Preparation and quality evaluation of a volatile oil microemulsion from *Flos magnoliae* and *Centipeda minima*

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Abstract. In order to improve the water solubility of the volatile oils extracted from *Flos magnoliae* (FM) and *Centipeda minima* (CM), they were prepared as a microemulsion (ME), which were then used in the development of an FM and CM volatile oil ME for the treatment of allergic rhinitis (AR). ME was prepared by phase inversion emulsification, and the prescription factors such as emulsifier, co-emulsifier, oil phase, Km, which represents the ratio of the mass of emulsifier to that of the co-emulsifier, and preparation factors such as temperature affecting the formation of the ME were selected according to the formation area of ME in a pseudo-ternary phase diagram. The quality of the ME was evaluated based on its appearance, particle size, Zeta potential and stability. The content of eucalyptol in ME was determined by gas chromatography-mass spectrometry (GC-MS). The cumulative permeability of the ME within 24 h was measured with a transdermal diffusion tester. The results revealed that the best formula for preparation of the ME was as follows: Castor oil polyoxyethylene ether (EL-40) was the emulsifier; the co-emulsifier was anhydrous ethanol; the Km was 2:1; the mixed phase of volatile oil and isopropyl myristate with mass ratio of 1:1 was used as oil phase; and the preparation temperature was 25˚C. The content of eucalyptol in the ME was 2.57 mg/g, and the cumulative permeability of the ME in 24 h was significantly increased compared with that of the reference oil solution. The appearance of the ME was uniform, and the solution was transparent. In conclusion, compared with traditional preparations, FM and CM volatile oil ME is a novel, improved and more effective preparation for the treatment of AR.

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Introduction

Allergic rhinitis (AR) refers to the non-infective inflammatory disease of the nasal mucosa caused by the release of IgE-mediated histamine following exposure to allergens by atopic individuals, and involves a variety of immune active cells and cytokines (1). The clinical manifestations of AR are usually nasal congestion, rhinocnesmus, rhinorrhea and sneezing. AR is the most common chronic refractory disease reported in recent years in otorhinolaryngology, and it is also one of the prioritized diseases for prevention and treatment (2). It is mainly caused by environmental changes, seasonal alternation, temperature change, wind, precipitation, pollen and dust (3). The disease is not lethal, but the nasal symptoms cause obvious discomfort, seriously affecting the physical health of the patient, their ability to work and study, and their quality of life. AR is difficult to control and leads to numerous long-term complications that may cause more severe diseases, such as nasopharyngeal carcinoma and tympanitis (4). Therefore, the identification of a safe drug for the treatment of AR is currently a research priority (2).

Bitongning drops are recognized in Volume 6 of the Drug Standard of the Ministry of Health of the People's Republic of China (5). The drops are made by steam distillation of *Flos magnoliae* (FM) and *Centipeda minima* (CM) to extract their volatile oil or aqua aromatica (6). Bitongning drops can clear nasal orifices, and therefore they are used for treating nasal congestion, acute and chronic sinusitis, AR and colds caused by wind chill (7-9). Pharmacological experiments have indicated that the volatile oils of drugs used in Traditional Chinese Medicine (TCM) have anti-inflammatory and anti-allergenic effects, and they are currently used to treat acute and chronic rhinitis, as well as AR. In particular, the volatile oils of FM and CM serve important roles in the treatment of AR (10,11). Due to the low and unstable extraction rate of volatile oil, constituents such as eucalyptol and geraniene D were easy to

volatilize (12). The majority of TCM volatile oils are used as a raw material only, and therefore few previous studies have reported the clinical application of its single preparation. The association between compatibility and pharmacodynamics of FM and CM has been rarely studied. Our previous study revealed that the combination of volatile oil from FM and CM may effectively relieve local inflammatory cell infiltration and cell necrosis of nasal mucosa in the nasal cavity of rats, thereby decreasing the histamine content in blood and improving the symptoms of AR (11). In Bitongning drops, the active component is aromatic water. Although aromatic water is mostly composed of volatile oil, it is a type of solution that is nearly saturated or contains water. Compared with pure volatile oil, the volatile oil content of aqua aromatica is lower and extremely perishable, which is an impediment for its production and storage on a large scale.

As a new drug carrier, microemulsions (ME) are transparent or translucent, have low viscosity, and are isotropic and thermodynamically stable systems. ME is spontaneously formed by an oil phase, water phase, emulsifier and co-emulsifier in an appropriate ratio (13,14). The particle size of ME ranges from 10-100 nm, and its characteristics include low viscosity, rapid absorption and targeted drug release, which can increase the solubility of volatile oil drugs, improve the bioavailability of drugs and decrease side effects (15-17). As the volatile oils of FM and CM are fat-soluble components, and their absorption is poor and their bioavailability is low, the common method of preparation results in poor absorption and low bioavailability. Therefore, in the present study, the volatile oils of FM and CM were prepared as an ME by phase conversion emulsification, and its basic properties, including stability, content and skin permeability were evaluated. The results led to the generation of a novel type of FM and CM volatile oil ME preparation with high stability, safety and loading capacity.

Materials and methods

Materials. FM volatile oil and CM volatile oil were produced by the Key Laboratory of New Drugs and Chinese Medicine Foundation Research, College of Pharmacy, Shaaxi University of Chinese Medicine (7,20). Eucalyptol (purity >99.5%) was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Hydrogenated castor oil polyoxyethylene ether (RH-40), castor oil polyoxyethylene ether (EL-40), isopropyl myristate (IPM) and isopropyl hexadecanoate (IPP) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Tween-80, Tween-20, glycerol, 1,2-propanediol, and oleic acid were purchased from Tianjin Tianli Chemical Reagent Co., Ltd. Polyethylene glycol-400 (PEG-400) was purchased Tianjin Kemiou Chemical Reagent Co., Ltd. Sudan III and methylene blue were obtained from Shanghai Xinsheng Test Chemical Technology Co., Ltd. Purified water, purchased from Wahaha Group Co., Ltd., was used throughout the experiments. All other reagents were of commercial analytical grade. A total of three three-month-old female bullfrogs (150±20 g) were supplied by Chengdu Dossy Experimental Animals Co., Ltd. Bullfrogs were housed in a transparent box with a water depth of 20 cm at 25˚C, 30-40% humidity and a 12-h light day cycle, without food for 3 days. Bullfrogs were sacrificed by marrow destruction, and the abdominal skin and subcutaneous fat was removed. The present study was approved by the Ethical Committee of Shaanxi University of Chinese Medicine.

