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Enhanced dissolution rate and oral bioavailability of Ginkgo biloba extract by preparing nano-particles via emulsion solvent evaporation combining freeze drying (ESE-FR)

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Abstract: Ginkgo biloba extract nano-particles were successfully prepared by the method of emulsion solvent evaporation combining freeze-drying (ESE-FR). By the single-factor method, the optimal parameters were determined: the concentration of GBE was 5 mg/mL, volume ratio of water to organic phase was 1:1, homogenate speed and time were 12000 rpm and 3 min, respectively, and homogeneous pressure and times were 500 bar and 3, respectively. The mean particle size (MPS) of GBE nano-emulsion prepared by the optimized conditions was 56.0 nm. SEM detection results indicated that the morphology of raw GBE was irregular. But the morphology of GBE nano-particles was close to the sphere and the particle size of GBE nano-particles was much smaller than that of raw GBE. The particle size of the freeze-dried powder was 277.0 nm. The characteristics of prepared GBE nano-particles were detected via XRD, DSC and TG, which showed that the crystals structure of GBE nano-particles was same to that of raw GBE. Through the test of solubility and dissolution in vitro, the solubility and dissolution of GBE nano-particles in artificial gastric juice, artificial intestinal juice and deionized water were obviously improved compared with that of raw GBE. The results of bioavailability showed that the Area Under Plasma Concentration Curves (AUC) of flavonoids and terpene lactones in GBE nano-particles were improved about 1.81 times and 1.75 times compared with that in raw GBE, respectively. In addition, the residual amounts of chloroform and ethanol were less than the ICH limit for class Ⅱ.

Keywords: Ginkgo biloba extract; Emulsion; Nano-particles; Dissolution rate; Oral bioavailability

Introduction

Ginkgo biloba has existed on the earth for more than 200 million years, which has been well known as the "living fossil".[1] In recent years, ginkgo biloba extracts (GBE) have been widely used as phytomedicine and a dietary supplement in the world.[2] The standardized preparation of GBE is Egb761, which contains 24% ginkgo flavonoids, 6% terpene lactones, and no more than 5 parts per million ginkgolic acids, in which ginkgo flavonoids (quercetin, kaempferol, isorhamnetin) and terpene lactones (ginkgolide A, ginkgolide B, ginkgolide C, bilobalide) are the bioactive components in GBE.[3] The chemical structures of these bioactive components are shown in Fig.1. Increasing research shows that the bioactive components in GBE can improve blood circulation, prevent thrombosis, enhance the capillary wall of the capillaries and protect nerve cells from harm of hypoxia.[4] GBE is used to treat mental disorder, such as concentration difficulties and memory impairment.[5] Moreover, GBE has significantly therapeutic effect on antioxidant,[6] antiasthmatic,[7] scavenge radicals,[8] wound healing [9] and neurodegenerative dementias associated with aging, Alzheimer's disease.[10] At present, the market has a wide variety of pharmaceutical products of GBE, such as granules, tablets, capsules, pills, injection, etc.,[11] in those productions Kinnado is one of the best-selling GBE pharmaceutical products.[12]

At present, the pharmaceutical products of GBE on the market are mostly oral agent and injection. Comparing with the injection, oral agent has the advantages of high safety and convenience. However, the poor aqueous solubility and low dissolution rate of GBE limits the body's absorption of the active ingredients in GBE after oral administration.[13] Recently, in order to improve the oral bioavailability, many researchers have investigated the methods of improving the aqueous solubility of the GBE. Wang and co-workers [14] enhanced dissolution rate and oral bioavailability of GBE by preparing solid dispersion via hot-melt extrusion. Tang et al.[15,16] improved the vitro dissolution of GBE by rapid self-emulsification. Moreover, Chen and colleagues [17] improved the oral bioavailability of GBE through preparing its phospholipids complexes and solid dispersions. Various approaches to overcome the poor aqueous solubility of drug candidates have been investigated in drug research and development, such as preparation of nano-particles,[18] crystal modification,[19] cyclodextrincomplexation[20] and pH

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modification.[21] In recent years, preparation of nano-particles has been used frequently in improving the water solubilityand bioavailability of the drug.[22] The preparation of nano-particles mainly includes bottom-up and top-down technologies.[23] Emulsification is a kind of bottom-up technology, which is usually used to prepare nano-particles. In the process of preparing nano-particle by emulsification technology, the organic phase containing drugs and water phase is fully mixed into nano-emulsion. Nano-emulsion droplet sizes fall typically in the range of 20–200 nm and show narrow size distributions. Next step, the nano-emulsion is processed through high pressure homogenization and the organic solvent is removed by rotary evaporation. Finally, the remaining aqueous phase is removed by an appropriate method.[24] At present, there have been several kinds of nano-particles such as genipin,[25] 10-hydroxycamptothecin,[26] Poly (lactide-co-glycolide) (PLG) [27] and puerarin[28] were produced by this technique.

In this work, the preparation technology of nano-particles of GBE was executed in experiment by the emulsion solvent evaporation and the freeze drying combination method (ESE-FR). The effect of various experimental parameters on particle size was investigated by single-factor method. These experimental parameters consisted of the concentration of GBE solution, volume ratio of water phase to oil phase, speed of homogenate and homogenate time. The optimal experimental parameters were selected. Then the nano-emulsion under the optimal experimental parameter was processed by high pressure homogenization. The influence of pressure and times of high pressure homogenization on the particle size were also studied, respectively. Lastly, the GBE nano-particle was prepared by vacuum freeze-dry. The raw GBE and the optimized GBE nano-particle were analyzed by SEM, FTIR, XRD, DSC, TG, HPLC, LC-MS/MS, dissolution test, residual solvent determination and bioavailability test.

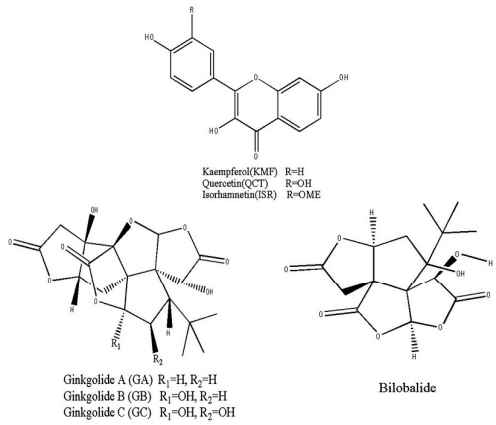


Fig.1 The chemical structures of quercetin, kaempferol, isorhamnetin, ginkgolide A, ginkgolide B, ginkgolide C and bilobalide.

Materials and Methods

Materials

GBE was purchased from Xi'an Xiaocao Botanical Development Co. Ltd. (Xi'an, PR China), with 24% flavonoids and 6% terpene lactones, and no more than 5 parts per million ginkgolic acids. Hydroxypropyl-β-Cyclodextrin (HP-β-CD) was obtained from Yuanye Bio Technology Co., Ltd. (Shanghai, China). Reference standards of quercetin (CAS: 117-39-5, QCT), kaempferol (CAS: 520-18-3, KMF), isorhamnetin (CAS: 480-19-3, ISR), bilobalide (CAS: 33570-04-6, BB), ginkgolide A (CAS: 15291-75-5, GA), ginkgolide B (CAS: 15291-77-7, GB), and ginkgolide C (CAS: 15291-76-6, GC) were all purchased from Shanghai Aladdin Biological Technology Co. Ltd. Ketoprofen (CAS: 22071-15-4) was purchased from National Institutes for Food and Drug Control (Beijing, China). Trichlormethane (purity > 99.5%), ethanol (purity > 99.5%) and the other reagents were all analytical grades.

