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Article

Nano-lipids based on Ginger Oil and Lecithin as A Potential Drug Delivery System

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Abstract: Lipid nanoparticles based lecithin are an interesting part of drug delivery system. However, the stability of lecithin nano-lipid is problematic due to the degradation of lecithin causing a decrease in pH. In this study, the modification of the conventional nano-lipid based soybean lecithin was demonstrated. Ginger oil derived Zingiber officinale was used along with lecithin, cholesterol and span 80 to fabricate nano-lipid (GL nano-lipid) using thin-film method. Through TEM and confocal microscope, GL nano-lipid exposes the liposome- like morphology. The average size of the resultant nano-lipid was 249.1nm with monodistribution (PDI= 0.021). The ζ-potential of GL nano-lipid was negative as similar to as prepared nano-lipid based lecithin. GL nano-lipid expressed the highly stable over 60 days of stor-age at room temperature in term of size, ζ-potential. A shift of pH value from alkaline to acid was detected in lecithin nano-lipid, while with the incorporation of ginger oil, pH value of nano-lipid dispersion was around 7.0. Furthermore, due to the rich of shogaol-6 and other active compounds in ginger oil, the GL nano-lipid is endowed with intrinsic antibacterial feature. In addition, the sulforhodamine B (SRB) assay and live/dead imaging revealed the excellent biocompatibility of GL nano-lipid. Notably, GL nano-lipid was capable to carry the hydrophobic agents as curcumin and perform a pH-dependent release profile. A subsequent characterization are a suitable potential for drug delivery system.

Keywords: lecithin; ginger oil; essential oil; nano-lipid; drug delivery system

1. Introduction

Lipid-based nano-carriers have been considered as a promising tool for drug delivery. Due to the natural structure of biological structure as cell membrane or vesicle, lipid nano-carriers are able to enter to cell via membrane fusion without compromising the intrinsic function of the carrier [1-4]. Lipid based nano-carriers have two separated parts: lipid matrix and aqueous phase; providing the excellent advantages in packing both hydrophobic and hydrophilic agents [1, 3]. In addition, the formation of lipid carrier is totally depended on physical interaction of lipid components via the control organic phase and aqueous phase leading to the self-assembly into defined structure [5]. Therefore, the preparation of lipid carriers can be easy to upgrade with various suitable large-scale production techniques [6].

Generally, the basic components of lipid carriers are lipid, the surface stabilizers, and/or lipid excipients [5-7]. Lipid plays a critical in the formation of the carrier's shape [7]. In fact, amphiphilic lipid such as phospholipid is the common choice for fabrication process because they can self-assemble into micelles structure in the aqueous environment and mimic the lipid layer of cell membrane [7-8]. Currently, edible lipids with the rich of phospholipid have generated much interest [4]. Lecithin is one of the most common natural edible lipid [9] and has been recognized as safe by the Food and Drug Administration

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[10]. Lecithin is a yellow-brown fatty substance, which can be found in both animal and plant [7]. Among lecithin, soy lecithin has got a lot of interest in drug delivery system due to the abundance sources as well as the cost of production [8]. Further, soybean lecithin contains the mixture of phospholipid compounds which are similar to lipid bilayer cell membranes [9-10]. Unlike the lecithin from animal or synthetic resources, along with 60-75% phospholipid, triglycerides and unsaturated fatty acid (linoleic, oleic, palmitic and α-linolenic acids) can be found in lecithin soybean [11]. These compositions shows the advance for health system, specialized on cardiac system, over other source of lecithin [11]. Various nano-lipid based lecithin have been introduced with their well-performance in drug delivery [9, 12]. However, currently, the concern about lecithin nano-carrier related to physicochemical stability after long-term storage [13-15]. The degradation of lecithin could induce the acidic environment for a parenteral solution, causing the serious problem to healthy [15].

Currently, research has emerged that essential oils (EEs) can also be inclusive in lipid formulations process [3, 16-19]. Together with monoterpenes and phenylpropenes, EEs also enrich in essential fatty acids, which can solve the problematic of lecithin carrier. The lipid carrier was prepared with essential oil of Cymbopogon flexuosus (Lemongrass), offering the greater stability at ambient temperature [16]. In addition, the combination of EEs could help to increase the encapsulated hydrophobic drug or vitamin, performing the sustainable released profiles as well as enhancing the cell permeation [3]. A. Meghea et al [17] found that lipid nano-carriers prepared with linseed oil showed the higher encapsulated drug and a gradual slow release as compared to convention lipid carrier. Kumar et al. [18] took the advance of lipid carrier with the addition of linseed oil in both permeability parameters and the therapeutic value of thiocolchicoside. The use of palm oil in the formulation from Lecithin/ Tween 80/ glycerol developed by Arbain et al. [19] exposed the high amount of quercetin encapsulation and efficiency for aerosols delivery. Another important factor to consider the participation of essential oil in the development of lipid carrier is the synergy between EE and loaded agent [3]. Ginger oil [20], frankincense oil [21], garlic oil [22], pomegranate seed oil [23], etc. displays various medicinal properties such as antimicrobial, antioxidant, anti-carcinogen, or sedative. Thus, essential oil have been developed the excellent strategy for fabricating lipid nano-carriers.

In this work, the modified conventional lecithin nano-lipid was prepared with ginger oils. Ginger oil is used as alternative medicine practice for long time and has affirmed as safe agents in food by FDA [17].Due to the lipophilic property, ginger oil could be located inside the lipid layer of lecithin nano-lipid. The composition into ginger oils include polyphenol (shogaol, gingerol, paradols..), alkaloids, saponins, tannins, glycosides, flavonoids as well as glycolipids and fatty acid, suggesting the golden therapeutic value of the developed system [24-25]. Notably, ginger oil had been proven the ability to prevent the damage of macromolecules inducing by free radicals [26]. Therefore, the strategy of the utilization of ginger oils as lipid constituent along with lecithin soy bean could be effective carrier for drug delivery system. In this study, the ginger oil was extracted from Vietnam ginger root (Zingiber officinale) and then was characterized by LC-MS and HPLC before using in the lipid fabricated process. The resultant GL nano-lipid batches were examined in term of size, zeta potential as well as stability over 60 days of storage at the room temperature. More importantly, to examine whether GL nano-lipid could be a potential carrier in drug delivery, the cytotoxic profile as well as the ability to carry hydrophobic bioactive mole-cules (curcumin) were also determined. This study may therefore have important implications for expand current concepts of drug delivery carrier with a multifunctional lipid nano-carriers based on essential oil.

