Journal of Research in Pharmacy

Research Article

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Development of astaxanthin-loaded nanostructured lipid carriers using a combination of cetyl palmitate and soybean oil

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Received: 18 December 2023 / Revised: 31 January 2024 / Accepted: 2 February 2024

ABSTRACT: The aim of this study was to formulate and evaluate the characteristics and stability of astaxanthin-loaded nanostructured lipid carriers (ASX-NLCs). Four ASX-NLC formulas were prepared using cetyl palmitate and soybean oil in ratios of 100:0, 90:10, 80:20, and 70:30, respectively. ASX-NLCs were prepared by high shear homogenization method, then characterized and evaluated for stability after 30 days of storage at 40 °C and 75% RH. Differential scanning calorimetry (DSC) analysis of solid lipid bulk (cetyl palmitate) and ASX-NLCs was also carried out. The characterization results showed that all ASX-NLCs had pH values suitable for skin application, with no significant differences between the four formulas, even after storage. All freshly produced ASX-NLCs yielded nanometer-sized particles with homogenous size distributions and provided quite good entrapment efficiency. DSC analysis results exhibited lower lipid crystallinity in ASX-NLCs compared to cetyl palmitate. After storage, there was an increasing trend in particle size and polydispersity index, while the entrapment efficiency and antioxidant activity decreased. However, ASX-NLC with a cetyl palmitate and soybean oil ratio of 70:30 showed an insignificant decrease in entrapment efficiency and antioxidant activity, thereby it was considered to have better stability than the other formulas.

KEYWORDS: Astaxanthin; nanostructured lipid carrier; cetyl palmitate; soybean oil; lipid crystallinity.

1. INTRODUCTION

Astaxanthin is a xanthophyll carotenoid that is naturally found in many microorganisms and marine animals such as salmon, lobster, and shrimp. The main source of astaxanthin for several human applications comes from *Haematococcus pluvialis*, a green microalgae. Astaxanthin has been widely used as a dietary supplement due to its antioxidant properties which can significantly reduce free radicals and help maintain the immune system [1, 2]. Astaxanthin provides antioxidant activities through direct radical scavenging as well as activation of cellular antioxidant defense systems [3].

Topical application of astaxanthin has several benefits for skin health, including antiaging effects, protecting the skin from ultraviolet (UV) radiation, increasing skin hydration, and healing wounds. However, its topical application has some limitations. Astaxanthin is highly lipophilic (logP 13.27) and has a high molecular weight (596.85 g/mol) [1], making it difficult to permeate into the deeper layers of the skin [4]. In addition, the chemical structure of astaxanthin which has conjugated polyene chains makes it susceptible to degradation. Astaxanthin tends to be sensitive to adverse conditions such as heat, light, oxygen, and metal ions [5]. Therefore, a delivery system is needed to improve the bioavailability of astaxanthin for topical use.

Several studies have been conducted to improve the delivery of astaxanthin. One of the promising delivery systems for astaxanthin with better stability and skin penetration is lipid nanoparticles. The first generation of lipid nanoparticles is solid lipid nanoparticles (SLN) which consist of a solid lipid matrix in which active molecules are incorporated. Solid lipids can provide a physical barrier to protect sensitive active substances. However, the highly ordered crystalline structure of solid lipids can be a weakness for SLN because it can reduce the drug loading capacity and drug expulsion can occur during storage, especially if the lipid matrix consists of similar molecules [6, 7].

How to cite this article: Zhihrotulwida D, Soeratri W, Rosita N. Development of astaxanthin-loaded nanostructured lipid carriers using a combination of cetyl palmitate and soybean oil. J Res Pharm. 2024; 28(6): 2038-2045.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.878

Nanostructured lipid carrier (NLC) is a modification of SLN which the lipid matrix consists of a mixture of solid lipid and liquid lipid. NLC was developed to overcome the limitations of SLN. The incorporation of liquid lipids in NLC will increase imperfections in the crystalline structure of solid lipids [6, 7]. Solid lipids can be mixed with liquid lipids preferably in a ratio of 70:30 up to 99.9:0.1 to produce NLC [8]. To obtain NLC with the desired physicochemical characteristics, the formula composition needs to be considered. Lipid mixtures have a significant impact on the chemical stability of sensitive active substances [7].

