Trop J Nat Prod Res, December 2023; 7(12):5509-5518

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Effect of Surfactant Combination on the Characteristics, Stability, Irritability, and Effectivity of Astaxanthin Nanoemulsion as Anti-Ageing Cosmetics

Vidya A. J. Yusuf[1,2], Widji Soeratri[2,3], Tristiana Erawati[2,3]*

[1]*Master Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Campus C Mulyorejo, Surabaya 60115, Indonesia*
[2]*Department of Pharmaceutical Sciences, Faculty Pharmacy, Universitas Airlangga, Surabaya, East Java-60115, Indonesia*
[3]*Pusat Unggulan IPTEK-Perguruan Tinggi (PUI-PT), Skin and Cosmetics Technology (SCT) Centre of Excellent, Universitas Airlangga, Surabaya East Java–601153, Indonesia*

ARTICLE INFO

Article history:
Received 05 October 2023
Revised 03 December 2023
Accepted 13 December 2023
Published online 01 January 2024

ABSTRACT

Astaxanthin is a xanthophyll carotenoid with antioxidant activity. The topical application of astaxanthin can enhance collagen density. However, its penetration into the skin is limited. Therefore, a delivery system, such as nanoemulsion, is needed. One factor influencing nanoemulsion formation is the selection of the type and concentration of surfactant. This study aims to determine the influence of surfactant combinations: Tween 80-Span 20 (F1), Tween 80-Span 60 (F2), and Tween 80-Span 80 (F3) in HLB system 14 on the characteristics, stability, irritability, and effectivity of astaxanthin nanoemulsion as an anti-ageing cosmetic. The nanoemulsion was prepared using the phase inversion composition (PIC) method. The pH values for all three formulas were within the range of normal skin pH (4-6), viscosity followed the order F1<F3<F2, droplet size F1<F3<F2, PDI for all three formulas was below 0.2, turbidity F3<F2<F1, %transmittance F3>F2>F1, interfacial tension F1<F3<F2, and zeta potential for all three formulas fell within the range of ±30 mV. Real-time tests showed that F3 was the most stable formula. All three formulas remained stable after centrifugation, did not cause irritation, and were able to enhance collagen density and skin elasticity in the order of F3>F1>F2. The characteristics of all three formulas meet the criteria for nanoemulsions with droplet sizes below 50 nm and polydispersity index (PDI) below 0.2 and without skin irritation. The Astaxanthin nanoemulsion with Tween 80-Span 80 (F3) surfactant combination was the most stable with better effects. Therefore, it has the potential for further formulation into anti-ageing cosmetic preparations.

Keywords: Anti-ageing, astaxanthin, characteristics, cosmetics, effectivity, irritation, nanoemulsion, stability

Introduction

Astaxanthin is a xanthophyll carotenoid naturally sourced from the green microalga *Haematococcus pluvialis*, producing a reddish-orange pigment.[1,2] Astaxanthin does not have pro-oxidative activity and does not convert into vitamin A.[3] So, it is highly effective in scavenging free radicals, with better antioxidant potential than vitamin E.[1,4] The antioxidant activity of astaxanthin is reported to be 10 times better than other carotenoids such as zeaxanthin, lutein, canthaxanthin, and *β*-carotene.[5] When used topically, astaxanthin can enhance collagen density by increasing tissue inhibition of metalloproteinases-1 (TIMP1) and reducing the expression of matrix metalloproteinase proteins (MMP1 and MMP3).[1]

Due to its physicochemical properties, astaxanthin is a relatively large molecule, with a molecular weight of 596.8 g/mol, has low solubility in water ($7.9x10^{-10}$ mg/L at 25^0C), and is highly lipophilic (log P 13.27).[6] This limits its penetration into the skin. Only small molecules with sizes <500 Da and lipophilic properties with log P values between 1-3 can penetrate through the stratum corneum.[7] Therefore, a delivery system like nanoemulsion is needed to enhance the penetration of astaxanthin into the skin, so enhance its effectiveness as an anti-ageing agent.

Nanoemulsion is a heterogeneous dispersion system of two immiscible liquids, such as oil and water, with an average droplet size of 20-200 nm, stabilised by a surface-active agent (surfactant).[8,9] Surfactants with low HLB (3-6) can form O/W nanoemulsions, while surfactants with high HLB (8-18) can form W/O nanoemulsions.[8] Specific combinations of surfactants with high and low HLB values are required to achieve stable nanoemulsion formation and to obtain the appropriate HLB for the surfactant system.[8,10,11] Previous studies have reported that O/W nanoemulsions with olive oil as the oil phase require an HLB value of 14.[11,12] This can be confirmed through physical stability testing of the system, such as the real-time and centrifugation tests.[13,14] To achieve this HLB value, a combination of surfactants with high HLB (such as Tween) and low HLB (such as Span) is needed. Tween and Span are nonionic surfactants that are relatively safer (less toxic) compared to ionic surfactants. They are minimally affected by pH, biocompatible, and have lower critical micelle concentration (CMC) values, which can reduce the concentration of surfactants used. Excessive surfactant amounts can lead to skin irritation after topical application.[8]

*Corresponding author. E mail: tristiana-e-m@ff.unair.ac.id
Tel: +628121671607

Citation: Yusuf VAJ, Soeratri W, Erawati T. The Effect of Surfactant Combination on the Characteristics, Stability, Irritability, and Effectivity of Astaxanthin Nanoemulsion as Anti-Ageing Cosmetics. Trop J Nat Prod Res. 2023; 7(12):5509-5518. http://www.doi.org/10.26538/tjnpr/v7i12.21

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Materials

Astaxanthin oleoresin (AstaLuxe® 5%, Evergen, Kendal), Ethanol 96% (PT. Brataco, Surabaya), Liquid paraffin (CV. Chemical Indonesia Multi Sentosa, Surabaya), Nipaguard® SCP (PT. Clariant Indonesia, Tangerang), Olive oil (Kimia Market, Bandung), Sodium chloride 0,9%, Sodium lauryl sulphate (SLS) 1%, Span 20 (CV. Total Equipment Pharmacy, Semarang), Span 60 (PT. Dwilab Mandiri Scientific, Bandung), Span 80 (PT. Dwilab Mandiri Scientific, Bandung), Tween 80 (PT. Brataco, Surabaya).

Methods

Preparation of Nanoemulsion

The O/W nanoemulsion formulation consists of Astaxanthin Oleoresin, Olive Oil, Tween 80 (T80), Span (S20, S60, S80), 96% Ethanol, Nipaguard (antimicrobial agent), and Distilled Water, it shown in Table 3. The method previously described by Erawanti (2015) was adopted for the formulation of the nanoemulsions used in this study.[11] Briefly, different combinations of the nanoemulsions (F1, F2 and F3) using a combination of Tween 80 (T80) and Span (S20, S60, and S80), respectively, in the ratio of T80-S20 (0.85:0.15), T80-S60 (0.91:0.09), and T80-S80 (0.91:0.09) were prepared at a surfactant to cosurfactant ratio of 6:1 (S_{mix}), and oil phase to S_{mix} of 1:9, (Table 1).[11] The O/W nanoemulsion was prepared by mixing 1% Astaxanthin Oleoresin, and Olive Oil (1:19). Then 96% Ethanol was added to the various mixtures: F1 (Astaxanthin oleoresin, olive oil, T80-S20), F2 (Astaxanthin oleoresin, olive oil, T80-S60), and F3 (Astaxanthin oleoresin, olive oil, T80-S80). The mixture and water phase was then heated to 50-60℃. Next, the mixture was stirred with a magnetic stirrer (Thermo Fisher Cimarec$^+$, US) at a speed of 600 rpm for 5 minutes. The water phase was added slowly (dropwise) to the mixture and stirred with a magnetic stirrer at a speed of 500 rpm for about 3 minutes and then was increased to 1000 rpm until the water phase was fully incorporated, resulting in a clear nanoemulsion. Finally, the antimicrobial agent (0.5% Nipaguard) was added to the nanoemulsion.

