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Liposome Formulation of Soybean Phosphatidylcholine Extract from Argomulyo Variety Soy to Replace the Toxicity of Injectable Phosphatidylcholine Solution Containing Sodium Deoxycholate

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Abstract: Subcutaneous injection lipolysis using phosphatidylcholine (PC) and sodium deoxycholate (SD) solution has been used in clinical practice with toxicity concerns of SD, a detergent emulsifier. Liposomal formulation of PCfrom local Indonesian soybean without SD was thought to be a safer alternative for this purpose.

The aim of this study was to develop injectable liposome formulations from soybean phosphatidylcholine (SPC) extract from Argomulyo variety soy. In addition, this study was aimed to compare the physical characteristics between the liposomes of SPC extract and the liposomes of purifiedSPC.

SPC extract was obtained by extraction-fractionation process from soybean powder. Liposome was prepared using thin film hydration (hand-shaking) method followed by a stepwise extrusion process through decreasing pore size extruder, i.e. 0.45 µm, 0.20 µm, and 0.10 µm. Liposomes were then characterized physically and microscopically.Laser scanning confocal microscopy (LSCM) with quinacrine dye andtransmission electron microscopy (TEM) without quinacrine dye was used for morphological confirmation.

The median size of liposomes fromSPC extract containing 26.2% of PC was 48.9 nm with polydispersity index of 0.35. The liposomes were anionic with mean zeta potential of -17.25 mV. Liposomes from purifiedSPC was larger with median size of 68.3 nm and polydispersity index of 0.16;the liposomes were cationic with mean zeta potential of 58.23 mV. Confirmation using TEM showed spherical structures covered with a single layer both from purifiedSPC and SPC extract.

Liposomes of SPC extract could satisfyingly be produced using local, Argomulyo variety soy. The liposomes were uniformly small, nanoparticle-sized, unilamellar, and negatively charged. These characteristics are suitable for subcutaneous injection to be used as lipolytic agent to replace the commercial PC plus SD solution.

Key words : lipolysis injection, liposome, soybean phosphatidylcholine,soybean lechitin.

Background

Phosphatidylcholine (PC) is the major constituent of phospholipid in human cell membranes. Phospholipids are ampiphilic molecules consisting of a polar head group and a lipophilic tail, making them suitable to be used as emulsifier, solubilizer, and liposome former.[1]

Intravenous PC injection has been indicated for various conditions, such as prevention of fat embolism, treatment of atheroma, hypercholesterolemia, and lipid deposits on arterial walls. Currently, PC injection is also popular for off-label indication, i.e.subcutaneous lipolysis injection in the field of dermatology and aesthetic surgery.[2,3]

Natural PC source like soybeans are highly preferable for application in pharmaceutical industries due to their emulsifying properties and non-antigenic nature. However, in order to make a soluble PC injection, a solvent is required, which in the case of lipolysis injection is sodium deoxycholate (SD), a detergent emulsifier.

The mechanism of fat reducing of subcutaneous PC/SD injection has not completely been understood. The formula may work as a detergent, causing cell membrane disruption and necrosis. SD has been thought to be the more active agent to cause fat cell necrosis.[4]Furthermore, recent studies suggest that SD may cause tissue destruction and fibrosis.[5,6]A study on fat tissues from lipoma showed that injection of PC/SD caused fat cell necrosis, which was confirmed by light and electron microscopy, immunostaining for active caspase-3 and in situ end-labeling assay.[7]

Although this commercial PC solution was effective in reducing fat, concerns on its safety have been raised due to the use of SD, which cause tissue necrosis and inflammation. However, a recent study confirmed the role of PC in inducing apoptosis in adipocyte cell culture.[8]

Liposomes are artificial vesicles formed by one or more concentric lipid bilayers separated by water compartments.[9]Phospholipids from soybean lecithin are widely used in liposomal drug delivery systems due to their safety and wide availability at relatively low cost for upscale production. Therefore, liposomal formulation of an injectable PC for subcutaneous delivery is an attractive option.

