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Original Article

Influence of stabilizers and cryoprotectants on the characteristics of freeze-dried PLGA nanoparticles containing *Morus Alba* stem extract

Kanyarat Srihaphon[1], Supaporn Lamlertthon[2], and Tasana Pitaksuteepong[1*]

[1] *Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

[2] *Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

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Abstract

This study aimed to investigate the effects of stabilizers and cryoprotectants on the characteristics of poly (lactic-co-glycolic) acid (PLGA) nanoparticles containing *Morus alba* stem extract (MSE). The stabilizers used in the preparation of the nanoparticles were polyvinyl alcohol (PVA) and poloxamer 188 at a concentration of 2% w/v. The effects on freeze-drying of cryoprotectants, including glucose, sucrose, maltose and mannitol at concentrations of 5% and 10% w/v, were investigated. The results showed that the presence or the absence as well as the type of stabilizer affected size and charge of the nanoparticles. They also affected the loading capacity and the release of MSE. In freeze-drying, the type of cryoprotectant greatly influenced the physical stability of the nanoparticles and the chemical stability of encapsulated MSE. The nanoparticles prepared using 2% w/v PVA as the stabilizer and freeze-dried in the presence of 5% sucrose were the optimal carriers of MSE.

Keywords: *Morus alba* stem extract, PLGA, nanoparticles, stabilizer, cryoprotectant

1. Introduction

Our previous studies found that *Morus alba* stem ethanolic extract (MSE) significantly inhibited various inflammatory mediators, including inducible nitric oxide synthase (iNOS), nitric oxide (•NO), prostaglandin-E_2 (PGE_2) and cyclooxygenase (COX)-2 in LPS-stimulated RAW 264.7 macrophage cell model (Soonthornsit, Pitaksutheepong, Hem stapat, Utaisincharoen & Pitaksuteepong, 2017; Wongwat, Srihaphon, Pitaksutheepong, Boonyo & Pitaksuteepong, 2019). The powerful capacity for scavenging •NO free radical was again observed *in vitro* in a nitric oxide radical scavenging assay with IC_{50} of 12.12 ± 1.71 µg/ml compared with L-ascorbic acid (IC_{50} of 85.10 ± 2.18 µg/ml) (Srihaphon, 2019). MSE also exhibited an ability to scavenge free radicals including DPPH and superoxide radicals with IC_{50} values of 32.07 µg/ml and 67.29 µg/ml, respectively. In addition, it was also found to effectively inhibit *Propionibacterium acnes*, which is known to play a major role in the pathogenesis of acne, with MIC and MBC values of 3.125 mg/ml and 12.5 mg/ml, respectively (Srihaphon, 2019). Moreover, the previous studies reported that the methanolic extract prepared from *M. alba* twigs could inhibit type II 5α-reductase enzyme in Bradford protein assay kit compared with finasteride (positive control) (Jang *et al.*, 2007). Therefore, MSE shows potential for the treatment of inflammatory acne. However, the clogged hair follicles caused by the accumulation of sebum are one of the major barriers limiting access of the extract to the target. Thus, nanocarriers are required.

Poly (lactic-co-glycolic) acid or PLGA is a synthetic co-polymer, consisting of lactic acid (LA) and glycolic acid (GA). It has been widely used in drug delivery applications (Jain, 2000; Semete *et al.*, 2010). In addition, it is used to prepare nanoparticles targeting the pilosebaceous unit, due to its biodegradability and biocompatibility. In addition, its

*Corresponding author
Email address: tasanap@nu.ac.th

hydrophobic features offer good compatibility with sebum (Reis, Martinho, Rosado, Fernandes & Roberto, 2014). Recently, Mittal *et al.* (2013) prepared the fluorescein isothiocyanate conjugated ovalbumin (FITC-OVA) loaded PLGA (Resomer RG 50:50 H) nanoparticles with the mean particle size of 170 nm using polyvinyl alcohol (PVA) as a stabilizer. These particles could significantly enhance hair follicle uptake, compared with an aqueous solution of FITC-OVA (Mittal *et al.*, 2013).