One of the widely used solid lipids for NLC preparation is cetyl palmitate [7]. Cetyl palmitate has a highly ordered crystal lattice arrangement, thus providing superior physical stability [9]. Vegetable oils, such as soybean oil, can be used as liquid lipids in NLC formulations. Using this type of oil can be beneficial because it may contain natural antioxidants that can help protect the active substances from oxidation [7].

In this study, astaxanthin-loaded nanostructured lipid carriers (ASX-NLCs) were made with several ratios of solid lipid and liquid lipid (100:0, 90:10, 80:20, and 70:30) using a blend of cetyl palmitate and soybean oil. This study was designed to formulate and evaluate the characteristics and stability of ASX-NLCs using various ratios of cetyl palmitate and soybean oil.

2. RESULTS AND DISCUSSION

2.1. Preparation and Characterization of Astaxanthin-loaded NLC

Four ASX-NLC formulas with different ratios of cetyl palmitate and soybean oil were successfully prepared by the high-shear homogenization method as described in the Materials and Methods section. ASX-NLCs were characterized by determining pH, viscosity, particle size, polydispersity index, and entrapment efficiency. The physicochemical characteristics of ASX-NLCs are shown in Table 1.

The pH measurement results were in accordance with the desired pH target based on the skin pH value range of 4.0 to 7.0 [10], therefore the ASX-NLC formulas can be used for topical administration. There were no significant pH differences between the four formulas. The viscosity of ASX-NLCs ranged from 132.8 ± 6.7 cP to 179.9 ± 9.2 cP. The formula without the addition of soybean oil (F1) had the highest viscosity and was significantly different from F4 which contained the largest amount of soybean oil.

All freshly produced ASX-NLCs yielded particles with sizes on the nanometer scale, between 373.8 ± 31.8 nm and 491.8 ± 35.1 nm. The four ASX-NLC formulas produced particle sizes that were not significantly different. For the particle size distribution, F4 provided the smallest polydispersity index and was significantly different from F2 which had the largest value. However, all formulas had a homogenous size distribution (the polydispersity index less than 0.3).

The entrapment efficiency obtained from each formula varied. F1 provided the highest entrapment efficiency, reaching 83.25 ± 4.10%. Entrapment efficiency is a parameter of the efficiency of nanoparticles to entrap active compounds within a lipid matrix [11]. Therefore, it can be seen that the lipid matrix containing cetyl palmitate can entrap astaxanthin efficiently. The ASX-NLC formulas containing soybean oil (F2-F4) had an increase in entrapment efficiency as the amount of soybean oil in the lipid mixture increased, but this increase was not significant in this study. Increasing the amount of liquid lipid made the lipid matrix crystals more disordered, resulting in more space to entrap astaxanthin in larger quantities [12].

Table 1. Physicochemical characteristics of ASX-NLCs with different solid lipid (cetyl palmitate) to liquid lipid (soybean oil) ratios. All data are expressed as mean ± SD (n = 3).

Parameter*	Formula (cetyl palmitate: soybean oil)			
	F1 (100:0)	F2 (90:10)	F3 (80:20)	F4 (70:30)
pH	6.68 ± 0.02	6.75 ± 0.01	6.65 ± 0.08	6.62 ± 0.08
Viscosity (cP)	179.9 ± 9.2[a]	152.6 ± 14.8	166.9 ± 5.5[b]	132.8 ± 6.7[a,b]
Particle size (nm)	491.8 ± 35.1	466.8 ± 41.8	444.0 ± 48.8	373.8 ± 31.8
Polydispersity index	0.212 ± 0.009	0.217 ± 0.013[a]	0.201 ± 0.013	0.171 ± 0.018[a]
Entrapment efficiency (%)	83.25 ± 4.10	62.73 ± 13.22	64.51 ± 8.15	68.52 ± 4.21

* Means with the same superscript letters in the same row are significantly different ($p < 0.05$) with Tukey HSD.

