Preparation and characterization of liposomes encapsulating *Calophyllum inophyllum* oil

Huu Trong Phan, Van Thanh Tran*

Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City

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Abstract:

***Callophyllum inophyllum* oil, also known as Tamanu oil, is reported to treat a wide range of skin problems such as acne, eczema, psoriasis, herpes, hemorrhoids, and injuries caused due to wounds, among others. Liposomes, which are effective carriers for topical treatment of dermal diseases, could enhance the therapeutic efficiency of Tamanu oil. Therefore, the purpose of this study was to formulate and characterize liposomes loading Tamanu oil. Liposomes encapsulating Tamanu oil with different ratios of Phospholipon 90G and L-α-lecithin were prepared using the thin-film hydration technique. Liposomal formulations were characterized in terms of aspect, particle size, size distribution, zeta potential, and morphology by using light microscope and dynamic light scattering analysis (DLS). Furthermore, the best formulation was tested with the storage stability after 30 days, and Tamanu oil loaded in the liposomes was identified as dictated in Vietnamese pharmacopoeia. The data demonstrated that an average liposome diameter of 53 nm with a narrow polydispersity (0.289) was obtained at a Phospholipon 90G to L-α-lecithin molar ratio of 4:6, and a Tamanu oil to phospholipid mass ratio of 1:3 approximately. In addition, according to the DLS results, the particle size and the zeta potential were quite stable at 2-8°C during 30 days of storage. The study achieved the promising results for developing a novel formulation containing Tamanu oil, which may be valuable to treatment of skin diseases.**

Keywords: ***liposome, Tamanu oil, thin-film hydration method.***

Classification number: *3.3*

Introduction

Calophyllum inophyllum L., Guttiferae, locally called Tamanu, is a tropical tree that is widely distributed throughout Africa, Asia, and Pacific countries. *Calophyllum inophyllum* oil extracted from the Tamanu nuts is composed of a mixture of lipids and other components including xanthone, flavon, and terpene derivatives. Traditionally, Tamanu oil has topically been used for skin care and to relieve skin problems for centuries. Ever since the first half of the 20th century, a number of researches have reported the pharmacological properties of Tamanu oil. Those include anti-inflammatory, antimicrobial, wound-healing, tissue-regenerative, and skin-protective properties. However, the direct application of pure Tamanu oil on the skin presents some disadvantages: The permeable efficiency of Tamanu oil across the skin is low due to the hydrophobicity of its lipid compositions; Tamanu oil is slightly rubefacient, so, long-term dermal exposure to the pure oil can lead to skin irritations; just like other oils, it can clog skin pores, often resulting in acnes and other skin infections [1, 2].

Tamanu oil came to be used in cosmetics about 40 years ago, and it was approved for clinical uses about 20 years ago [3]. Liposomes are used as a drug delivery system offering several benefits including biocompatibility, adjustable membrane to control their pharmacokinetic properties, increasing efficacy and therapeutic index of active agents [4, 5]. In terms of liposomal composition, liposomes are nanometric or sub-micrometric vesicles consisting of an internal aqueous core and one or more external phospholipid bilayer. This makes it possible for liposomes to load both hydrophobic and hydrophilic molecules. Hydrophobic compounds are inserted into a lipid layer, while hydrophilic compound can be entrapped in an aqueous center. This contributes to protecting these compounds from degradation and any other adverse environment al factors, thereby improving their stability. Moreover, owing to possessing a similar lipid bilayer with that of the skin, liposomes are easily attracted to dermal cells in different ways, for instance, adsorption, endocytosis, lipid exchange, or fusion. It is for this reason that the use of liposomes as a topical drug delivery system in the treatment of skin diseases facilitates the penetration of active ingredients into the deeper layer of the skin where their action should occur [6, 7]. Finally, the entrapment of drugs into liposomal vesicles overcomes some

*Corresponding author: Email: tranvanthanh@uphcm.edu.vn

inconveniences that are present in free drugs, such as irritation, unpleasant odor, clogging pores. Due to these benefits, liposomes are likely to be a promising choice for loading Tamanu oil in order to enhance clinical efficacy of the oil.

