Vietnam Journal of Science and Technology ***61*** *(6) (2023) 953-963*

doi:10.15625/2525-2518/17524

Fabrication of α-mangostin – loaded liposomes for cytotoxic-activity against A549 lung-cancer spheroids

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Received: 20 September 2022; Accepted for publication: 2 January 2023

Abstract. α-mangostin is a natural product isolated from the mangosteen pericarps with diverse biological activities, including cytotoxicity against cancer cells. However, the application of α-mangostin in cancer treatment is limited due to the high cytotoxicity and poor solubility in water. In this research, we fabricated α-mangostin-loaded liposomes from soybean lecithin and cholesterol to enhance the solubility in water of α-mangostin and reduce side effects in the treatment of cancer. The liposomal membrane uses Soybean Lecithin (SBL) as a phospholipid and Tocopherol (Toc) as a stabilizer, at 4 ratios of SBL/Toc are 1/1, 2/1, 4/1, 8/1. Experimental results showed that liposomes with SBL/Toc molar ratio of 4/1 had a mean size value of 105.8 ± 3.9 nm, and a polydispersity index (PDI) of 0.149. The encapsulation efficiency was 51.3 %. We believe that this formulation has the potential to be developed into an improved drug delivery system for α-mangostin. The lung carcinoma epithelial cells (A549) were cultured in microwell for 14 days to form spheroids. Then, spheroids were probed with a concentration of α-mangostin-loaded liposomes of 10 µM. The results showed the ability to significantly reduce cell viability after 36 hours of testing.

Keywords: liposome, A549, α-mangostin, DLS, 3D cell culture.

Classification numbers: 1.3.3, 1.2.4, 2.4.3

1. INTRODUCTION

Lung cancer is a kind of cancer that develops in lung tissues, typically in the cells that lining the airways. Currently, this disease is one of the main reasons led to death from cancer of both men and women, with an estimated 1.8 million deaths (18 %) [1, 2]. According to worldwide report, it estimated 19.3 million new cancer cases and almost 10.0 million lung cancer deaths occurred in 2020 [1]. The global cancer burden is expected to be 28.4 million cases in 2040, a 47 % rise from 2020 [1]. Therefore, it is the need of finding new therapeutic active ingredients for treatment of lung cancer.

Mangosteen, a type of fruit that grows in Asia, is well-known for a unique tropical flavor. For many years, people have used mangosteen fruits as a medicine to heal ailments including

colic, skin infections, and wounds [3]. One of the principal xanthones derived from the rind of the mangosteen fruits is called α-mangostin, which is a yellow compound discovered in 1855. This compound possesses a wide range of biological features, including anti-inflammatory activity [4], high antioxidant capacity [5], and killing cancer cells [6]. α-mangostin has the ability to prevent a variety of cell types from becoming tumors as HT-29 (colon), MCF-7 (breast), AsPC-1 and Capan-2 (pancreas) [7 - 9]. Inhibitory activity of α-mangostin is higher than previous inhibitors such as C75, epigallocatechin gallate (EGCG) and curcumin [10]. However, there are two main limitations on the usage of α-mangostin. First, this compound has poor water solubility (2.03×10^{-4} mg/L) [11], since the intracellular and extracellular environment are mostly fluid, the therapeutic effect of α-mangostin in its natural form will be low quality [12]. Second, the activity of α-mangostin can lead to high cytotoxicity to normal cells [13]. Therefore, the delivered methods and controlled release of α-mangostin to designed target areas are necessary.

Liposomes are proposed as a candidate to surmount these limitations of α-mangostin. They are spherical structures consisting of one or more phospholipid bilayers surrounding an aqueous core. The use of liposomes has many outstanding advantages such as biocompatibility and bioavailability[14]. Furthermore, the membrane composition of liposomes can be altered to control drug transport and release [15, 16]. For example, nano-sized dual-loading liposomes were prepared in Hong *et al.* study, and the dual-loading liposomes increased cytotoxic effects against colon cancer cells [17]. In another study, Chin *et al.* developed a liposome system to enhance the 1.8 - 8.0 fold of skin permeation rate of α-mangostin compared to the control [18].

