ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Silymarin Solid Lipid Nanoparticle containing Shellac Wax Fabricated with Hot Melt Emulsification

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ABSTRACT

Silymarin is a flavonoid extract isolated from seeds of the milk thistle (*Silybum marianum*). This compound has strong antioxidant and radical-scavenging properties. Additionally, silymarin also prevents UVB-induced skin carcinogenesis by pyrimidine dimer inhibition. Our study developed the solid lipid nanoparticle (SLN) fabricated by hot melt homogenization technique which contained silymarin and shellac wax. Type and amount of wax affected the morphology of SLN. The sonication at 40% amplitude for 45 sec was selected as the preparation condition for the 1% silymarin loaded in the used wax component. In order to investigate the effect of surfactants on homogeneity, particle size and rheological behavior, tween 80, solutol HS and lutrol F68 were employed as stabilizer in the selected preparation condition. The most efficient formulation was constructed to study the *in vitro* release and also shed snake skin permeation using a Franz diffusion cell. Surfactants influenced the particle size distribution whereas they had no influence on the mean particle size which was found in the range of 6-9 micrometer. The ζ-potential of the formula was rather high exhibited the pseudoplastic flow. Eventually, SLN could be promising to sustain the release of silymarin. These results implied that the optimal silymarin SLN was probably able to topically use for prevention of UVB-induced skin cancer.

Keywords: Silymarin, Solid lipid nanoparticle, Shellac wax, Hot melt emulsification

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ISSN: 0975-8585

INTRODUCTION

Silymarin is a group of flavonoids isolated from the fruits of Silybum marianum which has been used as a hepatoprotectant and in supportive treatment of patients with chronic inflammatory liver disorders. Recently, silymarin can be used to protect the skin from oxidative stress induced by ultraviolet (UV) irradiation [1]. Silymarin products have been supplied in different dosage forms such as capsules, tablets, liquids, powders, and creams [2,3]. The hydrophobic nature of silymarin chemical structure relates to its low aqueous solubility and bioavailability. Liposome, and lipid emulsion have been prepared to increase its bioavailability [4,5]. Solid dispersion of 1:4 silymarin to PEG 6000 increased the solubility of silymarin for 7.5-11 times [6]. Solid lipid nanoparticle (SLN) silymarin using Compritol 888 ATO as stabilizer with particle size 150 nm could increase the bioavailability [7].

Shellac wax is brownish wax obtained from manufacturing process as wasted product of shellac production [8,9]. Seedlac is a natural by-product obtained from Laccifer lacca which is the class of insect. Shellac's function is secreted to prevent blockage of cell pores and as an anti-constipate to alleviate the discomfort of the natural secretive activity of the insect [10]. This wax has been found in India, Thailand and other South East Asia. Shellac wax is obtained about 5% from a by-product of shellac manufacturing or collected from a first melting of crude as initial substance before processes to be shellac. Crude wax from the insect is melted and cooled to be a stick or film. From this process a stick lac is obtained which later is cracked to be a shellac wax. The shellac wax is refined to be shellac by three major processes including melting process, bleaching process and solvent extraction process [8].

Hot melt extrusion (HME) is a technique widely employed in plastic manufacturing and, nowadays, interestingly also applied in pharmaceutical process. HME has many advantages such as solvent free technique, less step of production process, no need many diluents or tableting compounds, homogeneous spreading and increasing for drug bioavailability [11,12]. The aim of this study is to determine the suitable condition and the effect of surfactant on the physical properties and release of silymarin from SLN containing shellac wax.

MATERIALS AND METHODS

Materials

Silymarin was purchased from Panjin Huacheng Pharmaceutical Co., Ltd. Tween 80, stearic acid and glyceryl monostearate were purchased from PC Drug Co., Ltd., Bangkok, Thailand. Lutrol F127 and solutol HS was obtained from BASF, Ludwigshafen, Germany. Shellac wax was purchased from Ake shellac Co., Ltd., Lumpang, Thailand. Potassium dihydrogen phosphate (Ajax Finechem, New South Wales, Australia), ethanol (lot no. 617W62, J. T. Baker Solusorb, Malaysia), citric acid (B/NO. AF609161, Seven Hills, NSW, Australia) and anhydrous Na_2HPO_4 (B/No.AF405300, Ajax Fineche, Australia) were used as received. Cellulose nitrate membrane with a pore size of 0.45 μm (Lot no.5020113060102363, Sartorius AG Weender Landstrasse 94-108 3707 Goettingen, Germany) was used as received. The dorsal part of shed

snake from Elaphae obsolete obtained from Saowabha Institute (Bangkok, Thailand) was stored at -4ºC prior to use for permeation study.

Methods

Determination of suitable condition for silymarin SLN preparation

The 0.4 g silymarin mixed with 7.5% shellac wax, glyceryl monostearate or stearic acid were heated at 75°C. For formulation containing 0%GMS, silymarin was dissolved in isopropranol before mixing. The 0.4g tween80 and 1.6 g Lutrol F68 were dissolved in distilled water and heated at 75°C and used as stabilizer. The total amount of component was 40 g. This prepared aqueous phase was pour into the oil phase and mixed with homogenizer (IKA® T25 digital Ultra-Turrax®) at rotation speed of 8,000 rpm for 15 min. The obtained systems were sonicated using Ultrasonicator (MODEL CV18 Serial ni. 18910184A). The amplitude and sonicating time were varied to determine the suitable condition. The obtained system was subsequently mixed with stirring overnight. The wax components of F1-F4 and optimum formula (Opt) as shown in Table 1 were prepared and evaluated finally.

Table 1 Composition of wax component

Formula	Wax
F1	-
F2	2% GMS
F3	2% Stearic acid
F4	1%GMS and 1%Steric acid
Opt.	2.5%GMS and 1%Steric acid

SLN preparation

The 0.4 g silymarin, 3 g shellac wax and 1 g glyceryl monostearate were heated at 75°C. The different amount of tween 80, solutol HS and lutrol F68 as shown in Table 2 were individually dissolved in distilled water and heated at 75°C. The total amount of component was 40 g. This prepared phase was poured into the oil phase and mixed with homogenizer (IKA® T25 digital Ultra-Turrax®) at rotation speed of 8,000 rpm for 15 min. The obtained systems were sonicated using Ultrasonicator (MODEL CV18 Serial ni. 18910184A) with amplitude of 40% for 45 s. The obtained system was subsequently mixed with stirring overnight.

Table 2 Composition of aqueous phase

Fomula	% Substance in aqueous
P1	1% tween 80 + 4% lutrol F68
P2	1% tween 80 + 2.5% solutol HS
P3	4% lutrol F68 + 2.5% solutol HS

* The oil phase contained: 0.4 g silymarin, 3 g shellac wax and 1g glyceryl monostearate

Evaluations of SLN

Images of dispersion (magnitude 100 times) were captured by inverted microscope (Nikon TE2000-5, Japan). The obtained dispersed systems were determined their pH using pH meter (Sartorius, Sartorius Mechatronics, Goettingen, Germany)(n=3). Viscosity and rheology at room temperature were evaluated using brookfield viscometer (DV-III Ultra programmable rheometer, Brookfield engineering laboratories, Inc., Middleboro, USA) performed in a cone-and-plate geometry with a cone no.40. The shear rates ranged from 0.1 up to $100s^{-1}$(n=3). Particle size determination of each formula was measured using laser scattering particle analyzer (LA-950, Horiba; Japan). Rheological behavior of the dispersion is expressed as N value (flowing parameter) of the Martin's equation. Zeta potential (ζ-potentia) was obtained by zata potential analyzer (ZetaPlus, Brookhaven; USA). The obtained SLNs were dried with freeze dryer (Labconco, Becthai Bangkok Equipment & Chemical Co., Ltd, Bangkok, Thailand). The surface topography of the prepared dried SLNs was determined using scanning electron microscope (SEM, Maxim 200 Camscan, Cambridge, England). Hot stage microscopy (stage from Mettler Toledo, Bangkok, Thailand and microscopy from Olympus, Bangkok, Thailand) was applied to observe the thermal behavior of dried SLNs under heating condition through microscopy.

