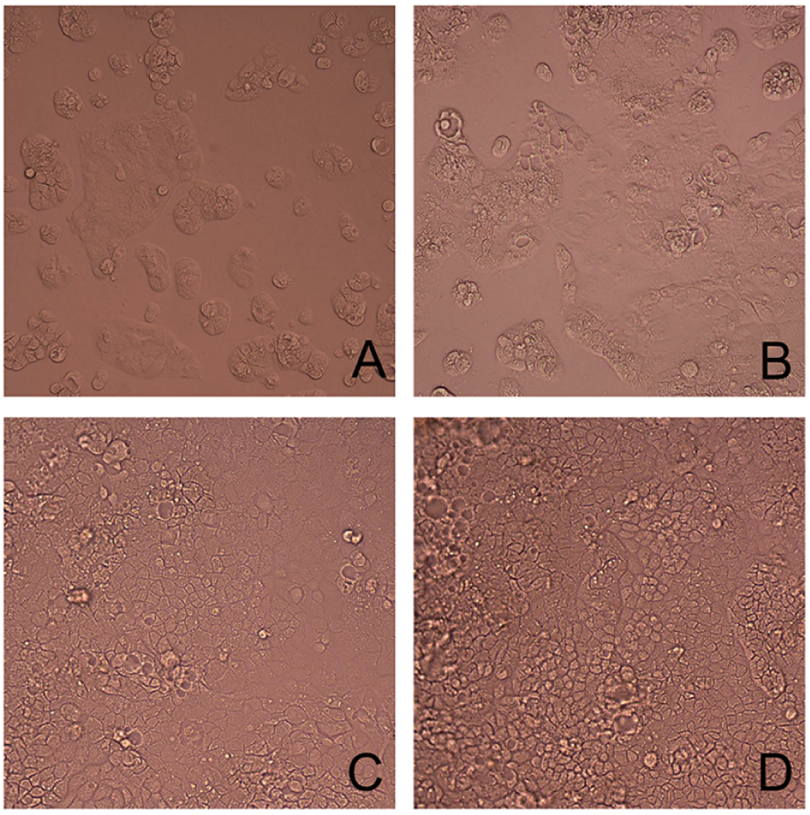

Figure 9. Morphology of Caco-2 cells cultured for 14 days [(A) 2 days, (B) 6 days, (C) 10 days, (D) 14 days].

Caco-2 cells in the Transwell. The cell TEER value on the Transwell increased with the increase in Caco-2 cell culture time (Figure 14). The TEER value was measured to be 395.39 ± 16.40 $\Omega \cdot cm^2$ after 21 days of culture. Normally, when the TEER value of Caco-2 cells exceeded 300 $\Omega \cdot cm^2$, it indicated that the cell growth could meet the monolayer membrane requirement for the test, and the subsequent uptake and transport experiment could be carried out.

The alkaline phosphatase ratio on the AP and BL sides could be used, besides the cell TEER value as described previously, to evaluate whether the Caco-2 monolayer cell model was successfully established. The alkaline phosphatase ratio of

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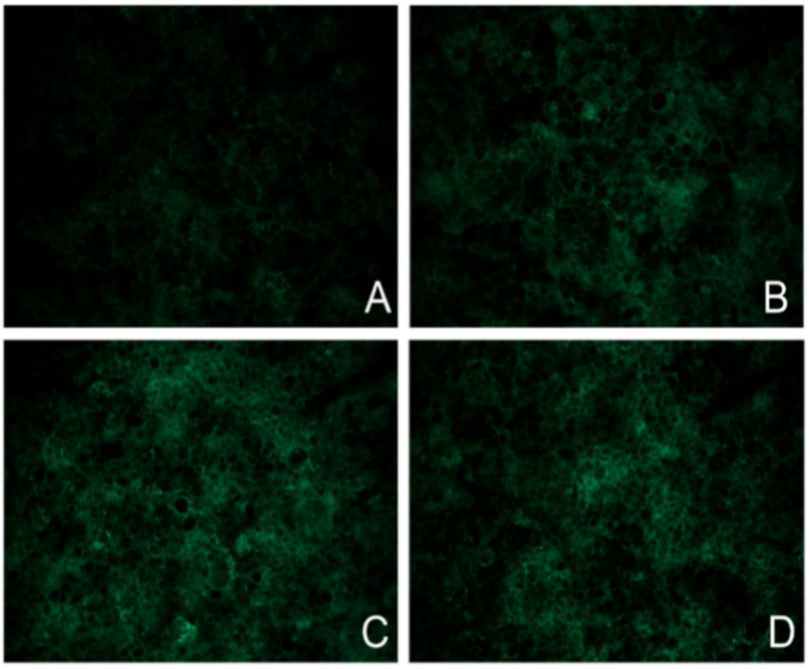

Figure 10. Binding of Caco-2 cells to fluorescent liposomes of *Zanthoxylum* alkylamides [(A) 30 min; (B) 60 min; (C) 90 min; (D) 120 min].

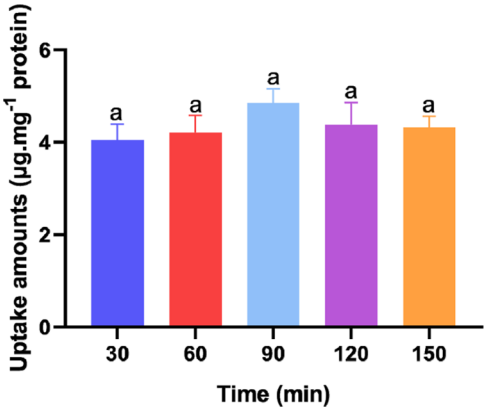

Figure 11. Effects of incubation time of liposome solution on the uptake of Caco-2 cells.