2.2. Differential Scanning Calorimetry Analysis

A differential scanning calorimetry (DSC) study was conducted to understand the effect of soybean oil incorporation on the melting behavior and the lipid crystallinity of ASX-NLCs. The DSC thermogram of cetyl palmitate and ASX-NLCs is shown in Figure 1. The DSC thermogram of cetyl palmitate showed a main peak (melting point) at 56.3 °C with 246.10 J/g melting enthalpy. A similar peak also appeared in the four ASX-NLCs, but the melting point decreased slightly as the amount of soybean oil in the ASX-NLC formula

increased. Likewise, the enthalpy of ASX-NLC also decreased when the amount of soybean oil increased. The melting enthalpy of each ASX-NLC was lower compared to cetyl palmitate because the melting process of the ASX-NLCs lipid matrix required less energy than in cetyl palmitate. Highly crystalline lipids, such as cetyl palmitate, require higher energy to overcome the lattice force [13]. Thus, it can be concluded that the lower melting enthalpy of ASX-NLC indicates a lower-ordered lattice arrangement of the lipid matrix.

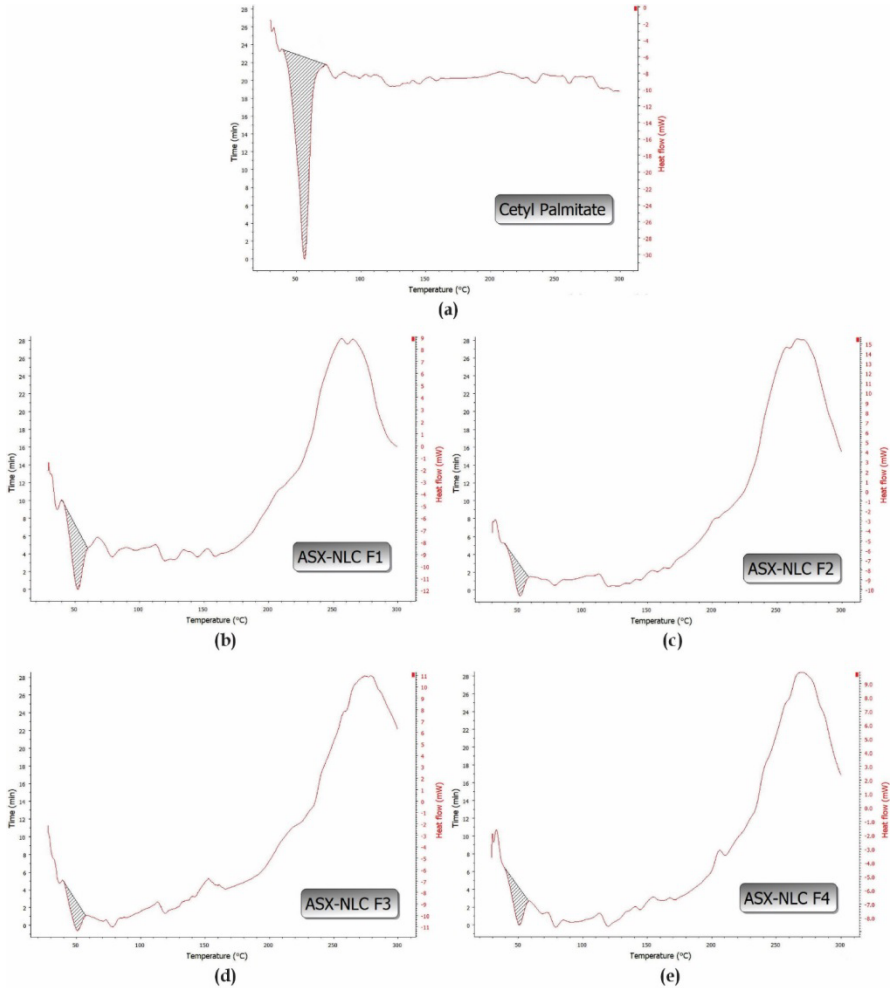

Figure 1. DSC thermogram of (a) cetyl palmitate and ASX-NLCs (b) F1, (c) F2, (d) F3, (e) F4.