Crude soybean lecithin contains 65-75% phospholipids, together with triglycerides and smaller amounts of other substances such as carbohydrates, pigments, sterols and sterol glycosides.[10] In order to make liposome preparation from soybean oil, a series of fractionation and purification steps are needed. Pure phosphatidylcholine is available commercially; however, to reduce cost and to give additional economic value, we choose to use Indonesian local soybean seeds, i.e. Argomulyovariety soy, which lipid content is equivalent to the imported ones.[11]

The aim of this study was to develop injectable liposomes formulation from soybean phosphatidylcholine (SPC) extractfrom Argomulyo variety soy. In addition, this study was aimed to compare the physical characteristics between the liposomes of SPC extract and the liposomes of purifiedSPC.

Materials and Methods

Preparation of soybean phosphatidylcholine- extract

Soybean seeds Argomulyo variety wasobtained from the Indonesia Research Institute for Legumes and Tuber Crops (ILETRI), Malang. Extraction of SPC was done in theAgro Industrial Technology Development Biomedical Laboratory, Serpong, South Tangerang.

Briefly, soybean (as fine powder) underwent solid-liquid extraction using *n*-hexane as the solvent to produce soybean oil. Then, lecithin was extracted by water degumming to produce crude soybean lecithin (CSL). The next step was lecithin purification, using acetone (to produce deoilized lecithin) and a series of ethanolfractionation. The final product was SPC extract. Phospholipid compositionof SPC extract was analyzed using high-performance liquid chromatography (HPLC) method. L-α-phosphatidylcholine (PC) from soybean with a purity of 99.9% (pure SPC)was purchased from Sigma-Aldrich, USA and was used as external standards for phospholipid analysis.

Thin layer chromatography

Qualitative analysis of SPC extract was done using thin layer chromatography (TLC) method. Silica gel plate (Merck GF254) was used as a bonded phase, while chloroform, methanol, and double distilled water (9:2.5:0.25 v/v) plus 25% ammonium hydroxide analytical grade (Merck) were used as mobile phase.

The deoilized lecithin, SPC extract, and pure SPC were eluted on the silica-gel chromatography column. The TLC plate then was visualized under the UV light at wavelength of 254 nm and 366 nm.

Analysis of phospholipids using high performance liquid chromatography (HPLC)

Analysis of phospholipids was done in the Agro Industrial Technology Development Biomedical Laboratoryand in the Laboratorium Kesehatan Daerah,Dinas Kesehatan Jakarta (District Laboratory of Jakarta Local Health Office).

Equipment for HPLC consisted of a chromatography chamber, HPLC system (Waters), UV cabinet (Camag), octadecyl (C18) column (Zorbax Eclipse Plus C18; 4,6 mm x 150 mm, 5µm), and a photodiode array (PDA) detector. The separation was performed using isocratic elution on an analytical octadecyl (C18) column. The mixture of methanol-acetonitrile (40:60 v/v) was used as mobile phase with a flow-rate set at 1 mL/min. Experiments were carried out at 30^{o}C. The samples were dissolved in methanol at a flow-rate of 1 mL/min. Injection volume was 30 µL and wavelength detection was set at 210 nm.

Liposome preparation

Liposome preparation of the SPC extract was done in the Laboratory of Medical Pharmacy, Department of Pharmacy, Faculty of Medicine, Universitas Indonesia, Jakarta.

Liposomeswere prepared using thin film hydration (hand-shaking) method followed by a stepwiseextrusion process. First, SPC extract was dissolved in methanol in a round-bottomed flask. Then, the solvent was evaporated by reduced pressureusing rotary evaporator to yield a thin lipid film inside the round-bottomed flask. Solvent evaporation was prolonged to 60 minutes to remove all solvent. The thin lipid film was hydrated with phosphate buffer saline (PBS) at pH 7.3-7.5 and agitated(hand-shaking) for 30 minutes to obtain multilamellar vesicles (MLV).The final concentration of purified SPC and SPC extract vary from 4000to 6500 ppm.

