Anthocyanin-loaded polymeric dextran nanoparticle as an anti-proliferative agent on human liver carcinoma cells

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Abstract. In this communication, anthocyanin-loaded dextran nanoparticles from *Clitoria ternatea* was synthesized and characterized to test its anti-proliferative activity on the human HepG2 liver cancer cell lines. By using dextran as an encapsulant polymer, the nanoparticles appeared to be spherical, with an average size of 45.5 ± 11 nm. The surface charge of the anthocyanin-loaded dextran nanoparticle was -4.39 mV, which slightly relative to free anthocyanin (-4.46 mV), which indicate good dispersion stabilities. The Fourier transform infrared analysis showed that the anthocyanins from *C. ternatea* was successfully encapsulated in dextran nanoparticles. Overall, the percentage of drug encapsulation efficiency was 3.03%. Based on the stability test, the anthocyanin-loaded dextran nanoparticle showed significantly better color stability index compared to free anthocyanin, particularly at the presence of light and temperature of 37°C and 50°C. In the anti-proliferation assay on HepG2 liver cancer cell lines, the viability of the cancer cells was significantly reduced after treatment with the anthocyanin-loaded dextran nanoparticle. The anti-proliferation activities of the nanoparticles were significantly better than free anthocyanin. Our findings revealed the ability of the anthocyanin-loaded dextran nanoparticle, in particular from *C. ternatea*, as an effective anti-proliferative agent against cancer cells. Nanoencapsulation with dextran significantly improve the efficacy and stability of the anthocyanins. Further investigations should be done to evaluate the *in vivo* efficacy.

Keywords: Anthocyanin nanoparticle, anti-proliferative activity, *Clitoria ternatea*, nano-encapsulation

INTRODUCTION

Cancer is a disease characterized by unregulated proliferation of cells, which results in the abnormal cells formation and uncontrolled growth of tissue. Globally, cancer is recognized as the second foremost cause of death and is expected to root an additional 9.6 million deaths in 2018. To date, about one in six deaths are due

to cancer (Bray *et al.*, 2018). Surgical resection, radiation or chemotherapy are among the standard protocol of treatments for cancer patients (Windchester *et al.*, 2010). However, the success rate for the cancer treatment is low as current treatments are often implicated with poor response, low survival rate and development of

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metastatic cells. Thus, there is a need to explore new approaches for the treatment of cancer (Zununi *et al.*, 2019).

Clitoria ternatea or blue pea can be found in Asian countries. It is a perennial climber crop native (Bhatia *et al.*, 2014) and rich with pigments, especially anthocyanins. The chemical exists in flavylium salts as their natural form (Kong *et al.*, 2003) and gives a color variety, including bright red, purple and blue, to fruits and vegetables (Wang *et al.*, 2008). Anthocyanins have been shown to give benefits in visual acuity, cancer, heart disease and age-related neurodegenerative disorders (Ray *et al.*, 2009). Besides, the epidemiologic studies suggested the anti-oxidant, anti-microbial and anti-inflammatory activities of anthocyanins can lower the risk of cardiovascular disease, diabetes, arthritis and cancer (Prior and Wu, 2006; Thanh *et al.*, 2020). Hwang *et al.* (2011) reported that anthocyanins can boost memory and the anti-carcinogen activities of the anthocyanins are competent to protect the liver.

The phenolic structure of anthocyanin has been associated with the antioxidant activity including the ability to scavenge superoxide, singlet oxygen, peroxide, hydrogen peroxide, and *hydroxyl* radical. These substances are categorized as reactive oxygen species (ROS) (Wang & Jiao, 2000). The antioxidant efficiency of anthocyanins was previously verified in many cell culture systems i.e. colon, endothelial, liver, breast, leukemic cells, and keratinocytes (Renis *et al.*, 2008; Parry *et al.*, 2006; Shih *et al.*, 2007; Yang *et al.*, 2019). Anthocyanins have demonstrated several anti-toxic and anti-carcinogenic properties by scavenging reactive oxygen species in the cell culture system, thereby enhancing the oxygen-radical absorbing ability of cells (Yang *et al.*, 2019). Therefore, the protective effects of anthocyanins are rather sensitive towards high temperature and light condition with relatively shorter viable shelf life (Karaoglan *et al.*, 2019; Gerald *et al.*, 2019). Nano-encapsulation could be a new approach to prolong the viable shelf life of the protective effects of anthocyanins in the context of cancer treatment or even prevention.

