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Formulation and Evaluation of Radiance Serum Containing Astaxanthin–Zeaxanthin Nanoemulsions as an Anti-Wrinkle Agent: Stability, Ex Vivo, and In Vivo Assessments

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Abstract: Reactive oxygen species (ROS), commonly known as free radicals, induced by UV radiation can compromise the dermal structure, leading to a loss of skin elasticity and subsequent wrinkle formation. A promising strategy to prevent and mitigate skin aging involves the use of topical formulations with potent antioxidant properties. Secondary metabolites such as astaxanthin and zeaxanthin are known for their robust antioxidant activities, which surpass those of tocopherol, offering significant benefits for skin health and protection against UV-induced damage. These properties suggest their potential application in anti-aging products. This study aims to evaluate the stability, ex vivo penetration, and in vivo efficacy of a radiance serum containing an astaxanthin–zeaxanthin nanoemulsion (AZ-NE) designed as an anti-wrinkle agent for topical application. The research was conducted in four stages: production of the astaxanthin–zeaxanthin nanoemulsion (AZ-NE), formulation of the AZ-NE radiance serum, stability, and efficacy testing. In this study, the formulated radiance serum demonstrated stability over three months under specified storage conditions. Ex vivo penetration studies indicated efficient diffusion of the active ingredients, with astaxanthin showing a penetration rate of 25.95%/cm^2 and zeaxanthin at 20.80%/cm^2 after 120 min. In vivo irritation tests conducted on human subjects revealed no adverse effects. Moreover, the serum exhibited substantial anti-wrinkle efficacy, with 15 female participants experiencing a wrinkle reduction of 80% to 93% over a 28-day period.

Keywords: astaxanthin–zeaxanthin nanoemulsions; stability assessment; skin penetration; anti-wrinkle efficacy

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1. Introduction

Reactive oxygen species, called free radicals, induced by UV radiation, can damage the structure of the dermis layer, causing the skin to lose its elasticity and leading to wrinkle formation. With the increasing incidence of skin damage, it is necessary to develop prevention strategies and therapies. One strategy to prevent and reduce aging levels is topical formulations with antioxidant effects [1,2]. Antioxidants are compounds that can donate one or two electrons to free radicals, inhibiting oxidation reactions in cells and minimizing cell damage. Antioxidants are available in various forms, including vitamins, minerals, and secondary metabolites [1,3].

Astaxanthin and zeaxanthin are secondary metabolites belonging to the group of xanthophylls, characterized by their lipophilic properties and strong antioxidant activity.

The DPPH radical scavenging assay demonstrated that astaxanthin exhibited significant antioxidant potential with an IC50 value of 17.5 ± 3.6 ppm, while zeaxanthin displayed even stronger antioxidant potential, with an IC50 value of 10 ppm. Both compounds are classified as very strong antioxidants, as their IC_{50} values are below the 50 ppm threshold [4–6].

Abd El-Baky (2004) reported that the combined use of these carotenoids results in a mutual enhancement of their free radical scavenging activities, providing greater protection against oxidative damage than when used individually. This enhanced efficacy may be due to the differing mechanisms of action of the two compounds, allowing them to more effectively address and neutralize a broader spectrum of reactive oxygen species (ROS). Recent findings have also shown that astaxanthin and zeaxanthin offer the most effective protection against UV-B radiation and inhibit radical peroxidation processes more efficiently than β-carotene [7].

In recent years, carotenoids such as astaxanthin and zeaxanthin have gained visibility and attracted attention for cosmetic and dermatological applications. Their antioxidant properties are much stronger than those of tocopherol, with positive effects on skin health and protection against UV radiation, which may have potential applications in anti-aging products [8,9]. In addition, topical astaxanthin and zeaxanthin applications have been reported in several clinical studies on skincare, including antioxidants, anti-aging, protection against UV irradiation, anti-wrinkle, hydration, and wound healing [4,5,9–15].

Astaxanthin and zeaxanthin have more potent antioxidant activity than β-carotene, as their polar ionone rings on both ends of their structures can quench free radicals and other reactive oxygen species (ROS), and the thirteen conjugated double, polyunsaturated bonds can remove high-energy electrons. Their amphipathic structure with polar-nonpolar-polar characteristics allows astaxanthin to be inserted into the bilayers of cell membranes, confines lipoperoxidation promoters to penetrate across the lipid bilayer, and thus reduces peroxidation-caused damage [16,17].

The synergistic combination of astaxanthin and zeaxanthin enhances the efficacy of preventing aging and improving skin brightening, surpassing the effectiveness of cosmetic therapies that rely solely on a single bioactive ingredient. However, the low bioavailability and solubility of carotenoids (astaxanthin and zeaxanthin) limit their use in topical formulations. In recent years, considerable research has been dedicated to investigating various delivery systems aimed at enhancing the properties of carotenoids. These systems, which have been employed to improve the functionality of lipophilic bioactive compounds, can be broadly categorized into lipid-based and polymer-based delivery systems. In particular, lipid-based nano-delivery systems, such as liposomes, solid lipid nanoparticles, nanostructured lipid carriers (NLCs), and nanoemulsions, have been shown to significantly enhance the solubility and stability of specific carotenoid classes. In a previous study, astaxanthin showed notable potential with an IC50 value of 8.62 ppm, while zeaxanthin nanoemulsions exhibited antioxidant activity with an IC50 value of 9.44 ppm, and their combination achieved an IC50 value of 5.85 ppm. Considering these findings, the formulation of innovative delivery systems for astaxanthin and zeaxanthin to enhance their solubility, stability, and consequently, penetration, is highly desirable [4,18,19].

Nanoemulsions are an innovative delivery system widely used in the cosmetic industry for encapsulating bioactive compounds with poor water solubility. These systems have been proven to improve the stability, textural properties, solubility, skin penetration, and efficacy of active ingredients in cosmetic formulations. Nanoemulsions are composed of extremely small droplets, which provide a larger surface area, allowing for better absorption of active compounds into the skin, making them highly effective for topical applications [20–22]. In cosmetic applications, nanoemulsions have been used to deliver active ingredients that target anti-aging, skin hydration, UV protection, and skin brightening. Furthermore, nanoemulsions provide the benefit of controlled release, enabling sustained delivery of active ingredients over time, which can improve long-term skin benefits [22–24].