In order to compare the crystallinity between the developed formulas, the parameter used is the crystallinity index. The crystallinity index is defined as the percentage of the lipid matrix that has recrystallized during storage time [14]. Based on the results of the DSC analysis, the crystallinity of each ASX-NLC was lower than bulk solid lipids, with the lowest one being ASX-NLC F4 which contained the highest amount of soybean oil. The ASX-NLC formulations were able to disrupt the perfect crystalline arrangement of cetyl palmitate and the incorporation of soybean oil further reduced the crystallinity of the lipid matrix. The melting point, enthalpy, and crystallinity index of each sample are listed in Table 2.

In the DSC thermogram of ASX-NLCs, there was also a peak in the temperature range between 230 °C and 300 °C. This was probably related to the autoxidation process of other ASX-NLC components (Tween 80 and Span 80). Oxidative demolition of the ethylene oxide chain was known to be a strongly exothermic process [15].

Table 2. Melting points, enthalpies, and crystallinity indexes of cetyl palmitate and ASX-NLCs.

Sample	Melting point (°C)	Enthalpy (J/g)	Crystallinity index (%)
Cetyl palmitate	56.3	246.10	100.00
ASX-NLC F1	51.7	30.23	12.28
ASX-NLC F2	50.8	20.18	8.20
ASX-NLC F3	49.8	18.06	7.34
ASX-NLC F4	50.2	16.84	6.84

2.3. Accelerated Stability Test

To determine the stability of the prepared ASX-NLCs, an accelerated stability test was performed for 30 days at 40 °C and 75% RH, which might correspond to 4 months of long-term stability [16]. The parameters evaluated for stability studies were pH, particle size, polydispersity index, entrapment efficiency, and antioxidant activity. After storage, the pH value of ASX-NLCs showed slight fluctuations that did not impact stability and remained within the target pH range for skin application. The pH measurement results of ASX-NLCs for F1 to F4 were 6.71 ± 0.09, 6.53 ± 0.03, 6.76 ± 0.07, and 6.69 ± 0.03, respectively.

The particle size and polydispersity index of ASX-NLCs before and after storage are shown in Figure 2. After storage, ASX-NLC particles from all formulas increased in size by 8.2-19.0% with a significant increase in F4. Even though the increase was significant, the particle size of F4 (444.7 ± 37.1 nm) was still the smallest among all formulas. Meanwhile, the largest particle size was found in F1 with a size of 542.4 ± 13.5 nm. The polydispersity index of all formulas also increased, with the largest increase in F2 (0.306 ± 0.072), but the increase was not significant ($p > 0.05$). The particle size of all ASX-NLCs remained homogeneous (polydispersity index ≤ 0.3).

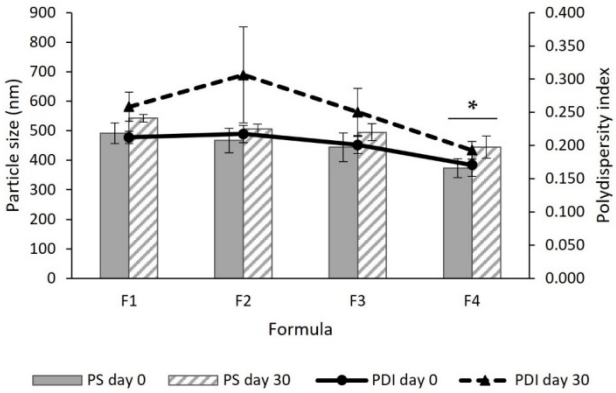

Figure 2. Particle size (PS) and polydispersity index (PDI) of ASX-NLCs before and after storage at 40 °C and 75% RH for 30 days (* $p < 0.05$).

The entrapment efficiency of ASX-NLCs for all formulas decreased after storage (Figure 3). A significant decrease occurred in F1 with a difference of 17.69% from the initial entrapment efficiency. This occurred because the lipid matrix only contains cetyl palmitate, without soybean oil. The highly ordered crystal lattice arrangement of cetyl palmitate can reduce astaxanthin entrapment because drug expulsion can occur after the polymorphic transition of cetyl palmitate crystals during storage [7, 9]. Meanwhile, F3 and F4 tended to maintain the entrapment efficiency with differences of only 1.91% and 1.04% respectively from the initial value. For F2, the difference was 7.89% from the initial entrapment efficiency. Increasing the amount of soybean oil appeared to maintain entrapment efficiency, especially in F3 and F4.

