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RESEARCH ARTICLE

THE EFFECT OF ULTRASONICATION TIME ON PARTICLE SIZE, POLYDISPERSITY INDEX AND STABILITY EVALUATION OF ANTHOCYANIN LIPOSOMES

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Abstract

Background: One of the drug delivery systems that acts as a targeting system is liposomes which have good characteristics so they can encapsulate and deliver drug compounds. One of the characteristics that need to be considered in liposomes is the particle size and polydispersity index. Several methods can be used to control these two characteristics, including the extrusion and the ultrasonication method.
Objectives: This research aims to see stability evaluation and effect of ultrasonication time and extrusion on particle size and polydispersity index (PDI) of Anthocyanin liposomes.
Methods: The liposome formula was prepared using Anthocyanin: DSPC: cholesterol: DSPE-PEG 2000 in a ratio of 1: 1.85: 1: 0.15 using the ethanol injection method. Anthocyanin liposome was divided into 3 treatments of varying ultrasonication times i.e. 5 minutes (L1), 15 minutes (L2), and 30 minutes (L3), and extrusion method as control (L0). All methods of anthocyanin liposomes size and PDI were done the stability evaluation based on storage temperature i.e 4ºC and 25ºC for 6 weeks
Results: The results of liposome characterization using particle size analyzer (PSA) showed that L0, L1, L2, and L3 had particle sizes of 152.1 nm, 150, 6 nm, 156.2 nm, 169.7 nm with the average PDI 0.379, 0.368, 0.450, 0.366 respectively. The statistical data (T-test) showed that there was no real influence on the particle size and PDI of Anthocyanin liposomes. However, Liposome treatment (L1) produced using the ultrasonication method for 5 minutes produces better particle size and PDI than other time variables. Moreover, the stability evaluation of anthocyanin liposomes size and PDI described the extrusion (control) and sonication method particularly 5 minutes of ultrasonication time were more stable than others due to no change size and PDI significantly at 4ºC and 25ºC for 6 weeks.
Conclusion: Both methods can reduce the particle size of anthocyanin liposomes and have a homogeneous particle distribution, particularly the ultrasonication treatment time of 5 minutes which is the same as the extrusion method and and also more stable at storage temperatures of 4ºC and 25ºC for 6 weeks.
Keywords: Anthocyanin, extrusion, liposome, particle size, ultrasonication.

INTRODUCTION

Anthocyanin is a secondary metabolite compound that is classified in the Flavonoid group. Anthocyanins have been studied extensively for their use as potential pharmaceutical ingredients, including as antidiabetic, anticancer, anti-inflammatory, antimicrobial, obesity, and prevention of cardiovascular disease, and most importantly, their activity as antioxidant compounds. Anthocyanins have weaknesses like antioxidant compounds in general, namely their low stability, so they need appropriate handling to protect their antioxidant activity. One form of targeting delivery that can overcome this weakness is the liposome delivery system. Liposomes are one of the right choices for encapsulating Anthocyanin compounds, which can protect the stability of Anthocyanins liposome, so that their activity and effectiveness as antioxidants can be maintained[1].

Liposomes have a diameter ranging from 20 nm to 1 µm which is influenced by several factors, such as the manufacturing method and composition. Generally, the composition of liposomes consists of phospholipids, both natural and synthetic, and cholesterol. Liposomes

have several advantages, which can encapsulate drugs to targeted cells or tissues, biocompatible, biodegradable and non-immunogenic, reduce the toxicity of encapsulated drugs, and increase the efficacy and therapeutic index of drugs[2,3].

As a drug delivery system, liposomes must meet the specified requirements before being used as a drug carrier, including physical, chemical and biological stability. One of the problems often faced by liposomes is the problem of particle size. Because particle size has a direct impact on liposome efficacy, it is a critical issue. The longer liposomes may remain in blood circulation, the smaller the particle size, and consequently, the greater the therapeutic effectiveness[4].

Apart from particle size, another parameter which is also closely related to the stability of liposomes is the polydispersity index, which is expressed as the particle size distribution in the sample. The numerical value of the polydispersity index ranges from 0.0 to 1.5. The greater the polydispersity index value, the more polydisperse the particles are with several particle size populations. The smaller the polydispersity index value describes the more uniform or homogeneous the formed particles, and indicates that a formulation is more stable, so that it can survive for a long period of time in blood circulation. In its application, drug delivery systems such as liposomes that use lipid-based carriers, have a polydispersity index value of ≤ 0.3 which is considered acceptable and shows a homogeneous population of phospholipid vesicles[5].