A previous study developed a nanoemulsion system to enhance the stability, solubility, and skin penetration of carotenoids (astaxanthin and zeaxanthin) for topical appli-

cation. The results demonstrated an improvement in the physicochemical stability and skin permeability of the functionalized nanoemulsion compared to the pure forms of carotenoids [25–28]. Accordingly, the objective of this research is to evaluate the stability, ex vivo penetration, and in vivo efficacy of a radiance serum containing a astaxanthin–zeaxanthin nanoemulsion, designed as an anti-wrinkle agent for topical administration.

2. Materials and Methods

2.1. Materials

The materials used in this study were astaxanthin (Fuji Chemical Industries, Toyama, Japan), zeaxanthin (Inner Mongolia Ever Brilliance Biotechnology Co., Ltd., Tongliao China), polyoxy-35-castor oil (BASF, Jakarta Barat, Indonesia), polyethylene glycol 400 (PEG 400) (Merck, Jakarta Timur, Indonesia), sunflower oil (Jan Dekker International, Amsterdam, The Netherlands), niacinamide, disodium ethylenediaminetetraacetic acid (EDTA), polyethylene glycol 12 (PEG-12) dimethicone, sodium hyaluronate (Thornhill Advanced Research Inc., Vaughan, ON, Canada), glycerine (Kemiko Indonesia, Bekasi, Indonesia), xanthan gum (Deosen Biochemical (Ordos) Ltd., Ordos, China), and d-alpha tocopheryl acetate (Zhejiang Medicine Co., Ltd., Hangzhou, China).

2.2. Methods

This research consisted of four stages, starting with production of the astaxanthin–zeaxanthin nanoemulsion (AZ-NE), formulation of the AZ-NE radiance serum, and stability and efficacy testing.

2.2.1. Production of the Astaxanthin–Zeaxanthin Nanoemulsion (AZ-NE)

Astaxanthin and zeaxanthin were formulated into a nanoemulsion delivery system to enhance the bioavailability and skin affinity of these bioactive molecules. The nanoemulsion, designated as AZ-NE, was prepared using the self-nanoemulsifying (SNE) method optimized in a previous study. This formulation utilizes the unique properties of nanoemulsions to improve the effectiveness of these compounds in skincare applications [17,21]. The preparation of the self-nanoemulsion involved mixing the oil phase, surfactant, and cosurfactant in the following ratio: sunflower oil, polyoxy-35-castor oil, and PEG 400 (1:8:1). Each component was stirred for 15 min, followed by an additional 30 min of stirring. Astaxanthin and zeaxanthin (in a 3:1 ratio) were then added and stirred until homogeneous, followed by sonication for 1 h. The formation of the nanoemulsion was confirmed by the appearance of a transparent mixture [29–31].

2.2.2. Determination of the Solubility of Astaxanthin

To determine the solubility of astaxanthin using UV-Vis spectrophotometry, standard solutions of astaxanthin were prepared in ethanol at concentrations of 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm. A total of 50 mg of free astaxanthin and astaxanthin nanoemulsions, respectively, was weighed and dissolved in 20 mL of aqua deionization. The solution was shaken using an orbital shaker at a speed of 150 rpm for 24 h. The absorbance value at the maximum wavelength (λ) of 475 nm was measured. All experiments were conducted in triplicate, and the solubility was calculated using the following Formula (1):

$$\text{Solubility}(\text{mg/mL}) = \frac{\text{Absorbance} \times \text{Dilution Factor}}{\text{Molar Absorptivity}} \times 100 \quad (1)$$

2.2.3. Determination of the Solubility of Zeaxanthin

To determine the solubility of zeaxanthin using UV-Vis spectrophotometry, standard solutions of zeaxanthin were prepared in ethanol at concentrations of 30 ppm, 40 ppm, 50 ppm, 60 ppm, and 70 ppm. A total of 50 mg of free zeaxanthin and zeaxanthin nanoemulsions, respectively, was weighed and dissolved in 20 mL of aqua deionization. The solution was shaken using an orbital shaker at a speed of 150 rpm for 24 h. The absorbance

value at the maximum wavelength (λ) of 475 nm was measured. All experiments were conducted in triplicate, and the solubility was calculated using the following Formula (2):

$$\text{Solubility}(\text{mg/mL}) = \frac{\text{Absorbance} \times \text{Dilution Factor}}{\text{Molar Absorptivity}} \times 100 \quad (2)$$

2.2.4. Determination of the Purity Levels of Astaxanthin

To determine the purity levels of astaxanthin using UV-Vis spectrophotometry, standard solutions of astaxanthin were prepared in ethanol at concentrations of 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm. A total of 50 mg of free astaxanthin and astaxanthin nanoemulsions, respectively, was weighed and dissolved in 25 mL of ethanol, which was subsequently diluted to reach a final concentration of 125 ppm. The absorbance value at the maximum wavelength (λ) of 475 nm was measured. All experiments were conducted in triplicate, and the purity levels were calculated using the following Formula (3):

$$\text{Puritylevels}(\%) = \frac{\text{Concentration of Active Compound}}{\text{Total Concentration}} \times 100 \quad (3)$$

2.2.5. Determination of the Purity Levels of Zeaxanthin

To determine the purity levels of zeaxanthin using UV-Vis spectrophotometry, standard solutions of zeaxanthin were prepared in ethanol at concentrations of 30 ppm, 40 ppm, 50 ppm, 60 ppm, and 70 ppm. A total of 50 mg of free zeaxanthin and zeaxanthin nanoemulsions, respectively, was weighed and dissolved in 25 mL of ethanol, which was subsequently diluted to reach a final concentration of 40 ppm. The absorbance value at the maximum wavelength (λ) of 450 nm was measured. All experiments were conducted in triplicate, and the purity levels were calculated using the following Formula (4):

$$\text{Puritylevels}(\%) = \frac{\text{Concentration of Active Compound}}{\text{Total Concentration}} \times 100 \quad (4)$$

2.2.6. Formulation of the AZ-NE Radiance Serum

The radiance serum was formulated to contain AZ-NE as the active substance, along with emollients, emulsifiers, solvents, preservatives, and fragrances as excipients. The AZ-NE radiance serum was prepared as a liquid serum, primarily consisting of an aqueous phase [25]. The formula of the AZ-NE radiance serum is presented in Table 1.

Table 1. Formula of AZ-NE radiance serum.

