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Full Length Research Paper

Preparation of triptolide ethosomes

Jin-Guang Chen[1,2]*, Yu Jiang[1] and Zhi-bo Yang[3]

[1]Department of Dermatology, People's Hospital of Zhongshan City, Zhongshan, 528403, China.
[2]Department of Dermatology, Tai Zhou Municipal Hospital, Taizhou, 318000, China.
[3]Department of Dermatology, Second Affiliated Hospital Hunan Chinese Medical College, ChangSha 410005, China.

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Filming-rehydration and ultrasonic method were combined to prepare ethosomes as a carrier of triptolide. A laser dynamic scattering instrument was used to determine the particle size of ethosomes. The average particle size was calculated based on the measurements of five different batches of ethosomes. To determine the entrapment efficiency, free triptolide was isolated by ultracentrifugation. The concentration of triptolide was then determined using high-performance liquid chromatography (HPLC). There was an inverse relation between the average size of the triptolide ethosomes and the concentration of ethanol, and the size increased as the phospholipid concentration increased; the entrapment efficiency of ethosomes increased with the increasing concentrations of ethanol and phospholipid. An optimal formulation was obtained when lecithin and ethanol were 2% (w/v) and 45% (v/v), respectively. Triptolide ethosomes exhibited a small particle size, even distribution and high entrapment efficiency (98%). Ethosomes have high entrapment efficiency, a good percutaneous permeability and qualities that make them particularly suitable to serve as a liposome drug carrier. The concentration of ethanol and phospholipids developed a positive impact on entrapment efficiency. Triptolide ethosomes with a ratio of 2% (w/v) lecithin to 45% ethanol (v/v) have, relatively, a small particle size, even distribution and high entrapment efficiency.

Key words: Ethosomes, triptolide, liposome.

INTRODUCTION

In Chinese traditional medicine, the extract of *Tripterygium wilfordii,* a medicinal plant, is being used by virtue of its various functions such as promoting blood flow, heat-clearing, detoxification, detumescence, and elimination of pathogens (Qian et al., 1995). Perusal of literature reveals that the extracts of *T. wilfordii* produce significant biological effect such as anti-inflammatory, immunological regulation, anti-tumor activity, curing rheumatic arthritis, dermatosis, nephritis of renal glomerulus, lupus erythematosus, nephrotic syndrome, rejection of organ transplantation, and asthma (Cantera et al., 2006; Zhang et al., 2007). However, its clinical application and generalization has been considerably limited due to its high toxic effects. Many patients experience various undesirable side effects on long-term consumption of the medicinal plant (Jia, 2006).

*Corresponding author. E-mail: jinguangchencn@126.com.

Hitherto, there are over 70 components that have been found in *T. wilfordii*, which include epoxy diterpenes, triterpenes, and alkaloid. Triptolide is a type of epoxy diterpene lactone compound. Besides triptolide possessing a strong anti-tumor activity, it also has the pharmacological activities of anti-inflammatory, anti-tumor, and immune regulation (Qian et al., 1995; Chen, 2001).

Nowadays, in the market, *T. wilfordii* class medicine mainly takes the form of orally-taken preparations like Glucosidorum Tripterygll Totorum, *T. wilfordii* dripping pills, *T. wilfordii* microcapsule and *T. wilfordii* oral liquid, large dose of which may increase toxicity to organism, or leads to partial loss of its effective constituent via the first-pass effect. Therefore, there is an urgent need to develop preparation with a precise curative effect and less toxic side effects reactions.

Ethosome is a neotype of liposome carrier in drug delivery system in recent years which has virtues of high deformability, high entrapment efficiency and