Although PLGA nanoparticles are extensively used in the field of smart drug delivery, their limitations include physical instability (aggregation /fusion nanoparticles) and chemical instability (drug leakage), especially when the particles are in suspension dosage form and are stored for long-term (De *et al.*, 1999). Thus, freeze-drying of the PLGA nanoparticles was considered in this study. A stabilizer and a cryoprotectant were used to prevent the aggregation of nanoparticles during preparation and freeze-drying. Although the effects of stabilizers (Dinda *et al.*, 2011; Hoda, Sufi, Cavuturu & Rajagopalan, 2018) and cryoprotectants (Fonte *et al.*, 2012; Jahan & Haddadi, 2015) have been widely investigated, their effects remain somewhat controversial depending on the encapsulated compounds investigated. In addition, there is no prior report on their effects on the characteristics of nanoparticles loading with plant extracts. Therefore, this study aimed to investigate the effects of stabilizers and cryoprotectants on the characteristics of PLGA nanoparticles containing MSE for the treatment of inflammatory acne.

2. Materials and Methods

2.1 Materials

Ethanolic extract of *M. alba* stem (MSE) was prepared as described in our previous study (Soonthornsit *et al.*, 2017). Oxyresveratrol was purchased from Sigma – Aldrich (St. Louis, USA). The oxyresveratrol content in the extract, when analyzed using HPLC, was found to be 15.06 ± 0.36% (w/w). Acetone, acetonitrile and methanol were purchased from RCI Labscan (Bangkok, Thailand). Poloxamer 188 was purchased from BASF Corporation (Ludwigshafen, Germany). PLGA in a 50:50 molar ratio (Resomer RG 502 H) was purchased from Evonik industries (Darmstadt, Germany). Polyvinyl alcohol (PVA) was purchased from the Nippon synthetic chemical industry Co., Ltd. (Bangkok, Thailand). Tween 80 was purchased from Srichand United Dispensary Co., Ltd. (Bangkok, Thailand).

2.2 Preparation of PLGA nanoparticles: Effects of stabilizers

Empty PLGA nanoparticles were prepared by the nanoprecipitation technique. Briefly, 90 mg of PLGA was dissolved in 5 ml of acetone and methanol (8:2). This organic phase was poured into 25 ml of water in the presence or absence of a stabilizer under stirring at 750 rpm for 5 min at room temperature. The stabilizer used was either 2% w/v PVA or 2% w/v poloxamer 188. After that, the organic solvent in the mixture was removed using a rotary evaporator at a controlled temperature of 45 ºC for 30 min. The resulting particles were isolated from the suspensions by centrifugation at 10,000 rpm for 30 min at 4 ºC (Kubota 5922, Kubota corporation, Tokyo, Japan) and washed twice using deionized water.

The PLGA nanoparticles containing MSE were prepared in the same way as the preparation of the empty PLGA nanoparticles, except that MSE (3 mg) was also dissolved in the organic phase (acetone-methanol (8:2)).

2.3 Characterization of PLGA nanoparticles

2.3.1 Particle size and zeta potential

The effects of stabilizers on particle size and zeta potential of the empty and MSE-loaded PLGA nanoparticles were measured using a particle size and zeta potential analyzer (Zeta PALS®, Brookhaven instrument, New York, USA). Following the washing described above, the particles were re-suspended in deionized water and then their particle size and zeta potential were measured.

2.3.2 Drug loading capacity

The prepared PLGA nanoparticles were dissolved using acetone and methanol (4:1) and analyzed for the amount of MSE by using HPLC. The HPLC analysis was performed using a C18 bonded-silica gel column (Gemini, 5 µm, 150 x 4.6 mm, Phenomenax, Torrance, USA) and a UV-Vis detector (SPD-10AVP, Shimadzu, Kyoto, Japan). The mobile phase consisted of acetonitrile and 0.0125 M phosphate buffer at pH 3 (1:3). Column oven temperature of 30 ºC, detection wavelength of 320 nm and flow rate of 1 ml/min were set. The injection volume was 20 µl, and the run time was set at 13 min. Drug loading capacity was calculated as follows:

$$\text{Loading capacity} = \frac{\text{The amount of MSE in PLGA nanoparticles}}{\text{The amount of PLGA nanoparticles}}$$

2.3.3 *In vitro* release study

After their final wash, MSE-loaded PLGA nanoparticles (72 mg) were re-suspended in 5 ml of 0.05 M phosphate buffer (pH 5.5), which was used as the release medium. This suspension was agitated at 100 strokes/min in an incubator shaker (GFL 1086 shaking water bath, Burg wedel, Germany), at a controlled temperature of 37 ºC. At specific time points, a 300 µl aliquot of the release medium was withdrawn and replaced by the same volume of fresh medium. The sample was then centrifuged at 10,000 rpm for 10 min at 4 ºC. The resulting clear supernatant was analyzed for the amount of MSE released, using HPLC. Each sample was tested in triplicate.

