Preparation and Characterization of Liposomes Containing Cinnamon and Cajuput Essential Oils

การเตรียมและการศึกษาคุณลักษณะของไลโปโซมที่ประกอบด้วยน้ำมันหอมระเหยอบเชยและเสม็ดขาว

Chayanee Wonglamthong, Parapat Sobharaksha, Ratana Indranupakorn*
Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University,Samutprakarn 10540
ชญานี วงศ์แหลมทอง, ปารภัทร โศภารักษ์, รัตนา อินทรานุปกรณ์*
คณะเภสัชศาสตร์ มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ สมุทรปราการ 10540
*Email : nong2indra@gmail.com

Abstract

The aim of the study was to prepare liposomes entrapping essential oils in order to decrease volatility and enhance stability. The combination of cinnamon (*Cinnamomum burmanii*) and cajuput (*Melaleuca Leucadendron*) oils (1:1 by volume) was selected for incorporation into liposome and was analyzed by a developed gas chromatography-mass spectrometry (GC-MS) method for two major compounds, cinnamaldehyde and eucalyptol. The liposomal formulations composed of phospholipid, cholesterol and oils mixture (2 mg/mL) were prepared using a thin film hydration method. The effects of different mass ratios of phospholipid/cholesterol (60:40, 70:30, 80:20, 90:10) and sonication time (0, 2 and 5 minutes) on oil entrapment efficiency were evaluated. The optimized formulation, containing the mass of phospholipid to cholesterol ratio of 80:20 and generated via sonication for 2 minutes, emerged the maximum amount of the two major components (88.40±0.22%) and was used for further preliminary stability studies at 4±2ºC and the room temperature, 25±2ºC, for a period of 2 months. Results showed that the essential oils in liposomal formulation were quite stable within 2 months at both storage temperatures and had good physical appearance. The percentage of oil retention in the liposomal formulations decreased by 8.78 and 12.83 at both temperatures, respectively. Findings suggested that the combination of cinnamon and cajuput oils which enhance antiacne-inducing bacterial activities and are stabilized in the liposomal formulation could be further developed to provide the anti-acne cosmetic products.

Keywords : Liposomes, cinnamon oil, cajuput oil, entrapment efficiency, GC-MS

บทคัดย่อ

วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาการห่อหุ้มน้ำมันหอมระเหยในรูปแบบไลโปโซมเพื่อลดสูญเสียจากการระเหยและเพิ่มความคงตัวของน้ำมันหอมระเหย ในการศึกษาครั้งนี้เลือกน้ำมันอบเชยผสมกับน้ำมันเสม็ดขาวใส่ในตำรับไลโปโซมและวิเคราะห์หาปริมาณซินนามาลดีไฮด์และยูคาลิปตอล ซึ่งเป็นสารสำคัญในน้ำมันหอมระเหยทั้งสองชนิดตามลำดับด้วยวิธี GC-MS ที่ทำการตรวจสอบความถูกต้องแล้ว สูตรตำรับไลโปโซมประกอบด้วยฟอสโฟไลปิด

24 กุมภาพันธ์ 2563 / 5 พฤศจิกายน 2563 / 30 ธันวาคม 2563
24 February 2020 / 5 November 2020 / 30 December 2020