Despite the many advantages of liposomes, their research and application remain to suffer various obstacles, including their instability. Cholesterol is normally a solution that is being explored to solve this problem. However, the application of cholesterol in liposomes may cause some psychological concern for patients because cholesterol is the cause of some cardiovascular problems such as myocardial infarction, and stroke [19]. For Alzheimer's patients, high cholesterol levels in the blood may hasten the development of beta-amyloid plaques, the clumpy protein deposits that harm to the brain [20]. Therefore, recent studies have been utilized tocopherol (Toc) to enhance liposome stability [21, 22]. Since it is not hazardous to the body at any volume, tocopherols give hopefulness for the potential to improve liposome quality [23].

In this study, we developed liposome dispersion systems from soybean lecithin (SBL) and Toc. The bioactive compound, α-mangostin, was isolated from the rind of the mangosteen. Then, the mean size, polydispersity index (PDI), and encapsulation efficiency of α-mangostin– loaded liposomes were determined. The cytotoxic potential of α-mangostin– loaded liposomes was tested through a live/dead assay in particular time and concentrations.

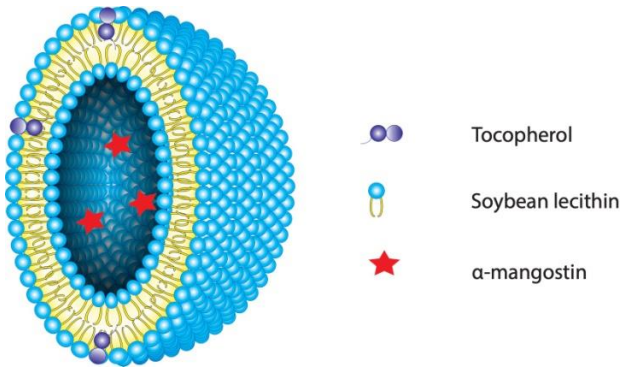

Figure 1. The structure of α-mangostin – loaded liposome.

2. MATERIALS AND METHODS

2.1. Materials

Soybean lecithin (SBL), extracted from soybean seeds was supplied by Aladdin (China). Tocopherol (Toc) was bought from Sigma Aldrich (MA, USA). The 100 nm polycarbonate membrane was purchased from Avanti® Polar Lipids (AL, USA). α-mangostin was extracted and isolated from mangosteen pericarp in previous study [24]. Chloroform was purchased from Chemsol (Vietnam). Methanol was bought from Merck (Germany). Distilled water was available in the laboratory. Dialysis membrane was supplied from Sigma Aldrich (MA, USA). Lung carcinoma epithelial cells (A594) cells was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Trypsin-EDTA 0.25%, trypsin neutralizer solution and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco (NY, USA). Phosphate buffered saline (PBS), fetal bovine serum (FBS), sodium hydroxide (NaOH), trimethylolpropane triacrylate (TMSPMA), polyethylene glycol diacrylate (PEGDA), photo initiator (PI) was bought from Sigma-Aldrich (MO, USA). Live/Dead® Viability/Cytotoxicity kits were ordered from Invitrogen (MA, USA). The Double Beam Spectrophotometer U-2900 machine was bought from Hitachi (Tokyo, Japan). SZ-100 Series Nanoparticle Analyzer machine was supplied from Horiba (Osaka, Japan).

