Enhancement Vehicles of Cardene Loading Poly(D,L-lactic-co-glycolic acid) Nanoparticles *in Vitro* Controlled Release for Biomedical Application

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***Abstract*—The goal of this research was to prepare a nanovehicles from Poly(D,L-Lactic-co-glycolic Acid) Nanoparticles (PLGA NPs) for delivering cardene. The cardene loading-PLGA NPs were fabricated by double emulsion and solvent evaporation process. PLGA has a monstrous interest to fabricate nanoparticles due to its good biodegradability and excellent biocompatibility. Drug loading and release profiles were performed as the model drug. The results appeared that cardene amount from 5.0 mg to 30.0 mg lead to the increase of the particle size, Polydispersity Index (PDI), and nanoparticle yield. The drug encapsulation efficiency was approximately 77% to 92%. The *in vitro* release from PLGA NPs was prolonged cardene concentration at periods more than 16 to 41 days. The cumulative drug release was a maximum of 40% to 70%. Moreover, the nanoparticles could be demonstrated biocompatible with the keratinocyte cells by MTT assay. The cardene loading-PLGA NPs were able to protect drug degradation and improve pharmacokinetics during transporting to the targeted organ. The cardene loading-PLGA NPs could be applied to prolonged drug delivery in biomedical applications.**

***Index Terms*—poly(D,L-lactic-co-glycolic acid), cardene, nanoparticles, controlled release**

I. Introduction

Nanotechnology in vehicles for biomaterial application has been nanoparticles for drug delivery to the protection of drug degradation and improved pharmacokinetics during transportation to targeted organs [1], as depicted in Fig. 1. Therefore, the researcher decided to study about the enhancement vehicles of cardene loading-Poly(D,L-Lactic -co-Glycolic Acid) Nanoparticles (PLGA NPs) *in vitro* controlled release for biomedical application.

PLGA NPs based controlled drug delivery are appropriated administration of drugs into the body in many ways. It is protected drugs to safely deactivation and maintained therapeutic drug concentrations. Including, PLGA NPs are improved pharmacokinetics during transport to the target organ for reducing the side effects of the drug [1], [2]. Cardene was a model drug in this work to study drug load and *in vitro* controlled release. Cardene is an antihypertensive drug class. it has been used clinically to treat high blood pressure and relaxes blood vessels leads to reduce pumping of the heart [3]. Fig. 1 shows examples of a conventional cardene for terminal hypertension, conventional dosage forms are degradable of the drug in 8 hrs. The half-live of cardene is short-acting which is insufficient for therapeutic levels. The ester linkage in the structure of cardene was degraded by hydrolysis reaction. [4], [5]. The drug concentrations are below than therapeutic concentrations, the patient has high blood pressure. Drug dose maintenance to therapeutic action is achieved by repeated administrations for relieving hypertension. Repeated administrations may result in a drug overdose, which the patient gets dangerous side effects more pronounced like slower heart rate and dizziness [6].

With the above motivation, the hypothesis of this research was the case that cardene loading-PLGA NPs could be the protection of cardene deactivation, control drug level in the body, and reduce side effects. The development of cardene pharmacokinetics is a classic problem in the pharmaceutical. A popular use of biopolymers that are enhanced to expand drug lives is established by pharmaceutical carriers [7]. There has shown significant potential as protection of drugs from deactivation and preservation of its activity during transport to the target organ. Among all the biomaterial indicated enormous potential as drug delivery [8]-[12] and as tissue engineering [13]-[15]. This study used PLGA nanoparticles as a drug delivery system because PLGA is polyester that a block copolymer of Poly(D,L-Lactic Acid) (PDLLA) and Polyglycolic Acid (PGA) [8]. This biopolymer was degraded by esterase enzyme and hydrolysis in the body with non-toxic by-products [16].

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The purpose was to prepare vehicles of PLGA NPs for prolonging the cardene delivery system.

