A Spray-Dried Self-Stabilizing Nanocrystal Emulsion of Traditional Chinese Medicine: Preparation, Characterization and *ex vivo* Intestinal Absorption

Jifen Zhang[1]* Wenxiu Xu[1] Fanjing Meng[1] Tao Yi[2]*

[1] College of Pharmaceutical Sciences, Southwest University, Chongqing, People's Republic of China
[2] Faculty of Health Sciences and Sports, Macao Polytechnic University, Macao, People's Republic of China

Pharmaceut Fronts 2024;6:e449–e458.

Address for correspondence Jifen Zhang, PhD, College of Pharmaceutical Sciences, Southwest University, 2 Tiansheng Road, Beibei District, Chongqing 400716, People's Republic of China (e-mail: zhjf@swu.edu.cn).

Tao Yi, PhD, Faculty of Health Sciences and Sports, Macao Polytechnic University, R. de Luís Gonzaga Gomes Macao 999078, People's Republic of China (e-mail: yitao@ipm.edu.mo).

Abstract

Salvia miltiorrhizae (Danshen, the rhizome of *Salvia miltiorrhiza Bge.*) and Chuanxiong rhizome (Chuanxiong, the rhizome of *Ligusticum chuanxiong Hort.*) are two traditional Chinese medicines that have been widely used for the treatment of cardiovascular and cerebrovascular diseases. However, formulation development is difficult due to the complexity of the active ingredients, particularly the water-insoluble tanshinones and volatile oil of Chuanxiong rhizome, which cannot be absorbed via oral administration in conventional dosage forms. This study aimed to develop a self-stabilized nanocrystal emulsion co-loading the water-soluble, insoluble, and volatile active ingredients of Salvia miltiorrhizae and Chuanxiong rhizome to improve the bioavailability of the drugs. In this work, a high-pressure homogenization method was used to prepare a self-stabilizing nanocrystal emulsion. The emulsion was then spray-dried using hydroxypropyl-β-cyclodextrin. The dispersibility and storage stability of the spray-dried emulsion, the particle size and morphology of the emulsion droplets, and the drug content and phase distribution of the reconstituted emulsion were evaluated. An everted intestinal sac model was established, and high-performance liquid chromatography was used to determine the concentration of six active components (ferulic acid, salvianolic acid B, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA) and to assess the cumulative uptake amount of the drug and the apparent permeability coefficient. A mixture of the crude materials of tanshinones extract, total salvianolic acid, ferulic acid, and volatile oil of Ligusticum Chuanxiong was used as a control. The results showed that the spray-dried emulsion can be easily reconstituted into a uniform submicron emulsion with no significant changes in particle size, morphology, and microstructure of the emulsion droplets compared with the original emulsion before drying. The self-stabilizing nanocrystal emulsion significantly improved the intestinal absorption of water-insoluble components (tanshinone IIA, cryptotanshinone, and ferulic acid), and volatile oil components (senkyunolide A and ligustilide). Overall, the spray-dried self-stabilizing nanocrystal emulsion represents a potential oral formulation for Salvia miltiorrhiza and Chuanxiong rhizoma.

Keywords
- Salvia miltiorrhizae
- Chuanxiong rhizome
- nanocrystals
- dry emulsion
- oral delivery
- tanshinones
- volatile oil

received
October 23, 2023
accepted
September 24, 2024
article published online
November 4, 2024

DOI https://doi.org/10.1055/s-0044-1791831.
ISSN 2628-5088.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Introduction

The development of oral compound formulations for traditional Chinese medicine (TCM) containing insoluble and volatile components has faced great difficulties, especially low oral bioavailability. Technologies, including lipid-based drug delivery systems,[1] solid self-microemulsifying drug delivery systems,[2] nanocrystals,[3] and polymer micelles,[4] have been used to improve the oral absorption of insoluble ingredients; however, their applications in compound formulations of TCM were challenging.[5] Firstly, the compound formula of TCM is complicated and contains multiple active components. The physicochemical characteristics of each component, such as solubility, pKa, and stability, vary greatly. Secondly, there are numerous macromolecular ineffective components, including proteins, polysaccharides, and resins. Additionally, the content of active components may be exceedingly low in TCM extracts, further complicating the application of these novel technologies. Thirdly, the absorption of water-insoluble and volatile components remains poor in conventional oral dosage forms, thereby diminishing their clinical efficacy. Furthermore, these nanoformulations frequently employ surfactants, which may impart specific toxic side effects and low drug loading, particularly for active ingredients with low content. The formulation process is complex and challenging to scale up.

Salvia miltiorrhizae (Danshen) and Chuanxiong rhizome (Chuanxiong) are both recognized for their effects on blood-activation and stasis-elimination, which are commonly used in China in pairs to treat cardiovascular and cerebrovascular diseases, such as ischemic stroke and coronary heart disease.[6,7] Studies have confirmed many active components in Salvia miltiorrhizae and Chuanxiong rhizome. The effective ingredients of Salvia miltiorrhizae are mainly divided into two groups. One is poorly water-soluble tanshinones, represented by tanshinone IIA and cryptotanshinone, and the other is soluble total salvianolic acid, represented by salvianolic acid B.[8,9] Both have demonstrated good effects on antiatherosclerosis, anticoagulant, antithrombotic, and antimyocardial ischemia and could improve coronary blood circulation and repair vascular endothelial cells.[10] The main active ingredients of Ligusticum rhizome are volatile oil and organic acids.[11,12] The volatile oil mainly comprises ligustilide, senkyunolide A, and other phthalides, which exhibit pharmacological activities including improvements in blood rheology, reductions in blood pressure, and anti-inflammatory and analgesic effects.[13,14] Organic acids, particularly ferulic acid, have been shown to inhibit platelet aggregation and the release of thromboxane-like substances, and have antihypertensive and antihyperlipidemic effects, thus preventing coronary heart disease and atherosclerosis.[15]

Tanshinones and the volatile oil of Chuanxiong rhizoma, as critical therapeutic ingredients, pose significant challenges to the formulation development of Salvia miltiorrhizae and Chuanxiong rhizoma due to their extremely low water solubility. Most formulations containing Salvia miltiorrhizae and Chuanxion rhizoma on the market were prepared by extracting active components with water first and then making the water extracts into granules, capsules, or tablets.[16] The amounts of tanshinones and volatile oil of Ligusticum Chuanxiong in these preparations were insufficient, sometimes even undetectable. Even if the amount of tanshinones and volatile oil of Ligusticum Chuanxiong could be increased by using ethanol as an extraction solvent, their clinical efficacy was still poor due to their extremely low oral absorption. Although microemulsions, solid lipid nanoparticles, and solid dispersions could improve oral absorption of tanshinones, these formulations were often limited to containing only monomer of tanshinone IIA or cryptotanshinone.[17–20] The volatile oil of Ligusticum Chuanxiong was usually encapsulated by cyclodextrins to lessen its volatilization.[21] Therefore, it is necessary to develop formulations containing various active ingredients, including water-soluble, insoluble, and volatile compounds of Salvia miltiorrhizae and Chuanxiong rhizoma, all of which have good oral absorption.

