Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 1 April 2024, 71-79
DOI: 10.20473/jfiki.v11i12024.71-79
Available online at https://e-journal.unair.ac.id/JFIKI/

Characterization and Stability Test of Hydrolyzed Collagen Glyserosomes

Nur Indah Safiul Fitria[1], Tristiana Erawati[2]*, Andang Miatmoko[2], Widji Soeratri[2]
[1]Master Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
[2]Department Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

*Corresponding author: tristiana-e-m@ff.unair.ac.id

Submitted: 5 March 2024
Revised: 10 April 2024
Accepted: 21 April 2024

Abstract

Background: *Hydrolyzed collagen is a protein obtained from enzymatic denaturation of collagen with a molecular weight of about 10 kDa, and it has been reported to produce anti-aging properties. Delivering hydrolyzed collagen into the dermis becomes a great challenge due to its large molecular weight, so glycerosome, a deformable vesicle containing glycerol as the edge activator, was developed to carry it into the dermis layer.* ***Objective****: The study aimed to determine the effect of increasing the concentration of glycerol and hydrolyzed collagen on the characteristics and stability of hydrolyzed collagen glycerosomes.* ***Methods****: Glycerosomes were composed of soy lecithin and prepared using a thin film lipid method. The lipid film was hydrated with phosphate-buffered saline pH 5 containing different glycerol concentrations (20% and 40%) and hydrolyzed collagen (2.5% and 5%). Then, characteristic tests and stability tests were carried out.* ***Results****: Hydrolyzed collagen glycerosomes had vesicle sizes of 170-180 nm, polydispersity index of 0.253-0.279, zeta potential values of -23.70 to -26.50 mV with deformability indexes of 2.25-3.49. The highest percentage of entrapment efficiency was 85.72%, achieved with a glycerol concentration of 40%. During the stability test at 25°C for 12 weeks, the hydrolyzed collagen glycerosomes did not experience pH and entrapment efficiency changes, but it increased the vesicle size.* ***Conclusion****: The use of 40% glycerol produced more deformable vesicles than 20% glycerol in hydrolyzed collagen glycerosomes; however, a formula improvement is required to improve the stability of glycerosomes.*

Keywords*: delivery system, glycerosome, glycerol, hydrolyzed collagen*

How to cite this article:
Fitria, N. I. S., Erawati, T., Miatmoko, A. & Soeratri, W. (2024). Characterization and Stability Test of Hydrolyzed Collagen Glyserosomes. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(1), 71-79 http://doi.org/10.20473/jfiki.v11i12024.71-79

P-ISSN: 2406-9388
E-ISSN: 2580-8303

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INTRODUCTION

Human skin protects the body from the surrounding environment and maintains body temperature (Aguirre *et al.,* 2020). The skin consists of three layers; in the dermis layer, some fibroblasts produce collagen and other protein matrics so that the skin becomes elastic (Lukić *et al*., 2021). Getting older and exposure to ultraviolet light (UV) and air pollution can cause skin aging. In skin aging, fibroblasts decline function, reducing collagen production (Jhawar *et al*., 2019). The decreased amount of collagen generates skin inelasticity and wrinkles, aging lines appearances, and skin dryness (Wang, 2021). So, topical delivery of hydrolyzed collagen (Cao *et al*., 2020) is needed to increase skin collagen levels and overcome skin aging.

Hydrolyzed collagen can be isolated from cattle, sheep, pigs, and marine (León *et al*., 2019). Hydrolyzed collagen is obtained from an enzymatic denaturation of collagen, which breaks down the protein into smaller weight molecules (Larde *et al*., 2023). Hydrolyzed collagen has anti-aging benefits for the skin by increasing the amount of collagen to increase the skin's elasticity (Aguirre *et al*., 2020). It also works on the dermis layer by filling in broken collagen to form collagen fibers (Ferdinando *et al*., 2022). Previous research (de Miranda *et al*., 2021) shows that hydrolyzed collagen supplements can increase skin elasticity compared to placebo. However, hydrolyzed collagen has a large molecular weight (Schmidt *et al*., 2019), which limits its topical delivery. Therefore, a delivery system, i.e., a deformable vesicle, is needed to effectively carry hydrolyzed collagen to the dermis layer.

