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JELLYFISH COLLAGEN HYDROLYSATE-LOADED NIOSOME FOR TOPICAL APPLICATION: FORMULATION DEVELOPMENT, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES

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Abstract: Vesicular carrier system, niosome, has become an attractive delivery system for active ingredients in cosmetics and pharmaceuticals. This study aims to prepare a niosome formulation containing collagen hydrolysate extracted from local jellyfish (*Rhopilema hispidum*) with promising antioxidant and antibacterial properties. Niosomes, which are composed of a mixture of non-ionic surfactants, was prepared by using coacervation and sonication techniques optimized using a D-optimal mixture experimental design (MED) to obtain a small particle size. The particle size of the niosome obtained was 111.2 nm with a polydispersity index (PDI) value of 0.256 and a zeta potential value of −6.62 mV. Transmission Electron Microscopy (TEM) study showed the formation of spherical bilayer vesicle with a size correlating well with Zetasizer analysis. The stability of the niosome formulation was studied over a 60-day period, during which changes in particle size from 104.5 to 386.5 nm were observed. The results showed no phase separation or creaming. Niosome obtained from this study was shown to exhibit antioxidant (65.9% DPPH scavenging activity and 34.6% metal chelating activity) and exhibited inhibitory activity against *Staphylococcus aureus* (up to 98.8%). The optimized niosome might be suitable for topical application.

Keywords: Jellyfish collagen hydrolysate, D-optimal mixture experimental design, niosome, nanoformulation.

Introduction

The right delivery system may be advantageous in increasing the rapid penetration of active ingredients by improving solubility, enhancing bioavailability and controlling the release of active ingredients (Basiri *et al.*, 2017). There are many types of delivery systema used in the cosmeceutical industry, including vesicular systems (such as liposomes and niosomes), emulsions, and nanoparticles (including solid lipid nanoparticles, dendrimers, cubosomes, and fullerene) (Lohani *et al*., 2014). Among the delivery systems, the niosome has become the ultimate choice in the cosmetics industry (Singh & Sharma, 2016).

Niosomes are made up of non-ionic surfactant vesicles, sized between 100 nm and 2 µm with double-layer configuration through self-assembly of non-ionic amphiphilic surfactant (which possessed both hydrophilic and hydrophobic properties) in the aqueous phase, leading to the formation of closed bilayer structures with an aqueous core (Basiri *et al*., 2017; Sahu *et al*., 2014). The presence of cholesterol and a charge inducer (i.e. dicetyl phosphate or dicetylpyrinidium chloride) assist in stabilizing the vesicle. It shares a similar structure as liposome by having a bilayer, except that the materials used to create niosome give higher stability, as well as more economical, thus giving niosome more advantage over liposome (Jacob *et al.*, 2017).

It has been widely used as a carrier system for delivering active compounds such as drugs and plant extracts (Alonso *et al.*, 2016). It has a lot of appealing features, including potent permeability with high loading capacity, and is also non-irritating to the skin (Khan & Irchhaiya, 2016). Niosome with small particle size provide wider surface area to interact with skin, thus enhancing the bioavailability of the active ingredients into the skin (Ghasemiyeh & Mohammadi-Samani, 2020).

Niosome has been applied in the cosmeceutical field for dermatological purposes (Singh *et al*., 2016). For example, L'Oreal was the first company that launched a niosome-based anti-aging product known as "Niosomes" and "Niosome Plus®" under the trade name of Lancome in 1987 and 1990 (Sharma *et al*., 2018). Dior also produced a niosome-based product named Dior's Capture™ (Manosroi *et al.*, 2012). The other niosome product for wrinkle treatment known as Anti-age response cream, marketed by Simply Man Match (Kaul *et al*., 2018).

Collagen hydrolysates are a combination of peptides with various chain lengths generated from collagen hydrolysis (Mazloomi *et al*., 2019). To acquire collagen hydrolysates with desired biological activities, collagen needs to undergo hydrolysis using different proteases at their optimal conditions including specific temperature, pH, and enzyme to substrate ratio (E/S) (Pongkai *et al*., 2017). Previous research has shown that jellyfish collagen hydrolysate (JCH) has significant biological activities, including antioxidant (Ding *et al.,* 2011), UV-protective (Zhuang *et al*., 2009), immunostimulant activity (Sugahara *et al*., 2006), anti-hyperlipidemic and anti-hypertensive (Liu *et al*., 2012). The presence of collagen in jellyfish was believed to be caused for these behaviour (Khong *et al*., 2016).

Therefore, this study was conducted to prepare a niosome formulation encapsulated in collagen hydrolysate using a combination of coacervation and sonication techniques and further optimized using MED to obtain a small particle size. The formulation was also tested for antioxidant and antibacterial activities and cytotoxicity against the 3T3 fibroblast cell line.