Pickering emulsion stabilized by solid particles at the oil–water interface of droplets has been reported for decades[22–24] and has attracted much attention in recent years due to the absence of surfactants, the reduction of potential adverse reactions caused by surfactants, and environmental friendliness. Nanocrystals of poorly water-soluble compounds, such as silybin,[25] quercetin,[26] curcumin,[27] and ursolic acid,[28] have been used as solid particles to stabilize Pickering emulsions. Pickering emulsions have been proven a promising oral delivery system for water-insoluble drugs due to higher oral bioavailability than crude material suspension and even nanocrystal suspensions. Previously, our group successfully developed a Pickering emulsion with puerarin nanocrystals as solid particles and volatile oil of Ligusticum Chuanxiong as an oil phase.[29] The adsorption of puerarin nanocrystals on surfaces of oil droplets of Ligusticum chuanxiong was confirmed by scanning electron microscope (SEM) and fluorescence microscope. The pharmacokinetic study in rats showed that the oral absorption of puerarin in this emulsion was significantly improved. The area under the plasma concentration–time curve for puerarin was increased by 1.6, 0.6, and 1.2-fold compared with the crude material, nanocrystal suspension, and Tween 80 stabilized emulsion, respectively.[29] Therefore, it is feasible to develop a self-stabilizing emulsion containing insoluble nanocrystals and herbal volatile oil without any additional stabilizer.

In this work, a self-stabilizing nanocrystal emulsion has been proposed co-loading active components of both Salvia miltiorrhizae and Chuanxiong rhizoma for the first time by the high-pressure homogenization method. A mixture of suspension of tanshinones nanocrystals and total salvianolic acid was used as the water phase, and a mixture of the volatile oil of Ligusticum Chuanxiong and ferulic acid was used as the oil phase. No other stabilizers were added. The emulsion was then spray-dried. The preparation process of the emulsion, the microstructures of the emulsion droplets, and the *ex vivo* intestinal absorption of the drugs by the emulsion after reconstitution were investigated. The spray-dried nanocrystal emulsion had many advantages, such as simple composition, no surfactants or other excipients,

containing most of the main active ingredients, high drug loading capacity, and a simple preparation process, which could serve as a reference for the development of new oral preparations containing both Salvia miltiorrhizae and Chuanxiong rhizoma.

Materials and Methods

Experimental Materials

Tanshinones extract (purity of 98%, mainly containing 42.94% of tanshinone IIA and 47.73% of cryptotanshinone) and ferulic acid of 98% purity were obtained from Nanjing Jingzhu Biotechnology Co. Ltd. (Nanjing, China). Total salvianolic acid extract (mainly containing 70.80% tanshinol acid B and 3.06% rosmarinic acid) was obtained from Xi'an Hongsheng Pharmaceutical Technology Co. Ltd. (Xi'an, China). The volatile oil of Ligusticum chuanxiong (mainly containing ligustilide 16.27% and senkyunolide A 6.45%) was purchased from Jiangxi Xuesong Natural Medicinal Oil Co., Ltd. (Ji'an, China). Hydroxypropyl-β-cyclodextrin (purity of 98%) was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). Reference standards of tanshinone IIA, cryptotanshinone, salvianolic acid B, ferulic acid, ligustilide, and senkyunolide A (purity > 98%) were all purchased from Chengdu Herbpurify Co., Ltd. (Chengdu, China). Methanol of HPLC (high-performance liquid chromatography) grade was purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Pentobarbital sodium was purchased from Absin (Los Angeles, California, United States). Acetone, methanol, chloroform, sodium chloride, potassium chloride, magnesium chloride, sodium bicarbonate, sodium dihydrogenphosphate, and calcium chloride of analytical grade were obtained from Chongqing Chuandong Chemical Group Co., Ltd. (Chongqing, China).

Preparation of Suspensions Containing Tanshinones Nanocrystals and Total Salvianolic Acid

Tanshinone nanocrystals were prepared using an antisolvent method. Briefly, 10 mg of tanshinones was dissolved in acetone and injected into 50 mL of pure water in a 3-neck flask placed in a sonication water bath at a stirring speed of 1,000 rpm. Ten minutes later, the suspension was degassed under reduced pressure for 10 minutes. The nanocrystals were prepared using a probe sonicator (Scientz-IID, Scientz, Ningbo, China) at 1,000 W for 3 minutes, with a working time of 3 seconds and a rest time of 3 seconds. The obtained nanocrystals were filtered through a 50-nm polycarbonate membrane. The residues were dispersed in 10 mL of aqueous solution, which contained 143 mg of total salvianolic acid (pH = 6.5) by bath sonication to obtain a suspension containing both tanshinones nanocrystals and total salvianolic acid. Meanwhile, 10 mg of tanshinones extract was added to 10 mL total salvianolic acid (aq, pH = 6.5) and then stirred to mix well, which was used as a control. The particle size and morphology were observed by optical microscope.

Characteristics of the Suspensions

Particle size and distribution of tanshinones nanocrystals with or without total salvianolic acid were determined by a Nanoseries ZS instrument (Zetasizer Nano-ZS, Malvern Instruments, Malvern, United Kingdom). The morphology of tanshinones nanocrystals and the mixture of crude materials of tanshinones extract and total salvianolic acid was observed by a Sigma 300 high-resolution field emission SEM (Zeiss, Oberkohen, GER).

Preparation of the Self-Stabilized Nanocrystal Emulsion

High-pressure homogenization is a common technique to prepare Pickering emulsion, and the resulting emulsions are more stable than those produced by sonication.[30] The formation and stability of the self-stabilized nanocrystal emulsion can be influenced by the particle size, concentration, aqueous pH, volume ratio of the oil phase to the water phase, and preparation method, and the wettability of solid particles, which was primarily determined by the properties of the active component and oil phase.[31] Here, a high-pressure homogenization was used to prepare the self-stabilized emulsion. The process was summarized as follows after assessing the factors including the volume ratio of the oil phase to the water phase, the aqueous pH, and the concentration of nanocrystals.[32] Briefly, 5 mg of ferulic acid was dispersed in 0.2 mL of violate oil of Ligusticum Chuanxiong, and the mixture was used as an oil phase. Tanshinones nanocrystals and total salvianolic acid were suspended in water as an aqueous phase. The oil phase was added into the aqueous phase drop by drop. The mixture was stirred at 1,000 rpm for 30 minutes at room temperature, followed by ultrasonication for 5 minutes at 500 W with 1 second of ultrasonication and 1 second of interval.

Preparation of Spray-Dried Self-Stabilizing Nanocrystal Emulsion

3 g of hydroxypropyl-β-cyclodextrin was added to 20 mL of the self-stabilized nanocrystal emulsion and stirred until dissolved completely. Afterward, the emulsion was spray-dried using a Mini Spray-Dryer (B-290, Büchi, Flawil, Swit) within 1 hour to achieve a spray-dried self-stabilized nanocrystal emulsion as a fine powder with a light red color. The inlet temperature was set at 120°C, and the outlet temperature was about 50°C. The airflow rate was 3.5 mL/min, and the airflow was 600 L/h.

Characteristics of Spray-Dried Self-Stabilizing Nanocrystal Emulsion

Redispersibility and Centrifugal Stability of Emulsion after Reconstitution

A total of 0.75 g of spray-dried powder was added to 5 mL of pure water in a vial and shaken gently for 30 seconds. The appearance of the reconstituted emulsion and particle size of emulsion droplets were compared with the original emulsion before drying. Particle size was determined using a laser particle size distribution analyzer (BT-9300HT, Better, Liaoning, Dandong, China). Zeta potential was determined after 2-fold dilution with pure water using a Nanoseries ZS instrument (Zetasizer Nano-ZS, Malvern Instruments, Malvern, UK). The reconstituted emulsion was centrifuged

at 1,800 × g for 15 minutes. The appearance including clear water phase, oil, and sediment, was observed.