โคเลสเตอรอลและน้ำมันหอมระเหยผสม (2 มก./มล.) และเตรียมโดยวิธี thin film hydration ทำการประเมินผลการเปลี่ยนแปลงสัดส่วนปริมาณฟอสโฟไลปิดกับโคเลสเตอรอลและระยะเวลาโซนิเคด (sonicate) ต่อประสิทธิภาพการเก็บกักน้ำมันหอมระเหยผสม พบว่าตำรับไลโปโซมที่ประกอบด้วยปริมาณฟอสโฟไลปิดกับโคเลสเตอรอลในสัดส่วน 80:20 และใช้ระยะเวลาโซนิเคด 2 นาที มีปริมาณสารสำคัญทั้งสองชนิดสูงสุด (88.40±0.22%) ซึ่งจะใช้ในการตรวจสอบความคงตัวเบื้องต้นที่อุณหภูมิ 4±2°C และที่อุณหภูมิห้อง 25±2°C นาน 2 เดือน ผลการทดลองพบว่าน้ำมันหอมระเหยผสมในตำรับไลโปโซมทั้งสองอุณหภูมิค่อนข้างคงตัวดีตลอดระยะเวลา 2 เดือน โดยมีปริมาณน้ำมันหอมระเหยผสมลดลงร้อยละ 8.78 และ 12.83 ที่อุณหภูมิทั้งสองตามลำดับ จากผลการศึกษา แสดงให้เห็นว่า น้ำมันอบเชยและน้ำมันเสม็ดขาวคงตัวได้ดีในตำรับไลโปโซม ซึ่งอาจพัฒนาเป็นผลิตภัณฑ์เครื่องสำอางที่ใช้ต้านเชื้อที่ก่อให้เกิดสิวต่อไปได้

คำสำคัญ : ไลโปโซม น้ำมันอบเชย น้ำมันเสม็ดขาว ประสิทธิภาพการเก็บกัก GC-MS.

Introduction

In the recent years, Eos (Essential oils) have received strong interest because of their potentially useful bioactivities[1] including antibacterial, antifungal, insecticidal, antioxidant and other medicinal properties such as anti-inflammatory,[2] analgesic,[3] sedative[4] and local anesthetic remedies.[5] Therefore, essentials oils are widely used in food, cosmetics and pharmaceutical industries due to these properties.[6] However, their components are sensitive to light, heat, moisture and oxygen, which convert to degradation products and loss of pharmacological properties.[7] The low stability and the high volatility of the Eos have limited their application in medicinal use. Some novel methods have been developed to decrease volatility, increase the stability of the active substances and enhance their bioactivities, among these is the encapsulation of the Eos in liposome.[8] In the past few decades, liposomes have received a lot of attention as an efficient entrapment of a large variety of both unstable lipophilic and hydrophilic agents inside vesicles which provide a wide range of advantages including high entrapment efficiency and long retention times.[9] Studies have shown that liposomes provide a feasible and efficient approach for incorporating natural compounds, such as Eo components, and the entrapped compound is more stable under the protection of the phospholipid bilayers, which is similar to biological membranes.[8]

The use of Eos in combination is a new approach to increase the efficacy and could lead to the optimization of bioactivities.[10] Preliminary studies[11] revealed that the three common Eos, CM (cinnamon), CP (cajuput) and P (Plai, *Z. cassumunar* Roxb) oils, have shown potent anti-acne-inducing bacterial activities against *S. epidermidis* (ATCC 14990) and *P. acnes* (DMST 14916). Moreover, the effect of two different blends of Eo components, including CM and CP, CM and P, CP and P oils (1:1 by volume), on the anti-acne-inducing bacterial activities were also investigated. The best synergistic antibacterial preparation composed of CM and CP oils showed particular efficacy, in comparison with others, with the same minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for both bacterial species at 1.024 and 0.512 mg/mL, respectively. The promising antibacterial activity of the blended CM and CP oils has encouraged researchers to use them as potential antimicrobial agents. Due to their susceptibility to degradation, encapsulation of the blended oils in liposome was proposed to be potentially useful technique to enhance stability and prolong retention time. This study is the first to report a preparation of effective blended CM and CP oils in liposome.

Objectives

In the present study, the combination of CM and CP oils (1:1 by volume) was selected for incorporation into liposome system. The effects of different quantities of phospholipid/cholesterol and sonication time on the physicochemical properties and the stability of liposome were evaluated. The major constituents of CM and CP oils, CH and ET, were chosen as indices for determination of entrapment efficacy.