Preparation of the GBE nano-particle

The GBE nano-particle was prepared by the method of ESE-FR. The preparation process of the GBE nano-particles is shown in Fig.2

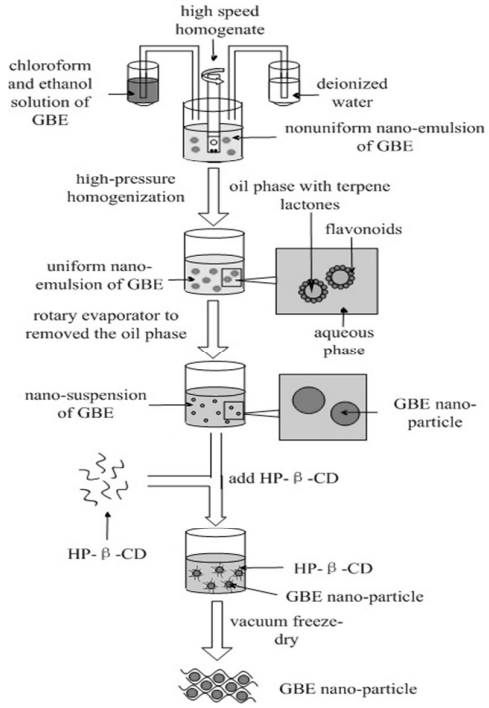


Fig.2 Diagram of experimental process to prepare the GBE nano-particles.

Preparation of the GBE nano-emulsion

In this experiment, the mixed solution of chloroform and ethanol (the volume ratio was 7:3) was chosen as the oil phase and the deionized water was selected as water phase. Firstly, the GBE was dissolved into the oil phase to form a solution with certain concentration. Secondly, the GBE solution was added into the deionized water slowly under the high-speed stirring state via homogenate. After a certain period of high speed homogenate, primary GBE nano-emulsion was formed. The GBE nano-emulsion was obtained after high-pressure homogenization by using Nano homogenize machine (AH100D, ATS Engineering Inc., Shanghai,

China), which was equipped with emulsion valve. Firstly the second-stage valve was regulate the pressure to the total pressure of 10% and then first-stage valve was increased the pressure to the total pressure.

Single-factor method was used to study the optimal operating parameters affecting particle size of GBE nano-emulsion during the preparation process. The parameters include two parts: (a) in the preparation of the primary GBE nano-emulsion process, there are four parameters: the concentration of GBE solution, volume ration of water phase to oil phase, speed of homogenate and time of homogenate, can affect the particle size of primary GBE nano-emulsion; (b) during the homogeneous process, the homogeneous pressure and times have an effect on the size of GBE nano-emulsion. The concentration of GBE solution were 1-5 mg/mL, the volume ration of water phase to oil phase were 1:1-9:1, the speed of homogenate were 8000-16000 r/min, the homogenate time were 1-9 min, the homogeneous pressure were 100-900 bar, the homogeneous times were 3-11, respectively. Example of implementation methods was as follows: when concentration of GBE solution was studied, the other factors were set under the following conditions: the volume ration of water phase to oil phase, speed of homogenate, homogenate time, homogeneous pressure and homogeneous times were set at 5:1, 12000 r/min, 5 min, 500 bar and 7, respectively. The same method was used to accomplish the investigation of other factors. Finally, the optimal condition was selected according to the minimum particle size of each factor. The GBE nano-emulsion was prepared under the optimum condition.

Preparation of GBE nano-suspension

The oil phase in nano-emulsion was removed by a rotary evaporator R201BL (SENCO, Shanghai, China) under the condition of temperature of 40 °C and vacuum pressure of -0.06 Pa. During the process, an equal volume of deionized water with GBE nano-emulsion was added into the GBE nano-emulsion. The GBE nano-suspension was obtained after removing the oil phase.

Lyophilization

After the GBE nano-suspension was obtained, the lyoprotectant was added into GBE nano-suspension and mixed fully. In this experiment, according to pre-experimental screening result and previous report,[29] the HP-β-CD was selected as the lyoprotectant, and the mass ration of the HP-β-CD and GBE in nano-suspension was 3:1. The GBE suspension was frozen at -40 °C to become a solid state. Finally, the GBE nano-particles were obtained after freeze-drying at -50 °C, -10 pa for 72 h.

Detection of main components

Detection of flavonoids

An aliquot 35 mg sample was mixed with 25 mL methanol - 25% hydrochloric acid solution (4:1), hydrolyzed for 30 minutes, cooled to room temperature rapidly, then transferred to a 50 mL volumetric flask and added methanol to scale line. All samples were filtered with 0.22 μm membrane before injected into an HPLC system(Waters Company, USA).The chromatographic column used Diamonsil C_{18} (200 mm×4.6 mm, 5 μm), with methanol - 0.4% phosphoric acid solution (52:48) as mobile phase, detection wavelength: 360 nm, the flow rate: 1.0 mL/min, the injection volume: 10 μL. The column temperature: indoor temperature.[30] The total flavonoids (TFs) were calculated according to Eq. (1):[31]

Total flavonoids (TFs) = (QCT+KMF+ISR)×2.51 (1)

Detection of the content of terpenoid lactones

150 mg sample was dissolved in 10 mL water which mixed with 50 μL 2% hydrochloric acid solution and warmed in 50 °C water bath. Then 2 mL ethyl acetate was added into the suspension of GBE and shaking fully, and the ethyl acetate layer was separated by centrifugation. This operation was repeated four times. The terpenoid lactones were measured by LC-MS/MS (Applied Biosystems, CA), equipping with electrospray mass spectrometry detector API3000 LC-MS/MS for quantitative analysis of terpenoid lactones. Chromatographic conditions: using Agilent Eclipse XDB-C_{18} chromatographic column (150 mm × 4.6 mm, 5 microns Agilent, USA), methanol: water = 7:3 (containing 0.2% ammonium acetate) as mobile phase, the flow rate been setting as 1.0 mL/min and injected sample volume been 5 μL. For operation in MS/MS mode, negative ion scanning; Under the condition of the thermal stability of ion source in front of the sample for 30 minutes; Multiple reaction monitoring ion to choose: ginkgo lactone A was m/z 407.5 to 351.3; Ginkgo lactone B was m/z 423.4 to 367.1; Ginkgo lactone C was m/z 439.5 to 383.3; Bilobalide was m/z 325.4 to 163.1. The other parameters were as following: nebulizing gas, curtain gas and collision gas were 12, 10 and 6 L/min, respectively, dwell time was 1.5 s, ion spray voltage was 5000 V, ion source temperature was 350 °C, declustering potentials (DP) were −42 V for ginkgolide A, −25 V for ginkgolide B, −28 V for ginkgolide C, −20 V for bilobalide, focusing potential (FP) and entrance potential (EP) were 400 and 10 V, respectively; collision energies (CE) were −25 V for ginkgolide A, −26 V for ginkgolide B, −24 V for ginkgolide C, −28 V for bilobalide, respectively; collision cell exit potentials (CXP) were −6 V for ginkgolide A, −7 V for ginkgolide B, −5 V for ginkgolide C, −5 V for bilobalide, respectively.[32] The total terpene lactones (TTLs) were calculated as the sum of BB, GA, GB and GC (Eq. (2)).

Total terpene lactones (TTLs) = BB + GA + GB + GC (2)

Characterization of GBE nano-particle

Particle size

Mean particle size (MPS) of GBE nano-emulsion and GBE freeze-dried powder was detected by laser light scattering using a Zeta PALS Analyzer (Brookhaven Instruments, Holtsville, NY, USA). The measurement was performed with angle detection at 90° in 4 mm diameter cells with field strength of 10 V/cm. The GBE freeze-dried powder was dispersed in pure water for detection. Every measurement was repeated three times.