Materials and Methods

Materials

The local jellyfish, *R. hispidum,* was purchased from a fisherman in Kukup, Johor, Malaysia. Papain (from papaya) was purchased from Acros Organics (St. Louis, MO, USA). 1,2-diphenyl-2- picrylhydrazyl (DPPH), L-(+)- and O-phthaldialdehyde (OPA), were purchased from Sigma-Aldrich (Munich, Germany). Acetic acid, tween 60, ethanol, sodium acetate, and potassium phosphate were obtained from Merck (Darmstadt, Germany). 2,4,6-tripyridyl-s-triazine (TPTZ) was obtained from Fluka Merck (Darmstadt, Germany). Ferrous sulphate ($FeSO_4.7H_2O$) was obtained from Friendemann Schmidt (Washington, USA). Ferric chloride ($FeCl_3$) was obtained from Qrec (New Zealand). All other solvents and reagents were of analytical or HPLC grade.

Preparation of Jellyfish Collagen Hydrolysate (JCH) from R. hispidum

The preparation of JCH was conducted according to Ab Aziz *et al*. (2020). Jellyfish tissue was cut into strips (1.5 – 2.0 cm), treated with alkaline distilled water followed by freeze-drying. The dried tissue was blended, and further hydrolyzed using papain enzyme and the collected hydrolysate fractions freeze dried and frozen at -20°C before further analysis.

JCH Solubility Study

A solubility study was conducted by dissolving JCH in 10 mL deionized water at different weight ranges (0.01-0.2 g). JCH was added into deionized water and stirred in a water bath at 60°C for 15 min on a magnetic stirrer (MS-H280-Pro, Scilogex LC, Rocky Hill, CT, USA) until all JCH was fully dissolved. These steps were repeated until precipitation and a cloudy solution was observed. The maximum weight of JCH that fully dissolved was recorded.

Preparation of Niosome Formulations

Niosome formulations were prepared using a combination of techniques (thin layer hydration method and sonication techniques) according to Thomas and Viswanad (2012) with modifications. Initially, the surfactants (Span 60, Tween 60 and lecithin), cholesterol, and a charge inducer (cetylpyrinidium chloride) were added into a beaker followed by the addition of absolute ethanol. This surfactant mixture was heated in a water bath at 60°C until all surfactants dissolved completely. The ethanol was then removed using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) for approximately 30 min until a white, thin layer was formed. In another beaker, an appropriate amount of deionized water was mixed with JCH. The mixture was then added to the surfactant mixture that was already prepared previously. Phenonip (as preservative) was finally added to the mixture and then sonicated using a probe sonicator (Omni Ruptor 4000, Omni International Inc, Kennesaw, GA) for 4 min at 20 W. These methods were repeated with different compositions of Tween 60 and cholesterol.

Experimental Design and Model Fitting

The composition of niosome formulations was optimized using a D-optimal mixture experimental design. The range for each component was determined based on the preliminary studies. D-optimal mixture experimental design was constructed to determine the effect of independent variables (Span 60 (A), Tween 60 (B), cholesterol (C) and lecithin (D) on a response (particle size). Twenty-four runs were developed and the results were evaluated statistically. The amount of cetylpyrinidium chloride, deionized water, absolute ethanol, JCH and phenonips were kept constant.

Since the factors are quantitative, experiments on at least two levels may be appealing when the response's curvature is predicted (Jacob *et al*., 2017). The factors were assessed at two levels (-1 and +1), demonstrating low and high ratios, respectively. The initial high and low ratios were dictated according to the hydrophilic and lipophilic characteristics of the components. The primary effects (A, B, C and D) indicate the average result of varying one component from a low to a high value at a time. The interaction terms (ABCD) illustrate how the response changes when all of these factors are transformed together. The level of independent proportions set is presented in Table 1.

Statistical analysis

The optimal niosome formulation compositions were determined based on conditions that resulted in the smallest particle size. The analysis of variance (ANOVA) and coefficient of determination (R^2) were conducted to identify the significant differences between the independent variables, expressed in terms of lack of fit. The significance of the equation parameters for each response can be determined with a probability of less than 0.05 ($p < 0.05$).

Validation of model

The adequacy of the chosen model was accessed by preparing various formulations at random. The percentage of the residual standard error (RSE) was calculated using the equation below:

$$\textbf{Residual standard error (\%)} = \frac{\textbf{Actual value} - \textbf{Predicted value}}{\textbf{Predicted value}} \times 100 \quad (1)$$

Table 1: Level of independent variables proportions

Independent variables	Lower limit (%)	Upper limit (%)
Span 60 (A)	0.47	1.42
Tween 60 (B)	0.47	1.42
Lecithin (C)	0.85	2.55
Cholesterol (D)	0.70	0.85

Physicochemical Characterization

Particle Size and Polydispersity Index Measurements

The particle size and polydispersity index (PDI) were determined using dynamic light scattering at an angle of 173° and a temperature of 25°C using a Nano ZS90 (Malvern Instruments, Malvern, UK). The niosome formulation containing JCH was inserted into a folded capillary and kept at a count rate of less than 500 kcps (kilo count per second) to yield less scattering intensity and to avoid multiple scattering. All measurements were taken three times in total.

Zeta-potential Measurement

The zeta potential of the niosomes was assessed with a Nano ZS90 (Malvern Instruments, Malvern, UK). The procedures were conducted on similar batches that were used for particle size analysis. Results were automatically calculated by the Zeta analyzer using the following Smoluchowski equation (Thomas & Viswanad, 2012).

$$M = \frac{ez}{\eta} \quad (2)$$

Where M is the mobility, e is the dielectric constant, z is the zeta potential and ŋ is the absolute viscosity of electrolyte solution.