Methods

CM and CP oils were obtained from Botanicssence Essential Oil and Tropicalife Co., Ltd., Thailand, respectively. L-alpha-lecithin from egg, cholesterol, cinnamaldehyde and eucalyptol were obtained from Sigma-Aldrich, Germany. Dichloromethane, methanol and hexane (AR grade) were purchased from Merck, Germany. All other chemicals were of analytical grade.

Liposome Preparation The liposomal formulations containing CM and CP oils were prepared by thin-film hydration technique as described by Liolios et al.[12] with some modifications. Twelve trial formulations were prepared using different proportions of phospholipid and cholesterol given in Table 1. Briefly, 80 mg of the blended CM and CP oils (1:1 by volume) was added to 100 mg of the lipid mixture of phospholipid (L-alpha-lecithin) and cholesterol at various mass ratios in round-bottom flask and dissolved in CH_2Cl_2:MeOH (3:1 by volume). The solvent was evaporated to dryness using a rotatory evaporator at 40°C to obtain a uniform and transparent film. The mixture was flushed with nitrogen gas in order to remove the residual solvent completely. The lipid film was suspended in phosphate buffer saline solution (pH 7), in hydration process, at 60°C for 2 hours for complete swelling. Then, liposome was reduced in size with various sonication times (Table 1). The entrapped Eos in liposome vesicles were separated from the unentrapped Eos by centrifugation[13] at 30000 rpm for 120 minutes. The clear supernatant was removed carefully to separate liposomes from non-entrapped Eos.

Liposome Characterization The amounts of Eos in sediment, after lysing liposomes with 20 mL of CH_2Cl_2:MeOH (1:1 by volume), were analyzed by developed GC-MS method. The loading capacity and entrapment efficiency were calculated using the following formulas.[14]

$$\% \text{ loading capacity} = \frac{\text{Amount of blended Eos in liposomes}}{\text{Amount of liposomal lipid content}} \times 100$$

$$\% \text{ entrapment efficiency} = \frac{\text{Amount of blended Eos in liposomes}}{\text{Amount of blended Eos fed initially}} \times 100$$

The Eos-loaded liposomes were also subjected to characterize for size and polydispersity indexes (PDIs) using particle size analyzer (Delsa® Nano C, Beckman Coulter, USA) at 25°C in triplicate. Liposome morphology was also evaluated by transmission electron microscopy (TEM). A drop of diluted liposome sample was negatively stained with 1% phosphotungstic acid and then examined using TEM (Tecnai T20 G2, Phillips, Japan) with an accelerating voltage of 80 kV at 71000X and 135000X.

Table 1 Composition of the liposomal formulations and duration of sonication for the preparation.

Composition	Formulations Number											
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
L-alpha-lecithin (mg)	60	60	60	70	70	70	80	80	80	90	90	90
Cholesterol (mg)	40	40	40	30	30	30	20	20	20	10	10	10
Blend CM and CP oils (mg)	80	80	80	80	80	80	80	80	80	80	80	80
Sonication time (min)	0	2	5	0	2	5	0	2	5	0	2	5

Liposome stability On the basis of oil entrapment efficiency, vesicle size and vesicle distribution, the optimized liposomal formulation was chosen to determine the preliminary stability. The studies were carried out by keeping L8 formulation, which stored in airtight sealed vials, for a period of two months at different storage conditions (4±2°C and 25±2°C) and continuously evaluated every week for two months in terms of changes in the appearance, mean particle size, PDIs, pH and percentage Eos retained in liposomes.

Quantitative Analysis of the Eos by Developed GC-MS (Gas Chromatography-Mass Spectrometry) Analysis of the blended and of the encapsulated oils were carried out using GC (Trace GC Ultra, Thermo Electron Corporation, USA), equipped with an autosampler (AI 3000, Thermo Electron Corporation, USA), a capillary column (ZB-5MSi, 30 m x 0.25 mm i.d., film thickness 0.25 μm, Phenomenex, USA) and a quadrupole MS detector DSQ (Thermo Electron Corporation, USA). The temperature program ranged from 60°C to 200°C with an increase rate of 6°C/min followed by 5 min under isothermal conditions. The injector was maintained at 250°C. Helium was the carrier gas at 1.2 mL/min. The ion source temperature was 250°C. The major constituents, CH and ET, were identified by matching their mass spectra with those reported in a computer library. Moreover, identification has been confirmed by comparing their retention times and mass spectra with those of pure reference standards. A quantitative analysis of CH and ET in blended oils of free and entrapped liposomes were carried out by external standard technique.

