Siti Sadiah et al /J. Pharm. Sci. & Res. Vol. 9(10), 2017, 1889-1893

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Preparation and Characteristics of Nanostructured Lipid Carrier (NLC) Loaded Red Ginger Extract Using High Pressure Homogenizer Method

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Abstract

This study presents the preparation of nanostructured lipid carrier (NLC) loaded extract of Zingiber officinale. Roxc. Var rubrum or Red Ginger (RG-NLC) using High-Pressure Homogenizer method with different the number of cycles. The RG-NLC formulation was characterized concerning particle size, polydispersity index (PDI), zeta-potential, particle shape and entrapment efficiency. The morphological study was performed by using Zetasizer Nano S and the entrapment efficiency analysis of NLC was performed using HPLC by detecting [6]-, (8)-, (10)-gingerol and (6)-shogaol as active biomarkers. The average particle size for RG-NLCs ranged from size 131 to 154 nm and a polydispersity index of 0.104 to 0.172. The zeta potential of the RG-NLC differed −33.00 ± 1.57 to -46.53 ± 0.38 mV and transmission electron microscopy showed that the particles were spherical in shape. Entrapment efficiency was higher more than 98% for (6)-gingerol, but other active biomarkers were less than 96 %.

Keywords: High-pressure homogenizer, nanostructured lipid carrier, red ginger

INTRODUCTION

Red ginger plant (*Zingiber officinale*. Roxc var rubrum) belong to family Zingiberaceae has been widely used in traditional oriental medicine, especially in Indonesia as JAMU. Several active components are present in ginger among them mayor active ingredients are gingerol and shogaol. These elements are used for a broad range of biological activities including anti-inflammatory, analgesic, antibacterial and anti-parasitic [1,2,3]. However, the lipophilic property of (6)-gingerol is unfavorable to be directly incorporated into the formulation. Hence as an alternative, NLC is developed as it is composed of a solid lipid matrix with the specific content of liquid lipid which is useful to increase the solubility of the lipophilic compound [4].

NLC was introduced at the end of the 1990s to overcome the potential limitations of SLN. NLC has been proposed as SLN of a new generation; they comprise particles with a solid lipid matrix with an average diameter in the nanometer range. NLC show a higher loading capacity for some active compounds, a lower water content of the particle suspension and avoid and minimize potential expulsion of active compounds during storage [4]. The carrier system can be used to overcome the observed limitations of conventional SLN, thus increasing the payload and preventing drug expulsion [5,6]. The molecular structure of SLN and NLC give the space to the drug, and the usual particles size were in the range approximately 10-1000nm. The small size of lipid nanoparticle ensures close contact with the target cell and able to increase the amount of drug penetration into the target cell, therefore providing greater efficacy as a delivery system [7]. NLC also has the potential to enhance solubility and improve bioavailability of the lipophilic drug.

Many different techniques for the production of lipid nanoparticles have been described in the literature. These methods are high-pressure homogenization, microemulsion technique, emulsification-solvent evaporation, emulsification-solvent diffusion method, solvent injection method, multiple emulsion techniques, ultrasonication, and membrane contractor technique [7].

High-Pressure Homogenizer (HPH) is one of the technological process aiming at the reduction in size and uniformity of fat globules, thus their even dispersion in the emulsion and an increase in emulsion stability. HPH is widely used in many industries including pharmaceuticals and nutraceuticals industry. It has many advantages compared to others method because its properties that are easy to scale up, avoidance of nonchemical solvents and short production time. Therefore, this study analyzes the effect of HPH on properties of the production of red ginger encapsulated nanostructured lipid carrier (RG-NLC), particularly on its size.

MATERIAL AND METHODS

2.1 Materials

Fat-free Soybean Phospholipids with 70% Phosphatidylcholine (Lipoid S75®), Polyoxyethylene sorbitan monooleate (Tween 80®), and Red ginger extract (*Zingiber officinale* roxc. var. rubrum) was obtained from Wellness Original Ingredient; (Bogor, West Java, Indonesia). Olive oil (Bratachem, Indonesia), Palm oil (Filma®, Indonesia). All other chemicals and reagents used in the study such as stearic acid and ethanol were of analytical grade. Distilled water was used throughout the experiment.

