Development of Liposomes Containing Oxyresveratrol: Comparative Study of Cholesterol and Tween 80 as Vesicle Stabilizer

การพัฒนาลิโพโซมที่บรรจุออกซีเรสเวอราทรอล: การศึกษาเชิงเปรียบเทียบของคลอเรสเตอรอลกับทวีน 80 ในการเป็นสารช่วยสร้างความเสถียรของถุงทรงกลม

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ABSTRACT

Oxyresveratrol, an anti-tyrosinase inhibitor, has been isolated heartwood extract of *Artocarpus lakoocha* Roxb and formulated in liposomes for topical delivery system by using Tween 80 or cholesterol as vesicle stabilizer. From the results, the liposomes consisting of Tween 80 had particle size in the range of 49-50 nm which was smaller than those of cholesterol. The polydispersity index of liposomes with Tween 80 had lower values in range of 0.179-0.296 while those with cholesterol showed values in range of 0.381-0.497. For zeta potential, liposomes with both vesicle stabilizer had negative values in the same range which were -28.0 to -34.1 mV. The entrapment efficiency (%EE), liposomes with high concentration of EPC had higher %EE than those of lower concentration. It was found that Tween 80 could be an alternative vesicle stabilizer of cholesterol in liposomes containing oxyresveratrol formation.

บทคัดย่อ

ออกซีเรสเวอราทรอลซึ่งเป็นสารที่มีฤทธิ์ยับยั้งเอนไซม์ไทโรซิเนส ได้ถูกแยกออกมาจากสารสกัดของแก่นมะหาดและเตรียมให้อยู่ในรูปแบบลิโพโซมโดยมีทวีน 80 หรือคลอเรสเตอรอลเป็นสารช่วยเพิ่มความคงตัวของถุงทรงกลม จากผลการทดลองพบว่า ลิโพโซมที่เตรียมจากทวีน 80 มีขนาดอนุภาคอยู่ในช่วง 49-50 นาโนเมตร ซึ่งเล็กกว่าลิโพโซมที่เตรียมจากคลอเรสเตอรอล ค่าดัชนีการกระจายตัวของอนุภาคลิโพโซมที่เตรียมจากทวีน 80 มีค่าแคบกว่า คืออยู่ในช่วง 0.179-0.296 ในขณะที่อนุภาคลิโพโซมที่เตรียมจากคลอเรสเตอรอลมีค่าอยู่ในช่วง 0.381-0.497 สำหรับศักย์ไฟฟ้าซีตา พบว่าสารช่วยทั้งสองชนิดให้ค่าศักย์ไฟฟ้าที่อยู่ในช่วงที่ใกล้เคียงกันคือ -28.0 ถึง -34.1 มิลลิโวลต์ ส่วนค่าการเก็บกักสารไว้ในลิโพโซมพบว่า ค่าการเก็บกักสารจะสูงขึ้นเมื่อเพิ่มความเข้มข้นของฟอสโฟลิปิด จากผลการวิจัยสรุปได้ว่า ทวีน 80 เป็นตัวเลือกที่สามารถนำมาใช้ในการเตรียมลิโพโซมเพื่อเก็บกักสารออกซีเรสเวอราทรอล

Key Words: Liposomes, Oxyresveratrol, Tween 80
คำสำคัญ: ลิโพโซม ออกซีเรสเวอราทรอล ทวีน 80

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Introduction

Liposomes have taken the interest of pharmacists for their potential to overcome the topical drug delivery challenges (Liu et al., 2007). Liposomes are microscopics structures consisting of a phospholipid bilayer encapsulating an aqueous core. The structures may be unilamellar, multilamellar or multivesicular (Chahar et al., 2012). Cholesterol is often included in the liposomes formulation to give more rigidity of the bilayer that may improve *in vivo* and *in vitro* stability of the liposomes (Tseng et al., 2007). Tween 80 has been used as a vesicle stabilizer in delivering *Hibiscus sabdariffa* (roselle) calyx extract into pig skin (Amnuakit et al., 2014). The part of the long hydrocarbon chain may behave like cholesterol in the liposomes. The heartwood extract of *Artocarpus lakoocha* Roxb which contains oxyresveratrol as an anti-tyrosinase inhibitor has been evaluated as a promising potential for using as an effective and economical skin-whitening agent (Tengamnuay et al., 2006).

Objective of the study

Our objective is to develop liposomes entrapping oxyresveratrol inside the vesicle and using Tween 80 to stabilize the bilayer comparing with cholesterol.