2. Results and discussion

2.1. Characterization of ginger oil

The yield of ginger oil from Zingiber officinale was found to be 0.7% on raw weight basic. The chemical volatile compositions of the resultant ginger oil were identified

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through gas chromatograph - mass spectrometry (GC-MS) analysis. 11 bioactive compounds were found in the obtained ginger oil within 55 minutes of retention time (fig 1A, fig S1), in which 5 compounds were identified by using NIST-14 Mass Spectral library (Table S1). Results from GC–MS profiles revealed that almost most compounds have a higher boiling point (over 80°C). The identified compounds were fatty acid ester, geraniol, gingerdiol, shogaols, gingerols, similar to those reported in literature [24, 25]. The most of abundance concentration in ginger oil is the compound with retention time of 40.41 min (63.13%). This compound has [M]+ peaks at m/z 276, a base peak at m/z137(cleavage of benzylic group, [M+H-C13H22O]+) and some mass fractions such as m/z 205 (corresponding to carbonyl group at C3) and m/z 119 (due to the loss of methyl group from the fragment 137), indicating this compound is 6-shogaol (Fig S2) [26]. The latter peak in GC with retention time of 41.55 is confirmed as [8]-Shogaol based on its mass spectrum (Fig S3) ([M]+ peak at m/z 304, base peak at m/z 137 and strong peak at m/z 205). This compound accounts for 6.29% of total volatile compound. The major compound present next to shogaol was found to be at retention time of 43.88 min (7.73%). The mass spectrum (Fig S5) shows [M]+ peaks at m/z 430 along with base peak at 137 (Fig S4). In addition, this spectrum has peak at m/z 278, 279 in the mid mass region, peak at m/z 131, 138, 151, 163 in the low mass region and peak at m/z 69, suggesting this compound to be [6]-Gingerdiol (2E)-geranial acetal. Also, with our extracted method, Geraniol, a new class of chemoprevention agents for cancer [27], was identified in this ginger oil with 1.95% (retention time of 23.57, mass spectrum at m/z 154 with the base peak at 69, (Fig S5)).

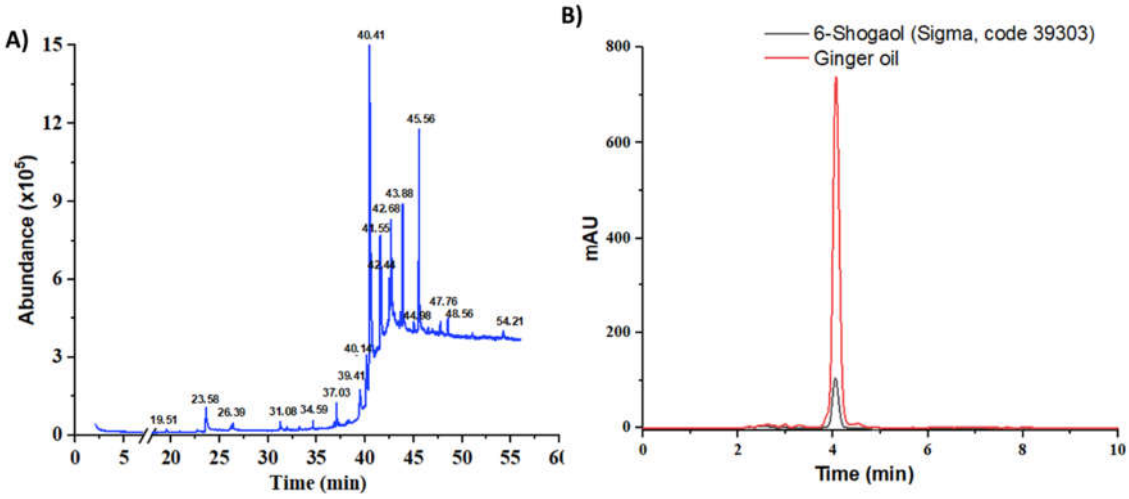

Figure 1. GC-MS chromatogram of edible ginger oil (A) and HPLC chromatograms of the representative ginger oil extracts in compared to standard 6-shogaol (B).

In this study, a 6-shogaol-rich ginger extract was our purpose. Therefore, the extracted process was per-formed with 2 solvent phase extractions; first with ethanol and heat to convert 6- gingerol to 6-shogaol, and then the second solvent fraction with petroleum ether – ethyl acetate (1:1) to increase the increase the concentration of 6-shogaol in ginger oil. HPLC method was conducted to estimate the amount of 6-shogaol in the ginger oil (Fig S6). The retention time of 6-shogaol in chromatographic program is at 4.194 ± 0,0059 min (RSD = 0,14%, tailing factor= 1.01, theoretical plates =4588) and the linear regression is 166129,76x + 22743,83 with R^2 = 0,9997 (Fig S6). In addition, HPLC chromatogram of ginger oil exhibited the strong and clear peak at similar position with standard shogaol (fig 1B), confirming that this program could be used to examine the concentration of shogaol in the extracted oil. The amount of 6-shogaol was increase from 0.22% in raw ginger material to 0.88% by mass after applying ethanol extraction with heat. By applying 2 solvent fractions, the concentration of 6-shogagol is about 2.33 % (23.33 µg per mg ginger oil), increase 2.65 times and 10.59 times as compared to single solvent applying and raw

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material, respectively. Taken together with GC-MS result, the volatile compounds are 3.69% by mass of ginger oil, in which shogaol compounds are approximately 70% by applying our extracted program.

2.2. Characterization of GL nano-lipid

GL Nano-lipid was fabricated by the thin film hydration method using lecithin, cholesterol, polysorbate 80 and ginger oil. TEM (fig 2A) shows that GL nano-lipid particles from non-extruded process appear bright against the background of the heavy metal stain, similar to liposome structure. However, owing to the stabilization problem of the morphology of nano-lipid in the dry-stage, nano-lipid was truncated shape. Therefore, to verify the structure of nano-lipid, carrier was stained with Dil C18. Inspection using confocal microscopy in the liquid stage shows that the red fluorescence signal is at the shell which emanated from the lipid bilayer (fig 2B). This morphology of GL nano-lipid was similar as liposome. In addition, the DLS result releaved that all the nano-lipid particles expose the circular shape with the homogeneous size. The hydrodynamic radii was 249.1nm (fig 2C). The polydispersity index (PDI) is 0.021. This confirms the relatively narrow size distribution of the GL nano-lipid in agreement with the confocal image analysis. The time correlation function of scattered light of GL nano-lipid has a single exponential decay (fig 2C inset), suguesting the high purity of GL nano-lipid, without any large particles in suspension. In addition, the participation of ginger oil in lipid phase does not induce any effect on the electrical characteristics of lecithin/cholesterol/ tween 80 colloidal nanoparticles. Soybean lecithin contains a mixture of various phospholipid [11]. Soy lecithin contains some acidic phospholipids such as phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol resulting in the negative charge value on the lecithin lipid droplet surface [12]. The conventional lecithin nano-lipid generates the negative zeta potential, about -54.5mV [9]. After adding ginger oil, the surface charge of GL nano-lipid is negative value (fig 2D). However, as compare to bare form of nano-lipid, the ζ-potential value of GL nano-lipid increases to -30.4mV, suggesting the interaction of ginger oil and the lipid layer [28].