Nanoemulsion Characterisation

pH and viscosity Measurement

The pH of the different nanoemulsion formulations was measured using a pH meter (Eutech pH 700, US) as described.[11] While the viscosity measurement was performed with an Ostwald viscometer as previously described.[15]

Droplet Size and PDI Examination

The droplet size and PDI of the formulations were examined using a Particle analyser (Delsa^{TM} Nano C, US) as described previously.[11]

Turbidity and %Transmittance Examination

Turbidity and %transmittance examination was conducted using a UV-Vis Spectrophotometer (Hitachi UH5300, Japan).[16] Approximately 3 mL of the sample was placed in a cuvette. The absorbance and %transmittance were measured at the maximum wavelength of 669 nm. Subsequently, the turbidity value was calculated using the following formula:

$$\text{Turbidity} = \frac{2.303 \text{ x absorbance}}{\text{Path length}} \qquad (1)$$

Interfacial Tension Examination

Interfacial tension examination of the formulations was carried out using a Du Nouy Tensiometer (Huazheng Electric HZZL-3, China).[17]

Zeta Potential Examination

In this procedure, 1 mL of the prepared nanoemulsion was diluted 100 times, injected into a disposable zeta cell (DT1060C) and analysed using a Particle analyser (Litesizer 500, US).[18]

Nanoemulsion Stability Test

Real-Time

Approximately 10 mL of the sample was placed in a vial and stored at room temperature (25^{0}C) for 3 months. Then, the formulations were observed for changes in colour, odour, consistency, and separation.[13]

Centrifugation Test

To assess the resistance of the nanoemulsions to external forces, approximately 10 mL of the sample was placed in a tube and centrifuged at a speed of 3000 rpm for approximately 30 minutes[14], using a Hettich Rotofix 32A, Centrifuge, Germany.

Irritability Test

Fresh fertile chicken eggs (not more than 7 days old) were placed in an incubator at a temperature of 37.8 ± 0.2℃ and a relative humidity of 58 ± 2%. The eggs were then manually rotated twice a day for 8 days. On the 8th day, the eggs are observed under light to verify the presence and position of the embryo and to separate infertile or damaged eggs. On the 9th day, the eggs were removed from the incubator for testing.[19,20] Test samples in the form of a liquid can be used directly without further dilution. Sodium lauryl sulphate (SLS) solution (1%) was used as a positive control, while the negative control was a 0.9% sodium chloride solution.[20] Furthermore, the air space in the egg was marked, and the shell was carefully opened. Then, the inner membrane was moistened with 2–3 mL of 0.9% sodium chloride solution. After that, the egg was returned to the incubator for a maximum of 30 minutes. The 0.9% sodium chloride solution was removed, and the inner membrane was detached. Subsequently, a silicone rubber ring was placed on the chorioallantoic membrane (CAM). Approximately 0.3 mL of the test sample was applied directly to the CAM surface inside the ring. Reactions occurring on the CAM were observed for 5 minutes. The time of the appearance of the endpoint, such as lysis, bleeding, or coagulation, was recorded.[19,20] Irritation Score (IS) was calculated from the formula:

$$\text{IS} = \left(\left(\frac{301-t_h}{300}\right) \text{x } 5\right) + \left(\left(\frac{301-t_l}{300}\right) \text{x } 5\right) + \left(\left(\frac{301-t_c}{300}\right) \text{x } 5\right) \qquad (2)$$

Where t_h = time of the first appearance of bleeding (s); t_l = time of first appearance of lysis (s); t_c = time of the first appearance of coagulation.[19]

Subsequently, the results obtained were classified based on irritation categories as follows:

Table 1: Irritation categories based on irritation score[19]

Irritation score	Irritation categories
0 – 0.9	Non-irritant
1 – 4.9	Slight irritant
5 – 8.9 or 5 – 9.9	Moderate irritant
9 – 21 or 10 – 21	Severe irritant

Effectivity Test

Ethical Approval

Ethical approval was obtained from the health research ethics committee of the Faculty of Nursing, Universitas Airlangga, with ethical approval number 2948-KEPK before the commencement of this study.

Selection of respondents

The population for the effectiveness testing consists of female respondents who are employees within the Universitas Airlangga environment. The sample is comprised of female respondents with the following inclusion, exclusion, and dropout criteria:

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 2: Inclusion, exclusion, and dropout criteria for respondents[21,22,23]

Inclusion criteria
1. Healthy women aged 35 - 45 years
2. Willing to sign an informed consent
3. Willing to discontinue the use of other products in the test area (such as lotion, body serum, etc.) one week before and during the testing
4. Willing to cooperate throughout the research
Exclusion criteria
1. Pregnant or breastfeeding
2. Have a history of allergies to the used substances.
3. Have tattoos and open wounds/cuts in the test area.
4. Using hormonal contraception in the last 3 months, antihistamines, non-steroidal anti-inflammatory drugs (NSAIDs), steroids, or laser therapy in the last 2 weeks, oral retinoids in the last 6 months, and topical retinoids in the last 2 months
5. Suffer from skin infections, atopic dermatitis, eczema, psoriasis, and skin cancer.
6. Smoke, consume alcohol, or use drugs.
Dropout criteria
1. Show allergic reactions to the test product during the study.
2. Do not use the test product.
3. Do not come to the research location for examination.
4. Withdraw from the study.

The determination of the sample size for respondents in this research used purposive sampling, which is a non-random technique for selecting respondents and does not require a fundamental theory or a predetermined number of respondents.[24] In this case, the researcher is free to select and determine the number of respondents according to the research objectives. Therefore, the sample size was 12 respondents. Subsequently, the respondents were randomly grouped into four treatment groups (3 test groups and 1 control group) using a lottery (double-blind) method so that each group consisted of 3 respondents.

Effectivity test method

The astaxanthin NE sample was applied to the back of the left hand of the respondent (2 drops) twice a day (morning and evening) for 4 weeks. The effectiveness test was conducted by measuring collagen density, which is assessed based on low echogenic band (LEB) value, and skin elasticity, which is assessed based on Young's modulus (E) value using the DermaLab Combo® (Cortex Technology, Denmark) High-Resolution Ultrasound Probe and Elasticity Probe instrument, respectively, before and after the use of the astaxanthin NE test sample, then compared to the control, which is astaxanthin oleoresin diluted with liquid paraffin (without NE).