In addition, when it comes to physicochemical properties of liposomes that influence skin permeation of active ingredient entrapped in liposomes, a few researches indicated that small-sized liposomal vesicles appear to bring out a higher degree of penetration, and some surfactants act as a skin penetration enhancer.

For these reasons, this study was aimed at the preparation and characterization of liposomes containing Tamanu oil. In this way, liposomal suspensions encapsulating Tamanu oil with various organic solvents dissolving the lipid phase, speed of rotor/stator homogenization, lipid compositions, and tween 80 concentration have been studied based on their particle size, size distribution, and stability in order to optimize the liposomal formulation loading Tamanu oil.

Then, the result would be useful in the creation of effective liposomal topical formulations loading Tamanu oil for cosmetic and dermatological applications.

Materials and methods

Materials

The *Calophyllum inophyllum* oil (CIO) was provided by the Traditional Medicine Institute in Ho Chi Minh city (Vietnam). Phospholipon 90G (PL) from soybean and L-α-Lecithin (LL) from egg yolk were purchased from Lipoid® and Calbiochem®, respectively. The solvents for phospholipids including chloroform, ethanol 90 percent, and petroleum ether (30-60) were purchased from Sigma Aldrich (Germany). Tween 80 which was used to enhance the solubility of phospholipids in ethanol was purchased from Sigma Aldrich (Germany).

Methods

Preparation of liposomes loading Tamanu oil:

Liposomes were prepared by the thin-film hydration technique. In brief, the lipid phase (consisting of accurately weighed quantities of CIO, and a PL-LL mixture in different molar ratios) was dissolved in organic solvent (ethanol, chloroform, or petroleum ether) in a round bottom flask; the organic solvent was then removed under reduced pressure by using a rotary evaporator (Buchi R-200/205) at 70°C, then a thin lipid layer appeared in the flask. Thus, the lipid film obtained was kept on evaporating for three hours to eliminate the trace of the organic solvent. Finally, the hydration of the film with 50 ml of distilled water was carried out on the rotary evaporator under fast spin, at 50°C, for one hour to favor the vesicle formation. Liposomes were stored at temperatures between 2-8°C.

Homogenization of prepared liposomes:

The prepared liposomal suspension was homogenized by using Rotor/ Stator Homogenizer, heated at 50°C (homogenization takes place at higher temperatures than phase transition temperatures of lipids) for 15 minutes. The rotor speed was set up at 11,000 rpm, 15,000 rpm, and 19,000 rpm in turns.

Measurement of liposome size and zeta potential:

The mean particle size, size distribution, and zeta potential analysis of the liposomes were determined at 25°C by Dynamic Light Scattering using a Malvern Zetasizer (Malvern Instrument Limited, Malvern, UK). Experiments were run in triplicate.

Storage stability studies:

The liposomal suspension was stored in darkness at 8°C and 25°C for 30 days. The storage stability of the liposomes was based on the change of both their particle size and polydispersity index (PDI) measured by the Malvern Zetasizer.

Light microscopy:

The liposomal vesicles were monitored for their morphological attributes with the help of a digital optical microscope at 100X Objective (Olympus, Moticam 1000, Japan).

Identification of Tamanu oil in liposomes:

Thin-layer chromatography identification test: 10 µl of standard solution (0.1 g of CIO dissolved in 0.5 ml diethyl ether), test solution (0.1 g of liposomes sediment dissolved in 0.5 ml diethyl ether), and placebo solution (0.1 g of a mixture of PL, LL, and tween 80, dissolved in 0.5 ml diethyl ether) were applied on a parallel line of the thin-layer chromatography plate coated with silica gel-G. The plate was placed in a pre-saturated chromatographic chamber with a solvent system consisting of a mixture of benzene and ethyl acetate (8:2). The chromatogram was developed with the developing solvent system until the solvent front had moved about three-fourths of the length of the plate. Thus, the plate was removed from the chamber, the solvent front was marked and dried at a temperature from 100-105°C for 5 minutes. The location of the spots on the plate was observed under UV light (at the wavelengths of 254 nm and 365 nm). Later, the plate was sprayed with a vanillin-sulfuric acid reagent, and heated at 100-105°C for 5 minutes. This step was carried out to detect the presence of other compositions that weren't visible under UV light. The test solution chromatogram should be mainly similar to that of the standard solution and different from that of the placebo solution.