Glycerosomes are deformable nanovesicles with a hydrophilic core surrounded by a lipid bilayer membrane with glycerol as the edge activator. Glycerosomes can load hydrophilic and lipophilic drugs (Zaki *et al.,* 2022). The lipid bilayer of glycerosomes is deformable, which can improve the permeability of the active ingredients (Gupta et al., 2020). As an edge activator, glycerol will move to the curved area of the lipid bilayer when the vesicles are under mechanical pressure resulting from the transepidermal osmotic gradient (Opatha et al., 2020) so that glycerosomes can pass through the intercellular skin gaps and reform to their original shape when reaching the deeper skin layers (Touitou and Natsheh, 2021). It has been reported that a glycerol concentration of 10% to 50% can successfully increase the skin penetration of vesicles (Gupta et al., 2020). In addition, the higher the concentration of glycerol, the higher the entrapment efficiency (Rani et al., 2021).

On the other hand, hydrophilic hydrolyzed collagen will be entrapped within the intraliposomal phase of the vesicles, and the higher the entrapped hydrolyzed collagen level, the higher the anti-aging efficacy will be since the skin collagen level will be increased. However, this vesicle aqueous core has limited volume capacity. Thus, the variation level of hydrolyzed collagen can affect the entrapment efficiency. Therefore, both parameters need to be evaluated in this research.

This study aims to determine the effect of increasing the concentration of glycerol and hydrolyzed collagen on the characteristics and stability of hydrolyzed collagen glycerosomes. The physicochemical characteristics of hydrolyzed collagen glycerosomes were evaluated for organoleptic properties, pH, vesicle size, polydispersity Index (PDI), zeta potential, deformability index, and entrapment efficiency (EE). In addition, the stability test of hydrolyzed glycerosomes was further observed for 12 weeks at 25°C.

MATERIALS AND METHODS

Materials

Hydrolyzed collagen was purchased from Fenchem, Nanjing, China. Soy lecithin (Phospholipids content 30%) was purchased from Himedia, Mumbai, India. Glycerol was purchased from Smart Lab, Tangerang Selatan, Indonesia. Dipotassium hydrogen phosphate, Potassium dihydrogen phosphate, and Sodium hydroxide were purchased from SAP Chemicals, Bandung, Indonesia. The aquadest was used as the solvent in this study.

Method

Preparation of hydrolyzed collagen glycerosomes

The hydrolyzed collagen glycerosomes were prepared using the formula as presented in Table 1, in which F1 contained 2.5% hydrolyzed collagen and 20% glycerol, F2 contained 2.5% hydrolyzed collagen and 40% glycerol, F3 contained 5% hydrolyzed collagen and 20% glycerol, and F4 contained 5% hydrolyzed collagen and 40% glycerol. Firstly, glycerosomes were prepared by dissolving soy lecithin in chloroform (1:10) in a round bottom flask. Then, the chloroform was evaporated entirely using a rotary vacuum evaporator (Buchi, Indonesia) at 100 rpm and 45°C for 45 minutes until a thin lipid layer formed at the bottom of the flask. A hydrating solution containing hydrolyzed collagen, glycerol, and phosphate-buffered saline (pH 5) was added to hydrate the thin lipid film by rotating the flask at 100 rpm and 45°C for 60 minutes. The mixture was

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E-ISSN: 2580-8303

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then sonicated for 20 minutes with a sonicator (Skymen, China) to obtain homogenous glycerosomes suspension (Gupta *et al*., 2020).

Characterization of hydrolyzed collagen glycerosomes

Organoleptic observation of hydrolyzed collagen glycerosomes

The physical appearance of the hydrolyzed collagen glycerosomes was organoleptically observed regarding their consistency, color, and odor (Table 2).

Morphology observation of hydrolyzed collagen glycerosomes

Morphological observations of hydrolyzed collagen glycerosomes were made using a scanning electron microscope (Thermo Fisher Scientific, US). Three drops of samples were placed in a holder and then dried for 24 hours. Then, the dried sample was coated with Au. The sample was then observed at 40.000x magnification (Gupta *et al*., 2020).

pH measurement of hydrolyzed collagen glycerosomes

The sample was diluted with distilled water (1:10), and the pH was measured using a pH meter (Horiba, Japan). The electrode was inserted into the sample solution until it showed a constant pH (Opatha *et al.*, 2020).