Statistical Analysis

The data from each experiment is presented as mean ± standard deviation (SD) values of three replicates (n=3). Subsequently, the data was processed using the IBM SPSS Statistics 20 with a confidence level of 95% ($\alpha = 0.05$).

Results and Discussion

Nanoemulsion Characterisation

Nanoemulsion is one of the delivery systems capable of transporting water-insoluble (lipophilic) active ingredients, enhancing skin penetration due to its small droplet size (20-200 nm) and large surface area. Additionally, it can protect against oxidation and hydrolysis, thus improving the stability of chemically unstable active ingredients like astaxanthin.[8,25,26] One influential factor in nanoemulsion formation is the choice of surfactant type and concentration. Selecting the right surfactant is crucial to ensure safety (avoiding irritation) and to ensure that the amount of surfactant used in the formulation is sufficient to stabilise the nanoemulsion droplets. Furthermore, the use of more than one surfactant (a combination) is also necessary to achieve the appropriate HLB (Hydrophilic-Lipophilic Balance) for the system.[8,10,11] The HLB system used in this research is 14.[11,12]

Table 3: Formula of Astaxanthin nanoemulsion

Materials	F1	F2	F3
Astaxanthin Oleoresin	0.05%	0.05%	0.05%
Olive oil	0.95%	0.95%	0.95%
Tween 80	6.5%	7%	7%
Span 20	1.2%	-	-
Span 60	-	0.7%	-
Span 80	-	-	0.7%
Ethanol 96%	1.3%	1.3%	1.3%
Nipaguard	0.5%	0.5%	0.5%
Aquadest	Ad 100%	Ad 100%	Ad 100%

Table 4: Characteristics of Astaxanthin nanoemulsion

Characteristic	F1 (T80-S20)	F2 (T80-S60)	F3 (T80-S80)	p-value $\alpha = 0.05$
pH	6.01 ± 0.01	6.15 ± 0.01	6.29 ± 0.01	0.027
Viscosity (mPa.s)	1.43 ± 0.01	1.59 ± 0.01	1.49 ± 0.00	0.023
Droplet size (nm)	19.8 ± 0.9	24.2 ± 0.6	23.9 ± 0.9	0.001
PDI	0.025 ± 0.006	0.070 ± 0.050	0.106 ± 0.071	0.224
Turbidity (%)	0.172 ± 0.001	0.164 ± 0.001	0.160 ± 0.001	0.025
%transmittance	17.9 ± 0.1	19.4 ± 0.2	20.2 ± 0.1	0.027
Interfacial tension (mN/m)	35.8 ± 0.1	39.0 ± 0.2	37.8 ± 0.1	0.027
Zeta potential (mV)	-20.8 ± 1.1	-24.7 ± 4.4	-25.9 ± 3.4	0.215

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The characterisation of astaxanthin nanoemulsion includes pH, viscosity, droplet size, PDI, turbidity, %transmittance, interfacial tension, and zeta potential, as shown in Table 4. In the Table 4 shows that the pH test results for the three formulas, F1, F2, and F3, were 6.01 ± 0.01, 6.15 ± 0.01, and 6.29 ± 0.01, respectively, which fall within the range of normal skin pH (4-6).[27] Therefore, it is expected not to irritate when used topically. Based on statistical analysis using the Kruskal-Wallis test, a p-value of 0.027 was obtained, which means there is a significant difference among the three formulas. Post hoc testing revealed that F1 significantly differs from F3, with the order of F1 < F2 < F3.

Furthermore, the viscosity test results were 1.43 ± 0.01, 1.59 ± 0.01, and 1.49 ± 0.00 mPa.s, respectively. Based on statistical analysis using the Kruskal-Wallis test, a p-value of 0.023 was obtained. Post hoc testing revealed that F1 significantly differs from F2, with the order of F1 < F3 < F2. Therefore, the difference in surfactant combinations affects viscosity, where the viscosity of the NE with the T80-S60 combination (F2) was higher than that of the T80-S20 (F1) and T80-S80 (F3) combinations. Similar results were also reported by Cho et al.[16] According to the literature, Span 60 is solid at room temperature due to its relatively longer saturated hydrocarbon chain. In contrast, Span 20 and Span 80 are liquid at room temperature, each having relatively shorter and unsaturated fatty acid chains.[28] This likely causes the viscosity of Span 60 to be higher than that of Span 20 and Span 80.

The droplet size test results were 19.8 ± 0.85 nm, 24.2 ± 0.85 nm, and 23.9 ± 0.9 nm, respectively. Based on statistical analysis using the One-way ANOVA, a p-value of 0.001 was obtained, which is less than 0.05. Post hoc testing with Tukey HSD revealed that F1 significantly differs from F2 and F3 in the order of F1 < F3 < F2. It can be seen that F1 and F3 have smaller droplet size compared to F2. This may be due to the interaction between the fatty acid chains of Span 20 and Span 80 (being smaller) than that of Span 60. The shorter (Span 20) or less saturated (Span 80) the chains, the smaller the chain-chain interactions. Conversely, the longer or more saturated the chains (Span 60), the greater the interactions.[29] The ability of Span to form nanoemulsions with small droplet sizes mainly depends on chain-chain interactions during the mixing process. The resulting droplet size also depends on the balance between droplet disruption and coalescence. Thus, when the oil-water interface is composed of Span 60, there will be a higher collision efficiency because the interactive hydrocarbon chains are exposed to the oil phase.[28] Consequently, more energy is required to break the bonds between its chains. Therefore, the formed droplets become larger.

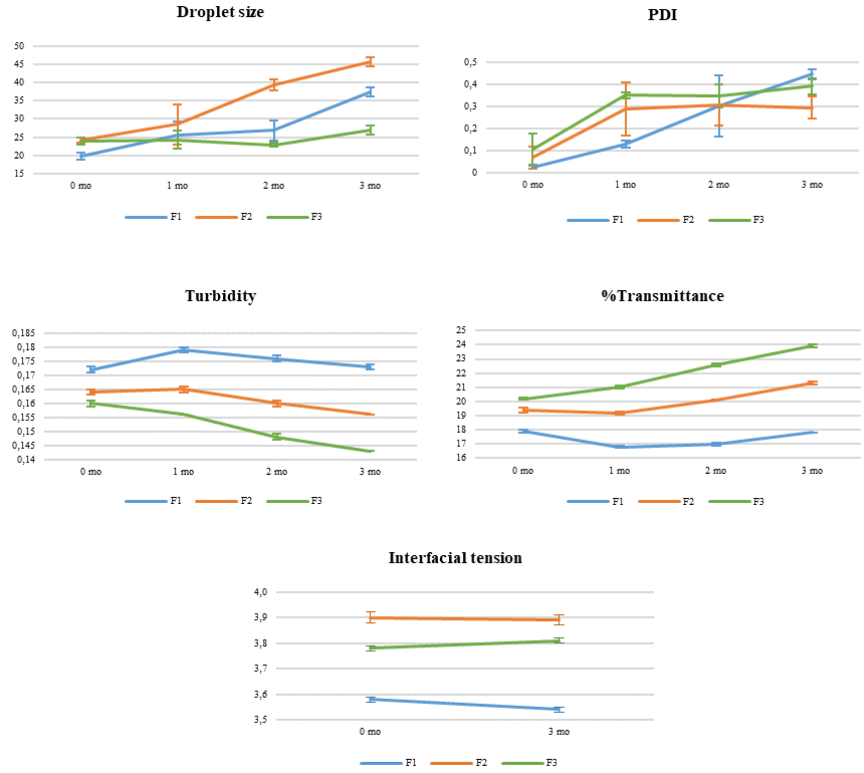

Figure 1: Graph of droplet size values, PDI, turbidity, %transmittance, and interfacial tension changes after real-time stability test