The developed GC-MS was validated in term of linearity, accuracy, precision and specificity parameters according to the International Conference on Harmonization (ICH) guideline.[15] For linearity study, the mixed standard solutions of CH and ET were prepared in the range of 0.25-4.00 mg/mL and injected into system at five levels of concentration in triplicate. The responses were measured as peak areas and plotted against concentrations to obtain the calibration curve. The accuracy of the proposed method was assessed by adding known amounts of CH/ET corresponding to three concentration levels (80, 100, and 120%) to the pre-analyzed sample. The experiment was conducted in triplicate. Accuracy was expressed as percent recovery by the proposed method. To study intra-day and inter-day precisions, three different concentrations of the CH/ET were analyzed, in six replicates for each level, on the same day and on five different days afterwards. The results were reported in term of relative standard deviation. The specificity of the developed method was evaluated by reference standards and test samples, verifying the absence of signal interference.

Data Analysis and Statistics. Data are expressed as mean ± SD. Statistical analysis was performed using SPSS 16.0. Statistically significant difference was considered at the level of $p<0.05$.

Results and Discussions

In order to study the influence of liposomal composition and sonication time on the retention time of entrapped oils, different vesicular formulations were prepared. The constituents in blended oils were analyzed by GC-MS which serves as a suitable and reliable method for the quality control of the chemical markers.[16] The developed GC-MS method for quantification of CH and ET in CM and CP oils has been validated according to ICH guidelines and shown to be reliable and reproducible (Table 2). The GC chromatograms showed sharp, symmetrical and well separated peaks for ET and CH in blended oils with consistent and convenient retention times of 10.68 and 18.18 min, respectively (Figure 1). The peak purity of analyzed components was accessed by comparing their respective mass spectra at different regions of the peaks. Results indicated the absence of signal interferences. The calibration curves of CH and ET showed good linear relationship in the concentration range of 0.28-4.26 and 0.26-4.18 mg/mL, respectively. The percentage of recoveries of both CH and ET were in the range of 98–102 and their RSDs of precision were less than 2 as recommend by ICH guidelines,[15] which indicated that the proposed method was precise and reproducible.

Table 2 Method validation and data

Analytes	Linearity			Accuracy		Precision	
	Slope	Intercept	r^2	Recovery (%)	RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
CH	66.11×10^6	0.40×10^6	0.9998	101.66	1.15	0.8025	1.2099
ET	49.03×10^6	3.74×10^6	0.9997	100.51	1.20	0.6745	1.1997

Quantitative Analysis of the Blended Eos The blended oils solution was analyzed by developed GC-MS. The results showed that the oils were composed of CH (28.95%) and ET (23.25%) as the main constituents (Figure 1). For the analyzed solution, the amount of both main compounds was 0.522 mg/mL.

Liposome Characterization L-alpha-lecithin from egg and cholesterol, most commonly used lipid in preparation of liposomes,[9,17] were chosen as the core components for the preparation of liposomes by the thin film hydration technique. Different ratios of phospholipid and cholesterol were incorporated with a fixed amount of blended Eos in various batches of the formulation using variables of sonication time (Table 1). The results showed that the phospholipid/cholesterol fed ratios and sonication times had an impact on both liposomal sizes and the amounts of entrapped Eos. The entrapment efficiency is the most important parameter from pharmaceutical viewpoint in liposome formulations. It was clearly depicted from the data shown in Table 3 that, overall, the oils entrapment efficiency agreed with the loading capacity profiles. The loading capacity and entrapment efficiency of the liposomal formulations with the same sonication time were increased on increasing the cholesterol content (10 to 20 mg), whereas on further increase in cholesterol content (20 to 40 mg) the entrapment efficiency was decreased. This might be due to the fact that, with increased cholesterol content, the bilayer hydrophobicity and stability of bilayers vesicles increased and the permeability decreased, which may lead to efficiently trapping the hydrophobic Eos into bilayers as vesicles formed. In contrast, higher amounts of cholesterol may compete with

the Eos for packing space within the bilayer, hence, excluding the Eos as the amphiphiles assembled into the vesicles.[18,19]