Screening of formula and preparation factors of ME

Emulsifiers. Representing an important component in the preparation of MEs, an emulsifier is a substance that can improve the surface tension between various phases in emulsion, and can form a uniform and stable dispersion system or emulsion (21). The hydrophilic and lipophilic balance (HLB) value of the emulsifier is the basic metric used for selecting a certain emulsifier (22). In the present study, RH-40, EL-40, Tween-80, and Tween-20, emulsifiers with HLB values between 8-18 that can effectively improve the surface tension of oil and water, were selected to investigate their effects on the formation of volatile oil ME. The mixed phase with 1:1 ratio of IPM to FM-CM volatile oil was used as the oil phase, and the preparation temperature was 25˚C. The different ratios of emulsifier to mixed oil phase examined were 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. ME was produced by stirring at constant temperature and uniform speed, adding ultra-pure water drop by drop and measuring the electrical conductivity. When the electrical conductivity of the system reached the highest point, it was defined as being an ME, and the dosage of emulsifier, mixed oil phase and water phase which can form the ME group was recorded (14,23). Data input was analyzed using Origin 8.0 drawing software (OriginLab Corporation) to generate pseudo-ternary phase diagrams.

Co-emulsifier. According to the screening results, EL-40 was selected as the emulsifier, the mixed phase of IPM and FM-CM volatile oil at a ratio of 1:1 was selected as the oil phase, the Km was 2:1, and the preparation temperature was 25˚C. The effects of the co-emulsifiers such as absolute ethyl alcohol, glycerol, 1,2-propanediol and PEG-400 on ME were investigated.

Oil phase. In the preparation of ME, the size and chain length of the oil phase molecules are important for the formation of ME. In the present study, IPM, IPP and oleic acid were selected as the components of the oil phase. According to the screening results of emulsifier and co-emulsifier, EL-40 was used as emulsifier, while anhydrous ethanol was used as co-emulsifier; the Km value was 2:1 and the preparation temperature was 25˚C. IPM, IPP, oleic acid and the FM-CM volatile oil of FM-CM were mixed in a certain proportion as the oil phase. The optimum oil phase for ME preparation was screened.

Km value. In the preparation of MR, the Km value is the ratio of emulsifier quality to co-emulsifier mass. The ability of ME formation varies with Km value. According to the screening results of the emulsifier, co-emulsifier and oil phase, EL-40 was used as emulsifier, anhydrous ethanol as co-emulsifier, and IPM and FM-CM volatile oil as mixed oil phase. The preparation temperature was 25˚C, the effects of Km values of 1:1, 2:1, 3:1 and 4:1 on ME formation were investigated.

Preparation temperature. Throughout the screening procedures of the above prescription factors, the basic prescription was determined, and the effects of different temperatures (25, 30, 40 and 50˚C) on the preparation of ME were investigated.

Characterization of ME. ME was characterized as described in previous studies (15,24-27).

Appearance. The copper mesh of the carbon-plated support film was placed on the sealing film, and a drop of sample

(~30 μl) was added to the support film. Following incubation for 5-10 min, the excess solution was removed from the edge of the filter paper with a pointed sheet of filter paper, and placed on the filter paper for 1 min and allowed to drain. Then, the dried supporting film was placed on the sealing film, and a drop of uranyl acetate dye solution was added for 90 sec. Subsequently, the excess dye solution was removed with a pointed filter paper, clip it on the filter paper and dried for 3 h. A JEM-1200EX transmission electron microscope (JEOL, Ltd.) was used for observation, as described previously (26).

Type identification. According to the principle of 'similar miscibility', 1 g/l of sudan III (an oil-soluble dye) and methylene blue (a water-soluble dye) solution were added at room temperature, and their diffusion rates in ME were directly observed by the naked eye for 1 min to determine ME. When the ME was of the water in oil type, the diffusion rate of Sudan III was increased compared with that of methylene blue, and when the ME was of the oil in water type, the diffusion rate of methylene blue was increased compared with that of Sudan III.

pH and refractive index. Substances delivered by nasal administration must have a suitable pH value, and the pH value of the ME in turn affects the stability of the system. The pH and refractive index of the freshly prepared ME were measured using a PHS-3C pH meter (Shanghai Yidian Scientific Instruments Co., Ltd.) and WYA-2W Abbe refractometer (Shanghai Instrument Electrophysical Optical Instruments Co., Ltd.). At room temperature (25˚C), ultra-pure water was used as the control for calibration. In total, the pH values of 3 batches of ME were determined, and the average value was calculated.

Particle size and Zeta potential. In total, 3 batches of 1 ml MEs were selected, and the particle size and zeta potential of the freshly prepared MEs were determined by Malvern Zetasizer NanoZS90 instrument (Malvern Instruments Ltd.). According to the basic definition of ME, the particle size of ME should be between 10-100 nm (28). Zeta potential measurement is the use of electrophoretic scattering to detect the potential of suspended particles in a specific solution environment. Its purpose is to detect the charged properties of the particle surface, including electrical properties and potential level, in order to predict the stability of the whole suspension system.

Physical stability. In total, 3 batches of the same ME quantity were placed in a centrifuge tube in a 416 low-speed centrifuge (Gene Company, Ltd.) and centrifuged at 1,890 x g for 30 min at 4˚C. Following centrifugation for 30 min, the appearance of ME was observed.