2.4 Freeze-drying process

Following the final wash, nanoparticles were freeze-dried either in the absence or in the presence of a cryoprotectant at a concentration of 5% w/v. The size of nanoparticles was measured before and after freeze-drying using a particle size analyzer.

2.5 Effect of cryoprotectants on the stability

The freeze-dried nanoparticles prepared as described above were stored under three different conditions: refrigerated temperature (5 ºC), room temperature (25 ºC) and elevated temperature (40 ºC) at 75 ± 5% RH for 12 weeks. The physical stability of the MSE-loaded PLGA nanoparticles was observed through changes in particle size, and the chemical stability was measured by analyzing the percentage MSE remaining by HPLC as described above.

2.6 Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). Statistical analysis used one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons (Graphpad Prism 6.0, Graphpad Software Inc., San Diego, USA). *P*-values less than 0.05 were considered significant.

3. Results and Discussion

3.1 Effect of stabilizers on the particle size and zeta potential

In the preparation process of PLGA nanoparticles, various stabilizers have been used to prevent aggregation of the particles: PVA, poly (vinyl pyrrolidone) (PVP), poloxamer 188, poloxamer 407, tween 80 etc. (Chun, Yoo, Yoon & Park, 2004; Jain, 2000; Kim, Jeong, Chun & Park, 2005; Lee *et al.*, 2005; Song, Lee, Choung, Cho, Ahn & Choi, 2006; Zhang, Cui, Bei & Wang, 2006). Based on our preliminary study, PVA and poloxamer 188 yielded particles with narrow size distributions and thus they were chosen as stabilizing agents in this study.

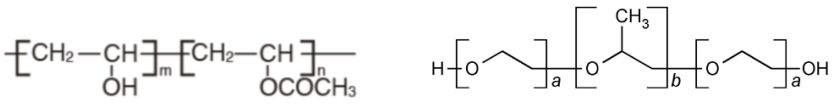
Both empty and MSE-loaded PLGA nanoparticles were prepared by using the stabilizer (either 2% w/v PVA or 2% w/v poloxamer 188) as well as without a stabilizer. It was found that on using the same stabilizer, the particle size of the empty PLGA nanoparticles was not different from that of the MSE-loaded PLGA nanoparticles (Table 1). However, absence or presence of the stabilizer affected the particle size. The particle size of both empty and MSE-loaded PLGA nanoparticles prepared in the presence of PVA (F1) was in the range of 198 -203 nm, which is larger than the particle size of those prepared in the presence of poloxamer **(**F2) (135-143 nm). The particle size of both empty and MSE-loaded PLGA nanoparticles prepared in the absence of any stabilizer (F3) was found to be the smallest (122-124 nm). These observations may be explained by the fact that PVA or partially saponified PVA (Figure 1A) is a copolymer of poly (vinyl acetate) and poly (vinyl alcohol) (Sahoo, Panyam, Prabha & Labhasetwar, 2002). The hydrophobic poly (vinyl acetate) part may be bound to PLGA polymer during formation of the nanoparticles. However, the poly (vinyl alcohol) part is hydrophilic and may stick out of the nanoparticle surface and increase the particle size of the nanoparticles. In contrast, poloxamer 188 is a triblock copolymer consisting of a central hydrophobic fragment of polypropylene oxide (PPO) and two hydrophilic chains of polyethylene oxide (PEO) at both sides (Figure 1B). The adsorption of the PPO part on the PLGA nanoparticle surface by hydrophobic forces increases the size of PLGA nanoparticles prepared using poloxamer as the stabilizer (Santander-Ortega, Jodar-Reyes, Csaba, Bastos-Gonzalez & Ortega-Vinuesa, 2006). However, as poloxamer 188 attaches to the particles via the central hydrophobic part and two hydrophilic parts stick out, the chains of hydrophilic parts of poloxamer 188 are shorter than those of PVA (Figure 2). In addition, the molecular weight of the PVA was higher than that of poloxamer 188. As a result, the particle size of the PLGA nanoparticles prepared using PVA was larger than the particle size of the PLGA nanoparticles prepared using poloxamer 188. The size of the PLGA nanoparticles prepared without a stabilizer was the smallest because these particles have no stabilizer on the PLGA nanoparticle surfaces.