Vesicle size and polydispersity index (PDI) measurement of hydrolyzed collagen glycerosomes

About 100 µL of the sample was added with 3 mL distilled water, then placed in a cuvette and mixed until homogeneous by pipetting. Then, vesicle size and PDI were measured using the dynamic light scattering method with the Beckman Coulter Delsa Nano Particle Analyzer (US) (Santuso *et al*., 2023).

Zeta potential measurement of hydrolyzed collagen glycerosomes

About 100 µL of the sample was diluted with 3 mL distilled water, then put into a cuvette and mixed until homogeneous by the pipetting. Then, the zeta potential was measured using an electrophoretic scattering method with Horiba Nanoparticle Analyzer SZ-100 (Japan) (Santuso *et al*., 2023).

Deformability index measurement of hydrolyzed collagen glycerosomes

To measure the deformability index of the sample, about 1 ml of the sample was passed through a polycarbonate membrane (Millipore®, Merck) with 50 nm of pore diameter using an Avanti mini extruder (Avanti Polar Lipid, US) for 5 minutes. Then, the vesicle size was measured. The deformability index was calculated using the following formula (Opatha *et al*., 2020):

$$D = J\left(\frac{rv}{rp}\right)^2$$

D: Degree of deformability
A: volume of sample extruded in 5 minutes (ml)
Rv: vesicle size after extrusion (nm)
Rp: pore size (50 nm)

The determination of entrapment efficiency of hydrolyzed collagen in glycerosomes

The percentage of entrapment efficiency (EE) was determined by analyzing the free protein/hydrolyzed collagen content using the Bradford reagent (Himedia, Maharashtra, India) (Sari *et al.*, 2021). Firstly, about 1 ml of the sample was centrifuged at 10,000 rpm for 10 minutes. Then 0.1 ml of the filtrate was taken, put into a tube, and added with 5 ml of Bradford reagent. The mixture was then incubated for 10 minutes. The absorbance of free hydrolyzed collagen was then measured using UV-Vis Spectrophotometry at the wavelength of 595 nm. The percentage of entrapment efficiency was calculated using the following formula (Opathe *et al*., 2020):

$$\%\ EE = \frac{Total\ amount\ of\ the\ drug\ added - Amount\ of\ the\ free\ drug}{Total\ amount\ of\ the\ drug\ added} \times 100$$

Stability test of hydrolyzed collagen glycerosomes

The stability test of hydrolyzed collagen was observed by maintaining samples at room temperature (25±1°C) and relative humidity of 60±5% for 12 weeks. At the end of the study period, the sample was determined for pH, vesicle size, and %EE (Fayalil *et al*., 2020).

Table 1. Formulation of hydrolyzed collagen glycerosomes

Material	Function	concentration			
		F1	**F2**	**F3**	**F4**
Hydrolyzed collagen	Active ingredient	2,5%	2,5%	5%	5%
Soy lecithin	Phospholipid	2%	2%	2%	2%
Glycerol	Edge activator	20%	40%	20%	40%
Phosphate buffer saline (pH 5)	Hydrating solution	Up to 100%	Up to 100%	Up to 100%	Up to 100%

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E-ISSN: 2580-8303

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Table 2. The organoleptic properties of hydrolyzed collagen glycerosomes

Formula	Organoleptic Parameter		
	Color	**Odor**	**Consistency**
F1	Yellow	Odorless	Liquid
F2	Yellow	Odorless	Liquid
F3	Yellow	Odorless	Liquid
F4	Yellow	Odorless	Liquid

Statistical data analysis

All experiments were determined in three replicates, and the values were presented as mean and SD. The physicochemical characteristics were analyzed using a One-Way Analysis of Variance (ANOVA), while the stability data were analyzed using the Paired T-test. The analysis was then followed by Tukey's post hoc test.

RESULTS AND DISCUSSION

The organoleptic properties of hydrolyzed collagen glycerosomes

The organoleptic properties of hydrolyzed collagen glycerosomes, such as color, odor, and consistency, were observed visually. The results showed that all hydrolyzed collagen glycerosomes had the same properties. All formulas produced odorless yellow color suspension and have a liquid consistency, as presented in Table 2. The yellow color occurs because of the natural color of the phospholipids and liquid hydrolyzed collagen used in the formula.