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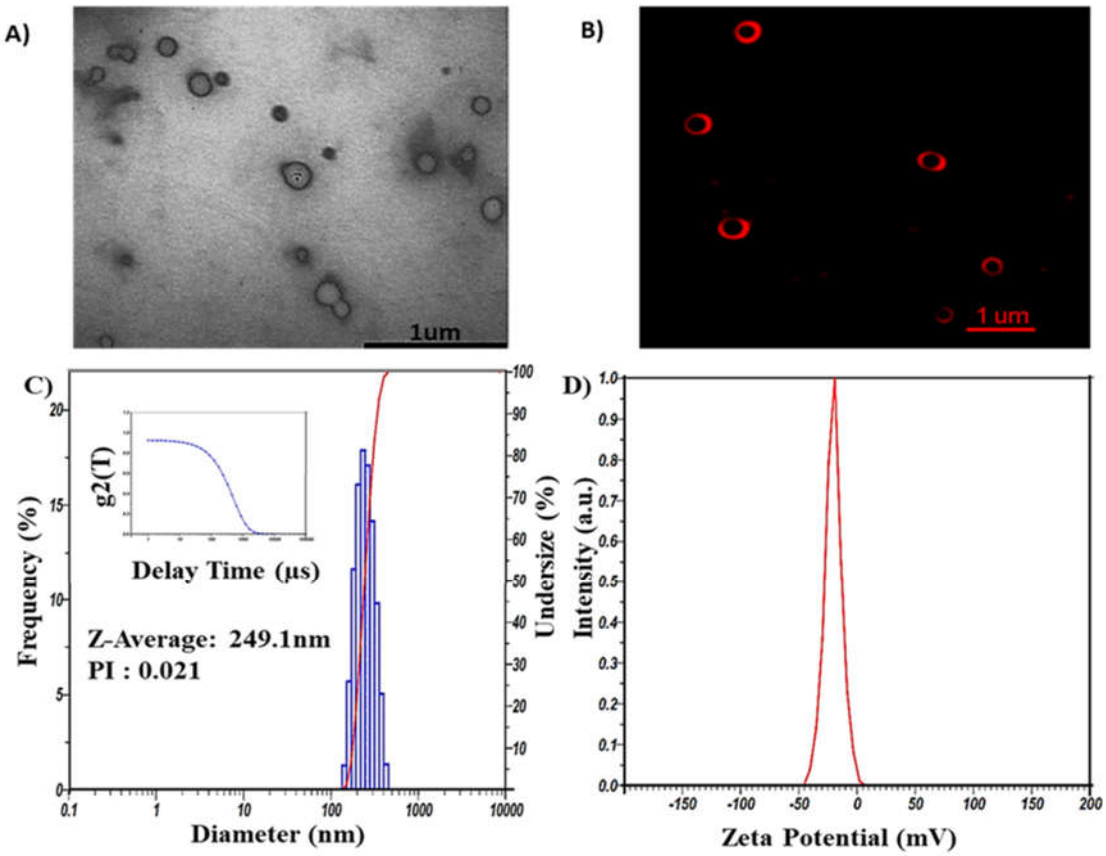

Figure 2. Morphology of GL nano-lipid via TEM image (A) and confocal microscopy image labeled with DiL C18 (B). Size distribution (C) and ζ-Potential values (D) obtained by DLS of GL nano-lipid at 25oC.

2.3. The physical stability study of GL nano-lipid

The physical stability of the dispersions plays a critical aspect to evaluate the potential application of nano-carrier. For the lipid carriers, size as well as size distribution play a critical point in the intravenous applications [6-7]. The unstable lipid droplet nanoparticles could induce a fat embolism [29-30]. Thus, the hydrodynamic size and zeta potential value of GL nano-lipid were exanimated during 60 days.

As shown in figure 3a, GL nano-lipid were stable with respect to the particle size distribution over 60 days of storage ($p>0.05$). Neither the intensity nor the number based particle size distributions revealed the occurrence of a second population as might be possible by particle aggregation. In general, samples stored at 25 °C showed a no-ticeable increase ($p < 0.05$) in PDI after 30 days of storage, but all are under 0.1, suggesting the monodistribution of nanoparticles [14]. ζ Potential varied between −30.4 mV and − 29.0 mV (fig. 3b) during 60 days. The non-significant different in ζ Potential reveals that GL nano-lipid stability was not lost during the entire storage. Since the absolute values of the measured zeta potentials are above the theoretically appointed 30 mV limit required for stability [31], we can conclude that GL nano-lipid are acceptable for the preparation of stable nano-lipid formulations.

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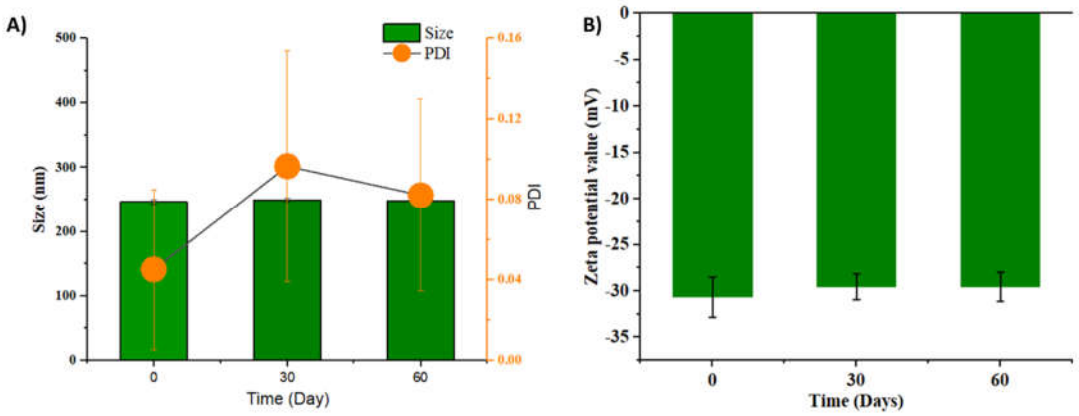

Figure 3. Hydrodynamic size PDI value (A) and zeta potential of GL nano-lipid solution during storage time: 0 day, 30 days and 60 days at room temperature (~25oC). Results are presented as mean ± standard deviation, (n = 9).

