

Physicochemical Characterization of Nanoemulsion Serum from Ethanol Extract of Kersen (*Muntingia calabura* L.) Fruit and Its Effectiveness as an Anti-aging Agent

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Abstract: Numerous studies indicate that kersen, or Jamaican cherry (*Muntingia calabura* L.) fruit, exhibits effective antioxidant properties and demonstrates anti-aging effects. Phenolic and flavonoid compounds in Kersen fruit extract contribute to its anti-aging bioactivity. This study aims to formulate a nanoemulsion-based serum utilizing the extract of kersen fruit to optimize its application as an anti-aging agent. The research involved formulating the extract into a nanoemulsion and characterizing it by assessing particle size, polydispersity index, zeta potential, transmittance, emulsification time, and entrapment efficiency, followed by formulation into the serum for evaluation. To assess the nanoemulsion delivery system's efficacy in enhancing the extract's potential, penetration profiles and in vitro inhibition of tyrosinase, elastase, and hyaluronidase were evaluated. The findings revealed that variations in oil, surfactant, and co-surfactant ratios significantly influenced the particle size, polydispersity index, entrapment efficiency, and emulsification time. Formulation F1 had the smallest particle size (195.07 ± 26.80 nm), polydispersity index (0.324 ± 0.13), zeta potential (-29.03 ± 1.0 mV), and high entrapment efficiency (74.16%). These features enabled effective delivery of active compounds, leading to the highest Q value and significant inhibition of tyrosinase (41.79%), elastase (29.08%), and hyaluronidase (27.65%). The nanoemulsion effectively facilitates the penetration of active compounds from kersen fruit through the skin and is effective as an anti-aging product.

Keywords: Anti-aging; characterization; Jamaican cherry fruit; nanoemulsion; serum; skin penetration.

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1. Introduction

Human skin is a complex body tissue that serves numerous functions and exhibits distinct characteristics unique to each individual. Notable alterations, such as premature aging of the skin, can significantly diminish an individual's self-esteem [1–3]. Premature skin aging represents an unprogrammed modification in the structural integrity of skin tissue, attributed to various contributing factors, with environmental elements being predominant. Indeed, environmental factors are recognized as primary catalysts for premature skin aging. Exposure to ultraviolet (UV) radiation from sunlight, commonly referred to as photoaging, greatly contributes to tissue damage and consequently facilitates an escalation in the levels of reactive oxygen species (ROS) [4].

Reactive oxygen species (ROS), elevated due to environmental factors, greatly influence the body's biochemical pathways. One pathway involves the breakdown of adipose tissue, lipids, and DNA, leading to angiogenic changes that trigger pro-inflammatory cytokines and neutrophil elastase production. Pro-inflammatory cytokines, such as interleukins and TNF- α , activate tyrosinase, stimulating melanin synthesis and causing hyperpigmented lesions (solar lentigo) while reducing collagen and elastin production. Dermal fibroblasts, essential for collagen synthesis, decrease with age, lowering collagen production. Neutrophil elastase further degrades elastin and collagen, promoting wrinkles and accelerating skin aging. Another pathway activates mitogen-activated protein kinase (MAPK), increasing AP-1 and NF- κ B levels, which regulate matrix metalloproteinases (MMPs) like collagenase and elastase [4,5]. These enzymes degrade collagen, elastin, and hyaluronic acid, weakening skin structure and leading to wrinkles, sagging, reduced elasticity, and overall skin aging [6,7].

Antioxidant compounds offer a way to prevent or repair skin aging caused by excessive ROS exposure. These molecules neutralize or eliminate free radicals, stopping them from causing harm. While the skin has its antioxidant system, prolonged exposure to environmental stressors can deplete it. Antioxidants work mainly by scavenging free radicals through electron or proton donation, stabilizing them, and making them harmless. Examples include phenolic compounds and flavonoids, which are rich in hydroxyl groups (-OH) and can easily stabilize ROS by transferring electrons or protons to free radicals. Natural antioxidants like these also help repair damaged skin by promoting new collagen synthesis and restoring the skin's structural integrity. Additionally, antioxidants inhibit inflammatory pathways, suppressing pro-inflammatory cytokines and enzymes to reduce inflammation and the aging effects on the skin.

The kersen plant (*Muntingia calabura* L.) is a rich source of antioxidants [8]. Research has highlighted its secondary metabolites, which act as antioxidants, and specifically its fruit, known for notable antioxidant activity. This is due to its high levels of ascorbate (vitamin C), vitamin A, and essential minerals like calcium and phosphorus. Additionally, kersen fruit is packed with bioactive compounds such as flavonoids, phenolics, saponins, tannins, terpenoids, steroids, alkaloids, and various other constituents [9–11]. Research indicates that Kersen fruit exhibits potent activity in inhibiting DPPH and ABTS free radicals, with an IC_{50} value of less than 50 μ g/mL. Furthermore, several studies have demonstrated that ethanolic extracts of Kersen fruit possess significant inhibitory effects on elastase, collagenase, tyrosinase, and hyaluronidase, with IC_{50} values of 37.08 μ g/mL, 203.47 μ g/mL, 188.75 μ g/mL, and 49.97 μ g/mL, respectively, categorizing these activities as strong [12–14]. These findings further underscore Kersen fruit's potential as an effective anti-aging and skin-brightening agent.

The phenolic and flavonoid compounds found in Kersen fruit (*Muntingia calabura* L.) are categorized as polar compounds, which pose challenges for diffusion through the skin [15]. This limited skin penetration can adversely affect the bioavailability of preparations derived from natural materials in the human body. Nanomaterials have been extensively studied for their application in the topical delivery of cosmeceuticals and pharmaceuticals for skin rejuvenation and anti-aging. Among the different approaches to tissue regeneration, the use of nanomaterials loaded with cosmeceutical and pharmaceutical agents has shown promise [16]. Nanoemulsion technology is gaining attention as a flexible and innovative method for delivering drugs and active ingredients across various industries like pharmaceuticals, cosmetics, and food science. It works by forming a stable, transparent, or slightly translucent mix of two immiscible liquids, usually oil and water, with nanometer-sized droplets (20 to 200 nm), stabilized by surfactants and co-surfactants. This approach can effectively bypass the skin's barrier and improve the penetration of active compounds into cells, leading to enhanced results [17]. Furthermore, the concentration of nanoparticles in serum formulations is anticipated to optimize their effectiveness as skin-brightening agents.