Morphology analysis

SEM (Quanta 200, FEI) was employed to analyze the particle shape of raw GBE, GBE nano-particles and GBE nano-particles without HP-β-CD. The raw GBE and GBE nano-particles were observed in dry samples. The GBE nano-particles without HP-β-CD were observed in liquid samples. GBE nano-particles were dispersed in deionized water, and the GBE nano-particles without HP-β-CD was obtained by centrifugal and the GBE nano-particles without HP-β-CD need to be washed with deionized water to remove the HP-β-CD. Each dried sample was onto the surface of an aluminum alloy sample table with conductive adhesive directly. The liquid samples were dripped into the foil which was stuck to the aluminum alloy station by using double-sided adhesive. Before the analysis, the samples were sputter-coated with gold under the argon atmosphere

Crystal structure analysis

The crystal structures of raw GBE, HP-β-CD, GBE nano-particles and physical mixture of raw GBE with HP-β-CD were detected via XRD, DSC and TG. X-ray diffraction analysis was used to detect the crystal form of the raw GBE, HP-β-CD, GBE nano-particles and physical mixture of raw GBE with HP-β-CD by using an X-ray diffractometer with a rotating anode (Philips, Xpert-Pro, Netherlands). The scanning range was from 5° to 60° with a step size of 0.02°, and the scanning rate was 5°/min. The samples were irradiated using a Cu target tube at 30 mA and 40 kV. Thermal stability analysis was employed to researched the heating stability of the raw GBE, HP-β-CD, GBE nano-particles, physical mixture of raw GBE with HP-β-CD by DSC (TA instruments, model, DSC 204), and TG (TGA, Diamond TG/DTA PerkinElmer, USA). The test temperature range of DSC was from 30 °C to 300 °C with a heating rate of 10 °C/min, and that of TG was from 30 °C to 600 °C with a heating rate of 10 °C/min.

Solubility analysis

The solubility of raw GBE, GBE nano-particles and physical mixture of raw GBE with HP-β-CD in artificial gastric juice (PH 1.2), artificial intestinal juice (PH 6.8) and deionized water (pH 7.2) was detected by using the HPLC method. According to the preparation methods in Pharmacopoeia of the People's Republic of China,[33] the artificial gastric juice and artificial intestinal juice were prepared. At the beginning of the experiment, excess of raw GBE, GBE nano-particles and physical mixture of raw GBE with HP-β-CD were added into three vials, in each vial been equipped with 3 mL artificial gastric juice, artificial intestinal fluid or deionized water. Then the vials were put in the water bath of 37 °C, 100 rpm/min for 48 h. After this, taking 1 mL suspension, centrifuging under 10000 rpm/min for 10 min, then taking 100 μL supernatant and diluting with methanol to 1 mL. The sample solution was detected by using HPLC after centrifuging under 10000 rpm/min for 10 min.

Dissolution analysis

The dissolution analysis of raw GBE, GBE nano-particles, physical mixture of raw GBE with HP-β-CD in the artificial gastric juice (pH 1.2), artificial intestinal juice (pH 6.8) and deionized water (pH 7.2) were performed by dialysis method. Raw GBE suspension (GBE concentration of 3.2 mg/mL, 7 mL), GBE nano-particles (GBE concentration of 3.2 mg/mL, 7 mL) and physical mixture of GBE with HP-β-CD (GBE concentration of 3.2 mg/mL, 7 mL) were separately placed in treated dialysis bags (MWCO3500; Sigma, St. Louis, USA) and then the dialysis bags were immersed in three identical beakers containing 200 mL artificial gastric juice, artificial intestinal juice or deionized water, respectively. Then the beakers were placed in a water bath at 37 °C, and the stirring rate was set at 100 rpm/min. At the time point of 2, 5, 10, 15, 25, 35, 45, 60, 75, 90, 120 min, the solution with 2 mL was taken out and filtered using 0.22 μm cellulose nitrate membrane. The removed solution was replaced with 2 mL fresh medium.

The dissolution rate was determined by plotting the cumulative amount of solute dissolved against time. Dissolution is calculated according to the following equation.[14]

$$D = \frac{C_n V_n + V_n \sum_{i=1}^{n-1} C_n}{M} \quad (3)$$

D= dissolution

C_n= drug concentration at each time interval

V_n= sampling volume at each time interval

M = total amount of active ingredients

Bioavailability analysis

Twelve male Sprague-Dawley rats, weighing 200-250 g, were divided randomly into two equal groups: reference group and experiment group. The animal use and care protocol was reviewed and approved by the ethics committee of the Harbin Medical University. All experiments were performed in compliance with relevant laws and followed institutional guidelines; Animals were housed under standard conditions of temperature, humidity and light with food and water provided freely and allowed to acclimatize in the laboratory for at least 1 week prior to the experiment. Before the experiment, all the rats were fasted for 12 h and water was given ad libitum. At the beginning of the experiment, the rats in reference group were given the raw GBE at a dose of 40 mg/kg according to the method of oral administration, and the rats in experiment group were given the GBE nano-particles with the same dose and method to the reference group.To determine the drug concentration and calculate the pharmacokinetic parameters, a series of blood samples were collected at the following times: 0.083, 0.167, 0.333, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0 h after dosing. The samples were immediately centrifuged at 3000 rpm for 10 min and plasma was separated from sediment cells with pipet. Samples were stored at -20 °C until analysis.

Plasma sample preparation

The plasma samples were extracted by using liquid-liquid extraction technique. 400 μL of plasma was added into an equal volume of 25% hydrochloric acid and put the plasma under vortex movement for 3 min. Then the plasma was hydrolyzed in a water

bath at 80 °C for 60 min. After this, the hydrolyzed sample was added 2 mL ethyl acetate and under vortex movement for 3 min. The mixed solution was centrifuged at 3000 rpm for 10 min to separate the ethyl acetate layer. Then 2 mL ethyl acetate was added into the remaining water layer and mixed by vortex for 3 min. The ethyl acetate layer was separated from the mixed solution again by centrifuged at 3000 rpm for 10 min, and was combined with the previous ethyl acetate layer. The obtained ethyl acetate was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 µL methanol, and then the solution was centrifuged at 15000 rpm for 10 min before been injected into HPLC and LC-MS/MS system.

Detection of residual solvents

The residual chloroform in the GBE nano-particle was analyzed by using an Agilent 7890A gas chromatograph (GC, Agilent Technologies, Palo Alto, CA, USA) with HP-5 5% phenyl methyl siloxane capillary column (30.0 m×250 mm×0.25 mm, nominal) equipping with an G1540N-210 FID detector. Peaks areas were used for obtaining quantitative data. The analysis conditions of GC for chloroform were as following: oven temperature was maintained at 40 °C for 5 min initially, and then raised at the rate of 20 °C/min to 200 °C maintaining for 2 min at last. Nitrogen was used as carrier gas at a flow rate of 30 mL/min. Then 10 mg of GBE nano-particles were dissolved by 1mL methanol and centrifuged at 8000 rpm for 5 min, and 1 µL samples were manually injected in split mode with a split ratio 25:1. Hydrogen gas and air flow rate were 30 mL/min and 400 mL/min, respectively. The conditions of GC analysis of ethanol were as follows: the oven temperature was initially maintained at 40 °C for 8 min and then ramped at a rate of 40 °C/min to 240 °C and held for 10 min. The injector temperature was set at 200 °C and the detector was 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 25 mL/min. Then 10 mg of GBE nano-particles were dissolved by 1 mL deionized water and centrifuged at 8000 rpm for 5 min. 1 µL samples were injected manually in split mode, with a split ratio 2:1. The hydrogen gas and air flow rates were 30 and 400 mL/min, respectively.

Results and discussion

Optimization Study

The optimal condition is chosen by using single-factor method and the optimization of experimental parameters was listed in Table 1 and Table 2. When determining one of the factors, the other factors were selected as fixed value. During the process of homogenate, the following factors affecting the particle size of primary GBE nano-emulsion were determined: the concentration of GBE solution, volume ration of water phase to oil phase, speed of homogenate and homogenate time. During the process of homogeneous, the optimal value of the homogeneous pressure and the homogeneous times were determined.