The morphological observations of hydrolyzed collagen glycerosomes

The morphology of hydrolyzed collagen glycerosomes was examined using SEM. Hydrolyzed collagen glycerosomes were observed as spherical vesicles with a size around 100 nm, as shown in Figure 1. In the aqueous environment, the phospholipids will spontaneously assemble into a bilayer membrane surrounding an inner water phase, forming spherical vesicles (Sun et al., 2022). The spontaneous drying process during sample preparation resulted in spherical vesicle formation. However,. However, further investigation is needed since the vesicles may be perturbed during this process.

The pH of hydrolyzed collagen glycerosomes

The pH measurement in (Figure 2A) shows that all glycerosomes have an average pH of 5.55. Varying the glycerol concentration and hydrolyzed collagen had no significant effects on pH parameters ($p>0.5$). In this study, phosphate-buffered saline with a pH of 5 was used as the hydrating solution, thus reflecting the pH of the hydrolyzed collagen glycerosomes. The pH of the sample is highly determined by considering the stability of hydrolyzed collagen, which is stable at pH 3.68-5.70 (León *et al.*, 2019). In addition, as a topical product, the pH of hydrolyzed collagen glycerosomes should correspond to the pH of human skin, 4.5-6.5 (Lukić *et al*., 2021).

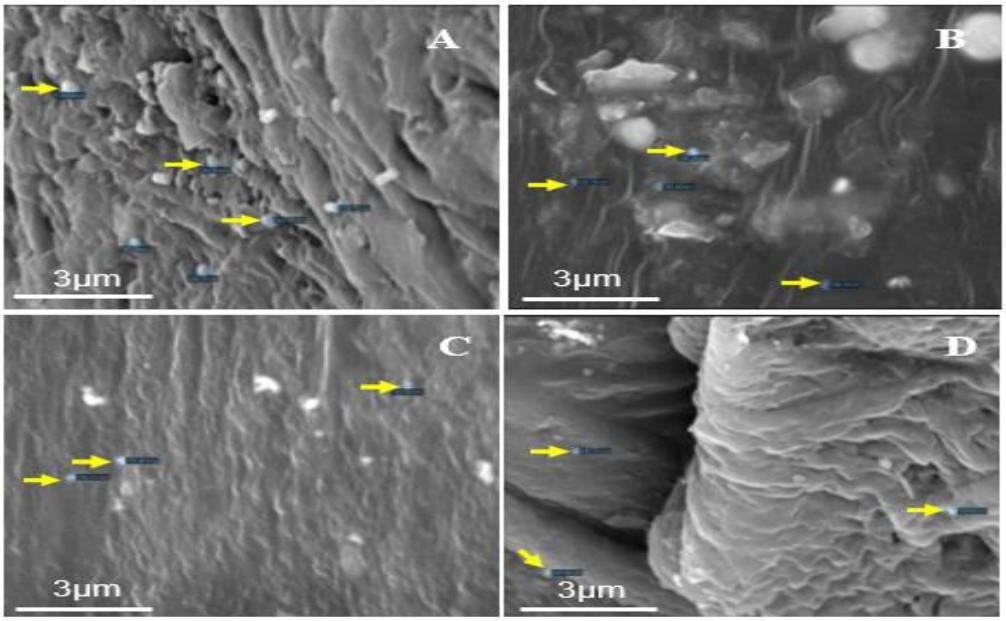

Figure 1. The morphological observations of hydrolyzed collagen glycerosomes (A) F1, (B) F2, (C) F3, (D) F4 by scanning electron microscopy. → = vesicle of glycerosomes