Morphology and Surface Structure of Emulsion Droplets

A drop of emulsion was added to the surface of the polycarbonate membrane of 50 nm and dried at room temperature. The samples were vacuum-coated with a gold-palladium film before observation. The morphology and surface structure of emulsion droplets were observed by SEM.

Drug Contents and Distributions in Emulsions

A total of 0.25 mL of emulsion was mixed with 1.25 mL of methanol–chloroform (1:2, v/v) by sonication for 10 minutes. Afterward, the mixture was fixed to 5 mL with methanol and filtered through a 0.22-μm microporous filter membrane. The filtrate was collected. The content of ferulic acid, salvianolic acid B, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA was analyzed by an HPLC method simultaneously.

A total of 4 mL of emulsion was centrifuged at 35,000 rpm, 4°C for 1 hour by a centrifuge (WX80011023, Thermo Fisher, Waltham, Massachusetts, United States). The oil phase and aqueous phase were separated gently using a micro syringe. Drugs in both phases were determined by an HPLC method.

The instrument used was HPLC (Agilent 1200, Santa Clara, California, United States) on a Waters Symmetry C18 column (4.6 × 250 mm, 5 μm). The mobile phases were 0.02% phosphoric acid (A) and acetonitrile (B). The gradient elution was: 0 to 10 minutes, 15 to 30% of B; 10 to 20 minutes, 30 to 60% of B; 20 to 30 minutes, 60 to 70% of B; 30 to 40 minutes, 60 to 70% of B. The column temperature was 30°C. The flow rate was 1 mL/min. The detection wavelength was 280 nm.

Storage Stability

The original nanocrystal emulsion and its spray-dried powder were stored at 25°C for 3 months. The appearances of the original emulsion and reconstituted emulsion were observed. For spray-dried emulsion, the redispersibility, particle size, and zeta potential of reconstituted emulsion droplets, as well as the drug content in the emulsion were measured.

Ex vivo Intestinal Absorption

Normal Sprague Dawley rats weighing 250 ± 20 g were supplied by Hunan Lake Kingda Laboratory Animal Co., Ltd., whose production license number was SCXK (Xiang) 2019-0004, and the laboratory animal qualification number was 430727220103123676. Rats were housed at the SPF-grade Laboratory Animal Center in the College of Pharmacies of Southwestern University. Rats were acclimated for at least 7 days prior to the study.

An everted intestinal sac model was established. Rats were fixed for 12 hours while allowed unlimited access to water and then anesthetized by peritoneal injection of pentobarbital sodium (50 mg/kg). The abdomen was shaved along the abdominal white line. A longitudinal midline incision of 3 to 4 cm was carefully made to expose the intestinal segments. The rat intestine was quickly excised to obtain approximately 10 cm of duodenum, jejunum, ileum, and colon, which were carefully placed in fresh Tyrode's buffer (a solution containing 8.0 g of NaCl, 0.2 g of KCl, 0.1 g of $MgCl_2$, 1.0 g of $NaHCO_3$, 0.05 g of NaH_2PO_4, 0.2 g of $CaCl_2$, and 1.0 g of glucose in 1 L of pure water) and continuously oxygenated until no intestinal contents were discharged. After cleaning, the intestinal segments were carefully inverted with a glass rod so that the mucosal side faces outward and the serous layer inward. The upper end was fixed in the sampling port, and the other end was ligated to obtain an everted intestinal sac.

The reconstituted emulsion obtained by dispersing 3 g of spray-dried powder in 30 mL of fresh Tyrode's buffer was used as a test sample. A crude material mixture of 8.5 mg of tanshinones extract, 173.0 mg of total salvianolic acid, 9.0 mg of ferulic acid, and 0.2 mL violate oil of Ligusticum Chuanxiong was added to 30 mL of Tyrode's buffer to be used as a control sample. The everted intestinal sac was placed vertically into a flask containing 30 mL of test or control samples, which were bubbled with 95% O_2 and 5% CO_2 and incubated in a 37°C water bath. The serosal side (inside) was filled with a blank Tyrode buffer (2 mL). The sample (250 μL) within the serosal side was taken out at 15, 30, 45, 60, 90, and 120 minutes, respectively, and added an aliquot of blank Tyrode buffer at 37°C. The sample was mixed with 0.75 mL of methanol by vortexing for 30 seconds and centrifuged at 12,000 rpm for 5 minutes. The active components (ferulic acid, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA) in the supernatant were determined using an HPLC method mentioned above. After 120 minutes, the intestinal sacs were taken out, washed, and blotted dry with filter paper. The length and radius of intestinal sacs were measured accurately to calculate the absorption area of the gut sac.

The drug's cumulative uptake amount (Q) and apparent permeability coefficient (P_{app}, cm·s^{-1}) were calculated according to the following Equation (1) and (2).[33,34]

$$Q = C_n V + \sum_{i-1}^{n-1} C_i V_i \quad (1)$$

Where C_n is the concentration of each component in samples at the nth time point, V is the total volume of sample solution in the gut sac, C_i and V_i are the concentration and volume of samples taken at each time point, respectively, and n is the number of time points.

$$P_{app} = \frac{\Delta Q}{60\Delta t \times A \times C_0} \quad (2)$$

Where Q was the total amount of drug absorbed (μg), A was the absorption area of the gut sac (cm^2), t was the sampling time (s), and C_0 was the initial concentration of each component (μg/mL).

Data Statistics

All data were expressed as mean ± standard deviation. SPSS 12.0 software (IBM, Armonk, United States) with a t-test was used for statistical analysis, with $p < 0.05$ being a statistically significant difference between groups.

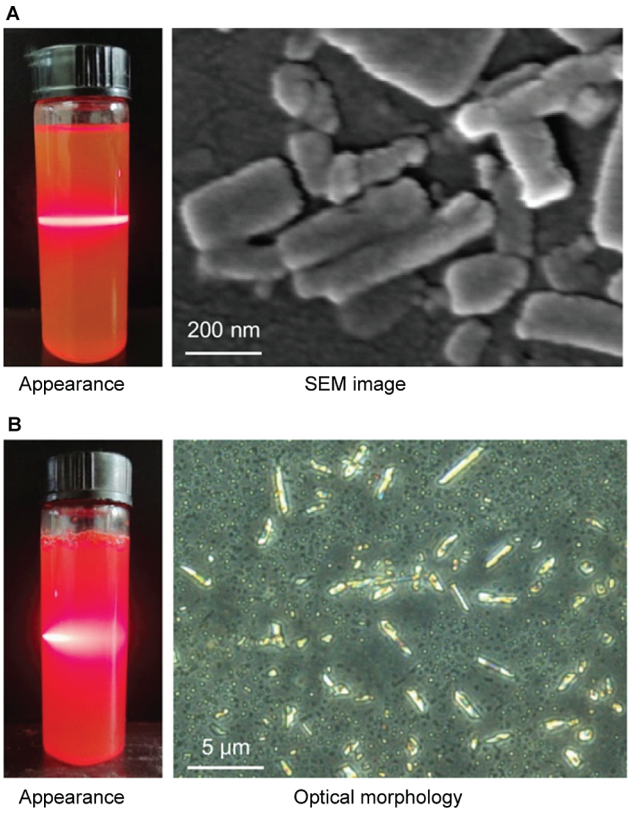

Fig. 1 Appearances and representative SEM images of nanosuspension (**A**), and appearance and optical morphology of the crude suspension containing both tanshinones nanocrystals and total salvianolic acid (**B**). SEM, scanning electronic microscopy.