In vitro release and permeation studies

The Franz® diffusion cells were used for the silymarin release study comprising the donor compartment with 2.1 cm diameter orifice and the receptor phase was stirred by a constantly spinning magnetic bar at 100 rpm. The receptor compartment was filled with 15 mL of receptor solution (phosphate-citrate buffer pH 5.5) at 32ºC. The membranes used in this study were cellulose nitrate membrane with a pore size of 0.45 μm and dorsal side of shed snake of Elaphae obsolete. One ml of 1% silymarin SLN dispersion and 1% silymarin ethanolic solution were added into the donor compartment. Cellulose nitrate membrane and dorsal shed snake were previously soaked with the receptor solution for 30 min to obtain the equilibrium with this medium. Then it was placed between the donor and receptor compartments. At appropriate time intervals, 0.5 mL of receptor solution was withdrawn. The amount of silymarin release was measured using UV-vis spectrophotometer (1100 series, Agilent, USA) at 289 nm. The volume of sample solution removed was replaced with an equal volume of fresh receptor solution. The cumulative amounts of silymarin released (total amount/surface area (mg/cm^2)) at each time interval were calculated using a calibration curve. All of the experiments were performed (n=6), and the mean release rate ± S.D. was calculated.

Statistic analysis

For the experimental measurements that were collected, the values were expressed as mean ± standard deviation (SD). Statistical significance of test values were examined using one-

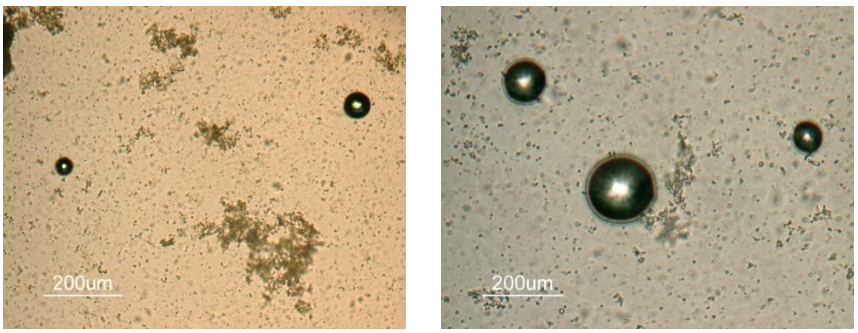
way analysis of variance (ANOVA). The significance level was set at $p < 0.05$. The analysis is performed using SPSS for windows, version 17.

RESULTS AND DISCUSSION

Increasing GMS could promote both the viscosity and the SLN formation as shown in Fig. 1. The addition of 1% stearic acid together with 2.5% GMS could obtain the proper SLN (Fig. 2). The higher amount of stearic acid did not improve the SLN property. The SLN morphology were not symetry for the system comprising stearic acid-shellac wax combination (Fig. 3). The SLN containing GMS together with shellac wax were spherical with the homogeneous small particles but the viscosity was rather high as the GMS amount was increased. The SLN containing GMS-stearic acid together with shellac wax were spherical with the homogeneous small particles therefore the system containing 2.5% GMS-1% steric acid and 7.5% shellac wax was selected.

The sonication intensity directly affected to the SLN particle size by decreasing the particle size as the amplitude was increased (Fig. 4). However the intesity higher than 40% did not further decreasing the particle size therfore 40% amplitude was selected for further study. The sonicating time was optimum at 45 min (Fig. 5) since the obtained SLN were homogeneous small spherical particles.

The selected condition as above mentioned was employed to prepare F1-4 and opt SLNs. The obtained SLNs are presented in Fig. 6. The particle size of F2 was smaller than that of F1 and 3. F2 contained GMS which had co-emulsifying property therefore it could promote the SLN formation. Particle size of F4 was rather smaller than F1 since there was GMS and stearic acid. Opt showed the larger particle since there was the high amount of wax but their particles were smaller than those of F1 therefore GMS could promote the smaller SLN.



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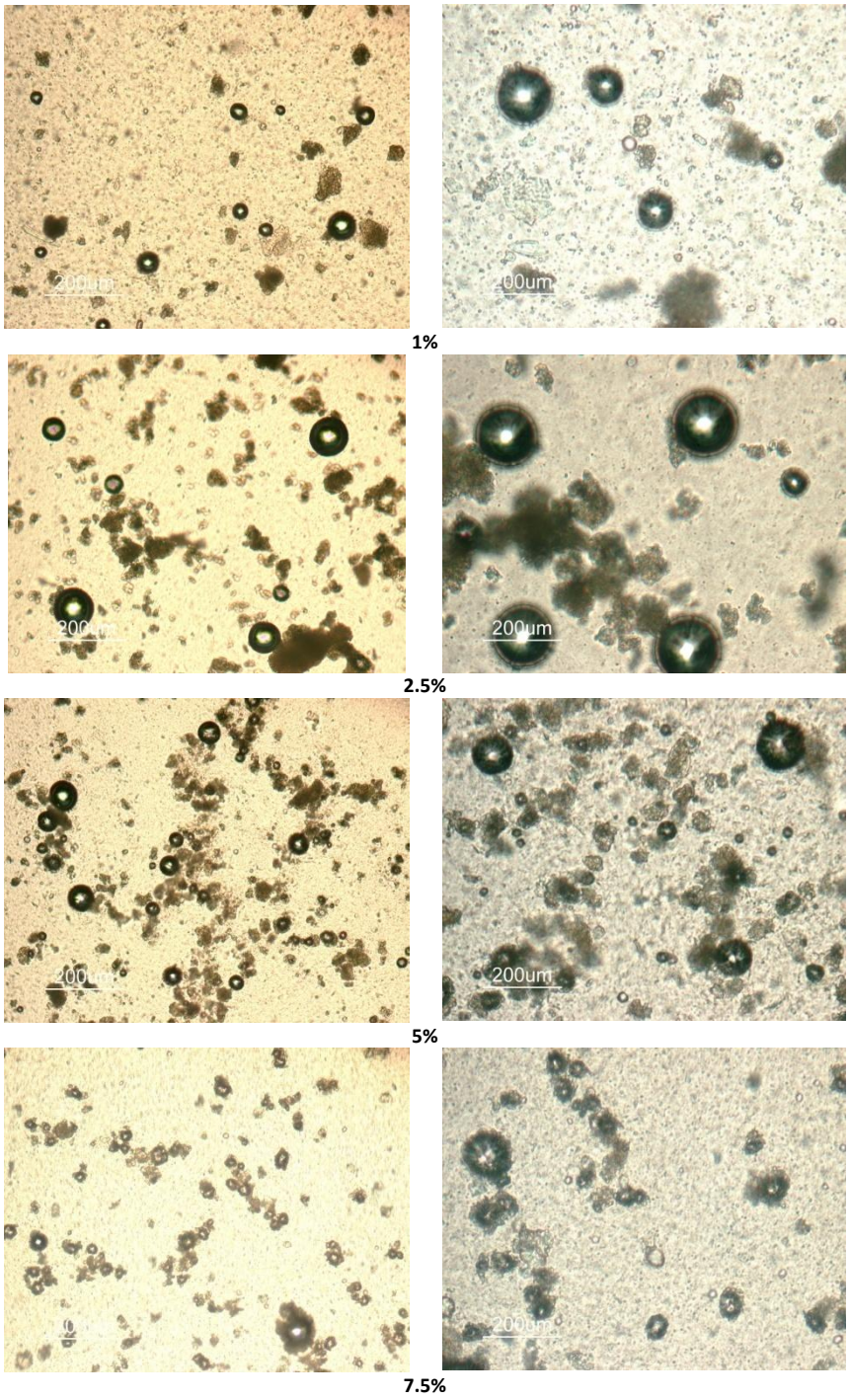