2.2 Preparation of red ginger extract-loaded NLC

Red ginger extract incorporated in nanostructured lipid carrier (RG-NLC) was prepared according to the method reported by Rahman *et al.* (2013) with some modifications.

Tween 80® was chosen as the surfactant. Initially, a certain amount of active compound (red ginger extract) was dissolved in a mixture of liquid lipid (palm oil and olive oil) and melted solid lipid (Lipoid S75®). The lipid melt containing active compound is then dispersed in a hot surfactant solution (temperature 5-10°C above the melting point of solid lipid) by high-speed stirring (IKA T-25 ULTRA-TURRAX® Stirring,) at 10 000 rpm for fifteen minutes. Emulsions are stored for one day and observed the physical changes. The most stable formulas are processed with HPH. The obtained emulsion is then passed through the high-pressure homogenizer (Niro-Soavi High-Pressure Homogenizer, 450 bar) following required cycles (0, 4, 6,12,16 &20 cycle), to produce RG-NLC. The minimum volume of the samples processed is 100 mL. All homogenizations are carried out above the transition temperature of the NLC. Subsequently, the NLC dispersion was cooled in ice water bath to room temperature (25±1°C) and stored at 4°C.

2.3 Particle Size, Zeta Potential, and Polydispersity Index Analysis

The particle size, zeta potential, and polydispersity index (PI) of RG-NLC was measured by photon correlation spectroscopy (PCS) (Dynamic Light Scattering, DLS, Zetasizer Nano NS, Malvern Instruments, Malvern, UK) at 25 ^{o}C using disposable plain folded capillary. Prior to measurements, all samples were diluted using distilled water and vortexed for 30 seconds to generate a suitable scattering intensity. Each measurement of RG-NLCs was performed in triplicate at 25 ^{o}C. Refractive indices of particles and water were 1.54 and 1.33 respectively, were used to calculate particle size distributions, zeta potential, and polydispersity index.

2.4 Transmission electron microscopy

Transmission electron microscopy was used to discover the shape and surface morphology of the nanoparticles. A drop of the diluted RG-NLC emulsion was placed on the surface of a carbon-coated copper grid after removal of excess liquid using a hydrophilic filter membrane. On drying at 25°C for up to one minute, the grid with a mesh size of 300 was then negatively stained with 2% uranyl acetate (w/v) for one minute and allowed to dry at room temperature. The RG-NLC sample was placed onto a sample holder, probed using a transmission electron microscope (Tecnai G2, FEI 120kv) and the image captured.

2.5 pH measurements

A calibrated pH meter was used each week to determine the pH of the RG-NLCs at room temperature, from the first day of production through to 5 weeks.

2.6 Sterilization by autoclaving

To observe the effect of sterilization on the particle size, zeta potential, and entrapment efficiency of RG-NLC, about 5.0 mL of RG-NLC was autoclaved at 121°C and 15 psi for 20 minutes.

2.7 Encapsulation efficiency and drug-loading capacity

The encapsulation efficiency and drug loading of RG-NLC were estimated after separation of red ginger extract and solid lipids from the aqueous medium by ultrafiltration. For this purpose, we used Centrisart® filter tubes (Sartorius AG, Göttingen, Germany) consisting of a filter membrane (molecular weight cutoff 300 kD) at the base of the sample recovery chamber. A 3 mL sample of undiluted RG-NLC was placed in the outer chamber, and the sample recovery chamber was fitted on top of the sample. The unit was closed and centrifuged by high-speed centrifuge at 20,000 × g for 15 minutes (himac CR 21G). The principle behind this process is based on RG-NLC remaining in the outer chamber separated from the aqueous phase while the aqueous phase filters into the sample recovery chamber through the membrane. The amount of gingerol in the aqueous phase was then evaluated by validated HPLC. The particles were evaluated by determining the amount of encapsulated [6]-gingerol, (8) gingerol, (10)gingerol, and (6)shogaol in NLCs using HPLC (Hitachi, Japan). The column used was Luna 5u C18 100Å (size 250 mm x 4.6 mm). The mobile phase consisted of acetonitril/water (60/40, v/v) and the flow rate was adjusted to 1 ml/min. The wavelength of detection was set at 260-300 nm. The calibration curve ranged from 20 to 100 µg/ml.