Results and discussions

Homogenization of liposomes loading Tamanu oil

The multi-lamellar vesicle (MLV) liposomes prepared by thin-layer hydration method are fairly large (several micrometers) and heterogeneous. In this way, it is compulsory to reduce and homogenize the liposomal particle size.

The particle size and size distribution of the liposomal vesicles obtained after homogenization using rotor/stator homogenizer at different rotor speeds are presented in Table 1.

The particle size of the non-homogenized liposomes was about four times larger than that of the homogenized liposomes. Furthermore, the rotor/stator homogenization decreased the size distribution of the liposomal vesicle, which related to the stability of the liposomes. These results showed that the homogenization played an important role in the preparation of liposomes.

The particle size and size distributions of liposomes obtained through homogenization at 15,000 rpm and at 19,000 rpm are mainly similar. Moreover, the homogenization at high speed may result in degradation of Tamanu oil and also lipid materials. Therefore, the speed of 15,000 rpm was chosen to homogenize liposomal vesicles.

Organic solvent used to dissolve the lipid phase

Solubility study proved that the lipid phase consisting of CIO, PL, and LL was dissolved well in chloroform or in a mixture of ethanol and petroleum ether (8:2). Yet it formed a suspension after being dispersed in ethanol.

Hypothesis: The majority of CIO compositions are lipophilic, which could dissolve the particles of phospholipid during the preparation of the thin-layer film. This might lead to a homogeneous layer film.

Therefore, ethanol, chloroform, and petroleum ether were chosen to dissolve the lipid phase. The particle size and size distribution of the liposomal vesicles were prepared by using these three different solvents as shown in Table 2. With regard to the particle size, there is no significant change between the formulations. Despite the increase in the polydispersity index, this value is a little small and is acceptable. Besides, the trace of the organic solvents such as chloroform, petroleum ether in a topical product can cause adverse side effects in cases of long-term application. As a result, ethanol was used as an organic solvent for dissolving the lipid phase.

Table 1. Particle size and polydispersity index of formulations homogenized at different speeds.

Formulation	Particle size (d.nm)	Polydispersity index
H0 (non-homogenized)	410.4	0.568
H11 (11,000 rpm)	119.26	0.292
H15 (15,000 rpm)	106.26	0.298
H19 (19,000 rpm)	106.06	0.279

Table 2. Particle size and polydispersity index of formulations prepared by using three different organic solvents dissolving the lipid phase.

Formulation	Particle size (d.nm)	Polydispersity index
A1 (ethanol)	110.70	0.309
A2 (chloroform)	114.04	0.270
A3 (ethanol:petroleum ether (8:2))	114.98	0.286

Encapsulation of Tamanu oil

Almost all the compositions in CIO are lipophilic. So, the CIO was added to the lipid phase (passive encapsulation method) and would be integrated into the phospholipid bilayer during the formation of the thin-layer film. The amount of CIO into the lipid phase was established by evaluating the stability (storage at a temperature between 2-8^{o}C for 30 days) of liposomal suspensions with a varying amount of CIO.

At concentrations of up to 24.1%, the liposomal suspensions obtained were stable during the storage phase (no surface phenomena were observed).

Optimization of lipid composition

The composition of the bilayer, in particular phospholipids, influences the fluidity as well as the stability of liposomes considerably. First of all, the stability of liposomal suspensions with different molar ratios was investigated on the basis of the centrifuge stability testing (centrifugation at 17,000 rpm for 30 minutes). It was seen that the change of the lipid molar ratio could result in the change in the stability of liposomal vesicles. To be more precise, the formulations containing a mixture of PL and LL with molar ratios from 8:2 to 4:6 presented a higher stability than the others. Then, the particle size and the size distribution of these formulations were measured in order to find out the best formulation. The results are displayed in Table 3.

The potential zeta is a good tool to investigate the stability of liposomal products. In this study, the incorporation of two kinds of phospholipids contributes to the augmentation of potential zeta (from -20 mV to -42 mV). This suggests that the incorporation of PL and LL improved liposome stability. The formulation with a mixture of PL and LL at the ratio of 6:4 gave the smallest mean particle size. Therefore, this ratio was chosen for the liposomal formulation loading CIO.

