INTRODUCTION

Vesicles are colloidal particles with a concentric bilayer formed by amphipathic molecules surrounding an aqueous compartment. These are useful carriers for delivering hydrophobic active substances that are bound to lipid bilayers and hydrophilic active substances that are encapsulated in the aqueous interior [1]. Liposomes were the first vesicle system discovered; however, they have several disadvantages, including cost and decreased stability at various pH levels. Niosomes are vesicle systems with non-ionic surfactants that can be made easily in the laboratory. Non-ionic surfactants have been shown to facilitate the transport of active substances through the skin [2]. Niosomes increase the time active substances can remain in the stratum corneum and epidermis [3], solving the problem of penetration of the active substance. In addition, niosomes may be used for the delivery of labile and sensitive active substances [4].

The skin is the largest organ of the body and is an important interface between the environment and the body's interior. It prevents dehydration, prevents penetration of harmful foreign materials and microorganisms, acts as a barrier against mechanical attacks, maintains constant body temperature, and transmits incoming stimuli. The skin is divided into three layers: The epidermis, the dermis, and the subcutaneous layer. The epidermis is the outermost part, consisting of the layered squamous epithelium. It also contains keratinocytes that produce filamentary keratin proteins as a protective barrier in combination with various lipid components. The epidermis consists of several layers: The basal layer (stratum basale), the Malpighi or spine (stratum spinosum) layer, the granular layer (stratum granulosum), and the horn layer (stratum corneum). During epidermal differentiation, changes are seen in lipid composition. Cholesterol, triglycerides, and phospholipids are found in the lower epidermal layers, while in the stratum spinosum and stratum granulosum, the lipids are packed into flat granules and include phospholipids, glycolipids, and free sterols. These polar lipids are converted back into neutral lipids in the intercellular space when the granular contents are released. The stratum corneum is rich in ceramides, free sterols, and free fatty acids [5].

Because the skin is the outermost organ of the body, it is the main target of physical toxic substances, such as ultraviolet (UV) rays, and chemicals, such as xenobiotics, which can affect the skin's structure and function. In many environments, both direct oxidants and oxygen catalysts/reactive oxygen species (ROS) may be directly or indirectly involved. ROS are believed to activate proliferation and signaling that may alter the apoptotic pathway, which may be involved in the pathogenesis of disease, including skin malignancies. The skin has a variety of mechanisms for dealing with toxic substances to eliminate their adverse effects, including potent antioxidants and oxidant-degradation systems. These defense mechanisms are very effective, but limited capacity can cause increased ROS in the skin that can encourage the development of the dermatological disease. One method of preventing or treating ROS-related diseases is to provide antioxidants to restore normal skin conditions [6].

Herbal antioxidants are skincare alternatives because of their more skin-friendly nature and relatively fewer adverse side effects, such as allergic reactions and compared to synthetic materials. The herbal-based antioxidant epigallocatechin gallate (EGCG) is one of the main polyphenols in green tea. Polyphenols are powerful antioxidants capable of counteracting H_2O_2 and superoxide anions, thereby preventing free-radical damage [7]. Topical EGCG modulates the biochemical pathways involved in the inflammatory response, cell proliferation, and the tumor-promoter response, and is also a marker of skin inflammation induced by mild UV light [8]. EGCG is soluble in water and ethanol [9], but it has low skin-penetrating ability [10]. In this study, to overcome the problem of low permeation of active substances through skin layers and to increase their stability, a topical antioxidant preparation based on niosomes was prepared.
MATERIALS AND METHODS

Materials
Crude extract of green tea leaf (Balitro), standard EGCG (Sigma Aldrich), non-ionic surfactant (Span® 60), cholesterol (Sigma Aldrich), dichloromethane, aqua demineralisata, hydroxypropyl methylcellulose (HPMC), glycerin, propylene glycol, methylparaben, propylparaben, citric acid, methanol pro analysis (Merck), ethanol pro analysis (Merck), uranyl acetate 0.2%, and ascorbic acid.

Tools
Rotary evaporator (Hahn Shin), analytical scale (Sartorius), magnetic stirrer (Eutech), UV-Visible (Vis) spectrophotometer (Shimadzu UV-1800), computer equipped with UV-Vis spectrophotometer (UVProbe), refrigerator, ultrasonicator (Kubota), hot plate, climatic chamber (Memmert), homogenizer, Zetazizer particle size analyzer (PSA) (Malvern), and transmission electron microscope (TEM) (JEOL).