Next, the liposomes underwent stepwise extrusion through decreasing pore size extruder, i.e. 0.45 µm, 0.20 µm (Minisart Filters, Sigma-Aldrich, USA) and 0.10 µm (LiposoFast Basic, Avestin, Canada). Extrusion was done eleven times to yield small, unilamellar, homogenous vesicles. The final liposomes werestored in a sterile brown flask at 4^{o}C.

Particle size analysis

Physical characteristic analyses of the liposomes were done in the Nanotech Laboratory, Jakarta. Liposome was stained with quinacrin dye and analyzed using a particle size analyzer (Delsa™ Nano, Beckman Coulter, USA) at 25^{o}C under the following conditions: Refractive index 1.3332; viscosity 0.8878, and scattering intensity 11480. Measurement was done in triplicate. Evaluation was repeated on day-1, day-4 and day-16 to test physical stability of the liposome.

Microscopic studies

Biological laser scanning confocal microscopy (LSCM) was performed in the Universitas Indonesia - Olympus Bioimaging Centre, Depok. Liposome samples were put under the confocal microscope (FV1200, Olympus, Tokyo, Japan) to confirm the presence of single bilayer membrane. Liposomes were stained with quinacrine, a hydrophilic fluorescence dye, prior to microscopic evaluation.

Transmission electron microscopy (TEM) was done in the Chemistry Department, Faculty of Mathematics and Natural Sciences, Gadjah Mada University, Yogyakarta, to confirm the presence of liposomal structures.

Liposomes were diluted before analysis with phosphate buffer pH 7.3- 7.5 (about 0.05 mg/mL) and then was placed on a 200-mesh formvar copper grid, allowed to adsorb. The excess buffer was removed by a filter paper. Negative staining was done with the addition of 2% phosphotungstic acid (PTA) and was left in contact with the sample for 2 min. The excess water was removed and the sample was dried at the room conditions. The grid was immediately imaged using an electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan) operating at 80 kV. Scanning began at low magnification (10,000 x) and then examined for finer details at higher magnification (20,000-80,000x).

Data Analysis

Data were presented as means and standard deviations. Mean differences were analyzed using t-test at an alpha level of 0.05. Statistical analysis was done using a software, SPSS for Windows PC version 17 (SPSS Inc., Chicago, USA).

Results and Discussion

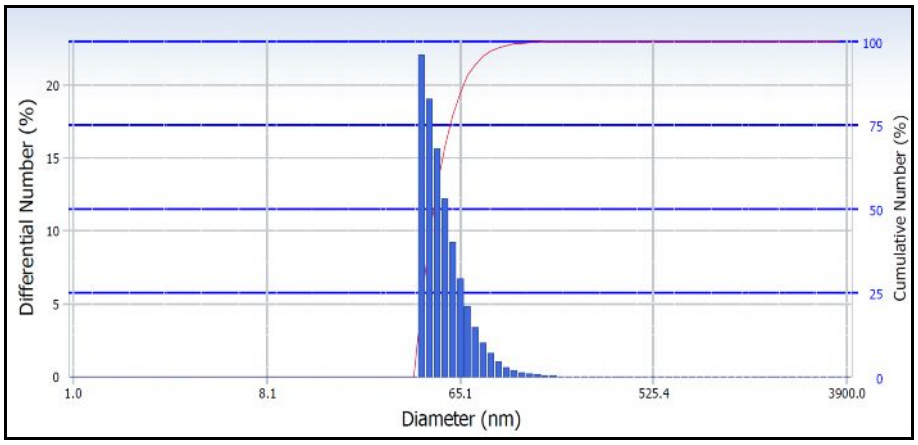
Analysis of SPC extractfrom Argomulyo variety

HPLC analysis showed that PC is the main phospholipid materials in the SPC extract. The percentage of PC based on peak area was 26.2%. A similar PC content was also found in other Indonesian local soy (Anjasmoro variety),which yielded a PC content of 26.1%.[12]As comparison, the purifiedSPC purchased from Sigma showed much higher purity (95.15%). PC content of more than 20% is considered sufficient for oral or topical formulation, but not for intravenous injection.[1]