The impact of different compositions in the liposome systems on the size was assessed because vesicular size has the ability to influence the penetration of Eos through the skin to the

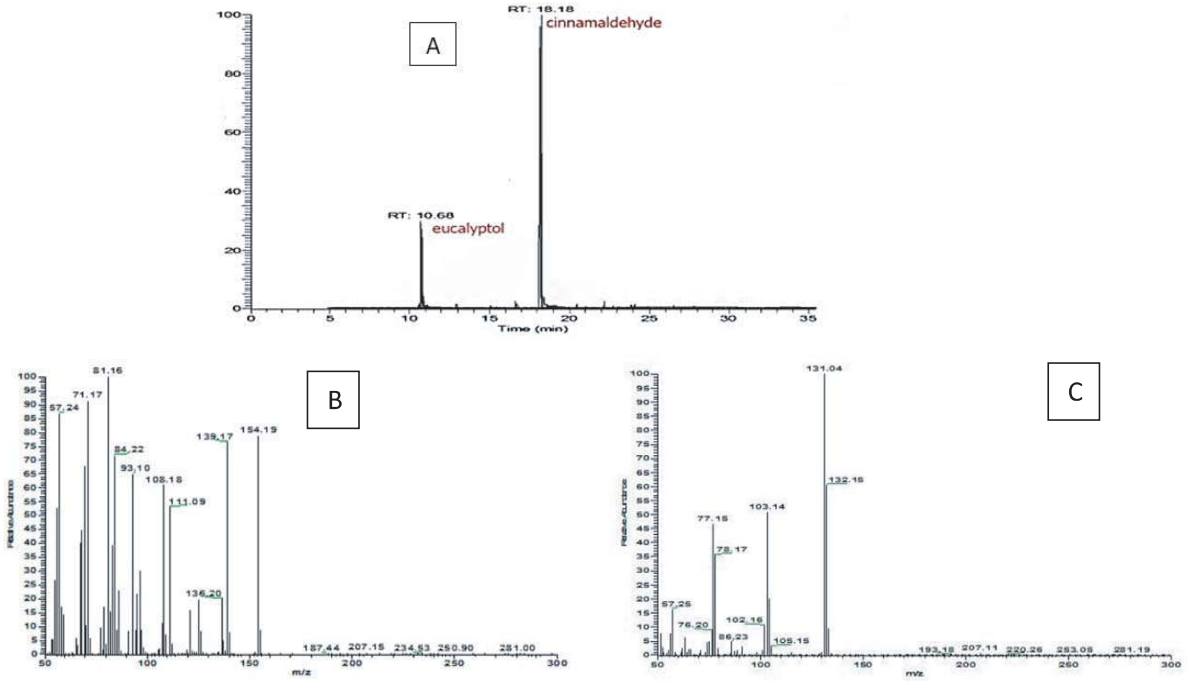

Figure 1 GC-MS chromatogram of the blended CM and CP oils containing CH and ET as main constituents at 18.18 and 10.68 minutes, respectively (A); electron impact mass spectra of CH (B) and ET (C).

Table 3 Particle size and size distribution of oil-loaded liposomes prepared by different proportions of phospholipid and cholesterol with various sonication time (mean ± SD, n = 3).