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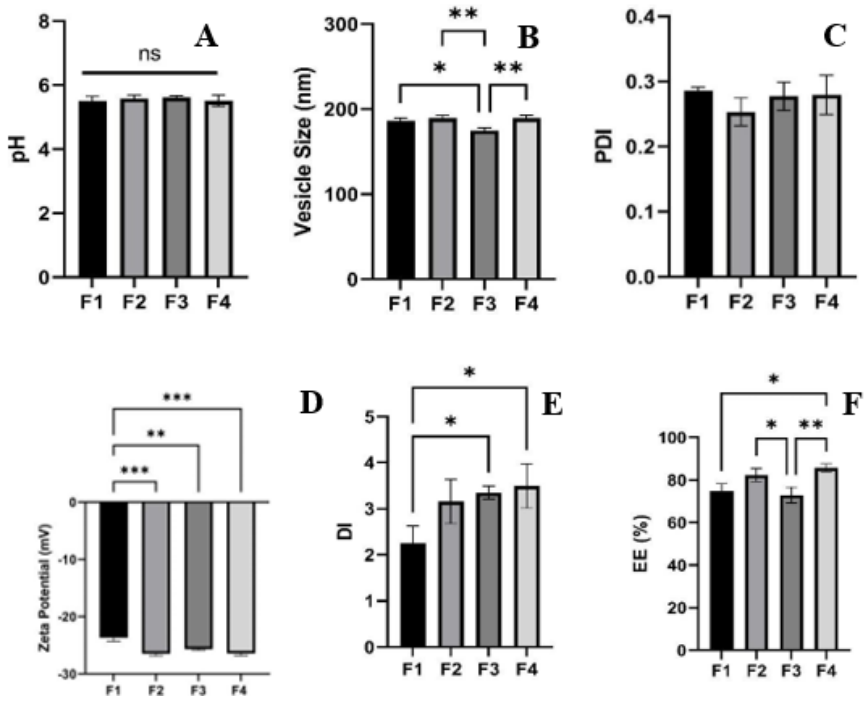

Figure 2. The evaluation of (A) pH, (B) vesicle size, (C) PDI, (D) zeta potential, (E) Deformability index, (F) %Entrapment Efficiency of hydrolyzed collagen glycerosomes

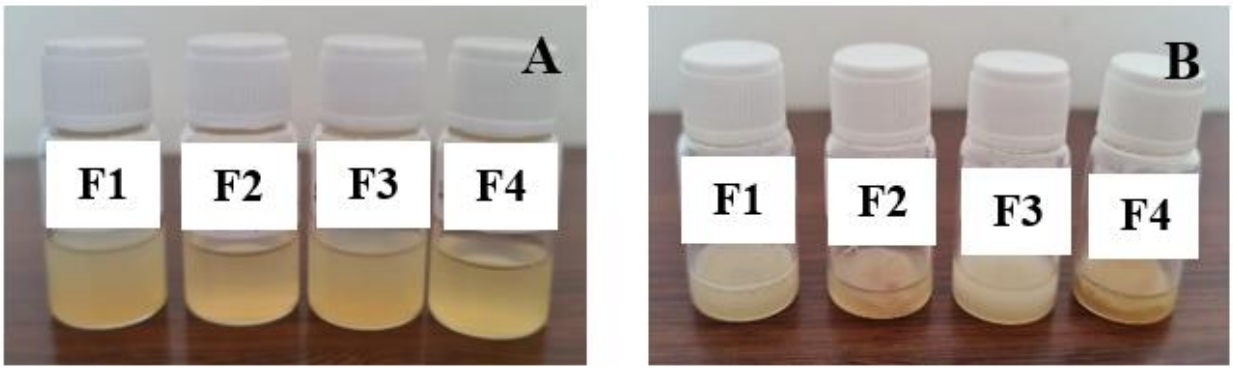

Figure 3. The organoleptic properties of hydrolyzed collagen glycerosomes (A) before and (B) after storage at a temperature of 25°C for 12 weeks

The vesicle size, Polydispersity Index (PDI), and zeta potential of hydrolyzed collagen glycerosomes

Designing a nanovesicle delivery system is essential to ensure penetration of the delivery system through deeper layers of the skin. The results show that the vesicle size of hydrolyzed collagen glycerosomes ranged from 170-180 nm, as presented in Figure 2B. Varying the concentration of glycerol and hydrolyzed collagen has a significant difference ($p<0.05$). It has been reported that increasing the edge activator level will increase the vesicles' size. Glycerol will damage the tight arrangement of phospholipids so that the phospholipids will stretch, causing the vesicles to become larger (Fatima et al., 2022).

In addition, the polydispersity Index (PDI) is a parameter of the size distribution homogeneity of glycerol vesicles, with a PDI value of 0.200 showing good homogeneity for the vesicles. The results showed that the PDI value of hydrolyzed collagen glycerosomes was 0.25-0.29, as shown in Figure 2C, which means all glycerosome formulas had homogeneous vesicle sizes. Varying the glycerol concentration and hydrolyzed collagen had no significant effects on the physical properties of the glycerosomes ($p>0.5$).