The polydispersity indexes (PDI) of the empty and the MSE-loaded PLGA nanoparticles F1, F2 and F3 were 0.04 ± 0.01, 0.15 ± 0.05 and 0.18 ± 0.02, respectively. These PDI values of all formulations are below 0.3, indicating narrow size distributions.

Table 1. The particle size and zeta potential of empty PLGA nanoparticles and *M. alba* stem extract (MSE) loaded PLGA nanoparticles when prepared using either 2% w/v polyvinyl alcohol or 2% w/v poloxamer 188 as a stabilizer as well as without a stabilizer (n=3).

PLGA nanoparticles	Stabilizer used	Particle size (nm)	Polydispersity index (PI)	Zeta potential (mV)
Empty	Polyvinyl alcohol	198.03 ± 5.49^{a}	0.04 ± 0.02	0.00 ± 0.00^{d}
	Poloxamer 188	143.17 ± 10.30^{b}	0.14 ± 0.01	-20.33 ± 3.76^{e}
	No stabilizer	122.33 ± 1.35^{c}	0.13 ± 0.02	-13.77 ± 2.27^{f}
MSE loaded	Polyvinyl alcohol	202.80 ± 3.27^{a}	0.04 ± 0.01	0.00 ± 0.00^{d}
	Poloxamer 188	135.20 ± 6.13^{b,c}	0.15 ± 0.05	-23.53 ± 2.32^{e}
	No stabilizer	123.93 ± 5.12^{c}	0.18 ± 0.02	-14.38 ± 1.96^{e,f}

Note: Mean values sharing the same superscript are not significantly different from each other ($p \leq 0.05$)



Molecular weight = 30,000-70,000 Dalton **Molecular weight = 7,680-9,510 Dalton**

Figure 1. Chemical structures of polyvinyl alcohol (left) and poloxamer 188 (right).

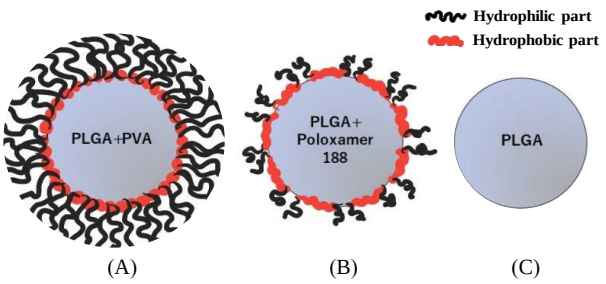


Figure 2. A schematic representation of the PLGA nanoparticles prepared using polyvinyl alcohol (A), or poloxamer 188 (B), or without a stabilizer (C).

Using the same stabilizer, the zeta potential of the empty PLGA nanoparticles was not different from that of the MSE-loaded PLGA nanoparticles (Table 2). Both empty and MSE-loaded nanoparticles prepared using either poloxamer 188 or without a stabilizer were negatively charged. The zeta potentials of the empty and MSE-loaded PLGA nanoparticles prepared using poloxamer 188 were found to be in the ranges -20.33 ± 3.76 mV and -23.53 ± 2.32 mV. The zeta potentials of the nanoparticles prepared in the absence of the stabilizer were in the ranges -13.77 ± 2.27 mV and -14.38 ± 1.96 mV. On the other hand, the zeta potential of both empty and MSE-loaded nanoparticles prepared using PVA was neutral.

The negative charge of the PLGA nanoparticles (~ 14 mV) is due to the terminal carboxylic groups in the PLGA polymer. Coating the nanoparticle surfaces with PVA decreases the zeta potential because the coating layer shields the surface charge of the PLGA nanoparticles (Sahoo *et al.*, 2002). However, in the case of poloxamer 188, a steric mechanism produced by the hydrophilic PEO chains is a barrier for the hydrophobic part to fully cover the whole surface of the nanoparticles (Santander-Ortega *et al.*, 2006). In addition, although poloxamer 188 is a non-ionic compound, this compound is synthesized in the presence of a strong base (Alexandridis & Hatton, 1995). The alkaline character of poloxamer 188 may cause ionization of carboxylic groups of PLGA polymer to some extent. Therefore, the negative zeta potential of PLGA nanoparticles prepared using poloxamer 188 was the largest over plain nanoparticles and nanoparticles prepared using PVA.