2.2. Methods

2.2.1. Synthesis of α-mangostin – loaded liposomes

Stock solutions of SBL 0.025 M and α-mangostin 0.003 M were prepared. The amount of Toc required was calculated from molar ratio, and prepared from 0.01 M Toc stock solution. The two lipids were combined in a 1.5 mL Eppendorf tube and 60 s vortex in order to mix the solution well. Liposomes were prepared by thin film hydration method. The SBL/Toc (with molar ratios 1:1; 2:1; 4:1; and 8:1) mixture was transferred to a 100 mL flask and put on a rotary evaporator with a water bath temperature of 30 °C, flask pressure 600 mbar, rotational speed 2 to slowly remove $CHCl_3$. A thin film of SBL, Toc, and α-mangostin was formed at the bottom of the flask. Then, 4 mL of distilled water was added and sonicated in 40 °C for 2 minutes to remove the film from the bottom of the flask. The result is a turbid suspension, which contains free α-mangostin and α-mangostin-loaded liposomes of various sizes and shapes. To reduce polydispersity and bring the liposomes to a common size, an extruder with a 100 nm Polycarbonate film was used. Each raw liposome sample was extruded through the membrane 50 times. After extrusion segment, the samples were transferred to new Eppendorf tubes and stored in the shade at room temperature (25 °C). To remove free α-mangostin in the mixture, a crude liposome suspension was dialyzed. Liposomes were transferred from the flask to a 14000 Dalton (20 cm × 10 cm) dialysis membrane sealed at both ends and placed in a 2L beaker. The volume of distilled water used was 1 L. The dialysis lasted 24 hours, and the water was changed every 2 hours.

2.2.2. Characterization of α-mangostin – loaded liposomes

At room temperature (25 °C), the polydispersity index (PDI) and mean size of the liposome samples were measured using a SZ-100 Series Nanoparticle Analyzer machine (Horiba, Japan) with a He/Ne laser (633 nm) and a 90-degree scattering angle. The output was the average of three measurements.

In order to calculate the encapsulation efficiency (EE %), the samples were added to a 100-mL round-bottom flask to undergo rotary evaporation till all water had been eliminated and just a

thin film was left in the flask. The liposomal suspensions were dispersed and the entrapped -mangostin was released by dissolving the lipids α-mangostin film in 3.5 mL of MeOH/$CHCl_3$ (3:1 [v/v]) and then sonicating the mixture in a bath for 2 minutes. The solution was then pipetted out of the flask and mixed with 2900 mL of MeOH/$CHCl_3$ (3:1 [v/v]) in an Eppendorf tube for 30 seconds. Afterwards, the mixture was poured into a glass cuvette. The sample's absorbance was measured at 243 nm in comparison to a MeOH/$CHCl_3$ (3:1 [v/v]) standard by using spectrophotometer U-2900 machine. The formulate might be used to compute the EE %.

$$\text{EE\%} = \frac{the\ amount\ of \propto -mangostin\ in\ the\ liposomes}{the\ total\ amount\ of \propto -mangostin} \times 100\ (\%) \quad (1)$$

α-mangostin – loaded liposomes were preserved and particle size and dispersion were monitored at 1 day, 4 days, 7 days, and 10 days. The ability of drug release in pH = 5.5 and pH = 7.4 was investigated by the dialysis method. α-mangostin – loaded liposomes were placed in a dialysate bag with a molecular weight of 14 000 Da. The system was placed in pH 5.5 and 7.4 buffers supplemented with 0.5 % Tween 80 and stirred at 100 rpm at 37 °C. After particular times, 1 mL of the mixture was withdrawn and replaced with the same volume of fresh buffer. Samples were analyzed by Double Beam Spectrophotometer U-2900 machine to calculate the cumulative amount of α-mangostin released. All data are the mean of 3 replicates.

2.2.3. Cell culture and 3D cancer spheroid formation

Lung carcinoma epithelial cells (A594) cells (American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM medium (consist of 10 % FBS, and 1 % PS). The conditions for maintaining the cells included 37 °C with 5 % CO_2. Exponentially grown cells were used for further experiments.