Methods
First, a calibration curve of standard solution containing standard EGCG in ethanol was prepared at concentrations of 7, 8, 10, 15, 20, and 24 ppm to give A (absorbance) of 0.2-0.8 at λmax=275 nm [11]. EGCG concentration in the crude extract of green tea was determined using the calibration curve.

Span 60 and cholesterol were dissolved in 50 ml of dichloromethane, placed in a round flask, and evaporated on a rotary evaporator at 39°C until dichloromethane completely evaporated and dry layers were formed. The formed thin layer was fed with nitrogen gas, and then allowed to stand for 24 hrs at room temperature before being hydrated to ensure the organic solvent was completely gone. The thin layer was then hydrated with a phosphate buffer solution of pH 5.5 with the help of glass beads to form 20 g of the niosome preparation in a rotary evaporator at a temperature of 60°C [12,13].

HPMC, glycerin, propylene glycol, methylparaben, propylparaben, and aqua demineralisata were carefully weighed. The gel was prepared by dispersing the polymer in warm aqua demineralisata (70°C) using a homogenizer at a rate of 1000 rpm. Methylparaben and propylparaben dissolved in the propylene glycol, glycerin, and 2% citric acid solution were added slowly to the polymer and aqua demineralisata mixture until the gel formed [14]. The niosome and gel-based suspensions with weight ratios of 1:1 were homogenized with homogenizer at a rate of 1000 rpm (Table 1).

Niosome characterization
The formed niosomes were characterized based on particle size and distribution, lamellarity, encapsulation efficiency, and zeta potential. The size and distribution of the particles were determined using a PSA, lamellarity was determined using a TEM, efficiency of encapsulation was determined using a UV-Vis spectrophotometer, and zeta potential was determined using the Zetasizer [11,15-18].

The size and distribution of the particles were measured by diluting 1 drop of the niosomes diluted in 30 ml of aquadest. The aqueous dispersion of the niosomes was then observed with a PSA.

Lamellarity was determined using a copper network to absorb the niosome particles from the suspension. They were then stained with 0.2% uranyl acetate in ddH₂O for 1 minute and dried. Specimens were observed under the TEM at 80 kV.

Table 1: Niosome formulations at various molar ratios (surfactant-to-cholesterol)

| Formulation | F1 (3:1) (mg) | F2 (2:1) (mg) | F3 (1:1) (mg) | F4 (0.5:1) (mg) |
|-------------|--------------|--------------|--------------|----------------|
| Span 60     | 1128         | 752          | 376          | 189            |
| Cholesterol | 338          | 338          | 338          | 338            |
| Green tea extract | 400        | 400          | 400          | 400            |

Notes: Total drug added: Amount of active substances inserted during preparation; Total free drug: Amount of free active substances in the supernatant.

Stability test
The ability of the vesicles to retain the active substance was determined by storing the niosomal suspension under different temperature conditions: 4±2°C (refrigerator), 25±2°C (room temperature), and 45±2°C (high temperature) for 12 weeks. Samples were periodically tested (every 2 weeks) for active substance content, in the manner described in the encapsulation efficiency procedure [19].

Antioxidant activity test with 1,1-diphenyl-2-picrylhydrazyl (DPPH)
The DPPH solution was prepared by dissolving DPPH in methanol (100 ppm), then 1 ml of extracts of various concentrations in methanol (2, 4, 6, 8, 10, and 12 ppm) and 1 ml of DPPH solution were homogenized and incubated in the dark. After 30 minutes, the absorbance was measured at λmax of DPPH in methanol (517 nm). The DPPH test was also performed on ascorbic acid as a control with the same concentration and niosomal gel preparations at concentrations equivalent to 2, 4, 6, 8, 10, and 12 ppm of green tea extract. The ability to counteract the DPPH radicals was calculated using the following equation [20]:

\[
\text{DPPH counteraction} (%) = \frac{A - B}{A} \times 100
\]

Notes: A: Absorbance of blank solution (1 ml DPPH 100 ppm + 1 ml methanol) at λmax DPPH=517 nm; B: Absorbance of ascorbic acid solution/extract/niosomal gel at λmax DPPH=517 nm.

RESULTS
Calibration curve
The linear calibration curve with r=0.9995 and linear regression equation y=0.00234 + 0.0263x was obtained, as seen in Fig. 1.