Ingredients	Formula (%)	Function
AZ-NE	5	Anti-aging active substance
Niacinamide	4	Moisturizer
Sodium hyaluronate	0.2	Humectants
Disodium EDTA	0.2	Chelating agent
Carbomer	0.2	Thickener
Xanthan gum	0.03	Thickener
Glycerine	3	Humectants
d-alpha tocopheryl acetate	0.6	Antioxidant
Phenoxyethanol	0.7	Antimicrobial
Fragrance	0.4	Fragrance
Distilled water	Ad 100	Carrier

2.2.7. Stability Test

The stability testing of the AZ-NE radiance serum was performed using accelerated stability testing methods. The tests spanned over a period of three months under different storage conditions: room temperature (25 ± 2 °C/75 ± 5% RH), climatic chamber

conditions (40 ± 2 °C/75 ± 5% RH), and refrigerator conditions (5 ± 3 °C). The primary aim of this study was to ensure the product's stability by evaluating various parameters, including organoleptic, pH, viscosity, microbial contamination, and heavy metal contamination [32,33].

2.2.8. Ex Vivo Permeation Test

The ex vivo permeation test was slightly modified from Nurdianti et al. (2023) [26]. Ex vivo permeation testing was performed using Franz diffusion cells with reticulated python skin as the barrier membrane. Approximately 0.5 g of AZ-NE radiance serum was applied to an area of approximately 2.8 cm^2 in the donor compartment. The receptor compartment contained 50 mL of pH 7.4 phosphate buffer. Throughout the operation of the Franz diffusion cells, the temperature was maintained at 37 ± 0.5 °C using a water jacket. Before the operation, 1 mL of the media was taken as a blank sample. Subsequently, 1 mL samples were collected from the receptor compartment at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min using a micropipette and were immediately replaced with an equal volume of pH 7.4 phosphate buffer. The samples were transferred into 5 mL volumetric flasks and homogenized. The absorbance was measured using a UV-Visible Spectrophotometer at maximum wavelengths of 488 nm for astaxanthin and 428 nm for zeaxanthin. The concentrations of astaxanthin and zeaxanthin that permeated into the receptor fluid were calculated at each sampling point.

2.2.9. Ethical Approval for Clinical Studies

The clinical trial was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and ICH Good Clinical Practice (GCP). Prior to the study, all protocols and amendments received approval from the ethics review board, specifically the Ethics Committee of Universitas Bakti Tunas Husada Tasikmalaya, with Ethics Number: 2778E.01/KEPK-BTH/X/2023 on 2 October 2023.

2.2.10. Skin Irritation Test

The irritation test was conducted to assess the compatibility of the cosmetic product with human skin as part of this study. The AZ-NE radiance serum was applied to the subjects' backs under occlusive patches for 48 h, with observations recorded at 1 h, 24 h, and 48 h post-application. Redness and its severity scale were visually assessed, and the intensity of redness was objectively measured. Additionally, the average irritation index was calculated by summing the score values and dividing by the number of subjects who completed the study [34]. Detailed information is provided in Tables 2 and 3.

Table 2. Skin irritation scoring system.

No.	Description of Irritant Response	Score
1.	No reaction	0
2.	Weak positive reaction (usually characterized by mild erythema across most of the treatment site)	1
3.	Moderate positive reaction (usually distinct erythema, possibly spreading beyond the treatment site)	2
4.	Strong positive reaction (strong, often spreading erythema with oedema)	3

Table 3. Average irritation index.

Average Irritation Index	Category
0.0–0.2	Non-irritant
0.2–0.5	Mild irritant
0.5–2.0	Moderate irritant
2.0–3.0	Severe irritant

2.2.11. Anti-Wrinkle Effectiveness

The effectiveness test of the anti-wrinkle AZ-NE radiance serum was conducted on 15 women (mean age 42 years). The study aimed to determine the product's efficacy in reducing wrinkles and improving skin texture. The study utilized test parameters, including wrinkle levels. Assessments were made using a smart skin analyzer device (Artificial Intelligent Image Analyzer, Bitmoji, Guangzhou, China) at baseline, day 7, day 14, day 21, and day 28. Approximately 0.5 mL of the test product was applied to the subjects' faces twice a day (morning and evening) as instructed [35]. The inclusion criteria for this study were females with signs of aging such as fine wrinkles and dry skin, those not using any other anti-aging products, those willing to apply the test product to the designated area as instructed by the researcher, and those willing to have the application area observed using a skin analyzer according to the study procedures. The exclusion criteria included acute or chronic skin diseases or dermatological conditions, sensitive skin or allergies to any components of the test product, and pregnancy or breastfeeding [36–39].

2.2.12. Statistical Analysis

The data are presented as means ± standard deviation (SD) from three independent replicates (n = 3). Statistical analyses of pH and viscosity were conducted using IBM SPSS software, version 29.0 for macOS. Given the dataset size, the Shapiro–Wilk test was applied to evaluate normality. For parametric data, one-way analysis of variance (ANOVA) was utilized, while the Kruskal–Wallis tests were used for non-parametric data.

3. Results

3.1. Astaxanthin–Zeaxanthin Nanoemulsion (AZ-NE)

The results of the astaxanthin and zeaxanthin nanoemulsion formulation using the self-nanoemulsifying (SNE) method are presented in Figure 1. The AZ-NE formulation was observed to be a viscous liquid with a red color and a characteristic odor of astaxanthin and zeaxanthin. Additionally, the AZ-NE exhibited a zeta potential value of −28.4 mV and a particle size of 20.5 nm.

Figure 1. Astaxanthin–zeaxanthin nanoemulsion.

3.2. Solubility of Astaxanthin and Zeaxanthin

The solubility results for free astaxanthin, free zeaxanthin, and astaxanthin–zeaxanthin nanoemulsions (AZ-NE) are presented in Table 4. Based on the data, the solubility values for free astaxanthin (0.06 ± 0.02 mg/mL), free zeaxanthin (0.08 ± 0.01 mg/mL), astaxanthin nanoemulsions (25.00 ± 0.21 mg/mL), and zeaxanthin nanoemulsions (30.21 ± 0.26 mg/mL) were determined.

Table 4. Solubility of astaxanthin and zeaxanthin in pure form and nanoemulsified.

Compound	Solubility in Water (mg/mL)
Free astaxanthin	0.06 ± 0.02
Free zeaxanthin	0.08 ± 0.01
Astaxanthin nanoemulsions	25.00 ± 0.21
Zeaxanthin nanoemulsions	30.21± 0.26

3.3. Purity Levels of Astaxanthin and Zeaxanthin

The purity levels of free astaxanthin, free zeaxanthin, and astaxanthin–zeaxanthin nanoemulsions (AZ-NE) are presented in Table 5. Based on the data, the purity of free astaxanthin is measured at 1.13 ± 0.02%, while free zeaxanthin has a purity of 40.30 ± 0.18%. The purity levels of astaxanthin and zeaxanthin in the nanoemulsions are determined to be 0.94 ± 0.67% and 34.5 ± 0.53%, respectively.