3.2 Effects of stabilizers on loading capacity

Loading capacities of MSE in the PLGA nanoparticles prepared using either PVA (F1) or poloxamer 188 (F2) was not mutually different, and were significantly lower than that of MSE in the nanoparticles prepared without stabilizer (F3) (Table 2). These results may be explained by the effects of PVA or poloxamer 188 covering the surfaces of PLGA nanoparticles. PVA and poloxamer 188 are non-ionic surfactants with hydrophilic head and hydrophobic tail parts. Generally, surfactants can form micelles in an aqueous solution by incorporating hydrophobic compounds within the micellar structures at the critical micelle concentration (CMC) that depends on the surfactant. A previous study has reported that the CMC of PVA at 20 ˚C is in the range 0.04-0.68% (Delbecq & Kawakami, 2014). Another previous study has demonstrated that the CMC at 25 ˚C of poloxamer 188 is found at 0.1% (Youan, Hussain & Nguyen, 2003). In this study, PVA and poloxamer 188 were used as the stabilizers at 2% concentration. Thus, the both may form micelles and incorporate the MSE within micellar structures, resulting in reduced loading capacity of MSE in the nanoparticles F1 and F2 (Figure 3).

3.3 Effects of stabilizers on *in vitro* release

MSE release from the nanoparticles was assessed in 0.05 M phosphate buffer solution (pH 5.5). The cumulative release data are shown in Figure 4. The release of MSE from the nanoparticles F1 and F2 was significantly faster than from nanoparticles of type F3. Burst release was also observed for both F1 and F2 formulations. The burst release may be due to extract located at the surface of the particles. Enhanced drug release by coating with a block copolymer (i.e. poloxamer series) has been reported (Kamel, Awad, Geneidi & Mortada, 2009), who explained that hydrophilic surfaces of the poloxamer coated nanoparticles resulted in a higher penetration of the release medium to the particles, leading to faster release of the entrapped compound. The hydrophilic nature of poloxamer 188 was stronger than that of PVA and gave a higher release rate, but without significant difference, of the extract from the particles prepared using poloxamer 188 (Figure 4).

3.4 Freeze-drying of MSE-loaded PLGA nanoparticles

As discussed previously, freeze drying is a useful method to improve the long term stability of nanoparticles. Thus, freeze drying of nanoparticles was performed in this study. Following the freeze drying, aggregation of nanoparticles, especially of F2 and F3 types, was observed (Figure 5). Aggregation of nanoparticles after freeze drying has been

Table 2. The loading capacity of the *M. alba* stem extract in PLGA nanoparticles when prepared using either 2% w/v polyvinyl alcohol (F1) or 2% w/v poloxamer188 (F2) as a stabilizer as well as without a stabilizer (F3) (n = 3).

Nanoparticle formulation	Loading capacity ± S.D. (µg extract/mg nanoparticles)
Polyvinyl alcohol (F1)	10.26 ± 0.39
Poloxamer 188 (F2)	10.33 ± 0.03
No stabilizer (F3)	20.09 ± 0.50

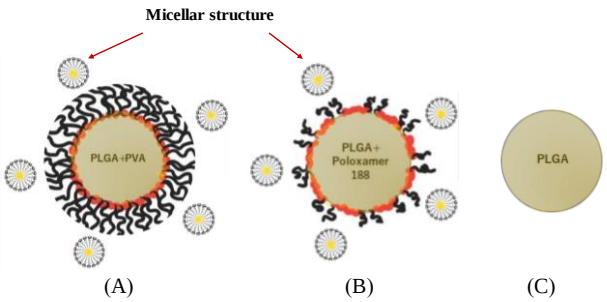


Figure 3. A schematic representation of the PLGA nanoparticles prepared using 2% w/v polyvinyl alcohol (A), or 2% w/v poloxamer 188 (B) or without a stabilizer (C) and micellar structures of polyvinyl alcohol and poloxamer 188.