Nanoparticles are solid particle with a size range of 10 to 200 nm (Khan *et al.*, 2019). Nanoparticles can be obtained via various preparation processes. The drug is dissolved and encapsulated or bound to a nanoparticle matrix as

a delivery medium. Drug-incorporated matrix nanoparticle is important to attain the site-specific action by controlling the particle size, surface properties and releases of the targeted drug (Mohanraj & Chen, 2006). Nanoparticle is a key aspect of nanomedicines whereby anticancer therapeutic drugs are encapsulated inside a fatty matrix of a suitable size for delivery whilst avoiding systemic side-effects (Khan *et al.*, 2019). Such a particle-matrix will allow sustained release of the chemotherapeutic over a long time scale and minimizing systemic side effects. Besides, the type of polymer used can be designed to deliver the therapeutic agent to the site specific targets. The polymers used including polyhydroxyalkanoate (PHA), poly-lactide-co-glycolide (PLGA), cyclodextrins and dextrans. Dextran is widely used for anticancer drug delivery purpose due to its excellent biocompatibility, non-toxic and non-immunogenic properties. The anticancer efficiency and selectivity of Goniothalamine can be enhanced by encapsulating the drug with pH-sensitive acetalated dextran nanoparticles (Braga *et al.*, 2020). Besides, dextran was also combined with polyacrylamide as matrices to create an anticancer nanocomposite (Telegeev *et al.*, 2017). The anticancer properties of dextran-cathecin and dextran-quercetin conjugates were also reported (Vittorio *et al.*, 2016; Oliver *et al.*, 2018).

In this study, in order to improve the anticancer properties and stability of *C. ternatea* derived anthocyanins, the nanoparticles were synthesized using dextran as an encapsulant. Synthesis and characterization of anthocyanin-loaded polymeric dextran nanoparticle were investigated. Moreover, its anti-proliferative activity was tested on human liver carcinoma cells, HepG2, in an attempt for the exploratory development of anthocyanin as a potential anticancer therapeutic approach.

MATERIALS AND METHODS

Plant sources

C. ternatea flowers, with no visible diseases, were collected at Jalan Tengker (N 2° 12' 3" E 102° 14' 21"), Bandaraya Melaka, Melaka, Malaysia. The samples were kept in ziplock bags, prior to

the cleaning process under flowing tap water. This process was done within 24 h after collection. Then, the flowers were dried in an incubator at 60°C until stable weight achieved. The dried flowers were ground into fine powder form by using a food blender (Panasonic, Japan). This powder was kept in a desiccator before further use.

Preparation of anthocyanin extract

In order to prepare an acidified ethanol, 1 M of acetic acid (Acros Organics, USA) was added to ethanol (Fisher Scientific, USA) until pH 4.5 was achieved. Next, 1 g plant powder was soaked in 20 mL acidified ethanol for 3 days. This mixture was stirred periodically. Then, the crude extract paste was obtained by drying the liquid extract under reduced pressure through a rotary evaporator. The temperature was set at 50°C. The anthocyanin test was performed by applying the extract to 2N hydrochloric acid (Acros Organics, USA) and ammonia solution (Acros Organics, USA) at equal volume (v/v). The presence of the blue-violet solution denotes a positive result for anthocyanin (Leong *et al.*, 2017).

Synthesis of anthocyanin-loaded dextran nanoparticles

Hundred microliter of 2% of pluronic F127 (Sigma-Aldrich, USA) solution was prepared in distilled water in an ice bath. After that, 200 mL of 1% anthocyanin extract was added into the aforementioned solution. Then, it was blended with a homogenizer (Heidolph, Germany) at 10,000 rpm for 2 min. Subsequently, 1 g of dextran (R&M, India) was mixed into the homogenized solution and further blended for 1 min at 10,000 rpm until it changed into transparent. This solution was subjected to a freeze dryer (Labconco, USA) and left to dry for 48 h. A blank control was integrated by substituting the anthocyanin extract with ethanol (Tan *et al.* 2019).