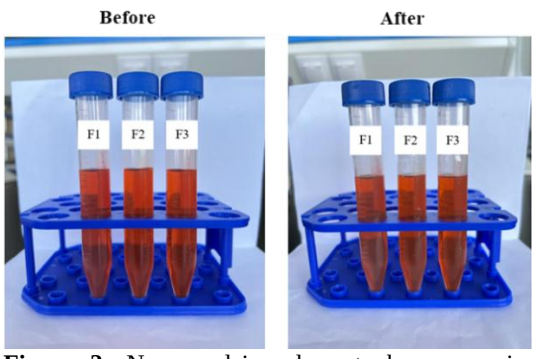

Figure 2: Nanoemulsion do not show any sign of phase separation after centrifugation test

Similarly, the PDI test results were 0.025 ± 0.006, 0.070 ± 0.050, and 0.106 ± 0.07, respectively, all of which were below the value of 0.2, indicating that the droplets were homogeneously distributed.[30] Based on statistical analysis using the One-way ANOVA, a p-value of 0.224 was obtained, which is greater than 0.05, indicating no significant difference among the three formulas.

The level of turbidity and %transmittance are some parameters determined for formulated nanoemulsions. The turbidity test results for the three formulas were 0.172 ± 0.001, 0.164 ± 0.001, and 0.160 ± 0.001, all falling below 1%. Based on statistical analysis using the Kruskal-Wallis test, a p-value of 0.025, which is less than 0.05, was obtained. Post hoc testing revealed that F1 significantly differs from F3, in the order of F3 < F2 < F1. A nanoemulsion is considered transparent when its turbidity value is <1%, translucent (light-penetrating) when ≥1%, and cloudy when ≥2%.[16]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 5: Droplet size values before and after real-time test

Formula	Droplet size (nm)				p-value α = 0.05
	0 mo	1 mo	2 mo	3 mo	
F1 (T80-S20)	19.8 ± 0.9	25.7 ± 3.7	26.9 ± 2.8	37.4 ± 1.3	0.042[a]
F2 (T80-S60)	24.2 ± 0.6	28.5 ± 5.4	39.4 ± 1.6	45.7 ± 1.3	0.015[b]
F3 (T80-S80)	23.9 ± 0.9	24.3 ± 2.4	22.9 ± 0.6	27.0 ± 1.3	0.072[b]

[a] Friedman test
[b] Repeated measures ANOVA

Table 6: PDI values before and after real-time test

Formula	PDI				p-value α = 0.05
	0 mo	1 mo	2 mo	3 mo	
F1 (T80-S20)	0.025 ± 0.006	0.131 ± 0.016	0.302 ± 0.137	0.446 ± 0.023	0.050[a]
F2 (T80-S60)	0.070 ± 0.050	0.288 ± 0.119	0.306 ± 0.094	0.295 ± 0.049	0.144[a]
F3 (T80-S80)	0.106 ± 0.071	0.350 ± 0.012	0.348 ± 0.052	0.391 ± 0.037	0.034[a]

[a] Repeated measures ANOVA

Table 7: Turbidity values before and after real-time test

Formula	Turbidity (%)				p-value α = 0.05
	0 mo	1 mo	2 mo	3 mo	
F1 (T80-S20)	0.172 ± 0.001	0.179 ± 0.001	0.177 ± 0.001	0.173 ± 0.001	0.032[a]
F2 (T80-S60)	0.164 ± 0.001	0.165 ± 0.001	0.160 ± 0.001	0.156 ± 0.000	0.032[a]
F3 (T80-S80)	0.160 ± 0.001	0.156 ± 0.000	0.148 ± 0.001	0.143 ± 0.000	0.029[a]

[a] Friedman test

The %transmittance test results were 17.9 ± 0.1, 19.4 ± 0.2, and 20.2 ± 0.1, respectively. Statistical analysis using the Kruskal-Wallis test showed a p-value of 0.027, which is less than 0.05. Post hoc testing revealed that F1 significantly differs from F3, with the order of F3 > F2 > F1. %Transmittance values are inversely related to turbidity values. The smaller the turbidity value, the more light will be transmitted.

Moreover, the values obtained were much lower than the %transmittance of water, which is 100%. These values may be due to the red colour of astaxanthin nanoemulsion, whereas water is colourless. When light waves pass through the nanoemulsion, they are selectively absorbed by the chromophore groups in the astaxanthin structure, a long polyene chain of 13 conjugated double bonds, at that particular wavelength. Therefore, less light is transmitted and reflected.[31,32]

The interfacial tension test results were 35.8 ± 0.1, 39.0 ± 0.2, and 37.8 ± 0.1, respectively. Statistical analysis using the Kruskal-Wallis test showed a p-value of 0.027, which is less than 0.05. Post hoc testing revealed that F1 significantly differs from F2, with the order of F1 < F3 < F2. Specific surfactant combinations result in lower interfacial tension than single surfactants.[33] Cho et al. reported that the combination of Span and Tween surfactants is more effective in reducing interfacial tension compared to Tween alone.[16] Based on the test results, it was observed that the interfacial tension of F1 and F3 was lower than that of F2. This result is supported by the droplet size test results, indicating that a lower oil-water interfacial tension results in smaller droplets. Therefore, the tendency for aggregation decreases, preventing various stability-related issues, such as creaming and sedimentation.[34]

Zeta potential represents the electrostatic charge on the droplet's surface. The sign and magnitude of surface potential are important parameters that determine the physicochemical properties of nanoemulsions, such as physical stability (aggregation), chemical stability, material interactions, and surface adhesion.[32] The zeta potential test results were -20.8 ± 1.1, -24.7 ± 4.4, and -25.9 ± 3.4 mV. They were within the range of ±30 mV, indicating good physical stability, with the repulsive forces being higher than the attractive forces.[30] Based on statistical analysis with One-way ANOVA, a p-value of 0.215 was obtained, which is greater than 0.05, indicating no significant difference among the three formulas.

Stability Test

Real-Time Test

Real-time stability testing is typically conducted over a longer duration to allow for significant product degradation under recommended storage conditions.[35] Friedman test analysis of the formulated NE, it was observed that the droplet size of F1 increased significantly with a p-value of 0.042 < 0.05. Repeated measures analysis with ANOVA indicates that the droplet size of F2 also significantly increased with a p-value of 0.015 < 0.05. However, there was no significant difference in droplet size for F3 with a p-value of 0.072 > 0.05, it shown in Table 5 and Figure 1. It can be observed that the droplet size of F3 remains stable after real-time test.