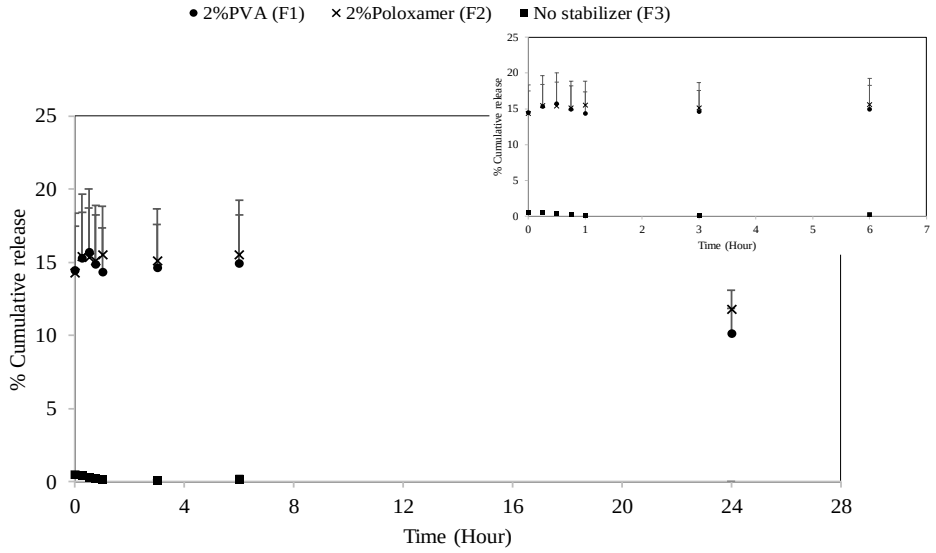


Figure 4. Release of *M. alba* stem extract from PLGA nanoparticles in 0.05 M phosphate buffer solution (pH 5.5).

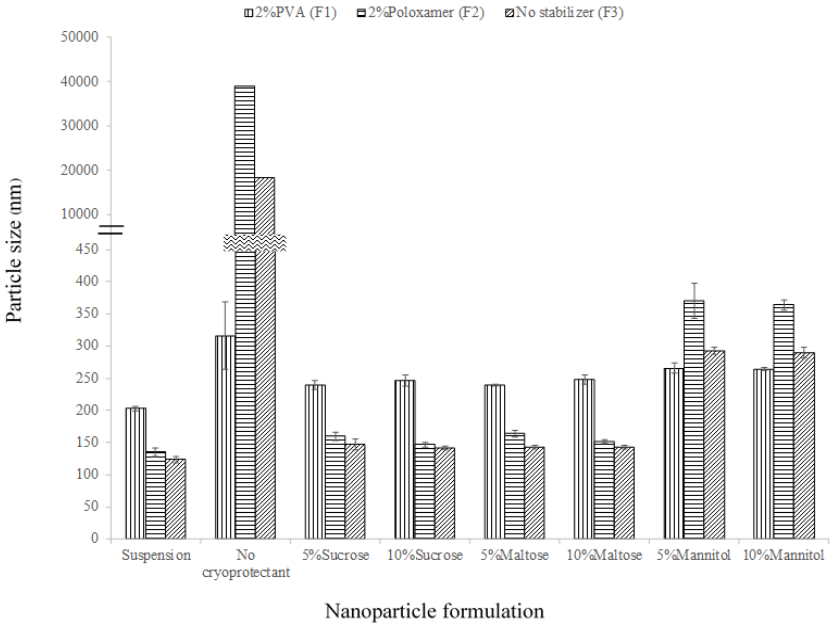


Figure 5. Effects of freeze drying and the cryoprotectants 5% w/v sucrose, 10% w/v sucrose, 5% w/v maltose, 10% w/v maltose, 5% w/v mannitol and 10% w/v mannitol on the particle size of the *M. alba* stem extract loaded PLGA nanoparticles prepared using either PVA (F1), poloxamer 188 (F2) or without a stabilizer (F3).

reported in other studies (Abdelwahed, Degobert, Stainmesse & Fessi, 2006; Chung, Lee & Lee, 2012). The aggregation results from many stresses in the freeze drying process, such as the stress from freezing and dehydration, which induces aggregation and fusion of the nanoparticles. A cryoprotectant was therefore added before freeze-drying. The most common cryoprotectant is sugar. It forms a glassy matrix at a specific temperature known as the glass transition temperature (T_g) and fixes the nanoparticles in this matrix. Thus, it protects the particles against mechanical stress and prevents aggregation. It also functions by lowering the T_g and preventing actual freezing.