Microscopic characterization

The ultra-structures of the anthocyanin-loaded dextran nanoparticles were observed under transmission electron microscopy (TEM) (Philips CM12, Netherlands). By using a clean dropper, one droplet of anthocyanin-loaded dextran nanoparticle solution was put on a carbon grafted

copper grid, prior to staining procedure by uranyl acetate. Then, the sample was dried at ambient temperature and viewed under TEM (Tong *et al.*, 2017).

Surface charge measurement

At a concentration of 10 mg/mL and a temperature of 25°C, the nanoparticle was suspended in deionized water for 90 s. Then, the surface charge of anthocyanin loaded dextran nanoparticles was assessed through a zeta sizer Nano ZS (Malvern Panalytical, UK) (Tan *et al.*, 2019).

Composition of the functional groups via Fourier transform infrared (FTIR)

Method of Tan *et al.* (2019) was applied. The samples used were anthocyanin extran, dextran and anthocyanin-loaded dextran nanoparticles. The samples were analysed using FTIR Spectrometer Spectrum RX I (Perkin Elmer, USA). All spectra were detected in the wavenumber range of 4000 – 400 cm⁻¹.

Anthocyanin release property

Anthocyanin-loaded dextran nanoparticles were suspended in phosphate buffer solution at a concentration of 10 mg/mL, pH 7.4. The solution was agitated on an orbital shaker at a rotational speed of 120 rpm, 37°C. At predetermined time gaps of 1, 2, 4, 8, 12, 24, 36, 48, and 60 h, 1 mL of sample was taken and analyzed through UV/Vis spectrophotometer. The amount of anthocyanin released was determined at 517 nm and quantified according to a calibration curve. The calibration curve was prepared by using *C. ternatea* derived anthocyanin as reference molecule. The standards were prepared at a range of concentrations (0–1000 µg/mL) by performing two-fold dilution. The analysis was performed in three replicate on separate occasions (Shaikh *et al.*, 2009).

Drug encapsulation efficiency.

The anthocyanin-loaded dextran nanoparticles were dissolved in PBS. Then, the solution was submerged for 15 min in the ultrasonic ice bath. The resulting solution was centrifuged at 20,000 rpm for 15 min. After that, the supernatant was quantified spectrophotometrically at 517 nm via UV/Vis spectrophotometer. The encapsulation

efficiency was measured according to the following equation;

$$EE (\%) = \frac{\text{Total amount of anthocyanin} - \text{free anthocyanin}}{\text{Total amount of anthocyanin taken for loading study} \times 100} \quad (1)$$

Stability of nanoparticles

The stability of anthocyanin-loaded dextran nanoparticle and free anthocyanin was studied at different temperature (37°C, 50°C, 80°C) and light condition. The sample was placed at 37°C, 50°C and 80°C for 24 h. The absorbance of all samples, before and after exposure to different conditions, were analysed at a wavelength of 362 nm.

Anti-proliferative activity of anthocyanin-loaded dextran nanoparticles

Cell culture

Liver cancer cells (HepG2) were maintained in minimal essential medium (MEM) [10% (v/v) fetal bovine serum and 1% (v/v) penicillin (100 U/mL)]. In a closed incubation chamber, the cell culture was exposed to 5% CO₂ atmosphere at 37°C for 48 h. Next, the attached cells were trypsinated and centrifuged at 3,900 rpm for 10 min. The cells were counted using a hemocytometer and diluted to get a concentration of 7×10³ cells/mL. Next, 100 µL of HepG2 cells were seeded in 96 well plates and further incubated at a similar temperature and CO₂ atmosphere as mentioned above. This procedure was performed to attach the cells to the bottom of the wells.