Formulation Code	L-Alpha-Lecithin: Cholesterol (mg)	Sonication Time (min)	Particle Size Diameter (nm)	Polydispersity Index	%Loading capacity	%Entrapment efficiency
L1	60:40	0	786.3±1.16	0.355±0.02	13.83±0.11	66.22±0.54
L2	60:40	2	367.9±1.97	0.241±0.02	14.47±0.09	69.32±0.44
L3	60:40	5	237.2±2.15	0.201±0.01	12.63±0.12	60.43±0.61
L4	70:30	0	816.7±2.35	0.460±0.01	14.72±0.08	70.49±0.38
L5	70:30	2	435.3±2.15	0.278±0.01	15.81±0.09	75.71±0.45
L6	70:30	5	321.7±1.47	0.191±0.01	13.91±0.05	66.63±0.23
L7	80:20	0	856.2±1.45	0.447±0.01	17.28±0.18	82.78±0.40
L8	80:20	2	456.9±1.25	0.287±0.01	18.35±0.10	88.40±0.22
L9	80:20	5	280.7±1.31	0.209±0.01	16.42±0.08	78.62±0.38
L10	90:10	0	876.8±1.26	0.487±0.01	16.49±0.05	79.00±0.26
L11	90:10	2	491.1±2.80	0.232±0.01	16.41±1.19	81.80±0.24
L12	90:10	5	221.2±2.08	0.201±0.01	15.62±0.11	74.83±0.52

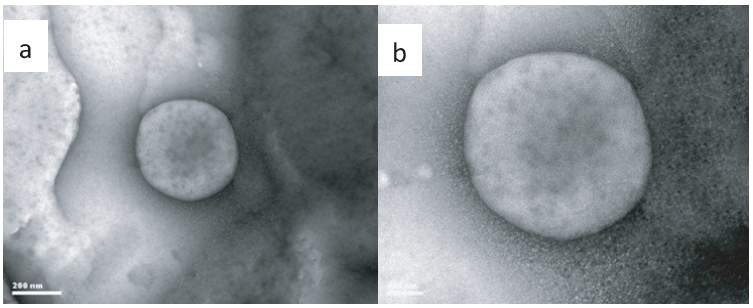

Figure 2 Transmission Electron Microscopic images of liposomal formulation, L8, entrapping cinnamon and cajuput oils at 71000X (Figure 2a) and 135000X (Figure 2b)

deeper layers. As shown in Table 3, all liposomes were produced with vesicle sizes ranging from 221.2 to 876.8 nm with PDI of 0.201-0.487. TEM photographs of the prepared nanoparticles (Figure 2) revealed the spherical or ellipsoidal vesicular structure of liposomes. These results can be attributable to the fact that the prepared liposomes are large unilamellar vesicles. Furthermore, the results indicated that the vesicle sizes and size distributions of liposomes were strongly dependent on sonication time. The increase of sonication time decreases the vesicle sizes and PDIs. This observation agreed with the study by Nam et al.[20] These results suggested that uniformity of the vesicular diameter increased with the sonication time. In drug delivery applications using lipid-based carriers, such as liposome, a PDI of $\leq$ 0.3 is considered to be acceptable and indicates a homologous population of phospholipid vesicles.[21] As illustrated in Table 3, the PDIs of different liposomal formulations after sonication were less than 0.3, indicating that the sonication method can prepare nano-size homogenous vesicle. Moreover, the sonication duration of two minutes was found to be appropriate to produce aggregate free liposomal suspension and showed high percentage of blended oils loading and entrapment in each level of cholesterol concentration. On the basis of the above results, L8 was the optimal formulation, as it showed the highest entrapment efficiency (88.40%) of Eos, had quite small vesicles (456.9 nm) and indicated the uniformity of the liposome diameter (PDI <3). Therefore, this formulation was considered as the model for further stability studies.