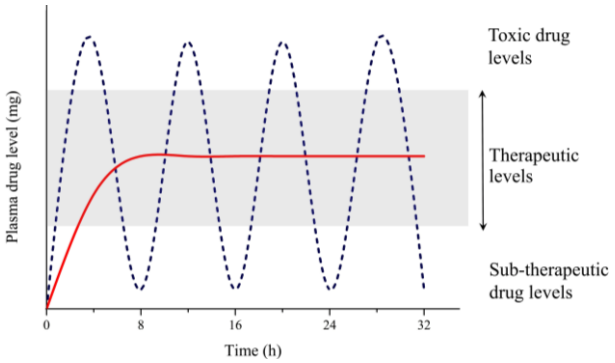

Figure 1. Drug concentrations at the site of therapeutic action as a conventional vasodilator drug (dash line) and PLGA NPs as a controlled released drug (solid line) [1].

II. Method

A. Preparation of the Cardene Loading-PLGA NPs

1) Materials

Poly(D,L-Lactic-co-Glycolic Acid) (PLGA; Mw 24,000-38,000 Da and 50: 50 ratio of PDLLA: PGA) and poly(vinyl alcohol) (PVA; Mw. ~31,000 Da) were purchased from Sigma-Aldrich (USA). Keratinocyte cells line, Dulbecco's Modified Eagle's Medium (DMEM), and MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich (USA). Cardene was obtained from Ramathibodi Hospital, Bangkok, Thailand. Dichloromethane and acetone were of analytical grade.

2) Method

The cardene loading-PLGA NPs were fabricated by double emulsion process. This research was studied various cardene amounts from 5.0 mg to 30.0 mg. 4.0 %w/v of PLGA concentration was dissolved in mixed solution acetone with dichloromethane 2.0 mL. The 1.0 mL aqueous solution of cardene and 3.0 %w/v of single emulsion stabilizer was subsequently mixed to this polymer solution. Next, this solution was again ultrasonicated (Model VCX 500, USA), under ice-cooling, forming a single emulsion. The single emulsion was added and ultrasonicated in 0.5 %w/v of PVA 30 mL as a secondary stabilizer for a double emulsion. The solvent evaporation of the double emulsion was stirred with a magnetic stirrer at 30 °C and 3 hrs. Finally, the cardene loading-PLGA NPs were collected by centrifugation (UNIVERSAL 320, Germany) and washed three times with DI water. The obtained cardene loading-PLGA NPs were drying by freeze-drying (Pirani 501, USA) and were stored at 4 °C for analysis nanoparticle characterization, encapsulation efficiency (%EE), and *in vitro* drug release. All of this results in this research were analyzed by a statistical package in terms of means and S.D.

B. Size and Surface Morphology Analysis

The cardene loading-PLGA NPs were analyzed particle size and polydispersity index (PDI) by dynamic light scattering (DLS; Malvern Zetasizer Nano ZSP, UK).

The cardene loading-PLGA NPs were to analyze the surface morphology of the produced nanoparticles with field emission scanning electron microscope (FE-SEM; JEOL JSM-7610F, Japan).

C. Nanoparticle Yield

The cardene loading-PLGA NPs were collected and weighed accurately. The percentage of yield (%Yield) can be described as (1) [17]:

$$\%\text{Yield} = \frac{\text{Mass of nanoparticles}}{\text{Total mass of PLGA and Drug}} \times 100 \quad (1)$$

D. Nanoparticle Settling Velocity

The cardene loading-PLGA NPs were studied settling velocity (υ) of nanoparticle suspended in a liquid by Stokes' law as (2) compare that of Brownian motion was calculated as (3) [18].

$$\upsilon = \frac{d^2 g(\rho_s - \rho_l)}{18\mu_l} \quad (2)$$

$$\chi = \sqrt{\frac{2k_B Tt}{\pi \mu_l d}} \quad (3)$$

where υ is the nanoparticle settling velocity by Stokes' law (nm/sec), χ is the average Brownian displacement (nm/sec), g is the gravitation acceleration (9.8 m/sec), ρ_s is the nanoparticle density (1.3 g/cm³), ρ_s and μ_l is the Di water density (1.0 g/cm³) and viscosity (8.9×10^{-4} Pa/sec) at room temperature, respectively, k_B is the Boltzmann constant (1.38×10^{-23} J/K), T is the room temperature (K), t is the time (1 sec), and d is the diameter of nanoparticles.