Table 5. Purity levels of astaxanthin and zeaxanthin in pure form and nanoemulsified.

Compound	Purity Levels (%)
Free astaxanthin	1.13 ± 0.02
Free zeaxanthin	40.30 ± 0.18
Astaxanthin nanoemulsions	0.94 ± 0.67
Zeaxanthin nanoemulsions	34.5 ± 0.53

3.4. AZ-NE Radiance Serum

The formulation results of the AZ-NE radiance serum are presented in Figure 2. The AZ-NE radiance serum was characterized as an orange-colored liquid and was found to be odorless.

Figure 2. AZ-NE radiance serum.

3.5. Stability of AZ-NE Radiance Serum

A stability study was conducted to verify the product's stability according to specific test parameters over a designated period, thus confirming its safety for use. The stability tests included accelerated stability testing over three months under various conditions: room temperature (25 ± 2 °C/75 ± 5% RH), climatic chamber conditions (40 ± 2 °C/75 ± 5% RH), and refrigerator conditions (5 ± 3 °C). Evaluations encompassed assessments of organoleptic properties, pH, viscosity, microbial contamination, and heavy metal contamination. The complete results of the stability tests for the AZ-NE radiance serum are presented in Tables 6–10 and Figures 3 and 4.

Table 6. Accelerated stability study of radiance serum in room temperature conditions.

Time (Days)	Organoleptic		
	Appearance	**Color**	**Odor**
0	Liquid	Orange	Odorless
7	Liquid	Orange	Odorless
14	Liquid	Orange	Odorless
21	Liquid	Orange	Odorless
28	Liquid	Orange	Odorless
35	Liquid	Orange	Odorless
60	Liquid	Orange	Odorless
90	Liquid	Orange	Odorless

Table 7. Accelerated stability study of radiance serum in climatic chamber conditions.

Time (Days)	Organoleptic		
	Appearance	**Color**	**Odor**
0	Liquid	Orange	Odorless
7	Liquid	Orange	Odorless
14	Liquid	Orange	Odorless
21	Liquid	Orange	Odorless
28	Liquid	Orange	Odorless
35	Liquid	Orange	Odorless
60	Liquid	Orange	Odorless
90	Liquid	Orange	Odorless

Table 8. Accelerated stability study of radiance serum in refrigerator conditions.

Time (Days)	Organoleptic		
	Appearance	**Color**	**Odor**
0	Liquid	Orange	Odorless
7	Liquid	Orange	Odorless
14	Liquid	Orange	Odorless
21	Liquid	Orange	Odorless
28	Liquid	Orange	Odorless
35	Liquid	Orange	Odorless
60	Liquid	Orange	Odorless
90	Liquid	Orange	Odorless

Table 9. Determination of quantitation limits for microbial contamination result.

Microbiology	Specifications	Observations	Remarks
Total Plate Count	NMT 10^3 colonies/mL	<10 colonies/mL	Complies
Yeast Mold Count	NMT 10^3 colonies/mL	<10 colonies/mL	Complies
Staphylococcus aureus	Negative/0.1 mL	Negative	Complies
Pseudomonas aeruginosa	Negative/0.1 mL	Negative	Complies
Candida albicans	Negative/0.1 mL	Negative	Complies

Table 10. Determination of quantitation limits for heavy metal contamination results.

Heavy Metals	Specifications	Observations	Remarks
Lead (Pb)	NMT 20 ppm	<0.0001 ppm	Complies
Arsenic (As)	NMT 5 ppm	<0.0001 ppm	Complies
Cadmium (Cd)	NMT 5 ppm	<0.0001 ppm	Complies
Mercury (Hg)	NMT 1 ppm	<0.0001 ppm	Complies

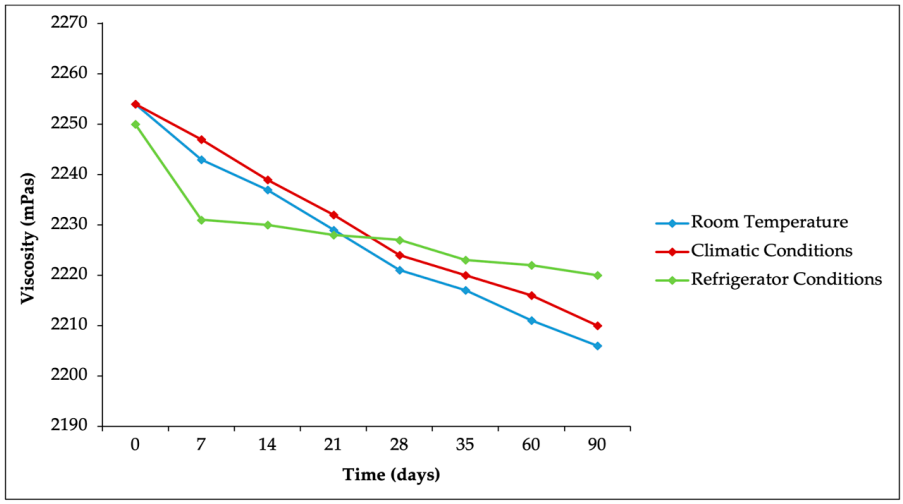

Figure 3. Results of the accelerated stability study on viscosity parameters.

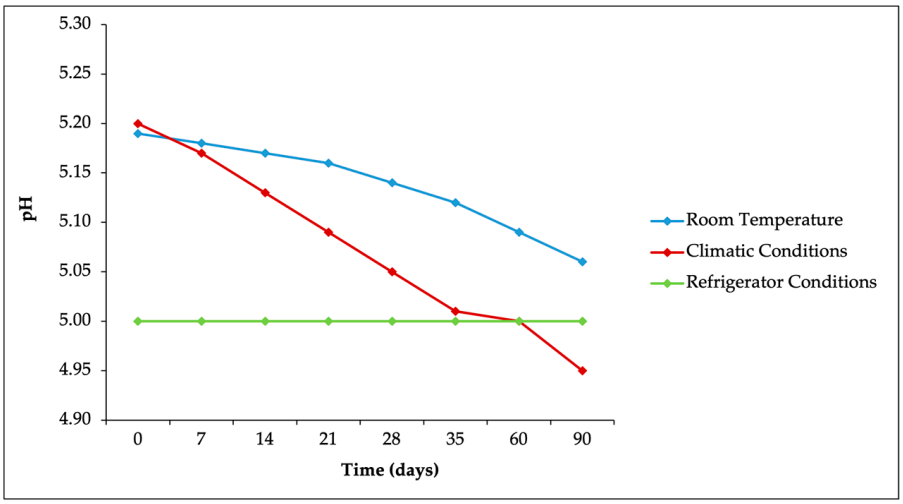

Figure 4. Results of the accelerated stability study on pH parameters.