Nanoemulsion delivery systems using plant extracts have shown strong success in various studies, mainly because they enhance the effectiveness of active plant compounds. For instance, Mahdi *et al.* designed a nanoemulsion serum from palm kernel oil esters as an anti-aging agent [18]. Fitri *et al.* developed nanoemulsions from *Centella asiatica* extract, which demonstrated good stability and antioxidant effectiveness [19]. Similarly, Nurdianti *et al.* formulated a nanoemulsion containing astaxanthin, a phytoconstituent, as an anti-aging agent [20]. These advancements highlight the potential of nanoemulsion technology to improve the efficacy of natural ingredient extracts across various applications.

The objective of this study is to formulate the ethanol extract of Kersen fruit into a nanoemulsion-based serum in order to optimize its use as an anti-aging and skin-brightening agent, ensuring its effectiveness against tyrosinase, elastase, and hyaluronidase enzymes. This study builds on our previous research results that have been reported and are expected to impact science [12,14,21].

2. Materials and Methods

2.1. Chemical reagents.

The chemical reagents used in this study included Elastase enzyme (Sigma-Aldrich, SLBV 9311), hyaluronidase enzyme (Sigma-Aldrich, H3506), tyrosinase enzyme (Sigma-Aldrich), N-Succinyl-Ala-Ala-Ala-p-nitroanilide/SANA (Sigma-Aldrich, S4760), L-Dopa (3-(3,4-Dihydroxyphenyl)-L-alanine) (Sigma-Aldrich), hyaluronic acid substrate, and Trizma base, all utilized in the anti-aging assay. Additionally, 70% ethanol was used as a solvent. Olive oil, Na CMC, phenoxyethanol, distilled water, and methylene blue were sourced from Sumber Rejeki Distributor, Indonesia. Polysorbate 80, PEG 400, gallic acid, and hydrochloric acid served as excipients in the nanoemulsion formula. Folin-Ciocalteu, KCl, potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Intraco Company and Sigma-Aldrich and used in chemical reactions for quantitative analysis. Circumcision skin membranes from a clinic were used as membranes in the penetration profile assay.

2.2. Plant materials.

This study used extracts of Kersen fruit (*Muntingia calabura* L.) sourced from Makassar, South Sulawesi (5°06'57.6"S 119°31'06.8"E). The Pharmaceutical Biology Laboratory at Almarisah Madani University analyzed the sample to confirm the plant species. It was identified as a Kersen fruit from the Muntingiaceae family, classified as *Muntingia calabura* L., with specimen number 0528/U/B/2023.

2.3. Instrumentation supplies.

The instrumentation used in this study included a rotary evaporator (IKA RV-10), a UV-Visible Spectrophotometer (Shimadzu UV-1900), a pH meter (LAQUA), an Analytical Scale (Mettler Toledo MX204), a Particle Size Analyzer (Malvern Nano ZS), an Oven (Mettler UN-55), an Incubator (Mettler IN-55), and a Magnetic Stirrer (B-One LMS-1-1500).

2.4. Extraction.

Kersen fruits were collected and wet-sorted, then washed thoroughly with running water. The fruits were then finely chopped to optimize the drying process. The drying process was subsequently carried out using a herb drying oven at a temperature of 40°C for 3 times 24 hours. The dried simplicia were then dry-sorted, powdered, and sieved using a 14-mesh sieve, after which the total weight of the simplicia was measured. One kilogram of powdered simplicia was extracted through maceration using 70% ethanol in a 1:10 ratio for 24 hours at room temperature, and away from light. The filtrate and residue were separated by filtering with filter paper. The residue was then re-macerated with the same solvent under the same conditions. The combined filtrates were evaporated using a rotary evaporator to obtain a thick extract [22].

2.5. Determination of total phenolic content.

A quantity of 10 mg of gallic acid was accurately weighed and subsequently dissolved in 10 mL of distilled water to prepare a standard solution with a concentration of 1000 ppm. This standard solution was then subjected to stepwise dilution to obtain final concentrations of 2.5, 5, 7.5, 10, and 12.5 ppm. Each dilution involved the addition of 50 µL of Folin-Ciocalteu reagent, followed by 50 µL of a 7.5% Na₂CO₃ solution, and then the total volume was adjusted to 200 µL using distilled water. The resulting mixtures were incubated for 30 minutes, after which their absorbance was measured using a microplate reader at a wavelength of 740 nm. Distilled water was used as a blank in this study. To determine the total phenolic content in the extract sample, a solution was prepared by dissolving 10 mg of the extract in 10 mL of distilled water. The procedure for measuring the total phenolic content followed the same method used for the standard curve [23].

2.6. Preparation of nanoemulsion from ethanolic extract of *M. calabura* fruit.

A nanoemulsion of the ethanolic extract of *M. calabura* fruit was prepared by homogenization with gradual mixing using a magnetic stirrer at 50°C. The composition is shown in Table 1 according to the ternary diagram optimization. The *M. calabura* fruit extract was combined with PEG 400 using a magnetic stirrer at 600 rpm for 5 minutes. Then,

Polysorbate 80 was added and mixed at the same speed for an additional 5 minutes. Finally, olive oil was added dropwise and mixed for 5 minutes.

Table 1. Nano-emulsion composition of ethanol extract of Kersen fruit.

Materials	F1	F2	F3
Kersen fruit extract	5%	5%	5%
Olive oil	0.026 mL	0.026 mL	0.026 mL
Polysorbate80	0.913 mL	0.852 mL	0.791 mL
PEG 400	0.061 mL	0.122 mL	0.183 mL

2.7. Nanoemulsion characterization.

2.7.1. Determination of particle size, polydispersity index, and zeta potential.

The resulting mixture was then characterized, including particle size, polydispersity index, and zeta potential, using a particle size analyzer, Beckman Colter Delsa™ Nano C. The sample was placed into a cuvette, then placed in the instrument holder and analyzed [22].

2.7.2. Emulsification time.

To determine the emulsification time, 50 µL of the sample was added to 25 mL of distilled water on a hot plate at $37 \pm 5^\circ\text{C}$. The mixture was then slowly stirred with a magnetic stirrer at 100 rpm. The emulsification time was measured from the addition of the sample to distilled water until homogeneity was achieved. The emulsification time for a nanoemulsion should be less than one minute [24].

2.7.3. Percent Transmittance.

The percent transmittance was determined using a UV-Vis spectrophotometer by mixing 100 µL of the nanoemulsion in 5 mL of distilled water using a vortex mixer for 1 minute. Transmittance was measured at a wavelength of 650 nm with distilled water as the blank. A high percent transmittance value indicates that the droplet size in the dispersion has reached the nano-scale with a homogeneous distribution. The transmittance requirement for nanoemulsion is >90% [24].