The combination of non-polar (chloroform), polar (methanol) and water (Bligh and Dyer's method) has been commonly used for lipid extraction from various biological materials.[13]However, chloroform was replaced by hexane in this study since it was known to be carcinogenic, non-biodegradable, and not environment friendly for drug development from natural products. [14]On the other hand, hexane is considered as a safer, less toxic and more biocompatible alternative.[15]

Liposome formulations from soybean extract can be done using pure phospholipids,[16,17] or unpurified soybean lecithin.[18,19] The cost of pure phospholipids is quite expensive and may limit the mass production of liposomal drug delivery systems.[20]Further purification on the local SPC extract was not done due to financial reasons and the possibility of PC loss during the process. The cost of liposome production from non-purified soybean lecithin is only 5% of the pure one;[21] thus, it is a more attractive choice. Increasing purity of SPC can be done using more sophisticated approaches, such as supercritical fluid extraction, solid phase extraction, column chromatography and even HPLC, which makes the pure SPC is much more expensive.[21]

Figure 1A. Distribution of size of liposomes from purified SPC extract.



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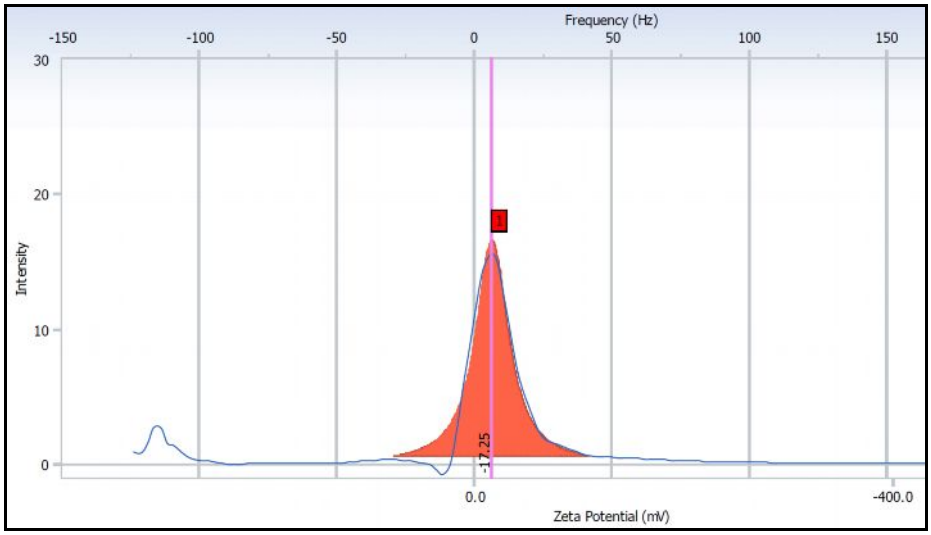

Figure 1B. Distribution of surface charge of liposomes from purified SPC extract.

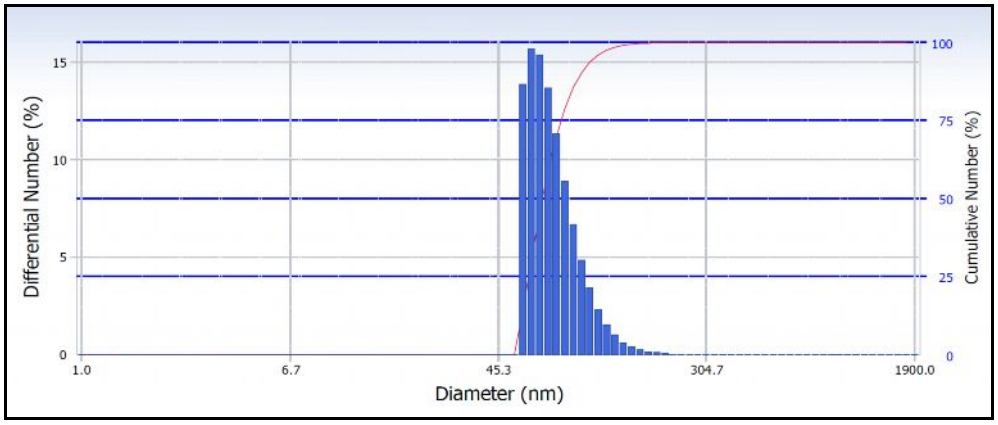

Figure 2A. Distribution of size of liposomes from pure SPC.