Analysis of cell proliferation

MTT assay was demonstrated to evaluate the viability of the cells treated with anthocyanin-loaded dextran nanoparticle, free anthocyanin and blank nanoparticle (dextran nanoparticles without loading of anthocyanin). At first, 100 µL of the yellow color MTT solution (1mL in 9 mL fresh medium) was added to each well and further incubated at 37°C for 24 h. After that, 100 µL of DMSO was pipetted to each well to dissolve the formazan crystal. The presence of viable cells was determined by the formation of a purple color of formazan crystals. Subsequently, the absorbance was quantified at 570 nm via a microplate reader (Varioskan Lux, Thermo Scientific, Finland). The mixture with blank nanoparticle was used to set

blank. The cell viability was verified by using the following equation;

$$\text{Cell viability (\%)} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank})}{(\text{Absorbance of control} - \text{Absorbance of blank})} \times 100 \% \quad (2)$$

The cell death was calculated by subtracting the cell viability (%) from 100%. A graph of cell death (%) versus test substance concentration was plotted.

RESULTS AND DISCUSSION

Characterization of anthocyanin nanoparticles

Dextran was applied to entrap anthocyanin in the form of nanoparticles. Dextran with tremendous aqueous solubility, biocompatibility and non-fouling properties is broadly employed as an encapsulant polymer for drug nanoparticles. Figure 1 shows the morphology of the anthocyanin-loaded dextran nanoparticles under TEM view. The nanoparticles appeared in spherical, with a size range from 40 to 110 nm. By average, the particle size was 45.5 ± 11 nm. As reported by Mudshinge *et al.* (2011), the nanoparticle solution has particles ranging from 1-200 nm in diameter, although the nanoparticles may not distribute evenly in the solution. In addition, there was no agglomeration observed in Figure 1. One of the factors contributed to such observation was the addition of pluronic F-127 as a stabilizer. This chemical was added to prevent the agglomeration of the nanoparticles by reducing their surface energy (Lee *et al.*, 2015).

Surface charge of the nanoparticles draws a thin layer of ions of the different charge to the nanoparticle surface. The combined ion layers travel together with the nanoparticles as it disperses throughout the solution. In this experiment, both anthocyanin-loaded nanoparticles and dextran have negative charges i.e. -4.39 mV and -4.46 mV, respectively. The high absolute value of zeta potential shows that high electric charge on the surface of the nanoparticles incorporated with a drug. This event causes strong repellent forces among particles, hence avoids the agglomeration event of the nanoparticles in buffer solution. In contrast, nanoparticles with low zeta potential will be

stacked accordingly to van der Waals inter-particle pulls. This event might be due to the weak ionic strength of the nanoparticles. Leary (2012) stated that small changes in ionic strength and pH can lead to huge effects in zeta potential.

Figure 2 exhibits all spectra of anthocyanin-loaded dextran nanoparticle, free anthocyanin and dextran under FTIR observation. First and foremost, the phenol O-H stretch for dextran, free anthocyanin, and anthocyanin-loaded dextran nanoparticle are 3286.51 cm^{-1} , 3259.17 cm^{-1} , and 3310.74 cm^{-1} , respectively. The C-H stretch absorptions can be seen just below 3000 cm^{-1} for all samples. These wavelengths symbolize the presence of saturated carbons. The peak above 3000 cm^{-1} is in fact from a hydroxyl group because of the broad gap that produced above the

wavelength. Data of the functional groups for all test samples were summarized in Table 1. Merlic *et al.* (2001) reported that this functional group represents the presence of the exchangeable of protons. It was noticed that between the 3 samples, the strongest hydroxyl group absorption was noticed in free anthocyanin at 3259.17 cm^{-1} compared to dextran (3286.51 cm^{-1}) and anthocyanin-loaded dextran nanoparticles (3310.74 cm^{-1}). It was reported by Gundala and Aneja (2000) that O-H groups serve as electron acceptors or hydrogen atom donors and reduce the leftover reactive oxygen species. Owing to the metal chelating and stabilizing properties of some phenolic compounds, free radicals are removed, thereby conferring strong antioxidant activity.

