Preliminary Study of Capsicum Extract-Loaded Liposomes and Ethosomes

Tun Chusut[1*], Laksana Charoenchai[1] and Natasa Vukovic[2]

[1] Sino-Thai Traditional Medicine Research Center, Faculty of Pharmacy, Rangsit University, Pathum Thani, 12000, Thailand
[2] Faculty of Pharmacy, University of Belgrade, Serbia
[*]Corresponding author, e-mail: tun@rsu.ac.th

Abstract

Objectives: The objectives of the study were to develop and determine physicochemical properties of capsicum extract-loaded liposomes and ethosomes. Methodology: The liposomes and ethosomes were prepared by modified ethanol injection technique and thin film hydration technique respectively. The physicochemical properties of all formulations such as particle size, polydispersity index, zeta potential and entrapment efficiency were investigate afterwards. Result and Discussion: The suitable liposomal formulation was formulation no. 27LS100 which was composed of phosphatidylcholine from soybean (SPC) and Tween 60 (80:20) and total lipid was 100 µg/ml. The mean particle size was smaller than 250 nm. The entrapment efficiency of capsaicin was 59%, and zeta potential value was -50 mV. In the case of ethosomes, formulations no. 15E20S4 and no. 16E30S4 showed the suitable results. It was composed of 4% SPC w/v and 20 – 30% ethanol v/v. The particle size of both formulations showed larger than 450 nm. The entrapment efficiency of them was 42 and 69% respectively. Zeta potential values were higher than -35 mV. Conclusions: All results of formulation no. 27LS100, 15E20S4 and 16E30S4 indicate that good physicochemical property of lipid-based nano-delivery systems for skin delivery. However, liposomal and ethosomal formulations need development and improvement with additives for increased stability and efficacy. The physicochemical properties of both liposomes and ethosomes such as morphology will be later determined. *In vitro* release, stability testing, and comparative study of the results of liposomes with ethosomes will be a further study.

Keywords**:** *Liposomes, Ethosomes, Capsaicin, Capsicum extract*

บทคัดย่อ

วัตถุประสงค์ การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาตำรับไลโปโซมและเอทโธโซมและประเมินหาคุณสมบัติทางเคมีและกายภาพ วิธีดำเนินการวิจัย การเตรียมไลโปโซมจะเตรียมจากการปรับปรุงวิธีการฉีดด้วยเอทานอลและเอทโธโซมจะเตรียมโดยวิธีทินฟิล์มไฮเดรชัน หลังจากนั้นจะทำการประเมินคุณสมบัติทางเคมีและกายภาพของตำรับทั้งหมดได้แก่ ขนาดอนุภาค ค่าศักย์ซีต้า ดัชนีการกระจายตัวของอนุภาคและประสิทธิภาพในการกับเก็บ ผลการวิจัย สูตรที่เหมาะสมที่สุดของไลโปโซมคือ สูตร 27LS100 ซึ่งประกอบไปด้วยฟอสฟาติดิลโคลีนจากถั่วเหลืองและทวีน 60 (80: 20) และความเข้มข้นไขมันรวมคือ 100 ไมโครโมล/มิลลิลิตร ซึ่งมีขนาดอนุภาคเล็กกว่า 250 นาโนเมตรประสิทธิภาพการกักเก็บแคปไซซิเท่ากับ 59% และค่าศักย์ซีต้า -50 มิลลิโวลต์ ในส่วนของเอทโธโซมนั้นพบว่าสูตรที่เหมาะสมที่สุดคือสูตร 15E20S4 และ 16E30S4 โดยมีส่วนประกอบของ SPC ร้อยละ 4 โดยน้ำหนักต่อปริมาตร และมีปริมาณเอทานอลร้อยละ 20 – 30 โดยปริมาตร ขนาดอนุภาคของทั้งสองสูตรนั้นมีขนาดใหญ่กว่า 450 นาโนเมตร ประสิทธิภาพการเก็บกักสารคือ ร้อยละ42 และ 69 ตามลำดับ ค่าศักย์ซีต้าสูงกว่า -35 มิลลิโวลต์ ข้อสรุป/ข้อเสนอแนะ ผลการทดลองทั้งหมดของสูตร 27LS100 15E20S4 และ 16E30S4 บ่งชี้ว่าทั้งสามสูตรนั้นมีคุณสมบัติทางเคมีและกายภาพที่ดีของระบบนำส่งอนุภาคนาโนสำหรับนำส่งทางผิวหนัง อย่างไรก็ตามสำหรับไลโปโซมและเอทโธโซมยังคงต้องพัฒนาสูตรต่อไปโดยการเพิ่มสารเติมแต่งเข้าไปเพื่อปรับปรุงความคงตัวและประสิทธิภาพต่อไป หลังจากนั้นจะทำการหาคุณสมบัติทางเคมีและกายภาพของทั้งไลโปโซมและเอทโธโซมอย่างเช่น ลักษณะสัณฐานวิทยา การศึกษาการปลดปล่อยในหลอดทดลองและการทดสอบความคงตัวและเปรียบเทียบผลของไลโปโซมกับเอทโธโซมในการศึกษาต่อไป