percutaneous permeability through the keratoderma barrier. Compared to conventional liposome, ethosome has a more stable structure and quality which not only can promote percutaneous absorptivity of medicine, increased drug storage in the skin, and drug mobility to the cells, but also has the characteristics of prolonged action and avirulent. Thus, it has become important in the development of new drug carriers (Fang et al., 2009; de la Presa et al., 2009). As a topical application carrier, ethosome has the characteristics of a stable structure and no obvious toxicity. Ethosome enables the drug to be released slowly and thus to increase its percutaneous capability and promote the curative efficacy, which endows ethosome with great potential in the development of biological and chemical percutaneous agents. Local application with the aid of ethosome can avoid the first-pass effect on liver and the degradation of gastrointestinal tract, maintain the stability of the medicine in focal zone, reduce the toxicity and untoward reactions, reduce the frequency of administration, and increase the clinical curative effect and compliance of patients. Many researchers have indicated that the ethosomes have rather stable structures and nature, which endow ethosomes with a great potential in the development of biological and chemical percutaneous agents. This paper provides further understanding of ethosomes as the carrier of externally applied agent, triptolide in order to bring the desirable biological activity of triptolide while reducing its toxic side effects. Filming-rehydration and ultrasonic methods were combined to prepare ethosomes, which has a small particle size, even distribution and a high entrapment efficiency.

MATERIALS AND METHODS

Preparation of ethosomes

Triptolide ethosomes was prepared by combining filming-rehydration method ultrasonic method. Ethosomes were comprised of 1 to 3% (w/v) lecithin, ethanol with volume fraction of 30 to 45% (v/v), Triptolide (0.1%) and water. Lecithin and triptolide were dissolved in a glass bottle, stirred and mixed well with a magnetic stirrer. Then, the glass bottle was connected with an injector and sealed. Later, ethanol was added without vaporization. Mixture was poured into a round bottom flask and prepared a thin film using roto-evaporator. The preceding procedure was repeated. Double-distilled water (100 ml) was added (methyl nicotinate ethosomes were obtained in this procedure) to rehydrate the film. It was thoroughly homogenized for 5 min using sonde-type ultrasonic instrument (with power of 300 w) followed by filtering the ethosomes using a 0.22 μm disposable filter. All the procedures in this test were carried out under nitrogen at room temperature. Drug add-in volume was regulated to reach the final concentration of 0.1% (triptolide) or 0.2% (methyl nicotinate). Triptolide was not added in the aforementioned preparation process to produce empty ethosome suspension.

Particle size analysis of ethosomes

Particle size was measured using the laser dynamic scattering Mastersizer 3000 (Malvern, England) immediately after diluting and filtering the ethosomes with a 0.22 μm filter. The average particle size of ethosomes was calculated based on the measurements of 5 batches of ethosomes.

Measurement of entrapment efficiency (EE) of ethosomes

Triptolide ethosomes were obtained by isolating the unentrapped part with ultracentrifuge (56000 rpm at 4°C for 40 min) after storing overnight at 4°C. Upper clear solution was removed and the sediments of triptolide ethosomes remained. Drug absorbed by the ethosomes was measured by HPLC U-3000 (DIONEX, USA). The entrapment efficiency was calculated following the formula:

$$\frac{D_{\mathrm{E}}}{D_{\mathrm{E}} + D_{\mathrm{S}}} \times 100$$

D_E: the drug content measured from the parvules.
Ds: the drug content measured from upper clear solution.

The results obtained were indicated as the average value ± standard deviation from 3 different batches of ethosomes.

HPLC analysis of samples

Chromatographic column used was μ-Bondapak C18 (250 mm × 4.6 mm, 5 μm) with the mobile phase as methanol to water (43:57), floating speed: The flow rate was 1 ml•min^{-1}; measurement wavelength: 219 nm; the column temperature was 30°C; volume: injectable sample volume was 20 μl; the ultraviolet detector with a wavelength of 219 nm was used: 219 nm; measuring range: 0.04 AUFS; and paper speed: 3mm/min. Theoretical number of plates were counted based on the peak value of triptolide.

Optimized design of ethosome prescription

The basic prescription and conditions for *Tripterygium* ethosome preparation were selected by single factor analysis, and then optimized by orthogonal experiment. The optimized prescription design includes three variables such as the concentration of phospholipid (A), the content of ethanol (B), and ultrasonic processing time (C) and they were counted as the major investigating factors, and 3 levels from each factor were taken. The experiment was carried out according to the orthogonal array, and the results were analyzed taking the corresponding entrapment efficiency EE (%) as target. The prediction schemes are presented in Tables 1 and 2.

RESULTS

Impact of phospholipid concentration on entrapment efficiency

The impact of different concentrations of phospholipids such as 1.0, 1.5, 2.0, 2.5 and 3.0% on the entrapment efficiency of ethosomes was evaluated. Results showed that both the particle size of ethosomes and the entrapment efficiency of *Tripterygium* ethosomes increased with the increasing concentration of phospholipids, the gathering probability of formed *Tripterygium* ethosomes increased accordingly (Table 3).