E. Nanoparticle Bulk Settling

The study of nanoparticles settling times in liquids. 50.0 mg of produced nanoparticles were dispersed in 10.0 mL of DI water. The colloidal stability of cardene loading-PLGA NPs dispersion was investigated variation in the measurements of average diameter and time with laser scattering techniques (DLS; Malvern Zetasizer Nano ZSP, UK).

F. Encapsulation Efficiency

The cardene loading-PLGA NPs were investigated the %EE by UV-Visible spectrophotometer (SPECORD 210 PLUS, Germany) at a wavelength of 359 nm, can be represented by the (4) [19]:

$$\%\text{EE} = 100 - \left(\frac{B}{A} \times 100\right) - C \quad (4)$$

where *A* is the total amount of cardene, *B* is the unencapsulated cardene in PLGA NPs, and *C* was the cardene adsorbed in aqueous solution.

G. In Vitro Drug Release

The cardene loading-PLGA NPs were determined 100 mg of nanoparticle suspended in a pH 7.4 of Phosphate-Buffered Solution (PBS) to mimic the body fluids in normal tissues. This solution was incubated at 37 °C and 70 rpm in the shaking water bath (GFL 1086, Germany). The supernatant release medium was pipetted 3.0 mL and added 3.0 mL new PBS back to the solution at a preset time following incubation. This pipette solution was investigated *in vitro* drug release by UV-Visible spectrophotometer at a wavelength of 359 nm, as calculated by (5) [20]:

$$\text{Cumulative release}(\%) = \left[\frac{M_i}{M_\infty}\right] \text{x } 100 \qquad (5)$$

where M_i is the cardene amount released from the PLGA NPs at time t and M_∞ is the total amount of cardene loading-PLGA NPs.

H. In Vitro Cytotoxicity Assay

Cell cytotoxicity was investigated by MTT assay. This protocol is a colorimetric reaction that leads to a transform of color based on metabolically active cells. The yellow tetrazolium salt is converted to purple formazan by the potentiality of viable cells. Keratinocyte cells were determined to study cytotoxicity of the cardene loading-PLGA NPs.

The 125 µg/mL of nanoparticles were sterilized by 6 watts UV radiation (Analytik Jena, Germany) on 254 nm wavelength band centered for 120 min and incubated in free bioactive compounds. During incubation, these cells were seeded at 1.0 x10^4 cell/well in 96-well culture plates containing DMEM with bioactive compounds. This incubation was added 0.5 mg/mL of MTT reagent at 37 ºC in 5% CO_2 incubator. The colorimetric reaction of cell viability was investigated using UV-visible spectrophotometer at a wavelength of 570 nm [7].

III. Results and Discussion

A. Preparation of the Cardene Loading-PLGA NPs

The research results appeared that the nanovehicles of cardene loading-PLGA NPs using *in vitro* controlled release was prepared by a double emulsion and solvent evaporation process. The cardene amounts may be a key factor influencing the nanoparticle characterization like particle size, surface morphology, nanoparticle yield, and nanoparticle settling velocity, %EE, and *in vitro* drug release.