3.6. Ex Vivo Permeation of AZ-NE Radiance Serum

Permeation testing was conducted to determine the rate and extent of absorption or penetration of the blank control (without active ingredients), free astaxanthin–zeaxanthin radiance serum, and astaxanthin–zeaxanthin nanoemulsion (AZ-NE) radiance serum. Ex vivo penetration tests indicated that the penetration of the blank control was measured at 0.00%, while free astaxanthin and free zeaxanthin achieved penetration rates of 10.06% and 6.19%, respectively. In contrast, the astaxanthin and zeaxanthin nanoemulsions demonstrated effective diffusion of the active ingredients through the skin layers, with rates of 25.95%/cm^2 and 20.80%/cm^2 after 120 min. The complete results of the permeation tests for the AZ-NE radiance serum are presented in Figures 5–9.

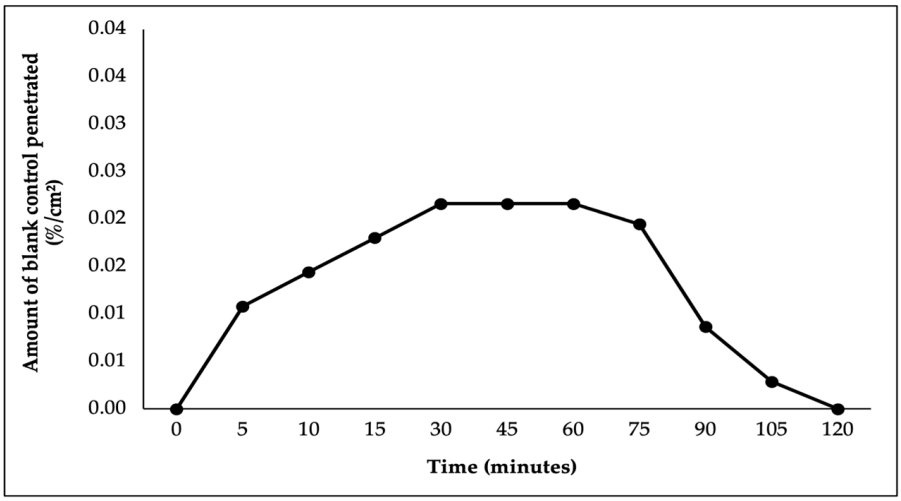

Figure 5. The amount of blank control that penetrated over a duration of 120 min is presented as a percentage per cm^2.

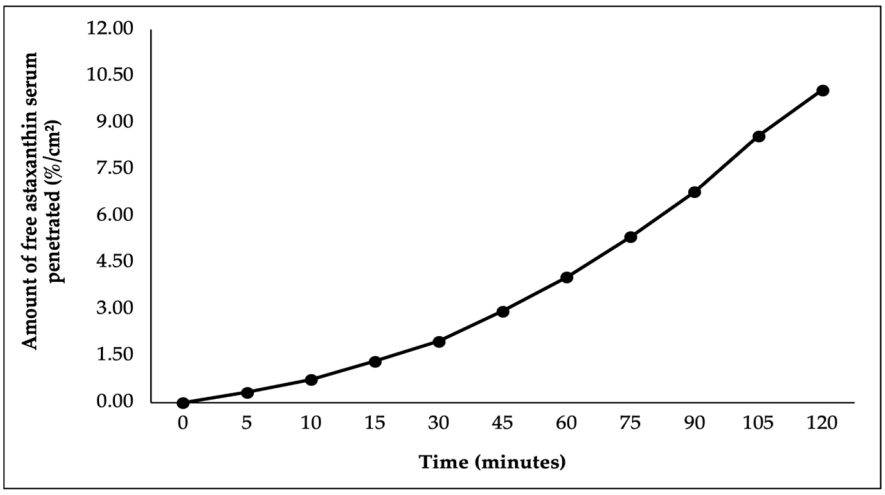

Figure 6. The amount of free astaxanthin in the radiance serum that penetrated over a duration of 120 min is presented as a percentage per cm^2.

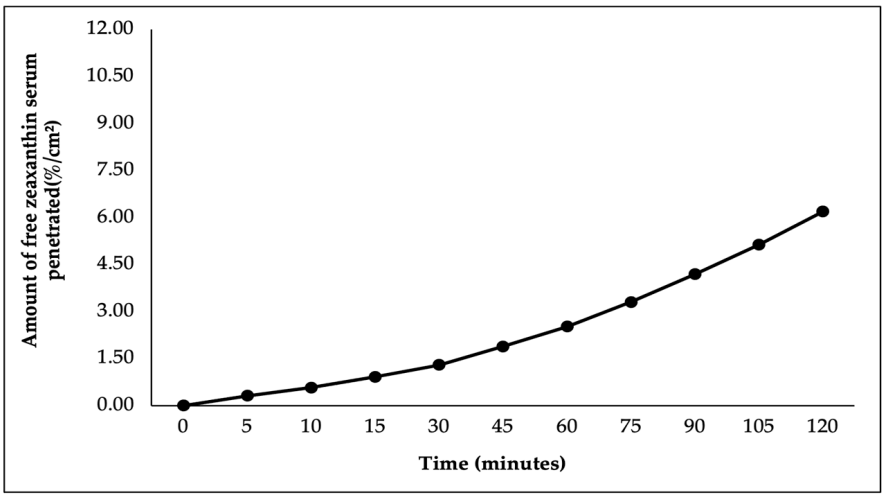

Figure 7. The amount of free zeaxanthin in the radiance serum that penetrated over a duration of 120 min is presented as a percentage per cm^2.