Table 1 Pre-emulsion of RG-NLC with variation of lipid composition (IKA,Ultra turax, 10000 rpm, 15 minutes)

Composition Lipid phase : aqueous phase	Formula code	Lipid phase (g)				Red Ginger Extract (JE96M) (g)	Aquaeous phase:(g)
		Liquid Lipid		Solid Lipid			
		Palm Oil	Olive Oil	Stearic Acid	Lipoid S 75		
1 : 1	F1	54	23	23	-	0.4	Sorbitol 4,5 g + Tween 80 1 g + Tiomerosal 0.0005% + Aquades Add 100g
1 : 2	F2	25	20	5	-	2	
1 : 2	F3	28	17	5	-	1	
1 : 4	F4	10	12.5	2.5	-	1	
1 : 4	F5	10	12.5	-	2.5	1	
1 : 4	F6	14	5.5	-	5.5	1	
1 : 4	F7	14	5.5	-	5.5	2	
1 : 4	F8	14	5.5	-	5.5	4	
1 : 8	F9	7	2.75	-	2.75	2	

The encapsulation efficiency (EE) was calculated using the following equations:

EE (%) = n1/n2 x 100 (1)

where;

n1 = total concentration of [6]-gingerol in red ginger extract (total amount of red ginger extract in starting soluition)

n2 = concentration of [6]-gingerol in encapsulated red ginger extract

Drug Loading (DL)%

= Total amount of gingerol encapsulated into NLC x 100
Total amount of lipid used in RG-NLC formulation

All measurements were performed in triplicate.

RESULT AND DISSCUSION

The pre-emulsion was made by differentiation of oil phase composition and aqueous phase, ie, 1:1, 1:2, 1:4 and 1:8. Differences in oil phase composition consisting of palm oil, olive oil, stearic acid and lechitin (Lipoid S75) and different concentrations of red ginger extract. The basis for the selection of palm oil and olive oil as liquid oil refers to research that has been done by Rahman, 2013 [8]. which formulated zerumbone NLC with zerumbon 0.4%. Rahman also uses lipoid S100 as a solid oil. Stearic acid is a solid fat commonly used in the manufacture of Solid Lipid Particles (SLN) [9]. In principle, the form of SLN and NLC is no different than its lipid-forming material. In the form of SLN only used solid lipid alone, while the NLC is used a combination of solid lipids and liquid lipids. SLN and NLC forms are dispersed as an emulsion preparation [10].

The results obtained from the use of stearic acid as solid lipids, unstable emulsions, and low exhaust stroke. In a comparison of an oil phase and water phase 1: 1 even produced a hard emulsion after storage for one day. The possibility of ginger extract with all its components is not compatible with stearic acid. On the use of Lipoid S 75 as solids, a reasonably stable emulsion, in the composition of palm oil: olive oil and lipoids S 75 = 14: 5.5: 5.5 and 1% extract concentration (F6). Furthermore, optimization is done by increasing the extracted content by 2% and 4%. It until the 2% concentration of the preparation is still stable, but at 4% concentration, it appears some ginger extract is not mixed. This result is no different from that of Rahman, 2013 [8]. which uses palm oil: olive oil: lipoid S 100 = 7: 3: 3 in zerumbone with levels of 0.4%. The novelty of this study is the level of red ginger extract as the active compound can be made up to 2% concentration. The importance of making this higher concentration considering the active ingredient used is a crude extract which of course on the application will produce a higher dose than the pure active compound. The use of crude extract as an active compound is based on the efficiency of the process undertaken which will undoubtedly be correlated with the cost/processing cost. Isolation of pure active components from crude extracts will have an impact on the high cost. Also, in vitro assay results comparing crude extracts with pure active compounds (6-8-, 10-gingerol, and 6-shogaol) showed that a red ginger extract especially exctract with a high 6-gingerol content had a potential that was not different than the pure 6-gingerol compound against the *Toxoplasma gondii* agent. Physical characteristics of the resulting formula as shown in Table 2.