Transmission Electron Microscopy (TEM) Measurement

The morphology of the niosome was examined using transmission electron microscopy (TEM, H-600, Hitachi, Tokyo, Japan). The samples were diluted with deionized water followed by negative-staining using 1% uranyl acetate solution for 1 min 30 s. Excess liquid was removed with filter paper and the sample was dried at room temperature before visualized using TEM.

pH Measurement

The pH of the niosome formulation was measured using a Delta 320 pH meter (Mettler-Toledo, Schawerzenbach) at room temperature (28 ± 1°C). The pH meter was calibrated with pH standard buffer solutions prior to sample pH measurements. The average value of the pH from three readings was taken as the results.

Stability Study

The niosome formulations were put into centrifuge tubes and centrifuged at 4000 rpm for 15 min right after preparation. Two samples of the optimized niosome were prepared and kept at different temperatures (25.0 and 45.0°C) separately for 60 days. The physical appearances of the formulation were examined to see whether any precipitates had developed.

Antioxidant Activity Analysis

DPPH Scavenging Activity Assay

The free-radical scavenging activity was measured using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) according to Zarei *et al*. (2014). Niosome solution was prepared by diluting 0.10 g niosome formulation with 10.00 mL deionized water, in order to prevent noise in UV-absorbance reading. 50.00 µL of niosome solution, 50.00 µL distilled water and 100.00 µL of 0.15 mM DPPH solution (in ethanol) were placed into a 96-well plate and mixed well. The mixture was incubated for 45 min in the dark at room temperature followed by absorbance measurement at 517 nm using a 96-well microplate reader (Labomed, model UVD-2950, USA). The control solution was prepared by mixing distilled water into DPPH solution at a 1:1 (v/v) ratio. The scavenging activity was calculated using the following equation.

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{(A_{Control} - A_{Sample})}{A_{Control}} \times 100 \quad (3)$$

Where $A_{Control}$ is the absorbance of the control solution and A_{Sample} is the absorbance of the samples at 517 nm.

Metal chelating activity assay

The metal chelating activity of the sample was measured according to Zarei *et al*. (2014). 100.00 µL of niosome solution (0.10 g niosome formulation diluted in 10.00 mL deionized water) was added into the 185.00 µL of deionized water containing 5.00 µL of 2.00 mM iron (II) chloride solution. Following that, 10.00 µL of 5.00 mM ferrozine solution was added and incubated for 10 min at room temperature. The absorbance was read using a 96-well microplate reader at wavelength 562 nm (Labomed, model UVD-2950, USA). The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation below.

$$\text{Metal chelating activity (\%)} = \frac{(A_{Control} - A_{Sample})}{A_{Control}} \times 100 \quad (4)$$

Where, $A_{Control}$ was the absorbance of the control solution and A_{Sample} was the absorbance of samples at 562 nm.

Antibacterial activity

The antibacterial activity against the bacteria *S. aureus* ATCC 6538 was conducted according to a previous study with minor modifications (Arulrajah *et al*., 2020). 100.00 µL of the optimized niosome and 100.00 µL of nutrient broth (consisting of 106 cfu/mL bacterial suspension) were added into the 96-well plate. The control solution was prepared using distilled water mixed with nutrient broth solution at 1:1 (v/v) ratio. The mixture was incubated at 37°C for 24 h followed by absorbance measurement using a 96-well microplate reader (Labomed, model UVD-2950, USA) at wavelength 600 nm. The bacterial growth inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = \frac{(A_{24\,h\,Control} - A_{0\,h\,Control}) - (A_{24\,h\,Sample} - A_{0\,h\,Sample})}{(A_{24\,h\,Control} - A_{0\,h\,Control})} \times 100 \quad (5)$$

Where $A_{24\ h\ Control}$ was the absorbance of the control at 24 h, $A_{0\ h\ Control}$ was the absorbance of the control at 0 h, $A_{24\ h\ Sample}$ was the absorbance of the sample at 24 h and $A_{0\ h\ Sample}$ was the absorbance of the sample at 0 h at 600 nm.

Cytotoxicity study

The cytotoxicity of niosome containing JCH was investigated against 3T3 fibroblast cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. 3T3 fibroblast cells were seeded in 96-well culture plates at 2 x 10^3 cells/well density and incubated for 72 h at 37°C. Each well was added with 20.00 µL of optimized niosome diluted in dimethyl sulfoxide (DMEM) which yield the final concentrations of 1.00 - 500.00 µg/ml. The same procedure was applied to the blank niosome (without JCH). These mixtures were further incubated for 3 h after the addition of 20.00 µL of MTT solution (5.00 mg/mL in PBS). Then the medium was added with DMSO to solubilize formazan purple salt. The plates were swirled for 10 min. These procedures were carried out three times. The absorbance was measured by using a microplate reader (Thermo Scientific, USA) at a wavelength of 570 nm. The cell viability (%) related to control wells with cell culture media was determined using the equation below:

$$\textbf{Cell viability (\%)} = \frac{(A_{Sample} - A_{Blank}) - (A_{Control} - A_{Blank})}{(A_{Control} - A_{Blank})} \times 100 \quad (6)$$

Where $A_{Control}$ was the absorbance of the control solution, A_{Blank} was the absorbance of the blank solution and A_{Sample} was the absorbance of samples at 570 nm

Results and Discussion

Solubility Study of JCH

The solubility study of JCH was conducted in order to determine maximum amount of JCH

that can be solubilized in deionized water for niosome preparation. Table 2 shows that maximum amount was 0.10 g JCH in 10 mL deionized water (1.0 % w/v).