2.7.4. Entrapment efficiency.

The entrapment efficiency test assessed the amount of active substance in the nanoemulsion. It was measured using absorbance readings from a microplate reader. A 2 mL sample of the nanoemulsion was centrifuged at 8000 rpm for 30 minutes. From the supernatant, 50 µL was diluted with phosphate buffer (pH 7.4) to a final volume of 1 mL. A 20 µL aliquot was transferred to a well plate, followed by the addition of 50 µL of Folin-Ciocalteu and 50 µL of Na_2CO_3 , up to 200 µL with phosphate buffer. The drug entrapment efficiency for each batch was calculated in terms of entrapment efficiency as per formula 1 [25].

$$\text{Entrapment Efficiency (\%)} = \frac{\text{loading drug-free drug}}{\text{loading drug}} \times 100\% \quad (1)$$

2.8. Preparation of nanoemulsion serum from ethanol extract of kersen fruit (*Muntingia calabura* L.).

The serum is formulated with a 2:1 ratio of nanoemulsion to serum base, as outlined in Table 2. Sodium carboxymethyl cellulose (Na-CMC) is first dispersed in heated water with

continuous stirring using a magnetic stirrer (50 rpm) until fully expanded; thereafter, phenoxyethanol is added. Subsequently, an ethanol extract derived from Kersen fruits was incorporated into the homogenized mixture. The resultant formulation undergoes comprehensive characterization through organoleptic evaluation, homogeneity assessment, pH measurement, viscosity analysis, spreading ability test, adhesion test, and emulsion type determination. Each formula was tested three times.

Table 2. Serum formulation.

Material	Weight/volume
Nanoemulsion of kersen fruit extract	30 mL*
Na CMC	0.675 g
Phenoxyetanol	0.075 mL
Distilled water	14.25 mL

*nanoemulsi: serum base (2:1)

2.8.1. Organoleptic.

Organoleptic testing was conducted through visual observation of the color, odor, and form of the obtained serum preparation [25].

2.8.2. Homogeneity.

The homogeneity of a preparation is assessed by applying a sample of the preparation to the surface of a glass slide, followed by the observation of any coarse particles or clumps. A preparation is considered homogeneous if no coarse particles or clumps are present in the preparation [25].

2.8.3. pH measurement.

The pH value of the preparation was determined using a digital pH meter (LAQUA) by immersing a calibrated anode into the preparation. The preparation is considered to meet the requirements if the pH falls within the skin pH range of 4.5-7.5 [25].

2.8.4. Viscosity analysis.

Viscosity measurements were performed using a Brookfield LV viscometer at a speed of 6 rpm (spindle no. 63). The viscosity standard for topical preparations is in the range of 2000-50000 cps [25].

2.8.5. Spreading ability.

The spreadability test was conducted by placing 0.5 g of serum between two glass slides of equal size. Successive loads (50 g, 100 g, 150 g, 200 g, and 250 g) were applied on top and allowed to stand for 1 minute. Each formula was tested three times, and the spread diameter was measured. A spreadability range of 5-7 cm is considered to indicate good consistency for topical preparations [25].

2.8.6. Adhesion times.

Adhesion testing is performed with an adhesion tester using a 0.5 g sample placed between two glass objects. Loads of 50 g, 100 g, 150 g, 200 g, and 250 g are applied for 5 minutes each. The serum is considered qualified if its adhesion lasts more than 4 seconds [25].

2.8.7. Emulsion type determination.

The emulsion type test is performed using the methylene blue staining method. A droplet of microemulsion serum was combined with a droplet of methylene blue on a glass slide. Observation of a homogeneous blue coloration in the external phase indicated an oil-in-water (O/W) emulsion [25].

2.9. Determination of the serum penetration profile of Kersen extract nanoemulsion.

The profile of penetration of the serum nanoemulsion formulation from kersen fruit extract was determined using the Franz diffusion method. The membrane selected to represent the penetration process was the prepuce skin, obtained from circumcision and cleaned of adhering fats. The skin membrane of the prepuce was cleaned by washing it with NaCl in order to remove fats attached to the skin before use. In the diffusion apparatus setup, the recipient fluid compartment was filled with Phosphate Buffered Saline at pH 7.4 up to a volume of 25 mL. A sample of 1 g was placed evenly on the diffusion membrane after adjusting the test conditions to a temperature of $37 \pm 2^\circ\text{C}$ and a speed of 200 rpm. Samples were taken at time intervals of 15, 30, 45, 60, 90, 120, and 180 minutes, with simultaneous replacement of the samples using a pH 7.4 buffer. The samples from the penetration test were then analyzed for their active substance concentration using a microplate reader, where 100 μL of the sample was mixed with 50 μL of Folin-Ciocalteu, 50 μL of 7.5% Na_2CO_3 , and pH 7.4 PBS, resulting in a final volume of 300 μL in the well plate, and incubated for 30 minutes. Absorbance was measured at the maximum wavelength of 740 nm and used to calculate the cumulative penetration amount and diffusion rate [25].

The cumulative number of phenolics that penetrated the diffusion area ($\mu\text{g}/\text{cm}^2$) was calculated using the equation.

$$Q = \frac{C_n.V + \sum_{i=1}^{n-1} C_i.S}{A} \quad (2)$$

With Q = Cumulative amount of substance passing through the membrane ($\mu\text{g}/\text{cm}^2$), C_n = Concentration of phenolics ($\mu\text{g}/\text{ml}$) at n minutes, V = volume of Franz diffusion cell (ml), $\sum_{i=1}^{n-1} C_i$ = The concentration penetrated the previous minute ($\mu\text{g}/\text{cm}^2$), S = Sampling Volume, and A = Membrane area (cm^2).

The rate of drug penetration per unit time (flux) is calculated based on Fick's law I:

$$J = \frac{M}{A.t} \quad (3)$$

With J = Flux ($\mu\text{g}/\text{cm}^2\text{h}$), M = Amount of active substance penetrated (μg), A = Membrane area (cm^2), and t = Time (hour).