Effect of the concentration of GBE solution

The concentration of GBE was set within the range from 1 mg/mL to 5 mg/mL. The MPS of primary GBE nano-emulsion with the increasing concentration of GBE was presented in Fig.3a. As shown in Fig.3a, the MPS of primary GBE nano-emulsion decreased from 96.6 nm to 62.2 nm with the concentration of GBE changing from 1 mg/mL to 3 mg/mL, and when the concentration of GBE solution was greater than 3 mg/mL, the MPS of primary GBE nano-emulsion almost unchanged. The reason for this phenomenon is that flavonoids can act as stabilizers of oil-in-water (O/W) emulsions through pickering stabilization. Due to the flavonoids existing as insoluble particles in the aqueous phase and tending to absorb on the oil-water interface, the flavonoids could provide an efficient steric barrier against the coalescence of emulsion oil globules.[34] However, when the concentration of GBE solution was higher than 5 mg/mL, the GBE nano-particles in suspension were easy to agglomerate and precipitate in the process of removing the organic phase. In order to improve the yield and ensure the quality of products, the GBE solution with 5 mg/mL was selected as the optimal concentration.

Effect of the volume ratio of water phase to oil phase

The MPS of primary GBE nano-emulsion with the increasing volume ratio of water phase to oil phase was shown in Fig.3b. From Fig.3b it can be seen that the MPS of primary GBE nano-emulsion increased gradually when the volume ratio of water phase to oil phase increased from 1:1 to 9:1.

Table 1 Experimental parameters of homogenate process.

No.	Concentration of GBE solution ($mg \cdot mL^{-1}$)	Volume ratio (v/v)	Speed of homogenate ($r \cdot min^{-1}$)	Homogenate time (min)	MPS of primary nano-emulsion (nm)
1	1	5	12000	5	96.6
2	2	5	12000	5	74.7
3	3	5	12000	5	62.2
4	4	5	12000	5	63.3
5	5	5	12000	5	61.5
6	5	1	12000	5	23.1
7	5	3	12000	5	43.2
8	5	5	12000	5	66.8
9	5	7	12000	5	85.4
10	5	9	12000	5	102.1
11	5	1	8000	5	114.9
12	5	1	10000	5	83.8
13	5	1	12000	5	52.2
14	5	1	14000	5	56.2
15	5	1	16000	5	57.6
16	5	1	12000	1	64.2
17	5	1	12000	3	48.4
18	5	1	12000	5	58.0
19	5	1	12000	7	68.1
20	5	1	12000	9	85.4

Table 2 Experimental parameters of homogenization process

No.	homogeneous pressure (bar)	homogeneous times	MPS of nano-emulsion (nm)
1	100	7	123.9
2	300	7	69.2
3	500	7	57.5
4	700	7	74.0
5	900	7	83.8
6	500	3	94.3
7	500	5	67.0
8	500	7	62.4
9	500	9	60.3
10	500	11	56.0

The reason for this phenomenon is that the increase of volume ratio of water phase to oil phase results in the decrease of concentration of flavonoids in the emulsion, and weakens the stability of the nano-emulsion. When the volume ratio was 1:1, the MPS was 23.1 nm, which was the smallest MPS. However, when the volume ratio of water phase to oil phase was lower than 1:1, the primary GBE nano-emulsion was heterogeneous and layered easily. So the optimal volume ratio of water phase to oil phase was 1:1.

Effect of homogenate speed and time

The MPS of primary GBE nano-emulsion with the increased homogenate speed were presented in Fig.3c, in which the homogenate speed was in the range of 8000-16000 rpm. It can be seen from Fig.3c that the MPS of primary GBE nano-emulsion changed from 114.9 nm to 52.2 nm with the homogenate speed increasing from 8000 rpm to 12000 rpm. When the homogenate speed was greater than 12000 rpm, the MPS of primary GBE nano-emulsion increased slightly. Therefore, the optimal homogenate speed was fixed to 12000 rpm.

The effect of the homogenate time on the MPS of primary GBE nano-emulsion was displayed inFig.3d. As shown in Fig.3d, the MPS of primary GBE nano-emulsion decreased from 64.2 nm to 48.4 nm when the homogenate time changed from 1 min to 3 min. While, when the homogenate time was greater than 3 min, the MPS of primary GBE nano-emulsion increased obviously. So the optimal homogenate time was fixed to 3 min.

The above results show that the longer the homogenate time, the faster the homogenization speed, the smaller the size of the nano-emulsion. But after the homogenate time and the homogenization speed reaching a certain value, the MPS of primary GBE nano-emulsion was increased. The reason for the effect of homogenate speed and time may be high speed transfer to the emulsion system of higher energy, the system tends to be unstable state.

From the above results, one can get the optimal experimental parameters in the process of homogenate, which were as following: the concentration of GBE solution was 5 mg/mL, volume ratio of water phase to oil phase was 1:1, homogenate speed was 12000 rpm and homogenate time was 3 min. Under the optimal conditions, the particle size of theprimary GBE nano-emulsion was 48.4 nm

Effect of homogeneous pressure and times

During the homogeneous process, the effect of the homogeneous pressure on the MPS of GBE nano-emulsion was investigated in this experiment. The MPS of GBE nano-emulsion with the increasing homogeneous pressure was shown in Fig.4a. It can be seen from Fig.4a that the MPS of GBE nano-emulsion decreased from 123.9 nm to 57.5 nm with the homogeneous pressure rising from 300 bar to 500 bar. However, when the homogeneous pressure was higher than 500 bar, the MPS of GBE nano-emulsion began to increase. So the optimal value of the homogeneous was 500 bar. That maybe because when the homogeneous pressure is higher than 500 bar, the energy delivering to the system is too large, resulting in instability of the system, the particles of the nano-emulsion occur agglomeration.

During the homogeneous process, the effect of the homogeneoustimes on MPS of GBE nano-emulsion was taken into account, and the results were shown in Fig.4b. It can be seen from Fig.4b that the MPS of GBE nano-emulsion reduced from 94.3 nm to 67.0 nm with the homogeneous times increasing from 3 times to 5 times. When the homogeneous times was over 3 times, the influence of the homogeneous times on the MPS of GBE nano-emulsion was unobvious. For improving the productivity, the homogeneous times was chosen 3. The MPS of GBE nano-emulsion prepared under the optimal conditions was 67.0 nm. The GBE freeze-drying powder was obtained after the nano-emulsion was processed through rotary evaporation and freeze-drying. GBE freeze-dried powder with was dispersed in the deionized water, and the MPS was detected by dynamic laser light scattering.The MPS of GBE freeze-drying powder without lyoprotectant was more than 1 μm, this is due to the agglomeration of GBE nano-particles during the process of freeze-drying. After testing and screening, the optimal MPS of GBE freeze-drying powder was 277.0 nm when the HP-β-CD was selected as the lyoprotactant and the mass ration of the HP-β-CD and GBE was 3:1. In addition to freeze-drying, atomization/freeze-drying is a commonly used method for the preparation of nano-particles, which may be worth pursuing in the future study.[35]

The particle size distribution of primary GBE nano-emulsion and GBE nano-emulsion was shown in Fig.4c. From Fig.4c it can be known that the particle size of primary GBE nano-emulsion was nonuniform, resulting in the system been unstable and the oil phase and water phase been easy to be stratified in a short time. The particle size distribution of the GBE nano-emulsion after homogenization was more uniform comparing with that of primary GBE nano-emulsion, and the system was more stable. Through above results can be seen, the MPS of GBE nano-emulsion was larger slightly than that of primary GBE nano-emulsion after homogenization, but the distribution of particle size of GBE nano-emulsion was concentrated, indicating that homogenization was an indispensable step in the prepared process of GBE nano-particles.