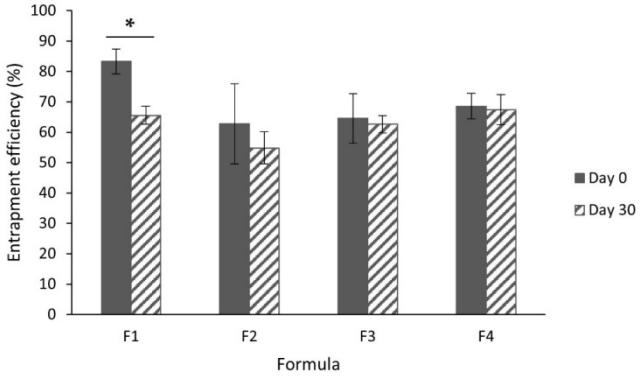

Figure 3. Entrapment efficiency of ASX-NLCs before and after storage at 40 °C and 75% RH for 30 days (* $p < 0.05$).

Antioxidant activity was evaluated before and after storage to determine whether the mixture of cetyl palmitate and soybean oil as a lipid matrix could maintain the antioxidant activity of ASX-NLC. In this study, antioxidant activity was expressed as the ability of ASX-NLC to scavenge ABTS free radicals. The difference in antioxidant activity of all ASX-NLC formulas before and after storage can be seen in Figure 4. ASX-NLCs from all formulas provided high ABTS scavenging rates, 97.52 ± 0.28% to 99.59 ± 0.19% initially. After storage, all ASX-NLC formulas exhibited a decrease in the scavenging rate from the initial value. Scavenging rates of F1, F2, and F3 significantly decreased by 14.54%, 9.81%, and 7.71%, respectively. Meanwhile, F4 decreased by 7.16%. Although all formulas had a decrease in scavenging rate, antioxidant activity tended to remain high because cetyl palmitate provides a physical barrier to protect astaxanthin [5].

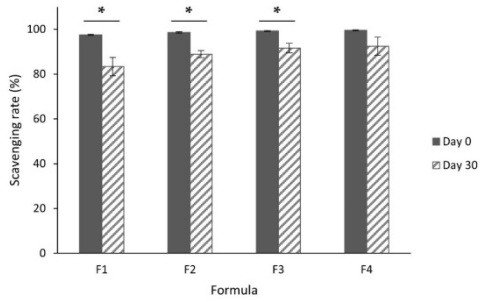

Figure 4. Antioxidant activity of ASX-NLCs before and after storage at 40 °C and 75% RH for 30 days (* $p < 0.05$).

3. CONCLUSION

In this study, ASX-NLCs were successfully prepared with several ratios of solid lipid and liquid lipid (100:0, 90:10, 80:20, and 70:30) using a blend of cetyl palmitate and soybean oil. All ASX-NLCs had pH values suitable for skin application. The difference in the ratio of cetyl palmitate and soybean oil affected the viscosity of ASX-NLC. The viscosity decreased as the amount of soybean oil increased. This had an impact on the particle size becoming smaller, as well as the polydispersity index. ASX-NLC from all formulas provided quite good entrapment efficiency, with the highest value in F1. After 30 days of storage at 40 °C, all ASX-NLCs showed an increase in particle size and polydispersity index, but the particle size distribution remained homogeneous. Meanwhile, the entrapment efficiency and antioxidant activity tended to decrease after storage. However, the addition of soybean oil was able to maintain entrapment efficiency after storage. This was in line with the results of DSC analysis which showed a decrease in lipid crystallinity as the amount of soybean oil increased, thereby preventing drug expulsion during storage. When the soybean oil content was increased to a lipid ratio of 70:30, the antioxidant activity of ASX-NLC did not show a significant decrease during storage. ASX-NLC F4 which had a cetyl palmitate and soybean oil ratio of 70:30 showed better stability compared to other formulas. This study was limited to the use of a combination of cetyl palmitate and soybean oil as a lipid matrix with four ratios ranging from 100:0 to 70:30. More ratio variations may be necessary to further investigate the effect of increasing the amount of soybean oil on the characteristics and stability of ASX-NLC.