Table 2: Solubility of JCH in 10 mL deionized water

JCH Amount (g)	Observation
0.01	Soluble
0.02	Soluble
0.03	Soluble
0.04	Soluble
0.05	Soluble
0.06	Soluble
0.07	Soluble
0.08	Soluble
0.09	Soluble
0.10	Soluble
0.11	Not soluble

Preparation of Nosome Formulations Containing JCH

The most important factors that affect the formation of niosome formulation are the choices of surfactant, additives including charge inducer, active ingredients and the hydration temperature and duration (Yeo *et al*., 2017). Ethanol was used to solubilize the surfactant mixture as it is an effective permeation enhancer in niosome making, whereby it is able to interact with a lipid molecule's polar-head group. This contributes to the reduction of lipid melting in stratum corneum. and increases liquid fluidity and permeability through cell membrane (Chen *et al*., 2019). Cholesterol was chosen in order to stabilize the niosome system by inhibiting the formation aggregate through electrostatic effects or repulsive steric which results in the formation of less leaky niosome (Debnath & Kumar, 2015; Gurjar *et al*., 2014). Spans and tweens are the most common surfactants in niosome development due to compatibility, low toxicity and stability compared to other surfactants (Chandu *et al*., 2012). For example, the mixture of Span 60 and Tween 60 could improve the hydrophobicity of the niosome membrane thus resulting in the formation of stable membrane (Junyaprasert *et al*., 2012). Lecithin was chosen as it enhances penetration of the formulation and prevents leakage of active ingredients from vesicles due to its high transition temperature (Li *et al*., 2014). Previous studies also suggested that lecithin and cholesterol can affect the size of microparticles (Jacob *et al*., 2017). Hence different ratios of these surfactants have been used to determine the best formulation with smallest particle size.

Fitting the Model

The compositional mean particle size of niosome formulations obtained experimentally based on D-optimal MED model is shown in Table 3. The minimum particle size obtained was 109.5 nm. The actual values for particle size were consistent with the predicted values.

The final equation to predict the particle size in terms of coded factor for reduced modified quadratic model is as follows:

$$Y = +186.98304A - 162.403949B + 4.91116C - 481.83627D + 14.68396AB + 52.16605AC + 29.07412BC + 399.03513BD + 142.76000CD - 84.40334ABC \times 100 \quad (7)$$

where A is Span 60, B is Tween 60, C is Lecithin and D is Cholesterol, respectively.

The comparison of the model's predicted particle to the actual particle from the experimental data reveals that the model was successful in defining the relationship between all of the independent variables with $R^2 = 0.9728$ (Figure 1).

ANOVA tests were carried out to investigate the effects of each independent variable using Design-Expert® software to assess the final model's suitability and significance (Table 4). The final reduced modified quadratic model had large *F*-values (39.76) and small *p*-values ($p < 0.0001$), and the lack of fit was not significant towards the response (particle size). These indicated a significant effect on the respective variable (Musa *et al*., 2013). Based on their coefficient and *p*-value of the linear mixture, all

Table 3: Experimental data and predicted and actual values of particle size obtained from the D-optimal mixture experimental design model

Run	Independent Variables (%)				Particle Size (nm)	
	A	B	C	D	Actual Value	Predicted Value
1	1.42	0.88	1.26	0.70	177.40	174.07
2	1.42	0.47	1.67	0.70	218.60	220.12
3	0.47	1.42	1.59	0.77	109.50	130.71
4	0.86	0.47	2.15	0.77	164.90	164.01
5	0.47	0.54	2.55	0.79	131.90	120.39
6	0.47	0.98	2.11	0.70	124.80	122.65
7	0.92	0.92	1.66	0.77	138.80	141.27
8	1.42	1.21	0.85	0.77	157.60	163.01
9	1.42	1.21	0.85	0.77	166.20	163.01
10	0.69	1.17	1.59	0.81	266.40	132.75
11	1.42	0.47	1.52	0.85	193.70	188.02
12	1.17	0.69	1.59	0.81	173.10	162.97
13	0.92	0.92	1.66	0.77	137.00	140.76
14	0.47	0.47	2.47	0.85	155.70	124.59
15	1.14	1.42	0.85	0.85	155.70	154.76
16	0.98	0.98	1.44	0.85	143.10	141.70
17	0.92	0.92	1.66	0.77	138.90	141.27
18	0.88	1.42	1.26	0.70	148.20	120.45
19	0.92	0.92	1.66	0.77	138.80	141.27
20	0.47	1.42	1.59	0.77	132.20	131.82
21	0.47	0.47	2.47	0.85	124.00	124.33
22	0.89	0.69	1.90	0.77	153.20	151.07
23	0.88	1.42	1.26	0.70	121.20	120.39
24	1.42	0.47	1.52	0.85	178.80	188.02

Note: The letters A–D represent the independent variables, where A is Span 60, B is Tween 60, C is Lecithin and D is Cholesterol, respectively. Other components were kept constant;10 mL deionized water, 0.1 g JCH, 0.01 g cetylpyrinidium chloride and 0.08 g phenonips

linear mixture components (A, B, C and D) from Equation 7 were acceptable in the response. The regression coefficient result for the final reduced model (Table 5) revealed that the predicted R^2 (0.9484) was in true agreement with the adjusted R^2 (0.9103).