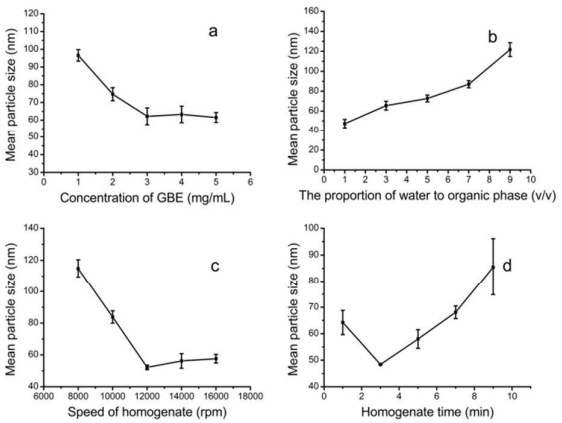


Fig.3 Influence of four parameters on the mean particle size of GBE nano-emulsion during the process of homogenate. (a) concentration; (b) volume ratio of water phase to organic phase; (c) speed of homogenate; (d) homogenate time.

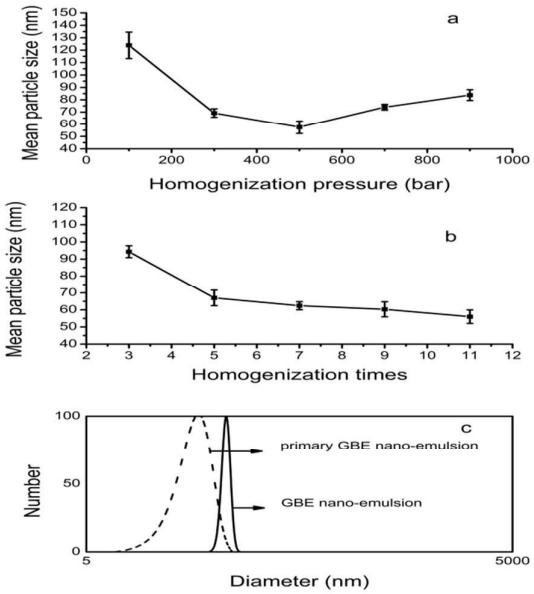


Fig.4 Influence of two parameters on the mean particle size of GBE nano-emulsion during the process of homogenization. (a) homogenization pressure; (b) homogenization times, and (c) normal distribution map of particle size of primary GBE nano-emulsion and GBE nano-emulsion.

Physicochemical characterization

Morphology analysis

The morphology of raw GBE, GBE nano-particles and GBE nano-particle without HP-β-CD was tested by SEM and the results were presented in Fig.5a, Fig.5b and Fig.5c. As shown in Fig.5a, the morphology of raw GBE exhibited various block shape, and the size of raw GBE was unequal. From Fig.5b it can be seen that the GBE nano-particles with HP-β-CD presented a structure like a net. The HP-β-CD with net structure had a good protective effect on GBE nano-particles, which preventing the aggregation of nano-particles. As shown in Fig.5c, the morphology of GBE nano-particles without HP-β-CD presented a fluffy and sparse state and nearly spherical, which had a smaller particle size than that of raw GBE.

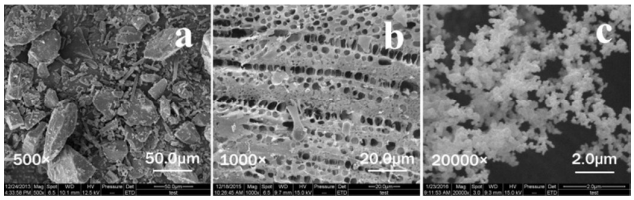


Fig.5 Pictures of scanning electron microscopy. (a) raw GBE; (b) GBE nano-particles; (c) GBE nano-particle without HP-β-CD.

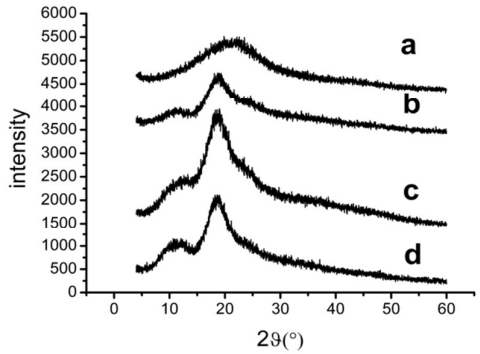


Fig.6 XRD results of each sample. (a) raw GBE; (b) GBE nano-particles; (c) physical mixture of GBE with HP-β-CD; (d) HP-β-CD.

Crystal structure

The crystal structure of raw GBE, HP-β-CD, GBE nano-particles and physical mixture of raw GBE with HP-β-CD was detected via XRD, DSC and TG. The result of XRD was shown in Fig.6. From Fig.6a it can be seen that there was no distinct peaks in the diffractogram of the raw GBE, indicating that raw GBE exists as amorphous state. This may be due to the complex composition of the raw GBE. As shown in Fig.6b, Fig.6c and Fig.6d, there were similar peaks at 2θ=19.82° for GBE nano-particles (Fig.6b) and physical mixture of GBE with HP-β-CD (Fig.6c) with that of HP-β-CD (Fig.6d). In the diffractograms of GBE nano-particles and the physical mixture of GBE with HP-β-CD, there were no other new crystals forming except the HP-β-CD. This result shows that the nano-particles exist as the amorphous state.

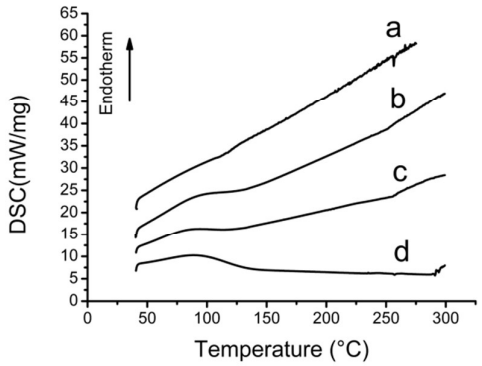


Fig.7 DSC thermograms of each sample. (a) raw GBE; (b) physical mixture of GBE with HP-β-CD; (c) GBE nano-particles; (d) HP-β-CD.

The DSC of raw GBE, HP-β-CD, GBE nano-particles and the physical mixture of raw GBE with HP-β-CD were presented in Fig.7. As shown in Fig.7a, for raw GBE, there was a small absorption peak at 257.02 °C, indicating that there were a few technical components of crystal morphology in raw GBE and the overall state of raw GBE was close to the amorphous state. It can be seen from Fig.7b, Fig.7c and Fig.7d that the absorption peaks of GBE nano-particles, the mixture of raw GBE with HP-β-CD, and HP-β-CD were unobvious, and the absorption peaks of GBE nano-particles and the physical mixture of raw GBE with HP-β-CD were similar to that of HP-β-CD, implying that the GBE in GBE nano-particles was same as that in physical mixture of raw GBE with HP-β-CD, closed to the amorphous state. This result is consistent with the test results of XRD.

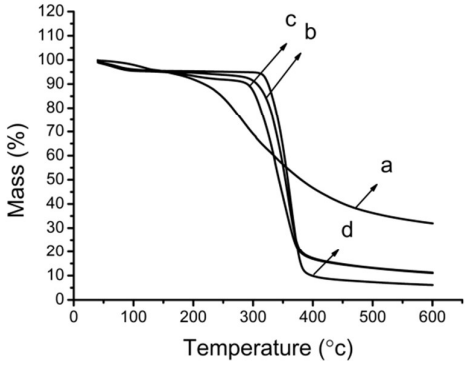
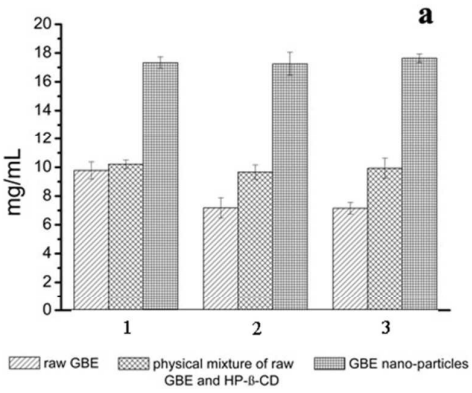


Fig.8 TG curves of each sample. (a) raw GBE; (b) GBE nano-particles; (c) physical mixture of GBE with HP-β-CD; (d) HP-β-CD.

