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Research Article

GREEN TEA EXTRACT IN AN EYELASH GROWTH ENHANCER GEL FORMULATION: STABILITY TEST, EYE IRRITATION TEST, AND HUMAN EYELASH GROWTH ACTIVITY

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ABSTRACT

Objective: To formulate a green tea extract (GTE), which is often used as a hair growth product, to produce an eyelash gel with good stability, effectiveness, and safety for growing eyelashes.

Methods: GTE was formulated into a gel. A stability test was performed at a high temperature (40±2°C), room temperature (25±2°C), low temperature (4±2°C), and a cycling temperature. An *in vitro* hen's egg test-chorioallantoic membrane assay was performed to evaluate potential eye irritation. An eyelash growth test was conducted by length measurement using an eyelash ruler before and after 2 mo of application in human volunteers.

Results: The GTE gel was stable in storage at high, room, and low temperatures and at cycling temperatures and did not cause eye irritation. Eyelashes grew significantly more in the test group than in the placebo group after 2 mo of application ($p<0.05$).

Conclusion: GTE gel provides a new, safe, and effective option for growing natural eyelashes.

Keywords: Green tea extract, Eyelash gel, Stability test, Hen's egg test-chorioallantoic membrane eye irritation test, Eyelash growth activity.

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INTRODUCTION

Long, thick, and full eyelashes stand for beauty and femininity in many cultures, whereas the loss of eyelashes has been associated with a loss of attractiveness and psychosocial problems [1]. Women often consider longer, thicker, and fuller eyelashes to be desirable and longer growth of eyelashes has been described as having a positive psychological effect [2]. Eyelash hair and scalp hair are basically terminal hair and have the same growth cycle (anagen, catagen, and telogen). The anatomy is also the same, but only scalp hair has arrector pili muscle [3]. The primary difference between eyelash hair and scalp hair is their growth pattern, i.e., the duration of growth of scalp hair is longer than 8 years, whereas that of eyelashes is approximately 5-12 months. The duration of the anagen phase is 6-8 years for scalp hair compared with 1-2 mo for eyelashes. The percentage of hair follicles in the telogen phase is only 5-15% for scalp hair and approximately 50% for eyelashes. In contrast to scalp hair, eyelashes are not sensitive to androgens [4], and therefore, are not susceptible to hair loss in response to androgen exposure. The differences in the cycle duration and the percentage of follicles in the telogen phase are the main reasons that eyelash hair cannot grow long like scalp hair. An understanding of these differences and similarities is useful when creating a product to treat eyelashes [3].

Green tea extract (GTE) has been used in many cosmetic products (e.g. anti-aging, eye creams, whitening, and acne care products) [5] and is also effective for hair growth in gel preparations [6]. Kwon *et al*. stated that green tea epigallocatechin-3-gallate (EGCG) caused an increase in *ex vivo* and *in vivo* human hair growth activity. This study indicated that EGCG caused hair growth by stimulating cell proliferation and causing an antiapoptotic effect on hair dermal papilla cells [7].

Various cosmetic products have been developed for many functions and applications. Cosmetic products for application around the eyes (e.g., mascaras and eye creams) and to hair (e.g., shampoos and hair tonic) may make contact with the eyes. Therefore, evaluating the eye irritation potential of a cosmetic product and its ingredients is critical to determine whether a product is safe for consumers to use for its intended and foreseeable applications in case of accidental exposure to the eye [8].

The Draize rabbit eye test has been the standard test used for 60 years to predict the human ocular irritation of cosmetic products. However, several aspects of the test have been criticized. These include the subjectivity of the method, the overestimation of human responses, and the method's cruelty [9]. In 2013, the EU banned the sale of all animal-tested cosmetic products. The ban applied to both the finished product and the raw ingredients [10-12]. Therefore, other *in vitro* eye irritation tests were developed to replace the *in vivo* methods. The hen's egg test-chorioallantoic membrane (HET-CAM) assay is an *in vitro* test developed by Luepke in 1985 to replace the Draize rabbit eye test for potential eye irritation [13,14]. The HET-CAM test permits the identification of irritant reactions that appear to be similar to those that occur in the eye using the standard Draize rabbit eye test. In the HET-CAM test, the three reactions observed are hemorrhage, lysis, and coagulation of the CAM. These are observed 5 minutes after direct application of the solution to the test subject at the exposed CAM [8,15].

In this study, GTE was formulated into a gel formulation. GTE gel stability using physical stability and a cycling test method and potential eye irritation using the *in vitro* HET-CAM method were evaluated, and eyelash growth activity was measured and compared to placebo gel in human volunteers.

METHODS

Materials

Ethanolic GTE was obtained from Balitro (Bogor, Indonesia). Aqua demineralisata, disodium ethylenediaminetetraacetic acid (EDTA), glycerin, sodium metabisulfite, and triethanolamine were purchased from PT. Brataco (Jakarta, Indonesia). Xanthan gum was obtained from Shandong Fufeng Fermentation Co. (Shandong, China) and isopropyl

Myristate from Oleon (Selangor, Malaysia). PEG-40 hydrogenated castor oil (HCO) was purchased from Corel PharmaChem (Gujarat, India) and Optiphen Plus™ from Saffire Blue Inc. (Ontario, Canada).