Formulation of niosomal gel
After dehydration and ultrasonication, a light brown niosomal preparation was formed (Fig. 2). After the gel base was homogenized with niosomal suspension (using the F3 formula), the niosome gel preparation was lighter brown than the niosomal suspension (Fig. 3).
Niosome characterization
The size of the majority of niosomes was 338.3 nm, and the particle distribution showed a polydispersity index of 0.349 (Fig. 4).

The TEM results showed the formation of visible vesicles. The active substance is illustrated by the black circles in the middle of the vesicles as seen in Fig. 5. The F1 encapsulation efficiency with the highest molar concentration, Span 60, had the highest encapsulation efficiency (77.80%) (Fig. 6 and Table 3). The niosomal suspension had a zeta potential −40 mV as seen in Fig. 7.

Niosome storage stability test
It was shown that the niosomes were most stable when stored at a temperature of 40°C (Table 4). The stability testing was done using formula F3.

Gel preparation evaluation
The color of the niosomal gel that formed was light brown, distinctive, and homogeneous (Fig. 8). The pH levels formulation of F3 were 4.99, 5.00, and 5.00, respectively. The pH was measured according to the pH range of the skin (4.5-6.5). The rheogram in Fig. 9 showed that the niosomal gel preparation was pseudoplastic. The results of the cycling test showed no changes in color or odor, and no visible syneresis. At low temperature, the niosomal gel preparation showed insignificant
changes in pH, organoleptic tests showed no changes, and the gel did not undergo syneresis (Fig. 10). At room temperature and at high temperature, pH level of the niosomal gel preparation decreased significantly, organoleptic tests showed no changes, and the gel did not undergo syneresis (Figs. 11 and 12).

Antioxidant activity
The DPPH test used ascorbic acid as a positive control and showed an inhibitory concentration 50% (IC₅₀) of 8.62 ppm for ascorbic acid. The green tea extract had an IC₅₀ of 9.18 ppm. In the niosomal preparation and niosomal gel forms, the IC₅₀ was slightly decreased at 11.97 and 12.51 ppm, respectively.

**DISCUSSION**

To manufacture the thin layer, the extract was mixed when the thin layer formed because this produces a higher absorption efficiency compared to addition of the extract during hydration. At the time of hydration, a phosphate buffer with a pH of 5.5 was used because the active substance of the extract is stable at acidic pH [21]. In the making of the gel, citric acid was added to achieve a pH corresponding to that of the skin (4.5-6.5).

Characterization of the niosomes showed that the resulting suspension had a uniform particle size. TEM results showed a less uniform vesicle size. This was probably due to fusion between vesicles formed during storage before the analysis. Although the results of F1 and F2 encapsulation efficiency were quite high, within 5 days of storage, F1 and F2 appeared to be separated, characterized by separation on the surface of the niosomal suspension. This was likely due to less rigid vesicles forming because the Span 60 molar ratio exceeded that of cholesterol.

The zeta potential (≤30 mV) indicated that the niosomal suspension was stable, and the formed particles tended to resist each other, with no tendency to form aggregates. The niosomal gel evaluation showed that the preparations were most stable when stored at low temperatures because the reaction was slowed,
and therefore changes were not significant. In addition, antioxidant activity was decreased as the extracts were made. This might be due to the reduced amount of free active substances of the extract due to the presence of lipid-extract complexes when dissolved in methanol.

CONCLUSION

The efficiency of niosome encapsulation containing green tea extract was increased with the greater molar concentration of Span 60. The highest encapsulation efficiency was obtained by the F1 formulation, with a molar ratio of Span 60-to-cholesterol of 3:1, that is, 77.80%. The encapsulation efficiency was 75.10% for F2, 63.33% for F3, and 56.39% for F4. However, F1 and F2, in which the molar ratio of Span 60 exceeded that of cholesterol, showed separation after 7 days of storage. The IC₅₀ of the extract decreased as the niosomal preparation and the gel were prepared. The IC₅₀ values for green tea extract, niosomal preparation, and niosomal gel were 9.18, 11.97, and 12.51 ppm, respectively. In addition, the niosomes with green tea extract (using formula F3) were most stable when stored at a low temperature (4±2°C). We suggest adding antioxidants to niosomal preparations to improve their stability, and continuing stability testing for up to 12 weeks. The effects of hydration duration should also be studied to determine the optimum time required to achieve the desired adsorption efficiency.

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