Thermodynamic stability. A total of 3 batches of ME were subjected to a heating-cooling cycle experiment. The 10 ml ME was placed in a centrifuge tube. Following continuous heating-cooling cycles between 40 and 4˚C for 6 times, its appearance was observed.

In total, 3 batches of ME were subjected to a freezing-thawing cycle experiment. The 10 ml ME was placed in a centrifuge tube and frozen in the refrigerator at -20˚C for 24 h. Upon thawing at room temperature for 24 h, after 6 consecutive cycles, its appearance was observed.

Detection of eucalyptol in ME

Preparation of reference substance. The weight of the eucalyptol reference substance was 0.2541 g, the volume of anhydrous ether was set as 5 ml as the reference substance solution, and the concentration of the reference liquid was 50.82 mg/ml.

Preparation of samples. Weighed ME (1.0 g in 5 ml) was added to a brown flask, followed by 5 ml of anhydrous ether. Following demulsification with Eddy for 1 min, centrifugation at 1,890 x g, at 4˚C for 10 min and filtration of the supernatant through a 0.22 μm microporous filter membrane, the sample solution was obtained.

Gas chromatography-mass spectrometry (GC-MS). An Agilent 7890GC/5977MS (Agilent Technologies, Inc.) was used to investigate the subsequent experimental methodology and the content of Eucalyptol, according to the manufacturer's protocol. In addition, the content of eucalyptol in ME was determined.

Chromatographic conditions. The chromatographic column used in the present study was a HP-5 quartz capillary column (30 m x 0.25 mm, 0.25 μm). The temperature of the injection port was 250˚C. The initial temperature of the oven was 55˚C (held for 2 min), with an increase of 8˚C/min until it reached 80˚C (held for 0 min), followed by an increase of 6˚C/min until reaching 160˚C (held for 2 min), and then an increase of 8˚C/min until reaching 200˚C (held for 0 min), with an increase of 3˚C/min until reaching a final temperature of 250˚C (held for 3 min). The total run time was 42.25 min. The sample was injected at 40:1 with high-purity helium as carrier gas and a constant flow rate of 1 ml/min.

Mass spectrometry conditions. The ion source was an EI source, the multiplication voltage was 1.5 kV, the electron energy was 70 EV, the mass scanning range was 28-555 m/z, and the solvent delay was 3 min.

Methodological investigation. The eucalyptol reference solution was prepared by pouring 25, 50, 100, 200, 400 or 800 μl of eucalyptol solution into brown volumetric bottles. Then anhydrous ether was added to a final volume of 10 ml to prepare reference solutions with concentrations of 0.12705, 0.2541, 0.5082, 1.0164, 2.0328 and 4.0656 mg/ml were prepared. Linear regression was performed using the concentration and peak area as abscissa and longitudinal coordinates with Microsoft Excel 2016 (Microsoft Corporation).

The same sample solution was injected repeatedly 6 times according to the above conditions. The relative standard deviation (RSD) of the peak area of eucalyptol was determined, and the precision of the method was investigated. In total, 6 samples were prepared in parallel with the same batch of sample solutions. According to the aforementioned conditions, the RSD of the peak area of eucalyptol was calculated, and the experiment method was repeated six times to investigate the repeatability. The same batch of ME solution was prepared according to the treatment method of the test sample, and the peak area of eucalyptol was determined at 0, 1, 2, 4, 6, 8, 10, 12 and 24 h. The peak area of eucalyptol RSD was calculated to investigate the stability of the ME within 24 h. A total of 9 samples of 0.5000 g ME were precisely weighed and divided into 3 groups. Eucalyptol reference solution was added according to the known levels of 50, 100 and 150%, respectively. Following mixing, the samples were analyzed according to the above chromatographic conditions, and the recovery and RSD value were calculated.

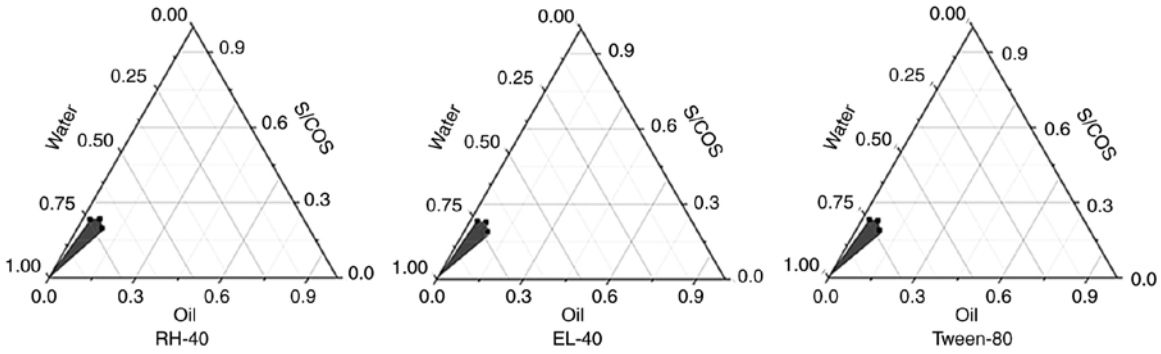

Figure 1. Pseudo-ternary phase diagram of different microemulsion emulsifiers.

Determination of eucalyptol. In total, 3 batches of samples were combined into a sample solution according to the treatment method of the sample solution, and the determination was performed according to the above chromatographic conditions.