Results and Discussion

Characteristics of Suspensions Containing Both Tanshinones Nanocrystals and Total Salvianolic Acid

Self-stabilizing nanocrystal emulsions are emulsions using small molecule nanocrystals as stabilizers. The size of the nanocrystals is a critical factor influencing the formation and stability of the emulsions. In general, the smaller the nanocrystal size, the higher the stability of the emulsion, whereas microsize particles were unable to form stable emulsions.[31] In this study, Tanshinones nanocrystals had an average particle size of 304.8 ± 36.03 nm and a polydispersity index (PDI) of 0.240 ± 0.030. The addition of total salvianolic acid did not affect the particle size of tanshinones nanocrystals (an average particle size of 348.17 ± 12.09 nm and a PDI of 0.186 ± 0.047, $p > 0.1$). As shown in ►**Fig. 1**, a colloidal solution with a light red color and an obvious Tyndall phenomenon was observed in the suspension containing tanshinones nanocrystals. The nanocrystals of tanshinones were regularly rod-shaped. However, in a crude mixture of the tanshinone extract and total salvianolic acid, particles were observed with the eyes and no straight light path appeared. Furthermore, long columnar particles of about several microns could be observed with a light microscope.

Redispersibility and Centrifugation Stability

As shown in ►**Fig. 2**, when the dry emulsion was redispersed in water, it could be easily reconstituted into a uniform emulsion,

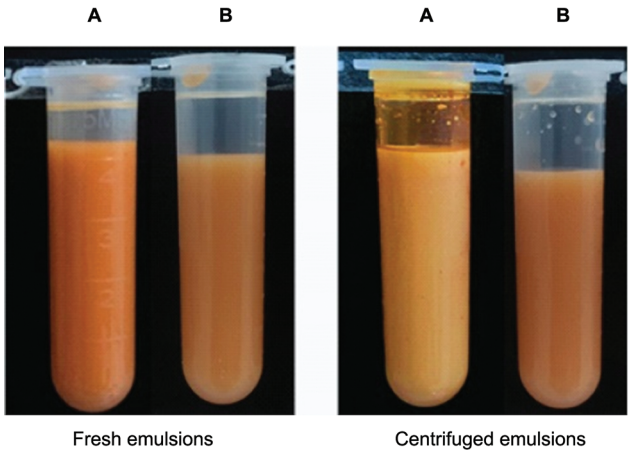

Fig. 2 Appearances of (**A**) original self-stabilized nanocrystal emulsion and (**B**) reconstituted emulsion from spray-dried powder when freshly prepared, or centrifugated at 1,800 × g for 15 minutes.

the same as the original emulsion before spray-drying. The particle size of the reconstituted emulsion droplets was 373.11 ± 11.01 nm, significantly larger than the original emulsion (252.69 ± 8.05 nm, $p < 0.01$). However, the PDI (0.224 ± 0.012) and zeta potential (-35.47 ± 1.57 mV) of the reconstituted emulsion droplets did not change significantly compared with the original emulsion (a PDI value of 0.240 ± 0.030 and zeta potential of -33.20 ± 1.22 mV, all $p > 0.1$).

After centrifugation, no precipitate or oil separated from the reconstituted emulsion; however, a small amount of oil phase appeared at the top of the original emulsion without drying, demonstrating a better stability of the reconstituted emulsion than the original emulsion. This may be due to the adsorption of hydroxypropyl-β-cyclodextrin on the surface of reconstituted emulsion droplets.

The Morphology and Surface Structure of Emulsion Droplets

The morphology of the original and reconstituted emulsion droplets was characterized using SEM. A blank emulsion without nanocrystals was prepared by sonicating a mixture of pure water and volatile oil of Ligusticum Chuanxiong, which was used as a control. As shown in ►**Fig. 3**, all emulsion droplets were nearly spherical. The droplet surface of a blank emulsion was very smooth (►**Fig. 3A**, **D**), however, apparent adsorbents were observed on the droplet surfaces of the self-stabilized nanocrystal emulsion (►**Fig. 3B**, **E**), indicating a different microstructure of the self-stabilized nanocrystal emulsion from that of the blank emulsion. The self-stabilized nanocrystal emulsion contained nanocrystals of tanshinones, however, nearly no rod-like crystals of tanshinones were observed (►**Fig. 3B**). It was hypothesized that one end of nanocrystals of tanshinones might partially be inserted into the Ligusticum chuanxiong oil emulsion droplets but was not completely dissolved in the oil, resulting in a fact that some of them remained embedded on the surfaces of the emulsion droplets. This hypothesis was supported by the results that the particle size of emulsion

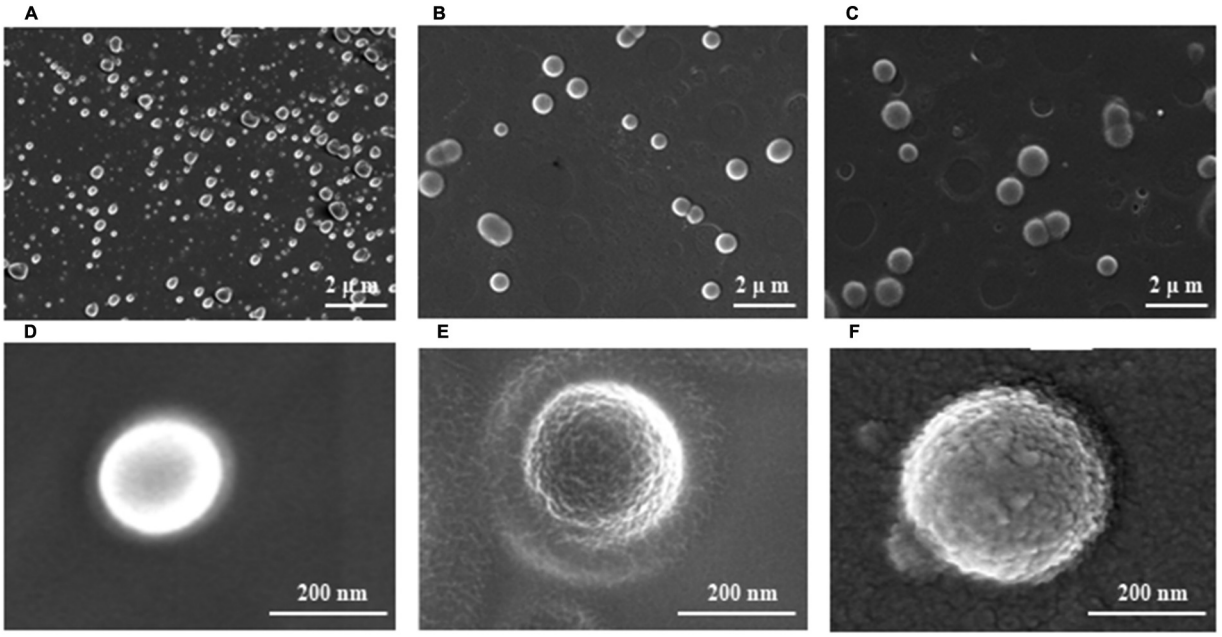

Fig. 3 Representative SEM images of the blank emulsion (**A**, **D**), the original self-stabilizing nanocrystal emulsion (**B**, **E**), and the reconstituted emulsion of spray-dried powder (**C**, **F**). SEM, scanning electronic microscopy.

droplets (252.69 ± 8.05 nm) was slightly smaller than that of tanshinones nanocrystals (304.8 ± 36.03 nm).