Methodology

Instrument and reagents

Egg yolk phosphatidylcholine (EPC) and cholesterol were purchased from Sigma (St. Louis, USA). Tween 80 (Polysorbate 80) was purchased from Croda (East Yorkshire, UK). The ultrasonic processor with timer and pulser was performed on Cole-Parmer EW-04714-50, 115 VAC (Illinois, USA). The zeta potential and particle size measurement was performed with Malvern Zetasizer Nano ZS (Worcestershire, UK). Morphology of the obtained liposomes was observed under a scanning electron microscope (SEM, Hitachi S-3400N, Japan) at an accelerating voltage of 20 kV. All other reagents and solvents were reagent grade and used without further purification. TLC was performed on silica gel GF_{254} (Merck). For column chromatography, silica gel (Merck 230-400 mesh) was used. The entrapment efficiency was performed on Agilent 1260 infinity HPLC system. NMR spectra were recorded with a Bruker Avance (^{1}H, 300 MHz) spectrometer. Chemical shifts are reported in ppm, and coupling constants are reported in Hz. All NMR spectra were obtained in deuterated dimethylsulfoxide (DMSO-*d6*) and referenced to the residual solvent peak. Mass spectra were obtained from Agilent LC 1200 coupled with Bruker microTOF.

Plant materials

The plant material of *A. lakoocha* was bought from local drugstore in Nonthaburi province, Thailand. The material was identified by comparison with the specimens at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimen of *A. lakoocha* (SRU 045) was deposited at Faculty of Oriental Medicine, Rangsit University, Pathumthani, Thailand.

Preparation of crude and partial-purified extracts

The dried heart wood powder of *A. lakoocha* 100 g was macerated with 95% ethanol 400 mL at room temperature for 7 day. The extract was filtered with Whatman No.1 and then evaporated

under reduced pressure with rotary evaporator to obtain 12 g of finally crude dark brown extract. The crude extract (2 g) was dissolved in CH_2Cl_2 : MeOH (7 : 3) (10 ml) . Then the mixture was subjected to silica gel column chromatography and eluted with CH_2Cl_2 : MeOH (7 : 3) to obtain dark brown extract (1.1 g).

Isolation of Oxyresveratrol

The crude extract (1.1 g) was dissolved in CH_2Cl_2 : MeOH (8 : 2) (5 ml). Then the mixture was subjected to silica gel column chromatography and eluted with mobile phase CH_2Cl_2 : MeOH (9 : 1). After that, fractions 15-19 were collected and evaporated to obtain a yellow residue. The residue was recrystallized in CH_2Cl_2 : MeOH (9 : 1) and stored in refrigerator at 14 °C for 24 h to obtain yellow solid 230 mg with melting point 196-198ºC. UV : λ_{max} 220, 302, 329 nm, IR : (KBr disc) 3290, 2925, 1610 cm^{-1} ^{1}H NMR : (300 MHz, DMSO-d_6) δ[ppm] 6.08 (br s, 1H), 6.25 (*dd*, J = 8.4, 2.4 Hz, 1H), 6.33(*d*, J = 2.4 Hz, 1H), 6.77 (*d*, J = 16.5 Hz, 1H), 7.15 (*d*, J = 16.5 Hz, 1H), 7.34 (*d*, J = 8.4 Hz, 1H)). ^{13}C NMR: (75.47 MHz, DMSO-d_6) δ[ppm] 158.5, 158.2, 156.1, 140.1, 127.3, 124.7, 123.3, 115.4, 107.4, 104.2, 102.7, 101.5 and MS (GC/MS): M^+ = 244 (บุญชู และคณะ, 2541).

Preparation of oxyresveratrol liposomes

As the main vesicle components, test liposomes composed of EPC, cholesterol, and Tween 80 at various weight ratios shown in Table 1.

Table 1 Composition of oxyresveratrol liposomes

No.	Name of chemicals	Formula 1	Formula 2	Formula 3	Formula 4
1	EPC (mg)	200	200	300	300
2	Propylene glycol (mL)	10.0	10.0	7.5	7.5
3	Tween 80 (mg)	25.0	-	25.0	-
4	Cholesterol (mg)	-	25.0	-	25.0
5	Oxyresveratrol (mg)	20.0	20.0	20.0	20.0
6	Water (mL)	9.75	9.75	12.15	12.15

The liposomes were prepared by modified ethanol injection method (Pinsuwan et al., 2012). Briefly, the lipid components were weighed and propylene glycol was added to dissolve all ingredients. The mixture was heated at 60°C until dissolved. Then it was added into the water phase

with stirrer to obtain white cloudy solution. The size of the vesicles was reduced by ultrasonic probe for 15 min. After that each formula was subjected to particle size, zeta potential, entrapment efficiency measurements and morphology observation.