Percutaneous permeability test. A marrow-destroying needle was inserted into the foramen magnum of the bullfrog, the spinal cord was transected left and right, and the brain was destroyed. The myelocytic needle was then withdrawn from the foramen magnum and inserted into the spinal canal to destroy the spinal cord. Death was confirmed by checking for complete relaxation of the limbs muscles. The abdominal skin of bullfrog was removed, and the fat and subcutaneous tissues were stripped, washed repeatedly with normal saline and fixed on the diffusion interface of the diffusion chamber, while the dermis was fixed to the reception tank. As the receptor fluid, 20% ethanol saline (Kunshan Hechuang Ultrasonic Instruments Co., Ltd) was used, which was injected into the reception tank following ultrasonic cleaning to remove bubbles. The temperature of the circulating water bath was 37±0.2˚C, and a constant speed of 350 rpm was employed to drain the bubbles in the receiving solution. Following balancing of the water bath for 30 min, 2.0 g ME and 2.0 g oil solution were placed into the supply tank. At 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h, 1 ml was sampled, and the same volume of blank receptor fluid was added to the reception tank and the bubbles were excreted (25,29-32). The ME solution was extracted with anhydrous ether 3 times, and the supernatant was collected and combined. The supernatant was centrifuged for 10 min in a high-speed centrifuge (335 x g, 4˚C), 1.5 ml supernatant was extracted with a disposable syringe and passed through a filter membrane of 0.22 μm, and the receiving solution at each time-point was obtained. The content of eucalyptol was determined according to the above chromatographic conditions, and the data were recorded. The cumulative transdermal volume was calculated according to the following formula:

$$Qn = (V \times Cn + \sum_{i-1}^{n-1} \times Vi \times Ci) / A$$

where Qn is the cumulative permeability per unit area at the time n, A is the effective transdermal area (superficial area =1.54 cm^2 and diameter =1.4 cm), Cn is the concentration of the drug measured at time n, V is the volume of the receiving pool (15 ml), and Vi is the volume of each sample. Cumulative transmittance was calculated using the formula Ln=Qn/W, where Ln is the cumulative transmittance and W is the content of eucalyptol in the sample.

Results

Screening of prescription and preparation factors of ME Emulsifiers. The data from the present study were collated to draw a pseudo-ternary phase diagram, as shown in Fig. 1, where the area of ME accounts for S_{RH-40} =0.0283, S_{EL-40} =0.0298 and $S_{Tween-80}$ =0.0287. The Tween-20 group could not form ME, and therefore the pseudo-ternary phase diagram could not be generated. The proportion of ME area in the EL-40 group was the largest. Therefore, EL-40 was selected as the emulsifier of FM-CM volatile oil ME.

Co-emulsifiers. A co-emulsifier can adjust the HLB value of the emulsifier and form smaller droplets, and an auxiliary emulsifier can improve the formation of an ME. The results of pseudo-ternary phase diagram in Fig. 2 indicate that the area of ME was $S_{anhydrous\ ethanol}$ =0.0782, $S_{1,2\text{-propanediol}}$ =0.0417, $S_{glycerol}$ =0.0414 and $S_{PEG-400}$ =0.0522. Anhydrous ethanol had the largest proportion of ME area; thus, it was selected as the co-emulsifier of FM-CM volatile oil ME.

Oil phase. The results of pseudo-ternary phase diagram showed that the proportion of ME area was S_{IPP} =0.0530, S_{IPM} =0.0766 and $S_{oleic\ acid}$ =0.0441. As the proportion of ME area was the largest in the IPM group, this was selected as the oil phase of FM-CM volatile oil ME. The results are presented in Fig. 3.

Km values. Different Km values have different effects on the formation of ME. According to the results of the pseudo-ternary phase diagram in Fig. 4, the area of ME was $S_{1:1}$ =0.0450, $S_{2:1}$ =0.0766, $S_{3:1}$ =0.0540 and $S_{4:1}$ =0.0398. When Km was 2:1, the ME area was the largest; thus, 2:1 was determined to be the best Km value.

Preparation temperature. By generating a pseudo-ternary phase diagram, the area of ME was $S_{25°C}$ =0.0766, $S_{30°C}$ =0.0696, $S_{40°C}$ =0.0650 and $S_{50°C}$ =0.0613, suggesting that 25˚C was the best preparation temperature. The results are shown in Fig. 5.

Preparation of optimal prescription ME. According to the above screening results, the best prescription of FM-CM volatile oil ME was as follows: Th emulsifier was EL-40; the co-emulsifier was anhydrous ethanol; the Km was 2:1; and IPM and FM-CM volatile oil were used as mixed oil phase at 1:1. The mixed emulsifier accounted for 25.81%; the

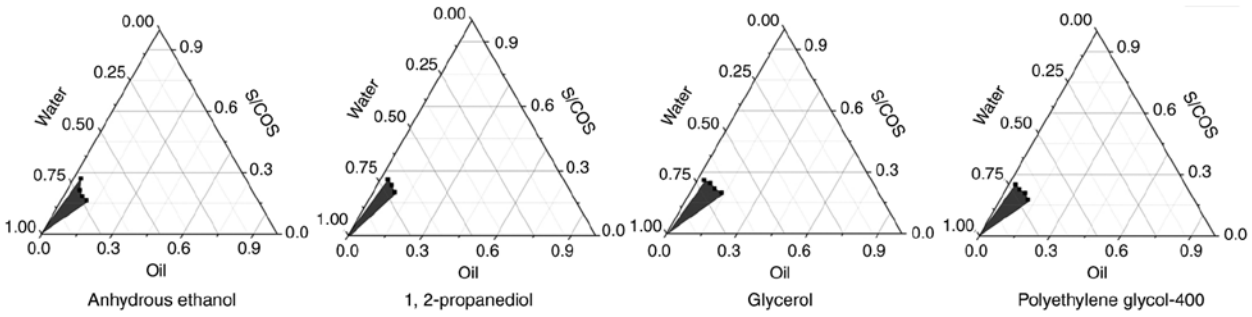

Figure 2. Pseudo-ternary phase diagram of different microemulsion co-emulsifiers.