คำสำคัญ: *ไลโปโซม เอทโธโซม แคปไซซิน สารสกัดจากพริก*

1. Introduction

Capsaicin is a main active compound in capsicum extract. It applies to the skin for pain caused by shingles, osteoarthritis, rheumatoid arthritis and fibromyalgia. It was also used topically for nerve pain (neuropathy) associated with diabetes and HIV, other types of nerve pain (neuralgia), and back pain (Wieland *et al*., 2005; Knotkova *et al*., 2008; Treede *et al*., 2013). However, a component of capsicum caused unpleasant effects such as stinging, burning and erythema when having a skin contact. Thus, the delivery system is applied to reduced side effects of capsaicin.

It has been reported that using of phospholipid vesicles as the carrier system for capsaicin would enable its improved therapeutic effect with reduced side effects such as liposomes and ethosomes (Thapa *et al*., 2013; Teixeira *et al*., 2015). Capsaicin was developed with other delivery systems such as nanostructure lipid carriers (Raza *et al*., 2014) and microemulsions (Duaungjit *et al.*, 2016). Most research studies focus on capsaicin but not capsicum extract. Thus, we will develop and focus on capsicum extract based on phospholipid vesicle such as liposomes and ethosomes in this study. Liposomes are phospholipid bilayer and spherical shape. They are safe and skin-friendly formulation. Besides, they can reduce toxicity by controlling release and improving drug permeation into deeper layers of the skin (Schreier and Bouwstra, 1994). That will enable its improved therapeutic effect with reduced side effects. Interestingly, ethosomes are mainly consisted of phospholipids, ethanol and water. The phospholipid bilayers in ethosomes are packed less tightly, possess a high degree of fluidity and are reported to penetrate deeper into the skin layers. Hence, the use of both liposomes and ethosomes as the carrier system for capsaicin encapsulation would enable its improved therapeutic effect with reduced side effects. In addition, the encouragement of using of capsicum extract can help add value to chili and generate revenue for the community. Hence, the delivery system based on phospholipid is interest and suitable for encapsulation capsicum extract.

2. Objectives

The objectives of this study were to develop and characterize physicochemical of capsicum extract-loaded liposomes and ethosomes to select the optimal ratio of compositons in liposomal and ethosomal formulations for further study.

3. Materials and methods

3.1 Materials

L-α-Phosphatidylcholine from soybean (SPC), L-α-Phosphatidylcholine from egg yolk (EPC) and cholesterol from lanolin (CHOL) were purchased from Sigma Aldrich (USA). Tween 60 was purchased from KAO (Japan). Capsicum extract was obtained from Kaewmungkorn Co., Ltd. (Thailand). Ultrapure water was obtained from Mirae ST (Korea). Acetic acid and acetonitrile were purchased from Fisher chemical (USA). Absolute ethanol was purchased from Carlo Erba Reagents (France).

3.2 Methods

Capsaicin determination

The amount of capsaicin in the capsicum extract and the vesicles were determined by HPLC at the wavelength of 280 nm on Agilent 1260 (Agilent Technologies, USA) with the isocratic mode. The mobile phase consisted of 1% acetic acid in water and acetonitrile (55:45) was pumped through C18 ACE Generix. The flow rate was 1.0 ml/min and the injection volume was 10 µl. The running time was 20 minutes at the room temperature.