The tested TG curves of raw GBE, HP-β-CD, GBE nano-particles and the physical mixture of raw GBE with HP-β-CD were presented in Fig.8, and it can be seen from Fig.8 that there did not appear suddenly weightless point in the TG of raw GBE. This result implied the raw GBE existed as amorphous state, which was consistent with the result of DSC. The masses of raw GBE, GBE nano-particles, the mixture of raw GBE with HP-β-CD decreased suddenly at the range of 300 °C to 380 °C, indicating that the existence of HP-β-CD resulted in the sudden decrease of the mass of GBE nano-particles and the mixture of raw GBE with HP-β-CD. Combined with the results from XRD and DSC, it implied that the raw GBE and GBE nano-particles existed as the amorphous state.

Solubility study



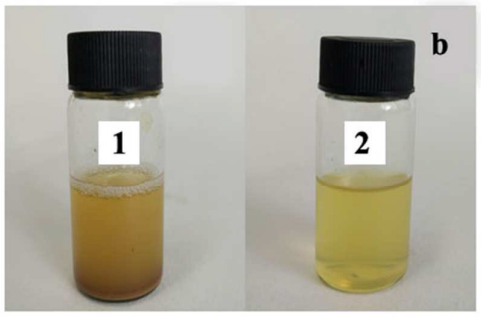


Fig.9 (a) Solubility of GBE in raw GBE, physical mixture of raw GBE with HP-β-CD and GBE nano-particles: (1) artificial gastric juice; (2) artificial intestinal juice; (3) deionized water. (b) The dissolution state of (1) raw GBE and (2) GBE nano-particles in deionized water with concentration of GBE was 5 mg/mL.

Fig.9a showed the results of solubility of GBE in raw GBE, GBE nano-particles and the physical mixture of raw GBE with HP-β-CD in artificial gastric juice, artificial intestinal juice and deionized water. From the testing results by HPLC can be known, the saturated solubility of GBE in raw GBE, GBE nano-particles and the mixture of raw GBE with HP-β-CD were 9.78 mg/mL, 17.33 mg/mL and 10.21 mg/mL in artificial gastric juice, respectively, whereas that were 7.18 mg/mL, 17.25 mg/mL, 9.66 mg/mL in artificial intestinal juice and 7.15 mg/mL, 17.64 mg/mL, 9.93 mg/mLin deionized water, respectively. From the above results can be drawn that the solubility of GBE in GBE nano-particles was much higher than that of in raw GBE, but that of in the mixture of raw GBE with HP-β-CD had no significant improvement compared with that of in raw GBE, indicating that the increase in solubility of GBE in GBE nano-particleswas not a result of the assistant dissolve effect of HP-β-CD. In previous researches,[36-40] the reasons resulting in the higher solubility of nano drug than the raw mainly have two types: (a) in the process of prepared nano-particles, the crystal type of drugs changes from crystalline state into amorphous state; (b) the particle

size of nano-particles is much smaller than that of raw, decreasing particle size leads to the increase in specific surface area. According to the Noyes Whitney equation,[41] the solubility of drug will be significantly increased with the increase of the specific surface area. In this study, according to the results of section "Crystal structure", one can know that before and after the preparation, the drug crystal was close to the amorphous form, so the improvement of solubility was mainly caused by the decreased particle size. In addition, the solubility of GBE in raw GBE, GBE nano-particles and the mixture of raw GBE with HP-β-CD in artificial gastric juice was higher than that in artificial intestinal juice and deionized water. This is due to artificial gastric juice containing a small amount of Tween, which has the assistant dissolve effect. In addition, the solution state of raw GBE and GBE nano-particles with equivalent mass in 5 mL deionized water were shown in Fig.9b, and from Fig.9b can intuitively see that the dissolved status of GBE nano-particles was significantly better than that of raw GBE.

Dissolution results

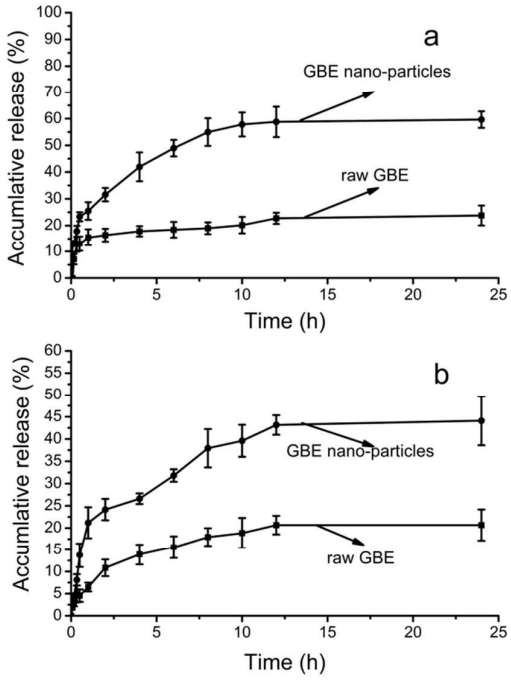


Fig.10 Dissolution profiles of raw GBE, GBE nano-particles in artificial gastric juice. (a) the accumulative release of total flavonoids; (b) the accumulative release of total terpene lactones.

The results of the dissolution for raw GBE and GBE nano-particles in artificial gastric juice were shown in Fig.10. From Fig.10a can be seen that in artificial gastric juice, the release of amount of flavonoids in raw GBE was close to the equilibrium at 12 h and reached to 20.57% at 24 h. While the release of flavonoids in GBE nano-particles was close to the equilibrium at 12 h and reached to 50.41%, improving 1.45 times comparing with that in raw GBE. As shown in Fig.10b, it can be found that the release amount of terpene lactones in raw GBE closed to the equilibrium state at 10 h, and the final release was 26.68%. The release amount of terpene lactones in GBE nano-particle closed to the equilibrium state at 10 h and the final release amount was 33.32%, which was 0.25 times more than that in raw GBE.

The dissolution results of raw GBE and GBE nano-particles in artificial intestinal juice were presented in Fig.11. It can be seen from Fig.11a that up to 12 h, the release amount of flavonoids in raw GBE reached to the equilibrium state with the final release of 23.78%. After 12 h, the release amount of flavonoids in GBE nano-particles reached to a balance level (59.64%0), which was increased by 1.51 times comparing with that in raw GBE. From Fig.11b can be seen that up to 12 h, the release of terpene lactones in raw GBE reached to the equilibrium state with the final release amount of 20.66%. For the terpene lactones in GBE nano-particles, after 12 h, the release amount reached to the equilibrium state with the final release of 44.11%, which was increased by 1.14 times comparing with that in raw GBE.