4. MATERIALS AND METHODS

4.1. Materials

AstaLuxe™ 5% Astaxanthin Oleoresin was purchased from PT. Evergen Resources (Kendal, Indonesia), cetyl palmitate from BASF (Düsseldorf, Germany), soybean oil from CV. INBI Nusantara Sejahtera (Gianyar, Indonesia), Tween 80 from KAO Corporation (Tokyo, Japan), Span 80 from Croda Singapore Pte. Ltd., propylene glycol from Dow Chemical Pacific (Singapore) Pte. Ltd., Nipaguard SCP (a blend of 2-phenoxyethanol and sorbitan caprylate) from Clariant International Ltd. (Muttenz, Switzerland), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥ 98% purity) from Sigma-Aldrich (St. Louis, MO, USA), and potassium persulfate from Merck (Darmstadt, Germany).

4.2. Preparation of Astaxanthin-loaded NLC

Astaxanthin-loaded NLC (ASX-NLC) was prepared using a high-shear homogenization method. ASX-NLC was prepared in several formulas with different ratios of solid lipid and liquid lipid as mentioned in Table 3. Cetyl palmitate, soybean oil, Span 80, and astaxanthin oleoresin were mixed, then heated at 65 °C until completely melted and stirred until homogenous (oil phase). In a separate container, Tween 80 and propylene glycol were mixed into distilled water, and then heated to a temperature of 65 °C while stirring until homogenous (aqueous phase). The aqueous phase was added to the oil phase slowly and stirred using Ultra-Turrax High Shear Homogenizer (IKA® T25 digital Ultra-Turrax®) at a temperature of 65 °C and a speed of 5000 rpm for 5 minutes in 3 cycles. Then the stirring speed was increased to 17000 rpm and stirred for 3 minutes in 3 cycles. Nipaguard SCP was added to the preparation and the preparation was cooled while stirring using a magnetic stirrer at a speed of 700 rpm until it reached room temperature.

Table 3. ASX-NLC formulas.

Component	Function	Concentration (%, w/w)			
		F1[a]	F2[b]	F3[c]	F4[d]
Astaxanthin oleoresin	Active substance	0.07	0.07	0.07	0.07
Cetyl palmitate	Solid lipid	5	4.5	4	3.5
Soybean oil	Liquid lipid	-	0.5	1	1.5
Tween 80	Surfactant	8.97	8.97	8.97	8.97
Span 80	Surfactant	11.03	11.03	11.03	11.03
Propylene glycol	Cosurfactant	10	10	10	10
Nipaguard SCP	Preservative	0.5	0.5	0.5	0.5
Distilled water	Water phase	64.43	64.43	64.43	64.43

[a] Solid:liquid lipid ratio of F1 = 100:0. [b] Solid:liquid lipid ratio of F2 = 90:10.
[c] Solid:liquid lipid ratio of F3 = 80:20. [d] Solid:liquid lipid ratio of F4 = 70:30.

4.3. Characterization of Astaxanthin-loaded NLC

4.3.1. pH

ASX-NLC was diluted 1:10 using distilled water, then the pH was evaluated using a pH meter Eutech™ pH 700 (Eutech Instruments Pte. Ltd., Singapore). The measurements were carried out in triplicate.

4.3.2. Viscosity

Viscosity testing was carried out using a Brookfield DV-I+ Viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a CP-51 spindle and a speed of 100 rpm. The measurements were carried out in triplicate.

4.3.3. Particle Size and Polydispersity Index

Particle size and polydispersity index analysis were performed using Delsa™ Nano C Particle Size Analyzer (Beckman Coulter, Inc., Brea, CA, USA). Approximately 50 mg of ASX-NLC was dispersed in 50 ml of distilled water. Then 2 ml of the dispersion was diluted with distilled water to 10 ml. The sample was placed in a cuvette and analysis was performed [17]. The measurements were performed in triplicate.