The extrusion and ultrasonication techniques are the most often employed techniques for reducing the particle size of liposomes. The liposome suspension is run through a filter membrane with a preset pore size using the extrusion process. Some of the advantages of the extrusion method are that it is relatively simple, can increase encapsulation efficiency, no phospholipid deposition is found, and various filter membrane sizes are available, so it can be used to produce liposome sizes as desired. Meanwhile, the ultrasonication method is a method that uses high frequency vibration waves which can increase the mixing and breaking down of larger particles, forming smaller vesicles in the form of multilamellar to unilamellar. In the ultrasonication method, time or duration is one of the important parameters that plays a role in reducing particle size. The longer the ultrasonication process, the energy received by the sample will spread to all parts of the solution, causing the breaking of chemical bonds and producing increasingly homogeneous particle sizes[6,7].

Based on research conducted by Lapinski *et al.*, it is said that although there are real differences in the average size and particle size distribution, both preparation methods still produce liposomes with the same molecular scale characteristics[8]. Based on this background, researchers were interested in seeing the effect of the length of time of the ultrasonication method on the particle size and polydispersity index of Anthocyanin liposomes. This research will also look at the comparison of particle size and index polydispersity of liposomes produced using the extrusion and the ultrasonication method.

MATERIALS AND METHODS

Study materials

The majority of chemicals or reagents such as ethanol 96%, disodium phosphat, sodium phospate monobase monohydrate, cholesterol were purchased from Sigma-Aldrich (St Louis, USA). In contrast, DSPC and DSPE-PEG 2000 were obtained from Lipoid Gmbh, Germany.

Preparation of phosphate buffer solution

Phosphate Buffer Solution 1 M was made by mixing 185 mg sodium phosphate monobasic monohydrate with 80 mg disodium Phosphate into a 200 mL beaker glass, and dissolved using Aquadest 100 mL. After that, sodium hydroxide was added as 200 µL to get pH 7.4[9].

Preparation of Anthocyanin Liposome

Anthocyanin liposomes were prepared with the formula Anthocyanin, DSPC, cholesterol, and DSPE-PEG 2000 with a ratio of 1 : 1.85 : 1 : 0.15 using the ethanol injection method. Liposomes were formulated in a mixture of 2 phases, namely the water phase and the organic phase. The organic phase consisted of phospholipids DSPC, DSPE-PEG 2000, and cholesterol which were dissolved into ethanol 96% at 50ºC. Meanwhile, for the water phase, the Anthocyanin compound was dissolved using a phosphate buffer solution at 50ºC. After that, all organic phace solution was taken by syringe and injected quickly into water phase (phosphate buffer solution) while stirring on a thinky homogenizer (ARM-310, Japan), until both phases become homogeneous. Then, the mixture of the two phases was left for 30 minutes to evaporate the remaining solvent used. The liposome suspension was put into a centrifuge tube and stored in the refrigerator for 24 hours[8].

Liposome formulation using the extrusion method

10 mL of liposome suspension was taken and then the particle size was reduced using the extrusion method. The liposome suspension was put into a centrifuge tube and labeled as L0 liposomes which served as a positive control. Moreover, the extrusion process is carried out using a mini extrusion set tool (T & T, USA). Liposomes were passed through a 200 nm polycarbonate filter membrane (T & T, USA) for 3 cycles and continued using a 100 nm polycarbonate filter membrane for 3 cycles. After that, the purification process was carried out using PD-10 by flowing liposome into a PD-10 column (Sigma-aldrich, USA) which had previously been eluted with phosphate buffer. The purification process was repeated three times. Liposomes that have been purified were characterized by spectrophotometry UV-VIS (Shimadzu, Japan) to calculate entrapment efficiency percentage, as well as particle size and polydispersity index analysis using the Particle Size Analyzer (Horiba SZ-100, Japan)[9].

Preparation liposomes using ultrasonication methods with various time lengths

15 ml of liposome suspension was taken and then the particle size was reduced using the ultrasonication method. The liposome suspension was divided into 3 parts and put into each centrifuge tube. Each centrifuge

tube contained liposomes 5 ml and was marked with liposomes L1, L2, and L3. Liposomes (L1) were subjected to an ultrasonication (Elmasonic, Germany) process with a time variation of 5 minutes, L2 (15 minutes), and L3 (30 minutes). After that, the purification process was carried out using PD-10 by flowing liposome into a PD-10 column which had previously been eluted with phosphate buffer. The purification process was repeated three times. Liposomes that have been purified were characterized by spectrophotometry UV-VIS and Particle Size Analyzer[10].