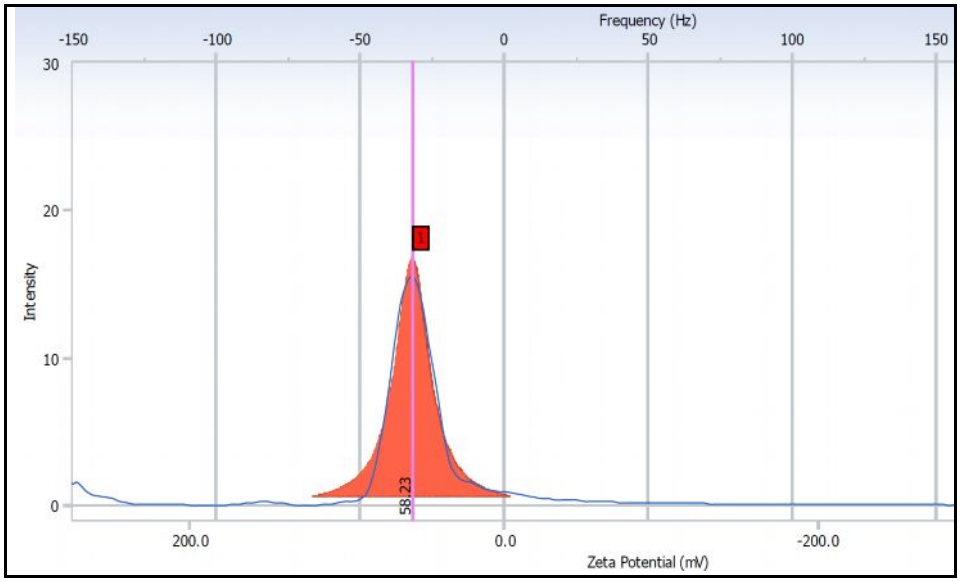

Figure 2B. Distribution of surface charge of liposomes from pure SPC.

Liposome characteristics

In one measurement, the median size of liposomes from SPCextract was 48.9 nm (10^{th}-90^{th} percentiles: 41.0-71.3 nm) with polydispersity index of 0.35. The liposomes were anionic with a mean zeta potential of -17.25 mV (Figure 1-B). Analysis of liposomes from purifiedSPC showed a median size of 68.3 nm (10^{th}-90^{th} percentiles: 55.7-96.8 nm), with a polydispersity index of 0.16. The liposomes were cationic with a mean zeta potential of 58.23 mV (Figure 2).

Table 1. Comparison of liposomes' characteristics between SPC extract (Argomulyo variety) and purified SPC

Variable*	**SPC Extract** (mean ± SD)	**PurifiedSPC** (mean ± SD)	***p* value**[#]
Diameter size (nm)	59.63 ± 0.3	77.70 ± 1.50	**0.018**
Polydispersity index	0.27± 0.08	0.17 ± 0.09	0.101
Zeta potential (mV)	-17.75 ± 0.50	56.70 ± 1.53	**<0.001**

*all measurements were done in triplicate; [#]t-test

To compare liposomes' characteristics from the SPC extract and purified SPC, measurements were done in triplicate. The mean diameter of liposomes from SPC extract was significantly smaller than the purified SPC. However, polydispersity indices were similar between SPC extract and purified SPC liposomes, confirming the uniformity of the particles was good. Zeta potential measurements showed different charges of liposomes from the SPC extract and purified SPC. The SPC extract produced anionic liposomes with a surface charge from -18.25 to -17.25 mV. On the contrary, the purified SPC resulted in cationic liposomes ranging from 55.17 to 58.23 mV (Table 1).

Size, surface charge (zeta potential), and number of bilayer membrane (lamellarity) of liposomes influence their biological effects. Therefore, physical characteristics of liposome products should be established before applying them for therapeutic use.