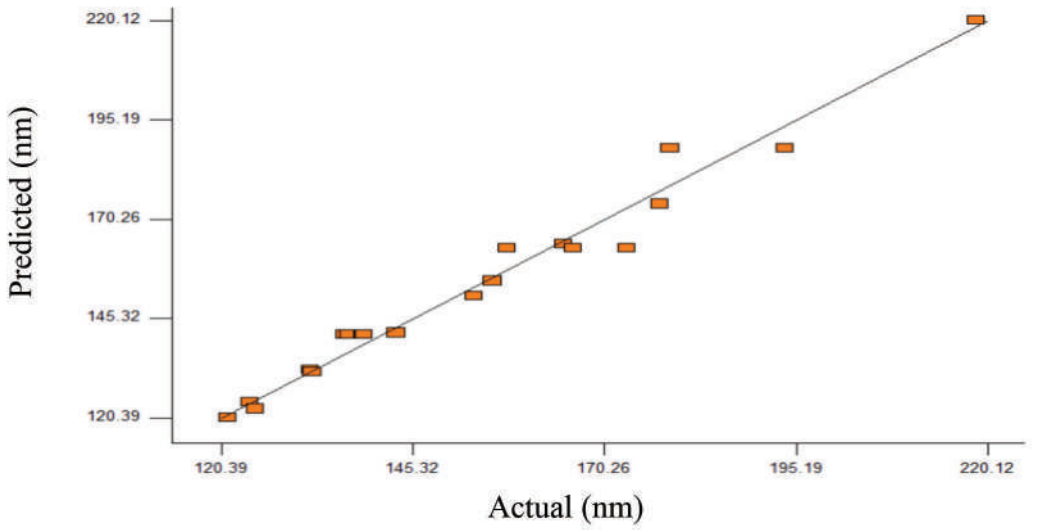


Figure 1: Scatter plot of predicted droplet size values versus actual particle size values from D-optimal mixture experimental design model

Table 4: Analysis of variance (ANOVA) results for all independent variables

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value	Significance
Model	12181.35	9	1353.48	39.76	< 0.0001	Significant
Linear mixture	11221.63	3	3740.54	109.89	< 0.0001	
AB	93.72	1	93.72	2.75	0.1280	
AC	10.50	1	10.50	0.31	0.5908	
BC	8.53	1	8.53	0.25	0.6274	
BD	561.65	1	561.65	16.50	0.0023	
CD	154.56	1	154.56	4.54	0.0589	
ABC	130.75	1	130.75	3.84	0.0785	
Residual	340.39	10	34.04			
Lack of fit	187.60	5	37.52	1.23	0.4137	Not significant
Pure error	152.79	5	30.56			
Cor total	12521.74	19				

Note: The letters A–D represent the independent variables, where A is Span 60, B is Tween 60, C is Lecithin and D is Cholesterol, respectively. Other components were kept constant;10 mL deionized water, 0.1 g JCH, 0.01 g cetylpyrinidium chloride and 0.08 g phenonips

Table 5: Regression coefficient results for the final reduced model

Source	Coefficient Estimate
A,Span 60	275.88
B, Tween 60	117.4
C, Lecithin	134.30
D, Cholesterol	-391.07
AB	-178.15
AC	39.02
BC	-33.08
BD	1245.90
CD	445.74
ABC	-465.66
SD	5.83
R^2	0.9728
Predicted R^2	0.9484
Adjusted R^2	0.9103
Adequate precision	24.173

Note: The letters A–D represent the independent variables, where A is Span 60, B is Tween 60, C is Lecithin and D is Cholesterol, respectively. Other components were kept constant;10 mL deionized water, 0.1 g JCH, 0.01 g cetylpyrinidium chloride and 0.08 g phenonips

D-optimal Analysis

For the optimization of niosome formulation, contour plot and three-dimensional surface graph were plotted using Design-Expert software to graphically present the effect of independent variables to the response variable (Figure 2 (a) and (b)). Particle size has been the main response to be considered in this study as cosmetics products with small particle sizes (20 – 200 nm) can improve the penetration of active ingredients through the skin (Roselan *et al*., 2020).

Figure 2 shows that as Span 60 concentration increases, so does the size of the particle. On the contrary, as the concentration of Tween 60 increases, the size of the particles decrease. Similar result was obtained by Junyaprasert *et al*., (2012) due to increase of hydrophilic-lipophilic balance (HLB) value from 14.9 (Tween 60) to 4.7 (Span 60). Furthermore, Span 60 has smaller hydrophilic head groups compared to Tween 60, which lead to smaller hydrophilic to hydrophobic area thus resulting in larger particle size (Basiri *et al*., 2017a).