The morphology and surface area of the reconstituted emulsion droplets (►**Fig. 3C**, **F**) were not different from those of the original emulsion droplets (►**Fig. 3B**, **E**), indicating that spray-drying did not affect the microstructure of emulsion droplets. However, SEM images showed that the droplet size of reconstituted emulsions was significantly larger than that of original emulsions, which was in line with the change in particle size that was determined using a laser particle size distribution analyzer. Cyclodextrins, as stabilizers, adsorbed onto the surface of oil droplets to form Pickering emulsions.[35–37] The hydroxypropyl-β-cyclodextrin, as a carrier added during spray-drying, may adsorb and wrap around the surface of emulsion droplets, thus slightly increasing the particle size of the emulsion droplet after reconstitution.

Drug Contents and Distributions in Emulsions

The amounts of active components (ferulic acid, salvianolic acid B, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA) in the original emulsion and reconstituted emulsion from the fresh powder were shown in ►**Table 1**. There was no significant change in the content of salvianolic acid B between the two groups ($p > 0.1$). However, the contents of ferulic acid, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA were reduced by 14.86, 18.37, 16.60, and 15.18%, respectively, in the reconstituted emulsion when compared with the original emulsion. These compounds had relatively good solubility in Ligusticum chuanxiong oil. During the spray-drying process, the high temperature may be associated with the volatilization of Ligusticum chuanxiong oil, leading to the reduced content of these drugs in emulsions.

The aqueous phase and the oil layers were separated by centrifugation. The distribution of the six components in the two phases was evaluated. As shown in ►**Table 2**, the distribution of ferulic acid, salvianolic acid B, and senkyunolide A in the reconstituted emulsion was similar to that of the original emulsion. The three compounds were much higher in the aqueous phase than the oil phase, especially salvianolic acid B, which was almost completely distributed in the aqueous phase due to its excellent solubility in water.

Ligustilide, cryptotanshinone, and tanshinone IIA were well distributed in the oil phase of both the original emulsion and reconstituted emulsion due to their high lipophilicity. However, their amount in the aqueous phase was significantly higher in the reconstituted emulsion than in the original emulsion ($p < 0.01$). Hydroxypropyl-β-cyclodextrin may entrap these compounds to form an inclusion complex during spray-drying or reconstitution process, thereby, increasing their water solubility. However, this speculation still needs to be verified by further studies.

It should be noted that the sum of cryptotanshinone and tanshinone IIA dissolved in oil and water was about 20 to 30% of the total amount in emulsions, respectively. It was suggested that the remaining cryptotanshinone and tanshinone IIA existed in nanocrystals because red solids were observed on the wall of centrifuge tubes after centrifugation. In our previous study, a chuanxiong oil emulsion stabilized by tanshinone IIA nanocrystals was prepared with an emulsion droplet size of about 10 μm. The tanshinone IIA nanocrystals could be adsorbed on the surface of the Chuanxiong oil droplet to stabilize the emulsion due to its appropriate three-phase contact angle and particle size.[38] This may be the reason why the emulsion in this study could remain stable even in the absence of stabilizers.

Table 1 Amounts of each active component in the original emulsion and the reconstituted emulsion from spray-dried powder ($n = 3$)

Active component	Amount in original emulsion (μg/mL)	Amount in RE from fresh dried powder (μg/mL)	Amount in RE from dried powder stored at 25°C for 3 months (μg/mL)
Ferulic acid	560.95 ± 1.80	477.56 ± 3.82[a]	485.24 ± 6.87
Salvianolic acid B	8,332.90 ± 18.37	8,270.30 ± 60.93	8,218.46 ± 45.18
Senkyunolide A	1,057.87 ± 0.65	863.50 ± 21.75[a]	877.28 ± 13.45
Ligustilide	1,869.25 ± 13.23	1,456.98 ± 39.82[a]	1,439.12 ± 28.79
Cryptotanshinone	372.61 ± 2.08	311.00 ± 7.67[a]	317.25 ± 6.41
Tanshinone IIA	293.15 ± 0.61	248.64 ± 6.40[a]	255.46 ± 4.89

Abbreviation: RE, reconstituted emulsion.
[a]$p < 0.01$ versus original emulsion.

Table 2 Contents of each active component in the oil phase and aqueous phase after centrifugation at 35,000 rpm for 1 hour ($n = 3$)

Component	Original emulsion		Reconstituted emulsion	
	Oil phase (μg/mg)	Aqueous phase (μg/mg)	Oil phase (μg/mg)	Aqueous phase (μg/mg)
Ferulic acid	5.33 ± 2.98	450.05 ± 22.40	4.18 ± 0.90	404.35 ± 13.04
Salvianolic acid B	–	8,353.57 ± 71.53	–	8,225.78 ± 127.18
Senkyunolide A	47.78 ± 4.72	143.42 ± 7.96	43.51 ± 5.83	156.60 ± 12.97
Ligustilide	92.92 ± 7.47	47.73 ± 3.84	87.54 ± 4.84	114.05 ± 8.23[a]
Cryptotanshinone	3.42 ± 0.24	4.73 ± 0.92	3.57 ± 0.23	12.32 ± 1.48[a]
Tanshinone IIA	4.79 ± 0.62	–	4.70 ± 0.56	2.72 ± 0.27[a]

Abbreviation: RE, reconstituted emulsion.
Note: – undetected.
[a]$p < 0.01$ versus the aqueous phase of original emulsion.

Stability

After 3 months of storage at 25°C, a clear oil film formed on the top of the initial liquid emulsion, and its bottom portion became partially transparent, indicating poor stability. In contrast, the spray-dried powder showed no significant change in appearance, and it was easily redispersed in water to form a uniform emulsion. The droplet size and zeta potential of the reconstituted emulsion were 386.47 ± 15.21 nm and −32.23 ± 1.51 mV, respectively, which did not change significantly ($p > 0.1$), whereas the PDI increased slightly to 0.418 ± 0.101. After 3 months of storage, each active component in the dried powder did not lose its content as shown in ►**Table 1**, indicating the storage stability of the spray-dried emulsion.

Ex vivo Intestinal Absorption

The *ex vivo* intestinal absorption of the self-stabilized nanocrystal emulsion was evaluated by determining the cumulative absorption amount (Q) and the apparent permeability coefficient (P_{app}) of the six active components, respectively. As shown in ►**Figs. 4** and **5**, the self-stabilized nanocrystal emulsion increased the Q and P_{app} values of the insoluble and volatile active components, including ferulic acid, senkyunolide A, ligustilide, cryptotanshinone, and tanshinone IIA, in different intestinal segments in comparison to a crude material mixture, indicating improved intestinal absorption of these compounds.

Cryptotanshinone and tanshinone IIA in the crude material mixture were not detected in the duodenum, jejunum, ileum, and colon due to their extremely low water solubility and intestinal permeability.[39] However, the Q value of cryptotanshinone ranged from 10 to 15 μg, and *P*app ranged from 0.71×10^{-6} to 1.0×10^{-6} cm/s. The Q value of tanshinone IIA ranged from 2.8 to 6.5 μg, and P_{app} ranged from 0.25×10^{-6} to 0.44×10^{-6} cm·s^{-1}, suggesting that the intestinal absorption of the two compounds in the four intestinal segments was significantly improved by the self-stabilized nanocrystal dry emulsion.