Table 2. Characterization of pre-emulsion (before unproccesed with *High pressure homogenizer)*

Formula	Organoleptic Characterization
F1	white, textur hard
F2	Light brown, non homogen
F3	White, textur hard
F4	Light brown, non homogen
F5	Light brown, non homogen
F6	**Broken white, homogen**
F7	**Broken white, homogen**
F8	Broken white, non homogen,
F9	Broken white, non homogen

*was observed after storage for one day (homogenized by Ultraturax, 10000rpm, 15 minutes)

Based on physical observation in Table 2, the formula F6 and F7 with palm oil composition: olive oil: lipoid S75 = 14: 5.5: 5.5 has the best stability, the emulsion seems still homogeneous in storage for one day. But on F8 even with the same lipid composition but with 4% extract of ginger to be not similar with the amount of extract of ginger floating on the surface after mixing with Ultraturax with speed 10.000 rpm for 15 minutes. Thus, the formulation of a further process with a high-pressure homogenizer (HPH) is a formula F7 containing red ginger extract is higher than F6.

The results obtained as shown in Table 3 show that homogenization process with high-pressure homogenizer has an authentic effect on nanoparticle formation. The average size of unprocessed particles with high-pressure homogenizer has particle size still in micron scale. But when homogenizing with a high-pressure homogenizer at four cycles up to 20 cycles has resulted in average particle size of 154.93 -131.60 nm. The size of the nanoparticles is getting smaller as the number of cycles increases. Small particle size may affect the distribution of nanoparticles since small particle sizes lead to a narrower polydispersity index and vice versa. With small particle size in NLC ginger extract will increase the potential of ginger extract so it has the advantage that can reduce the absorption by the liver, prolong the circulation time in the blood, and increase bioavailability. Small particles can also minimize phagocytosis by macrophages so that damage and cleansing by the body can be reduced [8].

A particle in the NLC shows a negative charge on its surface, expressed as a zeta potential. Zeta potential is a critical factor in the prediction and evaluation of colloidal dispersion stability. For full electrostatic stabilization, zeta potential must be higher than 30 mV or less than -30 mV [8]. The results obtained as shown in Table 3, zero zeta potential (F7A-F7E) ranged from -33.00 ± 1.57 mV to -46.53 ±

0.38 mV. It shows that by starting four cycles up to 20 cycles can produce reasonably stable particles. In contrast to the size of particles that look linear to the number of HPH cycles, the zeta potential is not direct. The highest zeta potential is generated by the number of 4 cycles precisely on the largest particle size and the highest polydispersity index, while the smallest zeta potential is at 16 cycles (F7D) with the smallest particle size and the most polydispersity index small.

Table 3. Effect of HPH cycle on RG-NLC properties (F7)

Formula	Average Particle size d. (nm)			Zeta Potensial (mV)			Polidispersi Index
F7 (non HPH))	1200.78	±	3.65	-12.98	±	1.47	0.329
F7A	154.30	±	5.38	-46.53	±	0.38	0.172
F7B	140.63	±	0.64	-39.03	±	0.65	0.149
F7C	136.80	±	0.17	-39.90	±	1.34	0.108
F7D	131.83	±	0.76	-33.00	±	1.57	0.104
F7E	131.60	±	0.55	-42.63	±	0.38	0.114

A=4 cycle, B=8 cycle, C=12 cycle, D=16 cycle, E=20 cycle

Generally, the zeta potential measurement is based on the mobility of drug electrophoresis in aqueous media. Emulsions with higher zeta potential appear to prevent particles from flocculation and aggregation due to electrostatic repulsion. However, according to Rahman,2013 electrostatic repulsion may also be due to the use of steric stabilizers in nanoparticle preformulation resulting in nanoparticle dispersion stability. In this study, the zirc zero potential is due to the lecithin content (lipoid S75) in the lipid matrix at constant concentration of emulsifier in the water phase [8].