Figure 1. The particle characteristic of 1% silymarin SLNs containing 7.5% shellac wax and different amount of GMS under inverted microscope (left: 200X; right: 400X)

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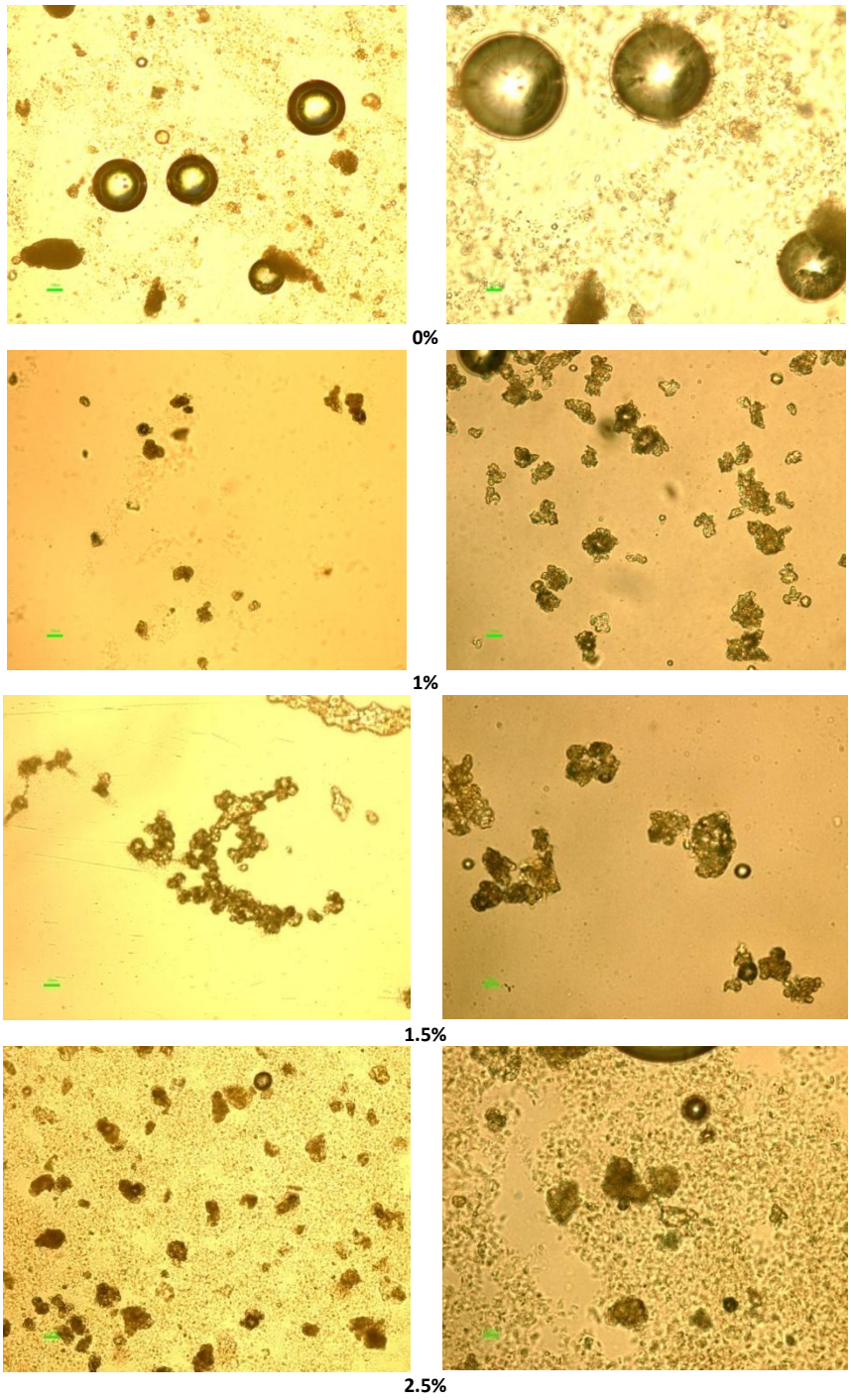

Figure 2. The particle characteristic of 1% silymarin SLNs containing 7.5% shellac wax, 2.5% GMS and different amount of stearic acid under inverted microscope (left: 200X; right: 400X)