Also, repeated measures analysis using ANOVA showed no significant difference in the PDI values of F1 after the real-time test (p=0.05). Also, the PDI values of F2 were not significantly different, with a p-value of 0.144 > 0.05. However, PDI values of F3 showed a significant difference with a p-value of 0.034 < 0.05, it shown in Table 6 and Figure 1.

In this study, the red color in Astaxanthin nanoemulsion leads to the selective absorption of light, resulting in an increase in absorbance values. Consequently, the turbidity values also increase.[36] Furthermore, an increase in droplet size in the red-colored nanoemulsion can reduce absorbance values and color intensity due to an enhancement in multiple light scattering.[32] Therefore, based on Friedman test analysis of the samples showed that the turbidity of F1 and F2 increased in the first month and then decreased in the second and third months, with p-values of 0.032 < 0.05 after the real-time test. However, the turbidity of F3 significantly decreased with a p-value of 0.029 < 0.05, it shown in Table 7 and Figure 1. Also, the %transmittance of F1 and F2 decreased in the first month and increased in the second and third months, with p-values of 0.032 and 0.029 < 0.05 after the real-time test, respectively. In contrast, the %transmittance of F3 significantly increased with a p-value of 0.029 < 0.05, it shown in Table 8 and Figure 1.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 8: %Transmittance values before and after real time test

Formula	%Transmittance				p-value
	0 mo	1 mo	2 mo	3 mo	α = 0.05
F1 (T80-S20)	17.9 ± 0.1	16.8 ± 0.1	17.0 ± 0.1	17.8 ± 0.0	0.032[a]
F2 (T80-S60)	19.4 ± 0.2	19.2 ± 0.1	20.1 ± 0.0	21.3 ± 0.1	0.029[a]
F3 (T80-S80)	20.2 ± 0.1	21.0 ± 0.1	22.6 ± 0.1	23.9 ± 0.1	0.029[a]

[a] Friedman test

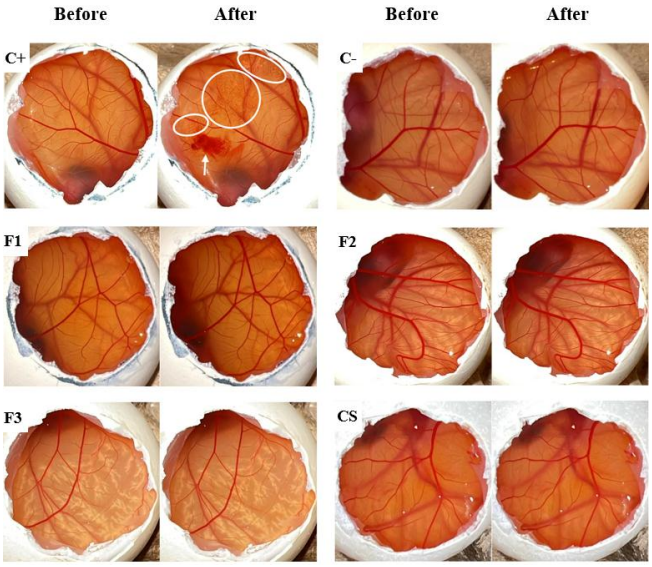

Figure 3: The irritation test result on the positive control (C^+), negative control (C^-), F1, F2, F3, control sample (CS), arrow symbols ↑ indicate bleeding and circle symbols O indicate lysis

Table 9: Interfacial tension values before and after real-time test

Formula	Interfacial tension (mN/m)		p-value
	0 mo	3 mo	α = 0.05
F1 (T80-S20)	35.8 ± 0.1	35.4 ± 0.1	0.102[a]
F2 (T80-S60)	39.0 ± 0.2	38.9 ± 0.2	0.184[b]
F3 (T80-S80)	37.8 ± 0.1	38.1 ± 0.1	0.102[a]

[a] Wilcoxon test
[b] Paired T-test

Table 10: The results of the irritability test of Astaxanthin NE

Treatment group	Irritation score
Positive control	8.15 ± 0.13
Negative control	0.00 ± 0.00
F1 (T80-S20)	0.00 ± 0.00
F2 (T80-S60)	0.00 ± 0.00
F3 (T80-S80)	0.00 ± 0.00
Sample control*	0.00 ± 0.00

*Without NE

Based on statistical analysis using the Wilcoxon test, the interfacial tension values for F1 and F3 did not significantly differ with p-values of 0.102 > 0.05 after the real-time test. Furthermore, using the Paired T-test, the interfacial tension values of F2 also did not differ significantly with a p-value of 0.184 > 0.05, as shown in Table 9 and Figure 1.

Therefore, it can be assumed that the nanoemulsion formulated with the T80-S80 surfactant combination (F3) was the most stable formula compared to T80-S20 (F1) and T80-S60 (F2) in the HLB system of 14 after storage at room temperature for 3 months. Literature reports that when surfactant combinations have the same side chains, such as T80-S80 (F3) (both oleic acid), they are more likely to mix easily.[11,37] On the other hand, the combination of T80-S60 (F2) has different side chains, namely oleic acid (unsaturated, C18:1) and stearic acid (saturated, C18:0), making it less likely to mix, even though they have the same number of hydrocarbon chains. Additionally, for the T80-S20 (F1) surfactant combination, its stability is more influenced by the difference in the HLB values of the two surfactants, which is 6.4, falling within the moderate range,[38] despite having different side chains of oleic and lauric acids.

Centrifugation Test

Centrifugation is commonly performed to test the stability of nanoemulsions regarding phase separation. A kinetically stable nanoemulsion should maintain its homogeneity during high-speed centrifugation.[9] The results of the centrifugation test revealed that all three formulas remained stable and do not show any sign of phase separation after being centrifuged at a speed of 3000 rpm for approximately 30 minutes (Figure 2).

Irritability Test

The irritability test result is shown in Table 10. The irritation score of CAM when treated with SLS 1% (positive control) was 8.15 ± 0.13, which is within the range of 5.0 to 8.9 (moderate irritation). The occurrence of irritation is characterised by bleeding or coagulation, as depicted in Figure 3. On the other hand, the irritation score for CAM when treated with 0.9% sodium chloride (negative control), the test samples (F1, F2, F3), and the control sample were 0.00 ± 0.00, indicating no irritation. The irritation test results indicate that none of the three formulas induce any irritation reactions when compared to the positive control (SLS 1%). SLS is an anionic surfactant, meaning it's a negatively charged surfactant formed out of a combination of saturated/unsaturated hydrocarbon chains or hydrophilic groups with strong acids such as sulphate (-O-SO3) or sulphonate (-SO3)[39], which is responsible for the irritation of SLS. SLS has the potential to eliminate cells and DNA components while also causing harm to collagen and glycosaminoglycans.[40] On the skin, SLS can disrupt cell barriers and damage cell proteins by forming positively charged side groups.[39] An SLS solution in water will swell and disrupt the stratum corneum, affecting the lipid and protein structure. SLS can also break down and expand the α-keratin structure, thereby increasing the surface area and thickness of the stratum corneum. The incorporation of SLS into the lipid structure can reduce the ability of lipids to bind to each other, leading to lipid fluidisation between cells and the removal of lipids. Furthermore, SLS can increase Transepidermal Water Loss (TEWL), possibly due to increased blood flow and skin temperature in cases of irritation.[41] Several studies also suggest that SLS has a direct effect on corneocytes and denatures the keratin structure through direct binding. As a result, repeated doses of SLS can lead to dry, cracked skin and even contact dermatitis.[39]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