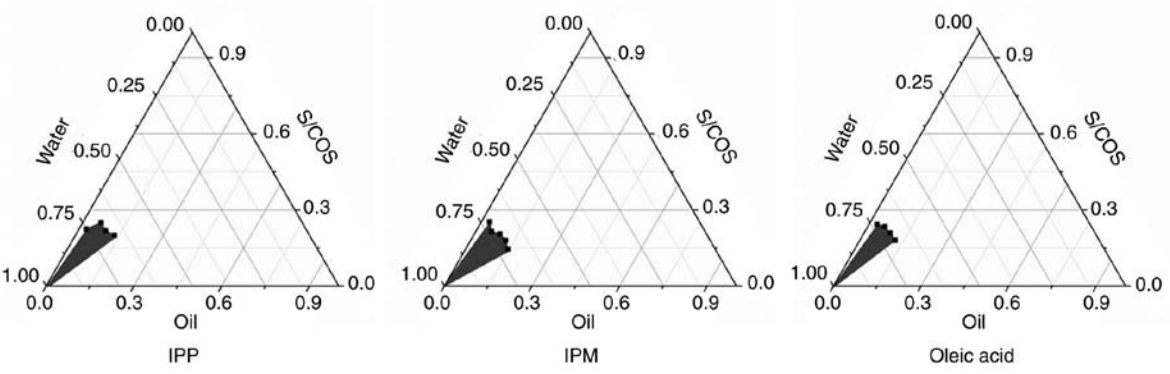

Figure 3. Pseudo-ternary phase diagram of different microemulsion oil phases.

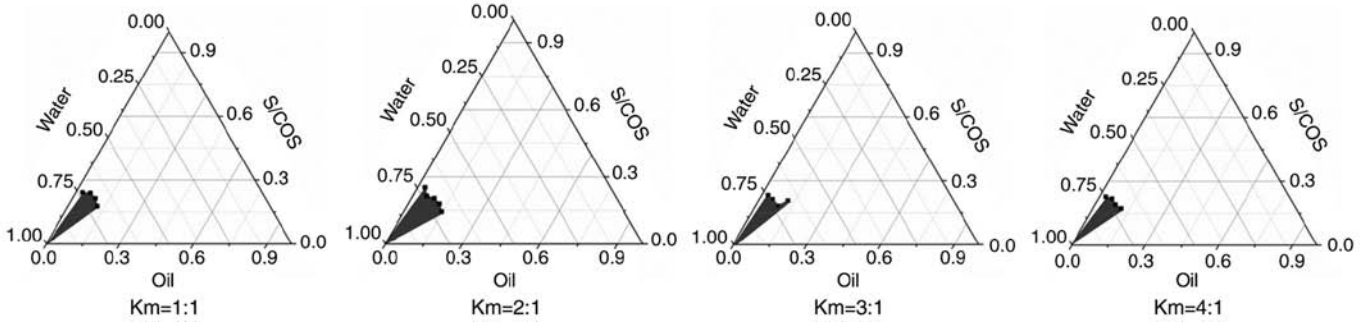

Figure 4. Pseudo-ternary phase diagram with different microemulsion Km values.

mixed oil phase accounted for 2.81%; and the aqueous phase accounted for 71.38% of the total ME system. The preparation temperature was 25˚C. The water representing 71.38% of the system was slowly added to form clear and transparent ME droplets.

Characterization

Morphological observation. The results of the observation of the appearance and morphology of the ME are presented in Fig. 6. The ME was a sphere with a round appearance and uniform particle size distribution.

Identification of ME type. The methylene blue diffusion rate of the ME prepared in the present study was markedly increased compared with that of Sudan III, which indicates that it was an O/W ME (Fig. 7).

pH and refractive index. The pH value of ME was 5.67±0.01 and the refractive index was 1.4198±0.0011 nd at 25˚C.

Particle size and Zeta potential. As shown in Fig. 8, the particle size distribution of ME was uniform and the average particle size was 14.62±0.4576 nm. The mean polydispersity index value was 0.0747±0.0265 (n=3), and when there was only one peak in the range of 10-100 nm, the average potential was -4.06±0.0702 mV (n=3). These results indicated that the ME met the requirement of particle size, and the system was stable.

Physical stability. The appearance of the 3 batches of ME following high-speed centrifugation for 30 min remained uniform, clear, and transparent, and there was no stratification observed, demonstrating that the centrifugal stability of ME was good (Fig. 9A).

Thermodynamic stability. After 6 heating-cooling and freezing-thawing cycles, the 3 batches of ME remained clear and transparent, and there was no stratification, indicating that the thermodynamic stability of ME was good (Fig. 9B).

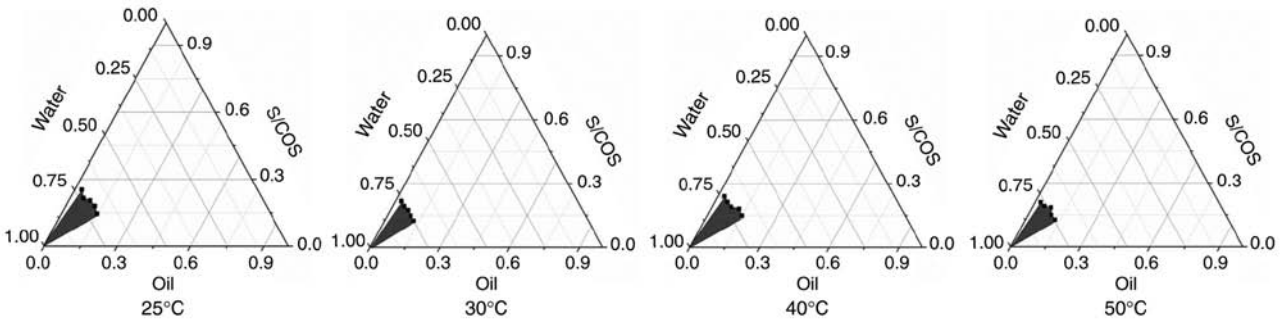

Figure 5. Pseudo-ternary phase diagram of different microemulsion preparation temperatures.