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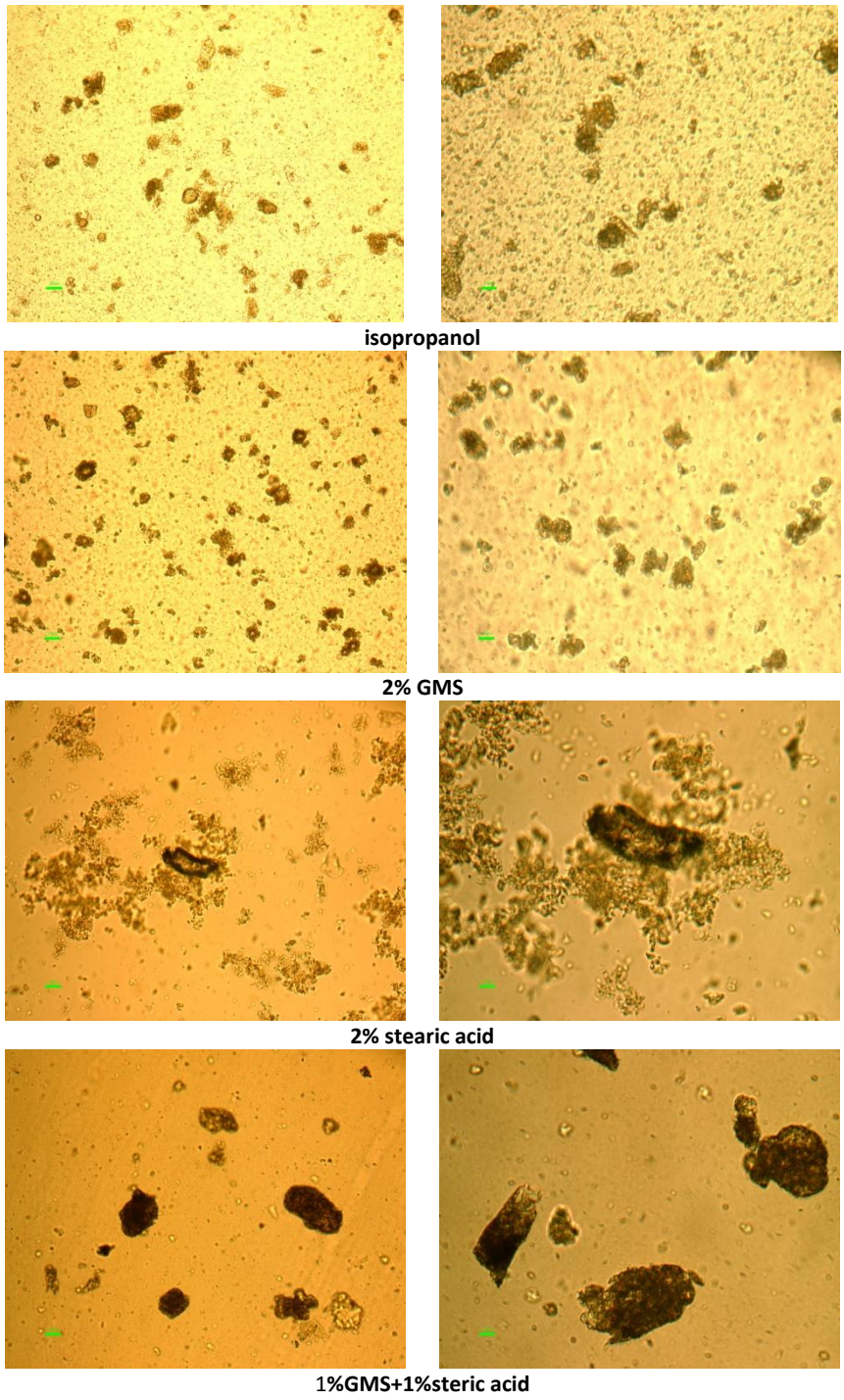

Figure 3. The particle characteristic of 1% silymarin SLNs containing 7.5% shellac wax and different amount of other compounds under inverted microscope (left: 200X; right: 400X)

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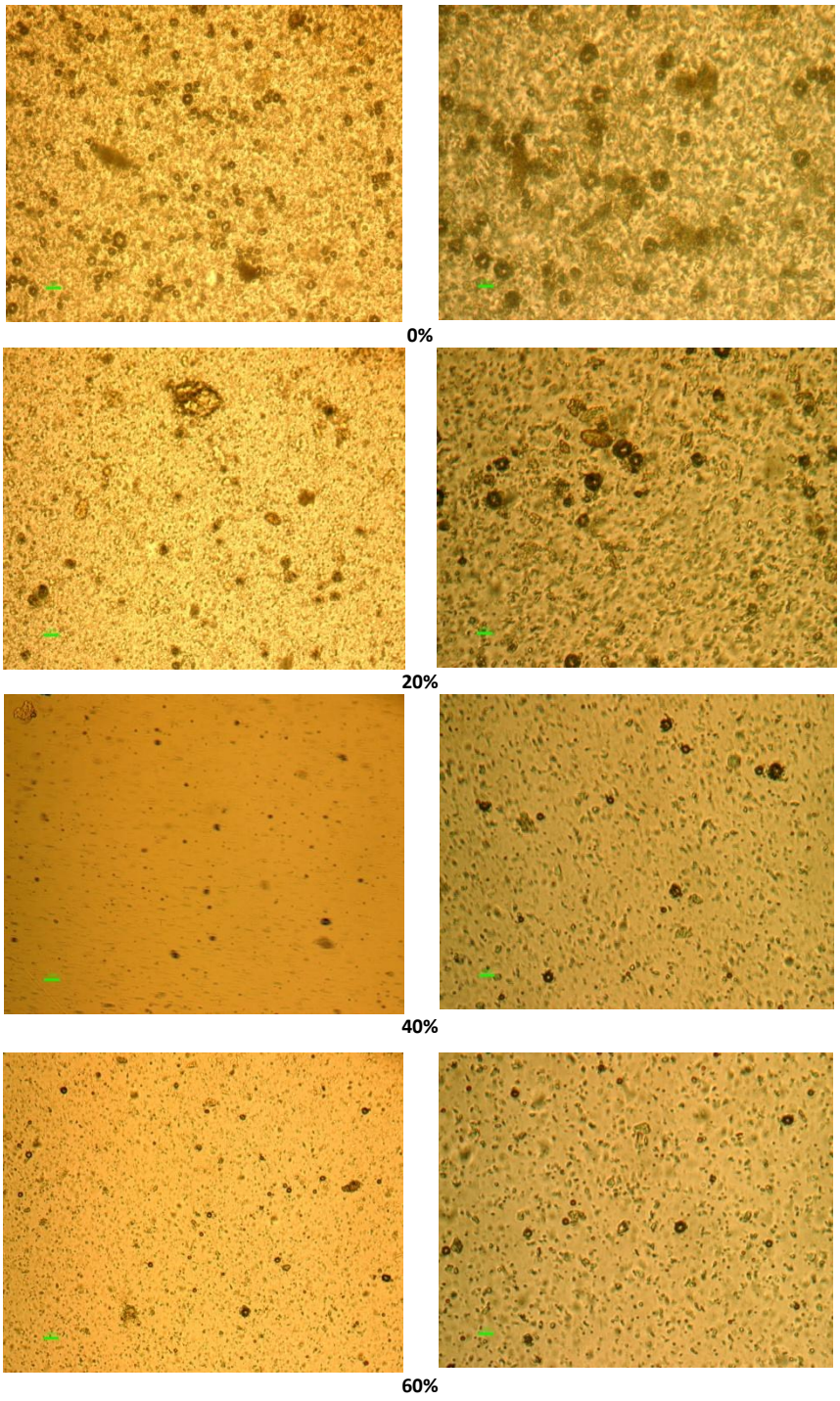

Figure 4. The particle characteristic of 1% silymarin SLNs after sonicating with different amplitude for 15 sec under inverted microscope (left: 200X; right: 400X)

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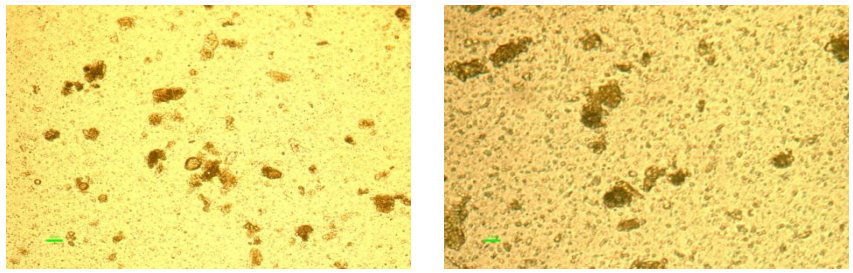