Influence of tween 80 on physical properties of liposomes

The application of liposomes as a tropical and transdermal drug delivery system necessitates some specific

Table 3. Particle size distribution and potential zeta of different formulations with various molar ratios of PL and LL.

Formulation	Particle size (d.nm)	Polydispersity index	Potential zeta (mV)
B0 (10 PL: 0 LL)	143.08	0.333	-20.3
B2 (8 PL: 2 LL)	118.18	0.302	-42.0
B3 (7 PL: 3 LL)	114.06	0.309	-48.7
B4 (6 PL: 4 LL)	107.88	0.301	-53.7
B5 (5 PL: 5 LL)	110.70	0.309	-56.7
B6 (4 PL: 6 LL)	110.22	0.298	-56.8

Table 4. Particle size and polydispersity index of formulations with and without different concentrations of tween 80.

Formulation	Particle size (d.nm)	Polydispersity index	Potential Zeta (mV)
C0 (0% w/w)	-	-	-
C1 (5% w/w)	109.8	0.301	-53.7
C2 (10% w/w)	102.74	0.276	-54.5
C3 (15% w/w)	97.38	0.418	-53.9
C4 (20% w/w)	106.32	0.455	-54.2

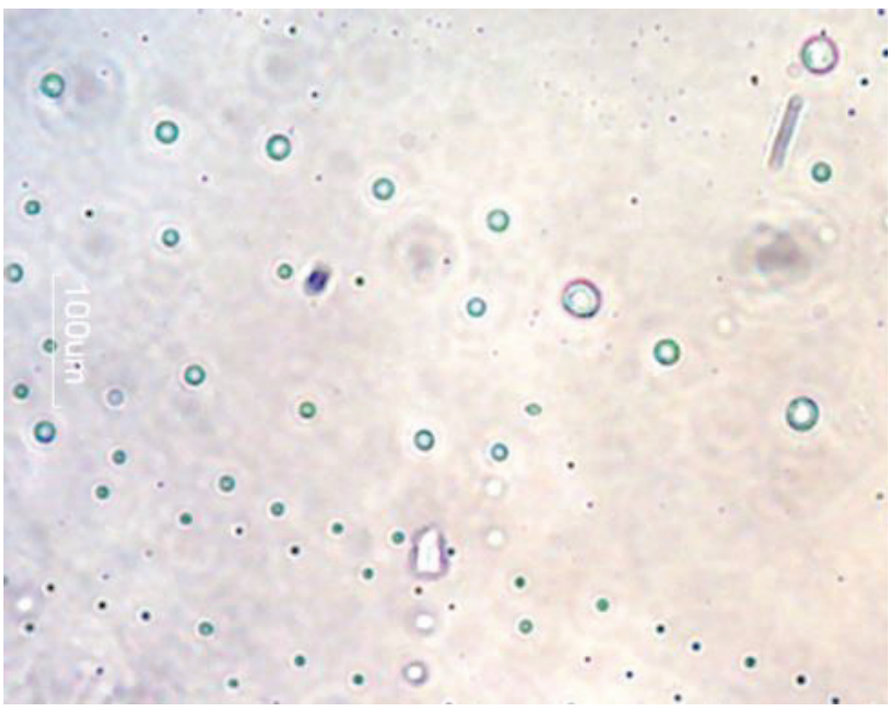


Fig. 1. Optical microscope image at 100X objective of the liposomal formulation.

properties such as elasticity of liposomes. It was reported that the surfactant acted as an "edge activator" which enhanced the flexibility of liposomes. This helps the encapsulated agent to penetrate to the deeper layer of the skin. In the present paper, the effect of tween 80 is evaluated as well as that of its concentration on the particle size, and the size distribution of liposomal vesicles is also evaluated, which is summarized in Table 4. The film of the tween 80-free formulation (C0) did not completely detach during the hydration because of the hydrophobicity of the film compositions. The use of the surfactant allowed the film to become more fluid and to form vesicles easily. At concentrations up to 10% (w/w), tween 80 was not only beneficial to the hydration, but it also contributed to the reduction in the particle size and the size distribution of liposomal vesicles. However, at the range from 15-20% (w/w) of tween 80 in the liposomal formulation, liposomal particles became more heterogeneous. Therefore, the content of 10% (w/w) of tween 80 was chosen to be added to the formulation for preparation of liposomes encapsulating the CIO.