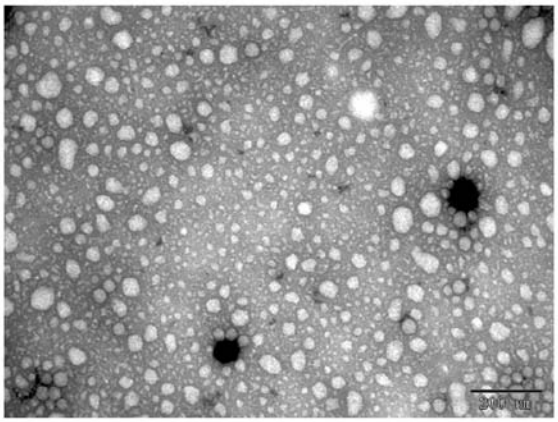

Figure 6. Transmission electron micrograph.

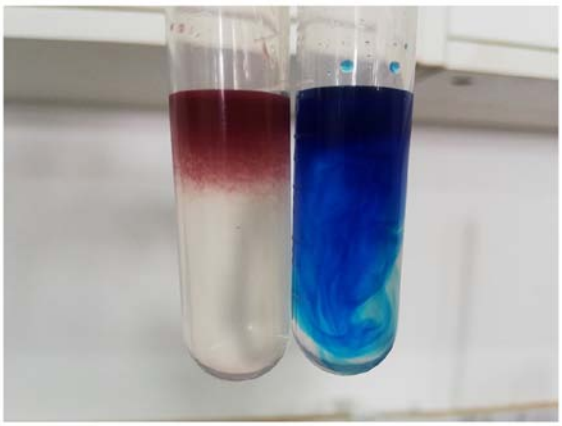

Figure 7. Identification of microemulsion type.

Methodological investigation. The calibration curve of the peak area and concentrations for eucalyptol was linear, ranging from 0.12705-4.0656 mg/ml. The calibration curve was Y=37,4632X + 2x10^8 (R^2=0.9992), where Y represents the eucalyptol peak area and X represents the concentration of eucalyptus reference substance. The chromatograms of the reference substance and the sample solution are shown in Fig. 10. In the precision experiment, the RSD of the eucalyptol peak area was 1.36%, demonstrating that the precision of this method was good. In the repeatability experiment, the RSD of the eucalyptol peak area was 2.20%, indicating that this method has good repeatability. In the stability experiment, the RSD of the eucalyptol peak area was 1.93%, indicating that the sample remained stable for 24 h. In the sample recovery experiment, the mean recovery rate was 99.27%, and the RSD value was 2.91%. These results showed that the accuracy of the experimental observations was high.

Determination of eucalyptol concentration. According to the results of sample content determination, the mean content of eucalyptol in the ME was 2.57 mg/g, as shown in Table I.

Percutaneous permeability test. The curve was generated by considering the cumulative permeability Q_{24} (μg/cm^2) as the longitudinal coordinate and the sampling time t as the abscissa. Linear regression analysis of the obtained curve was performed. The linear slope represents the steady penetration rate *J*ss (μg/cm^2/h). The results are shown in Table II, Fig. 11 and Table III.

These results indicated that the cumulative permeabilities of eucalyptol in the ME and oil solution were 301.0800±2.80 and 73.3491±2.19 μg/cm^2, respectively, and the steady state penetration rates were 9.4349 and 2.0082 μg/cm^2/h, respectively. The cumulative permeability of the ME was increased 4.10-fold compared with that of the oil solution, and the steady penetration rate was increased 4.70-fold compared with that of the oil solution, which indicated that ME could effectively promote the transdermal absorption of the drugs.

Discussion

Bitongning drops serve an important role in the clinical treatment of AR due to their active ingredient, the aromatic water of FM and CM, and previous studies have shown that their therapeutic effect on AR is due to their volatile oil components (33-37). The aim of the present study was to increase the drug concentration in a standard AR treatment method by replacing traditional aromatic water with volatile oil. Volatile oils have an important therapeutic effect in numerous TCM treatments, and are an indispensable functional ingredient (38). According to the theory of TCM, volatile oils function as 'aromatic strings'. An aromatic string is called 'Fangxiangzouchuan' in TCM. It can be interpreted as 'the fragrance of the medicine can be dispersed everywhere', the curative effect is exact (39). However, volatile oils are a fat-soluble component with poor water solubility that can be highly irritable. In the preliminary experiments in the present study, the best compatibility ratio of the volatile oils from FM

Table I. Eucalyptol content in microemulsions.

Sample number	Sampling quantity, g	A1	A2	Ā	Eucalyptol content, mg/g	Average content, mg/g	RSD, %
1	1.0167	412958743	383474502	398216622.5	2.60	2.57	1.19
2	1.0012	381962376	405573769	393768072.5	2.54		
3	1.0032	398652865	391974721	395313793	2.56		

A_1; A_2; Ā; RSD, relative standard deviation.