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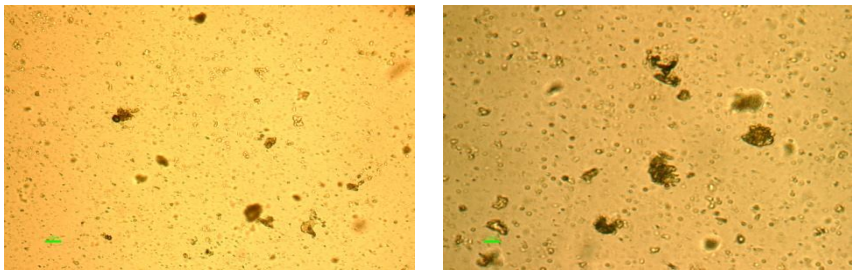

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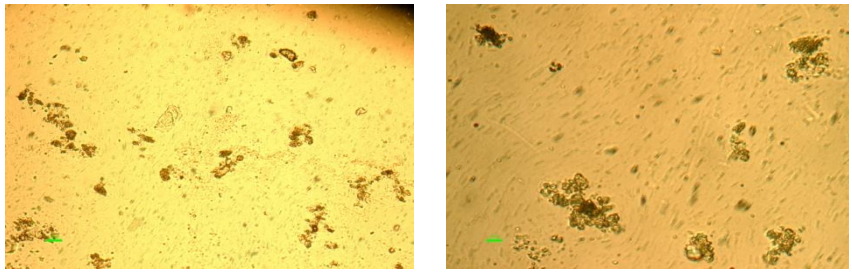

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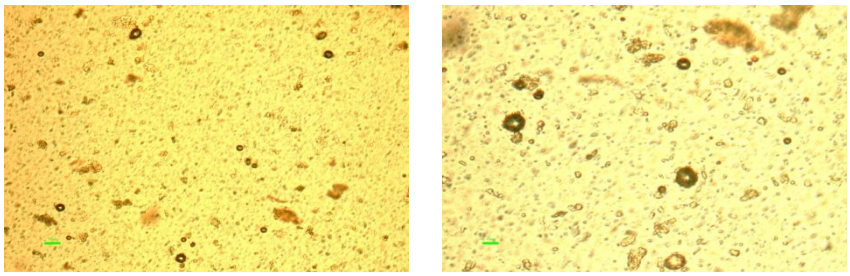

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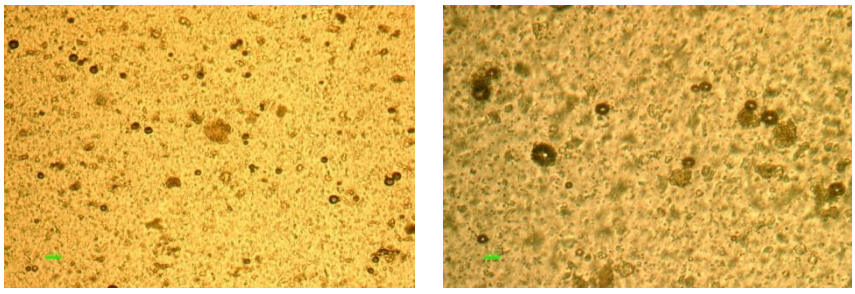

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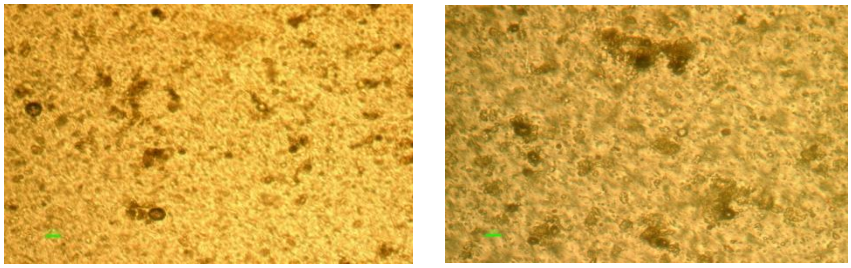
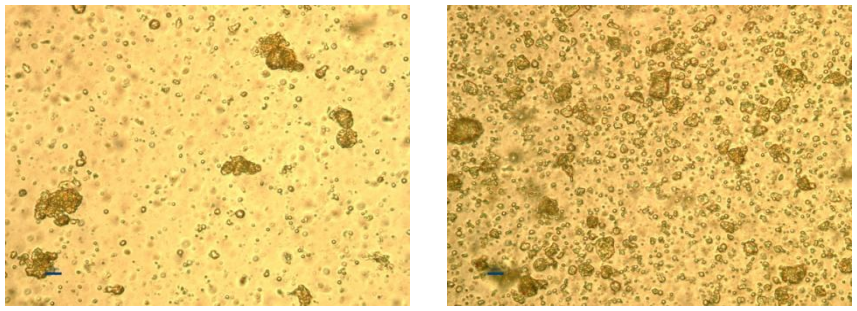
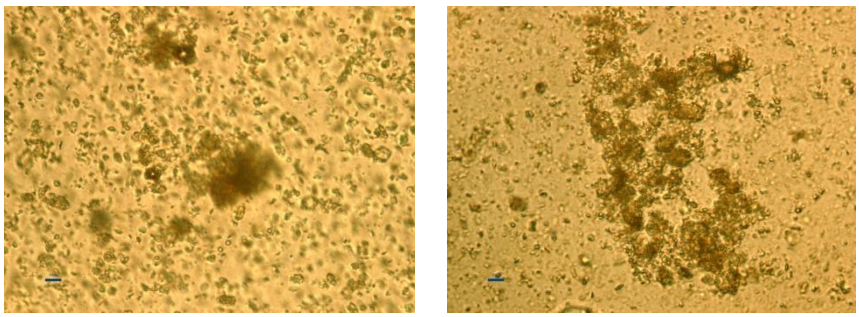
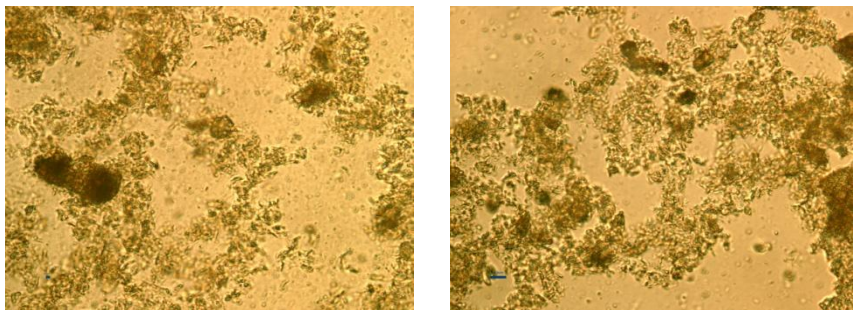


Figure 5. The particle characteristic of 1% silymarin SLNs after sonicating with amplitude of 40% for different time under inverted microscope (left: 200X; right: 400X)