The fabrication of the 3D artificial tumor in the microwells followed the two steps. The microwell system first is prepared by photochemical hydrogel polymerization. PEGDA was dissolved in PBS buffer at a concentration of 10 % (w/w), and PI was added at a concentration of 0.05 % (w/w) to create a PEGDA solution for each experiment. Before being applied in the experiment, the slides (22 × 22 mm from Microglass) were given a TMSPMA treatment. Pour 50 to 100 mL of PEGDA solution onto a petri dish, then lay the slide on top of it. It was placed under UV for 1 min to form microwells. Before cell seeding, the microwell – hydrogels were plated in the six – well plate and cleaned by PBS. Then, the cell solution was prepared with a concentration of 2×10^5 cells/mL and inserted into the plate with microwells. A549 cells were slowly seeded to drop into and fill up the bottom of the microwells to fill up the bottom of the microwells. After seeding, the hydrogel was kept stable for 5 minutes for cells deposited inside the microwell, and excess cells in devices were removed by cleaning with DMEM culture medium. Cells were cultured in a DMEM medium at 37 °C in a 5 % CO_2 incubator for 10 days for cancer spheroid growth. The DMEM cultures were refreshed every 2 days and the formation of cancer spheroids was observed by using a microscope at particular times: day 1, day 4, day 7 and day 10. Cell viability of spheroids after 10 days culture in microfluidic system is presented by live/dead assay.

2.2.4. Cell viability

Cancer spheroids were cultured in DMEM medium in the microwells as explained in the previous section. Following the manufacturer protocols, cancer spheroids were then stained with Live/Dead assay kit (Invitrogen R37601). The images were taken using an Olympus fluorescent IX83 (Japan). Fluorescence images showing the cell viability are analyzed using ImageJ software (National Institute of Health, Maryland, USA).

2.2.5. Statistic

All experiments were used student's t-tests for statistical analysis and the data is presented by OriginPro 2022 (OriginLab, MA, USA). Trials were performed 3 biological replicates for mean and standard deviation demonstrated as error bars.

3. RESULTS AND DISCUSSION

3.1. Characterization of α-mangostin – loaded liposomes

The characterization of liposomes was assessed based on the mean size value and the PDI of nanoparticles. The properties of liposomes synthesized from different ratios of SBL and Toc were measured immediately after being generated; the results are shown in Figure 2A. Liposomes in all samples had mean size around 105 nm. The SBL/Toc ratio 1/1, 2/1, 4/1, and 8/1 have the measured size of 101.7 ± 3.5 nm, 103.2 ± 2.8 nm, 105.8 ± 3.9 nm, and 108.6 ± 2.8 nm, respectively. The PDI of liposome was measured 0.156, 0.139, 0.149, and 0.138, which correspond to SBL/Toc 1/1, 2/1, 4/1, and 8/1 ratios.

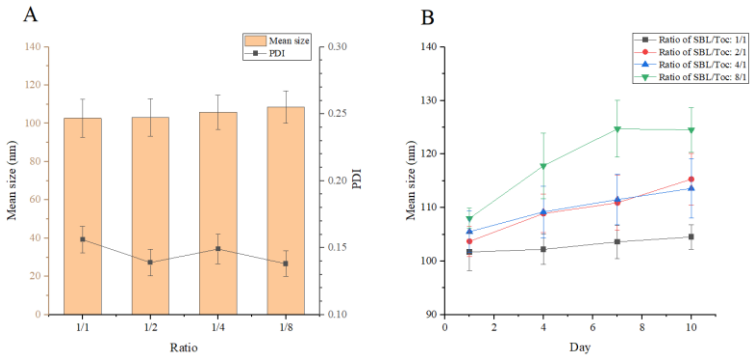

Figure 2. The size and stability of synthesized α-mangostin – loaded liposomes. (A) Mean size and DPI of α-mangostin – loaded liposomes synthesized with different molar ratios of SBL/Toc. (B) The size change of liposomes in 10 days period.