A major disadvantage of nano-lipid based on lecithin soy bean however is the critical chemical stability of lipid layer after long time storage. Lecithin is known to degrade on exposure to air, and for that degradation to cause a decrease in pH [32-33]. To test this, GL nano-lipid were re-hydrated and their pH was measured as a function of time in the compared to lecithin nano-lipid. For the freshly preparation, a pH value of 7.2 was measured in both GL nano-lipid and bare form. However, it is clear that the pH of bare nano-lipid samples decrease with time ($p>0.05$) (fig 4a). This is in consistent with the results obtained in lecithin liposome [13-14]. Despite the unchanged in the size and the PDI <0.1 after 3 months, liposome formulation based soy phosphatidylcholine exposed the degradation causing the increase of the acidic level of the dispersion [14]. With the addition of ginger oil, the lecithin nano-lipid dispersion exhibit only a very minor decrease in pH, no pH lower than 6.5 was found, not even after store for 60 days. Images of bare nano-lipid and GLnano-lipid during storage are shown in figure 4b. The stored sample of bare nano-lipid at day 60 is turbid compared to the fresh sample. GL nano-lipid was similar in appearance to its freshly prepared sample during the entire storage. Along with pH of dispersion, the increased turbidity in lecithin nano-lipid could be due to the degradation of lecithin into the low solubility of phospholipid hydrolysis product. The stable in pH value of GL nano-lipid might be attributed to the inclusion of the ginger oil which have various anti-oxidants.

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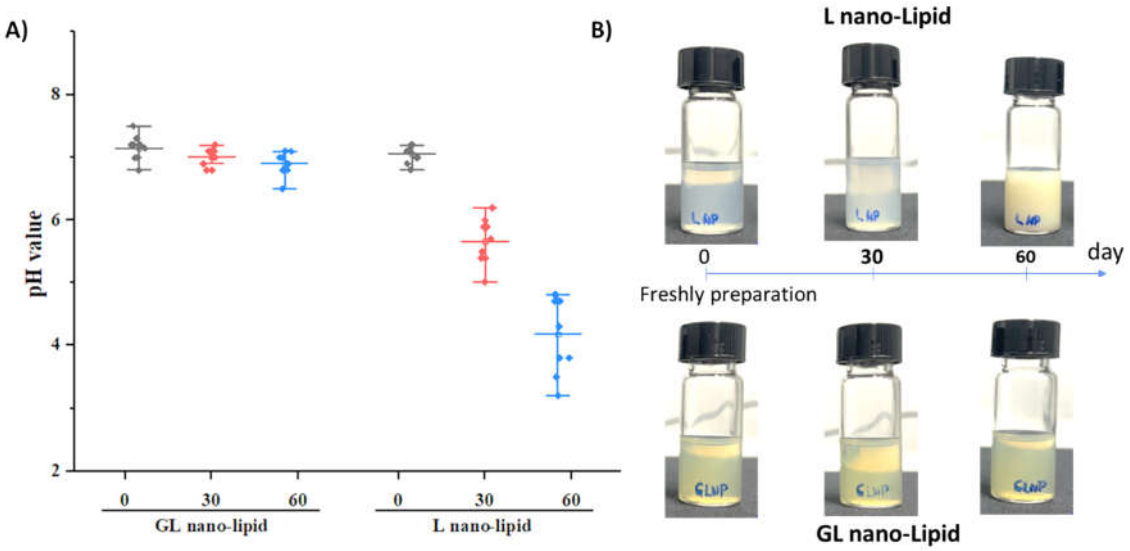

Figure 4. pH value (A) and visual observations of GL nano-lipid and L nano-lipid solution during storage time: 0 day, 30days and 60 days at 25oC. Boxes represent pH value with a probability between 25 and 75%, the line ▬ inside the box is for the median pH value of solution, a bullet is for data.

To prove the ability of ginger oil in protection of GL nano-lipid, pyrogallol assay was perfomed. For this test, pyrogallol was oxidized by hydrogen peroxide (H_2O_2) with the help of HRP enzyme [34]. Previous studies have mentioned the ability of ginger oil in cancel the activity of oxidant due to the abundance of polyphenol compounds such as 6-shogaol or gingerol [25-26]. Thus, the ginger oil or GL nano-lipid was first incubated with hydrogen peroxide before adding pyrogallol and HRP enzyme. After 30 min, the orange-brown solution and precipitate was appeared in the control sample (Fig S7), indicating the formation of Pyrogallol-quinone (oxidative product of pyrogallol). Nothing changed in the color was detected in case of ginger oil and GL nano-lipid. In other word, both ginger oil and GL nano-lipid showed a stronger scavenging activity in inhibiting H_2O_2.

This study suggest that the inclusion of the ginger oil could prevent the oxidation of lipid composition resulting in the enhancement of the stability of nano-lipid as compared to raw form.

2.4. Biocompability of GL nano-lipid

Lecithin nano-lipid has been proved with the excellent biocompaility [8]; however, ginger oil is cytotoxic dependent dose [35]. Thus, the cytotoxicity profile of GL nano-lipid was assessed with MSC cells. First, MSC cells were exposed to various concentration of GL nano-lipid (fig 5a). GL nanolipid exhibit excellent bio-availability with the upper limit of GL nano-lipid. The viability of MSC cels was above 95% in respected to non-treated cell after 24h exposing to 5mg/ml GL nano-lipid. The growth retardation of MSC cell was detected ($p<0.05$) with 10mg/ml GL nano-lipid. The survival percentages were 88.07±3.46%, terming relatively harmless according to ISO 10993. However, it is worth sense for longer time exposure to nanoparticles. We therefore extended the culture time to 72h with 5mg/ml GL nano-lipid (fig 5b). From the acute toxicity test with MSC cells, 5mg/ml GL nano-lipid was used in our subsequent experiments. The viability of MSC cells after 72h continuous exposure to nanoparticles was identical as compared to 24h exposure time or a further 48h culture. To verify the cytotoxic profile of GL nano-particles, dual staing AO/PI was conducted. As shown in figure 5c, the MSC cells after 72h incubation with nanoparticles showed well-maintained cell survival, in agreement with SRB result. MSC cells cultured with Hepes buffer or with GL nano-lipid exhibited the homogeneous a fibroblastic morphology with the well-adhesion. After 72h exposure to nanoparticles, the density of the cell was indistinguishable from negative control. The above data reveal

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that the GL nano-lipid has excellent biocompatibility, which is favorable as a potential candidate carrier for drug.