Table 1. Optimization of *Tripterygium* ethosome preparation by orthogonal array.

Number	A% (w/v)	B% (v/v)	C (min)
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

A, Phospholipids concentration %(w/v); B, ethanol content % (v/v); C, ultrasonic time (min).

Table 2. Optimization of *Tripterygium* ethosome preparation by orthogonal array factor – level.

Level	A% (w/v)	B % (v/v)	C (min)
1	1.5	35	5
2	2.0	40	8
3	2.5	45	10

NoA, Phospholipids concentration % (w/v); B, ethanol content %(v/v); C, ultrasonic time (min).

Impact of ultrasonic processing time on entrapment efficiency

With different ultrasonic processing time (2, 5, 8, 10 and 12 min), results on the percent entrapment efficiency of the drug increased correspondingly with increasing ultrasonic processing time, and reached the peak (66.07 ± 1.25) at 8 min. But different ultrasonic processing time did not cause obvious change in the particle size (Table 4).

Impact of ethanol content on entrapment efficiency and the average particle size

With different amounts of ethanol (30, 35, 40, 45 and 50%) used in the preparation of *Tripterygium* ethosomes, results showed that the entrapment efficiency increased with increasing percent of ethanol (Table 5), while the average particle size and the gathering probability among ethosomes showed decreased tendencies, and the percutaneous effect of the drug increased when the particle size became less than 100 nm. Thus, on the premise of stable ethosome structure, ethanol content should be maximized to increase the percutaneous capability.

Optimization of preparation process of *Tripterygium* ethosomes

Based on the results obtained by single factor analysis, considering the concentration of phospholipid (A), the content of ethanol (B), and ultrasonic processing time (C) as major investigating factors and 3 levels from each factor, the experiment was carried out according to the orthogonal array. The experimental results were analyzed with the corresponding entrapment efficiency (EE) as target (Table 6). The impact order of the three factors from high to low was A > B > C, and factor A: average IIj > average IIIj > average Ij; factor B: average IIIj > average IIj> average Ij; factor C: average Ij > average IIIj > average IIj. Based on the medium values, the most optimized prescription was $A_2B_3C_1$, that is, 2.0% (w/v) of phospholipid 2.0% (w/v) + 45% (w/v) of ethanol + 5 min of ultrasonic processing time. The entrapment efficiency of the produced *Tripterygium* ethosomes based on this specific prescription was 97.9 ± 4.5%.

Evaluation of the quality of *Tripterygium* ethosomes

Ten grams of *Tripterygium* ethosomes was taken from 3 batches of samples, and centrifuged at 3000 r/min^{-1} for 30 min. No delamination was observed. Three batches of samples were placed in an incubator at 60°C or at -10°C for 24 h, and then brought to room temperature. No delamination was observed. These proved that ethosomes were comparatively stable and not easily delaminated or precipitated at normal temperature. Observed with eyes, visual observation of the ethosomes was found to be ivory white suspl with proper viscosity and good ductility, and the colloid was smooth and evenly-distributed. After probe-type ultrasound, it turned into transparent colloid solution.

Percutaneous capability through micro porous membrane (orifice diameter of 0.15 μm) after deformation under external pressure (0.1 to 0.3 MPa) was analyzed. First, the time consumed by 5 ml flexible liposome colloid solution in penetrating through the filter membrane was observed. Then, the time taken by 5 ml water in penetrating through the filter membrane was observed. The relative passing rate (P) was calculated: P = Vethosomes / Vwater × 100%.

The experimental results showed that with the increasing pressure, value of P was increased, and P value of *Tripterygium* ethosomes produced in the current experiment reached 91% under external pressure of 0.3 MPa, which indicated that *Tripterygium* ethosomes have better deformability.

Average particle size is the important parameter which affects the curative effect of the medicine. Five batches of *Tripterygium* ethosome were separately prepared according to the most optimized prescription. The average particle size of *Tripterygium* ethosomes after dilution was measured as 51.8 ± 4.7 nm using laser particle analyzer

Table 3. Impact of phospholipid concentration on entrapment efficiency (n = 5, $\bar{X}$ ± S).