For liposome stability, all samples were kept in 10 days to investigate the change of liposome size. Liposome samples with an SBL/Toc ratio of 1/1 had a relatively stable mean size. After 4 days, the mean size was slightly different change from 101.7 ± 3.5 nm to 102.3 ± 2.8 nm. After 10 days, the average sample size was 104.5 ± 2.3 nm. Meanwhile, the liposome sample with the ratio SBL/Toc 8/1 showed quite large particle size and dispersion when kept for 10 days. Particle size increased from 108.6 ± 2.8 nm to 124.3 ± 4.2 nm. This shows that the liposome sample with the ratio SBL/Toc 8/1 has low tocopherol stabilizer content, and showed less stable. The two SBL/Toc ratios are 2/1 and 4/1 for liposomes whose size were quite stable during the period. However, the liposome sample with an SBL/Toc ratio of 2/1 had a more unstable mean size, it increased from 103.7 nm to 108.9 nm at day 4 and increased to 115.6 ±4.8 nm at day 10. Therefore, SBL/Toc ratio of 4/1 was selected for further research, requiring a smaller amount of tocopherol to prepare, consistent with the ability for control release of α-mangostin.

Further, the EE % results of liposomes with SBL/Toc ratio 4/1 was approximately 51.3 %. Compared with the study of Hosseinali *et al.*, with a ratio of 5/1, the EE % is approximately 28.0 % [23], showing that the method of making liposomes containing α-mangostin in this study is a potential method.

The drug release capacity of α-mangostin and α-mangostin loaded liposomes were shown in Figure 3A. In the first 20 minutes, the release ability of α-mangostin was 42.3 % and increased sharply to nearly 98 % after 12 h and remained unchanged for 96 h. For α-mangostin loaded

liposomes, in the first 20 minutes, the drug release is low, approximately 20 %. After 12 h, the release a-mangostin ability of liposomes was 52.4 % and it continued to increase to 72.8 % after 96 h. It showed the expected drug release ability of α-mangostin loaded liposomes.

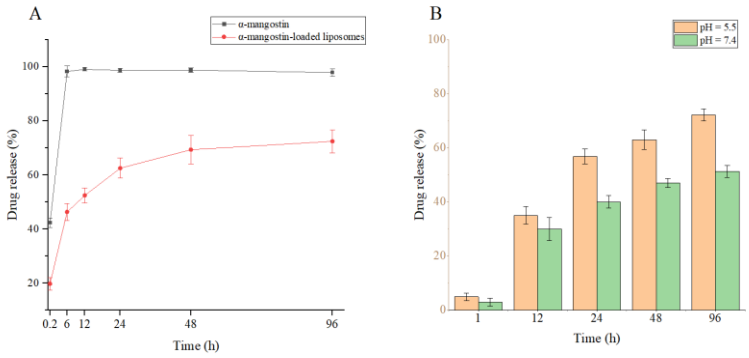

Figure 3. Drug release profiles of α-mangostin loaded liposomes. (A) The ability of drug release during particular time. (B) The effect of pH environment on drug release over time.

Previous cancer studies have shown that the environment around cancer cells has a lower pH than the environment around normal cells [25]. Therefore, this is an important factor to determine the working ability of the drug delivery system. This study also evaluated the release of liposomes containing α-mangostin when stored in buffer solutions with pH = 7.4 and pH = 5.5. According to Figure 3B, in the first hour, the medium with pH 5.5 showed a higher rate of α-mangostin release than in the pH medium of 7.4, although the difference was not significant. This difference was clearly seen within 12 h with the drug release rate of the medium pH = 5.5 was 38.1 % and that of pH = 7.4 was 31.2 %. After 24 h, the ability to release α-mangostin from liposomes at pH = 7.4 and 5.5 was 42.3 % and 58.4 %, respectively. Thereafter, the release of α-mangostin at both pH levels was slowed down and maintained at a steady rate for 96 h. However, the drug release ability at pH = 5.5 still reached nearly 70 % greater than that at pH = 7.4. This confirmed that liposomes work effectively with cancer cells and release fewer α-mangostin around normal cells.