Characterization of Anthocyanin Liposomes

Standard curve analysis of anthocyanin using Spectrophotometry UV-VIS

The absorbance value of Anthocyanin was measured using a spectrophotometry UV-VIS at 281.4 nm. The results of measuring the absorbance of anthocyanin will produce a linear regression equation[11].

Analysis of particle size and polydispersity index of anthocyanin liposomes using Particle Size Analyzer (PSA)

Analysis of the particle size and polydispersity index of Anthocyanin liposomes was carried out using a Particle Size Analyzer (Horiba SZ-100) to determine the particle size and polydispersity index[12].

Entrapment efficiency percentage of anthocyanin liposomes

The percentage of entrapment efficiency of Anthocyanin liposomes was measured using the Spectrophotometry UV-VIS and analyzed in the form of absorbance values for each variable, then calculated using the formula[13]-

$$\%EE = \frac{Td - Fd}{Td} X100$$

Whereas; Td = Total drug, Fd= Free drug

Stability evaluation of anthocyanin-loaded lipsomes

After being stored in PBS at 4°C for six weeks, the mean size and size distribution of the liposomes were measured weekly using PSA to examine the liposomes' colloidal stability under storage. The above treatment was also carried out at a storage temperature of 25°C for six weeks.

Data analysis

The Independent Sample T-test was selected as the analytic type. The purpose of this study's Independent Sample T-test is to compare the particle size and polydispersity index produced by the extrusion and the ultrasonication method and determine whether variations in ultrasonication time have an impact on these properties of liposomes[14].

RESULTS AND DISCUSSION

Anthocyanin Standard Curve Characterization Using Spectrophotometry UV-VIS

Calculation of Anthocyanin levels was carried out using the UV-VIS Spectrophotometry calibration curve method which were prepared from pure Anthocyanin with concentrations of 10, 20, 30, 40, and 50 ppm at 281.4 nm then were measured to get the linear regression equation. The standard curve results for pure anthocyanin compounds. From this curve, the linear regression equation is obtained i.e. b=0.0155, a= 0.0567, and r^2=0.9988. The value (r) obtained is almost close to 1 showing the linear regression equation, so it can be said that absorbance and concentration have a very strong correlation. The Anthocyanin standard curve image also shows that the concentration is directly proportional to the absorbance value, the greater the concentration of the Anthocyanin standard solution, the higher the absorbance value produced[15].

Table 1: Characterization of anthocyanin liposomes (%EE).

Formula	Absorbance (λ=281.4 nm)	% EE
L_0	0.4478	94.95
L_1	0.4220	95.28
L_2	0.3888	85.71
L_2	0.3833	85.78

Characterization of anthocyanin lipsoome (%EE) using Spectrophotometry UV-VIS

Entrapment efficiency is the percentage of active substance which is the ratio between the initial amount of drug and the amount of free (not trapped) drug in the formula. The greater the entrapment efficiency value, the greater the ability of phospholipids to protect the active substance from external influences that can damage the active substance, so that the bioavailability of the active substance will also increase[16,17]. The anthocyanin liposomes that had been formed were then analyzed using spectrophotometry UV-VIS at 281.4 nm to determine the percentage of entrapment efficiency. The results obtained can be seen in Table 1.

Table 1 shows the results of characterizing the entrapment efficiency of Anthocyanin liposomes such as L0 liposomes prepared using the extrusion method was obtained by 94.95%. Meanwhile, for liposomes L1, L2, and L3 which used the ultrasonication method with varying times were gained by 95.28%, 85.71%, and 85.78% respectively.

Characterization of anthocyanin lipsoome particle size and polydispersity index (PDI)

The analysis method of particle size and PDI used particle size analyzer (PSA) SZ-100 Horiba. The measurement method is non-invasive, fast, and can well measure the distribution of molecules and particles in the submicron region down to lower than 1 nm[18,19]. Which produce data in the form of diameter, molecular weight and particle size distribution[20].

Table 2: Characterization of anthocyanin liposome using extrusion method.

	Formula			
Method	**Size (nm)**	**Size Average (nm)**	**PDI**	**PDI Average**
Extrusion	153.2	152.1	0.371	0.379
(control)	151.0		0.388	

Table 3: Characterization of anthocyanin liposome using ultrasonication method with time variation (minutes).