Methods

Formulation and preparation of the gel

Quantities of all materials were prepared as indicated in Table 1. Sodium metabisulfite and disodium EDTA were dissolved in 5 ml of aqua demineralisata and added to a mixture of xanthan gum and glycerin in aqua demineralisata with continuous stirring. A mixture of isopropyl myristate and PEG-40 HCO was added to the gel along with triethanolamine and Opthiphen Plus™, and the mixture was stirred until it was homogeneous. For the GTE gel, GTE was dissolved in 5 ml of aqua demineralisata before it was added.

Stability test

Physical stability tests were performed by storing the gel at high temperature (40±2°C), room temperature (25±2°C), and low temperature (4±2°C) for 3 mo. In addition, a cycling test was performed over 6 cycles (1 cycle=24 hrs in low temperature+24 hrs in high temperature) to monitor the stability of the GTE gel in changing temperatures. Organoleptic properties (color, visual appearance, and odor), pH, and homogeneity were observed every 2 weeks over the 3 mo test period and before and after the cycling test. A stable result at an accelerated temperature over 3 mo indicated the product would be stable for 1 year at room temperature [16].

HET-CAM test

Fresh, clean, fertile Leghorn chicken eggs, weighing 40-50 g and aged 7-10 d, were obtained from Balitnak (Bogor, Indonesia). These eggs were candled to detect the viability and development of the embryos before use. Defective eggs were discarded. The air space of the egg was marked, and the outer layer of the shell opened to expose the inner CAM membrane. The inner CAM membrane was opened to expose the CAM itself. The test solution was applied directly to the CAM to observe the response. For this test, the eggs were divided into three groups: Negative control, positive control, and treatment (n=3 per group).

For the negative control, 0.3 ml of 0.9% NaCl solution was applied directly to the CAM to provide a baseline for the assay endpoints. No response was expected.

For the positive control, 0.3 ml of 1% sodium dodecyl sulfate (SDS) was applied directly to the CAM. A hemorrhage response was expected.

For the treatment group, 0.3 ml of the GTE gel was applied directly to the CAM.

Effects were assessed within 5 minutes. The time point was noted when one of the following effects occurred: Hemorrhage, lysis, or coagulation. An irritation score (IS) was calculated, and the test item was classified from the score. The following formula was used to generate an IS:

$$IS=\left[\left(\left(\frac{(301-\text{Hemorrhage time})}{300}\right)\times 5\right)+\left(\left(\frac{(301-\text{Lysis time})}{300}\right)\times 7\right)+\left(\left(\frac{(301-\text{Coagulation time})}{300}\right)\times 9\right)\right]$$

Where hemorrhage time was the time (s) at which hemorrhage reactions started on the CAM; lysis time was the time (s) at which vessel lysis occurred on the CAM, and coagulation time was the time (s) at which coagulation formation began on the CAM [11]. After treatment, the IS was calculated and the irritation effect determined according to the following scheme: 0-0.9=No irritation; 1-4.9=Slight irritation; 5-9.9=Moderate irritation; and 10-21=Severe irritation [14].

***In vivo* trial of the eyelash gel in human volunteers**

In this trial, we used a randomized, double-blind, placebo-controlled method to test the GTE gel in human volunteers. The test subjects were divided into two groups: Placebo and test group. Each group consisted of 10 healthy females, aged 18-45 years, without any conditions of the eyes, eyelashes, or skin at the application area. The gel was applied in a thin layer at the base of the upper eyelashes daily at bedtime using an eyelash brush. The eyelash growth length was measured after 2 mo of application using an eyelash ruler [17] and compared to the length on the first day of the trial.

All tests concerning humans were ethically approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia, Indonesia, with regard to human rights and welfare in medical research (No: 649/UN2.F1/ETIK/2016). Volunteers provided written informed consent after comprehension of the study protocol for the effects that may occur at the area where the gel was applied.

Statistical analysis

Data were analyzed using SPSS version 22 software (IBM, Armonk, NY, USA). An independent t-test was used for normal and homogeneous data distribution, and a non-parametric Mann–Whitney test with a confidence level of 95% was used for irregular and homogenous/not homogenous data distribution. A result of $p<0.05$ was considered statistically significant.

Table 1: Compositions of the GTE and placebo gels

Materials	Quantity (%; w/w)	
	GTE gel	Placebo gel
GTE	2.5	-
Glycerin	6	6
Xanthan gum	0.8	0.8
Isopropyl myristate	4	4
Sodium metabisulfite	0.08	0.08
PEG-40 HCO	0.5	0.5
Disodium EDTA	0.1	0.1
Triethanolamine	0.17	0.17
Opthipen plus™	1.5	1.5
Aqua demineralisata	Ad 100	Ad 100

GTE: Green tea extract, HCO: Hydrogenated castor oil, EDTA: Ethylenediaminetetraacetic acid

RESULTS AND DISCUSSION

GTE, the active ingredient in this study, was produced using a macerated extraction method with 70% ethanol as the solvent. Compared to the use of water, the use of a mixture of ethanol and water resulted in the extraction of more phenol and flavonoid content from the green tea leaves [18,19].