The increase of lecithin content slightly reduced the particle size of the niosome. This result contradicts what was reported by previous studies (Jacob *et al*., 2017; Thomas & Viswanad, 2012). Cholesterol also did not affect the particle size of the niosome. This result is opposite to that what was reported by Thomas and Viswanad (2012). Despite non-significant effects of both lecithin and cholesterol on the particle size of niosome, the presence of these two components is very crucial for membrane stability and permeability of active ingredients (Aggarwal *et al.*, 2013).

Verification of Model

Validation of the model was conducted to determine the sufficiency of the final obtained model (Table 6). Four different niosome formulations were designated. The discrepancy between the actual and predicted response values was insignificant as their residual standard error (RSE) was below 5.00%, which indicated that the model was fitted to the system (Wahgiman *et al*., 2019). The predicted and actual values of particle size for optimized niosome formulation was 143.54 nm and 120.62 nm, with RSE of 1.18% (Table 7). From the result obtained, it indicates that a good model was successfully developed.

Physiochemical Characterisation of the Optimized Noisome

The particle size distribution of the optimized niosome containing JCH obtained was 120.62 ± 0.50 nm which is valid with the predicted value (118.2 nm) as its RSE is 1.18%. The polydispersity index (PDI) of 0.256 ± 0.008 was obtained, indicating a monodisperse system and zeta potential value of −6.62 ± 0.007 mV. The PDI value was used for the measurement of particle size distribution in the delivery system. The near-zero PDI value demonstrates a

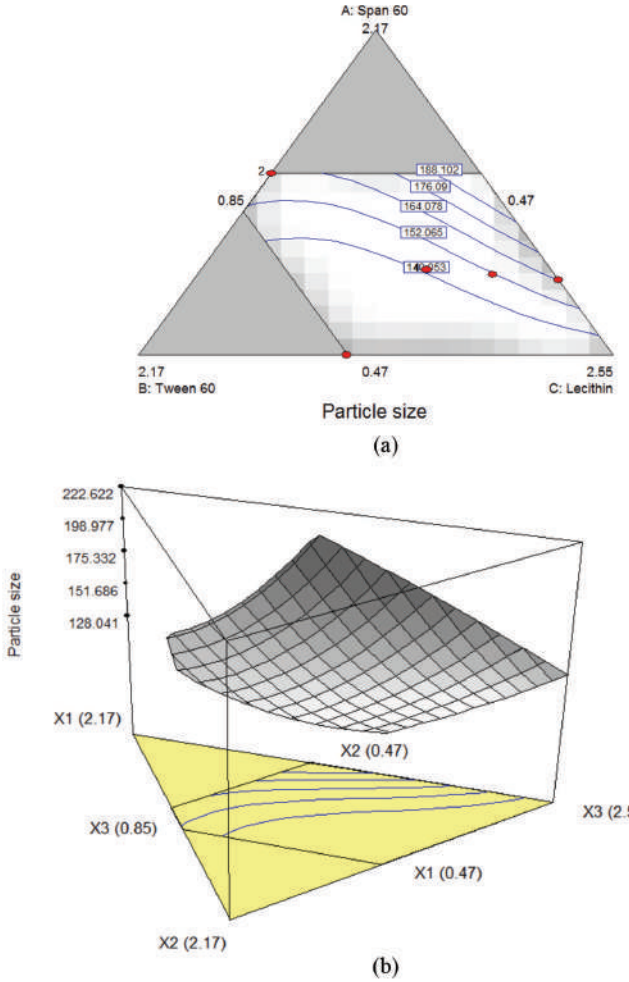


Figure 2: (a) Contour plot and Three-dimensional surface (b) showing the interaction effect between four variables (Span 60, Tween 60, lecithin and cholesterol) on the response (particle size)

Table 6: Validation set

Run	Span 60 (%)	Tween 60 (%)	Lecithin, (%)	Cholesterol, (%)	Particle Size (nm)		RSE (%)
					Actual	Predicted	
Validation							
1	0.52	1.10	1.94	0.70	120.70	121.02	0.26
2	0.50	1.41	1.50	0.85	140.90	143.40	1.74
3	0.86	0.90	1.80	0.70	134.50	138.62	2.97
4	0.60	1.20	1.76	0.70	115.60	119.51	3.27

Note: RSE is the residual standard error. Other components were kept constant;10 mL deionized water, 0.1 g JCH, 0.01 g cetylpyrinidium chloride and 0.08 g phenonips

Table 7: Optimized niosome formulation containing JCH

Span 60 (%)	Tween 60 (%)	Lecithin, (%)	Cholesterol, (%)	Particle Size (nm)		RSE (%)
				Actual	Predicted	
0.58	1.23	1.73	0.71	120.62	118.2	1.18

Note: RSE is the residual standard error. Other components were kept constant;10 mL deionized water, 0.1 g JCH, 0.01 g cetylpyrinidium chloride and 0.08 g phenonips

monodispersed system; meanwhile, a PDI value of ±1 implies a polydispersed system (Roselan *et al*., 2020). Zeta potential value determines the electrokinetic potential of a particle and the most favourable value is ±25 mV, indicating the stability of a delivery system (Che Sulaiman *et al*., 2016). Zeta potentials between ±5 and ±15 mV lead to minimal flocculation, whereas potentials between ±5 and ±3 mV lead to maximum flocculation (Ahmad *et al*., 2014).