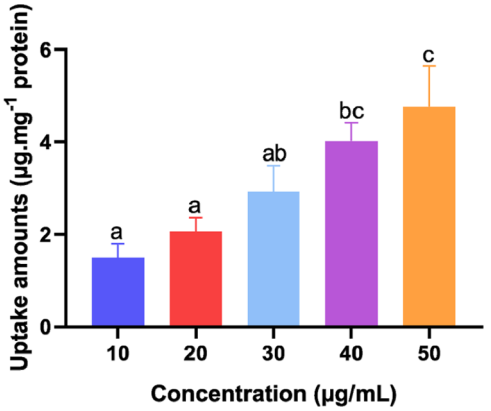

Figure 12. Effect of *Zanthoxylum* alkylamides content in liposomes on the uptake of Caco-2 cells.

Caco-2 cells cultured for 3 days in Transwell chambers was 1.33 ± 0.11, whereas the alkaline phosphatase ratio of Caco-2 cells cultured for 21 days was 4.25 ± 0.18 (Figure 15). This result

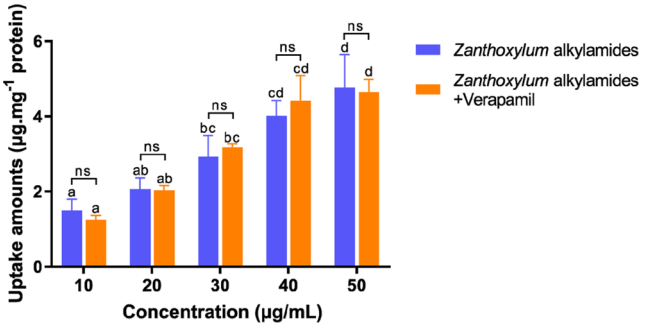

Figure 13. Effect of verapamil on the uptake of liposomes of Caco-2 cells.

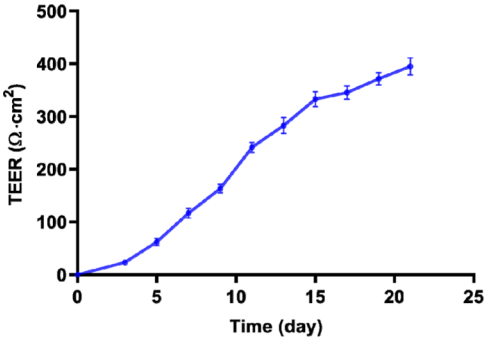

Figure 14. TEER value of the Caco-2 monolayer cell model.

indicated that polarization occurred on both sides of the membrane of the Caco-2 monolayer and could be used in the uptake transporter assay.

Generally, a P_{app} value greater than 1.0×10^{-5} cm/s indicated that the substance could be completely absorbed; a P_{app} value between 0.1×10^{-6} and 1.0×10^{-6} cm/s indicated that the absorption rate of the substance was 1−100%; and a P_{app} value $\leq 1.0 \times 10^{-7}$ cm/s indicated that the substance could not be absorbed. Further, the P_{app} ratio $P_{app(AP \rightarrow BL)}/P_{app(BL \rightarrow AP)}$ of the

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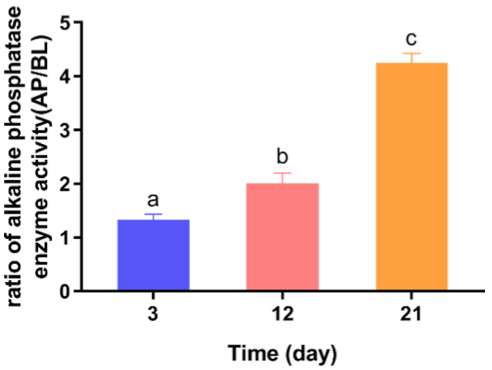

Figure 15. Ratio of alkaline phosphatase.

AP side to the BL side between 0.5 and 2.0 indicated that the absorption mechanism of the substance transport was mainly passive transport.[33] The $P_{app(AP-BL)}$ and $P_{app(BL-AP)}$ of *Zanthoxylum* alkylamides were lower than those of *Zanthoxylum* alkylamides liposomes in the Caco-2 monolayer cell model (Table 5). This suggested that the preparation of *Zanthoxylum* alkylamides into liposomes improved its absorption. Further, the values of $P_{(AP-BL)}/P_{(BL-AP)}$ for both the *Zanthoxylum* alkylamides and the *Zanthoxylum* alkylamides liposomes were less than 1.5 in the Caco-2 monolayer cell model, indicating that the absorption of *Zanthoxylum* alkylamides and its liposomes was consistent with passive absorption.

Table 5. Permeability Coefficient of *Zanthoxylum* Alkylamides Liposomes

	$P_{app(AP-BL)}$ ($\times 10^{-6}$ cm/s)	$P_{app(BL-AP)}$ ($\times 10^{-6}$ cm/s)	$P_{(AP-BL)}/P_{(BL-AP)}$
Zanthoxylum alkylamides	1.14 ± 0.24	1.00 ± 0.24	1.14
Zanthoxylum alkylamides liposomes	2.00 ± 0.66	1.43 ± 0.25	1.40

3. CONCLUSIONS

In this study, the Box−Behnken RSM method was applied to investigate the preparation process of *Zanthoxylum* alkylamides liposomes using *Zanthoxylum* alkylamides as the raw material. The optimal parameters were as follows: *Zanthoxylum* alkylamides of 15 mg, phospholipid−raw material ratio of 6.14, phospholipid−cholesterol ratio of 8.51, sodium cholate of 33.80 mg, and isopropyl myristate of 29.49 mg, which could achieve a theoretical EE of 90.23%. The fit of the model was good.