Figure 1. TEM micrograph of anthocyanin-loaded dextran nanoparticle

Figure 2. Functional groups in (A) dextran, (B) free anthocyanin, and (C) anthocyanin-loaded dextran nanoparticle based on FTIR analysis.

Release property of anthocyanin nanoparticles

The release of anthocyanin from the dextran nanoparticles was observed at 37°C for 60 h (Figure 3). The amount of anthocyanin released in the medium was quantified based on the standard calibration with a R_2 value of 0.928. As observed, the release was slow and incremental throughout the experimental period. The anthocyanin release profile showed zero burst impact. The release of drug achieved plateau phase at 48 h with a total release of 48.35%. The drug released has reached the minimum effective concentration as the time increased. The results showed that most of the anthocyanin was found in the internal structure of the polymer matrix. The drug release behaviour of nanoparticles is related to the polymer matrix structure and also the physicochemical properties of the drug (Soppimath *et al.*, 2001). The slow drug release property of nanoparticles decreases the degradation of the drug, thus prolonging its bioavailability (Aditya *et al.*, 2015). In overall, the drug release property of anthocyanin-loaded

dextran nanoparticles provide a constant drug release to the medium, without a rapid increase of drug concentration. The results also showed that dextran is an ideal polymer to encapsulate anthocyanins, which retard the burst release effect of anthocyanin to the test medium. This control release property is very important to ensure long term therapeutic effect.

Drug encapsulation efficiency

There was approximately 3.03% of encapsulation efficiency recorded by anthocyanin-loaded dextran nanoparticle. The percentage explained that 3.03% of anthocyanin was successfully entrapped in the polymeric dextran nanoparticle. Low concentration of anthocyanin inside the nanoparticle can reflect the low percentage of encapsulation efficiency. As reported by Lee *et al.* (2015), the increase in encapsulation efficiency is in line with the increase of drug concentration used in the formulation. The solubility of the polymer and use of co-solvent is also influenced the drug encapsulation efficiency (Bhalekar *et al.*,

2009). Besides, the low encapsulation efficiency can be caused by low stirring speed during the nanoparticles synthesis. The encapsulation efficiency can be improved by increasing the stirring speed, in order to create high shear stress which facilitates the dispersion of viscous droplets of anthocyanins (Lee *et al.*, 2015).

Figure 3. Drug release study of anthocyanin loaded nanoparticles

Stability of anthocyanin-loaded dextran nanoparticle

A comparison of the pigment stability between anthocyanin-loaded nanoparticles and free anthocyanin was exhibited in Table 1. In general, both samples were in blue and more stable in dark condition (amber glass). However, with light exposure, the color of the free anthocyanin started to fade and recorded lower pigment stability index than the anthocyanin-loaded nanoparticles. The similar events were observed at 37°C and 50°C ($P < 0.05$). Hence, the anthocyanin-loaded nanoparticles were significantly stable than free anthocyanin. At 80°C, the stability indexes for both samples were relative ($P > 0.05$).

The pigment stability of anthocyanin is reliant on several factors including pH, light, temperature, as well as its structure (Laleh *et al.*, 2006). In this experiment, two factors were considered i.e. light exposure and also temperature. For instance, the exposure of anthocyanin extracted from *C. ternatea* to light can destruct the anthocyanin pigments. Our results were in line with Bakhshayeshi *et al.* (2006). Under light exposure, the flavylium cation is increasingly constructed, however, in dark condition, the amount of chalcone denotes higher than flavylium cation. Generally, flavylium cation is a basic structure of anthocyanins which engages with the red color. Thus, the active construction of

flavylium cation drives into color changes in *C. ternatea*, which originally in purple hue color. Our results showed there was better color or pigment stability index obtained from anthocyanin-loaded dextran nanoparticle. In conclusion, the dextran that encapsulated the anthocyanin significantly improved the stability of anthocyanin itself.