Time (Minutes)	Formula			
	Size (nm)	Size Average (nm)	PDI	PDI Average
5	150.0	150.6	0.369	0.368
	151.2		0.367	
15	155.3	156.2	0.511	0.450
	157.1		0.389	
30	169.7	169.7	0.338	0.366
	169.7		0.394	

Based on particle size measurements that have been carried out using PSA (Particle Size Analyzer), the results obtained can be seen in Table 2 (Extrusion Method) and Table 3 (Ultrasonication Method). Table 3 shows the results of liposome characterization using the PSA, with the particle size average were obtained by L0 152.1 nm, L1 150.6 nm, L2 156.2 nm, and L3 169.7 nm. Meanwhile, the results of PDI average were 0.379, 0.368, 0.450, and 0.366 respectively. Liposomes are one of the most frequently used forms of drug delivery systems in the last few decades because they are considered the most ideal model for imitating biological membranes in the body, so they can be used to deliver drug compounds, vaccines or other compounds. Generally, the composition of liposomes is composed of natural or synthetic phospholipids and cholesterol[21,22]. The use of liposomes is not limited only to the delivery of synthetic drugs, but has also been used as a delivery agent for secondary metabolite compounds from plants[23]. In this research, the extrusion method and ultrasonication method were used to reduce the particle size of the liposomes produced. The extrusion method is a method of reducing particle size, which the liposome solution is passed through a filter membrane of a certain size, resulting in the desired particle size.

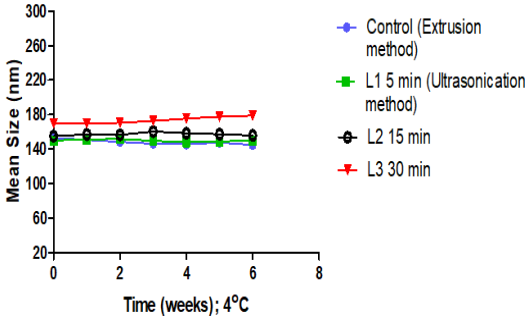

Figure 1: Colloidal stability of anthocyanin liposomes and control size at 4°C.

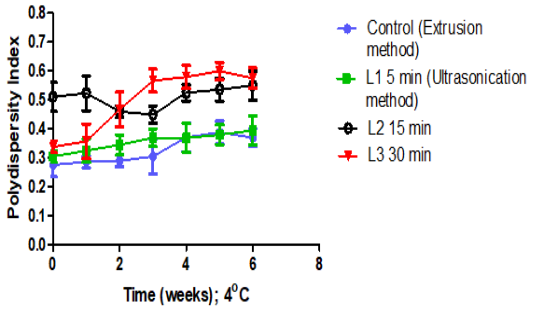

Figure 2: Colloidal stability of PDI of anthocyanin liposomes and control at 4°C.

The resulting particle size is usually of the SUV (Small Unilamellar Vesicles) type. Meanwhile, the ultra-sonication method is a method of reducing particle size using high frequency vibration waves which can increase the process of mixing and breaking up larger particles, forming smaller vesicles in the form of unilamellar or multilamellar. Apart from reducing particle size, the two methods also aim to compare the particle sizes produced by each method.

In previous research by Ong *et al.*, stated that the extrusion method was the most efficient method for reducing the size of liposomes, which can produce homogeneous and controlled particle sizes[24]. This is also in line with research by Pasaribu *et al*. stating that after undergoing the extrusion process, the particle size that will be produced was in the range or close to the pore size of the extrusion membrane[25]. In the ultrasonication method, the resulting particle size did not differ significantly, which the smallest particle size was produced from the sonication process for 5 minutes. However, the results obtained are inversely proportional to theory, stating that the longer the ultrasonication process time, the energy received will be evenly distributed to all parts of the solution, thus causing the particles to become more homogeneous.

It is possible that the particle size of anthocyanin liposomes may decrease with longer ultrasonication time, but this is not always the case. In some studies, a longer ultrasonication duration can produce larger liposome particle sizes. This is based on research by Dzakwan & Priyanto stating that the length of ultrasonication time can affect particle size which the longer the sonication process, the greater the mechanical energy released. This energy can damage and break down the vesicles, then cause the phospholipid double layers to fuse together, thereby inducing re-agglomeration[26].

As stated in the research of Dzakwan and Priyanto, the results showed that a sonication duration for 8 minutes was the optimal time to reduce particles to nano size, this is because a sonication duration more than 8 minutes will cause the cavitation energy to increase and be released into heat energy, which can later induce particle agglomeration. In addition, in research by Essa, it was shown that the maximum reduction in particle size was obtained in the first 10 minutes during the ultrasonication process[27].

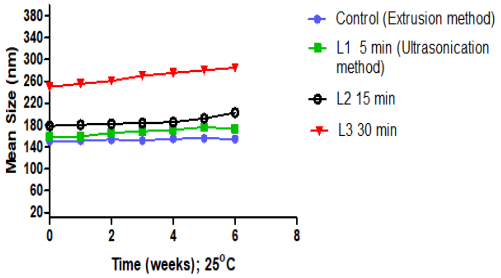

Figure 3: Colloidal stability of anthocyanin liposomes size and control at 25°C.