The stability, particle size, ζ-potential, and other indexes of the *Zanthoxylum* alkylamides liposomes were evaluated. The study showed that the prepared liposomes could be preserved for 14 days with the content of *Zanthoxylum* alkylamides less than 2 mg/mL. Also, the preservation temperature was lower than 25 °C, and their particle size of 155.47 ± 3.16 nm and ζ-potential of −34.11 ± 4.34 mV could satisfy the requirements.

The uptake properties of *Zanthoxylum* alkylamides liposomes were investigated in the Caco-2 cells. The results showed that the *Zanthoxylum* alkylamides liposomes were taken up and absorbed by the Caco-2 cells and had a better uptake performance compared with that of the unembedded *Zanthoxylum* alkylamides and conformed to the passive uptake.

4. METHODS

4.1. Chemicals and Experimental Materials. All chemicals were purchased at the highest purity available and used without further modification. *Zanthoxylum* oleoresin was purchased from Xuemailong Food Spice Co. Ltd. (Zhengzhou, China). Soy lecithin was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Sodium cholate and coumarin 6 were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Cholesterol and isopropyl myristate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA). Fetal bovine serum (FBS) was purchased from ExCell Bio (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). The Caco-2 cell line was purchased from the Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences (Shanghai, China).

4.2. Isolation and Purification of *Zanthoxylum* Alkylamides. The crude extract of *Zanthoxylum* alkylamides was obtained through the extraction of *Zanthoxylum* oleoresin by leaching with organic solvents, and then isolated and identified in our previous study.[34−36] Also, the purity of the *Zanthoxylum* alkylamides was high, as detected using the area normalization method. The content of three sanshools was 5.9, 87.2, and 6.9%, respectively. Figure 16 indicated that the purified *Zanthoxylum* alkylamides was mainly composed of hydroxy-ε-sanshool, hydroxy-α-sanshool, and hydroxy-β-sanshool. Meanwhile, the molecular weights of three sanshools were all 263, and they were isomers of each other.

4.3. Preparation of *Zanthoxylum* Alkylamides Liposomes. An appropriate amount of soybean phospholipid was weighed in a 100 mL round-bottom flask, mixed with 20 mL of anhydrous ethanol, and ultrasonicated at 40 °C until it was clarified and transparent. Then, cholesterol, sodium cholate, and isopropyl myristate were added in sequence, and the mixture was ultrasonicated until it was clarified. An appropriate amount of *Zanthoxylum* alkylamides was dissolved in anhydrous ethanol, added to the round-bottom flask mentioned earlier, and ultrasonicated at 40 °C for 15 min. The round-bottom flask was placed on the rotary evaporator, and the anhydrous ethanol was removed until a film was formed on the inside of the flask. An appropriate amount of ultrapure water was added at 60 °C, and the round-bottom flask was shaken gently to remove the film from the wall and mixed to form a milky white solution. Next, the round-bottomed flask was placed in a 50 °C water bath and

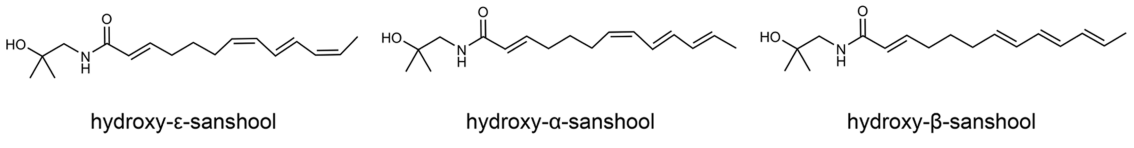

Figure 16. Chemical structures of hydroxy-ε-sanshool, hydroxy-α-sanshool, and hydroxy-β-sanshool.

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subjected to magnetic stirring for 20 min. While still hot, the solution was filtered with a 0.2 μm microporous filter membrane three times to obtain the liposome solution of *Zanthoxylum* alkylamides.[37]

4.4. Encapsulation Efficiency (EE) of *Zanthoxylum* Alkylamides Liposomes. A liposome solution of *Zanthoxylum* alkylamides was prepared at a theoretical concentration of 1 mg/mL, and 1 mL of the liposome solution was aspirated and filtered through a 0.22 μm microporous membrane. Then, 0.1 mL of the filtrate was emulsified with anhydrous ethanol and fixed to a volume of 10 mL.[38] The upper layer of the solution was aspirated and filtered through the 0.22 μm microporous membrane and then analyzed using Thermo Fisher UltiMate 3000 HPLC system (Thermo Fisher Scientific, Inc., Waltham, MA). The samples were analyzed on a Thermo Fisher Accucore C18 column (4.6 × 150 mm^2, 2.6 μm) (Thermo Fisher Scientific, Inc., Waltham, MA) at 35 °C with a mobile phase flow rate of 0.5 mL/min. A gradient mobile phase composed of methanol (A) and water (B) was used for the HPLC. The linear gradient program of phase A started from 70%, increased to 90% from 0 to 12 min, and further increased to 100% when the time was 15 min. The detection wavelength was 254 nm.

The EE of the liposomes of *Zanthoxylum* alkylamides was calculated using the following equation:

$$EE(\%) = C_1/C_2 \times 100\%$$

where C_1 refers to the content of *Zanthoxylum* alkylamides in the liposomes after filtration using a microporous filtration membrane and C_2 refers to the added amount of *Zanthoxylum* alkylamides in the formulation.