There are two possible reasons for the significant increase in absorption. First, most of the tanshinones were present as nanocrystals in emulsions. It was known that nanocrystals could improve oral absorption of poorly water-soluble drugs by increasing saturation solubility and dissolution rate, allowing a higher concentration gradient between the diffusion layers and increased adhesion to the mucosa, thus prolonging drug preservation and absorption time.[40–42] Recent studies have demonstrated that nanocrystals could also be taken up directly by cells through endocytosis

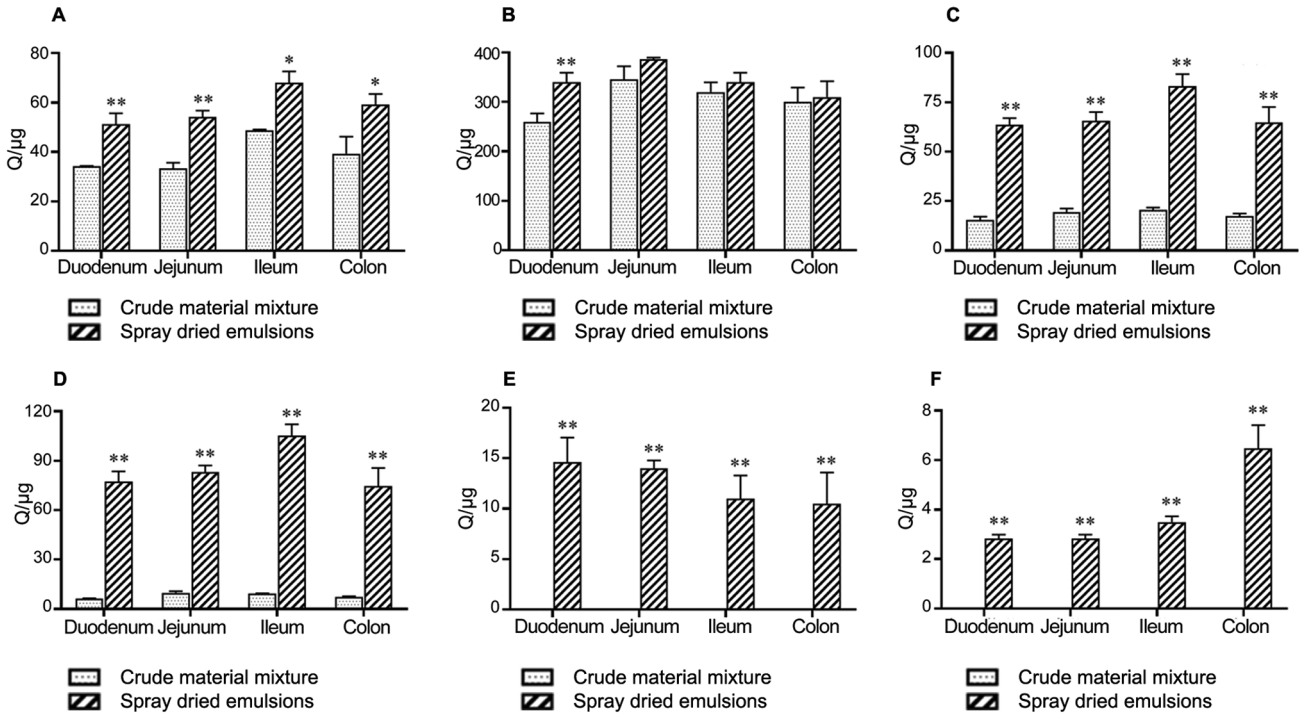

Fig. 4 The cumulative absorption amount of (**A**) ferulic acid, (**B**) salicylic acid B, (**C**) senkyunolide A, (**D**) ligustilide, (**E**) cryptotanshinone, and (**F**) tanshinone IIA at 120 minutes in different intestinal segments of rats by a crude material mixture and spray-dried emulsions ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus a crude material mixture. *Q*, cumulative absorption amount.

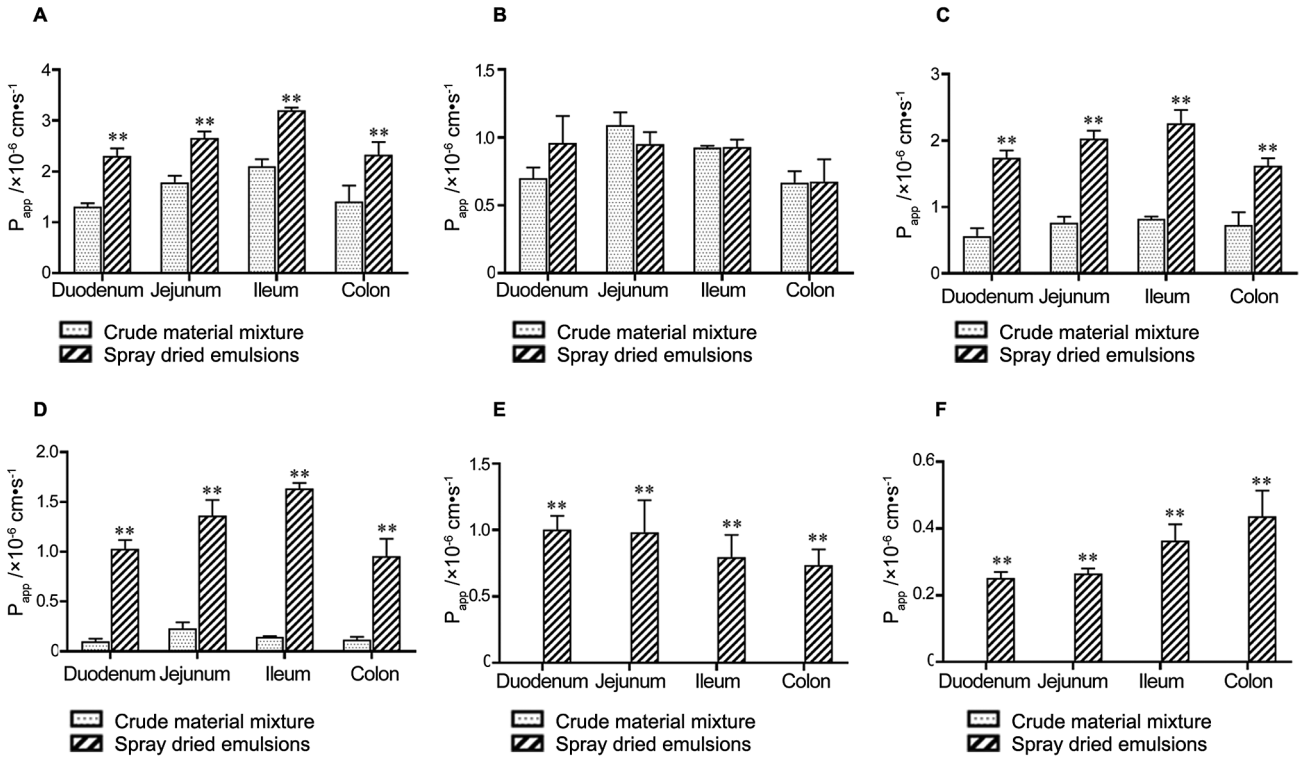

Fig. 5 The apparent permeability coefficient of (**A**) ferulic acid, (**B**) salicylic acid B, (**C**) senkyunolide A, (**D**) ligustilide, (**E**) cryptotanshinone, and (**F**) tanshinone IIA at 120 minutes in different intestinal segments of rats by a crude material mixture and spray-dried emulsions ($n = 4$). $^{**}p < 0.01$ versus crude material mixture. P_{app}, apparent permeability coefficient.

without dissolving in the digestive fluid.[43–45] On the other hand, partial tanshinones were dissolved in Ligusticum chuanxiong oil, which allowed it to be phagocytosed by intestinal cells together with oil droplets.