The five types of HPH cycles in general both four cycles up to 20 cycles result in particle size, polydispersion index and zeta potential eligible. Therefore, Formula with four-cycle become one of choice with consideration of process efficiency. The particle shape (F7A) is observed by a transmission electron microscope (TEM) within 15 days of production. As shown in FIG. 1, NLC is a relatively straight round, nanometer-shaped size of 154.8 nm, and has a relatively narrow size distribution. measurements with TEM are based on high vacuum exposure to electron beam columns [11].

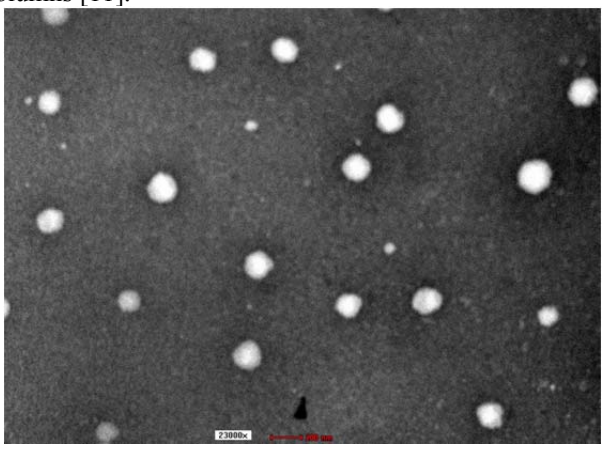

Fig 1 : TEM of RG-NLC (F7A)

The particle size, polydispersity index and zeta potential of RG-NLC (F7A) increased to 173.13 ± 0.52 nm, 0.24 ± 0.025 μm and −40.23 ± 0.41 mV respectively, and entrapment efficiency was lowered to 94.53% after one month of storage at 40°C. However, only very slight changes in mean particle size, polydispersity index, zeta potential, and entrapment efficiency was found in samples stored at 4°C and left to stand at room temperature before analysis. This implies that RG-NLC stored either refrigerated or at room temperature are physically stable and do not flocculate or coalesce under these conditions. On the other hand, the pH of the RG-NLC suspension decreased to 3.7 for samples stored at 40°C, while the pH of those stored in refrigerated conditions and at room temperature were approximately 2.5 and 2.8, respectively. This suggests that the pH of RG-NLC will decrease that particle size may not be affected by temperature during the first month of storage. Particle size also seemed not to be affected by the experimental methods, which included high-speed centrifugation, filtration, freeze-drying, and diluting buffer solutions at different pH values. This shows that the physical characteristics of aqueous RG-NLC dispersions were indeed stable, although transitions of dispersed lipid from a metastable to a stable form may occur slowly on storage and cause expulsion of the compound from solid lipid nanoparticles is possible

Efficiency value of ginger extract adsorption was determined based on 4 active components of 6-gingerol, 8-gingerol, 6-shogaol and 10-gingerol by HPLC method. Determination of permeate etched concentration was done by measuring the precipitate of nanoparticles formed after centrifugation at 20,000 rpm for 30 minutes. The results are shown in Table 4.

Table 4 : The Encapsulation Entrapment RG-NLC (F7A)

Active compound of RG-NLC	Content in Red Ginger Extract (mg/g)	Content in NLC (mg/g)	EE (%)
(6)-gingerol	14.92	2.71	98.68
(8)-gingerol	3.31	0.485	79.94
(6)- shogaol	1.17	0.208	96.57
(10)-gingerol	4.86	0.26	29.37