4.5. Optimization Preparation of *Zanthoxylum* Alkylamides Liposomes. The process optimization of *Zanthoxylum* alkylamides liposomes was carried out with the Box–Behnken RSM method,[39] using EE as the evaluation index. The effects of factors such as phospholipid–feedstock ratio (A), phospholipid–cholesterol ratio (B), sodium cholate dosage (C), and isopropyl myristate dosage (D) on the EE of liposomes were examined, and the process of liposome preparation was optimized. The Box–Behnken test factors and levels are depicted in Table 6.

Table 6. Box–Behnken Test Factors and Levels

	the level of factors		
factors	−1	0	1
phospholipid–feedstock ratio	5	6	7
phospholipid–cholesterol ratio	6	8	10
sodium cholate (mg)	10	30	50
isopropyl myristate (mg)	5	25	45

4.6. Characterization of *Zanthoxylum* Alkylamides Liposomes. *4.6.1. Stability.* The liposomal solutions of *Zanthoxylum* alkylamides with theoretical contents of 1, 2, and 4 mg/mL were prepared. The aforementioned samples were placed at 4, 25, and 40 °C, respectively, for 21 days, sampled at 7-day intervals, and filtered through a 0.8 μm microporous filter membrane to determine the *Zanthoxylum* alkylamides content and average particle size.[40]

4.6.2. Morphology, Particle Size, and ζ-Potential. The *Zanthoxylum* alkylamides liposome solution with a theoretical content of 1 mg/mL and a fluorescent liposome solution of *Zanthoxylum* alkylamides containing 0.1% coumarin 6 were prepared, and the properties of the liposome solutions were observed.

The *Zanthoxylum* alkylamides liposome solution with a theoretical composition of 1 mg/mL was prepared, and a portion of the liposome solution was diluted with an appropriate amount of ultrapure water. The ζ-potential and particle size distribution of the liposome samples were determined using a ζ-potential and particle size analyzer.[41] A separate portion of the liposome solution, diluted with an appropriate amount of ultrapure water, was dripped onto a copper mesh. It was allowed to dry at room temperature, and then the liposome samples were stained by adding one drop of 2% phosphotungstic acid solution. After drying at room temperature, the microscopic morphology of liposomes was observed under a JEM-1400 plus transmission electron microscope (TEM) (Japan Electronics Co., Ltd. Tokyo, Japan).[42]

4.7. Characterization of the Uptake of *Zanthoxylum* Alkylamides Liposomes in Caco-2 Cells. *4.7.1. Caco-2 Cell Culture.* Caco-2 cells were cultured in DMEM high-sugar culture medium containing 10% FBS, 1% double antibiotic (penicillin and streptomycin), 1% nonessential amino acids, and 1% glutamine, and passaged once in 2–3 days.[43]

4.7.2. Effect of Zanthoxylum Alkylamides Liposomes on the Proliferation Rate of Caco-2 Cells. Caco-2 cells with good growth status were adjusted to 8 × 10^4 cells/mL suspension, inoculated uniformly in 96-well cell culture plates at a volume of 100 μL per well, and cultured at 37 °C in a 311 CO_2 incubator (Thermo Fisher Scientific, Inc., Waltham, MA) for 24 h. Then, the culture medium was aspirated, the cells were washed with phosphate-buffered saline (PBS) solution two times, and the cell culture medium containing liposomes with different contents of 0, 25, 50, 100, and 200 μg/mL of *Zanthoxylum* alkylamides was added to each well. After 6 h of culture, 100 μL of the culture medium was aspirated, the cells were washed with PBS solution three times, and the cell culture medium containing 10% Cell Counting Kit 8 (CCK-8) solution was added to determine the cell proliferation rate.

4.7.3. Effects of Zanthoxylum Alkylamides Liposomes on the Growth Morphology of Caco-2 Cells. Caco-2 cells with good growth status were adjusted to a cell suspension of 8 × 10^4 cells/mL, inoculated uniformly at a volume of 1 mL per well in 24-well cell culture plates, and cultured at 37 °C for 24 h. Then, the culture medium was aspirated, the cells were washed twice with PBS, and the cell culture medium containing liposomes with different contents of 0, 25, 50, 100, and 200 μg/mL of *Zanthoxylum* alkylamides was added into each well. After 6 h of culture, the morphology of the cells was observed under an IX53 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

4.7.4. Uptake of Zanthoxylum Alkylamides Liposomes in Caco-2 Cells. Caco-2 cells in good growth conditions were adjusted to a density of 4 × 10^4 cells/mL, and 2 mL of the cell suspension was added to each well of the 12-well cell culture plates. After being cultured at 37 °C for 24 h, the culture medium was aspirated and washed with PBS two times before adding a new culture medium to continue the culture. The fluid was changed every other day in the first week of the Caco-2 cell culture and every day in the second week. The cell morphology was observed under the inverted microscope on days 2, 6, 10, and 14 of the Caco-2 cell culture. After 14 days of Caco-2 cell culture, a Hank's balanced salt solution (HBSS) of liposomes of *Zanthoxylum* alkylamides containing fluorescent dye coumarin 6 at a concentration of 50 μg/mL was added, incubated in a cell

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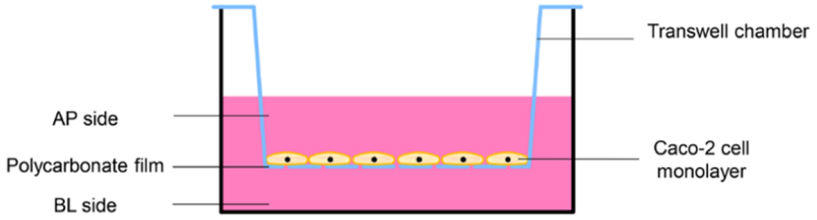

Figure 17. Schematic of the tanswell.

culture chamber at 37 °C for 30, 60, 90, and 120 min, and observed under an inverted fluorescence microscope.