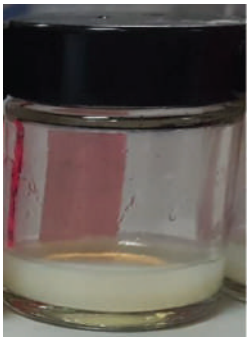
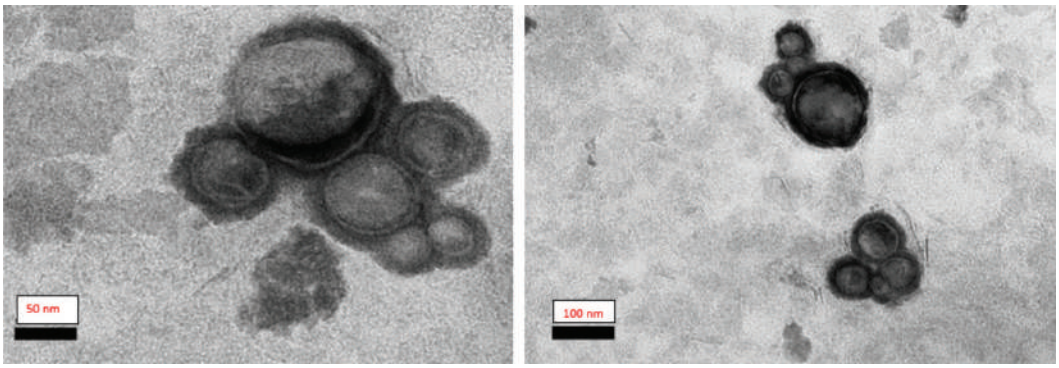


Figure 3: The presentative of the physical appearance of optimized niosome

The optimized niosome exhibited zeta potentials within the minimal flocculation, indicating that the formulation does not have adequate stability due to low electrostatic stabilization. From the visual observation in Figure 3 the niosomes containing JCH were whitish, fluid, slightly translucent, and homogenous with no observation of phase separation and JCH precipitation after 60 days of storage.

Figure 4 shows the formation of a spherical bilayer vesicle of the niosome containing JCH. It was found that the size obtained by TEM analysis correlated well with the particle size obtained from Zetasizer analysis. The TEM images also showed a non-aggregated and smooth spherical structure with no uniform size.

The pH value of the optimized niosome was 6.80 ± 0.05 indicating its suitability for topical use, as described by Syed Azhar *et al*. (2018), who indicated that the pH values would be within the 4.0-7.0 range. The optimized niosome was also found to be stable upon centrifugal force tests. Storage conditions at 25°C and 45°C, and the mechanical test are regular stability tests



(a) (b)

Figure 4: TEM images of niosome at different magnification (a) x 50000 (b) x 25000

subjected to cosmetics products. Samples were stored for 60 days (25°C and 45°C) observed for the absence of phase separation as an indication of its stability. The stability of optimized niosome formulation under different conditions were tested to ensure that the niosome formulation tolerates condition under market storage. The stability of the niosome formulation at 25°C was further studied through observation of changes in its particle size over 60 days. The particle size of the niosome formulation increased after 60 days, from 104.5 to 386.5 nm (within the nano-sized range). No distinct changes were observed during 60 days indicating that the niosome formulation was stable.

Antioxidant Activity of Optimized Niosome Formulation

DPPH (1-1-diphenyl-2-picrylhydrazyl) is a free and stable radical used for assessing the anti-radical function and the ability of antioxidant molecules to serve free-radical scavenger or hydrogen donors (Tavano *et al*., 2014). Conversely, metal ion chelating activity was studied as compound with Fe^{2+} chelating activity that can inhibit the formation of free radicals by reducing the ion concentration thus protecting against oxidative damage and related diseases (Alsarra *et al*., 2005). As shown in Figure 5, the antioxidant activities of optimized niosome formulation, including DPPH radical scavenging and metal ion chelating activities, were measured at different concentrations (1.25, 2.50, 5.00 and 10.00 mg/mL). At highest concentration, optimized niosome exhibited 65.9% DPPH scavenging activity ($p < 0.001$) and 34.6% metal chelating ($p < 0.05$), activities that might be contributed by potent antioxidant effect of JCH. JCH exhibited antioxidant activities including DPPH radical scavenging and metal ion-chelating assays up to 28% and 83%, respectively (Ab Aziz *et al*., 2020). This shows that the niosome formulation successfully preserved the antioxidant activities of JCH.