2.10. Determination of the in vitro activity of serum nanoemulsion of Kersen fruit extract.

2.10.1. Anti-tyrosinase activity.

The tyrosinase inhibition activity was carried out based on the previously reported procedure [9]. The sample used for testing was the diffusion sample of the serum of the nanoemulsion of the Kersen fruit extract. A 40 μL aliquot of the sample was mixed with 40 μL

of tyrosinase enzyme solution and 80 μL of phosphate buffer solution in a well plate, then homogenized and incubated for 10 minutes at 25°C. The mixture was then reacted with 40 μL of L-DOPA substrate and incubated again at 25°C for 30 minutes. Afterward, the absorbance was measured at a wavelength of 490 nm using a microplate reader. The same process was performed for the blank (without sample extract) and negative control (phosphate buffer solution) at 490 nm.

2.10.2. Anti-elastase activity.

The elastase inhibition activity was carried out according to the previously published procedure. A 100 μL sample was mixed with 30 μL of elastase enzyme solution and 50 μL of Tris-HCl buffer solution in a well plate, then incubated for 10 minutes at 25°C. The mixture was reacted with 20 μL of SANA substrate and incubated again at 25°C for 30 minutes. The absorbance was measured at a wavelength of 405 nm using a microplate reader. The same process was conducted for the blank (without sample extract) and negative control (Tris HCl buffer solution) at a wavelength of 405 nm [26].

2.10.3. Anti-hyaluronidase activity.

The hyaluronidase inhibition activity was determined using a modified protocol from Sigma-Aldrich and Thring *et al.* A 100 μL sample was mixed with 300 μL of hyaluronidase enzyme and 300 μL of hyaluronic acid substrate, and the mixture was then made up to 1 mL with phosphate buffer B in a 96-well plate. The mixture was incubated for 24 hours at 37°C. After 24 hours, 200 μL was transferred to a well plate, then 100 μL of albumin solution was added and incubated for an additional 10 minutes, after which absorbance was measured at 600 nm. The same process was carried out without the sample to measure the blank at a wavelength of 600 nm. The absorbance was then measured using a Microplate Reader [26].

3. Results and Discussion

This study focuses on the ethanol extraction of kersen fruit, which previous research suggests may have anti-aging and skin-lightening properties. The research findings are applied in this study through various stages, including extraction, formulation, and quality control using different testing methods. The initial stage involved the maceration extraction process with a 70% ethanol solvent concentration, achieving an optimized extraction yield of 40.70%. Furthermore, the extract was formulated into a nanoemulsion to enhance the effectiveness of the phenolic and flavonoid compounds present in the kersen fruit extract.

The next step is to create a nanoemulsion serum as part of developing a natural-ingredient anti-aging product using kersen. We prepared nanoemulsions using high-pressure homogenization, followed by comprehensive characterization, including particle size determination, polydispersity index assessment, zeta potential measurement, emulsification time analysis, transmittance evaluation, and entrapment efficiency quantification. The results of the characterization are presented in Table 3 and Figure 1.

Table 3. Characteristics of nanoemulsions from Kersen extract.

Characteristic	F1	F2	F3
Particle size	195.07 $\pm$ 26.80*	254.73 $\pm$ 5.10*	300.3 $\pm$ 34.67*
Polydispersity index	0.324 $\pm$ 0.13 ^c	0.558 $\pm$ 0.09*	0.310 $\pm$ 0.12 ^a
Zeta Potensial (mV)	-29.03 $\pm$ 1.0 ^b	-30.06 $\pm$ 2.41 ^a	-32.37 $\pm$ 7.14*
Emulsification time (s)	24.39 $\pm$ 0.73*	26.71 $\pm$ 1.21*	29.62 $\pm$ 1.05*

Characteristic	F1	F2	F3
Transmitan (%)	98.79±0.92 ^{bc}	98.35±0.56 ^{ac}	98.31±1.66 ^{ab}
Entrapment efficiency (%)	74.16±1.91 [*]	87.85±2.01 ^c	90.18±0.88 ^a

*The symbol indicates that there is a significant difference between the formulas (p-value <0.05, n=3, Tukey one-way ANOVA). The alphabetical differences indicate that the results do not differ significantly between the samples (p-value >0.05, n=3, Tukey one-way ANOVA).