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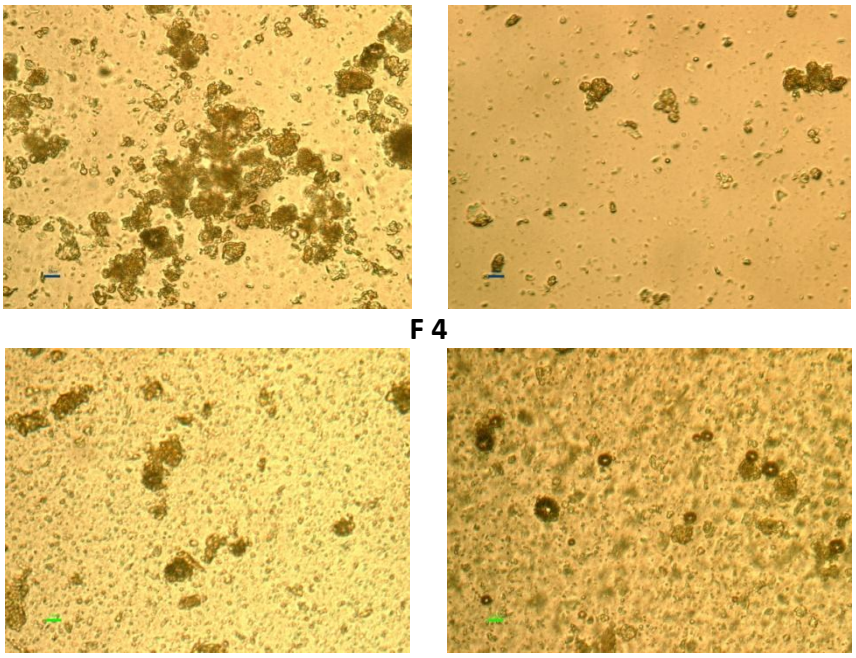


Figure 6. The particle characteristic of 1% silymarin SLNs before (left) and after (right) sonication at 40% amplitude for 45 sec under inverted microscope (left: 200X; right: 400X)

For formula comprising 1% silymarin and 7.5% shellac wax, type and amount of other waxs inflluenced the ζ-potential as shown in Table 3. The ζ-potential of F2 was apparently higher than that of F3 since the large polar graoup of GMS of F2 was dominant than that of stearic acid in F3. The ζ-potential value of F4 was between F2 and 3. The rather high ζ-potential of Opt was due to the high amout of GMS and also included stearic acid in the component. The highest ζ-potential of F1 was owing to the fatty acid component in shellac wax. Shellac wax composes of four fatty materials including fatty acid esters (70-82%), free fatty alcohols (8-14%), free fatty acids (1-4%) and hydrocarbons (1-6%). The fatty esters and fatty alcohols are from the interaction of some fatty acid component. The fatty acids were sarealeuritic acid, shellolic acid and jalaric acid. These compounds can interact together to form a many of ester products and also some of them transforms to be the fatty alcohols [8]. GMS or stearic acid might shield the charge of these fatty acids of shellac wax in the other fornula. The viscosity was in the range of 7-46 cPs. The viscosity of Opt was highest (Table 3). Most of them showed the pseudoplastic flow. Figure 7 exhibited the silymarin in vitro release from opt SLN comparing with that from ethanol solution. Apparently, SLN could prolong the silymarin release into phosphate-citrate buffer pH 5.5.

ISSN: 0975-8585

Table 3. Physical properties of silymarin SLNs

Formula	Mean size(μm)	ζ-potential	Viscosity (cPs)	N	Rheology flow
F1	18.94	-21.34±2.76	7.31±0.13	1.1134	Newtonian
F2	5.58	-20.37±2.82	8.99±0.65	1.1147	Newtonian
F3	26.06	-13.73±2.32	28.66±1.32	1.6228	Pseudoplastic
F4	14.34	-15.79±2.73	28.83±0.39	1.4947	Pseudoplastic
Opt.	22.91	-20.66±2.44	45.71±0.76	2.0900	Pseudoplastic

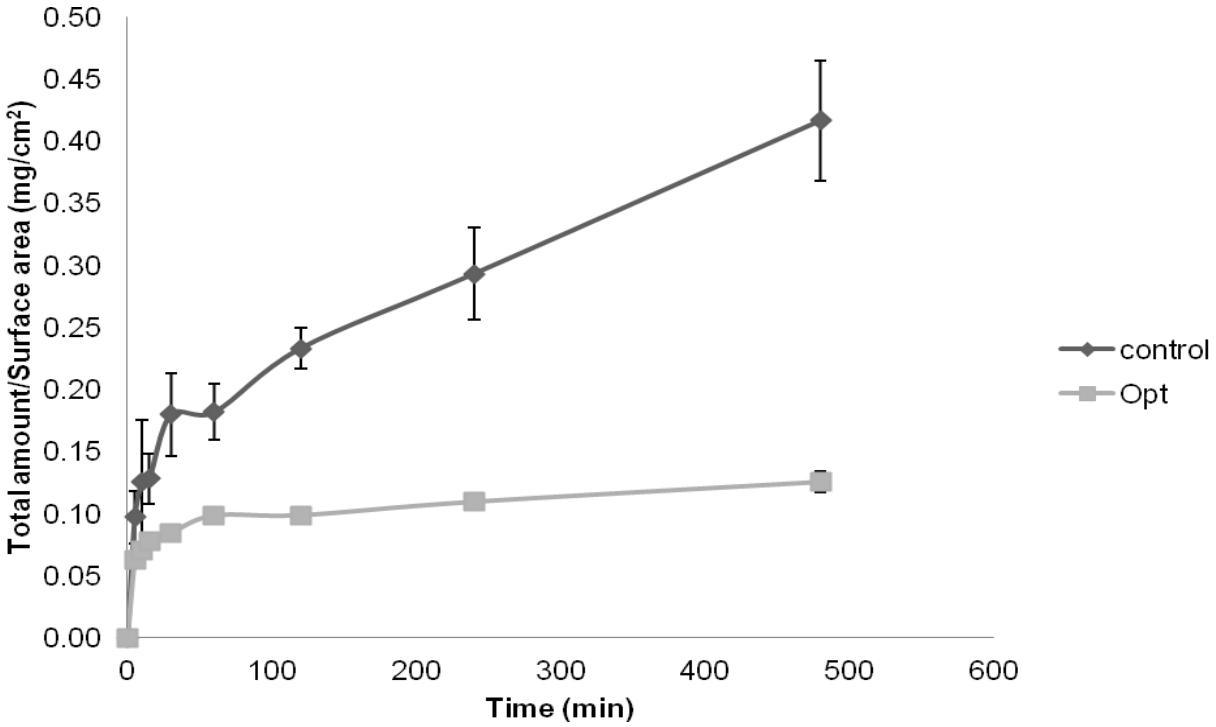


Figure 7. Cumulative silymarin release from ethanol solution and optimum formula through cellulose nitrate membrane.

All silymarin SLNs were rather viscous yellowish dispersions. The physical properties of the obtained SLN (P1-3) are shown in Table 4. The pH and viscosity values of P3 were higher and different significantly from those of P2 and P1, respectively. The results should relate to the effect of lutrol added into the P3. The SLN particle size was 6-9 μm. However the particle size distribution of P2 was smallest corresponding to the homogeneous of particle under inverted microscope as presented in Fig. 8.