Preliminary Stability Studies Responses obtained from different parameters of L8 during stability period are as shown in Table 4 and 5. Stability results indicated that no significant change in the physical appearance and the vesicle size of L8 was slightly increased, by around 13.51% and 16.48%, after storage at 4±2°C and 25±2°C over 2 months respectively. However, it showed narrow size distribution (PDI 0.286–0.327). The increase of size might be caused by the aggregation and fusion of vesicles itself. Moreover, the amount of the entrapped Eos in liposome was slightly decreased, about 8.78 and 12.83% after storage at 4±2°C and 25±2°C over 2 months respectively. It could be observed that no changes in appearance and slight differences in the value of pH were present. Results suggest that keeping the liposomal product in refrigerant conditions minimizes stability problems of liposomes.

Table 4 Stability studies of L8 at 4±2°C.

Week	Particle Size (nm)	Polydispersity Index	pH	%Entrapment efficiency
0	456.8±4.5	0.286±0.003	5.78±0.04	88.40±0.22
1	459.2±3.8	0.287±0.002	5.65±0.10	88.30±0.38
2	461.7±3.1	0.289±0.002	5.70±0.14	88.11±0.31
3	478.3±1.8*	0.293±0.005	5.63±0.15	85.75±0.16*
4	482.6±1.9*	0.295±0.004*	5.62±0.07	85.28±0.29*
5	496.1±3.8*	0.301±0.003*	5.52±0.07	82.74±0.44*
6	498.4±3.4*	0.303±0.002*	5.62±0.12	82.55±0.24*
7	503.2±1.9*	0.304±0.001*	5.61±0.04	82.17±0.35*
8	518.5±2.9*	0.309±0.002*	5.81±0.08	79.62±0.25*

Data are given as mean ± SD (n = 3). *Values are significantly different, identified based on the 95% confidence interval of Week 0 values.

Table 5 Stability studies of L8 at 25±2°C.

Week	Particle Size (nm)	Polydispersity Index	pH	%Entrapment efficiency
0	456.8±4.5	0.286±0.003	5.78±0.04	88.40±0.22
1	461.2±4.3*	0.291±0.003*	5.53±0.04	88.21±0.17
2	465.7±3.6*	0.294±0.002*	5.59±0.17	87.64±0.29*
3	481.3±1.6*	0.299±0.002*	5.73±0.08	85.75±0.12*
4	488.8±1.4*	0.302±0.004*	5.51±0.05	83.96±0.15*
5	499.4±2.4*	0.309±0.003*	5.57±0.05	82.55±0.50*
6	506.3±3.4*	0.313±0.004*	5.71±0.08	80.85±0.36*
7	525.7±1.9*	0.320±0.005*	5.69±0.21	78.96±0.28*
8	532.1±2.9*	0.327±0.004*	5.76±0.07	75.57±0.21*

Data are given as mean ± SD (n = 3). *Values are significantly different, identified based on the 95% confidence interval of Week 0 values.

Conclusions and Perspectives

The present study showed that liposomal formulation containing the blended of CM and CP oils was successfully prepared by thin film hydration technique using different ratios of L-alpha-lecithin from egg and cholesterol. Among the various formulation, the liposomal formulation, L8, comprising L-alpha-lecithin from egg, cholesterol 9:1 ratio displayed good entrapment efficiency (88.40%). The stability studies for a period of 2 months also showed that liposomal dispersions maintained 91.22% of the blended Eos content at 4±2°C. This leads to the conclusion that the entrapment of the blended Eos in liposome increased the oil stability. The further *in vitro* skin permeation of the optimized liposomal formulation should be tested for evaluation suit of applying as topical delivery system for the blended of CM and CP oils. The performed studies contribute to the development of the optimal preparation method and composition of the investigated system and provide useful information to develop the prepared liposomal formulation to the commercialized anti-acne cosmetic products.