Preparation of liposomes containing capsicum extract

Liposomes containing capsicum extract were prepared by modified ethanol injection technique (Maitani *et al*., 2001) with the amount of capsaicin in formulations was defined 0.025% (w/v). The oil phase was composed of SPC or EPC, CHOL and capsicum extract dissolved in ethanol with a variation of total lipids and ratio. The water phase was composed of Tween 60 dissolved in the water and propylene glycol (7:3). Both phases were sonicated until the composition dissolved completely and mixed them together in round bottom flask. After that, the ethanol was evaporated by evaporator (Buchi, Switzerland) and obtained liposomes. Table 1 shows the compositions of capsicum extract-loaded liposomes.

Table 1. Composition of capsicum extract-loaded liposome formulations

Formulation No.	Total lipids (µmol/ml)	Ratio	Composition			
			SPC	EPC	Tw60	CHOL
1LS20	20	Weight	84	-	16	-
2LS30	30	Weight	84	-	16	-
3LS40	40	Weight	84	-	16	-
4LS20	20	Weight	80	-	20	-
5LS30	30	Weight	80	-	20	-
6LS40	40	Weight	80	-	20	-
7LS20	20	Molar	7	-	-	3
8LS30	30	Molar	7	-	-	3
9LS40	40	Molar	7	-	-	3
10LS20	20	Molar	4	-	-	1
11LS30	30	Molar	4	-	-	1
12LS40	40	Molar	4	-	-	1
13LE20	20	Weight	-	84	16	-
14LE30	30	Weight	-	84	16	-
15LE40	40	Weight	-	84	16	-
16LE20	20	Weight	-	80	20	-
17LE30	30	Weight	-	80	20	-
18LE40	40	Weight	-	80	20	-
19LE20	20	Molar	-	7	-	3
20LE30	30	Molar	-	7	-	3
21LE40	40	Molar	-	7	-	3
22LE20	20	Molar	-	4	-	1
23LE30	30	Molar	-	4	-	1
24LE40	40	Molar	-	4	-	1

Table 2. Composition of capsicum extract-loaded ethosome formulations

Formulation No.	% SPC(w/v)	% Ethanol(v/v)
1E00S0.5	1	0
2E10S0.5	1	10
3E20S0.5	1	20
4E30S0.5	1	30
5E00S1	2	0
6E10S1	2	10
7E20S1	2	20
8E30S1	2	30
9E00S2	3	0
10E10S2	3	10
11E20S2	3	20
12E30S2	3	30
13E00S4	4	0
14E10S4	4	10
15E20S4	4	20
16E30S4	4	30

Preparation of ethosomes containing capsicum extract

Capsicum extract-loaded ethosomes were prepared by thin film hydration technique (Abdulbaqi, 2016) with the capsaicin concentration was fixed at 0.0025% (w/v). First of all, the oil phase, capsicum extract and variation with the amount of SPC were dissolved in ethanol and then they were sonicated for 30 minutes. After that, the ethanol was evaporated by rotary evaporator and formed lipid film in round bottom flask. Then the lipid film was hydrated by different concentration of the hydroethanolic solution in the round bottom flask and rotated flask at 60 rpm at the room temperature until lipids film removed from round bottom flask. Lastly, the colloidal dispersions were sonicated for 30 minutes and obtained ethosome formulations. The compositions of capsicum extract-loaded ethosomes are shown in Table 2.

Particle size and zeta potential

Particle size, size distribution and zeta potential of liposomes and ethosomes containing capsicum extract were measured by NanoPlus (Particulate Systems, USA) at 25 ± 0.2°C. The samples were diluted 50 folds with ultrapure water. The laser diffractometry (LD) data obtained were evaluated using volume distribution as diameter (*d*) values of 10%, 50%, 90% and Span values. The diameter values that indicate the percentage of particles possessing a diameter equal to or lower than the given value. The Span value is a statistical parameter useful to appraise the particle size distribution and calculated applying the following equation (Teeranachaideekul *et al*., 2007).

Entrapment efficiency (%EE)

Entrapment efficiency of liposomes and ethosomes containing capsicum extract were determined by ultrafiltration centrifugation technique (Zhang *et al*., 2008). They were diluted with the water: propylene glycol (7:3). After that, the centrifugal filter tube (Amicon® Ultra0.5; Millipore, USA) with a molecular weight cut off of 30KDa was added sample 500 µl and centrifuged at 10,000 rpm for 10 minutes. Free drug which passed through the membrane into collector tube was analyzed HPLC. The entrapment efficiency of capsaicin was calculated as followed.

$$\%\,EE = \left[\frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}}\right] x\ 100$$

3.3 Statistics

All reported data are mean ± standard deviation (S.D.). A significance of difference was evaluated using student's t-test and one way ANOVA at $p < 0.05$.