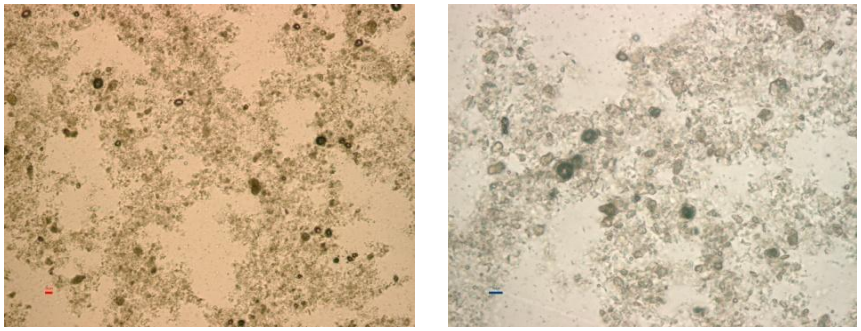
SEM micrograph indicated that P2 was compact particle whereas that of P1 was not smooth and that of P3 exhibited the flake on particle surface (Fig. 9). P2 contained tween 80 and solutol HS comprising separated hydrophilic part and hydrophobic part at different sides of their molecules therefore the complex film formation could effectively form and stabilize the

inner oil droplet. The wider size distribution of P1 and P3 was evident (Table 4). All SLNs exhibited the pseudoplastic flow indicated that the viscosity of dispersion decreased with increasing shear rate.

Table 4 Effect of stabilizer on physical properties of silymarin SLN

Parameter	P1	P2	P3	Statistics P <0.05
Viscosity (cPs)	86.94±0.92	88.94±2.08	163.50±1.14	significant
pH	4.65±0.10	4.85±0.10	5.13±0.08	significant
Particle size (μm)	6.47±0.55	8.70±1.32	8.58±0.68	insignificant
ζ-potential (mV)	-37.48±1.08	-38.84±1.24	-28.97±1.42	insignificant
Rheology	pseudoplastic	pseudoplastic	pseudoplastic	N/A
Inverted microscope	various size wide spread	similar size wide spread	various size wide spread	N/A
SEM	strip surface	flake surface	strip and flake surface	N/A
Melting point (ºC)	71.67±2.89	70.00±0.00	71.67±2.89	insignificant

Melting point of three SLNs was similar as shown in Table 4, therefore the type of stabilizer did not influence the melting behavior of dried SLNs. The used waxe showed the dominant effect on the melting point of dried SLNs. By comparison the wax should melt first due to its lowest melting point. The ζ-potential of prepared SLNs was not different. Tween 80 could form the intrapolymeric connection and decrease the interpolymeric connection promoting the recoil structure [13,14] therefore the solid particles were dispersed in water easier and viscosity was lowered. This penetrating effect of water also promoted the ionization of fatty acid therefore the ζ-potential was higher for the formula containing tween 80. This rather high ζ-potential could protect the particle agglomeration owing to its effective repulsive force. From the literature, a minimum ζ-potential of higher than −60.0mV is required for excellent physical stability and of higher than −30.0 mV for good physical stability [15]. Therefore P1 and P2 should show the good physical stability. Typically, the agglomeration was due to Van der waals force and charge interaction. P2 was selected for further study because of its small particle size distribution and homegeneous dispersion.



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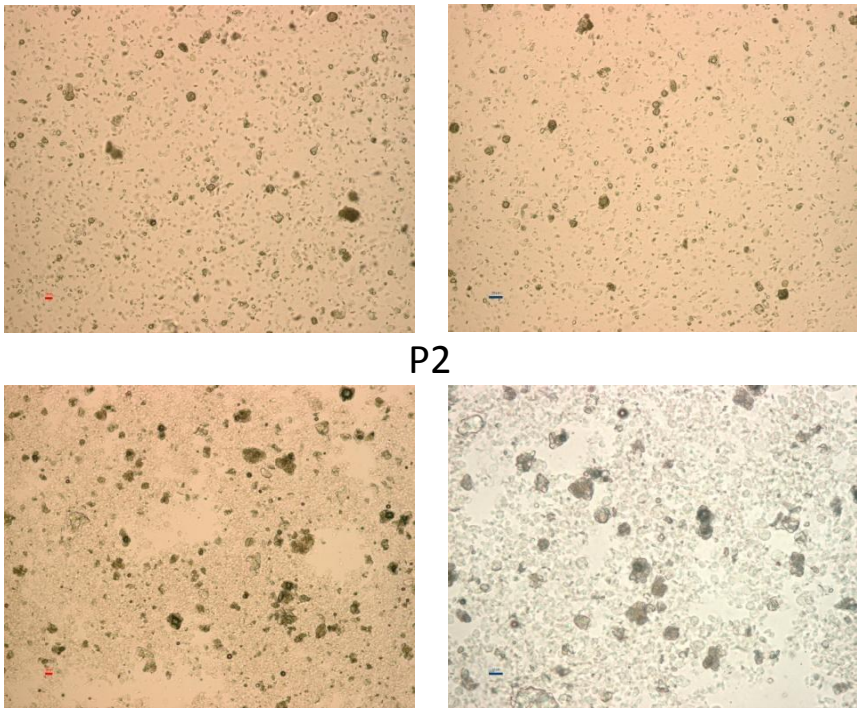
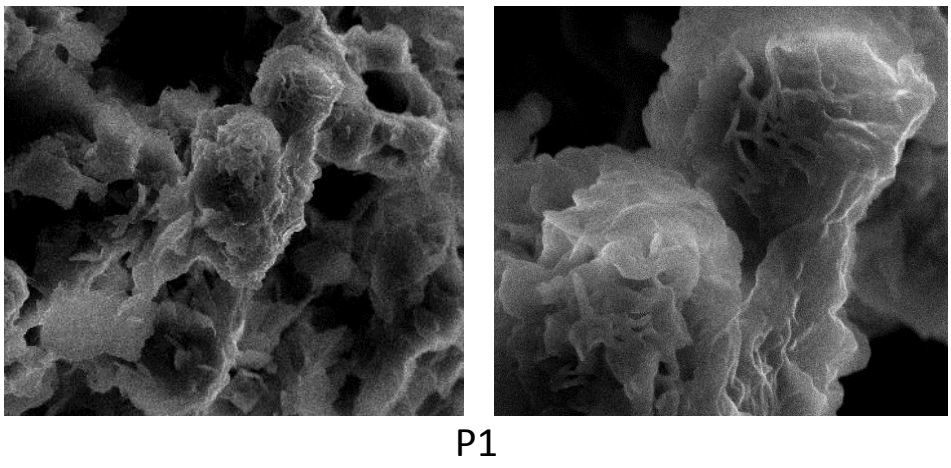


Figure 8. The particle characteristic of silymarin SLNs (P1-3) under inverted microscope (left: 200X; right: 400X).