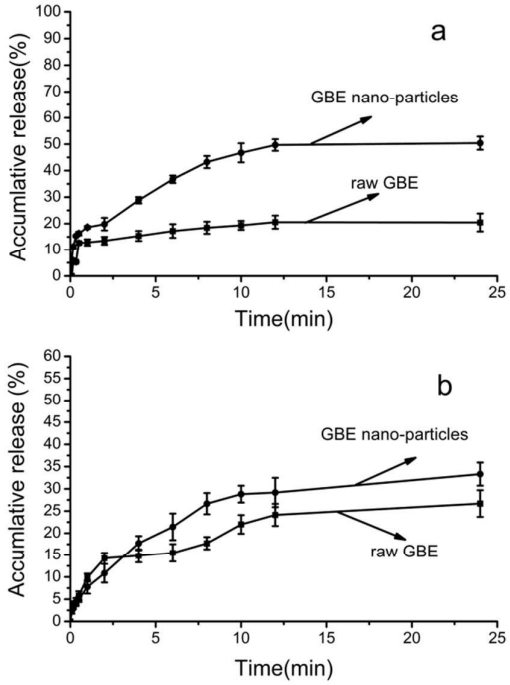


Fig.11 Dissolution profiles of raw GBE, GBE nano-particles in artificial intestinal juice. (a) the accumulative release of total flavonoids; (b) the accumulative release of total terpene lactones.

In addition, the dissolution results of raw GBE and GBE nano-particles in deionized water were presented in Fig.12. From Fig.12a can be seen that in deionized water, after 4 h, the release amount of flavonoids closed to the equilibrium state (20.61%). Meanwhile, the release amount of flavonoids in GBE nano-particles in deionized water reached to the maximum value of 78.53% at 6 h. It was noting that the release amount of ginkgo flavones in GBE nano-

particles decreased comparing with that in 6 h, and tended to 71.46%, which was 2.47 times higher than that in raw GBE. As shown in Fig.12b, the dissolution rate of terpene lactones in raw GBE, after 8 h, the release tended to the equilibrium state with the final release of 29.56%. For the terpene lactones in GBE nano-particles, the release almost reached to equilibrium state with release of 50.62% at 12 h, which was 0.71 times higher than that in raw GBE.

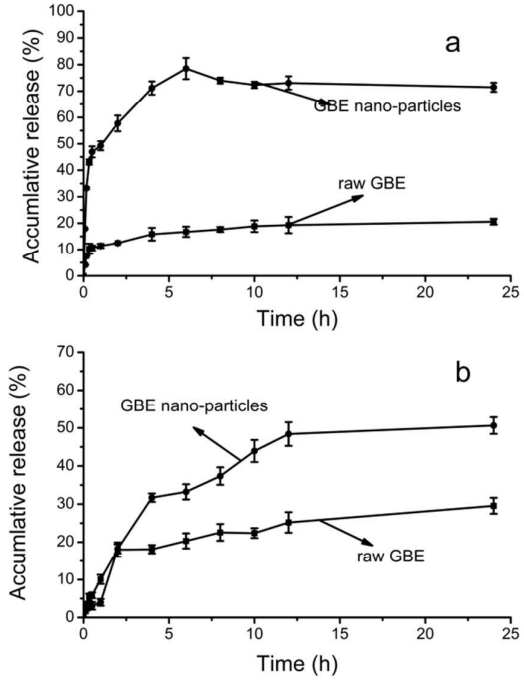


Fig.12 Dissolution profiles of raw GBE, GBE nano-particles in deionized water. (a) the accumulative release of total flavonoids; (b) the accumulative release of total terpene lactones.

In the three systems, the saturated solubility and dissolution of ginkgo flavones and terpene lactones in GBE nano-particles are higher than that in raw GBE. This because the particle size of GBE nano-particles is much smaller than that of raw GBE, and the decrease of particle size leads to an increase in the surface area, which increases the solubility and dissolution.

Results from the results above, it can be known, in the three systems, due to the poor solubility of raw GBE, the release amount of ginkgo flavones and terpene lactones in vitro dissolution shows unobvious. But for GBE nano-particles with outstanding water solubility, the release amount of ginkgo flavones and terpene lactones in vitro dissolution realizes a significant difference, in which the release amount of ginkgo flavones in artificial gastric juice shows the minimum and the maximum in deionized water, owing to the different pH of three systems. The dissolution rate of ginkgo flavones and terpene lactones could be inhibited under the acidic condition. Therefore, the release amount of ginkgo flavones and terpene lactones increases with the elevated pH in these three systems.

Bioavailability analysis

The plasma concentration of ginkgo flavones in plasma for reference group and experiment group were presented in Fig.13a. As shown in Fig.13a, the charts appeared two peaks for the two groups. For the reference group (raw GBE), the first peak appeared at 0.5 h with plasma concentration of 3.80 μg/mL and the second peak at 4 h with plasma concentration of 2.70 μg/mL. For the experiment group (GBE nano-particles), the first peak was located at 0.083 h with plasma concentration of 8.34 μg/mL and the second peak at 0.5 h with plasma concentration of 6.79 μg/mL.

Moreover, the drug concentration of terpene lactones in plasma for reference group and experiment group were presented in Fig.13b. It can be seen from Fig.13b that the charts appeared three peaks for the two groups. For the reference group, the first peak appeared at 1 h with plasma concentration of 0.11 μg/mL, and the second peak was located at 4 h with plasma concentration of 0.09 μg/mL. In addition, the third peak lied at 8 h with plasma concentration of 0.05 μg/mL. For the experiment group, the first peak appeared at 0.167 h with plasma concentration of 0.18 μg/mL and the second peak was located at 1 h with plasma concentration of 0.13 μg/mL. Meanwhile, the third peak lied at 4 h with plasma concentration of 0.12 μg/mL.

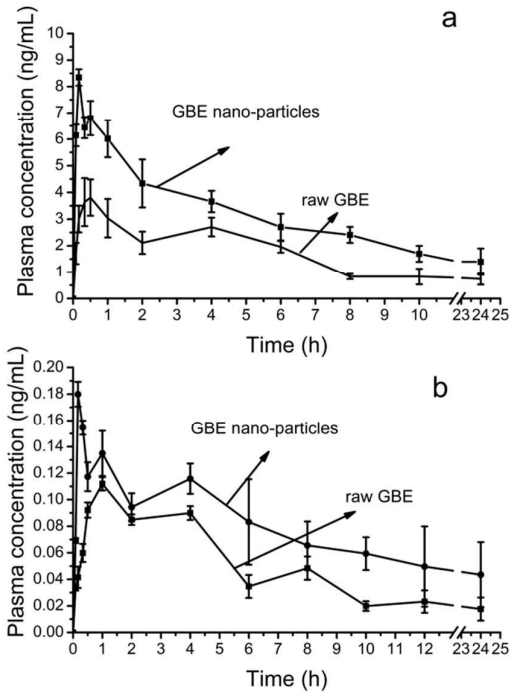


Fig.13 Plasma concentration of raw GBE, GBE nano-particles. (a) the plasma concentration of total flavonoids; (b) the plasma concentration of total terpene lactones.

From the above results, one can find that the detected plasma concentration of ginkgo flavones and terpene lactones in the rats of experiment group were higher than that in the rats of reference group, and the absorption rate to ginkgo flavones and terpene lactones of the rats in the experiment group was faster than that in the reference group. This is because the particle size of GBE nano-particles is much smaller than that of raw GBE, which enhances the water solubility of GBE nano-particles, thereby improving the absorption effect and rate in the rats. Meanwhile, the plasma concentration curves of ginkgo flavones and terpene lactones in raw GBE and GBE nano-particles appearing two peaks owes to the ginkgo flavones are mainly composed of quercetin, kaempferol and isorhamnetin, and the absorption rate to these three components is different in vivo. When a component has been absorbed to reach the maximum value, the other ingredients may just began to absorb, when the absorption of first component declines, that of the other components reaches maximum, leading to the two peaks in the plasma concentration curves. For the same reason, terpene lactones contain the components of ginkgolide A, ginkgolide B, ginkgolide C and bilobalide, the absorption rate of these substances is different in vivo, leading to the three peaks occur in the plasma concentration of the rats. The oral relative bioavailability of GBE nano-particles was calculated by the way of comparing the corresponding AUC values of the two groups. The result demonstrated that the AUC values of the flavonoids and terpene lactones in GBE nano-particles was about 1.81 and 1.75 times compared with raw GBE, respectively. Therefore, the oral bioavailability of the GBE nano-particles was improved significantly compared with the raw GBE.