3.2. Formation of 3D cancer spheroids

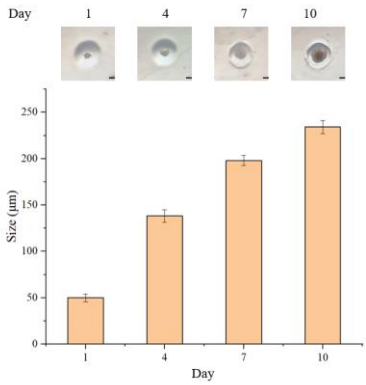

Figure 4. Formation of A549 cancer spheroids in 10 days. (Scale bar = 100 µm).

The modified bottom glass surface helped the hydrogel microwells adhere more firmly to the glass and do not drift during the cell culture and drug testing process [26, 27]. The formed

microwells had a cone curve shape with a design surface diameter of 400 µm. In 3D microwell culture, the shape and size of culture microwells determined the cell culture efficiency and spheroids formation [28]. Cubical or cylindrical microwells were commonly applied in 3D cell culture, however, curved microwells improve oxygen and nutrition delivery to spheroids [29]. In addition, in each curved microwell, cells tend to form and grow form a single combination [28, 29]. As illustrated in Figure 4, after seeding A549 into microwells with concentration of 2 x 10^5 cells/ml and cultured for 10 days, cell aggregates spontaneously formed inside the microwells within one day with diameter of 50 µm and further grew into 3D spheres in following days. Bright field optical microscope images were taken at different timepoints to confirm the spherical structure of A549 spheroids (Figure 4). In stationary media, growths of cancer sphere diameters of 139.3 µm, 198.5 µm and 238.2 µm were found on day 4^{th}, day 7^{th} and day 10^{th} respectively. During spheroids culture process, the medium was changed after 24 h and after 10 days, cancer spheroids were formed and grown in stability.

3.3. Drug testing of α-mangostin – loaded liposomes

The effects of α-mangostin free and α-mangostin – loaded liposomes on the cell viability were evaluated with different concentration (0.1, 1, 2, 5, 10, 20 µM).

Figure 5A shows the effect of different drug concentrations on the cell viability of A549 cancer spheroids. Experimental results showed that, when increasing the concentration of α-mangostin and α-mangostin – loaded liposomes, the cell viability decreased sharply. When A549 cancer spheroids were tested at two concentrations of 0.1 µM and 1 µM α-mangostin, the high viability was presented as 78.1 % and 69.7 %, respectively. With the same concentration of mangostin – loaded liposomes, the cell viability is 70.7 % and 67.7 %, respectively. At the 2 µM α-mangostin and α-mangostin – loaded liposomes, the viability decreased continuously to 63.4 % and 51.1 %, respectively. The cell viability dropped to 57.2 % with the concentration of 5 µM α-mangostin, while it declined sharply to 32.9 % at 5 µM α-mangostin – loaded liposomes. It decreased to 27.2 % and 20.1 %, respectively at the two highest concentrations of α-mangostin–loaded liposomes (10 µM and 20 µM). However, the cell viability of 10 µM and 20 µM α-mangostin free just dropped to 40.2 % and 34.6 %, respectively. The decline in cell viability suggests that the use of α-mangostin – loaded liposomes allowed α-mangostin penetrate into A549 cancer spheroids better than free α-mangostin.