B. Size and Surface Morphology Analysis

In the first study, the effect of cardene amounts that influenced the size of nanoparticles was investigated. The results indicated that 5.0 mg, 10.0 mg, 20.0 mg, and 30.0 mg of cardene amount in this work were prepared to obtain less average particle size 200 nm by DLS technique, as illustrated in Fig. 2. The amount of 5.0 mg to 20.0 mg cardene had appeared a narrower size distribution with a particle size of ~170 nm to ~176 nm. In the case of 30.0 mg of the cardene amounts was prepared an average particle size of ~184 nm and abroad size distribution with nanoparticle sizes ranging from ~30 nm to ~650 nm due to the charge are driven against the rising electrostatic potential between polymeric and drug [4], [5], [8]. In addition, the high amounts of cardene in the solution was increased the hydrophilic substance amounts in aqueous solution. It is a weak interaction of polymer layer and drug layer; which polymer chains can easily move in a polymeric matrix led to the increased size of produced nanoparticles. As a result, the increase of cardene amounts from 5.0 mg to 30.0 mg was increased size and a broad range of size distributions.

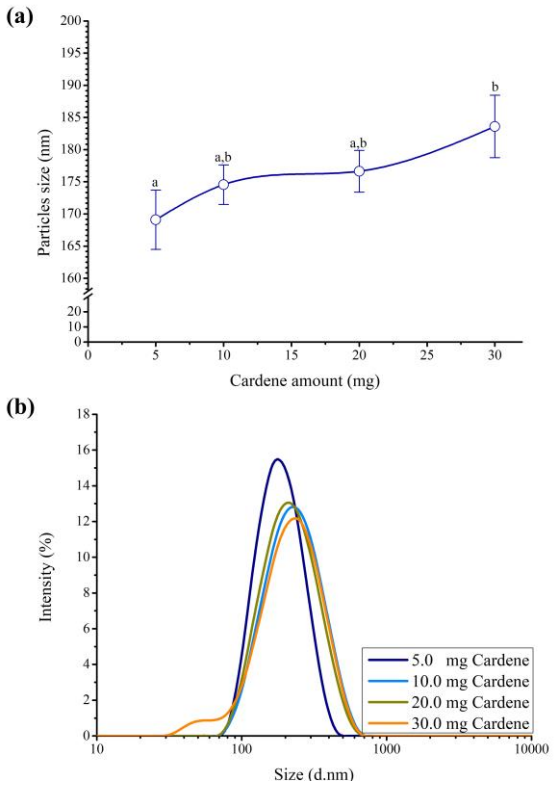

Figure 2. Particle sizes (a) and size distributions (b) of the cardene loading-PLGA NPs (n = 3); ($^{a, b}$ are significantly different at P≤ 0.05; compared with the nanoparticles in each group).

Fig. 3. shows SEM micrographs of different the cardene amounts. The cardene loading-PLGA NPs were still the same size and size distributions according to DLS technique. The SEM images were observed a few produced nanoparticles with diameters higher than 500 nm. The obtained result of PDI values, the produced nanoparticles in this research had size distributions of 100 to 600 nm in diameter, as shown in Fig. 2 (b). The surface morphology of nanoparticles was spherical with smooth surfaces. However, the SEM micrographs observed capillary bridging between nanoparticles. The capillary bridging was probably resulting from an incomplete PVA washing process before collecting nanoparticles. Nevertheless, all cases of nanoparticles in this research were produced it have homologous properties in each group since PDI values were less than 0.3 indicates monodispersity [21]. It is presented systematically in Table I.

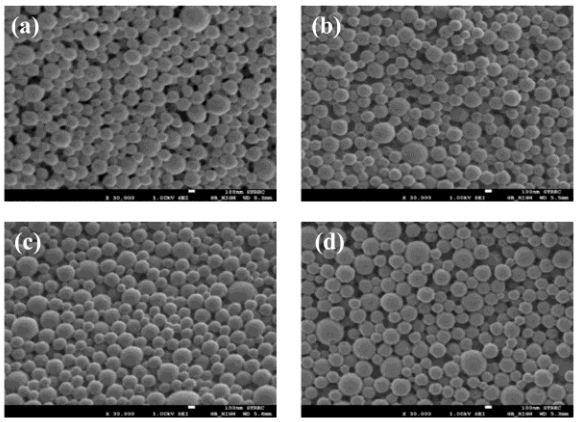

Figure 3. The influence of the cardene amounts of 5.0 mg (a), 10.0 mg (b), 20.0 mg (c), and 30.0 mg (d) on the morphology of the cardene loading-PLGA NPs from SEM micrographs (magnification 30,000x).