Intestinal absorption of ligustilide and senkyunolide A was also significantly improved by the self-stabilizing nanocrystal emulsion. The Q and P_{app} values of ligustilide in the four intestinal segments were 10.2 to 12.4 times and 5.9 to 10.9 times higher than that of the crude material mixture. Compared with ligustilide, the intestinal absorption of senkyunolide A was slightly improved, with the Q value and P_{app} being 3.4 to 4.1 times and 2.3 to 3.1 times higher than that of the raw material mixture. Microemulsions have been reported to be effective in improving oral absorption of insoluble drugs.[46,47] When Ligusticum chuanxiong oil is prepared into an emulsion of about 300 to 400 nm, it is dispersed in water in tiny droplets, making it easier to get close to the digestive fluid. At the same time, the droplets are better dispersed has a larger contact area with the digestive fluid, which greatly enhances the absorption of the compounds, particularly for ligustilide, which is less soluble in water than senkyunolide A.

The intestinal absorption of ferulic acid in the self-stabilized nanocrystal emulsion was slightly improved, with only a 40 to 60% increase in Q value and a 50 to 75% increase in P_{app} values compared with the raw material mixture. Ferulic acid is soluble in Ligusticum Chuanxiong oil, allowing it to be phagocytosed by intestinal cells along with oil droplets, resulting in enhanced intestinal absorption. Ferulic acid has a certain lipophilicity and exhibits better water solubility (0.62 mg/mL) when compared with tanshinones and Ligusticum chuanxiong oil[48] and therefore is less difficult to be absorbed orally than tanshinones and Ligusticum chuanxiong oil. Thus, the self-stabilized nanocrystal emulsions induced the improvement of intestinal absorption of ferulic acid was limited.

Litter or no noticeable improvements in the intestinal absorption of salvianolic acid B were observed with the self-stabilizing nanocrystal emulsion, except in the duodenum, where the Q value was increased by 40.7%. This may be related to the fact that salvianolic acid B was hardly dissolved in Ligusticum Chuanxiong oil, which prevented it from being phagocytosed together with oil droplets. The intestinal permeability of salvianolic acid B was also not improved by the self-stabilizing nanocrystal emulsions either.

Conclusion

Salvia miltiorrhizae and Chuanxiong rhizoma are commonly used in TCM formulas to treat cardiovascular and cerebrovascular diseases. However, the water-insoluble tanshinones and volatile oil of Ligusticum Chuanxiong were usually absent or difficult to absorb in current formulations. In this study, the main active components of Salvia miltiorrhizae and Chuanxiong rhizoma, including tanshinones, total salvinolic acid, ferulic acid, and volatile oil of Ligusticum chuanxiong, were all co-loaded into the self-stabilizing nanocrystal emulsion for the first time. The prominent advantage of this new emulsion was self-stabilizing, because there were no excipients, except water and active ingredients. The self-stabilizing nanocrystal emulsion was further solidified by spray-drying with hydroxypropyl-β-cyclodextrin as a carrier to obtain a dry emulsion. The self-stabilizing nanocrystal emulsion significantly improved the *ex vivo* intestinal absorption of water-insoluble components (ferulic acid, cryptotanshinone, and tanshinone IIA) and volatile components (senkyunolide A and ligustilide) compared with crude materials, showing a promising high oral bioavailability. Pharmacokinetic and pharmacological evaluations should be performed to verify the advantages of high oral bioavailability and the resulting better therapeutic effects of multicomponents, which are currently underway. The microstructure and self-stabilizing mechanism of the self-stabilized nanocrystal dry emulsion should also be investigated in the near future.

Ethical Approval

The present study was approved by the Institutional Animal Care and Use Committee of Southwest University (IACUC-20230427-02).

Funding

This research was funded by the Key Project of Traditional Chinese Medicine Research Project of Chongqing Health Commission (Grant No. 2023ZDXM034), the Science and Technology Development Fund of Macao Special Administrative Region (Grant No. 0061/2023/RIA1), and Macao Polytechnic University Research Fund (Grant No. RP/FCSD-01/2023).

Conflict of Interest

None declared.

References

1 Yi T, Zhang J. Effects of hydrophilic carriers on structural transitions and *in vitro* properties of solid self-microemulsifying drug delivery systems. Pharmaceutics 2019;11(06):267
2 Xiao L, Yi T, Liu Y, Zhou H. The *in vitro* lipolysis of lipid-based drug delivery systems: a newly identified relationship between drug release and liquid crystalline phase. BioMed Res Int 2016; 2016:2364317
3 Rocha B, de Morais LA, Viana MC, Carneiro G. Promising strategies for improving oral bioavailability of poor water-soluble drugs. Expert Opin Drug Discov 2023;18(06):615–627
4 Babadi D, Dadashzadeh S, Osouli M, Abbasian Z, Daryabari MS, Sadrai S. Biopharmaceutical and pharmacokinetic aspects of nanocarrier-mediated oral delivery of poorly soluble drugs. J Drug Deliv Sci Tech 2021;63;
5 Wang YH, Zhang JF. Progress in the application of oral absorption promotion technology for insoluble ingredients in traditional Chinese medicine compounding [In Chinese]. Zhongchengyao 2021;43(06):1559–1564
6 Zhang C, Zhang L. Analysis of prescription rules for the prescriptions containing Salvia miltiorrhiza and Ligusticum chuanxiong drug pair based on TCM inheritance support platform [in Chinese]. Chinese Journal of Ethnomedicine and Ethnopharmacy 2022;31(19):96–100
7 Bai M, Liu S, Zhang JL, et al. Study on the mechanism of drug pair Ligusticum chuanxiong-Salvia miltiorrhiza in treating cardiac cerebrovascular diseases based on network pharmacology and molecular docking [in Chinese]. China Pharmacist 2022;25(01):18–26, 48
8 Shan XX, Hong BZ, Liu J, et al. Review of chemical composition, pharmacological effects, and clinical application of *Salviae*