The zeta potential values of hydrolyzed collagen were around -20 mV, as presented in Figure 2D. Varying the concentration of glycerol and hydrolyzed collagen also had no significant difference ($p<0.5$). Zeta potential is related to glycerosome stability during storage. It has

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been reported that the zeta potential value of around ±30 mV indicates that the vesicles are stable during storage (Apriani., 2022).

The deformability index of hydrolyzed collagen glycerosomes

The deformability index is essential to determine the permeability of the delivery system (Rasheed *et al.*, 2022). Glycerol, as an edge activator, can make the lipid bilayer flexible by changing the orderly arrangement of phospholipids, thereby reducing the transition temperature of the bilayer membrane (Miatmoko *et al*., 2021). The results of the measured deformability index show that the deformability index value is 2.25 to 3.49 (Figure 3E). Varying the concentrations of glycerol and hydrolyzed collagen resulted in a significant difference in the deformability index ($p<0.5$). Hydrolyzed collagen glycerosomes containing 20% glycerol with deformability index values of 2.25-3.15 had a lower deformability index than hydrolyzed collagen glycerosomes containing 40% glycerol with deformability index values of 3.35-3.49. This result shows that the higher the glycerol level, the more flexible the glycerosomes produced (Leonyza and Surini., 2019). Variations in the concentration of hydrolyzed collagen influence the deformability index values.

The entrapment efficiency of hydrolyzed collagen in glycerosomes

Entrapment Efficiency (EE) is the value of the concentration of a drug trapped in a glycerosome vesicle (Opatha *et al*., 2020). As an edge activator, glycerol is important in increasing the EE value (Md *et al*., 2021). The EE results of hydrolyzed collagen glycerosomes differed significantly ($p<0.05$), as shown in Figure 3F. Hydrolyzed collagen glycerosomes containing 40% glycerol with EE values of 82.26-85.73% had higher EE than hydrolyzed collagen glycerosomes containing 20% glycerol with EE values of 72.90-74.90%. Glycerol, as an edge activator, can disrupt the stability of the lipid bilayer and increase its fluidity and elasticity. A high EE% indicates the successful formulation of a delivery system (Khan *et al*., 2022). Variations in hydrolyzed collagen concentration did not affect trapping efficiency. Hydrolyzed collagen is not all trapped in the vesicle because the compartments are limited, so the EE value has reached a maximum value.

The stability of hydrolyzed collagen glycerosomes

The stability test was evaluated by storing samples for 12 weeks at a temperature of 25±10°C with a humidity of 60±5% RH. After 12 weeks, the organoleptic properties of hydrolyzed collagen glycerosomes were evaluated. As shown in Figure 2, all formulas were still odorless; however, the visual appearance of several formulas has changed, and the F1 and F3 became cloudy, and F2 and F4 remained yellow (Figure 3B). Furthermore, lump-like sedimentation was observed in F2 and F4, as presented in Figure 3B. This sedimentation may occur because of the sticky nature of glycerol (Sharma *et al*., 2023). The pH of glycerosomes did not change ($p>0.05$) before and after the stability test, except for F4 (Figure 4A). The remaining pH can be maintained because phosphate buffered-saline pH 5 was used as a hydrating agent in the formula. This pH is related to the pH stability of hydrolyzed collagen, 3.68-5.70 (León *et al*., 2019), and the pH of the skin, 4.5-6.5 (Lukić *et al*., 2021).

In addition, the vesicle size of hydrolyzed collagen was significantly different before and after storage ($p<0.05$). The vesicle size became larger after 12 weeks of storage at room temperature (Figure 4B). This is probably due to the low zeta potential value (-26.50 mV until -23.70 mV), so the vesicles experience agglomeration (Apriani., 2022). There were negligible changes in %EE of hydrolyzed collagen glycerosomes before and after the stability test ($p>0.05$). In (Figure 5) the entrapment efficiency remains unchanged during 12 weeks of storage. If the phospholipid concentration is low, glycerosome leakage may occur (Zhu *et al*., 2022).

Based on this stability test, variations in glycerol concentration affected vesicle size and entrapment efficiency. The pH and entrapment efficiency remain unchanged in hydrolyzed collagen glycerosomes with hydrolyzed collagen concentrations of 2.5% and 5%. However, vesicle size changed with increased glycerol concentration.