C. Nanoparticle Yield

The yield is the percentage successfully produced nanoparticles in this work. Table I describes the yield of nanoparticles produced by the various amount of cardene was ~ 64% by calculated as follows (1) [17]. It can be extrapolated that, as the amounts of cardene increases, was the effect on a low influence of nanoparticle yield.

TABLE I. PDI VALUES AND PERCENTAGE OF YIELD OF THE CARDENE LOADING-PLGA NPS NCH (N = 3)

Cardene-loading (mg)	PDI values	Yield (%)
5.0	0.152±0.032	64.98±1.75[b]
10.0	0.185±0.027	63.48±1.55[b]
20.0	0.232±0.042[a]	65.11±0.92[b]
30.0	0.257±0.053[a]	66.33±1.04[b]

Remark: [a, b] are significantly different at P≤ 0.05; compared with the nanoparticles in each group.

D. Nanoparticle Settling Velocity

The cardene loading-PLGA NPs have studied the action or process of precipitating via Stokes' law and Brownian motion equation. Stokes' law, the nanoparticles were transferred down to bottom, which leads to precipitating under gravitational force. Conversely, the common of nanoparticles move randomly in a fluid according to Brownian motion. The random movement of nanoparticles can be suspended when immersed in a liquid environmental. The calculation on Stokes' law is less than the calculation on the Brownian motion equation, lead to nanoparticles produced opposite of precipitation, which was calculated as follows (2) and (3), respectively [18].

The effect of cardene amounts has a direct influence on precipitating because it affects the size of cardene loading-PLGA NPs, as appeared in Fig. 2 (a). The diameter of nanoparticles has an enormous influence on Stokes' law, as observed (2) [18]. The results appeared that the increase of cardene amounts led to the increase of the nanoparticle diameter, resulting in an increased of precipitating via Stokes' law. However, all study of Brownian displacement has more calculated value than settling velocity of Stokes' law. As a result, the nanoparticles produced can spread immersed in a liquid environmental, as illustrated in Fig. 4. The small size of particles in this work can easy suspended in a liquid. The large size of particles has a strong force of gravity than nanoparticles. The large microparticles larger can easily precipitate under gravitational force, therefore it needs to a label as "*shake well before use*" [18].

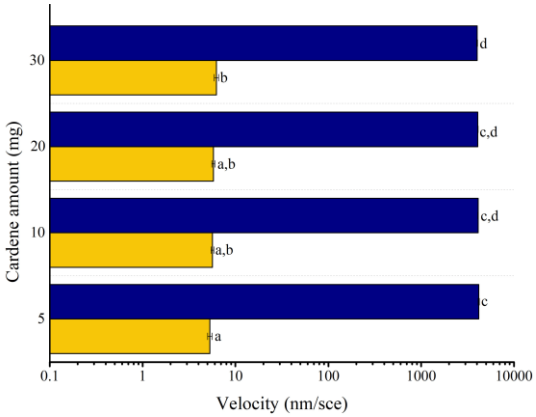

Figure 4. Settling velocity (■) and Brownian displacement (■) of the cardene loading-PLGA NPs (n = 3); ([a-d] are significantly different at P≤ 0.05; compared with the nanoparticles in each group).

E. Nanoparticle Bulk Settling

The result of settling velocity indicated that produced nanoparticles were not settling in DI water. In this study, for confirmation of nanoparticles settling times for 24 hrs. by laser scattering techniques and the results are appeared in Fig. 5. The cardene loading-PLGA NPs remained rather stable at room temperature, as demonstrated by the investigating of average diameter were softly increased with approximately 32 nm (<21 %) after 24 hrs.