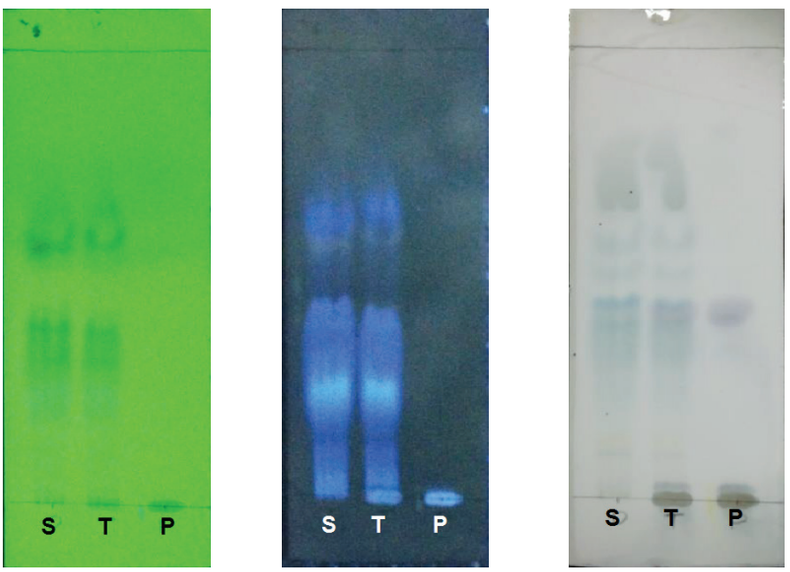
Characterization of the final liposomal formulation

Physical appearance of liposomal suspension: The liposome suspension is homogeneous, and light green in color. The morphological attributes of liposomes before homogenization, as observed through an optical microscope, is shown in Fig. 1. Although the liposomes viewed by using the optical microscope were giant liposomes, the optical microscope image provided the morphology of liposomes loading CIO. The image showed that Tamanu oil (green color) was encapsulated into the liposomal bilayer.

Storage stability: The stability results which displayed minimal changes of particle size and size distribution of the final liposomal formulation are summarised in Table 5.

Table 5. Particle size and polydispersity index of the final liposomal formulation before and after storage for 30 days at the different temperatures.

Formulation	Particle size (d.nm)	Polydispersity index	Potential zeta (mV)
S0 (day 0)	106.26	0.289	-49.8
S01 (30 days stored at 2-8°C)	100.9	0.300	-43.0
S02 (30 days stored at 25-30°C)	108.2	0.301	-51.4



UV at 254nm UV at 365nm Vanilin-Sulfuric

Fig. 2. Chromatograms of the standard solution (S), the test solution (T), and the placebo solution (P), stained under a UV light at 254 nm, 365 nm, and with Vanilin-Sulfuric respectively.

The particle size and the size distribution of liposomal suspension had slightly modified after 30 days of storage. The liposomal vesicles entrapping Tamanu oil were fairly stable during the storage even at temperatures between 25-30°C.

Identification of Tamanu oil into liposomes: The chromatograms (Fig. 2) showed that each separated spot obtained from the test solution corresponds to that of the standard solution. As a result, all components of CIO were encapsulated into the liposomal vesicles.

Concluding remarks

In the present study, liposomal formulation trapping *Calophyllum inophyllum* oil was optimized with the lipid phase comprising 65.9% (w/w) of the Phospholipon 90G: L-α-lecithin combination in the 6:4 molar ratio, 10% (w/w) of tween 80, 24.1% (w/w) of CIO. The average size of the prepared liposomes was small (mean diameter of 102.74 d.nm) and homogeneous (PDI of 0.276). The high negative charge of liposomal vesicles (-54.5 mV), and the minimal modification of particle size as well as the polydispersity index of liposomal vesicles after the storage stability studies indicated a good stability of suspension of liposomes encapsulating Tamanu oil. Interestingly, ethanol was used as a solvent dissolving the lipid phase in order to avoid the toxicity of the trace of organic solvent.

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