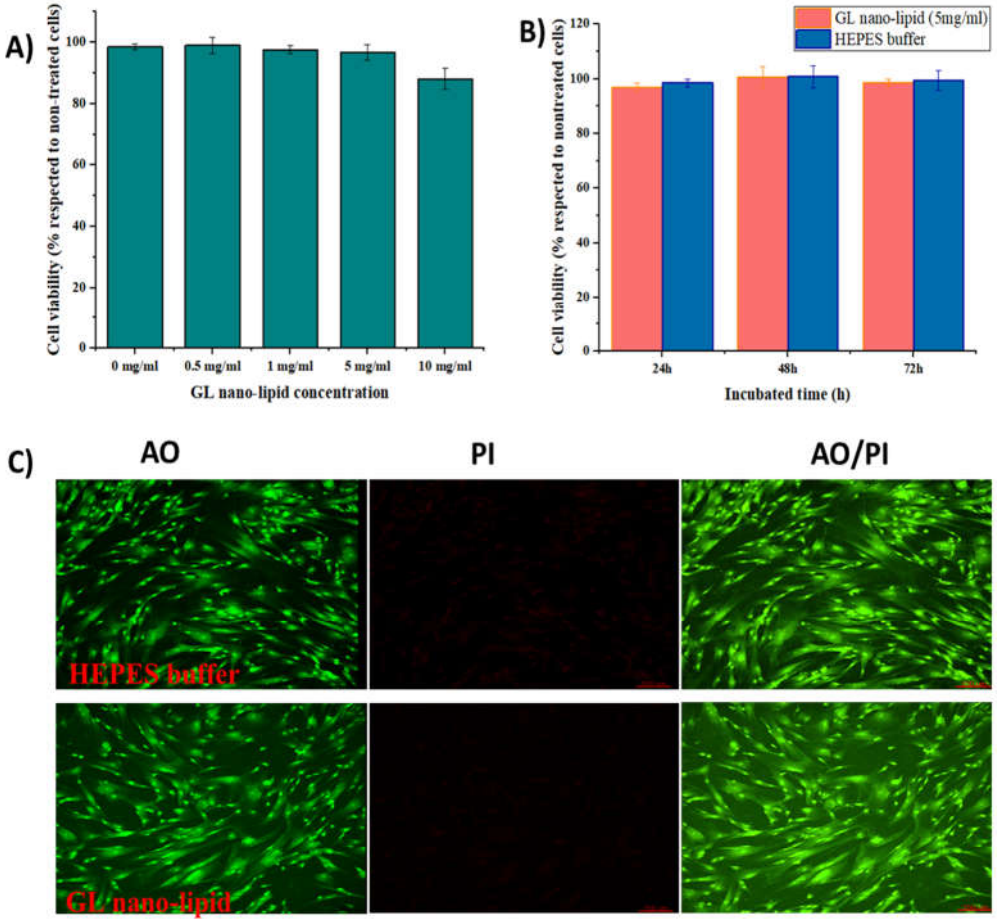

Figure 5. A) The cytotoxicity of MSC cells treated with different GL nano-lipid concentrations (0, 0.5, 1, 5 and 10 mg/ml) in respected to non-treated MSC cells. B) The cytotoxicity of MSC cells in function of time when incubated with 5mg/ml GL-nanolipid and HEPES buffer. Results are presented as mean ± standard deviation (n = 3). C) Con-focal microscopy images of MSC cells upto 72 h incubation at 37 °C with GL nano-lipid (5mg/ml) and HEPES buffer. AO: green color; PI: Red color; AO/PI: Merged color; Scale bar = 150µm.

2.5. Anti- bacteria property of GL nanolipid

Escherichia coli and Staphylococcus aureus were acted as model to prove the intrinsic antibacterial feature of GL nano-lipid. Both positive controls (Ampicillin) and (Streptomycin) exhibits their strong sensitive to bacteria (Fig S8). The anti-bacterial of ginger oil has previously been reported [24-26, 36]. In this study, with 1.16 mg/ml ginger oil, the inhibition zone of 14.83±0.58 mm diameter clear zone was noticed against E. coli and 12.77 ± 1.65 mm clear zone recorded against S. aureus. No inhibition zone forms in case of L nano-lipid. In the same concentration with L nano-lipid, GL nano-lipid (10mg/ml) displays the inhibition zone with the diameter of 17.20±0.76 mm and 15.87±0.47 mm to E.coli and S. aureus, respectively. Thus, the addition of ginger oil into lecithin nano-lipid (L nano-lipid) showed the ability to inhibit the growth of bacteria.

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Table 1. Disc diffusion assay of GL nano-lipid against some pathogenic bacteria.

Test sample	Concentration	Inhibition zone (mm)	
		E.*coli*	S.*aureus*
Ginger oil	1.16 mg/ml	14.83 ± 0.58^{D}	12.77 ± 1.65^{C}
L nano-lipid	10mg/ml	6^{D}	6^{D}
GL nano-lipid	10mg/ml	17.20 ± 0.76^{C}	15.87 ± 0.47^{B}
Ampicillin	10µg/ml	21.19 ± 0.70^{B}	23.89 ± 1.56^{A}
Streptomycin	25µg/ml	27.4 ± 0.96^{A}	23.84 ± 0.15^{A}
HEPES buffer		6^{D}	6^{D}

Values are mean ± SD of 3 independent replicates

The significant difference between variable means was presented by words A, B, C, and D following least significant difference (LSD) test, 95% confident level.