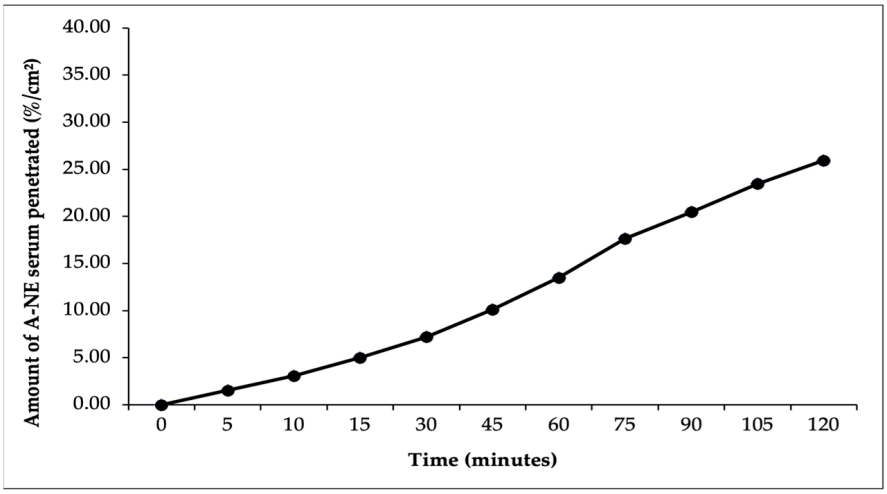

Figure 8. The amount of astaxanthin nanoemulsions (A-NE) in the AZ-NE radiance serum that penetrated over a duration of 120 min is presented as a percentage per cm^2.

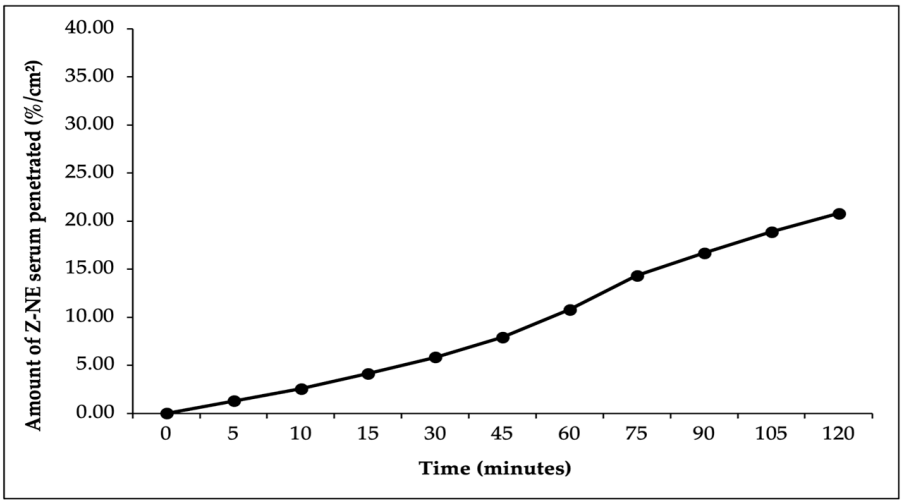

Figure 9. The amount of zeaxanthin nanoemulsions (Z-NE) in the AZ-NE radiance serum that penetrated over a duration of 120 min is presented as a percentage per cm^2.

3.7. Clinical Studies

3.7.1. Skin Irritation of AZ-NE Radiance Serum

The irritation test of the radiance serum was conducted to evaluate the compatibility of a cosmetic product on human skin in a study involving 20 women (mean age 38 years). The testing lasted for 48 h, with observations made at 0, 24, and 48 h. The test results were subsequently assessed using the scoring system outlined in Table 11. The study findings indicated that the AZ-NE radiance serum did not induce irritation on human skin. This conclusion was supported by observations made during the application period, revealing no erythema, oedema, or other signs of skin irritation among the study subjects, with a Primary Irritation Index (PII) value of 0. This study demonstrated the safety of using the AZ-NE radiance serum.

Table 11. The results of the irritation test of the radiance serum on human subjects.

Subject No.	Subject	Age (Years)	Score	Remarks
1	NHT	46	0	Non-irritant
2	YWT	45	0	Non-irritant
3	RKA	33	0	Non-irritant
4	LKA	25	0	Non-irritant
5	RHT	42	0	Non-irritant
6	DBA	26	0	Non-irritant
7	SNA	27	0	Non-irritant
8	DRH	45	0	Non-irritant
9	ASH	48	0	Non-irritant
10	ESI	34	0	Non-irritant
11	EMN	48	0	Non-irritant
12	TTH	33	0	Non-irritant
13	NNI	45	0	Non-irritant
14	EEI	40	0	Non-irritant
15	OEA	47	0	Non-irritant
16	RDS	28	0	Non-irritant
17	HIA	28	0	Non-irritant
18	ARH	36	0	Non-irritant
19	ERH	42	0	Non-irritant
20	TAH	42	0	Non-irritant

3.7.2. Anti-Aging Activity

The effectiveness test of the AZ-NE radiance serum as an anti-wrinkle agent was conducted on 15 subjects, utilizing test parameters including wrinkle levels, assessed with a skin analyzer device. The results indicated a significant reduction in wrinkle levels after 28 days of product use. A significant decrease in wrinkles was experienced by all subjects, with a percentage reduction ranging from 80% to 93% and an average reduction of 84%. Detailed results are presented in Table 12.

Table 12. Anti-wrinkle effectiveness test of the radiance serum on human subjects.

Subject No.	Subject	Age (Years)	Observations					Decrease (%)
			Day 0	Day 7	Day 14	Day 21	Day 28	
1.	DRH	45	32	25	13	5	2	93
2.	ELS	38	31	18	19	10	4	87
3.	YLW	45	41	22	13	11	7	83
4.	RHY	42	38	25	18	10	5	88
5.	NLH	46	23	27	26	12	4	81
6.	TTH	36	19	20	12	9	3	82
7.	RKM	35	22	17	15	8	3	86
8.	TAW	42	20	10	8	9	4	82
9.	ERH	42	23	19	17	8	4	84
10.	ARH	36	43	27	19	12	6	85
11.	EMN	48	22	21	19	13	4	80
12.	ASH	48	23	21	19	14	4	83
13.	ENE	40	34	22	19	13	6	82
14.	UUJ	50	43	34	30	19	8	82
15.	NNE	45	25	18	17	12	4	86

4. Discussion

Astaxanthin and zeaxanthin are synthesized by the alga species *Haematococcus pluvialis* [39]. This alga belongs to the division Chlorophyta, class Chlorophyceae, order Volvocales, family Haematococcaceae, and genus *Haematococcus*. The secondary metabolites produced by this alga are carotenoids, specifically astaxanthin and zeaxanthin [10]. Astaxanthin and zeaxanthin are natural compounds projected to gain popularity in the

coming years due to their potent antioxidant effects, which can prevent deoxyribonucleic acid (DNA) damage and enhance mitochondrial function [17,40]. They are effective in anti-aging and could reduce skin damage caused by UV radiation [11–14]. Additionally, they can activate the nuclear factor erythroid 2-related factor (Nrf2) pathway to stimulate the production of other antioxidants, promote skin regeneration by controlling inflammation and enhancing collagen synthesis, inhibit matrix metalloproteinases (MMPs), and aid in wound healing [15,16]. These carotenoids are being developed to maximize their potential as cosmetic raw materials with anti-wrinkle effects. The process begins with the production of astaxanthin–zeaxanthin nanoemulsions (AZ-NE), followed by serum formulation, stability testing, ex vivo penetration, irritancy testing, and histopathological and in vivo efficacy testing of the AZ-NE radiance serum.