References

1. Chouhan S, Sharma K, Guleria S. Antimicrobial activity of some essential oils - present status and future perspectives. Medicines. 2017;4(3):58-78.
2. Da Silvai GL, Luft C, Lunardelli A, Amaral RH, Melo DA, Donadio MV, et al. Antioxidant, analgesic and anti-inflammatory effects of lavender essential oil. An Acad Bras Cienc. 2015;87(2)(Suppl.):1397-408.
3. Sarmento-Neto JF, Do Nascimento LG, Felipe CF, De Sousa DP. Analgesic potential of essential oils. Molecules. 2016;21(1):20-48.
4. Rabbani M, Sajjadi SE, Sadeghi M. Chemical composition of the essential oil from *Kelussia odoratissima* Mozaff. and the evaluation of its sedative and anxiolytic effects in mice. Clinics. 2011;66(5):843-8.
5. Tsuchiya H. Anesthetic agents of plant origin: a review of phytochemicals with anesthetic activity. Molecules. 2017;22(8):1369-403.
6. Bhavaniramya S, Vishnupriya S, Al-Aboody MS, Vijayakumar R, Baskaran D. Role of essential oils in food safety: antimicrobial and antioxidant applications. Grain Oil Sci Technol. 2019;2:49-55.
7. Turek C, Stintzing FC. Stability of essential oils: a review. Compr Rev Food Sci Food Saf. 2013;12:40-53.
8. Sherry M, Charcosset C, Fessi H, Greige-Gerges H. Essential oils encapsulated in liposomes: a review. Liposome Res. 2013;23(4):268-75.
9. Bulbake U, Doppalapudi S, Kommineni N, Khan W. Liposomal formulations in clinical use: an updated review. Pharmaceutics. 2017;9,12-44.
10. Bassolé IHN, Juliani HR. Essential oils in combination and their antimicrobial properties. Molecules. 2012;17:3989-4006.
11. Wonglamthong C, Indranupakorn R, Rattanaroatpong T, et al. Study of antioxidant and anti-acne inducing bacterial activities of cinnamon, cajuput and plai essential oils. Agricultural Sci J. 2016;47(2)(Suppl.):141-4.
12. Liolios CC, Gortzi O, Lalas S, Tsaknis J, Chinou I. Liposome incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and *in vitro* antimicrobial activity. Food Chem. 2009;112:77-83.
13. Pasha K, Banu S. Formulation and evaluation of glimepiride liposomal drug delivery system. IJRBS. 2017;4(3):39-44.
14. Zhang J, Froelich A, Michniak-Kohn B. Topical delivery of meloxicam using liposome and microemulsion formulation approaches. Pharmaceutics. 2020;12(3):282-305.

15. ICH Expert Working group. Validation of Analytical Procedures: Text and Methodology Q2(R1), ICH Harmonised Tripartite guidelines. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. [internet]. 2005 [Cited 2020 Oct 22];1-15. Available from database.ich.org/sites/default/files/Q2%28R1%29%20guideline.pdf.

16. Peng C, Zhang T, Zhao G, Wang S. Analysis on fat-soluble components of *Sinapis semina* from different habitats by GC-MS. J Pharm Anal. 2013;3(6),402-7.

17. Yusaf R, Nawaz R, Hayat S, Khursheed A, Zafar N, Ahmad, A, et al. Structural components of liposomes and characterization tools. Indo Am j Pharm. 2014;4(8),3559-67.

18. Kirby C, Clarke J, Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*. Biochem J. 1980;186(2):591-8.

19. Shilakari Asthana G, Asthana A, Singh D, Sharma PK. Etodolac containing topical niosomal gel: Formulation development and evaluation. J Drug Deliv. [internet]. 2016 [Cited 2020 Oct 22]; 9324567. Available from https://pubmed.ncbi.nlm.nih.gov/27478643/. Doi:10.1155/2016/9324567.

20. Nam JH, Kim SY, Seong H. Investigation on physicochemical characteristics of a nanoliposome-based system for dual drug delivery. Nanoscale Res Lett. [internet]. 2018 [cited 2020 Oct 22]; 13(1):101. Available from https://pubmed.ncbi.nlm.nih.gov/29654484/. Doi:10.1186/s11671-018-2519-0.

21. Chen M, Liu X, Fahr A. Skin penetration and deposition of carboxyfluorescein and temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application. Int. J. Pharm. 2011;408(1-2),223–34.