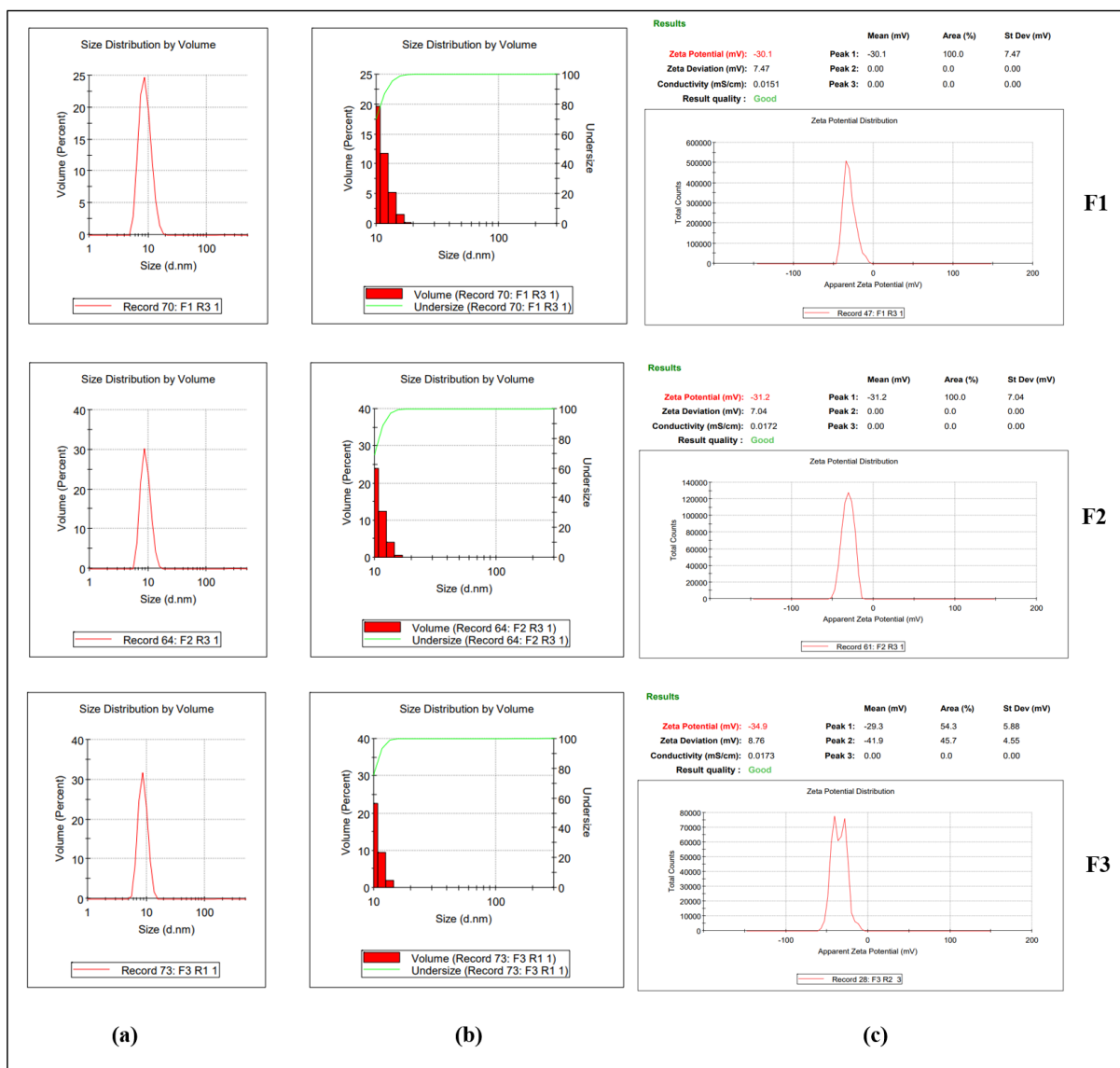


Figure 1. Characteristics of Kersen fruit nanoemulsion, including (a) particle size; (b) polydispersity index; (c) zeta potential.

An essential aspect of the nanoemulsion formulation process is optimizing the oil-to-water phase ratio and using suitable emulsifiers. The resulting nanoemulsion shows a small particle size (less than 200 nm) and a low polydispersity index (± 30 mV), indicating stability, along with a negative zeta potential that reflects favorable dispersion properties [19,20]. The ethanol extract nanoemulsion derived from Kersen fruit comprises three primary components: the oil as the drug carrier, the surfactant functioning as the emulsifier that facilitates the integration of oil in water by forming an interfacial film layer to ensure stability, and the co-surfactant, which enhances drug incorporation and aids in the nanoemulsification process [16,18].

The study used olive oil as the oil phase because of its high oleic acid content (56-85%), an unsaturated fatty acid known for its effective self-emulsifying properties and high drug-

loading capacity [27]. Polysorbate 80 was used as a non-ionic surfactant with a Hydrophilic-Lipophilic Balance (HLB) value of 15, making it suitable for nanoemulsion formulation, given that an ideal HLB value exceeds 10, ensuring stability for O/W emulsions while remaining safe for physiological applications [28]. Polysorbate 80 reduces nanoemulsion particle size by enhancing surfactant adsorption at the oil-water interface and lowering surface tension, thereby aiding the formation of nanoemulsions with smaller particle sizes [29]. The co-surfactant PEG 400, possessing a high HLB value (greater than 10), contributes to promoting the spontaneous formation of nanoemulsions and augmenting the solubility of solutes within the dispersion medium by improving the flexibility of the layer enveloping the droplets [28].

Table 3 shows that the particle sizes of the three formulas fall within the nanoparticle size range (50-500 nm), where F1 has the smallest particle size compared to F2 and F3 (Figure 1). This small droplet size can increase the contact surface area and facilitate the permeation of active ingredients. Statistically, there is a significant difference in the nanoemulsion particle sizes of the Kersen fruit extract from the three formulas ($p < 0.05$). Nanoemulsion droplet size correlates with skin penetration; smaller particles enhance delivery of active substances to deeper skin layers. The results indicate that a higher amount of polysorbate 80 results in smaller particle sizes. Polysorbate 80 can prevent particle size increases by reducing surface free energy and forming a mechanical barrier. Meanwhile, PEG 400 in the formulation as a co-surfactant helps prevent phase separation, as indicated by the most homogeneous polydispersity index of the nanoemulsion shown by F3 among the other formulas, although not significantly different ($p > 0.05$). The polydispersity index indicates the homogeneity of droplets in a system. An index value of < 0.5 indicates homogeneous droplets, while a value of > 0.5 indicates varying droplet sizes and results in variable penetration of the active substance. Furthermore, the preparation was characterized for zeta potential. A standard zeta potential value of ± 30 mV indicates system stability; values in this range indicate repulsion between particles, which prevents aggregation [30].

The zeta potential values of the three formulas are close to ± 30 mV (Figure 1). The use of the surfactants Polysorbate 80 and PEG 400, which are nonionic surfactants, tends to lower the zeta potential values because these surfactants do not have charges on their hydrophobic groups. Statistically, there is no significant difference in the zeta potential values of the three formulas ($p > 0.05$). In addition to determining particle size using a particle size analyzer, the formation of nanoemulsion droplet sizes can be observed by examining the clarity of the produced mixture and based on the % transmittance determined using a spectrophotometer [31]. Transmittance percentage influences the formation of dispersion in nanoemulsions and visually serves as a qualitative indicator of dispersion spontaneity. Transmittance approaching 100% indicates a clear and transparent dispersion with nanometer-sized particles. The transmittance results of nearly $> 90\%$ from the three formulas indicate that the particle sizes have reached the nanoparticle level and show a clear solution, which is consistent with the research conducted by Buya *et al.* and Mobarak *et al.* [32,33].

Another notable characteristic observed in the produced nanoemulsions is the emulsification time, which demonstrates the ability of the nanoemulsion to form spontaneously in less than one minute. The results presented in Table 3 indicate that emulsion formation occurs rapidly across all three formulations. The process of creating nanoemulsions in each formulation is facilitated by polysorbate 80 acting as the surfactant and PEG 400 serving as the co-surfactant [34]. These components function by reducing the interfacial tension between the aqueous and oil phases. The co-surfactant intercalates between the surfactant molecules,

generating additional space that enhances structural expansion, thereby increasing fluidity and promoting a more efficient formation of nanoemulsions [34–36].

The entrapment efficiency was calculated to quantify the amount of active substance encapsulated within the nanoemulsion formulation. A higher adsorption efficiency value indicates a greater concentration of the active substance. According to the results in Table 3, the efficiency values for the three formulations exceeded 60%, meeting the desirable encapsulation-efficiency criteria for nanoemulsions. Among the three formulations, F1 showed the lowest encapsulation efficiency, which can be attributed to its smaller particle size relative to the other two formulations. Particle size significantly influences the ability to retain compounds within the droplets. Overall, the combination of these three components yielded a nanoemulsion with the anticipated characteristics suitable for delivering compounds found in Kersen extract. These findings are consistent with previous studies [19], which indicated that a blend of olive oil, Polysorbate-80, and PEG 400 can yield an optimal nanoemulsion formulation in terms of particle/droplet size, emulsification time, drug loading, and percentage transmission.