From the above results, the method of ESE-FR achieved good results in improving the oral bioavailability of GBE. In addition to GBE, this method also significantly improved the oral bioavailability of genipin, [25] 10-hydroxycamptothecin, [26] Poly (lactide-co-glycolide) (PLG) [27] and puerarin. [28] In recent years, preparation of GBE solid dispersion via hot-melt extrusion[14] and phospholipid complexes[17] had improved its oral bioavailability obviously. However, comparing with the method of ESE-FR, the operation of preparing GBE phospholipid complexes and solid dispersion are very complex and not conducive to the expansion of production scale. These two methods cost much higher than the method of ESE-FR. In addition, the preservation of GBE phospholipid complexes is difficult. In the process of preparing GBE solid dispersion by hot melt extrusion, due to the difference between the melting point of the drug and the auxiliary materials, the concentration distribution of solid dispersion is difficult to ensure uniformity.

The detection of residual solvents

In the actual production process, the residual solvent is also considered in drug production. In this work, the GBE nano-particles were prepared successfully by using emulsion solvent evaporation method, which used the International Conference on Harmonization (ICH) class Ⅱ solvent ethanol and chloroform with low toxicity. Fig.14a and Fig.14b showed the chromatograms results of 10 mg/mL ethanol solution of the GBE nano-particles and 0.05 mg/mL chloroform of ethanol solution, respectively. From Fig.14a and Fig.14b it can be seen that, there were two solvent peaks, the retention time of chloroform was 3.130 min after confirmation. Meanwhile, from Fig.14b, the peak of chloroform was significantly smaller than that of chloroform in Fig.14a, the percentages of residual chloroform was about 0.00217% in GBE nano-particles. The residual chloroform content in GBE nano-particles was less than the ICH limit for chloroform in class Ⅱ solvents of 0.006% for solvents.

Fig.14c and Fig.14d presented the chromatograms results of 0.05 mg/mL ethanol of deionized water solution and 10 mg/mL deionized water solution of the GBE nano-particles, respectively. In Fig.14c, there was a peak at retention time of 2.192 min, which represented ethanol after confirmation. From Fig.14d, the peak of ethanol was significantly smaller than that of ethanol in Fig.14c, the percentages of residual ethanol was about 0.07% in GBE nano-particles. The residual ethanol content in GBE nano-particles was less than the ICH limit for ethanol in class Ⅱ solvents of 0.5% for solvents. GBE nano-particles fully meet the requirements of pharmaceutical use.

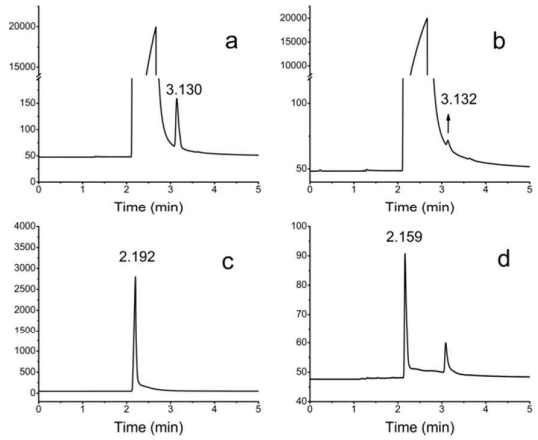


Fig.14 Gas chromatograms of (a) chloroform standard solution; (b) GBE nano-particles; (c) ethanol standard solution; (d) GBE nano-particles.

Conclusions

By using the method of ESE-FR, the GBE nano-particles were prepared successfully. By means of a single factor method, the optimal parameters were as follows: the GBE concentration was 5 mg/mL, volume ratio of water to organic phase was 1, homogenate speed was 12000 rpm, homogenate time was 3 min, homogeneous pressure and times were 500 bar and 3 times, respectively. According to pre-experimental screening result, the HP-β-CD was selected as the lyoprotectant, and the mass ration of the HP-β-CD and GBE in nano-suspension was 3:1. The particle size of prepared GBE nano-particles was 56 nm under the optimal parameters. One has come to the conclusion by means of the following test: (a) the detection of XRD, DSC and TG shows that the GBE nano-particles have the same crystal type with raw GBE. (b) By detecting the

solubility, dissolution in vitro and bioavailability, it can be known that the saturated solubility of GBE nano-particles is 1.77 times, 2.47 times and 2.40 times of raw GBE in artificial gastric juice, artificial intestinal juice and deionized water, respectively. And the release percentage of GBE nano-particles in vitro has been greatly improved compared with that of raw GBE. By detecting the bioavailability, the plasma concentration of GBE nano-particles in the rats is much higher than that of raw GBE, and the absorption rate of GBE nano-particles in the rats is significantly accelerated comparing with that of raw GBE. (c) the results of solvent residue show that the residual amounts of chloroform and ethanol are 0.00217% and 0.07%, respectively, completely consistent with the requirements of ICH. In a word, the method of ESE-FR has great potential value to the preparation of oral GBE drugs.

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Enhanced dissolution rate and oral bioavailability of Ginkgo biloba extract by preparing nano-particles via emulsion solvent evaporation combining freeze drying (ESE-FR)

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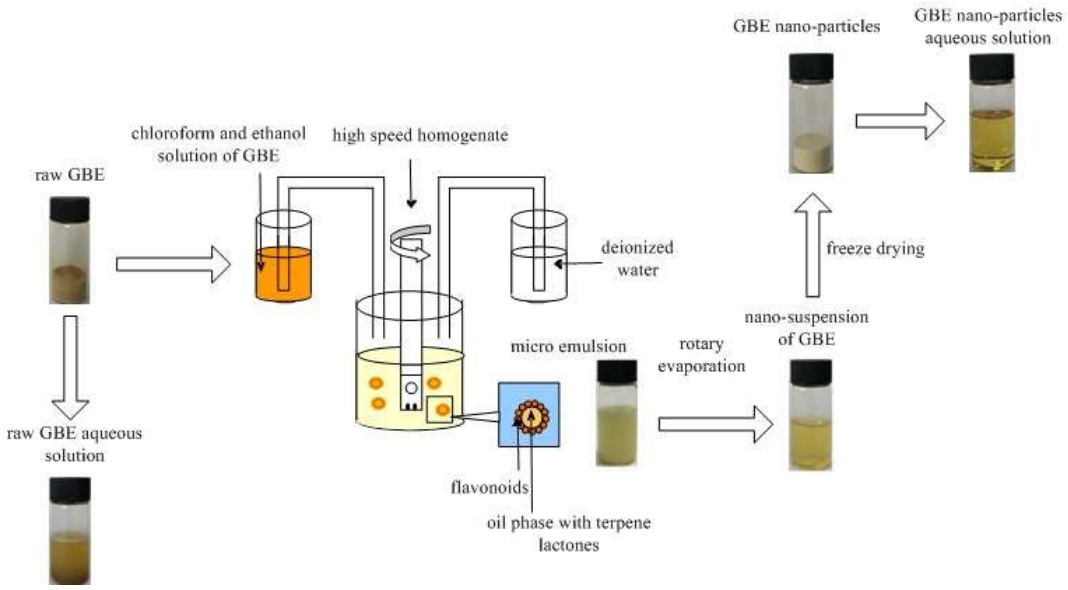
Graphical Abstract



Ginkgo biloba extract (GBE) nano-particles were successfully prepared by the method of emulsion solvent evaporation combining freeze drying. The solubility, dissolution rate and oral bioavailability of GBE nano-particles were significantly improved compared with raw GBE. According to the results above, the method of emulsion solvent evaporation and freeze-drying combined has great potential value to the preparation of oral GBE drugs.

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