Entrapment Efficiency

Five hundred microliter of Sephadex G-50 swelled in deionized water was filled in the 1.0 mL-syringe barrel which contains a small cotton ball to support the gel. Then the syringe barrel was inserted into an Eppendorf tube and spun at 3,000 rpm for 3 min to remove excess water from the gel. Then the syringe barrel containing Sephadex gel was transferred into a new Eppendorf tube. Four hundred microliter of the liposomal preparation was applied to the Sephadex bed and spun at 1,500 rpm for 3 min. Two hundred microliter of water was added to the syringe barrel. The system was spun again at 3,000 rpm for 3 min (Torchilin et al., 2003). The separated material was added with 0.20 mL of Triton-X100 to break the liposome and transfer into 10.0 mL volumetric flask. The volume of mixture was adjusted with mobile phase of HPLC to determine the content of oxyresveratrol. For the analysis of total content of oxyresveratrol, 0.40 mL of the liposomal preparation was mixed with 0.20 mL of Triton-X100 to break the liposome and transfer into 10.0 mL volumetric flask. The volume of mixture was adjusted with mobile phase of HPLC and subjected to analyze for oxyresveratrol content. HPLC condition composed of column Ace C-18 (4.6 X 15 cm.), mobile phase (acetronitrile : 1% acetic acid, 48 : 52), flow rate 0.8 mL/min, UV detector at 327 nm, and injection volume 10 µL.

Results

The purification of *A. lakoocha* crude extract with silica gel column chromatography provided the partially purified extract. Then some portion was further purified with silica gel column chromatography with different solvent systems. The positive fractions were combined and removed the solvent to obtain a residue which was further purified by recrystallization to give desired product, oxyresveratrol. The compound was identified by spectroscopic methods and compared the results with literature data (บุญชู และคณะ, 2541).

For the preparation of liposomes, the modified ethanol injection method was chosen to make nanoparticles. In this experiment, egg yolk phosphatidylcholine (EPC) was used with either cholesterol or Tween 80 as vesicle stabilizer. All formula was conducted with the same method to obtain slightly cloudy pale-yellow dispersion system (Figure 1).

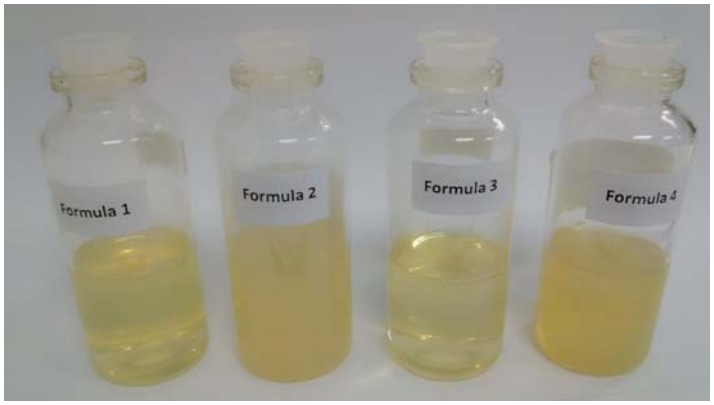

Figure 1 Liposomes of oxyresveratrol

The particle size, polydispersity index, zeta potential and entrapment efficiency of each formula were measured and showed in Table 2.

Table 2 PS, PI, ZP, and EE% of oxyresveratrol liposomes

Formulation	PS ± SD (nm)	PI ± SD	ZP ± SD (mV)	EE% ± SD
1	49 ± 1.00	0.296 ± 0.02	-28.0 ± 0.5	83.32 ± 0.38
2	189 ± 0.96	0.381 ± 0.07	-30.6 ± 0.4	79.64 ± 0.23
3	50 ± 0.58	0.179 ± 0.02	-34.1 ± 0.6	88.67 ± 0.31
4	510 ± 3.21	0.497 ± 0.01	-33.7 ± 1.2	95.43 ± 0.36

Abbreviation: PS, particle size; PI, polydispersity index; ZP, zeta potential; EE%, entrapment efficiency