2.6. GL nano lipid as curcumin delivery

Next, curcurmin – bioactive compound with various pharmaceutical properties such as anti-cancer, anti-inflammation, wound healing, etc, was selected as a drug model to investigate whether GL nanolipid could have ability to encapsulate drugs. The appearance of 2% Cur loaded GL nano-lipid (Cur@GL) had a transparent organe-yellow color (fig 6A), confirming a good dispersion in aqueous system. Entrapment efficacy (EE) and drug loading capacity (DL) are two main parameters to evaluate the ability of carrier [37]. 85.97 % ± 4.03% (EE) curcumin was loaded into GL nano-lipid. The DL value was 16.72% ± 0.78%. The particle size of Cur@ GL are about 250.2 nm obtaining via DLS. Intrinsic green fluorescence of curcumin has been utilized by previous reports [38]. Therefore, to further prove the successful encapsulation, confocal image was conducted. Following incubation with Dil, Cur@ GL expresses dual fluorescent channels (fig 6B, fig S9), suggesting that Cur was encapsulated into GL nano-lipid. In addition, the charge surface of Cur@ GL was indistinguishable from GL bare form; however, the value is higher (-20.2 mV), indicating the interaction between curcumin and lipid layer of nanoparticles [28]. This value proposes the moderate stability of the colloidal system [14, 31]. Interestingly, GL nano-lipid performed the different released behavior in a biological model. In the agreement with zeta potential value, the hydrodynamic size of Cur@ GL did not show any changed after 10 days incubation in physiology buffer, PBS 7.4 (Fig S9). In addition, the amount of curcumin was 84.13 % ± 4.79% as compared to the initial curcumin loading. This EE value after 10 days was similar to the EE values of fresh prepared sample ($t=0.476$, $p=0.66 > 0.05$, $n=5$), suggesting the high stability of Cur@ GL system.

Drug release profile is another parameter to examine the carrier. The curcumin release pattern from GL nano-lipid was conducted under two pH values (5.5 and 7.4) which mimics the cellular enviroment of normal and tumor tissue, respectively [39]. As shown in figure 6C, the curcumin was faster release when the system was in acidic acid (pH 5.5). In the first 2h, curcumin leaking from the carrier was quite similar at both pH. After that, cur-cumin was stablely released in physiological buffer (pH 7.4) upto the first 10h (insertion in Fig. 6C). In case with acidic environment, GL nano-lipid provided a linear release profiles for curcumin. Within 50h, there was ~70% of curcumin was released in acidic pH, which was double amount curcumin release in a pH 7.4. This pH-dependent behavior of GL nano-lipid was totally agreed with previous report [14]. In the acidic environment, the degradation of soy lecithin was accelerated [13]. The lipid layer could be markedly weak-

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ens and subsequently, the higher amount of cur was escaped from carrier. This pH-dependent drug-release profile of GL nano-lipid could reduce the toxic of drug to normal tissue, and could help to increase the accumulation of drug at tumor site.

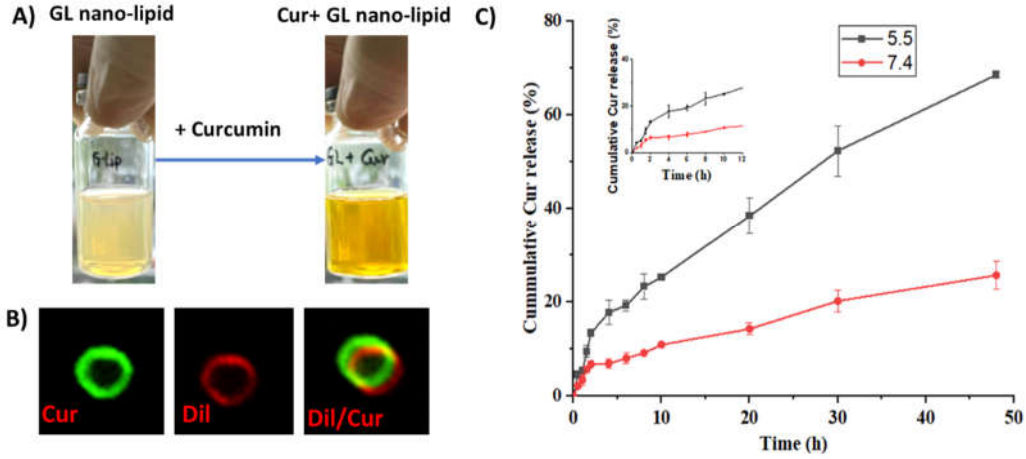

Figure 6. A) Visual observations and confocal microscopy image (B) of of Cur/GL nano-lipid. C) In vitro release profiles of Curcumin from GL nano-lipid at pH 7.4 (red line) and at pH 5.5 (black line) in 37°C. Data was presented as mean± SD (n=3).

Next, the release kinetics of curcumin from GL nano-lipid was examined. 5 different models which are known as the suitable model for liposome/nano-lipid [40]were used fit the experimental data using KinetDS [41]. Based on R^2, AIC, BIC and RSME values, a better fit of experimental data into the model.

Table 2. The Estimated parameters, R^2, AIC, BIC and RSME values obtained from fitting experimental release data at pH 5.5 and pH 7.4.

	Parameter	Zero order	First order	Higuchi	Korsmeyer-Peppas	Weibull
pH= 5.5	R^2	0.9627	0.0894	-0.0054	0.9931	0.9931
	AIC	70.3998	153.55	110.486	72.9361	79.5854
	BIC	71.3696	154.52	111.456	73.9059	80.5552
	RSME	4.5915	146.759	24.3971	5.1033	6.7324
pH = 7.4	R^2	0.9808	0.0899	0.0189	0.9888	0.99
	AIC	43.3975	132.69	90.6057	50.3217	53.1504
	BIC	44.3673	133.66	91.5755	51.2915	54.1202
	RSME	1.4905	61.5375	10.6559	1.989	2.2378

As shown in table 1 and figure S11, both first order as well as Higuchi model did not suitable with the released data. The release data at two different pH conditions were fitted adequately with zero order, Korsmeyer-Peppas, and Weibull models with R^2 values higher than 0.95. All AIC, BIC and RSME values suggested that the release of Cur@ GL fitted to zero-order kinetics at both conditions. Zero order drug delivery system can be useful to enhance the therapeutic values and prevent the side effect of the drug [40, 42]. Due to releasing drug at a constant rate, the concentration of drug in the bloodstream could be maintained for long time; consequently, reduce the dosing frequency [42]. The pattern release of curcumin from GL nano-lipid suggested that GL nano-lipid could be designed for sustainable delivery of hydrophobic agents (chemotherapy drug, bioactive compound). Altogether, the obtained data exhibited that GL nano-lipid can successfully encapsulate the hydrophobic drug and perform a pH-dependent release profile, indicative of potentially carrier for drug delivery.