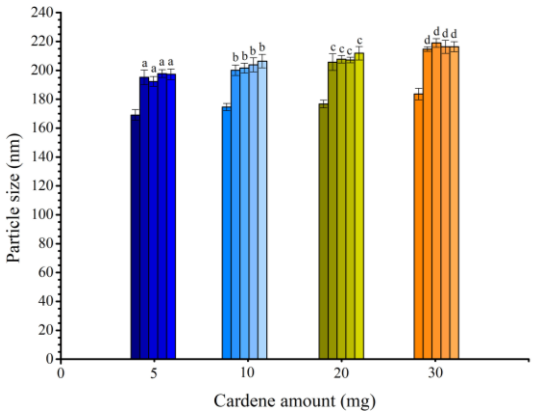

Figure 5. Particle size as a function of time (1, 5, 10, 16, and 24 hrs.) the cardene loading-PLGA NPs (n = 3); ([a-d] are significantly different at P≤ 0.05; compared with the nanoparticles in each group).

The particle sizes were observed in Fig. 5 presented the results corresponding to the settling velocity test performed on dispersions DI water. The investigate of nanoparticles stable observed no significant increased average diameter throughout the test period. Only 1 to 5 hrs. of the test period, the produced nanoparticles were increased the particle size due to swelling of the nanoparticles. The stability of nanoparticles, a possible explanation could be from the steric stabilization of polymeric molecules adsorbed on the nanoparticle. The produced nanoparticles were the narrow size distribution (Fig. 2 (b)) and PDI values less than 0.3 (Table I) lead to

the monodispersity of stabilizer molecules. The stabilization was achieved steric monodispersity of cellulose molecules as a single stabilizer attached to the inner surface of nanoparticles and PVA molecules as a secondary stabilizer forming a coating on the outer surface of nanoparticles. The steric of stabilizer established a repulsive force that leads to separates the nanoparticles from another one [22].

F. Encapsulation Efficiency

The %EE is the percentage of cardene amounts that are successfully entrapped into the PLGA NPs, which calculated by (4). In order to determine the %EE of nanoparticles was depending on the cardene amount. The results appeared that the increase of the cardene amount led to the decrease of the %EE, as shown in Fig. 6. However, the 30.0 mg of the cardene amounts had a minimum of the %EE (76.81%); conversely, it had high drug entrapment about ~23.04 mg. High amounts of cardene in the solution were driven off the polymeric matrix via the electrostatic potential on the surface. Moreover, the cardene entrapment in nanoparticles was affected by the aqueous phase, which aqueous volume consistent whose drug solubility decreases when the cardene amounts increase.

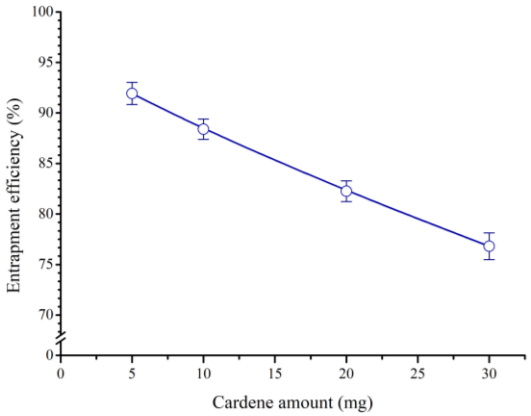

Figure 6. The entrapment efficiency of the cardene loading-PLGA NPs (n = 3).

G. In Vitro Drug Release

In this work enhanced the PLGA NPs as controlled release vehicles of cardene. The produced nanoparticles were incubated at 37°C and 70 rpm of a shaking water bath in pH 7.4 of PBS to mimic the body fluids in normal tissues. Fig. 7 shows the *in vitro* cardene releases profiles illustrated the controlled release of cardene from nanoparticles in period time from 16 to 41 days. The high amount of cardene was increased the release times. The 5.0 mg of the cardene amounts had a maximum of the %EE (91.93%); conversely, it had the least amount of cardene load in the PLGA NPs influence less release time. However, the 5.0 mg of cardene amount was obtained high cumulative release since the less total amount of cardene loading-PLGA NPs can be calculated as (5) [20]. In this work, the cardene loading-PLGA NPs have indicated the *in vitro* cardene releases from nanoparticles as prolong drug delivery vehicles.