The co-pigment complexes are exothermic and sensitive to temperature (Roobha *et al.*, 2011). It has been shown that the rise in temperature shifted the anthocyanin equilibrium towards the chalcone form. Hence as the temperature increase, there will be a large decrease in absorbance. The anthocyanin-loaded dextran nanoparticle was more stable due to the self-agglomeration of anthocyanins that prevents the hydration of anthocyanins, preserves the flavylium cation and enhances the stability of anthocyanins (Gris *et al.*, 2009).

Analysis of cell proliferation

The anti-proliferative efficiency of anthocyanin-loaded dextran nanoparticles was tested against the human liver carcinoma cells, HepG2 (Figure 4). The free anthocyanin and encapsulated anthocyanin were added to the culture medium of HepG2 cells and incubated for 24 h. The MTT assay was done to evaluate the anti-proliferative potential of the anthocyanin-loaded nanoparticles and free anthocyanin. The HepG2 cells treated with the anthocyanin-loaded dextran nanoparticles showed significantly higher cell death compared to free anthocyanin at a concentration range from 15.6-125.0 µg/mL. For free anthocyanin, the highest cell death recorded was 33.4% at 250.0 µg/mL. In contrast, by nano-encapsulation with dextran, the highest cell death recorded was 67.3% at 62.5 µg/mL. Nano-encapsulation with dextran significantly improved the anti-proliferative significantly improved the anti-proliferative activity of *C. ternatea* derived anthocyanins. It is noticeable that the cell death recorded was reduced when the anthocyanin-loaded dextran nanoparticles were tested at 125.0 and 250.0 µg/mL. This is due to the agglomeration of nanoparticles when the dissolved nanoparticles were tested at high concentration. Agglomeration refers to adhesion of the nanoparticles to each other by weak forces leading to the formation of sub-microparticles (Pellegrino *et al.*, 2017). The agglomerated

particles reduce the absorption of the drug to the cancer cells, thus reducing the therapeutic efficiency (Limbach *et al.*, 2005; Lankoff *et al.*, 2012). The agglomeration of the nanoparticles can be avoided by addition of surfactant type stabilizing agents (Mehta *et al.*, 2009).

The formation of nanoparticles helps to sustain the release of the anthocyanin and thus improving the drug absorption to the cancer cells.. Nano-encapsulation with dextran prolongs the bioavailability of anthocyanins. The nanoparticles also lower the degradation of anthocyanin in cancer cells treatment. Dextran might increase the bioavailability of anthocyanins, thus enhances its anti-cancer properties. Similar observation was reported by other researchers. A study reported

by Esmatabadi *et al.* (2015) signified a comparison between raw curcumin and curcumin grafted-dendrosome nanoparticles on cell viability of SW480 colon carcinoma and Huh7 hepatome cells after incubation time for 48 h. The treatment of cell lines with curcumin loaded-dendrosome significantly reduced cell viability of SW480 and Huh7 cells more than free curcumin. Cell sensitivity to curcumin was greatly increased once the cells were treated with curcumin loaded-dendrosome. As a result, curcumin loaded-dendrosome increased in bioavailability than free curcumin. Besides, the loading of cisplatin in dextran nanoparticles also improved the anti-tumor activity of metastatic breast cancer.

Table 1. Anthocyanin stability index at different temperature and light conditions.

	Colour stability index				
	Amber glass	Light glass	37°C	50°C	80°C
Anthocyanin-loaded dextran nanoparticle	0.7675	0.7540	0.9151	0.58688	0.3522
Free anthocyanin	0.6819	0.4580	0.5952	0.40606	0.3459

Figure 4. Cell death analysis of human liver carcinoma cells, HepG2 after incubated with free anthocyanin and anthocyanin-loaded dextran nanoparticles.

CONCLUSION

A competent nanoparticle-based drug delivery system which involved anthocyanin and dextran was successfully developed. The nano-encapsulation of *C. ternatea* derived anthocyanins with dextran also significantly improved the light and heat stabilities of the anthocyanins. The anti-proliferative activity of the anthocyanin-loaded dextran nanoparticles were significantly better than free anthocyanins on HepG2 cell lines. Further investigation must be conducted to test the anti-proliferative activity of the anthocyanin-loaded dextran nanoparticles on more cancer cell lines to explore their pharmaceutical potential.

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