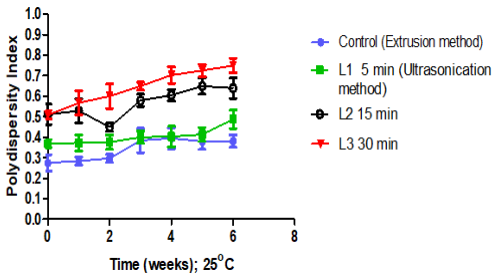

Figure 4: Colloidal stability of anthocyanin liposomes PDI and control at 25°C.

This opinion is also supported by research by Singh *et al*., who said that a duration of 10 minutes was sufficient to reduce particle size[28].

Table 2 and Table 3 also show the results of measuring size and PDI of Anthocyanin liposomes. The numerical value of the polydispersity index number ranges from 0.0 (for samples with perfectly uniform particle size) to 1.5 (for highly polydisperse samples with a large population of particle sizes). From the four liposome variables, the PDI was obtained from 0.368-0.450 and met the requirements for a good PDI value. This is in line with the opinion of Refai *et al*., which stated that the polydispersity index of acceptable liposomes should have a value below 0.7 indicating good dispersion homogeneity[29]. In addition, according to Jarrar *et al*., it is said that PDI values ranging from 0 - 0.5 reflect the uniformity of the particle size of homogeneous dispersed colloids and vesicles[30].

Stability evaluation of anthocyanin liposomes

Over the course of six weeks, and under liposome-storage settings at 4°C, there were very little changes in the mean size of each the length of ultasonication method but no change of the extrusion method as control. All methods showed that the size and PDI of anthocyanin liposomes is stable at 4°C each week which there is no significant change (Figure 1 and 2). This is related to the storage temperature of liposome constituents, namely phospholipids and cholesterol, which all stable raw materials are stored at -20°C.

On the other hand, all methods showed that the size and PDI of anthocyanin liposomes is unstable at 25°C which is characterized by increasing the particle size and PDI of liposomes for 30 minutes (L3) of ultrasonication time and followed by L2 (15 minutes). Otherwise, extrusion method (control) and L1 (5 minutes) are more stable than others which the size and PDI do not change significantly as seen in Figure 3 and 4. From the results of the Independent Sample T-Test test analysis, it shows that the particle size has a significance value smaller than α (0.05), which means that H0 is rejected, meaning that there is an influence of the length of ultrasonication time on the liposome particle size. However, the polydispersity index shows a significance value that is greater than α (0.05), which means that H0 is accepted. It means that there is no effect of the length of ultrasonication time on the polydispersity index of liposomes. Based on the results of the analysis, there are 2 pairs that have a significance value greater than α (0.05), namely the L0 and L1 liposome pair and the L0 and L2 liposome pair, with significance values of 0.354 and 0.102 respectively. This means that the hypothesis H0 is accepted, which there is no real difference between pairs of liposomes. Meanwhile, the other 4 pairs such as liposomes L0 and L3, L1 and L2, L1 and L3, and L2 and L3, have significance values which are smaller than α (0.05), namely 0.004, 0.001, 0.004, and 0.029. This means that hypothesis H0 is rejected, where there are real differences between treatment pairs. Because the results we want to see in this research are the comparison of particle sizes produced from the extrusion method and the ultrasonication method, resulting in 2 out of 3 pairs showing a significance value greater than α (0.05), it can be stated that there is no real difference in particle size of liposomes produced using the extrusion and ultrasonication method. Meanwhile, for PDI, the significance value obtained for all pairs is greater than α (0.05), which means H0 is accepted.

Limitations of the study

The limitation of this study is the lack of data on the release of anthocyanin liposomes in vitro and in vivo because this research is still ongoing. Besides that, there are no results looking at the morphology of liposome size using SEM because this research did not receive funding from sponsors which the cost of SEM results is quite expensive.

CONCLUSIONS

It can be concluded that there is no real difference in particle size and polydispersity index of anthocyanin liposomes using the extrusion or ultrasonication method. The preparation of anthocyanin liposome (L1) using the ultrasonication method for 5 minutes is smaller particle sizes and PDI than L0 liposomes (control) using the extrusion method.

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AUTHOR'S CONTRIBUTION

Tungadi R: Writing original draft, review, methodology, data curation, literature survey, editing.

DATA AVAILABILITY

Data will be made available on request.

CONFLICT OF INTEREST

None to declare.

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