Caco-2 cells cultured for 14 days were washed three times with HBSS. Then, 1 mL of liposomal HBSS solution of *Zanthoxylum* alkylamides (containing 0.5% DMSO) with a content of 20 μg/mL was added, placed in a cell culture incubator at 37 °C, and incubated for 0, 30, 60, 90, 120, and 150 min to examine the effect of the incubation time of liposomal solution on the Caco-2 cells. Meanwhile, Caco-2 cells cultured for 14 days were washed three times with HBSS, and then 1 mL of liposomal HBSS solution of *Zanthoxylum* alkylamides (containing 0.5% DMSO) with the contents of 0, 10, 20, 30, 40, and 50 μg/mL was added. And then, the cells were incubated at 37 °C for 90 min to investigate the effect of the concentration of the liposomal solution on the uptake of Caco-2 cells. Moreover, the liposomal HBSS solution (containing 0.5% DMSO) of *Zanthoxylum* alkylamides was prepared at concentrations of 0, 10, 20, 30, 40, and 50 μg/mL, separately. Verapamil, a P-glycoprotein inhibitor, was added at a final concentration of 100 μmol/L to obtain the sample solution. And then, 1 mL of verapamil-containing HBSS solution of *Zanthoxylum* alkylamides liposomes at different concentrations were added in the Caco-2 cells which have been cultured for 14 days, and incubated at 37 °C for 90 min to examine the effect of verapamil on the uptake of Caco-2 cells.

At the end of the aforementioned incubation of Caco-2 cells, the cells were washed twice with HBSS solution precooled to 4 °C. Further, 2 mL of HBSS solution was added to each well. The cells in the wells were collected with a cell scraper, transferred to 10 mL centrifuge tubes, and placed on ice. The cells were lysed with a SCIENTZ-IID ultrasonic cell crusher (Scientz Biotechnology Co., Ltd. Ningbo, China) under an ultrasonic power of 300 W, 2 s of ultrasonication, and 1 s of pause for 5 min to obtain the cell suspension. The cell suspension was divided into two portions. One portion was used to determine the protein content of the cell suspension by the bicinchoninic acid (BCA) assay method. The other portion was mixed with acetonitrile solution and centrifuged at 12,000 rpm for 15 min at 4 °C to precipitate the protein. The supernatant was aspirated and filtered through a 0.22 μm micropore filter membrane. Then, the samples were analyzed by HPLC using Thermo Scientific UltiMate 3000 HPLC system with a Thermo Fisher Accucore C18 column (4.6 × 150 mm^2, 2.6 μm packing material) at 35 °C. Acetonitrile was used as the mobile phase A, and a mixture of 0.5% acetic acid and water was used as the mobile phase B. The elution was performed for 25 min in a 40:60 equilibrium elution. The flow rate of the mobile phase was 0.5 mL/min, the wavelength was 254 nm, and the injection volume was 5 μL. The uptake of *Zanthoxylum* alkylamides in Caco-2 cells was expressed as μg (*Zanthoxylum* alkylamides)/mg (cell protein).[44]

4.7.5. Absorption of Zanthoxylum Alkylamides Liposomes in Caco-2 Cells. Caco-2 cells in good growth condition and logarithmic phase were taken and digested with trypsin. The concentration of cell suspension was adjusted to 2 × 10^5 cells/mL. Then, 1.5 mL of cell suspension was added to the apical (AP) side of the six-well plate Transwell chambers (Figure 17), followed by 2.6 mL of DMEM high-glucose culture medium containing 10% FBS. The cell culture plates with Transwell chambers were cultured in a 37 °C, 5% CO_2 cell culture incubator. The cell culture medium was changed every 48 h in the first week and every 24 h after 1 week for 21 days.[45,46]

During the establishment of the Caco-2 monolayer cell model, the cell transepithelial electrical resistance values were measured with a RE1600 Digital conductivity meter (Jingong Hongtai Technology co., Ltd. Beijing, China) on days 0, 3, 6, 9, 12, 15, 18, and 21. The transepithelial electrical resistance (TEER) value of the Caco-2 monolayer cell model was calculated as follows:

$$\mathrm{TEER} = (\mathrm{TEER}_{\text{assay wells}} - \mathrm{TEER}_{\text{blank wells}}) \times S_0$$

In this equation, TEER value is expressed in Ω-cm^2 and S_0 is the Transwell single-well membrane area of 4.67 cm^2.

The cell cultures of the AP and BL sides on days 3, 12, and 21 after cell inoculation in Transwell chambers were collected. The alkaline phosphatase activity in the cell cultures of the two sides was detected using the Alkaline Phosphatase Kit (Beyotime Biotech. Inc. Shanghai, China), and the ratio of the alkaline phosphatase activity in the cell cultures of the two sides was calculated.

Caco-2 cell plates (21 days of culture, TEER value >300 Ω·cm^2), which had been confluent into monolayers and were successfully modeled, were selected, and the cell culture medium was aspirated from the two sides.[47] Next, 1.5 and 2.6 mL of HBSS solution was added to the AP and BL sides, respectively, the cell culture medium was incubated in the incubator for 30 min, and the two sides were aspirated off. The outside and inside of the Transwell chamber were washed with HBSS solution three times.