In this study, glucose, sucrose, maltose and mannitol were chosen to serve as cryoprotectants, and they were used at the concentrations of 5% and 10% w/v. The results show that the freeze-dried nanoparticles prepared using 5% w/v glucose or 10% w/v glucose as cryoprotectant were hygroscopic. Consequently, they were excluded from this study. Adding a cryoprotectant was found to greatly reduce the aggregation of nanoparticles during freeze drying (Figure 5). Among the cryoprotectants investigated, sucrose and maltose at concentrations of 5% and 10% w/v were the best for maintaining the size of nanoparticles. Thus, sucrose and maltose at a concentration of 5% w/v were selected to serve as cryoprotectants in further experiments.

3.5 Effect of cryoprotectants on physical stability

Following the previous stability test, the physical stability of the MSE-loaded PLGA nanoparticles was observed based on changes in particle size and size distribution. At 12 weeks, all of the freeze-dried nanoparticles stored under elevated temperature were found to be hygroscopic and thus the particle sizes were determined only at 0 and 4 weeks (Figure 6C). Under refrigerated and room temperature, the changes in particle size of MSE-loaded PLGA nanoparticles of F1-F3 types, prepared using sucrose or maltose at a concentration of 5% w/v as a cryoprotectant, were lesser than of the particles stored under elevated temperature (Figure 6A-C). However, at 12 weeks, the freeze-dried nanoparticles F2 and F3 made with 5% maltose and stored under room temperature were prone to aggregate. Thus, the freeze-dried MSE-loaded nanoparticles F1-F3 prepared using 5% w/v sucrose as a cryoprotectant were further investigated for chemical stability.

3.6 Effect of cryoprotectants on chemical stability

The chemical stability of the MSE loaded PLGA nanoparticles was detected based on oxyresveratrol by HPLC analysis. Type of stabilizer and temperature affected the stability of MSE that was loaded in the particles. The PLGA nanoparticles prepared using 2% w/v PVA as stabilizer and freeze-dried in the presence of 5% sucrose had the best formulation. The preservation of these particles in refrigerated temperature is recommended. After storage for 12 weeks, the percentage of MSE remaining in these freeze-dried nanoparticles was 96.57 ± 7.15 (Figure 7). In a preliminary study, these particles also demonstrated promising slow-down of the growth of *Propionibacterium acnes*.

4. Conclusions

The stabilizers and cryoprotectants influenced the characteristics of PLGA nanoparticles encapsulating the stem extract of *M. alba*. The most promising formulation had nanoparticles prepared by nanoprecipitation using 2% w/v PVA as the stabilizer and the freeze-drying performed in the presence of 5% sucrose.

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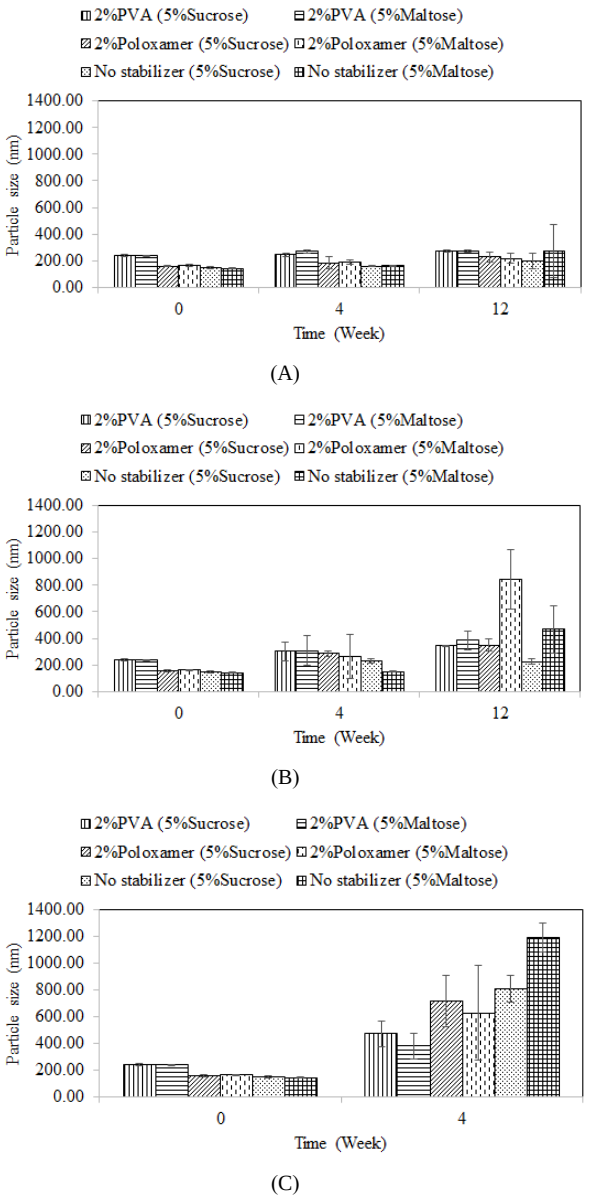