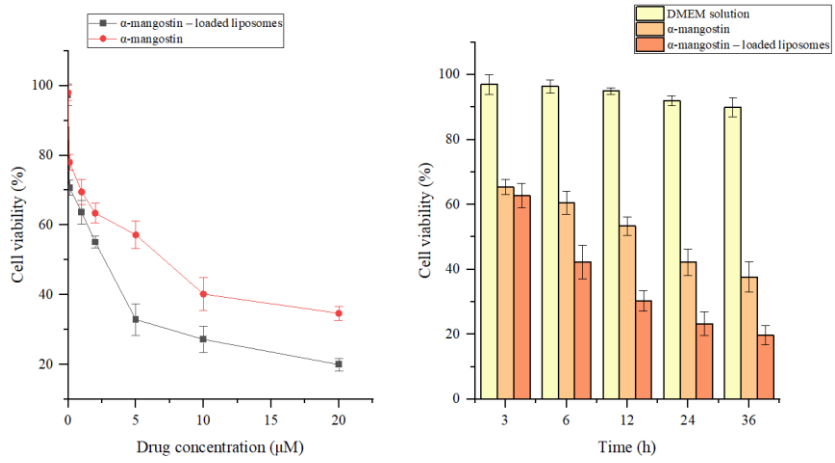

Figure 5. Drug testing of α-mangostinfree and α-mangostin – loaded liposomes on A549 cancer spheroids. (A) Cytotoxic effects ofα-mangostinfree and α-mangostin-loaded liposomes on A549 cells at different drug concentrations. (B) Cell viablity of A549 cells in cancer spheroid at differnt time point when treating with α-mangostinfree and α-mangostin – loaded liposomes solution10 µM.

Figure 5B shows the cytotoxic effect of 10 μM α-mangostin – loaded liposomes and α-mangostin free on A549 cancer spheroids at particular times. After 3 h treated with α-mangostin free, cell viability was reduced to 65.4 % and decreased to 60.4 % after 12 h, while in α-mangostin – loaded liposomes, after 3 h and 12 h, it declined to 62.4 % and 30.3 %, respectively. The viability in α-mangostin – loaded liposomes declined to nearly 20 % after 36 h. However, the cell viability was 37.7 % when spheroids were treated with α-mangostin free for 36 h. This result demonstrated the expected cytotoxic potential of α-mangostin loaded liposomes. The significant reduction of cell viability in α-mangostin – loaded liposomes can base on the nanoscale of liposomes that improve the drug entering into the cell [30]. The effectiveness of α-mangostin – loaded liposomes were examined in a study by Chen *et al*. The liposome was utilized to deliver α-mangostin with the result of increased distribution of transferrin α-mangostin liposomes in the brain [31].

4. CONCLUSIONS

α-mangostin – loaded liposomes were effectively synthesized using the thin film hydration process, with liposomal membrane composition containing SBA and tocopherol. The SBA/Toc molar ratio was varied to find a formula with small size and stability. Based on the experimental results, the molar ratio of SBL/Toc 4/1 (mean size = 105.8 ± 3.9 nm, PDI = 0.149) was selected. The drug release ability of α-mangostin – loaded liposomes has been tested, showing that liposomes have a high drug release rate after 24 h and they are able to perform better in lower pH environments. The cytotoxicity of α-mangostin – loaded liposomes has been tested on A546 cancer spheroids, which can more closely mimic clinical and in vivo tumors. The test results showed that α-mangostin - loaded liposomes reduced the cell viability by nearly 30 % after 12 h and after 36 h, the viability was only 18.9 %. The results showed that liposomes can help α-mangostin to enter and attenuate the cell viability of cancer spheroids. In addition, based on these results, α-mangostin – loaded liposome is an expected candidate to need further research to find therapeutic applications.

Acknowledgements. The research funding from Basic scientific development program in the field of chemistry, life science, earth science and marine science for the period of 2017 - 2025 through the Ministry of Science and Technology of Vietnam (Grant number: DTDLCN.68/22) was acknowledged.

CRediT authorship contribution statement. Tran Dinh Thiet: Investigation, Formal analysis. Pham Thu Uyen: Writing-original draft, Writing-review and edit, Methodology, Formal analysis, Nguyen Thanh Duong: Methodology, Formal analysis, Funding acquisition and Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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