4. Results and Discussion

Physical appearance

All formulations showed orange colloid after preparation. After 1week storage, the liposomal formulations were composed of EPC separated (13LE20-24LE40) due to capsicum extract perturb the forming of EPC phospholipid bilayer. Moreover, the liposomal formulations were composed of cholesterol and SPC (7LS20-12LS40), they were found aggregation. It is possible that they lacked steric repulsion of nonionic surfactant in the formulation (Kronberg *et al*., 1990). The suitable ratio of capsicum extract liposomes was 80:20 (Weight ratio). They were considered with the results of physicochemical properties and stability after 1 week storage. Thus formulations no. 6LS40 were chosen and developed with the increase in total lipids and capsicum extract concentrations (25LS60 – 28L2S100). Both the increase in total lipids 60 to 100 µmol/ml (25LS60 – 27LS100) and the increase in capsicum extract (28L2CS100) in formulations were still stable.

All formulations of ethosomes were unstable except 12E30S2, 15E20S4 and 16E30S4. Interestingly, the high content of ethanol in ethosomal formulations helped to disperse the lipid, and it affects their stability. Also the total lipids increased the ethosomes had more stable. Figure 1 showed stable formulations of liposomes and ethosomes after 1 week storage.

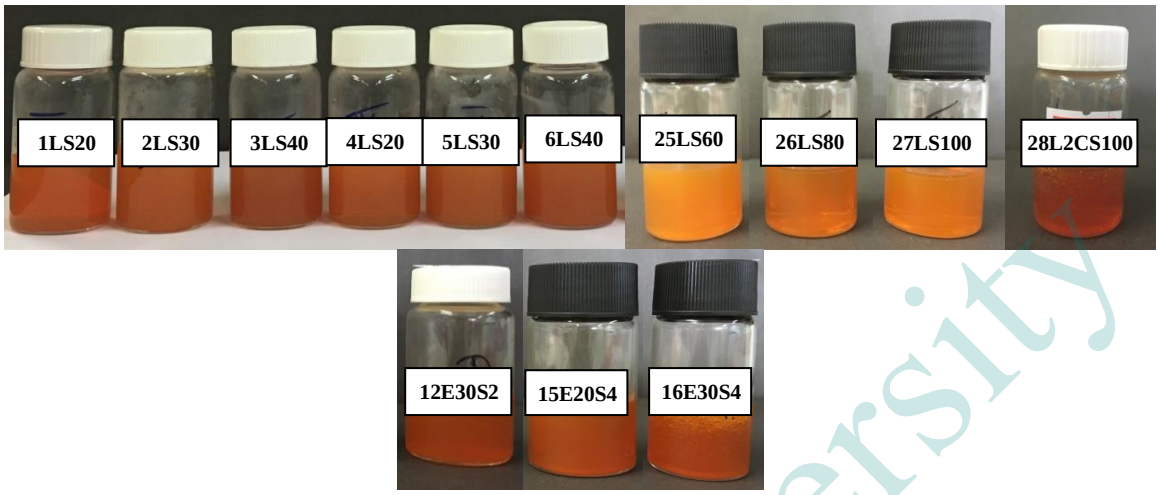

Figure 1. Physical appearance of liposomes and ethosomes formulations after 1 week storage