The efficiency of the adsorption is expressed as the percentage of active ginger ingredients determined by HPLC. Higher absorption efficiency was obtained at 6-gingerol and 6-shogaol in the range 96.57 -98.68% reflected that almost all ginger oleoresin trapped within the lipid carrier. But the smallest value of occurrence occurs in 10-gingerol because of only 29.37% only. In this formulation, the oil phase comprises palm oil on the highest composition then olive oil and S75 lipoids in the same form. When the ultra-centrifugation process occurs the separation back into 3 phases, namely the bottom water phase, the phase of the nanoparticles in the middle and the liquid oil phase at the top. The color of the liquid oil is browner than liquid oil when before it is formulated. This

means that there are components of ginger extract that may be interested in the liquid oil phase. And it is probable that 10-gingerol is much interested in the liquid oil phase because of its lowest polarity compared to the other three active compounds.

To prove it is necessary to re-determine the level of gingerol in the oil phase.Sufficiently good encapsulation efficiency values obtained particularly for the 6-gingerol, 8-gingerol and 6-shogaol content can be attributed to the structure of a particular portion of the NLC, the combined use of a solid lipid matrix with liquid lipids gives rise to a less compact structure, thereby being able Compared with the form of SLN (solid lipid Nanoparticles) formed only from dense lipids alone, the size of the nanoparticles produced in this NLC form is smaller. Research conducted by Ratcharin et al., 2012 [12], created SLN ginger extract with stearic acid as solid lipid, chromophore RH 40 as surfactant and ethanol as cosurfactant, and made by microemulsion technique only able to produce particle size in the range of 453.10 - 551.7nm. While Rosli N.A *et al.,* 2014, in the formation of 6-gingerol NLC with ultrasonication technique also resulted in particle size in the range of 100-250nm with a 6-gingerol absorption efficiency of 92.7%. This result corroborates the research that NLC form with High-pressure homogenizer technique can also produce smaller particle size than SLN form and when compared with ultrasonication technique, this HPH result yields a slightly higher gradient to accommodate loading ginger oil.

Compared with the form of SLN (solid lipid Nanoparticles) formed only from dense lipids alone, the size of the nanoparticles produced in this NLC form is smaller. Research conducted by Ratcharin *et al,* 2012 [12]. produced SLN ginger extract with stearic acid as solid lipid, cromophor RH 40 as surfactant and ethanol as cosurfactant, and made by microemulsion technique only able to produce particle size in the range of 453.10 - 551.7nm. While Rosli N.A *et al*, 2014 [13]., in the formation of 6-gingerol NLC with ultrasonication technique also resulted in particle size in the range of 100-250nm with a 6-gingerol absorption efficiency of 92.7%. This result corroborates the research that NLC form with High pressure homogenizer technique can also produce smaller particle size than SLN form and when compared with ultrasonication technique, this HPH result yields a slightly higher gradient efficiency on 6-gingerol. The difference with NLC produced by Rosli N.A *et al* 2014 [13] is on the use of solid lipids and their liquid lipids. Rosli *et al* used glyceryl monostearate as a solid lipid and virgin coconut oil as a liquid lipid. One more advantage of this research with previous research is NLC ginger extract produced mengadung concentration of ginger extract that is used high enough that is 2% if compared it which only 0,7%.

CONCLUSIONS

In the present study, red ginger extract loaded nanostructured lipid carrier with particle size under 200 nm could be successfully obtained. High-Pressure Homogenizer Methods can significanty lower particle size. The average particle size for RG-NLCs ranged from size 131 to 154 nm and a polydispersity index of 0.104 to 0.172. The zeta potential of the RG-NLC reached −33.00 ± 1.57 to -46.53 ± 0.38 mv and transmission electron microscopy showed that the particles were spherical. Entrapment efficiency was higher more than 98% for (6)-gingerol, but other active biomarkers were less than 96 %.

ACKNOWLEDGMENTS

Supported by research funded by Ministry of Research, Technology and Higher Education of the Republic of Indonesia. I thank also to Laboratory of Nanotecnology, Ministry of Agricultural , Bogor –Indonesia, and Laboratory of Tropical Biopharmaca Research Center, Bogor Agricultural University, Bogor – Indonesia for all support my research.

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