Antibacterial Activity

Figure 6 shows that the antibacterial activities of optimized niosome against *Staphylococcus aureus* were up to 98.8% at different concentrations (1.25, 2.50, 5.00 and 10.00 mg/mL). The inhibition might be contributed by Phenonip, which is a mixture of paraben and phenoxyethanol and is commonly used as preservative in cosmetic products (Dieu *et al*., 2019). *S.aures* is the most common bacteria present on human skin and has been associated with wound infection, pleuropulmonary, skin rash, infective endocarditis and bacteremia (Dong *et al*., 2020). Therefore, inhibition activity against this particular bacteria can protect against these complications.

Cytotoxicity Analysis

The toxicity of optimized niosome was tested on the mouse embryonic fibroblast cell line (3T3) by measuring reduced cell viability by increasing niosome concentration. Figure 7 shows the IC_{50} value (or known as inhibition of cell viability by 50%) of the blank niosome (without JCH) and optimized niosome (with JCH). The MTT assay results indicated that the relative viability decreases to below 85% as the sample concentration increases to a maximum concentration of 500 µg/mL. The survival of the

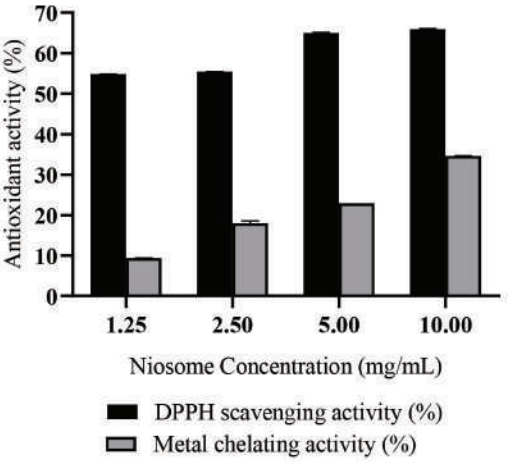


Figure 5: Antioxidant activities (DPPH scavenging and metal chelating activities) of optimized niosome formulation (pH 6.8 ± 0.05)

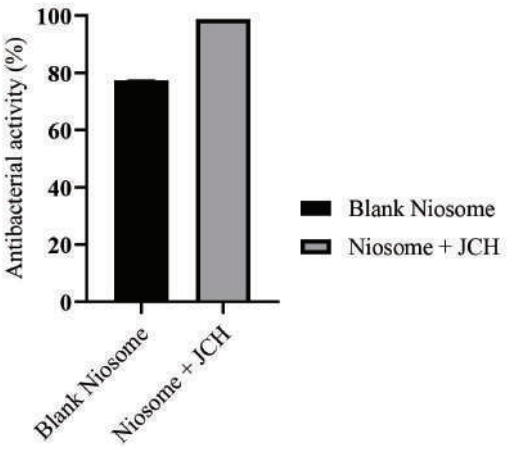


Figure 6: Antibacterial activities of blank niosome (without JCH) and optimized niosome (containing JCH) formulations against *S.aureus*

cells for empty niosome is higher compared to the optimized niosome. However, both niosome formulations exhibit IC_{50} value > 500 µg/mL, indicating that optimized niosome formulation is not toxic to normal skin cells and safe for topical application. Previous studies have reported that lesser toxicity has been observed in niosome made up of ester-based surfactants (Tweens and Spans) compared to that composed of ether-based surfactants (Brijs) (Nematollahi, *et al*., 2017). Each surfactant consist of different lengths of polyoxyethylene and hydrocarbon chains. Although polyoxyethylene and hydrocarbon chains do not significantly affect cell proliferation, the bond, particularly ester that links the alkyl chain to the polyoxyethylene group, can dramatically inhibit cell proliferation (Abdelkader *et al*., 2014). The enzymatic degradation of the ester bonds can lead to inhibition of cell proliferation (Chen *et al.,* 2019). On the other hand, Nematollahi *et al*. (2017) reported that the usage of cholesterol in niosome formulation results in lower cytotoxicity as well. Hence Span 60, Tween 60 and cholesterol have been great choices as niosome components.

Conclusion

This study revealed that optimized niosome formulation encapsulated JCH exhibit good antioxidant activities, 65.9% DPPH scavenging activity and 34.6% metal chelating activity and high inhibitory activity against *Staphylococcus aureus* (up to 98.8%). The optimized niosome also exhibits a small particle size and maintains its stability within 60 days. The optimized niosome with antioxidant and inhibitory properties against *S. aureus* strain suggest its potential application in topical administration. Further studies on characterization may be conducted in the future as well.

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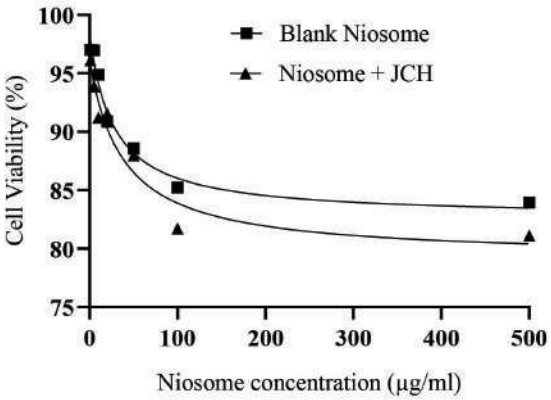

Figure 7: Toxicity effect of the blank niosome (without JCH) and optimized niosome (containing JCH) on 3T3 cells after 24 h exposure

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