The average and range of size are important parameters of the liposomes intended for therapeutic application. In this study, most liposomes from SPC extract were small, 90% of them sized less than 72 nm. This is typical for conventional liposomes that are either neutral or negatively charge. On the contrary, liposomes from purified SPC were larger. However, in terms of drug efficacy this different may not be clinically significant. The ideal size of liposomes for intravenous drug delivery systems is 50-100 nm. Larger liposomes increase the possibility of reticuloendothelial system (RES) uptake in the blood circulation.[22]

The surface charge of the liposomes represents an index of the interaction magnitude between particles. Large negative or positive zeta potential indicates the low tendency of the liposomes to aggregate with each other. On the contrary, low zeta potential may indicate no force to prevent particles' aggregation.[23] In our study, the low negative charge of the liposomes from SPC extract may indicate the less stability of these liposomes compared to the liposomes of purified SPC ones. However, this stability refers to the injectable liposomes into the circulation, whereas our liposomes are intended for subcutaneous administration with local biological effect. Conversely, cationic liposomes are typically preferable for gene delivery allowing them to interact with genetic material inside the nucleus due to the negatively charged nucleic acids.[24]

Microscopic studies

The presence and morphology of liposomes were further confirmed by TEM, which verified the presence of round liposomes of less than 200 nm width. The unilamellar lipid bilayer of the liposomes can be clearly seen at high magnification (Figure 3).

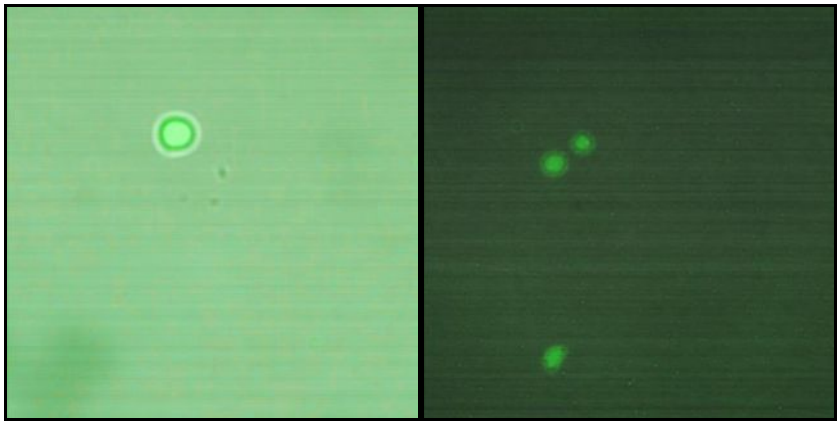

Figure 3. Membrane bilayer of a small unilamellar vesicle (SUV) as seen by laser scanning confocal microscopy (LSCM) with quinacrine dye. Left: Liposome of purified SPC (magnification 60x with 5x zoom); right: liposomes of pure SPC (magnification 6x with 2x zoom).The bilayer membrane structure is seen as a strong green circle line separating the water content of the liposome at the center and the surrounding liquid system

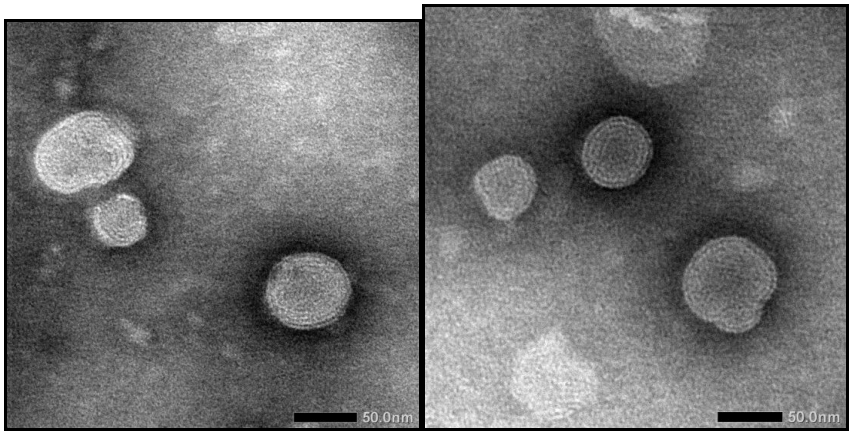

Figure 4. Microscopic comparison of liposomes from pure SPC (left) and purified SPC extract (right) using transmission electron microscopy without staining. Magnification 80,000 x.