AP → BL direction transfer: The HBSS solution on the AP and BL sides was aspirated, and 1.5 mL of liposomal HBSS solution containing 50 μg/mL *Zanthoxylum* alkylamides was added to the AP side of the Transwell cell culture chambers as the supply pool. Further, 2.6 mL of the blank HBSS solution was added to the BL side as the receiving pool. The cell culture plate with Transwell was incubated in an IS-RDV1 air-bath thermostatic oscillator (STIK Instrument Equipment Co., Ltd. Shanghai, China) at a temperature of 37 °C and a rotational speed of 50 rpm, and 600 μL of samples were taken from the BL side at the 30th, 60th, 90th, 120th, 150th, and 180th min for subsequent analytical assays. Next, 600 μL of blank HBSS solution prewarmed to 37 °C was added for replenishment. Meanwhile, 600 μL of liposomal HBSS solution was added to the BL side as a receiving pool at a concentration of 50 μg/mL and used as a control. The aforementioned test procedure was repeated.

BL → AP direction transfer: The HBSS solution on the AP and BL sides was aspirated, and 2.6 mL of liposomal HBSS

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solution containing 50 μg/mL of *Zanthoxylum* alkylamides was added to the BL side of the cell culture plate as the supplying pool. Also, 1.5 mL of blank HBSS solution was added to the AP side as the receiving pool. The cell culture plate with Transwell was incubated in an air-bath thermostatic oscillator at 37 °C and a rotational speed of 50 rpm. Further, 400 μL was sampled from the AP side at the 30th, 60th, 90th, 120th, 150th, and 180th min for subsequent analytical assays. Next, 400 μL of blank HBSS solution prewarmed to 37 °C was added for replenishment. Meanwhile, 400 μL of liposomal HBSS solution was added to the AP side at a concentration of 50 μg/mL and used as a control. The aforementioned experimental procedure was repeated.

The sample solutions taken out at each time point were mixed with acetonitrile to precipitate the proteins and then centrifuged at 4 °C and 12,000 rpm for 15 min. An appropriate amount of the supernatant was aspirated, and the content of *Zanthoxylum* alkylamides was determined by HPLC. The apparent permeability coefficients (P_{app}) were calculated as follows:[48]

$$P_{app} = (dQ/dt)/(A \cdot c_0)$$

where dQ/dt denotes the rate of appearance of *Zanthoxylum* alkylamides at the receiving end, c_0 denotes the initial concentration of *Zanthoxylum* alkylamides in the supply cell, and A is the membrane area of the Transwell (4.67 cm^2).

4.8. Statistical Analysis. Unless otherwise stated, the data were obtained from three parallel determinations, and the test results were expressed as mean ± standard deviation. Duncan comparisons were made between multiple groups using one-way analysis of variance (ANOVA), and a P value < 0.05 indicated a statistically significant difference. The experimental data were plotted and analyzed using GraphPad Prism 8 after preliminary statistics in Excel. Graphical editing was done using GraphPad Prism 8 and Microsoft PowerPoint 2016.

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Author Contributions

R.W. and X.L. conceived and designed the research; R.W., C.R., and Q.L. performed the experiments; R.W. analyzed data and wrote the original manuscript; and X.L. supervised the study. All authors discussed the results, edited the manuscript, and gave approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Liu, Y. Y.; Shen, X.; Zhang, Y.; Cao, W.; Wang, K.; Xu, S. Z.; Wang, S. W. Analysis of chemical constituents of volatiles and petroleum ether extract from the branch of *Zanthoxylum bungeanum* by GC-MS. *Asian J. Chem.* **2014**, *26*, 3863−3867.

(2) Wansi, J. D.; Tcho, A. T.; Toze, F. A. A.; Nahar, L.; Martin, C.; Sarker, S. D. Cytotoxic acridone and indoloquinazoline alkaloids from *Zanthoxylum poggei*. *Phytochem. Lett.* **2016**, *17*, 293−298.

(3) Ombito, J. O. Phytochemistry and pharmacology of the genus *Zanthoxylum* (Rutaceae): a review. *Nat. Prod. J.* **2021**, *11*, 21−43.

(4) Shu, J.; Zhang, X. Y.; Song, S.; Fan, J. P. Research progress on exiraction and application of *Zanthoxylum bungeanum* essential oil. *China Condiment* **2023**, *48*, 203−207.

(5) Albin, K. C.; Simons, C. T. Psychophysical evaluation of a sanshool derivative (alkylamide) and the elucidation of mechanisms subserving tingle. *PLoS One* **2010**, *5*, No. e9520.

(6) You, Y. M.; Zhou, M.; Wang, Q. Q.; Ren, T.; Liu, X. Antioxidant activity *in vitro* of sanshool from Sichuan peppers (*Zanthoxylum bungeanum*). *Food Sci.* **2015**, *36*, 27−31.

(7) Tsunozaki, M.; Lennertz, R. C.; Vilceanu, D.; Katta, S.; Stucky, C. L.; Bautista, D. M. A 'toothache tree' alkylamide inhibits Aδ mechanonociceptors to alleviate mechanical pain. *J. Physiol.* **2013**, *591*, 3325−3340.

(8) Chung, Y. C.; Chien, C. T.; Teng, K. Y.; Chou, S. T. Antioxidative and mutagenic properties of *Zanthoxylum ailanthoides* Sieb & zucc. *Food Chem.* **2006**, *97*, 418−425.