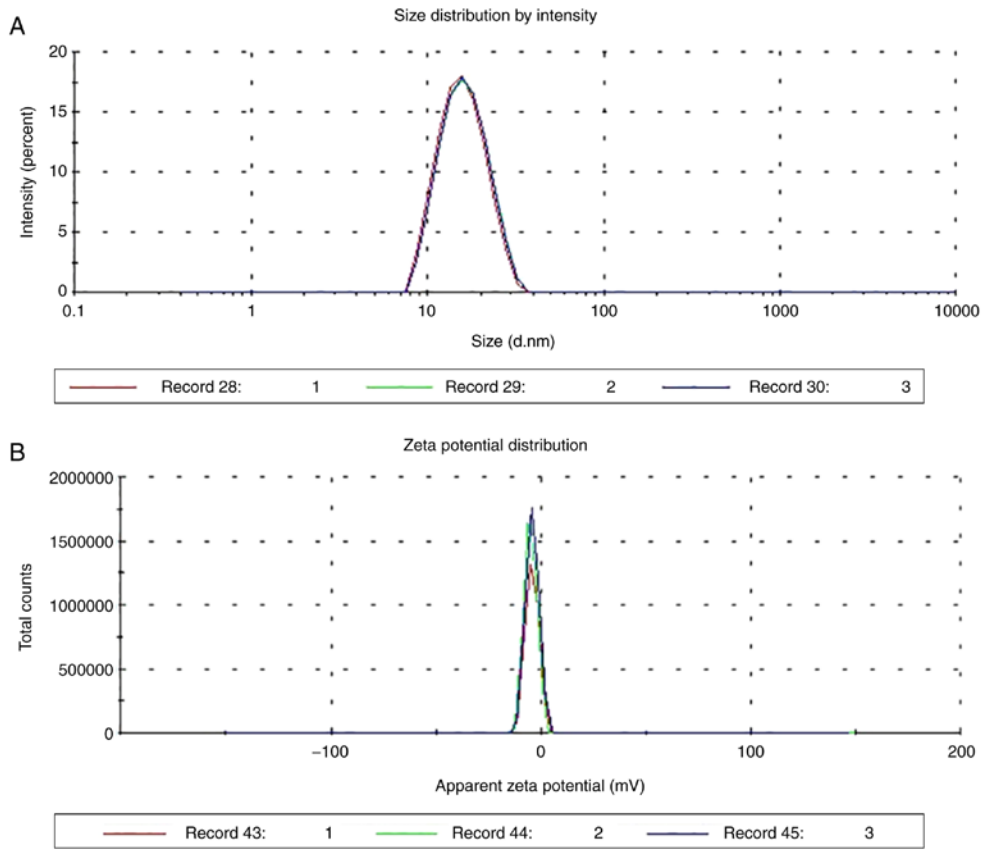

Figure 8. Particle size and Zeta potential distribution of ME. (A) Particle size distribution of ME. (B) Zeta potential distribution of ME. ME, microemulsion.

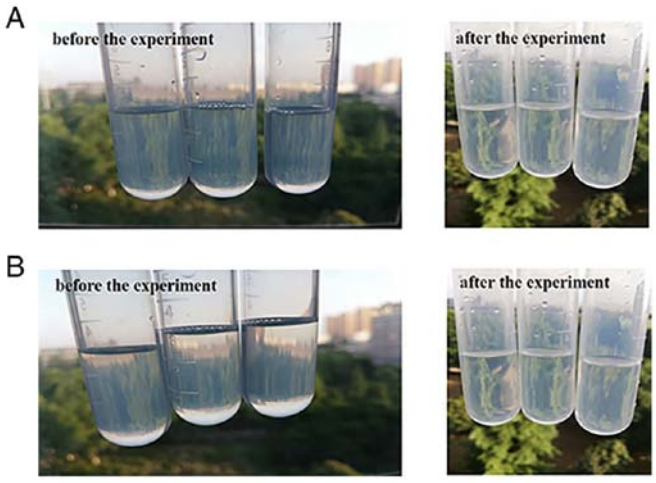

Figure 9. Thermodynamic stabilization of microemulsion.

and CM in the treatment of AR was screened, and the results revealed that the best proportion was 7:1 FM: CM. In the present study, an ME was generated to improve bioavailability, to resolve the issues of poor absorption and low solubility normally associated with volatile oils, and to decrease drug toxicity, irritation, and adverse reactions.

The ME was prepared by water titration with different emulsifiers, co-emulsifiers, oil phases, Km values, and preparation temperatures. The area of the ME was mapped using a pseudo-ternary phase diagram, and the particle size was combined with the size of the ME The best formula for the preparation of ME was determined by centrifugal stability and other factors. When the Km value increased from 1.0 to 2.0, the area of ME increased, which was primarily due to the increase of emulsifiers ratio, emulsifiers can effectively reduce oil/water interfacial tension and decrease the interfacial film tension formed by ME, thereby enhancing the emulsification ability to

Table II. Qn and Ln per unit area of ME at each time-point (n=3).

	Eucalyptol Qn, μg/cm^2		Ln, %	
Time-point, h	ME group	Oil solution group	ME group	Oil solution group
0.5	54.2507±0.4277	21.3442±1.0544	1.06	0.42
1	85.4878±3.2467	31.9255±3.0570	1.66	0.62
2	101.6378±1.1280	36.3763±1.2314	1.98	0.71
4	120.9467±1.5481	39.6531±0.4615	2.35	0.77
6	133.4428±1.4452	42.5442±0.3600	2.60	0.83
8	145.0037±3.2474	45.3398±0.3371	2.82	0.88
10	160.0303±0.4325	50.6580±1.5446	3.11	0.99
12	173.7156±3.5513	60.7943±0.3699	3.38	1.18
24	301.0797±2.8009	73.3491±2.1919	5.86	1.43

Data are presented as the mean ± standard deviation. Qn, cumulative permeability; Ln, cumulative transmittance; ME, microemulsion.