Further study using TEM confirmed the spherical structures covered with a single layer both from purified PC and SPC extract. The average size of the particles was 59.77± 15.66 and 57.98±11.96 nm, respectively. Some irregular spherical particles were also observed (Figure 4).

Liposome reproducibility

The United States Pharmacopeia <420>(USP <429>)stated that samples should be measured at least three times and reproducibility must meet specified guidelines. The coefficient of variation (CV) must be less than 20% at the D50 (median liposome diameter) and less than 30% at the D10 and D90 (10^{th} and 90^{th} percentile of liposome diameter) when following the USP <429> requirements for particle less than 10 µm at D50.[25] In our study, the CV of one preparation (intrameasurement reproducibility) from SPC extract was 13% for D10 and D50 and 14% for D90, whereas the highest CV of liposome preparation from purifiedSPC was 2% for D10, D50 and D90. The CV for two different preparations (intermeasurement reproducibility) of purified SPC extract liposomes was 14% for D10 and 15% for D50 and D90. These values were within the limit set by the USP <429>.We did not distinguish the intermeasurement reproducibility between the SPC extract and purified SPC.

Liposome stability during storage

Liposomes from the SPC extract was stored at 4°C. Physical characteristics were analyzed at Day-1, Day-4, and Day-16. Liposome diameter was reduced signficantly after 16 days of storage, but the polydispersity index did not significantly altered. Interestingly, the negatively charge liposomes at baseline (-19,34 mV) turn to cationic liposomes on Day-16 (20.76 mV) (Table 2).

Table 2.Effect of storage on physical characteristics of the liposomes from SPC extract.

Variable	Day-1	Day-4	Day-16	*p* value
Diameter size (nm)	48.20	37.80	37.07	<0.05[a]
Polydispersity index	0.31	0.30	0.27	NS
Zeta potential (mV)	-19.34	-12.11	20.76	<0.05[b]

[a]significant difference between Day-1 and Day-4 and Day-1 and Day-16; [b] significant difference between Day-1 and Day-16; NS: not significant.

Stability of liposomes during storage is a point of concern. In this study, storage may reduce the particle size slightly and may change the surface charge of the liposomes. For therapeutical use, this alteration would need further confirmatory studies, as positively charge liposomes may interact with genetic materials inside the cells and can be used for gene therapy.

Efficacy of injectable liposomes in animal model

In this study, we do not particularly test the liposome formulation *in vivo*. However, another group of investigators has tested our liposomal SPC extract to anobese rat model *(Rattus norvegicus)*as a preliminary study. In this study, they used 36 male, obese rats of Wistar strain, aged 90 days and have body weight off 200 g. Eighteen rats were injected with liposomes (4.5 mg/mL)while another 19 were given placebo (normal saline), once a week for two weeks. The rats were sacrified a week after the last injection. The subcutaneous abdominal fat were removed and weighed.They found that rats injected with our liposomes of the SPC extract significiantly reduced abdominal subcutaneous fat compared to the control group (Table 3).[26]

Table 3. Effect of subcutaneous injection of liposomes from SPC extract [24]

Group	n	Abdominal subcutaneous fat Mean ± SD	*p* value
Control rats	18	2.18 ± 0.44 g	0.001
Treated rats	18	1.13 ± 0.23 g	

Conclusion

As conclusion, liposomes of soybean phosphatidylcholine extract could satisfyingly be produced using local, Argomulyo variety soy. The liposomes were uniformly small, nanoparticle-sized, unilamellar, and negatively charged. These characteristics are suitable for subcutaneous injection to be used as lipolytic agent. Further confirmatory studies and clinical trial are still needed.

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