The resulting nanoemulsion was refined into a serum formulation to improve its applicability. The ethanol extract nanoemulsion derived from Kersen fruit was incorporated into a Na-CMC gel base at a ratio of 2:1. This ratio was used to produce a nanoemulsion serum with physical characteristics that are easy to apply topically. Sodium carboxymethyl cellulose (Na-CMC) is used as a gel base to obtain preparations with the viscosity expected for serum preparations because it exhibits neutral characteristics, stable viscosity, resistance to microbial growth, and the ability to form a transparent gel. A 4.5% Na-CMC concentration was used to create a nanoemulsion serum with optimal viscosity for topical application, staying within the typical 2-6% range for gel bases. Phenoxyethanol was used as a preservative at 0.5% to inhibit microbial proliferation. Its amphiphilic properties, which allow solubility in both aqueous and oleaginous phases, make it particularly effective in water-based formulations incorporating emulsions, such as nanoemulsions [28]. The final formulation underwent comprehensive physical characterization to ensure optimal user experience. The results of this characterization are detailed in Table 4, which includes organoleptic properties, homogeneity, pH, viscosity, and spreadability.

Table 4. Characteristics of the resulting nanoemulsion serum of kersen fruit extract.

Characteristic	F1	F2	F3
Organoleptic	Form: Semi-solid Color: brown Odor: specific	Form: Semi-solid Color: brown Odor: specific	Form: Semi-solid Color: brown Odor: specific
Homogeneity	Homogenous	Homogenous	Homogenous
pH	6.40±0.23	6.55±0.31	6.72±0.2
Spreadability (cm)	5.2±0.26	5.7±0.15	5.3±0.29
Viscosity (cPs)	2900±28.6*	3700±76.4*	9200±57.7*

*The symbol indicates that there is a significant difference between the formulas (p-value <0.05, n=3, Tukey one-way ANOVA). Among the dependent variables in the evaluation formulas, only the viscosity parameter showed a significant difference compared to the pH and spreadability parameters. Despite this difference, it still complied with the viscosity requirements for a semi-solid dosage form.

An organoleptic assessment was performed to evaluate the characteristics of the formulation post-preparation. This evaluation encompassed visual analysis of shape, color, and odor. The findings indicate that the formulation has a semi-solid, gel-like consistency, a brown hue, and a distinct fragrance, with no coarse particles that may interfere with application. Notably, there is no discernible organoleptic difference among the three formulations. The pH

values of the three formulations align with the standard pH range stipulated by Indonesian National Standard (SNI) 164399-1996 for topical preparations, which is between 4.5 and 7.5 [37]. Formulations with pH levels that are excessively acidic or alkaline can have detrimental effects on the skin. Specifically, a formulation that is overly acidic may lead to skin irritation, whereas one that is too alkaline can cause dryness and scaliness of the skin [38].

An additional characteristic evaluated in the assessment of the formulations is spreadability and viscosity. This evaluation aims to provide insights into the serum's ability to spread upon application, which is indicative of its ease of application on the skin. An effective topical formulation should demonstrate a spreadability range of 5-7 cm, as highlighted in the data presented in Table 4. The capacity of the formulation to spread is directly influenced by its viscosity. Acceptable viscosity levels are established between 2000 and 50000 cPs [37]. The findings indicate that all three formulations comply with the established viscosity standards.

The characterization test results form the basis for advancing to *in vitro* studies that assess the ability of the nanoemulsion system to enhance the penetration of phenolic or flavonoid compounds from Kersen extract using Franz Diffusion Cells. This apparatus measures drug release kinetics from topical formulations under conditions mimicking human skin. The experiments maintain a temperature of $37 \pm 2^\circ\text{C}$ with continuous stirring at 200 rpm. The receptor compartment is filled with Phosphate Buffered Saline (PBS) at pH 7.4, simulating human bodily fluids, while the donor compartment contains the test serum sample, separated by a skin membrane derived from human circumcision. This configuration allows for the evaluation of the Kersen extract nanoemulsion's penetration into the dermis, the primary site for anti-aging effects.

Diffusion testing was conducted over three hours, with samples taken at 15, 30, 45, 60, 90, 120, and 180 minutes. Each time a fluid sample was collected from the receptor compartment, it was immediately replaced with an equal volume of initial fluid to maintain a consistent volume. The absorbance of the samples was then measured for further analysis to determine the cumulative penetration amount (Q) and diffusion rate (Flux), as shown in Figure 2.

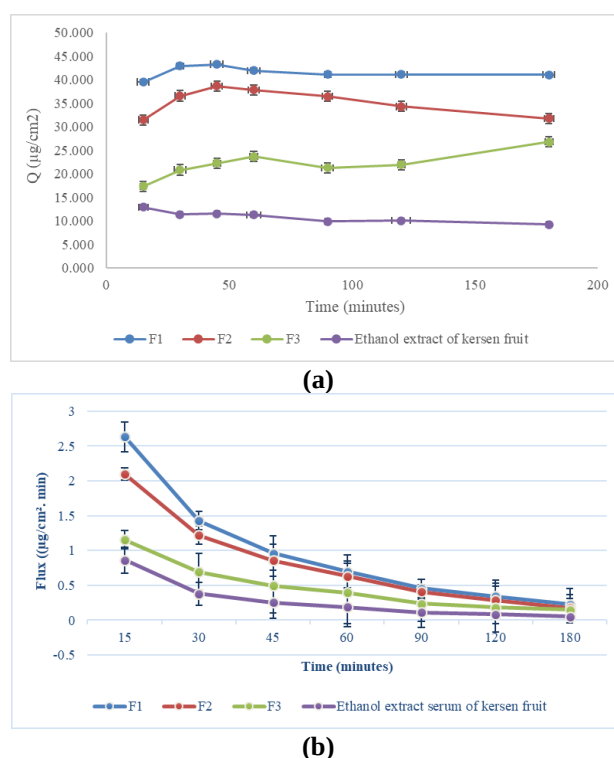


Figure 2. (a) the cumulative amount (Q); (b) flux (J).