(9) Wang, R.; You, Y. M.; Liu, X. Effect of *Zanthoxylum* alkylamides on glucose metabolism in streptozotocin-induced diabetic sprague-dawley rats. *Appl. Biol. Chem.* **2021**, *64*, 11.

(10) You, Y. M.; Ren, T.; Zhang, S. Q.; Huang, K.; Liu, X. Effect of *Zanthoxylum* alkylamide on intestinal microbiome in diabetic rats. *Acta Nutr. Sin.* **2017**, *39*, 170−176.

(11) Vera Candioti, L.; De Zan, M. M.; Camara, M. S.; Goicoechea, H. C. Experimental design and multiple response optimization. Using the desirability function in analytical methods development. *Talanta* **2014**, *124*, 123−138.

(12) Mora-Huertas, C. E.; Fessi, H.; Elaissari, A. Polymer-based nanocapsules for drug delivery. *Int. J. Pharm.* **2010**, *385*, 113−142.

(13) Andrade, S.; Loureiro, J. A.; Pereira, M. D. C. Influence of *in vitro* neuronal membranes on the anti-amyloidogenic activity of gallic acid: implication for the therapy of Alzheimer's disease. *Arch. Biochem. Biophys.* **2021**, *711*, No. 109022, DOI: 10.1016/j.abb.2021.109022.

(14) Guimarães, D.; Cavaco-Paulo, A.; Nogueira, E. Design of liposomes as drug delivery system for therapeutic applications. *Int. J. Pharm.* **2021**, *601*, No. 120571, DOI: 10.1016/j.ijpharm.2021.120571.

(15) Beltrán-Gracia, E.; Lopez-Camacho, A.; Higuera-Ciapara, I.; Velazquez-Fernandez, J. B.; Vallejo-Cardona, A. A. Nanomedicine review: Clinical developments in liposomal applications. *Cancer Nanotechnol.* **2019**, *10*, No. 11, DOI: 10.1186/s12645-019-0055-y.

(16) Bouchemal, K.; Briancon, S.; Perrier, E.; Fessi, H. Nano-emulsion formulation using spontaneous emulsification: Solvent, oil and surfactant optimization. *Int. J. Pharm.* **2004**, *280*, 241−251.

(17) Mirhosseini, H.; Tan, C. P.; Hamid, N. S. A.; Yusof, S. Optimization of the contents of Arabic gum, xanthan gum and orange oil affecting turbidity, average particle size, polydispersity index and