Lecithin % (w/v)	1.0	1.5	2.0	2.5	3.0
EE (%)	85.1 ± 2.8	87.1 ± 3.7	89.1 ± 4.7	90.1 ± 2.9	94.5 ± 3.4

Table 4. Impact of ultrasonic processing time on entrapment efficiency.

Ultrasonic processing time (min)	2	5	8	10	12
EE (%)	52.11 ± 2.74	61.75 ± 2.64	66.07 ± 1.25	61.08 ± 3.51	56.31 ± 1.39
Particle size (nm)	76.71 ± 7.23	74.23 ± 6.92	80.54 ± 7.99	81.09 ± 8.23	69.97 ± 5.52

Table 5. Impact of ethanol content on entrapment efficiency (n = 5, $\bar{X}$ ± S).

Ethanol % (v/v)	30	35	40	45	50
EE (%)	37.26 ± 1.21	44.32 ± 1.25	57.72 ± 2.32	70.05 ± 3.17	75.41 ± 2.35

Table 6. The result of optimization of *Tripterygium* ethosome preparation process.

Number	A	B	C	EE (%)
1	1	1	1	89.20
2	1	2	2	91.45
3	1	3	3	92.31
4	2	1	2	95.11
5	2	2	3	96.59
6	2	3	1	97.65
7	3	1	3	93.64
8	3	2	1	95.79
9	3	3	2	94.01
Ij	272.96	277.95	282.64	
IIj	289.35	283.83	280.57	
IIIj	283.44	283.97	282.54	
Average Ij	90.99	92.65	94.21	
Average IIj	96.45	94.61	93.52	
Average IIIj	94.48	94.66	94.18	
Rj	5.46	2.01	0.69	

A, Phospholipids concentration % (w/v); B, ethanol content % (v/v); C, ultrasonic time (min). Average IIj - Average Ij = Rj5.46; Average IIIj - Average Ij = Rj2.01; Average Ij - Average IIj = Rj0.69.

and the distribution of ethosomes was found to be tight (Figure 1).

Measurement of triptolide content of the samples

A stock of standard *Tripterygium* was prepared by accurately weighing 4 mg of standard *Tripterygium* which was in a 100 ml volumetric flask, and dissolved in methanol. Different volumes of the stock such as 1, 2, 4, 8 and 10 ml were, placed in 10 ml volumetric flasks, and diluted with methanol. After filtering with millipore, 10 µl from each concentration was separately injected into liquid chromatography. The respective peak areas were recorded. The standard sample was injected thrice. Standard curves were drawn and regressed, with average value peak area (A) as axis Y and sample volume (µg) as axis X. The equation of linear regression