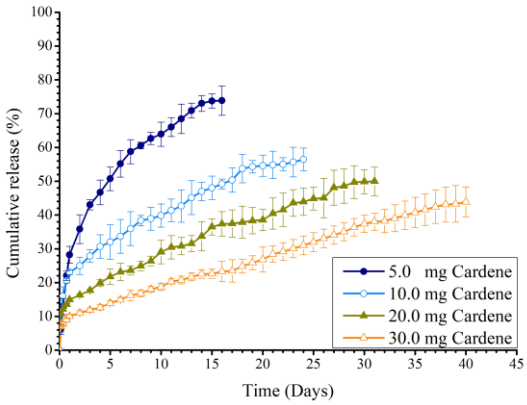

Figure 7. The *in vitro* release profiles of the cardene loading-PLGA NPs incubated in test media with 20.0 mL PBS at pH 7.4 and 37 °C to mimic the body fluids in normal tissues (n = 3).

H. In Vitro Cytotoxicity Assay

From MTT assay, to study the cytotoxicity of 125 mg/mL cardene loading-PLGA NPs were incubated with keratinocyte cells (normal cells) for 24 h and 37 ºC. The addition of cardene amount was expanded absorbance purple formazan of MTT reagent. The increased of purple absorbance was indicated higher relative cell viability after 24 hrs. The result was produced nanoparticles in this work do not demonstrate the toxicity of the keratinocyte cells. The cardene loading-PLGA NPs could be demonstrated biocompatible with the keratinocyte cells, as illustrated in Fig. 8.

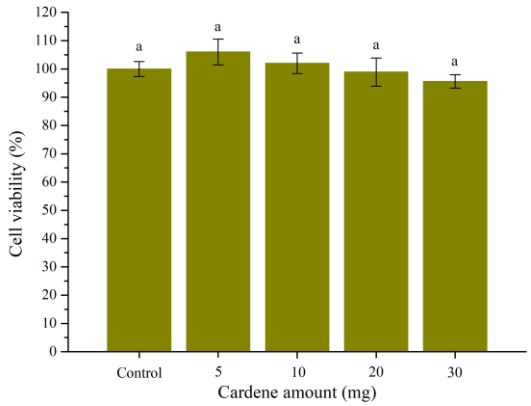

Figure 8. The cell viability of the cardene loading-PLGA NPs incubating keratinocyte cells individually for 24 hrs. at 37 ºC by using MTT assay (n = 3); (a is significantly different at P≤ 0.05; compared with the nanoparticles in each group).

IV. Conclusion

The study of cardene amount was able to prepare nanovehicles of the cardene loading-PLGA NPs for controlled release systems. The morphological of nanoparticles presented a spherical shape and smooth surfaces which had the particle size from 170 nm to 184 nm. The nanoparticles showed the times of stabilizers after 24 hrs. The percentage of cardene encapsulated within nanoparticles was approximately 77% to 92%. The PLGA nanoparticles prolonged the release of cardene for up to 16 days in vitro. The 5.0 mg to 30.0 mg of cardene amounts

was non-cytotoxicity for the keratinocyte cells with produced nanoparticles. The results were indicated that the enhancement of cardene loading-PLGA NPs could be controlled release for drug delivery nanovehicles in biomedical applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

N. Soomherun designed and performed the experiments with support from all authors, conducted the research, analyzed the data, and co-wrote the paper; N. Kreua-ongarjnukool, S. T. Niyomthai and S. Chumnanvej were involved in planning and supervised the research, processed the experimental data, co-wrote the paper, reviewing, editing, and contributed to the interpretation of the results; all authors had approved the final version and provided critical feedback on the paper.

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