4.3.4. Entrapment Efficiency

The entrapment efficiency of ASX-NLC was determined indirectly by the centrifugation method. About 1 g of ASX-NLC was centrifuged at 15,000 rpm for 90 minutes at 4°C (BOECO Centrifuges M-240R,

Boeckel + Co (GmbH + Co) KG, Hamburg, Germany). The supernatant obtained was diluted in ethanol at a ratio of 1:100. The amount of free astaxanthin in the supernatant was measured using a UV-visible spectrophotometer (Hitachi UH5300, Hitachi High-Tech Corporation, Tokyo, Japan) at a wavelength of 474.6 nm. Entrapment efficiency (EE) was calculated by the following equation (Eq. 1) [18]:

(Eq. 1) $$\text{EE (\%)} = \frac{\text{The total amount of ASX} - \text{Free ASX}}{\text{The total amount of ASX}} \times 100$$

4.4. Differential Scanning Calorimetry Analysis

The thermal behaviour of cetyl palmitate and lyophilized ASX-NLC was examined by Differential Scanning Calorimetry (DSC) using a Linseis DSC PT 1000 (Linseis Messgeraete GmbH, Selb, Germany). The samples (5–7 mg) were placed in sealed standard aluminum pans and heated from 30 °C to 300 °C at a constant rate of 10 °C/min. An empty aluminum pan was used as a reference.

The parameter used to compare the lipid crystallinity between formulations is the crystallinity index (CI%). The CI% of ASX-NLC was calculated according to the following equation (Eq. 2) [19]:

(Eq. 2) $$\text{CI\%} = \frac{\text{Enthalpy}_{\text{NLC}}}{\text{Enthalpy}_{\text{bulk}}} \times 100\%$$

4.5. Antioxidant Activity Test

Evaluation of antioxidant activity was carried out using the ABTS free radical scavenging method as part of the stability test parameters. Before determining antioxidant activity, ASX-NLC was demulsified to provide a complete release of ASX [20]. ASX-NLC (0.5 mL) was added with 4.5 mL of ethanol and demulsified by ultrasonication for 30 minutes. Then the sample was centrifuged at 2,000 rpm for 10 minutes. The supernatant obtained from demulsification was used for the antioxidant activity test.

The $ABTS^{\bullet+}$ free radical stock solution (7mM) was prepared by mixing 14 mM ABTS solution and 4.9 mM potassium persulfate solution in a ratio of 1:1 (v/v). The mixture was placed in the dark at room temperature for 16 hours. The $ABTS^{\bullet+}$ free radical stock solution was diluted with ethanol to obtain an $ABTS^{\bullet+}$ working solution which gave an absorbance of 0.70 ± 0.02 at 753 nm. Then, 1.5 mL of sample obtained from demulsification was mixed with 1.5 mL of $ABTS^{\bullet+}$ working solution and incubated in the dark at room temperature for 90 minutes. The absorbance of each sample was measured by a UV-visible spectrophotometer at 753 nm. Ethanol was used as a blank control. Antioxidant activity was calculated as % scavenging rate using the following equation (Eq. 3):

(Eq. 3) $$\%\ \text{scavenging rate} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100\%$$

where A_{blank} was the absorbance of ABTS solution mixed with ethanol (absorbance 0.70 ± 0.02) and A_{sample} was the absorbance of ABTS solution mixed with sample [21].

4.6. Accelerated Stability Test

An accelerated stability test was performed by storing ASX-NLC in a climatic chamber (Climacell MMM, MMM Medcenter Einrichtungen GmbH, München, Germany) for 30 days at 40 °C and 75% RH [16]. The stability parameters evaluated before and after storage were pH, particle size, polydispersity index, entrapment efficiency, and antioxidant activity.

4.7. Statistical Analysis

All results were expressed as mean ± standard deviation (SD) and analyzed statistically. Multiple comparisons were performed using one-way ANOVA followed by Tukey HSD post hoc test. For the stability study, paired-sample t-tests were performed to determine the statistical significance between data before and after storage. A p-value < 0.05 was considered to be statistically significant.

Acknowledgements: The authors are grateful to PT. Evergen Resources for the support by providing AstaLuxe™ 5% Astaxanthin Oleoresin.
Author contributions: Concept – D.Z., N.R., W.S.; Design – D.Z., N.R.; Supervision – N.R., W.S.; Resources – D.Z., N.R.; Materials – D.Z.; Data Collection and/or Processing – D.Z.; Analysis and/or Interpretation – D.Z., N.R.; Literature Search – D.Z.; Writing – D.Z.; Critical Reviews – D.Z., N.R., W.S.
Conflict of interest statement: The authors declared no conflict of interest.

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