miltiorrhizae Radix et Rhizoma and prediction of its Q-markers [in Chinese]. Zhongguo Zhongyao Zazhi 2021;46(21):5496–5511
9 Feng KR, Li WX, Wang XY, et al. Chemical components and pharmacological action for *Salviae miltiorrhizae* Radix et rhizoma and predictive analysis on quality markers [in Chinese]. Chinese Traditional and Herbal Drugs 2022;53(02):609–618
10 Mao ML, Xie LY, Luo WK, et al. Research progress on pharmacological mechanisms of Danshen (*Salviae miltiorrhizae* Radix Et Rhizoma) and its active ingredients on cardiovascular system [in Chinese]. Zhonghua Zhongyiyao Xuekan 2023;42(07):120–124
11 Han W. Advances in chemical constituents and pharmacological effects of *Ligusticum chuanxiong* [in Chinese]. Zhongguo Xiandai Zhongyao 2017;19(09):1341–1349
12 Cai SJ, Fang JZ. Research progress of Chuanxiong (Chuanxiong Rhizoma) and its drug pairs [in Chinese]. Zhonghua Zhongyiyao Xuekan 2024;42(08):244–248
13 Yan H, Zhou Y, Tang F, et al. A comprehensive investigation on the chemical diversity and efficacy of different parts of *Ligusticum chuanxiong*. Food Funct 2022;13(03):1092–1107
14 Du JC, Xie XF, Xiong L, Sun C, Peng C. Research progress of chemical constituents and pharmacological activities of essential oil of *Ligusticum chuanxiong* [in Chinese]. Zhongguo Zhongyao Zazhi 2016;41(23):4328–4333
15 Li D, Rui YX, Guo SD, Luan F, Liu R, Zeng N. Ferulic acid: a review of its pharmacology, pharmacokinetics and derivatives. Life Sci 2021;284:119921
16 Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. Beijing: China Pharmaceutical Science and Technology Press; 2015:714,727–729,972
17 Zhong C, Lin Z, Ke L, et al. Recent research progress (2015-2021) and perspectives on the pharmacological effects and mechanisms of tanshinone IIA. Front Pharmacol 2021;12:778847
18 Ashour AA, Ramadan AA, Abdelmonsif DA, El-Kamel AH. Enhanced oral bioavailability of tanshinone IIA using lipid nanocapsules: formulation, *in-vitro* appraisal and pharmacokinetics. Int J Pharm 2020;586:119598
19 Pan Y, Bi HC, Zhong GP, et al. Pharmacokinetic characterization of hydroxylpropyl-beta-cyclodextrin-included complex of cryptotanshinone, an investigational cardiovascular drug purified from Danshen (Salvia miltiorrhiza). Xenobiotica 2008;38(04):382–398
20 Hu L, Xing Q, Meng J, Shang C. Preparation and enhanced oral bioavailability of cryptotanshinone-loaded solid lipid nanoparticles. AAPS PharmSciTech 2010;11(02):582–587
21 Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. Beijing: China Pharmaceutical Science and Technology Press; 2015:853,923,1253
22 Zhao W, Ruan B, Sun X, Yu Z. Preparation and optimization of surface stabilized cryptotanshinone nanocrystals with enhanced bioavailability. Front Pharmacol 2023;14:1122071
23 Xia TH, Xue CH, Wei ZH. Physicochemical characteristics, applications and research trends of edible Pickering emulsions. Trends Food Sci Technol 2021;107:1–15
24 Low LE, Siva SP, Ho YK, Chan ES, Tey BT. Recent advances of characterization techniques for the formation, physical properties and stability of Pickering emulsion. Adv Colloid Interface Sci 2020;277:102117
25 Yi T, Liu C, Zhang J, Wang F, Wang J, Zhang J. A new drug nanocrystal self-stabilized Pickering emulsion for oral delivery of silybin. Eur J Pharm Sci 2017;96:420–427
26 Wang Z, Dai B, Tang X, et al. Fabrication and *in vitro/vivo* evaluation of drug nanocrystals self-stabilized Pickering emulsion for oral delivery of quercetin. Pharmaceutics 2022;14(05):897
27 Zembyla M, Murray BS, Sarkar A. Water-in-oil Pickering emulsions stabilized by water-insoluble polyphenol crystals. Langmuir 2018;34(34):10001–10011
28 Liu YG, Xia HP, Guo SY, Lu XY, Zeng CX. Development and characterization of a novel naturally occurring pentacyclic triterpene self-stabilized Pickering emulsion. Colloid Surface A 2022:634
29 Zhang J, Zhang J, Wang S, Yi T. Development of an oral compound Pickering emulsion composed of nanocrystals of poorly soluble ingredient and volatile oils from traditional Chinese medicine. Pharmaceutics 2018;10(04):170
30 Wu J, Ma GH. Recent studies of Pickering emulsions: particles make the difference. Small 2016;12(34):4633–4648
31 Zhang J, Dong F, Liu C, Nie J, Feng S, Yi T. Progress of drug nanocrystal self-stabilized Pickering emulsions: construction, characteristics *in vitro*, and fate *in vivo*. Pharmaceutics 2024;16(02):293
32 Meng FJ. Construction and evaluation of intestinal absorption effect of nanocrystalline submicroemulsions as active components of guanxinning [in Chinese]. Chongqing: Southwest University; 2023
33 Cheng M, Yuan F, Liu J, et al. Fabrication of fine puerarin nanocrystals by box-Behnken design to enhance intestinal absorption. AAPS PharmSciTech 2020;21(03):90
34 Chen P, Zhao M, Chen Q, Fan L, Gao F, Zhao L. Absorption characteristics of chitobiose and chitopentaose in the human intestinal cell line Caco-2 and everted gut sacs. J Agric Food Chem 2019;67(16):4513–4523
35 Zhang J, Wang Y, Wang J, Yi T. A novel solid nanocrystals self-stabilized Pickering emulsion prepared by spray-drying with hydroxypropyl-β-cyclodextrin as carriers. Molecules 2021;26(06):1809
36 Hu JW, Yen MW, Wang AJ, Chu IM. Effect of oil structure on cyclodextrin-based Pickering emulsions for bupivacaine topical application. Colloids Surf B Biointerfaces 2018;161:51–58
37 Liu MQ, Wu L, Li HY, et al. Formation mechanism of Pickering emulsions induced by self-assembly of medium chain triglycerides and α-cyclodextrin [in Chinese]. Yao Xue Xue Bao 2016;51(03):469–474
38 Wang F. Study on the factors affecting the preparation of self-stabilized Pickering lotion with nanocrystals of three insoluble components in Tongmai Recipe [in Chinese]. Chongqing: Southwest University; 2018
39 Chen B, Jia XB. Research on biopharmaceutics characters of tongmaifang's optimization components. Paper presented at International Conference on Agricultural and Natural Resources Engineering; August 3, 2011; Singapore
40 Lu Y, Qi J, Dong X, Zhao W, Wu W. The *in vivo* fate of nanocrystals. Drug Discov Today 2017;22(04):744–750
41 Junyaprasert VB, Morakul B. Nanocrystals for enhancement of oral bioavailability of poorly water-soluble drugs. Asian J Pharm Sci 2015;10(01):13–23
42 Zhou Y, Du J, Wang L, Wang Y. Nanocrystals technology for improving the bioavailability of poorly soluble drugs: a mini-review. J Nanosci Nanotechnol 2017;17(01):18–28
43 Gao W, Lee D, Meng Z, Li T. Exploring intracellular fate of drug nanocrystals with crystal-integrated and environment-sensitive fluorophores. J Control Release 2017;267:214–222
44 Shen B, Shen C, Zhu W, Yuan H. The contribution of absorption of integral nanocrystals to enhancement of oral bioavailability of quercetin. Acta Pharm Sin B 2021;11(04):978–988
45 Guo M, Wei M, Li W, et al. Impacts of particle shapes on the oral delivery of drug nanocrystals: Mucus permeation, transepithelial transport and bioavailability. J Control Release 2019;307:64–75
46 He CX, He ZG, Gao JQ. Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs. Expert Opin Drug Deliv 2010;7(04):445–460
47 Alkrad JA, Assaf SM, Hussein-Al-Ali SH, Alrousan R. Microemulsions as nanocarriers for oral and transdermal administration of enoxaparin. J Drug Deliv Sci Technol 2022;70:103248
48 Wang F, Wang S, Yi T, Zhang JF. Effects of drug-oil properties on fabrication of drug nanocrystalline self-stabilizied Pickering emulsions [In Chinese]. Zhongguo Zhongyao Zazhi 2017;42(19):3739–3746