The self-nanoemulsifying drug delivery system (SNEDDS) is a homogeneous anhydrous liquid mixture composed of oil, surfactant, co-surfactant, and active ingredients [39]. In this study, sunflower oil was used as the oil phase, polyoxy-35-castor oil served as the surfactant, PEG 400 functioned as the co-surfactant, and astaxanthin–zeaxanthin acted as the active ingredient. The appropriate type and ratio of the oil phase, surfactant, and co-surfactant are critical parameters for the formation of nanoemulsions. The ratio of oil, surfactant, and co-surfactant was 1:8:1, following the guidelines and methods for nanoemulsion preparation from previous research [29]. The resulting nanoemulsion was a thick, orange liquid with a distinct aroma, a zeta potential of −28.4 mV, and a particle size of 20.5 nm (see Figure 1). The zeta potential indicated the surface charge characteristics of the nanoparticle system. This zeta potential value suggested good stability, implying that the particles were dispersed by the repulsive forces between similarly charged particles. The AZ-NE radiance serum had a zeta potential of approximately −28.4 mV, indicating a moderately negative charge [26,41].

The solubility results for free astaxanthin, free zeaxanthin, and astaxanthin–zeaxanthin nanoemulsions (AZ-NE) are presented in Table 4. Based on the data, the solubility values for free astaxanthin (0.06 ± 0.02 mg/mL), free zeaxanthin (0.08 ± 0.01 mg/mL), astaxanthin nanoemulsions (25.00 ± 0.21 mg/mL), and zeaxanthin nanoemulsions (30.21 ± 0.26 mg/mL) were determined. The solubility of astaxanthin in the nanoemulsion is nearly 416 times higher than in its pure form, while the solubility of zeaxanthin in the nanoemulsion is approximately 377 times higher compared to its free form. These findings suggest that the astaxanthin–zeaxanthin nanoemulsion can effectively enhance both penetration and bioavailability.

The purity levels of free astaxanthin, free zeaxanthin, and astaxanthin–zeaxanthin nanoemulsions (AZ-NE) are presented in Table 5. Based on the data, the purity of free astaxanthin is measured at 1.13 ± 0.02%, while free zeaxanthin has a purity of 40.30 ± 0.18%. The purity levels of astaxanthin and zeaxanthin in the nanoemulsions are determined to be 0.94 ± 0.67% and 34.5 ± 0.53%, respectively. The lower purity of astaxanthin and zeaxanthin in the nanoemulsions compared to their free forms indicates the presence of additional components such as surfactants and emulsifiers, which are essential for stabilizing the formulations and enhancing their solubility in aqueous environments. Although the purity of astaxanthin in the nanoemulsion is only 0.94 ± 0.67% and that of zeaxanthin is 34.5 ± 0.53%, these formulations may provide improved bioavailability and absorption compared to their free forms, thereby illustrating the effectiveness of nanoemulsion technology in maximizing the functional benefits of these carotenoids despite the trade-off in purity.

The nanoemulsion was then formulated into a cosmetic serum, which primarily consisted of an aqueous phase. The base included suitable emollients, emulsifiers, solvents, preservatives, and fragrances. The formula for the prototype of the developed radiance serum is shown in Table 1. The formulation results indicated that the produced serum was in liquid form, orange in color, and odorless (see Figure 2). The quality parameters of Radiance Serum AZ-NE were evaluated through stability tests, irritation tests, and anti-aging effectiveness tests. The parameters for the stability tests of Radiance Serum

AZ-NE included organoleptic properties, pH, viscosity, and microbial and heavy metal contamination. Organoleptic testing of the AZ-NE radiance serum involved observations of its appearance, color, and odor. Based on the observations presented in Tables 6–10, it was evident that the AZ-NE radiance serum exhibited stable organoleptic properties over a three-month storage period under room temperature conditions (25 ± 2 °C/75 ± 5% RH), climatic chamber conditions (40 ± 2 °C/75 ± 5% RH), and refrigerator conditions (5 ± 3 °C), with a liquid consistency, orange color, and odorless quality.

Topical products such as serums are recommended to maintain a pH within the range of 4–6. A reference pH range of 4.5 to 5.5 is considered normal for women, and 4 to 5.5 is considered normal for men. Formulating facial serums within this pH range is beneficial as it supports the skin barrier function [42]. The pH measurements of the radiance serum are detailed in Figure 3. According to Figure 3, during storage at room temperature (25 ± 2 °C/75 ± 5% RH), climatic chamber conditions (40 ± 2 °C/75 ± 5% RH), and refrigerator conditions (5 ± 3 °C), the pH of the radiance serum decreased from 5.19 ± 0.04 to 5.06 ± 0.03 and from 5.20 ± 0.02 to 4.95 ± 0.03, respectively. This pH decrease might be attributed to the influence of CO_2 in the formulation, where atmospheric CO_2 reacted with the aqueous phase of the serum, leading to acid formation. However, despite the pH decrease, the formulated AZ-NE radiance serum remained within the acceptable pH range, specifically around pH 5, in accordance with the standards specifying a pH range of 4–8 [43]. In particular, there were no significant differences in pH values across the three storage temperature variations, indicating that the formulation maintained a consistent level of acidity under varying conditions ($p > 0.05$).