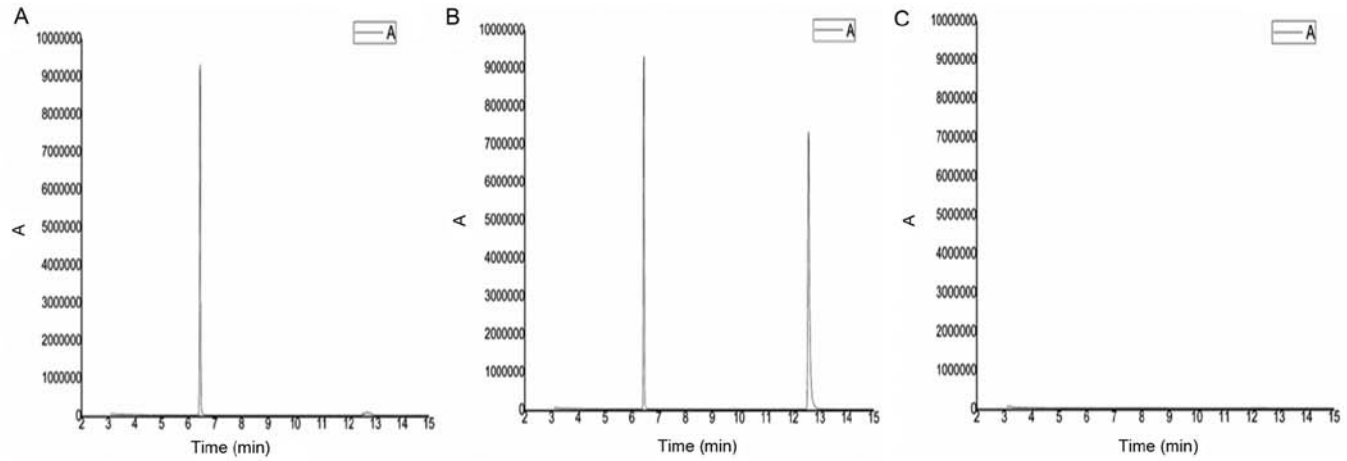

Figure 10. Gas chromatography-mass spectrometry chromatogram. (A) Reference substance. (B) Microemulsion sample. (C) Blank solvent sample. A, peak area

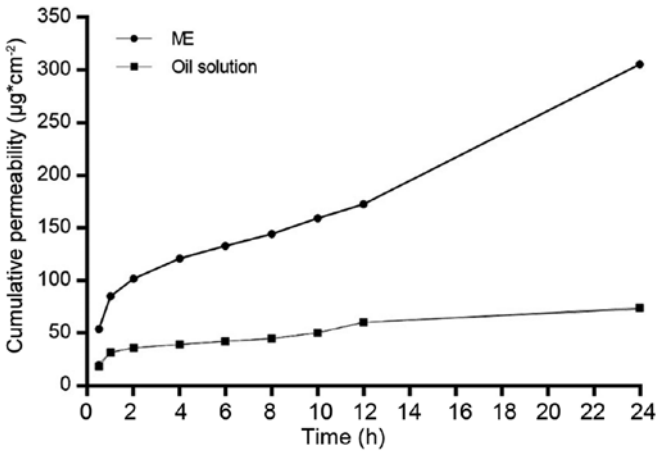

Figure 11. Percutaneous permeation curve of microemulsion and oil solution. Data are presented as the mean ± standard deviation (n=3).

form a larger ME area. When studying Km, it has been observed that when the Km value increased from 2.0 to 4.0, possibly due to the increase of emulsifiers to a certain value, an increase in viscosity and expansion of the gel region and double continuous zone were observed, which led to the decrease of the formation area of ME (40). When examining preparation temperature, the area of ME formation was observed to decrease with the increase in preparation temperature, since volatile oil was used as part of the mixed oil phase. It was hypothesized that this may have been caused by the increase in volatilization of the drug with the increase in temperature. Therefore, 25˚C was selected as the most suitable temperature for ME preparation. However, this hypothesis requires further experimental verification. The ME was identified as an O/W ME, and its particle size, pH value and refractive index all met the requirements of an ME. It is generally considered that the absolute value of Zeta potential in a stable-dispersion system should be >30 mV, and the larger the absolute value is, the more stable the system is (41). However, the absolute value of Zeta potential in the present study was <30 mV. It has been reported that the Zeta potential of ME may be low (41), which does not indicate that ME is unstable. Instead, the primary reason for this is that the ME is an uncharged system. The emulsifier EL-40 in ME is a non-ionic solubilizer, which can be used in the emulsification of vegetable, animal, and mineral oils. It can also effectively

Table III. Comparison of transdermal permeation parameters between ME and oil solution groups (n=3).

Group	Cumulative permeability - time equation	Steady penetration rate Jss, $\mu g/cm^2/h$	Cumulative permeability, $\mu g/cm^2$
ME group	Q=9.4349t+70.971 (R^2=0.9758)	9.4349	301.0800±2.80
Oil solution group	Q=2.0082t+29.604 (R^2=0.9119)	2.0082	73.3491±2.19

Data are presented as the mean ± standard deviation. ME, microemulsion.

promote the transdermal absorption of drugs and decrease skin irritation (42). The co-emulsifier anhydrous ethanol may decrease the polarity of the aqueous phase and surface tension, thus compensating for the poor fluidity of the oil-water interface film when a emulsifier is used alone, and when combined with emulsifiers, the preparation of ME is improved (43,44). Previous studies reported that adding the volatile oil of TCM to the oil phase of ME could improve transdermal permeation (45-47). In the present study, according to preliminary experiments, the drug with mass ratio of 1:1 and IPM were used as mixed oil phase to promote drug absorption. During the *in vitro* transdermal comparison between ME and oil solution in the present study, it was identified that the cumulative and steady permeabilities of ME were significantly increased compared with those of the oil solution group, indicating that ME may effectively promote the transdermal absorption of volatile oil. These results provide a reference for follow-up development and research of ME.

In the present study, the preparation method and quality of ME were examined systematically. It was identified that the *in vitro* transdermal performance of ME was significantly increased compared with that of the oil solutions, and that its properties were stable. In order to further demonstrate the good bioavailability of ME, *in vivo* pharmacokinetic studies or cell experiments will be conducted in the future. Notably, in the preparation process of ME, the specific formula used and preparation process factors have important effects on the formation of ME. Consequently, the selection of the appropriate emulsifier, co-emulsifier, oil phase, Km, and preparation temperature are important.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YL performed the experiments and data analysis, and wrote the manuscript. JZ performed the experiment and participated in the analysis of the experimental data. YS conceptualized the study and analyzed the experimental data. JT and YW performed experiments. XZ, DG and MY conceptualized the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Shaanxi University of Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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