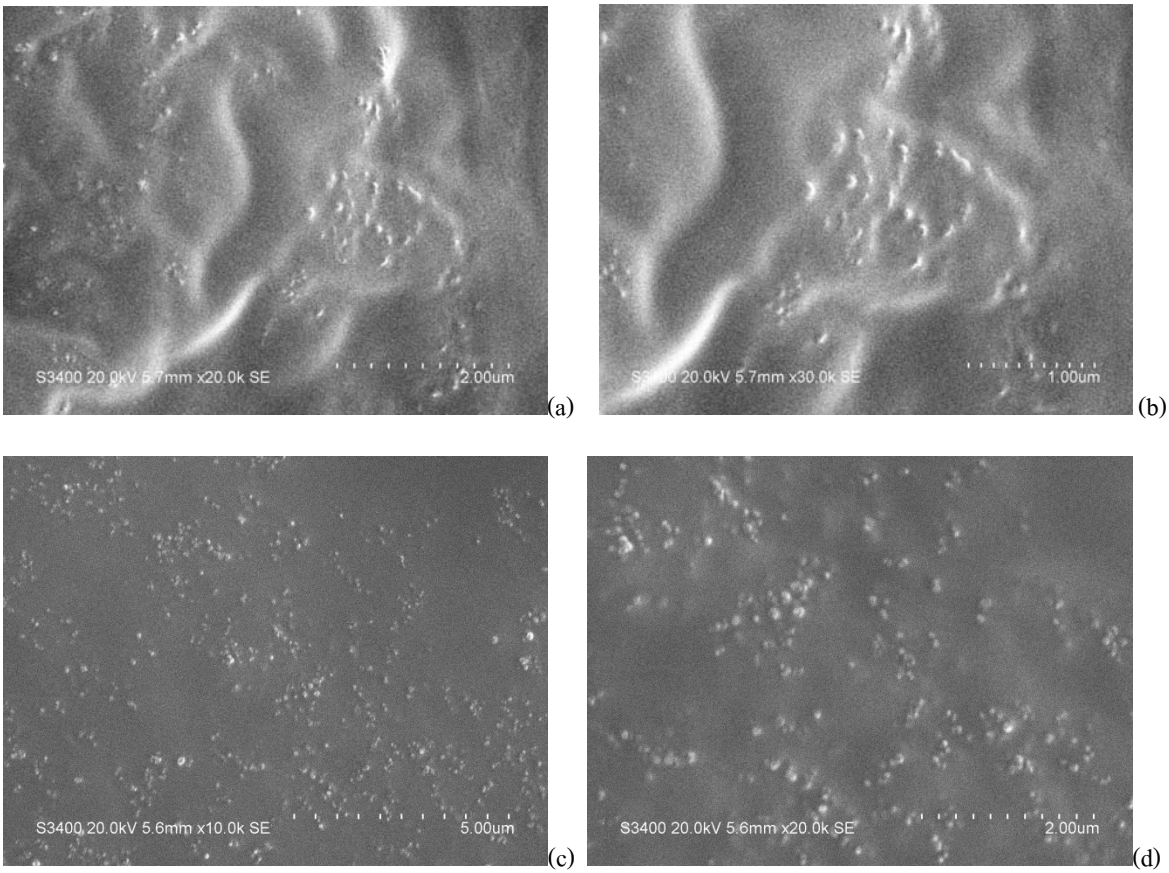

Figure 2 Scanning electron microscope pictures of liposomes (a) formula 1 (Tween 80) at 2 µm (b) formula 1 at 1 µm (c) formula 2 (cholesterol) at 5 µm, and (d) formula 2 at 2 µm.

Discussion and Conclusions

According to morphological analysis, all formula seemed to have a round vesicle or spherical shape of the liposomes. Figure 2 shows pictures of liposomes formula 1 and 2 observed under SEM.

From our study, the liposomes consisting of Tween 80 had particle size in the range of 49 - 50 nm which was smaller than those of cholesterol. Furthermore, increasing in concentration of EPC significantly affected the particle sizes of liposomes forming with cholesterol but was not affected by Tween 80. Next, the polydispersity index (PI) value is a value calculated for size distribution. The higher value means that the sample has broad size distribution. The PI values of formula that used Tween 80 were lower than those that used cholesterol which mean Tween 80 showed some influence in this case. This might be that Tween 80 molecule has a single hydrocarbon chain close to lipophilic part of EPC. It could promote formation of small vesicle of liposome and stabilize the vesicle. In addition, because the polyethoxylated sorbitan which is a polar part of Tween 80 faced to aqueous phase of the liposome and the aqueous environment, it could prevent particle aggregation by steric effect leading to the more stable system (Sinko, 2006). For the physical stability, all formulas were stable more than 1 month at room temperature storage. Oxyresveratrol is an anti-tyrosinase which potent than kojic acid, a

standard anti-tyrosinase, 10 times (*in vitro*). This compound has high polarity that can easily dissolve in water and suitable for making in liposome delivery system. Next, Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. For zeta potential values, all formulas gave a comparable value in range of -28.0 ± 0.5 to -34.1 ± 0.6 mV. This high zeta potential makes high physical stability due to repulsive force between particle surface. For the entrapment efficiency (%EE), the quantity of material entrapped inside liposomes, liposomes with high concentration of EPC had higher %EE than those of lower concentration. This synergism of good anti-tyrosinase and anti-oxidant in a good delivery system as liposomes can make this innovation brightening the skin and give radiance. Moreover, Tween 80 can be obtained from commercial supplier, stored in room temperature and the price is cheaper than cholesterol.

According to the results, liposomes which contained Tween 80 and oxyresveratrol exhibited many good characteristics including a small particle size, low PI, high zeta potential and good stability. Further investigation on formulation and skin penetration are in progress in our laboratory.

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