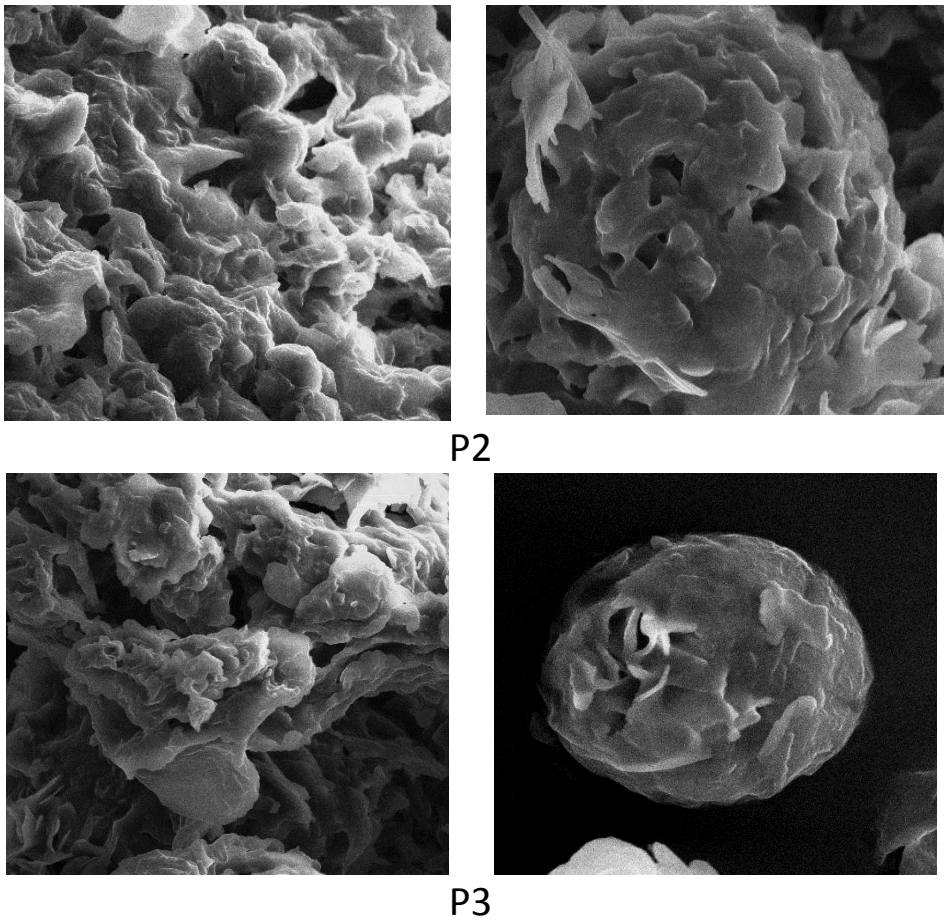


Figure 9. SEM photomicrograph of silymarin SLNs (P1-3) (left: 500X; right: 2000X).

In vitro release of silymarin from P2 through synthetic membrane and permeation through shed snake are shown in Figure 10 and 11, respectively. The silymarin release and permeation from P2 was apparently slower than that from the silymarin ethanolic solution. At 8 h, the silymarin release and permeation were 0.03-0.11 mg/mL and 0.02-0.04 mg/mL, respectively. From above result, the preparation into SLN should prolong silymarin release after employing as topical preparation. These results implied that the optimal silymarin SLN was probably able to topically use for prevention of UVB-induced skin cancer.

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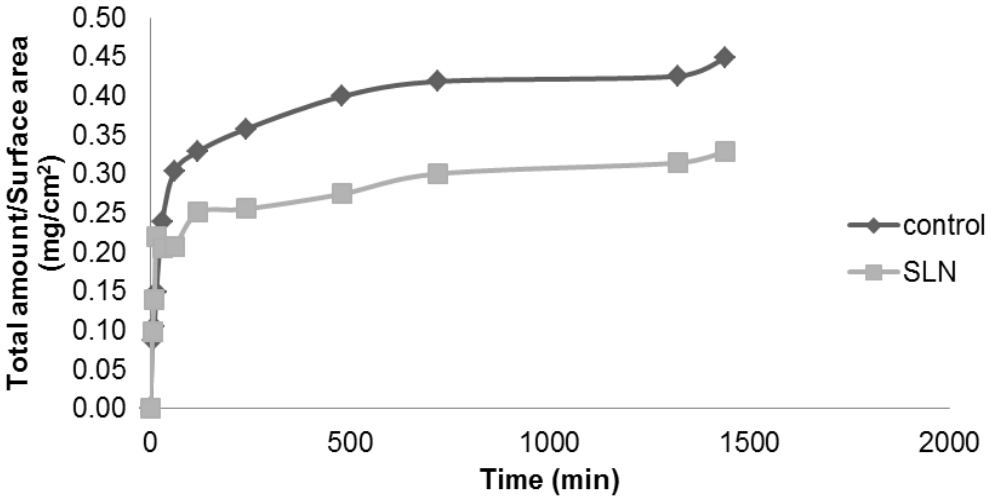

Figure 10. Cumulative silymarin release from ethanol solution and P2 through cellulose nitrate membrane.

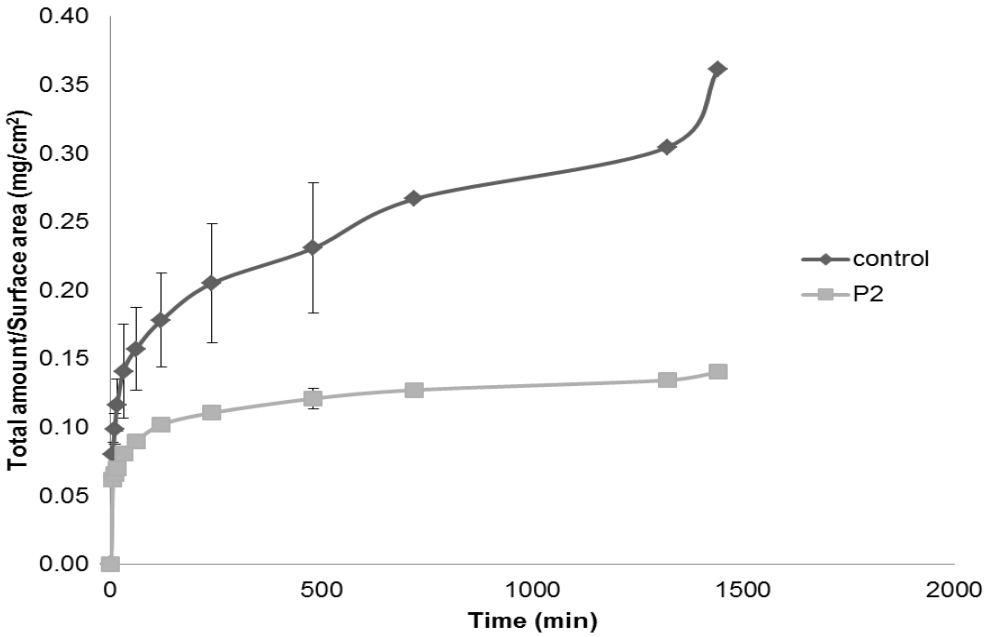

Figure 11. Cumulative silymarin release from ethanol solution and P2 through shed snake.

CONCLUSION

Silymarin solid lipid nanoparticles (SLN) were fabricated by hot melt homogenization technique using shellac wax and also glyceryl monostearate as wax matrix. The sonication with higher amplitude and time was able to decrease the SLN particle size. The sonication at 40% amplitude for 45 sec was the preparation condition. Surfactants influenced the particle size

distribution whereas they had no influence on the mean particle size which was found in the range of 6-9 micrometer. The ζ-potential of the formula was rather high. The obtained SLN exhibited the pseudoplastic flow. Eventually, SLN could be promising to sustain the release of silymarin from both in vitro release test and permeation study through the shed snake skin.

ACKNOWLEDGEMENTS

This research project thanks to Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. Miss Jongjan Mahadlek, Mr. Jesada Eaimsri and Miss Sasimaporn Bunklang are acknowledged for their valuable assistance on this work.

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