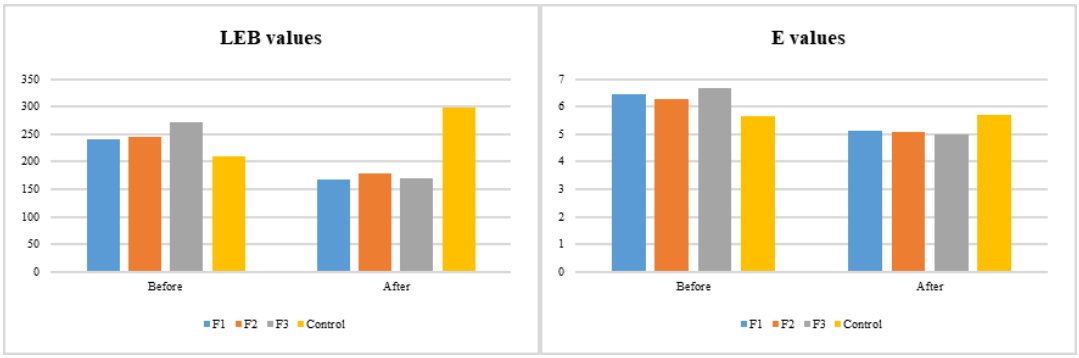

Figure 4: Histogram of LEB and E values changes after effectivity test

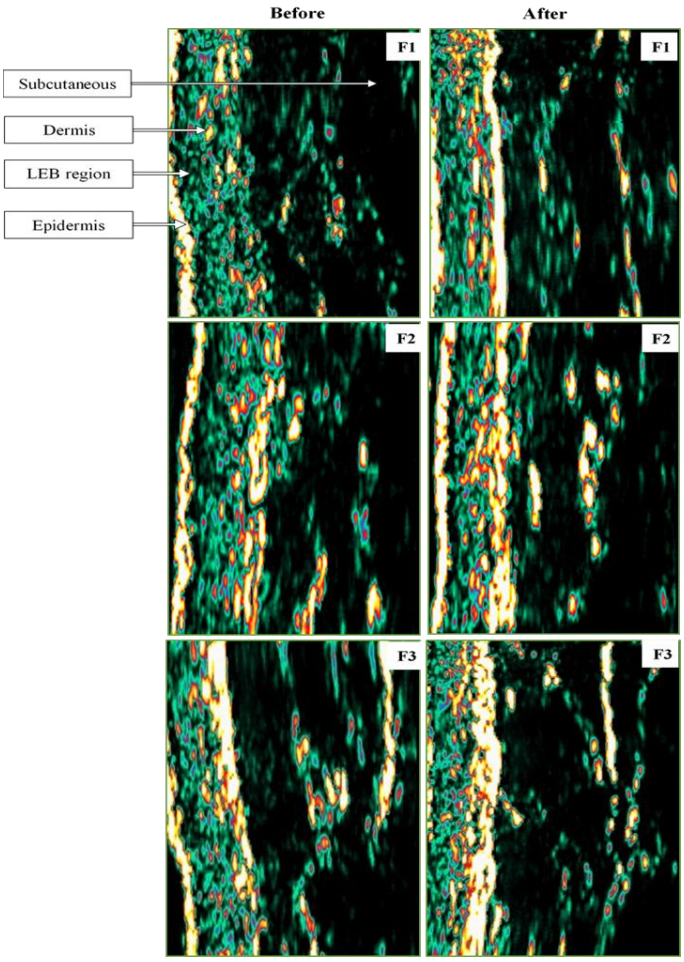

Figure 5: Ultrasound images of respondents' skin before and after treatment using nanoemulsion F1, F2, F3

In this study, all three nanoemulsion formulas use Tween and Span surfactants, which are nonionic. Unlike anionic surfactants, nonionic surfactants are not electrically charged, and their binding to proteins is limited or absent due to weak hydrophobic interactions, which do not cause protein denaturation. Additionally, nonionic surfactants can more easily penetrate the stratum corneum compared to anionic surfactants, which strongly bind to the stratum corneum and tend to cause less irritation to the skin.[42,43]

Effectivity Test

The thickness of LEB is considered to reflect the level of skin ageing and can be used to observe the severity of photoaging caused by collagen degeneration. Therefore, larger LEB values indicate a greater propensity to reduce collagen density.[44,45] Furthermore, Young's modulus (E) value indicates skin stiffness, where the lower the E value, the higher the skin elasticity.[46] Statistical tests using the Paired T-test showed that all three Astaxanthin nanoemulsion formulas (F1, F2, and F3) significantly improve collagen density with p-values of 0.003, 0.000, and 0.013, respectively (Table 11 & Figure 4A). Additionally, all three formulas also significantly enhance skin elasticity with p-values of 0.043, 0.045, and 0.038, respectively, after using the test samples for 1 month (Table 12 & Figure 4B). This result may be due to the very small droplet sizes (<50 nm), homogeneous droplet distribution, and low viscosity of all three formulas. According to the literature, small droplet size and low PDI values can increase the surface area, enhancing the permeation of astaxanthin through the epidermis and dermis.[34,47] The smaller the particle size, the greater the amount of astaxanthin present in the stratum corneum, thus increasing the amount that diffuses into the epidermis and dermis. Viscosity also affects the release of astaxanthin from the formulation. Lower viscosity leads to more significant movement of astaxanthin within the nanoemulsion, facilitating faster release.[48] Additionally, the aqueous phase, serving as the continuous phase, can cause the swelling of cells in the stratum corneum, widening the channels through which astaxanthin molecules pass.[34] The olive oil used as the oil phase also acts as a good enhancer, increasing the fluidity of the lipid barrier between cells by forming separate domains that disrupt the continuity of the stratum corneum, thereby inducing penetration through the stratum corneum.[49] On the other hand, the group of respondents using only the control sample experienced a decrease in collagen density as there was no system to deliver astaxanthin into the dermis.

Subsequently, a statistical test was conducted using the Kruskal-Wallis test to examine the differences among the formulations by comparing the delta values of each parameter in the effectiveness test. As shown in Table 13, the LEB values of the three formulations differ significantly, with a p-value of 0.042. Furthermore, the post-hoc test results indicate that the LEB value of F3 was significantly different from F2. However, the values for elasticity do not differ significantly, with p-values of 0.466. Thus, in terms of effectiveness in increasing collagen density, the ranking would be F3 > F1 > F2.