density in orange beverage emulsion. *Food Hydrocolloids* **2008**, *22*, 1212−1223.
(18) Iwabu, J.; Watanabe, J.; Hirakura, K.; Ozaki, Y.; Hanazaki, K. Profiling of the compounds absorbed in human plasma and urine after oral administration of a traditional japanese (Kampo) medicine, Daikenchuto. *Drug Metab. Dispos.* **2010**, *38*, 2040−2048.
(19) Liu, Q. Y.; Fang, G. S.; Liu, X.; Lin, J. C. *In vivo* absorptive dynamics of *Zanthoxylum* alkylamide in rat intestines. *J. Northwest A&F Univ.* **2015**, *43*, 57−62.
(20) Bezerra, M. A.; Santelli, R. E.; Oliveira, E. P.; Villar, L. S.; Escaleira, L. A. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **2008**, *76*, 965−977.
(21) Pinot, M.; Vanni, S.; Pagnotta, S.; Lacas-Gervais, S.; Payet, L. A.; Ferreira, T.; Gautier, R.; Goud, B.; Antonny, B.; Barelli, H. Polyunsaturated phospholipids facilitate membrane deformation and fission by endocytic proteins. *Science* **2014**, *345*, 693−697.
(22) Hussain, A.; Singh, S.; Sharma, D.; Webster, T. J.; Shafaat, K.; Faruk, A. Elastic liposomes as novel carriers: Recent advances in drug delivery. *Int. J. Nanomed.* **2017**, *12*, 5087−5108.
(23) Monteiro, N.; Martins, A.; Reis, R. L.; Neves, N. M. Liposomes in tissue engineering and regenerative medicine. *J. R. Soc. Interface* **2014**, *11*, No. 20140459, DOI: 10.1098/rsif.2014.0459.
(24) Sinatra, S. T.; Teter, B. B.; Bowden, J.; Houston, M. C.; Martinez-Gonzalez, M. A. The saturated fat, cholesterol, and statin controversy a commentary. *J. Am. Coll. Nutr.* **2014**, *33*, 79−88.
(25) Yingchoncharoen, P.; Kalinowski, D. S.; Richardson, D. R. Lipid-based drug delivery systems in cancer therapy: What is available and what is yet to come. *Pharmacol. Rev.* **2016**, *68*, 701−787.
(26) Kraft, J. C.; Freeling, J. P.; Wang, Z.; Ho, R. J. Y. Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. *J. Pharm. Sci.* **2014**, *103*, 29−52.
(27) Olusanya, T. O. B.; Ahmad, R. R. H.; Ibegbu, D. M.; Smith, J. R.; Elkordy, A. A. Liposomal drug delivery systems and anticancer drugs. *Molecules* **2018**, *23*, No. 907, DOI: 10.3390/molecules23040907.
(28) Liu, W. L.; Liu, W.; Liu, C. M.; Liu, J. H.; Zheng, H. J.; Yang, S. B.; Su, J. H. Preparation and evaluation of easy energy supply property of medium-chain fatty acids liposomes. *J. Microencapsulation* **2011**, *28*, 783−790.
(29) Thant, Y.; Wang, Q. L.; Wei, C. M.; Liu, J.; Zhang, K. Y.; Bao, R.; Zhu, Q.; Weng, W.; Yu, Q. T.; Zhu, Y.; Xu, X. M.; Yu, J. N. TPGS conjugated pro-liposomal nano-drug delivery system potentiate the antioxidant and hepatoprotective activity of Myricetin. *J. Drug Delivery Sci. Technol.* **2021**, *66*, No. 102808.
(30) Braun, A.; Hammerle, S.; Suda, K.; Rothen-Rutishauser, B.; Gunthert, M.; Kramer, S. D.; Wunderli-Allenspach, H. Cell cultures as tools in biopharmacy. *Eur. J. Pharm. Sci.* **2000**, *11*, S51−S60.
(31) Borchardt, R. T.; Hidalgo, I. J.; Raub, T. J.; Borchardt, R. T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, gastroenterology. *AAPS J.* **2011**, *13*, 323−327.
(32) Artursson, P.; Palm, K.; Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Delivery Rev.* **2012**, *64*, 280−289.
(33) Deng, L. Y.; Zhang, J. H. Preparation and release of nattokinase microcapsules and evaluation of absorption in Caco-2 cells model. *Sci. Technol. Food Ind.* **2019**, *40*, 115−121.
(34) Wang, R.; Rao, C. L.; Liu, Q. Y.; Liu, X. Degradation and transformation mechanisms of *Zanthoxylum* alkylamides exposed to UVB light. *Foods* **2023**, *12*, 4392.
(35) You, Y. M.; Ren, T.; Zhang, S. Q.; Shirima, G. G.; Cheng, Y. J.; Liu, X. Hypoglycemic effects of *Zanthoxylum* alkylamides by enhancing glucose metabolism and ameliorating pancreatic dysfunction in streptozotocin-induced diabetic rats. *Food Funct.* **2015**, *6*, 3144−3154.
(36) Wang, S.; Xie, J.; Yang, W.; Sun, B. Preparative separation and purification of alkylamides from *Zanthoxylum bungeanum* maxim by high-speed counter-current chromatography. *J. Liq. Chromatogr. Relat. Technol.* **2011**, *34*, 2640−2652.
(37) Feng, Y.; Sun, C.; Yuan, Y.; Zhu, Y.; Wan, J.; Firempong, C. K.; Omari-Siaw, E.; Xu, Y.; Pu, Z.; Yu, J.; Xu, X. Enhanced oral bioavailability and *in vivo* antioxidant activity of chlorogenic acid *via* liposomal formulation. *Int. J. Pharm.* **2016**, *501*, 342−349.
(38) Oswald, M.; Platscher, M.; Geissler, S.; Goepferich, A. HPLC analysis as a tool for assessing targeted liposome composition. *Int. J. Pharm.* **2016**, *497*, 293−300.
(39) Chouhan, M.; Sharma, R.; Dashora, K. Development of optimized formulation of liposome using 3-factor Box-Behnken Design. *Int. J. Life Sci. Pharm.* **2021**, *11*, 7−13.
(40) Liu, G. Y.; Hou, S. L.; Tong, P. H.; Li, J. P. Liposomes: Preparation, characteristics, and application strategies in analytical chemistry. *Crit. Rev. Anal. Chem.* **2022**, *52*, 392−412.
(41) Wang, L.; Chen, Y.; Yang, Y. Y.; Lu, B. J.; Zhang, J. Preparation and stability of blueberry anthocyanin liposomes. *J. Northwest A&F Univ.* **2022**, *50*, 85−94.
(42) Chattopadhyay, S.; Modesto-Lopez, L. B.; Venkataraman, C.; Biswas, P. Size distribution and morphology of liposome aerosols generated by two methodologies. *Aerosol Sci. Technol.* **2010**, *44*, 972−982.
(43) Ma, Y.; Liu, Y.; Wei, M.; Song, F.; Liu, Z.; Pi, Z. Studies on intestinal transport of ginsenoside compatibility with *Veratrum nigrum* via Caco-2 cell mono layer model coupled with UPLC-ESI-MS method. *Chem. Res. Chin. Univ.* **2015**, *31*, 914−918.
(44) Liu, G. H.; Liu, X. L.; Tan, M. E.; He, C. H. Preparation of saussureae involucratae herba extract loaded solid lipid nanoparticles and uptake of rutin and chlorogenic acid in Caco-2 cells. *Chin. J. Inf. Tradit. Chin. Med.* **2018**, *25*, 76−82.
(45) Chen, L.; Lu, X.; Liang, X.; Hong, D.; Guan, Z.; Guan, Y.; Zhu, W. Mechanistic studies of the transport of peimine in the Caco-2 cell model. *Acta Pharm. Sin. B* **2016**, *6*, 125−131.
(46) Pietzonka, P.; Rothen-Rutishauser, B.; Langguth, P.; Wunderli-Allenspach, H.; Walter, E.; Merkle, H. P. Transfer of lipophilic markers from PLGA and polystyrene nanoparticles to Caco-2 monolayers mimics particle uptake. *Pharm. Res.* **2002**, *19*, 595−601.
(47) Ding, X.; Hu, X.; Chen, Y.; Xie, J.; Ying, M.; Wang, Y.; Yu, Q. Differentiated Caco-2 cell models in food-intestine interaction study: Current applications and future trends. *Trends Food Sci. Technol.* **2021**, *107*, 455−465.
(48) Chen, P.; Zhao, M. Y.; Chen, Q. M.; Fang, L. Q.; Gao, F.; Zhao, L. M. Absorption characteristics of chitobiose and chitopentaose in the human intestinal cell line Caco-2 and everted gut sacs. *J. Agric. Food Chem.* **2019**, *67*, 4513−4523.

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