Viscosity measurements were conducted to observe changes in consistency during the storage of the formulation. The viscosity measurements are presented in Figure 4. Based on Figure 4, a decrease in the viscosity of the formulation was observed during storage at room temperature (25 ± 2 °C/75 ± 5% RH), climatic chamber conditions (40 ± 2 °C/75 ± 5% RH), and refrigerator conditions (5 ± 3 °C) ($p > 0.05$). This reduction in viscosity could be attributed to increased water absorption due to higher temperatures and humidity, making the formulation more reactive to air. Despite this decrease, the viscosity values remained within the required standard range of 800 to 2000 mPas [44]. In particular, there were no significant differences in viscosity across the three storage conditions, suggesting that the formulation maintained its consistency regardless of the temperature environment ($p > 0.05$).

In this study, microbial and heavy metal contamination were investigated in AZ-NE radiance serum. Measurements of microbial contamination included total plate count, yeast and mold count, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. The objective of these tests was to ensure that the cosmetic serums produced during the formulation processes met the contamination limits specified for cosmetic products. Microbial contamination poses health risks due to the presence of pathogenic bacteria. The results of microbial contamination testing are presented in Table 9, demonstrating compliance with the requirements. Furthermore, the results of heavy metal contamination testing are found in Table 10. The tests showed no presence of heavy metals such as arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg). Heavy metal contamination refers to pollution by metallic and metalloid elements with high atomic and specific weights, which are toxic to living organisms. Evaluating microbial and heavy metal contamination is a critical step in ensuring the safety of cosmetic products [32,33].

Numerous animal models have been employed as alternatives to human skin for evaluating percutaneous permeation of substances, including pigs, rats, guinea pigs, and snakes. The use of shed snakeskin as a barrier membrane in in vitro permeation studies was first proposed by Higuchi and Kans. Shed snakeskin is non-living tissue that can be harvested without harming the animal, lacks hair follicles, and presents less variability compared to human or animal skin. Various permeation studies have utilized shed skins from different snake species, including *Elaphe obsoleta*, *Python reticulatus*, and *Ophiophagus hannah*. Haigh et al. (1998) examined the effects of species, anatomical sites, and body

regions of the shed snakeskin on permeability measurements and their correlation with human skin performance. They reported a significant correlation with human skin, supporting the potential use of shed snakeskin as a model membrane for permeation studies despite anatomical and chemical differences [45–48].

In this study, ex vivo penetration testing was conducted to simulate and measure the ability of astaxanthin–zeaxanthin nanoemulsions (AZ-NE) in serum to penetrate skin layers using a controlled model system with shed skin from *Python reticulatus*. The penetration test results, presented in Figures 5–9, indicated that AZ-NE from the serum gradually diffused into the phosphate buffer medium at pH 7.4. After 120 min, the penetration of astaxanthin was measured at 25.95%/cm^2, and zeaxanthin at 20.80%/cm^2. Over the 120 min period, both astaxanthin and zeaxanthin showed a steady increase in penetration, with astaxanthin reaching 1.53%/cm^2 at 5 min and gradually rising to 25.95%/cm^2, while zeaxanthin started at 1.27%/cm^2 and increased to 20.79%/cm^2. In contrast, free astaxanthin demonstrated a penetration rate of 10.06%, and free zeaxanthin exhibited a penetration rate of 6.19%, both of which were significantly lower than their nanoemulsified counterparts. The comparison between free forms and nanoemulsified forms indicates that the nanoemulsion formulation of astaxanthin and zeaxanthin (AZ-NE) significantly enhances the penetration and diffusion of active ingredients through the skin layers compared to their free forms. These results suggest that nanoemulsions not only improve the solubility and stability of active ingredients but also enhance their bioavailability, making them more effective for dermatological applications aimed at improving skin health and combating oxidative stress. Therefore, the use of nanoemulsion technology is a promising approach for developing more effective formulations in skincare products [49–51].

The human irritation study aimed to evaluate the potential irritation caused by the AZ-NE radiance serum on human skin. Assessing skin irritation is crucial for determining the safety of cosmetic products prior to their broader application among the human population. This study included 20 women (mean age 38 years) with varying skin types, such as dry, normal, oily, and combination skin. The AZ-NE radiance serum was applied to the subjects' backs, and signs of irritation, including erythema, oedema, or a burning sensation, were monitored. Observations were recorded over a 48-h period at 0, 24, and 48 h post-application [34]. The results of this irritation study on human subjects are presented in Table 11. The study findings indicated that the AZ-NE radiance serum did not induce irritation on human skin. This conclusion was supported by observations made during the application period, revealing no erythema, oedema, or other signs of skin irritation among the study subjects, with a Primary Irritation Index (PII) value of 0. This study demonstrated the safety of using the AZ-NE radiance serum.

The efficacy of an anti-wrinkle product was evaluated through a clinical study involving human subjects. This study aimed to determine the product's ability to reduce the appearance of wrinkles and improve skin texture [17,52,53]. The objective of this anti-wrinkle efficacy study was to evaluate the performance of the AZ-NE radiance serum in reducing signs of aging in humans. The testing focused on reducing wrinkle levels in 15 women (mean age 42 years) who regularly used the serum for 28 days. The results of this study are presented in Table 12. The reduction in the number of wrinkles from day 0 to day 28 showed positive results. A significant decrease in wrinkles was experienced by all subjects, with a percentage reduction ranging from 80% to 93% and an average reduction of 84%. The most substantial reduction in wrinkles was observed during the first week of use, with more than a 50% reduction experienced by some subjects. The number of wrinkles on day 0 ranged from 19% to 43%, while on day 28, it decreased to between 2% and 8%. Significant wrinkle reduction was observed in both younger and older subjects, proving the effectiveness of this serum across various age ranges.

In this study, the mechanism of astaxanthin–zeaxanthin nanoemulsions as anti-wrinkle agents was also investigated. These nanoemulsions enhance the delivery of carotenoids, providing potent antioxidant protection against free radicals, which damage collagen and elastin. Furthermore, by improving hydration and reducing moisture loss, the nanoemul-

sions help maintain skin structure, firmness, and elasticity, thereby reducing the appearance of wrinkles and enhancing overall skin elasticity [52–54]. These results support the claim that the radiance serum is effective in reducing wrinkles and improving skin condition. The serum was deemed safe for use on various skin types and ages, offering hope for those seeking an effective anti-wrinkle solution.

5. Conclusions

The AZ-NE radiance serum for topical application was successfully evaluated for stability, ex vivo penetration, and in vivo efficacy. The results of these assessments demonstrated that the AZ-NE radiance serum is stable, safe for skin application, and effective as an anti-wrinkle treatment, with all subjects experiencing a decrease in wrinkles ranging from 80% to 93% over a 28-day period and an average reduction of 84%.

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