The findings regarding the penetration profile of the anti-aging serum derived from the ethanol extract of Kersen fruit (*Muntingia calabura* L.) are illustrated in Figure 2. This figure presents the cumulative amount (Q) of compounds permeating per area per unit time (minute) (Figure 2a) over a duration of 3 hours (180 minutes), quantified as gallic acid, alongside the flux per unit time (Figure 2b). The data indicate that the highest penetration amount (Q) was observed in formulation F1, followed by formulations F2 and F3. This observation is attributed to the smaller particle size of F1 relative to F2 and F3 (Table 3), which enhances the optimization of compound penetration through the skin layers, despite F1 exhibiting a lower %EE value compared to F2 and F3. This finding is consistent with the research conducted, which demonstrated that reduced particle sizes facilitate improved diffusion capabilities.

Further diffusion tests revealed that the penetration of compounds into the skin layers was detectable as early as 15 minutes, with an increase observed until 45 minutes, after which a decline was noted; however, statistical analysis indicated that this decrease was not significant ($p > 0.05$). This result underscores the efficacy of the nanoemulsion delivery system in promoting phenolic compound penetration through the skin, which showed a statistically significant difference ($p < 0.05$), with values 2 to 4 times greater than serum preparations not formulated as nanoemulsions. Smaller particles in nanoemulsions form, allowing the active compound to penetrate the skin's stratum corneum more effectively. This system is small enough to pass through the denser skin barrier, enhancing the penetration of active ingredients deeper into the dermal layers by transcellular and paracellular mechanisms. Smaller particles have a larger surface area, allowing more active molecules to interact with the skin's surface and accelerating diffusion. As the nanoemulsion serum is applied to the skin, the active ingredients are gradually released over time, increasing bioavailability by allowing prolonged absorption rather than rapid release and potential loss of efficacy [34,39,40].

In addition to the Q value, the flux values indicate that serum preparations using the nanoemulsion delivery system show a faster diffusion rate than the extracted serum (without nanoemulsion). The nanoemulsion particle size ranges from 20 to 500 nm, facilitating efficient transport and penetration of phenolic compounds into the skin layers. The particle size of the nanoemulsion also significantly influences the diffusion rate (flux) of compounds in F1 (Figure 2b), which is higher than in F2 and F3 due to its smaller particle size (Table 3).

Overall, the observed decrease in flux value after the initial minute (Figure 2b) suggests that the penetration rate during the first minute is more rapid than in subsequent minutes. According to the research by Marwah *et al.* [41], this phenomenon may arise because the active substances present in the lower layer during the initial phase have not fully dissolved in the receptor compartment, potentially inhibiting the entry of active substances into the receptor compartment in the following minutes.

The research subsequently progressed to evaluate the inhibitory activities of the diffusion assay results in vitro across all formulations of nanoemulsion serum derived from the ethanol extract of Kersen fruit (*Muntingia calabura* L.), specifically formulations F1, F2, and F3—alongside serum from the ethanol extract of Kersen fruit and a control serum without extract (blank sample). Tyrosinase is an enzyme essential for skin pigment synthesis, a process known as melanogenesis, and it catalyzes two distinct reactions: the hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to DOPA quinone. Tyrosinase within skin tissue is activated by ultraviolet (UV) radiation from sunlight, which subsequently accelerates melanin production [42]. Elastase is a proteolytic enzyme capable of hydrolyzing various protein types, including the supporting and structural proteins of

connective tissue, such as collagen, elastin, proteoglycans, and keratin. The degradation of elastic fibers occurs readily through the action of elastase, which is activated by UV exposure (photoaging) or ROS [43,44]. Hyaluronidase acts as an endoglycosidase, facilitating the breakdown of hyaluronic acid (HA) into monosaccharides by cleaving its glycosidic bonds through the hydrolysis of (1-4) hexosaminidic bonds, resulting in bepolysorbate, N-acetyl-D-glucosamine, and D-glucuronic acid residues. Hyaluronic acid is a glycosaminoglycan and a principal component of the extracellular matrix, playing a critical role in maintaining skin elasticity. The hydrolysis of HA by hyaluronidase reduces its viscosity and increases its permeability. Aging correlates with a decline in skin moisture levels maintained by hyaluronan (HA), which binds and retains water molecules. As aging progresses, the amount of HA present in the skin diminishes, resulting in dryness and the formation of wrinkles [45]. Prolonged UV exposure can activate these three enzymes, leading to considerable effects, including wrinkle formation, loss of elasticity, sagging, and ultimately, skin aging [6,26]. Consequently, the inhibition of these enzymes may play a vital role in preventing premature aging.

The tyrosinase enzyme inhibition activity was assessed using mushroom tyrosinase and L-DOPA as the substrate. The method for quantifying inhibition relies on the enzymatic conversion of L-tyrosine to L-DOPA, resulting in dopachrome, which has a light purple color measurable at 480 nm [46]. Nur *et al.* noted that flavonoids, phenolic compounds, and tannins in the ethanol extract of Kersen fruit may act as depigmentation agents by inhibiting tyrosinase activity [12].

The results from the tyrosinase inhibition assay (Figure 3a) showed peak inhibition at 45 minutes for all formulations, followed by a decline until 180 minutes, although this decrease was not statistically significant ($p > 0.05$). This aligns with the diffusion test results (Figure 2a), which indicated the highest Q value at 45 minutes, suggesting a correlation between polysorbate and increased Q value and enhanced inhibition activity. Formulation F1 demonstrated the highest inhibition at 41.79%, significantly differing ($p < 0.05$) from formulations F2 and F3, as well as the ethanol extract serum of Kersen fruit and the serum base control. The tyrosinase inhibition activity of the nanoemulsion formulation was 2.2-2.8 times greater than that of the conventional serum containing only the ethanol extract, highlighting the nanoemulsion system's effectiveness in improving the penetration of compounds from the Kersen fruit extract.

The elastase inhibition activity test assesses the serum's ability to inhibit elastin degradation, a key factor in skin aging, by Porcine Elastase. When elastase activates in the presence of a substrate, it produces a yellow color measurable at 405 nm [47]. Results shown in Figure 3b align with the inhibition observed against tyrosinase and correlate with diffusion test outcomes (Figure 2a). Peak inhibition occurred at 45 minutes (F1) with inhibition activity of 29.08%, after which activity declined, though this decrease was not statistically significant. The serum demonstrated an inhibition capacity 2.4 to 2.9 times greater than the control (5.78%), which was a serum extract not formulated as a nanoemulsion.