From the results of the difference tests between the formulas, it could be suggested that the nanoemulsion formulated with a surfactant combination of T80-S80 (F3) is the most effective compared to T80-S20 (F1) and T80-S60 (F2) in the HLB system 14. Also, F3 was the most stable formula, as its droplet size did not significantly differ after storage at room temperature for 3 months. As indicated in Figure 5, the thickness of the LEB region in F3 becomes smaller after 1 month compared to F1 and F2, indicating an increase in collagen density. This is because both Tween 80 and Span 80 have oleic acid side chains that act as enhancers. Oleic acid can interact and modify the lipid domain of the stratum corneum by disrupting the packing and structure of the lipid bilayer.[50] When oleic acid is used together with ethanol (cosurfactant), it can cause lipid extraction and form water channels, making the stratum corneum more permeable.[51,52]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Conclusion

This study was designed to obtain a formula for the formulation of a nanoemulsion with the best physical characteristics using different physical parameters tests and the formula that showing better biological activity (irritability and effectivity). Results of the study showed that the Astaxanthin nanoemulsion formulated using a Tween 80-Span 80 surfactant combination (F3) exhibited the best physical characteristics and biological effectiveness. This formula holds the potential for the formulation of stable and effective anti-ageing cosmetic preparations.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 11: The results of collagen density of Astaxanthin NE

Treatment group	Respondent	LEB values (µm)		p-value α = 0.05
		Before	After	
F1	SS	237	158	0.003[a]
	AN	211	145	
	DS	276	198	
F2	KI	171	105	0.000[a]
	SI	263	198	
	ED	303	237	
F3	DR	250	145	0.013[a]
	LR	303	184	
	ND	263	184	
Control	NS	263	342	0.031[a]
	AI	263	382	
	AK	105	171	

[a] Paired T-test

Table 12: The results of skin elasticity values of Astaxanthin NE

Treatment group	Respondent	E values (MPa)		p-value α = 0.05
		Before	After	
F1	SS	6.4	4.5	0.043[a]
	AN	6.3	5.2	
	DS	6.7	5.7	
F2	KI	6.5	5.4	0.045[a]
	SI	4.9	4.1	
	ED	7.5	5.8	
F3	DR	6.7	5.7	0.038[a]
	LR	6.2	4.2	
	ND	7.1	5.1	
Control	NS	5.8	5.9	0.667[a]
	AI	5.3	5.4	
	AK	5.9	5.8	

[a] Paired T-test

Table 13: Comparison of the delta values of each parameter in the effectiveness test

Parameter	Delta values (Δ)			p-value α = 0.05
	F1	F2	F3	
LEB values	-74.3 ± 7.2	-65.7 ± 0.6	-101.0 ± 20.3	0.042[a]
E values	-1.33 ± 0.49	-1.2 ± 0.46	-1.67 ± 0.58	0.466[a]

[a] Kruskal-Wallis test

Acknowledgement

The author extends appreciation to the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, for the support and facilities provided during the research.

References

1. Chou H, Lee C, Pan J, Wen Z, Huang S. Enriched Astaxanthin Extract from *Haematococcus pluvialis* Augments Growth Factor Secretions to Increase Cell Proliferation and Induces MMP1 Degradation to Enhance Collagen Production in Human Dermal Fibroblasts. Int J Mol Sci 2016;17:1-13.
2. Lima SGM, Freire MCLC, Oliveira VdaS, Solisio C, Converti A, de Lima ÁAN. Astaxanthin delivery systems for skin application: A review. Mar Drugs 2021;19:1-17.
3. Davinelli S, Nielsen ME, Scapagnini G. Astaxanthin in skin health, repair, and disease: A comprehensive review. Nutrients 2018;10:1-12.
4. Yoshihisa Y, Rehman Mur, Shimizu T. Astaxanthin, a xanthophyll carotenoid, inhibits ultraviolet-induced apoptosis in keratinocytes. Exp Dermatol 2014;23:178–83.
5. Naguib YMA. Antioxidant Activities of Astaxanthin and Related Carotenoids. J. Agric. Food Chem 2000;48:1150-1154.
6. National Library of Medicine – Astaxanthin. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Astaxanthin. Accessed 21 February 2023.
7. Kim DM, Hyun SS, Yun P, Lee CH, Byun SY. Identification of an emulsifier and conditions for preparing stable nanoemulsions containing the antioxidant astaxanthin. Int J Cosmet Sci 2012;34:64–73.
8. Gajendiran A, Abraham J. Nanoemulsions for Drug Delivery. In: Keservani RK, Sharma AK, editors. Nanodispersions for Drug Delivery. New Jersey: Apple Academic Press; 2019.
9. Tayeb HH, Felimban R, Almaghrabi S, Hasaballah N. Nanoemulsions: Formulation, characterisation, biological fate, and potential role against COVID-19 and other viral outbreaks. Colloids Interface Sci Commun 2021;45:1-19.
10. Azeem A, Rizwan M, Ahmad FJ, Iqbal Z, Khar RK, Aqil M, Talegaonkar S. Brief / Technical Note Nanoemulsion Components Screening and Selection : a Technical Note. AAPS PharmSciTech 2009;10:69-76.
11. Erawati, T. Para Methoxycinnamic Acid (PMCA) in Nanoemulsion Drug Delivery Systems Using Soybean Oil, Corn Oil, and Virgin Coconut Oil (VCO) [Dissertation]. Surabaya: Universitas Airlangga; 2015.
12. Deapsari F, Erawati TM, Soeratri W. Penetration of Ubiquinone (Q10) Nanoemulsion Using Olive Oil Through Rat Penetration of Ubiquinone (Q10) Nanoemulsion Using Olive Oil Through Rat Skin. Int J Pharm Clin Res 2017;9:169-72.
13. Mayangsari FD, Erawati T, Soeratri W, Rosita N. Characteristics and Physical Stability of NLC-Coenzyme Q10 in Sleeping Mask with Patchouli Oil. J. Farm. Dan Ilmu Kefarmasian Indones 2021;8(2):178-186.
14. Navarro-Pérez YM, Cedeño-Linares E, Norman-Montenegro O, Ruz-Sanjuan V, Mondeja-Rivera Y, Hernández-Monzón AM., Gonzáles-Bedia, MM. Prediction of the physical stability and quality of O/W cosmetic emulsions using full factorial design. J Pharm Pharmacogn Res 2021;9:98–112.
15. US Pharmacopeia. The United States Pharmacopeia, USP 39/The National Formulary, NF 34. Rockville, MD: US Pharmacopeia Convention; 2016.
16. Cho YH, Kim S, Bae EK, Mok CK, Park J. Formulation of a cosurfactant-free O/W microemulsion using nonionic surfactant mixtures. J. Food Sci 2008;73:115–21.
17. Ernoviya, Afriadi. Formulation and In-Vitro Penetration Test of Ketoconazole Nanoemulsion. Indones. J. Pharm. Clin. Res 2020;3(1):1–7.
18. Ullah N, Amin A, Alamoudi Rana A, Rasheed SA, Alamoudi Ruaa A, Nawaz A, Raza, M, Nawaz T, Ishtiaq S, Abbas SS. Fabrication and Optimisation of Essential-Oil-Loaded Nanoemulsion Using Box–Behnken Design against *Staphylococcus aureus* and *Staphylococcus epidermidis* Isolated from Oral Cavity. Pharmaceutics 2022;14(1640):1-22
19. Cazedey ECL, Carvalho FC, Fiorentino FAM, Gremião MPD, Salgado HRN. Corrositex®, BCOP and HET-CAM as alternative methods to animal experimentation. Brazilian J. Pharm. Sci 2009;45:759–766.
20. ICCVAM. Recommended Test Method Protocol: Hen's Egg Test–Chorioallantoic Membrane (HET-CAM) Test Method. ICCVAM Test Method Eval. Rep 2010;13:B30–B38.
21. Ayuningtyas IN, Mun'im A, Sutriyo. The Study of Safety and Skin Whitening Efficacy of Melinjo (*Gnetum gnemon* L.) Seed Extract-Loaded Lipid Particle Gel. Pharmacog Res. 2018;10(4):432–436.
22. Bhardwaj V, Namkoong J, Tartar O, Diaz I, Mao J, Wu J. *In Vitro* and *Ex Vivo* Mechanistic Understanding and Clinical Evidence of a Novel Anti-Wrinkle Technology in Single-Arm, Monocentric, Open-Label Observational Studies. Cosmetics 2022;9(80);1-15.
23. Plyduang T, Atipairin A, Yoon AS, Sermkaew N, Sakdiset P, Sawatdee S. Formula Development of Red Palm (*Elaeis guineensis*) Fruit Extract Loaded with Solid Lipid Nanoparticles Containing Creams and Its Anti-Aging Efficacy in Healthy Volunteers. Cosmetics 2022;9(3):1-24.
24. Etikan I, Musa SA, Alkassim RS. Comparison of Convenience Sampling and Purposive Sampling. Am. J. Theor. Appl. Stat 2016;5(1):1-4
25. Dasgupta N, Ranjan S. Food Nanoemulsions: Stability, Benefits and Applications. Environmental Chemistry for a Sustainable World. Springer, 2018, 19–48
26. Gurpreet K, Singh SK. Review of nanoemulsion formulation and characterisation techniques. Indian J Pharm Sci 2018;80:781–89.
27. Ali SM, Yosipovitch G. Skin pH: From basic science to basic skin care. Acta Derm Venereol 2013;93:261–67.
28. Márquez AL, Palazolo GG, Wagner JR. Water in oil (w/o) and double (w/o/w) emulsions prepared with spans: Microstructure, stability, and rheology. Colloid Polym Sci 2007;285:1119–28.
29. Peltonen, L., Hirvonen, J., Yliruusi, J. The effect of temperature on sorbitan surfactant monolayers. J. Colloid Interface Sci 2001;239:134–138.
30. Ahmad J, Nollet Leo ML. Fundamental Aspects of Food-Grade Nanoemulsions. In: Ahmad J, Nollet LML, editors. Nanoemulsion in Food Technology: Development, Characterisation, and Applications. Boca Raton: CRC Press; 2022.
31. Czamara K, Adamczyk A, Stojak M, Radwan B, Baranska M. Astaxanthin as a new Raman probe for biosensing of specific subcellular lipidic structures: can we detect lipids in cells under resonance conditions? Cell. Mol. Life Sci 2021;78:3477–3484.
32. Zhang Z, McClements DJ. Overview of Nanoemulsion Properties: Stability, Rheology, and Appearance. In: Jafari SM, McClements DJ, editors. Nanoemulsions: Formulation, Applications, and Characterisation. United States: Elsevier; 2018.
33. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nano-emulsions. Adv Colloid Interface Sci 2004;108–109:303–18.
34. Elmataeeshy ME, Sokar MS, Bahey-El-Din M, Shaker DS. Enhanced transdermal permeability of Terbinafine through