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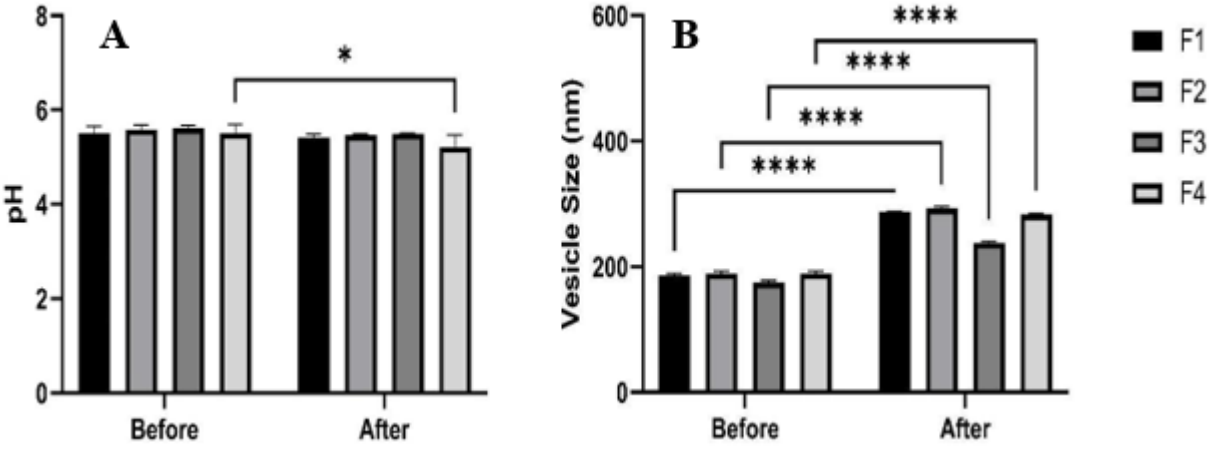

Figure 4. The pH (A) and vesicle size (B) hydrolyzed collagen glycerosomes before and after storage at a temperature of 25ºC for 12 weeks

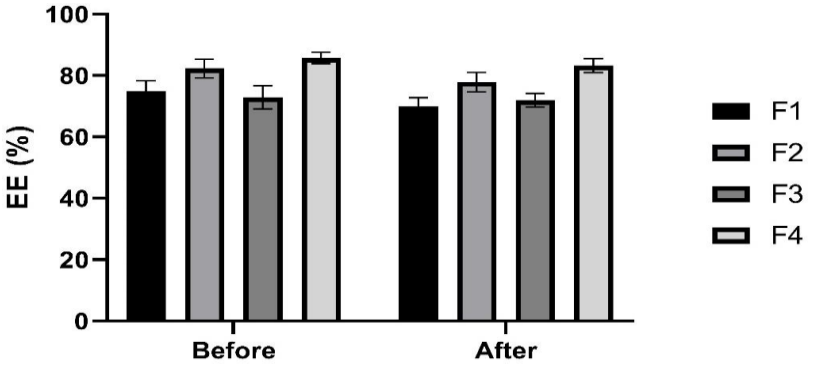

Figure 5. The results measurement of %EE of hydrolyzed collagen glycerosomes before and after storage for 12 weeks at a temperature of 25ºC

CONCLUSION

In conclusion, the use of glycerol affected the physical characteristics and stability of hydrolyzed collagen glycerosomes. Furthermore, this study also indicates that the concentration of hydrolyzed collagen had no effect on them, and a 40% glycerol concentration provided better physicochemical characteristics of hydrolyzed collagen glycerosomes than a 20% glycerol concentration at concentrations of hydrolyzed collagen of 2.5% and 5%.

AUTHOR CONTRIBUTIONS

Conceptualization, N. I. S. F., T. E., A. M., W. S.; Methodology, N. I. S. F., T. E., A. M.; Validation, N. I. S. F., T. E., A. M.; Formal Analysis, N. I. S. F., T. E., A. M.; Investigation, N. I. S. F., T. E., A. M.; Resources, N. I. S. F., T. E., A. M.; Data Curation, H.D.M N. I. S. F., T. E., A. M.; Writing - Original Draft, N. I. S. F., T. E., A. M.; Writing - Review & Editing, N. I. S. F., T. E., A. M.; Visualization, N. I. S. F., T. E., A. M.; Supervision, N. I. S. F., T. E., A. M.; Project Administration, N. I. S. F., T. E., A. M.; Funding Acquisition, N. I. S. F., T. E., A. M.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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