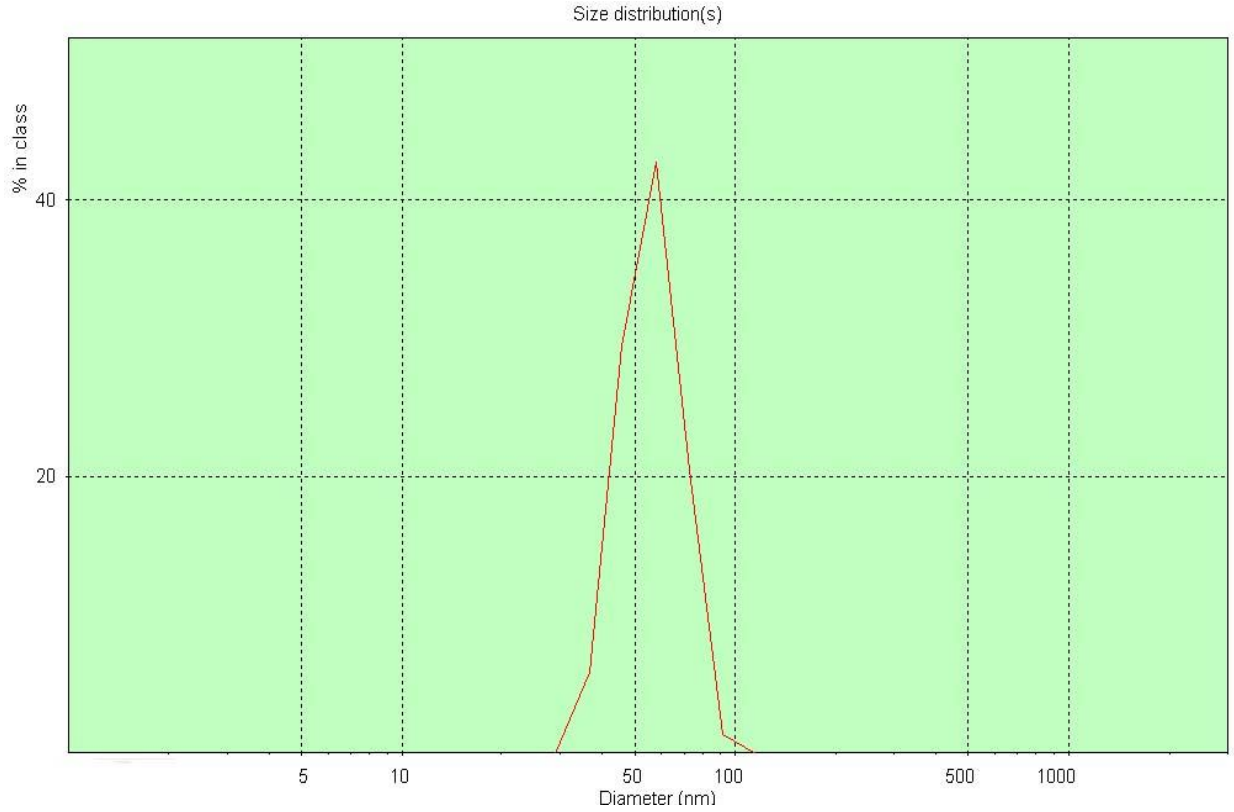

Figure 1. *Tripterygium* ethosome particle size and its size distribution. The average size was 51.6 nm, and the poly index was 0.342.

was found to be: A = 75.3152 + 211.8861X, r = 0.9999, linear range is: 0.04 to 0.4.

Triptolide ethosome sample (1 ml) from 3 batches prepared as per optimized prescription was separately drawn into a 100 ml measuring flask, diluted with methanol and mixed well. A sample of 20 µl was injected into high-performance liquid chromatography (HPLC), and, the peak area was recorded and their values were subjected to regression equation. The marked contents of the 3 batches of samples were calculated as 99.10, 96.84 and 97.92%, respectively.

Test of precision

Same sample solution was precisely taken, and the application of sample was repeated 5 times. The peak area of triptolide was measured, and its RSD obtained as 1.06% (n = 5).

Measurement of entrapment efficiency

According to the optimized prescription, 3 batches of samples of Triptolide ethosomes were prepared. The unentrapped part was removed using ultracentrifuge at 56000 r/min for 45 min after storing overnight at 4°C. The upper clear solution was removed, and the parvules of Triptolide ethosome were kept. The drug content was measured using HPLC. The entrapment efficiency was calculated using the following formula:

$$\frac{D_E}{D_E + D_S} \times 100$$

D_E is the drug content measured from ethosome parvules, and Ds is the drug content measured from upper clear solution.

The results were indicated as average value ± SD from 3 different batches of ethosomes. The average entrapment efficiency in this study was 97.9 ± 4.5% (n = 3). To our knowledge, there has been no report about the entrapment efficiency of ethosome.

DISCUSSION

As a vesicle-structured neotype of drug carrier, ethosome has the characteristics of good deformability, high entrapment efficiency, good permeability, and good stability, which enable them to effectively carry the drug

through the skin, and to penetrate through cuticle to get into deeper layers (even into the blood), and meanwhile, to provide the effective internal transmission in the cell for lipophilic drugs. It was the good fluidity and deformability given by ethanol that enabled ethosomes to carry drug to penetrate through the eyelet, 1/5 to 1/10 less than its size without much change in shape under the pressure of hydration, so as to penetrate through cell membrane and release drug more effectively than liposomes (Godin and Touitou, 2003). Generally, ethosome comprises of high concentration of low molecular weight alcohol (ethanol, propylene glycol, and isopropyl alcohol), phospholipid and water, and sometimes the addition of cholesterol (zhu, 2003). Ethosomes containing high concentration of ethanol could increase the flexibility and fluidity of lipid bilayer so that the liposomes with ethanol become apt to deform and penetrate through the unstuck cuticle; in addition, the interaction of ethanol and cuticle could increase the solubility of the drug in the cuticle, decrease the phase-transmission temperature of the cuticle, change the dense arrangement of lipid molecule, and increase the fluidity and flexibility of ethosome membrane, making ethosome deform during the process of transmission through skin, easier to pass the gap smaller than its size, and successfully reach to the deep layers of the skin (but as phospholipid was apt to be dissolved by ethanol, the concentration of ethanol in ethosome should not be too high, generally, not higher than 45%) (Touitou et al., 2000; Barry, 2001).