novel nanoemulgel formulation; Development, *in vitro* and *in vivo* characterisation. Futur. J. Pharm. Sci 2018;4:18–28.
35. Bajaj S, Singla D, Sakhuja N. Stability Testing of Pharmaceutical Products. J Appl Pharm Sci 2012;2:129–138.
36. McClements DJ, Jafari SM. General Aspects of Nanoemulsions and Their Formulation, in: Jafari SM, McClements DJ, editors. Nanoemulsions: Formulation, Applications, and Characterisation. United States: Elsevier; 2018.
37. Kim C. Advanced Pharmaceutics: Physicochemical Principles. United States: CRC Press; 2004.
38. Takamura A, Minowa T, Noro S, Tadamichi Kubo T. Effects of Tween and Span Group Emulsifiers on the Stability of o/w Emulsions. Chem Pharm Bull 1979;27:2921–26.
39. Dhruv D. The Study of Sodium Lauryl Sulfate (SLS) Toxicity. J. Clin. Toxicol 2023;13(4):1–5.
40. Prebeg T, Omerčić D, Erceg V, Matijašić G. Comparison of Sodium Lauryl Sulfate and Sodium Lauryl Ether Sulfate Detergents for Decellularization of Porcine Liver for Tissue Engineering Applications. Chem. Eng. Trans 2023;100:745–750.
41. Wilhelm KP, Surber C, Maibach HI. Effect of sodium lauryl sulfate-induced skin irritation on in vitro percutaneous absorption of four drugs. J. Invest. Dermatol 1991;96:963–967.
42. Pezron I. Skin–Surfactant Interactions. Encycl. Surf. Colloid Sci. Third Ed. 2015:6645–6657.
43. Yuan CL, Xu ZZ, Fan MX, Liu HY, Xie YH, Zhu T. Study on characteristics and harm of surfactants. J. Chem. Pharm. Res 2014;6(7):2233–2237.
44. Cortex Technology. DermaLab Combo Instruction Manual. Denmark: Cortex Technology; 2021.
45. Gniadecka M, Gniadecki R, Serup J, Sondergaard J. Ultrasound structure and digital image analysis of the subepidermal low echogenic band in aged human skin: Diurnal changes and interindividual variability. J. Invest. Dermatol 1994;102(3):362–365.
46. Abdul Jabbar O, Kashmoola MA, Al-Ahmad BEM, Mokhtar KI, Muhammad N, Abdul Rahim R, Qouta LA. The effect of flaxseed extract on skin elasticity of the healing wound in rabbits. IIUM Med. J. Malaysia 2019;18(1):5–12.
47. Sharma B, Iqbal B, Kumar S, Ali J, Baboota S. Resveratrol-loaded nanoemulsion gel system to ameliorate UV-induced oxidative skin damage: from in vitro to in vivo investigation of antioxidant activity enhancement. Arch. Dermatol. Res 2019;311:773–793.
48. Annisa R, Mutiah R, Hakim A, Rahmaniyah DNK. Formulation design and evaluation of hydrocortisone-loaded nanoemulsion and nanoemulsion gel for topical delivery. AIP Conf. Proc 2019;2120.
49. Abdellatif AA, Abou-Taleb HA. Optimization of nano-emulsion formulations for certain emollient effect. World J. Pharm. Pharm. Sci 2015;4(12):1314–1328.
50. Ramadon D, McCrudden MTC, Courtenay AJ, Donnelly RF. Enhancement strategies for transdermal drug delivery systems: current trends and applications, Drug Delivery and Translational Research. United States: Springer; 2022.
51. Dragicevic N, Maibach HI. Percutaneous penetration enhancers chemical methods in penetration enhancement: Modification of the stratum corneum, in: Dragicevic, N., Maibach, H.I. (Eds.), Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement: Modification of the Stratum Corneum. Germany: Springer; 2015.
52. Touitou E, Godin B, Karl Y, Bujanover S, Becker Y. Oleic acid, a skin penetration enhancer, affects Langerhans cells and corneocytes. J. Control. Release 2002;80:1–7.