Figure 6. Particle size of the *M. alba* stem extract loaded PLGA nanoparticles prepared in the presence of polyvinyl alcohol (F1), or poloxamer 188 (F2), or in the absence of a stabilizer (F3). The cryoprotectant added in the freeze drying process was 5% w/v sucrose or maltose. The storage conditions were refrigerated temperature (A), room temperature (B), and elevated temperature (C). The particle size, as indicator of physical stability, was measured at 0, 4 and 12 weeks.

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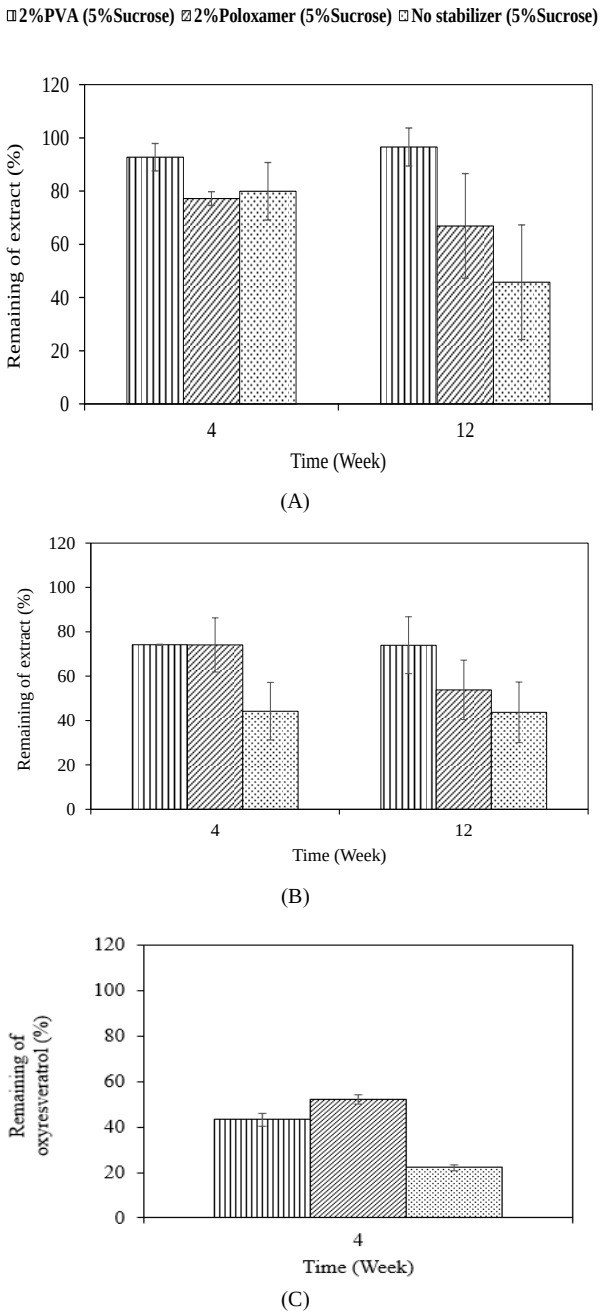

Figure 7. Percentage of the *M. alba* stem extract remaining in PLGA nanoparticles prepared in the presence of polyvinyl alcohol (F1), or poloxamer 188 (F2), or in the absence of a stabilizer (F3). The cryoprotectant added in the freeze drying process was 5% w/v sucrose. The storage conditions were refrigerated temperature (A), room temperature (B), and elevated temperature (C), for 4 and 12 weeks.

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