The work presented in this paper reflects the preparation of *Tripterygium* ethosomes based on optimal prescription. Single factor method was used to select the basic prescription and conditions for *Tripterygium* ethosome preparation considering phospholipid concentration, ethanol content and ultrasonic processing time as factors, and orthogonal experiment was carried out to design the optimized prescription for preparing ethosomes with entrapment efficiency as target, and then the quality of produced *Tripterygium* ethosomes was primarily analyzed. Result showed that the impacts of three factors on entrapment efficiency from high to low were: Concentration > ethanol content > ultrasonic processing time; the particle size of ethosome decreased with the increasing concentration of ethanol while it increased with the increasing concentration of phospholipid. The results obtained from the analysis of the average particle size by laser dynamic scattering equipment displayed that the particle size increased with the increase of phospholipid concentration and decreased with the increase of ethanol concentration, while the impact of the ultrasonic processing time on the average particle size was not found. Different concentrations of phospholipid and ethanol show impacts on the particle size of ethosome to various degrees where the particle size of ethosomes show an inverse relation with the concentration of ethanol and directly proportional to the concentration of the phospholipid. Entrapment efficiency and stability are two important factors that could be correlated with particle size and distribution, and they could directly influence the activities of ethosomes in the organism (Esposito et al., 2004). In this paper, it was found that the entrapment efficiency of the drug increased with the increasing concentration of phospholipid and ethanol content; and it increased with the extension of ultrasonic processing time, reaching its peak at 8 min. In addition, the aggregation probability among ethosomes increased with the increase of phospholipid concentration while it decreased with the increasing concentration of ethanol. As the particle size over 100 nm has a negative impact on percutaneous capability of the drug, phospholipid concentration must well be controlled, and the ethanol content should be increased as high as possible on the premise of the stability of ethosomes, so as to increase the percutaneous capability of the agent (Esposito et al., 2004).

In our study, the particle size of ethosome decreased more obviously compared to those of general liposomes. Presumably, the addition of ethanol in the prescription may give rise to a change in the nature of electrical charge, which strengthens the space stability of vesicle to some degree and shrinks its size. *Tripterygium* is a fat-soluble drug which has relatively high entrapment efficiency. In the present study, it was found that the entrapment efficiency of *Tripterygium* ethosomes was over 85%. Entrapment efficiency is usually measured with the method of dialysis or ultracentrifugation, and if entrapment efficiency measured by ultracentrifugation is lower than dialysis, it indicates that the drug has been partially lost due to the deformation and disruption of the lipid layers in the process of ultracentrifugation (López-Pinto et al., 2005). In the present study, the entrapment efficiency of *Tripterygium* ethosomes was over 85%, when the ethosomes were prepared with the mixture ratio of 45% (v/v) of ethanol to 2% (w/v) of phospholipid. The particle size of ethosomes became smaller, the distribution was better and the entrapment efficiency of *Tripterygium* ethosomes reached as high as 98%. Results by MIC, MBC and antibiosis-seasoning experiment proved that the speed rate and the percutaneous capability of ethosomes was higher and the time lag was shorter, compared with those of liposomes, which makes the drug to exert its effects better in antibiosis and the treatment of deep mycotic infection (Godin and Touitou, 2005).

Based on the physiologic barrier and limitation in reaching effective component to the lesion areas, this study introduces a new method for the preparation of ethosome with lecithin, ethanol and water, which has advantages of even distribution of particles, small particle size and high entrapment efficiency. Ethosomes prepared by combined thin-film hydration and ultrasound technology that make them particularly suitable to serve as liposome drug carriers. Therefore, this study may provide

a new orientation for the research and development of external triptolide agents.

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