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3. Materials and Methods

3.1. Materials

Ginger plant (Zingiber officinale) was collected at Duc Trong District. Lam Dong Province in April 2019. The plants were identified by Dr. Nguyen Ngoc Tuan (Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City) and the reference specimens were stored at Institute of Applied Materials Science (IAMS). Soy Lecithin (CAS number 8002-43-5) was ordered from Tokyo Chemical Industry (TIC, Tokyo, Japan). Tween 80 (CAS number 900 5-65-6), Cholesterol (Code 110190025) were come from Acros (USA). Curcumin (CAS number 458-37-7) was acquired from Merck (Singapore). Cell culture reagents were originated from Gibco (USA) and Sigma Aldrich (Singapore). All the chemical solvents in analytical study and nano-lipid fabrication were analytical grade and purchased from Fisher Scientific (USA). All the solvents in extracted process were obtained from Chemsol (Viet Nam).

3.2. Ginger oil preparation

3.2.1. Ginger oil extraction

After collection and identification, the ginger root was cleaned with tap water and then dried at room temperature for 1 week. The ginger root was applied into planetary ball mill (PM 100, RETSCH) for 30 min with 200 rpm at 25 ^{o}C. The resultant powder (50g) was soaked in ethanol (80%, Chemsol) in soxhlet extractor under 50oC for 24h. Vacuum filtration with Whatman filter paper (Grade No. 41) was used to collect the supernatant. The first crude extract was obtained through the Rotary Evaporators (N-1200A V-WD, EYELA). The co-solvent, petroleum ether – ethyl acetate (1:1), was applied into the ethanol crude (ratio 1g crude extract: 20ml co-solvent). The supernatant was then collected through centrifugation at 10000 rpm, 25 ^{o}C (HERMLE). The precipitation was repeated with co-solvent for 3 times. The supernatant in each extracted repetition was merged before removing solvent by Rotary Evaporators resulting the yellow oil sample. The oil sample took into oven vacuum (VOS-301SD, EYELA) at 40 ^{o}C for 1 hours to further removing solvent.

3.2.2. Characterization of ginger oil

Gas chromatography-Mass spectrometry (GC-MS): The composition of the resultant ginger oil was determined by GC-MS (GC Agilent 6890N, MS 5973 inert) equipping with HP5-MS (0.25mm, 0.25mm, 30m). The sample (25 µl) was mixed with 5 ml n-hexane following 0.45 micron filter before injecting to system. The injection volume was 1.0 µL. The inlet pressure by Helium gas was 9.3 psi. The temperature program was as follows: initial temperature was 50 ^{o}C for 2 minutes, then increased to 80 ^{o}C at 2 ^{o}C/min, increased to 150 ^{o}C at 5 ^{o}C/min, increased to 200 ^{o}C at 10 ^{o}C /min and finally increased to 300^{o}C at 20 ^{o}C/min.

High Performance Liquid Chromatography (HPLC): HPLC (Flexar/ParkinElmer equipped with PDA) using VDSpher PUR 100 C18 column (250 × 4.6 mm, 5 µm) was conducted to qualify the amount of 6-shogaol in the ob-tained ginger oil. [6]-shogaol (Sigma, code 39303, lot BCBZ1777) was used for constructing the standard curve. Standard agent or sample were dissolved in methanol and filtered with 0.45 micron filter. The HPLC program for 10 minutes at detecting wavelength of 230 nm was: injection volume 20 µl, the column flow rate, 1.0 mL/min. The mobile phase was followed gradient elution with acetonitrile (A, MeCN, HPLC grade, Fisher Scientific) and water containing 0.1% H_3PO_4 (B, HPLC grade, Acros Organics) as follows: 0-3.5 mins 82 %A, 3.5-4.5 mins 65 % A, 4.5 – 6 min 60 %A, 6-10 min 80 %A.

3.3. Nano-lipid preparation

3.3.1. GL nano-lipid preparation

GL nano-lipid was prepared following the conventional thin film technique. Briefly, soy lecithin (90 mg), ginger oil (5.8 mg), cholesterol (31.8 mg), tween 80 (31.8mg) were

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dissolved separately in 10 ml chloroform (synthesis grade, Fisher Scientific). All the prepared solution was transferred into a 500 ml round bottomed (RB) flask and chloroform was added upto 50ml. The flask was placed into sonication bath for 1-2 min to make homogenous solution. A thin lipid film was formed in the RB flask through the Rotary Evaporators (N-1200A V-WD, EYELA). The hydration process was done with 10mM HEPES buffer (code 15630080, Gibco). After 5h for stirring under room temperature with high speed (1500 rpm), five freeze-thaw cycles (-80oC – 40oC) was applied. The obtained solution was cen-trifugation at 12000rpm. The supernatant was collected and freeze-dried for further study.

3.3.2. Characterization of nano-lipid

TEM: The GL nano-lipid solution (0.5 mg/ml) was mixed with 2% uranyl acetate. This mixture was dropped on the copper grid (Ted Pella, Inc., USA) and then dried at room temperature. TEM (JEM-1400, Japan) was operating at 300 KV for monitoring the morphology of sample.

Confocal microscopy: GL nano-lipid (0.5 mg/ml) was mixed with Dil C18 (5) (Invitrogen™) at the ratio 200:1 w/w). This solution was dropped on the slide and observed under confocal microscope (Andor, England) at 640 nm emission.

Size, zeta potential: Size and Zeta potential were monitored under Zetasizer Nano SZ (SZ-100, Horiba). For size measurement, 1mg/ml GL nano-lipid was prepared. The measurement was at 25 °C with general mode analysis. For zeta potential, 0.5mg/ml GL nano-lipid was prepared and then loaded into zeta potential cell with palladium electrodes. All measurements were performed at least three independent trials.

Stability testing: GL nano-lipid (1 mg/ml) was stored in 25 ml injection vial (Viet Nam) at room temperature. At the determined time, some parameters such as hydrodynamic size, zeta potential as well as pH value of the suspension were analyzed. Each time point was inclusive 9 repetitions. The stability of GL nano-lipid in the oxidant condition was tested using pyrogallol assay. 4.2 ml GL nano-lipid (100ppm), ginger oil (100ppm) and HEPES buffer were incubated with 0.32ml H_2O_2 (0.02%) separately. After 20mins incubation at room temperature, 0.64 ml pyrogallol (50mg/ml) was added into each vial. Then, 0.2ml HRP enzyme (10 mg/ml in PBS buffer) was added. After 30 min incubation and mixing, the color of solution was recorded.