Particle size analysis and zeta potential

The physicochemical properties of all formulation were shown in Table 3. All liposomal formulations showed the mean particle size (z-ave) smaller than 350 nm were observed. They are suitable for topical application. PI values were less than 0.2 that indicate narrow size distribution of the vesicles. The z-ave and PI values of liposomal formulations tended to increase when total lipids increased that indicate phospholipid concentrations playing a role in the size of vesicle (Touitou *et al*., 2000). The mean particle size of formulations no. 1LS20 to 6LS40 was composed of Tween 60 showed smaller than the mean particle size of formulations no. 7LS20 - 12LS40 which consisted of cholesterol. It is due to nonionic surfactant such as Tween 60 which affected to reduce the polymeric wall thickness of liposomes (Palumbo *et al*., 2002) and caused the particle size reduction. Span values of liposomal formulations were low values, and obtained volume distribution diameter of 90% was smaller than 510 nm. This result confirmed narrow size distribution of liposomal formulations corresponding with their PI values. The ethosomal formulations showed the particle sizes were in the range 480 to 700 nm and that were larger than the liposomes ($p < 0.05$). Not only PI value but also Span values of ethosomes were higher than liposomes. In this study, the ethosomal formulations had surfactant-free which surfactant played a key role in particle size. Hence, the absence of surfactant involves the large particle size. Additionally, when increasing the ethanol concentrations, the particle sizes were smaller. It is possible that the presence of ethanol causes a modification of the net charge of the system and confers it some degree of steric stabilization that may finally lead to a decrease in the size of vesicles (Lasic *et al*., 1998; Touitou *et al*., 2000). The zeta potential of liposomal formulations and ethosomal formulations were in the range -35 to -62 mV and -26 to -52 mV respectively. They approximate ± 30 mV that indicate good physical stable of colloidal dispersions (Souto and Müller, 2005). Furthermore, the zeta potential was lower when increased the ethanol content contrast with increased total lipids that caused the zeta potential of ethosomes was higher.

Entrapment efficiency

Both the entrapment efficiency of liposomal formulations and ethosomal formulations were higher when increasing the lipid content. %EE of liposomal formulations no. 27LS100 shows the highest entrapment efficiency (59%) and a statistically significant difference ($p < 0.05$) from the other formulations. Formulations no. 28L2CS100, the encapsulation did not increase when the capsicum extract increased two fold. %EE of ethosomal formulation increased about two folds when increased the lipid content to 4% w/v. However, ethosomal formulations showed lower encapsulation of capsaicin when increasing the ethanol content. It is due to the high ethanol concentrations which can decrease the membrane thickness of the vesicles, corresponding to the formation of a phase with interpenetrating hydrocarbon chains (Lopez-Pinto, Gonzalez-Rodriguez and Rabasco, 2005).

Table 3. Physicochemical properties of liposomes and ethosomes containing capsicum extract

Formulation No.	z-ave (nm)	PI	Zeta potential	%EE	Physical appearance
1LS20	120.60 ±1.23	0.103 ± 0.009	-49.66 ± 0.85	21.79 ± 4.39	Orange colloid
2LS30	154.93 ±1.24	0.135 ± 0.014	-62.02 ± 1.57	41.60 ± 1.99	Orange colloid
3LS40	140.13 ±0.90	0.177 ± 0.005	-64.73 ± 1.36	44.80 ± 0.69	Orange colloid
4LS20	121.27 ±0.76	0.088 ± 0.016	-53.46± 3.76	27.14 ± 2.68	Orange colloid
5LS30	150.30 ±0.66	0.119 ± 0.005	-57.54 ± 0.26	43.07 ± 1.61	Orange colloid
6LS40	192.50 ±0.70	0.141 ± 0.012	-59.07 ± 0.21	47.39 ± 2.27	Orange colloid
7LS20	150.30 ±1.20	0.128 ± 0.015	-57.87 ± 2.33	-	Aggregation
8LS30	183.93 ±1.68	0.147 ± 0.015	-43.65 ± 1.98	-	Aggregation
9LS40	216.20 ±0.98	0.182 ± 0.004	-40.23 ± 0.54	-	Aggregation
10LS20	146.13 ±2.54	0.091 ± 0.004	-61.63 ± 3.34	-	Aggregation
11LS30	188.20 ±1.04	0.145 ± 0.014	-53.86 ± 1.87	-	Aggregation
12LS40	236.83 ±0.76	0.186 ± 0.008	-57.57 ± 2.35	-	Aggregation
13LE20	-	-	-	-	Separation
14LE30	-	-	-	-	Separation
15LE40	-	-	-	-	Separation
16LE20	-	-	-	-	Separation
17LE30	-	-	-	-	Separation
18LE40	-	-	-	-	Separation
19LE20	-	-	-	-	Separation
20LE30	-	-	-	-	Separation
21LE40	-	-	-	-	Separation
22LE20	-	-	-	-	Separation
23LE30	-	-	-	-	Separation
24LE40	-	-	-	-	Separation
25LS60	306.73 ± 4.45	0.197 ± 0.016	-35.54 ± 1.47	46.83 ± 2.05	Orange colloid
26LS80	315.00 ± 1.11	0.181 ± 0.008	-52.30 ± 0.67	51.52 ± 2.46	Orange colloid
27LS100	249.90 ± 0.26	0.207 ± 0.010	-49.58 ± 0.29	59.36 ± 0.73*	Orange colloid
28L2CS100	322.00 ± 3.30	0.170 ± 0.004	-47.43 ± 0.53	48.85 ± 0.60	Orange colloid
1E00S0.5	-	-	-	-	Separation
2E10S0.5	-	-	-	-	Separation
3E20S0.5	-	-	-	-	Separation
4E30S0.5	-	-	-	-	Separation
5E00S1	-	-	-	-	Precipitation
6E10S1	-	-	-	-	Separation
7E20S1	-	-	-	-	Separation
8E30S1	-	-	-	-	Separation
9E00S2	-	-	-	-	Precipitation
10E10S2	-	-	-	-	Seperation
11E20S2	-	-	-	-	Seperation
12E30S2	481.3 ± 8.6*	0.285 ± 0.019	-26.56 ± 11.08	37.45 ± 1.98	Orange colloid
13E00S4	-	-	-	-	Precipitation
14E10S4	-	-	-	-	Separation
15E20S4	686.1± 13.3*	0.346 ± 0.005	-51.21 ± 1.45	69.26 ± 0.12	Orange colloid
16E30S4	487.0 ± 6.0*	0.262 ± 0.015	-37.05 ± 1.64	42.10 ± 0.28	Orange colloid