The hyaluronidase enzyme inhibition test evaluated the effectiveness of ethanol extract serum from Kersen fruit nanoemulsion in inhibiting hyaluronidase, which degrades hyaluronic acid (HA). This was measured by the turbidity generated when HA reacts with an albumin solution, quantifiable at 600 nm [26,48]. Results shown in Figure 3c reflect a consistent pattern with the diffusion test, indicating an increasing trend in inhibition percentages during the first 45 minutes (27.65%), followed by a slight decline that was not significant. F1 showed the

highest inhibition percentage among F1, F2, and F3 and was significantly higher than the non-nano emulsified serum extract, with inhibition 5 to 8 times higher.

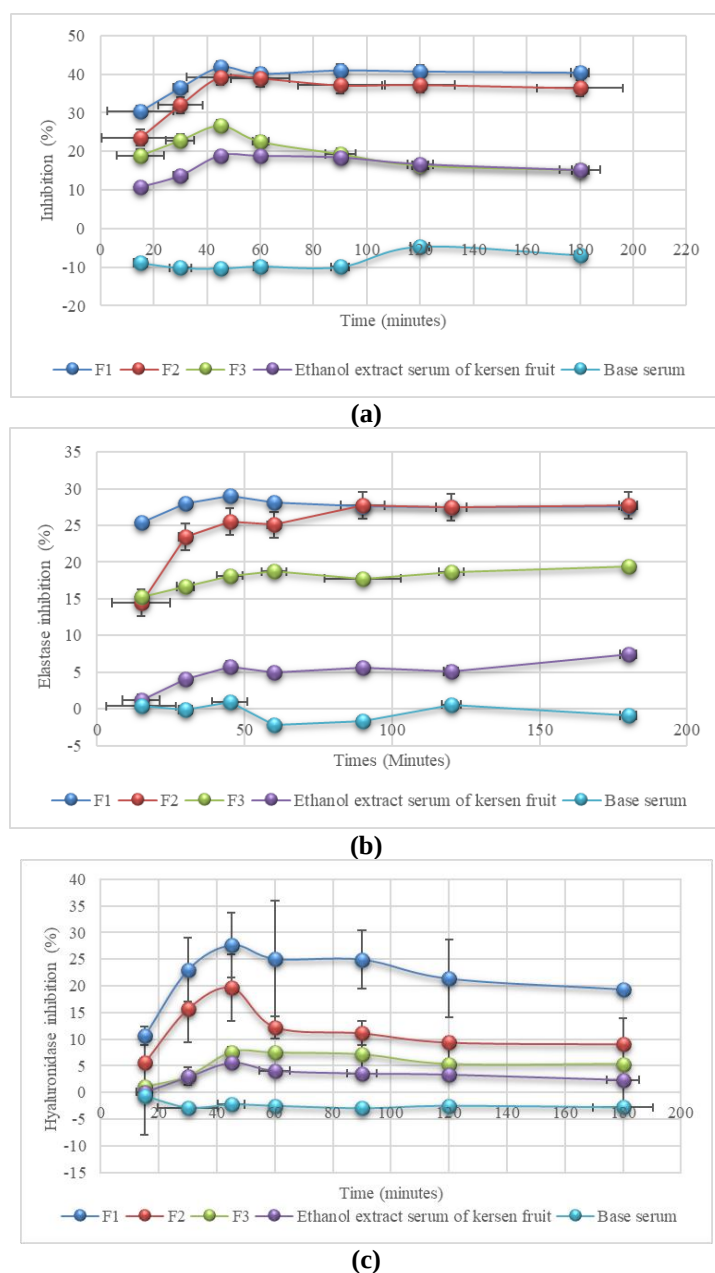


Figure 3. Profile of the inhibitory effects of a Kersen fruit ethanol extract-derived nanoemulsion on (a) tyrosinase; (b) elastase; (c) hyaluronidase enzymes.

The design of an anti-aging preparation formula involving nanotechnology with active ingredients from kersen fruit ethanol extract has been described as significantly influencing anti-aging effects. Several studies have shown that natural extracts can be effectively formulated using nanoemulsion-based drug delivery systems. Ghazy *et al.* demonstrated this with *Lawsonia inermis* extract, which was assumed to have high efficacy as an antimicrobial [49]. At the same time, Safe *et al.* reported similar results with *Achillea millefolium* and *Crocus sativus* flower extracts, highlighting the enhanced antimicrobial potential of the nanoemulsion formula [50]. Ismail *et al.* also found that nanoemulsions made from mustard and flaxseed oils achieved stable formulas and improved the penetration of active substances, leading to greater effectiveness [51]. The use of nanotechnology, particularly nanoemulsions, in developing natural extract formulations is now being applied across various pharmaceutical preparations.

Nanoemulsions made from natural extracts hold great potential in formulations, especially for cosmetics. Their formulation can improve the solubility and stability of active compounds while boosting skin absorption. This makes them an innovative choice for creating cosmetic products that are effective, safe, and eco-friendly, aligning with the growing demand for natural solutions.

4. Conclusions

The nanoemulsion serum formula containing ethanol extract of kersen fruit has been successfully developed. The physical evaluation parameters of formula F1, including particle size (195.07 ± 26.80), polydispersity index (0.324 ± 0.13), zeta potential (-29.03 ± 1.0), and percentage transmittance (98.798%), demonstrate superior characteristics compared to formula 2 (F2) and formula 3 (F3). The smaller particle size of F1 contributes to a better cumulative profile of active substances and an improved diffusion rate (Flux). In vitro studies show that the physicochemical properties and diffusion rate of active substances in F1 are linked to its effectiveness in inhibiting tyrosinase, elastase, and hyaluronidase enzymes, measured over 0 to 180 minutes. Evaluation results reveal that F1 nanoemulsion serum inhibits these enzymes within a range of 25-50%, showing a significant difference from F2 and F3 with a p-value < 0.05 . The nanoemulsion serum formula, derived from ethanol extract of kersen fruit, holds potential as a reference for enhancing production and optimizing cosmetic formulations. This study still requires more development to validate its in vivo effectiveness and build confidence in its benefits and use as an anti-aging cosmetic.

Author Contributions

Conceptualization, S.N. and A.S.; methodology, A.S. F.J.S. and M.I.; software, H.F.F. and E.D.F.; validation, A., S.S., and M.W.; formal analysis, A.N.A.; investigation, H.F.F. and E.D.F.; resources, S.N.; data curation, N.S. and M.W.; writing—original draft preparation, A.S. and F.J.S.; writing—review and editing, A.N.A.; visualization, A. and N.S.; supervision, S.N.; project administration, M.I.; funding acquisition, S.N. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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