3.4. Fabrication of curcumin loading GL nano-lipid

3.4.1. Preparation of Cur@GL nano-lipid

Curcumin- GL nano-lipid (Cur@GL nano-lipid) was prepared following the thin film method. Curcumin (2%, 3.1 mg) was dissolved into chloroform and then mixed with solution containing soy lecithin, ginger oil, cholesterol, tween 80. The procedure was similar as GL nano-lipid fabrication. The unloaded curcumin was removed by centrifugation method (15000 rpm in 30 min at 25 °C, Hermele Z32HK). The supernatant was collected and then free-dryed. The yellow powder was kept at 2-8 °C for further study.

3.4.2. Characterization of Cur@GL nano-lipid

After lyophilizing, Cur@GL nano-lipid was re-solved into DI water to perfome some measurements such as size, zeta potential. The morphology of Cur@GL nano-lipid was proven under confocal microscopic with the help of Dil C18 (5).

The amount of curcumin that encapsulated into GL nano-lipid was determined by HPLC as described in the previous study [38]. Trixton X (1 mM) was added into Cur@GL nano-lipid. The centrifugation was applied to collect curcumin. Curcumin was then re-dissolved into absolute ethanol (HPLC grade, Fisher). The entrapment efficiency (EE) and drug loading capacity (DL) were calculated using the following equation:

$$\text{EE}\ (\%) = \frac{\text{Amount of curcumin determined by HPLC}}{\text{Initial amount of curcumin}} \times 100\% \quad (1)$$

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$$\text{DL}\ (\%) = \frac{\text{Amount of curcumin determined by HPLC}}{\text{Total mass of the composition in GL nano} - \text{lipid}} \times 100\% \quad (2)$$

The release behavior of curcumin from GL nano-lipid was followed the previous study [21]. In brief, Cur@GL nano-lipid (0.5ml) was loaded into di-alysis tube 3500kD (Spectra/Por) and then soaking into physiological buffer (pH 5.5 and pH 7.4), PBS (1X, Gibco). At the determined time, 0.5ml media was withdrawn and the equal volume of fresh media was paid back. The amount of curcumin releasing from the system was estimated by HPLC method. The release kinetics model was performed using KinetDS 2.0 [41]. The release equation with best goodness-of-fit was evaluated based on the values of reduced chi-square statistic and coefficient of determination shown by KinetDS result.

3.5. In vitro cytotoxic test

The toxicity of GL nano-lipid was tested with hMSC - Human Mesenchymal Stem Cells (passage number: 04, PT-2501, Lonza – Switzerland). hMSC was seeded on 96 well plate with density of 2x104 cells/well and cultured with completed Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham media (Sigma Aldrich, D8900) supplying 1% Penicillin-Streptomycin (Sigma Aldrich, P4333), 7.5% sodium bicarbonate (Sigma Aldrich, S5761) and 10% Fetal Bovine Serum (Sigma Aldrich, F7524). After 24h incubation, the culture media was withdrawn, and re-placed the new culture media containing various concentration of GL nano-lipid (0- 10mg/ml). All the cell was cultured under normal conditions: 5% CO_2 and 90% humidity. Sulforhodamine B (SRB) assay was applied to identify the toxicity of material following the instruction of Abcam (ab235935). Further, live/dead staining via dual Acridine Orange (AO)/ propidium iodide (PI) staining was conducted to give more the evidence for toxic profile.

3.6. Anti-bacteria testing

Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC8739) were selected as models for Kirby-Bauer disk diffusion susceptibility test. 100µl inoculum (107 CFU/ml) was spread on the surface agar plate using a steri-lized spreader until to obtain the dry surface. Several 6-mm filter paper disk (WHA2017006, sterilized) was placed on the agar surface. 20µl of each sample (ginger oil, GL nano-lipid, convention lecithin nano-lipid, ampicillin, streptomycin, and HEPES buffer) dropped on the paper disk. The plates were cultured at 37 ^{o}C in the incubator. The diameter zone of transparent around the paper disk was recorded after 24h incubation. If the diameter zone was greater than 6.0 mm, it was concluded with anti-bacteria property. The experiment was repeated 3 times.

3.7. Statistic test.

The data were presented as mean ± standard deviation. The graph for GC-MS, cell cytotoxic, stability data and release profiles were conducted with OriginPro (2021, v9.8). For the analysis, multiple comparisons (ANOVA test) was performed together with Tukey's multiple comparison test or least significant difference (LSD). All the statistic test was at a confidence level of 95%

5. Conclusions

Despite the excellent carrier based lecithin nano-lipid, the application of these vesicles are still hindered in drug delivery system due to the oxidation of lecithin during the storage. This study provided the alternative procedure for fabrication of nano-lipid based lecithin. The 6-shogaol rich oil extracting from Zingiber officinale following 2 solvent phase extractions, confirming by HPLC and GC-MS, was used as the composition in the fabricated process. The addition of ginger oil in the lipid phase with lecithin allowed the formation of functional lipid nanoparticles. Applying the film hydration technique without extrusion, the size of nanoparticles was around 250nm and homogenous distribution, confirming by DLS and confocal imaging. Through zeta potential value, it suggested that

ginger oil was blended to lipid layer phase. Also, the addition of ginger oil did not induce any significant effects on particle size distribution and zeta potential over 60 day's storage at room temperature. Notably, the fast decreasing the pH value of dispersion was noted over time for lecithin nano-lipid. Soy lecithin is mixture of phospholipid, mainly polyunsaturated fatty acids, which are easy to be degraded, causing the release of fatty acid into aqueous resulting in the generation of acidic pH. In our case, the pH value of GL nano-lipid dispersion was around 7.0-7.2 in the same storage condition with lecithin nano-lipid. We would assume that the introduction of ginger oil with the rich of anti-oxidant compounds could minimize the oxidation of soy lecithin. In addition, the modification of the conventional lecithin nano-lipid with ginger oil showed antibacterial activity against both gram (-) and gram (+) bacteria. Also, non-significant change in viability and morphology of Mesenchymal stem cells (MSCs) culturing with GL nano-lipid as compared to negative control revealed its excellent biocompatibility. Specially, the modified lecithin nano-lipid particles were able provide a sustained-release formulations to curcumin – hydrophobic bioactive agent. This study will endow the new strategy on drug delivery system based soy lecithin.

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