* = p value < *0.05*

Table 4. Volume distribution diameters in nanometer (nm) (*d*10%, *d*50% and *d*90%) and Span values on the day of preparation

Formulation No.	*d10*%	*d*50%	*d*90%	Span
1LS20	81	123	189	0.882
2LS30	98	157	258	1.022
3LS40	85	142	250	1.164
4LS20	80	123	190	0.897
5LS30	94	152	251	1.036
6LS40	119	205	328	1.018
7LS20	90	155	271	1.168
8LS30	118	183	293	0.955
9LS40	124	222	417	1.319
10LS20	95	148	232	0.930
11LS30	114	191	368	1.333
12LS40	130	252	500	1.469
13LE20	-	-	-	-
14LE30	-	-	-	-
15LE40	-	-	-	-
16LE20	-	-	-	-
17LE30	-	-	-	-
18LE40	-	-	-	-
19LE20	-	-	-	-
20LE30	-	-	-	-
21LE40	-	-	-	-
22LE20	-	-	-	-
23LE30	-	-	-	-
24LE40	-	-	-	-
25LS60	183	347	569	1.114
26LS80	176	336	646	1.399
27LS100	127	278	525	1.429
28L2CS100	193	346	609.1	1.202
1E00S0.5	-	-	-	-
2E10S0.5	-	-	-	-
3E20S0.5	-	-	-	-
4E30S0.5	-	-	-	-
5E00S1	-	-	-	-
6E10S1	-	-	-	-
7E20S1	-	-	-	-
8E30S1	-	-	-	-
9E00S2	-	-	-	-
10E10S2	-	-	-	-
11E20S2	-	-	-	-
12E30S2	215	535	1401	2.216
13E00S4	355	1140	3256	2.543
14E10S4	-	-	-	-
15E20S4	-	-	-	-
16E30S4	268	513	1028	1.480

5. Conclusion

The suitable formulation of liposomes was formulation no. 27LS100 which composed of SPC and Tween 60 (80: 20) with total lipids concentration 100µg/ml. The mean particle size was smaller than 250 nm. This formulation demonstrated the highest entrapment efficiency (59%) and a good physical stability indicated by zeta potential value (-49.58). In the case of ethosomes, the suitable formulations were formulations no. 15E20S4 and 16E30S4. They were composed of 4% SPC (w/v) and 20 – 30% ethanol (v/v). The mean particle size was larger than 450 nm. The entrapment efficiency of both formulations no. 15E20S4 and 16E30S4 were 42 and 69%, respectively. Zeta potential values were higher than -35 mV. In conclusion, formulation no. 27LS100, 15E20S4 and 16E30S4 seem promising of lipid-based nano-delivery systems for skin delivery. This is preliminary study of liposomal and ethosomal formulations. However, liposomal and ethosomal formulations need development and improvement with additives for increased stability and efficacy later. The physicochemical properties of both liposomes and ethosomes such as morphology will be later determined. *In vitro* release, stability testing, and comparative study of the results of liposomes with ethosomes will be a further study.

6. Acknowledgements

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