Each component in this formulation was chosen in advance to produce a fine quality gel. Xanthan gum was chosen as the gelling agent because it is commonly used in drug, cosmetic, and food products. Xanthan gum is non-toxic, not an irritant, and stable over a large range of pH values (3-12). Glycerin was used as a humectant and solubility cosolvent, and sodium metabisulfite was used as an antioxidant to prevent GTE oxidation. Disodium EDTA was used as a chelating agent to prevent oxidation caused by metal and improve the anti-oxidant properties of sodium metabisulfite. Isopropyl myristate was used as a humectant with a penetration enhancer and non-sticky properties. PEG-40 HCO was used as a solubilizer for isopropyl myristate, which is an oil-soluble ingredient. Triethanolamine was used as a pH balancer. Ophtiphen Plus™ (a mixture of phenoxyethanol, caprylyl glycol, and sorbic acid) was used as a preservative. The combination of phenoxyethanol and sorbic acid offered a wide range of antibacterial and antifungal ability [20].

The organoleptic characteristics of the finished GTE gel were a light-green color (Pantone 2421 U), pH of 5.99, and viscosity of 20973

cps (Fig. 1). The finished GTE gel was easily spread on the skin and dried quickly. The pH was suitable for skin pH (4.5-6.5).

Evaluation of the GTE gel continued with a physical stability test conducted in various temperatures for 3 mo. Organoleptic properties, pH, and homogeneity of the GTE gel were observed periodically. After 3 mo of testing at low, room, and high temperature, there were no significant differences in the organoleptic properties compared to the first day of the test. The color and odor remained the same and were homogenous. The pH decreased in value but remained within the acceptable range of skin acidity (4.5-6.5). Fig. 2 shows the pH chart during the 3 mo stability test. A cycling test was performed to monitor the stability of the GTE gel in changing temperatures. The results of this test indicated that the gel was stable in fluctuating temperatures. In addition, the GTE gel was still homogeneous and the color and odor still the same as before the test. As an antioxidant agent, sodium metabisulfite effectively prevented oxidation of GTE, which was susceptible to oxidation, especially at high temperatures. These results indicated that GTE gel would be stable for 1 year at room temperature [15].

The HET-CAM test results indicated a large difference between the positive control (SDS 1%), negative control (0.9%), and GTE gel. The positive control (SDS 1%) induced major vascular hemorrhage of the CAM. 22 seconds after application of the SDS 1% solution, a small hemorrhage started to form. After 5 minutes, the hemorrhage continued to form and affected most of the vasculature of the CAM. Conversely, application of the negative control (NaCl 0.9%) had no effect on the CAM. Application of the GTE gel caused a minor hemorrhage after 277 s, but no lysis or coagulation appeared after 5 minutes. The ISs for positive control, negative control, and GTE gel were 4.63, 0, and 0.39, indicating the positive control would be slightly irritating to the eyes (1-4.9) and negative control, and GTE gel would not be irritating to the eyes (0-0.9). The GTE gel showed no potential irritation to eyes because the excipients for the GTE gel formula were non-irritating and non-toxic. In addition, some were registered as generally recognized as safe ingredients [20]. Fig. 3 presents the results regarding the eye irritation potential of GTE gel.

After a 2 mo trial in human volunteers, the eyelash length was significantly different between the test and placebo groups. In the placebo group, there was no growth of the eyelashes. In the test group, the eyelashes grew and the length was longer than on the first day of the trial by a mean of 0.0012±632 m ($p<0.05$; Table 2). Fig. 4 presents the results of a volunteer's eyelash after 8 mo application of the GTE gel. This indicated that GTE was the primary cause of this result. Catechins in the GTE, including EGCG as the major component, may play the primary role in inducing eyelash hair growth.

Fig. 1: The finished green tea extract gel

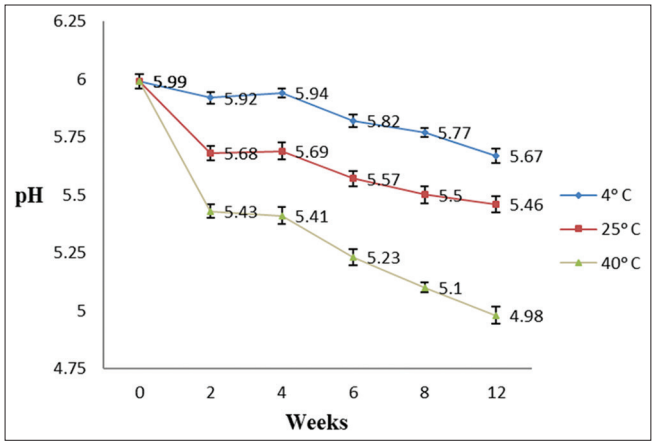

Fig. 2: pH profile over 12 weeks of storage at low temperature (4±2°C), room temperature (25±2°C), and high temperature (40±2°C). Data represent mean (±SD) (n=3)

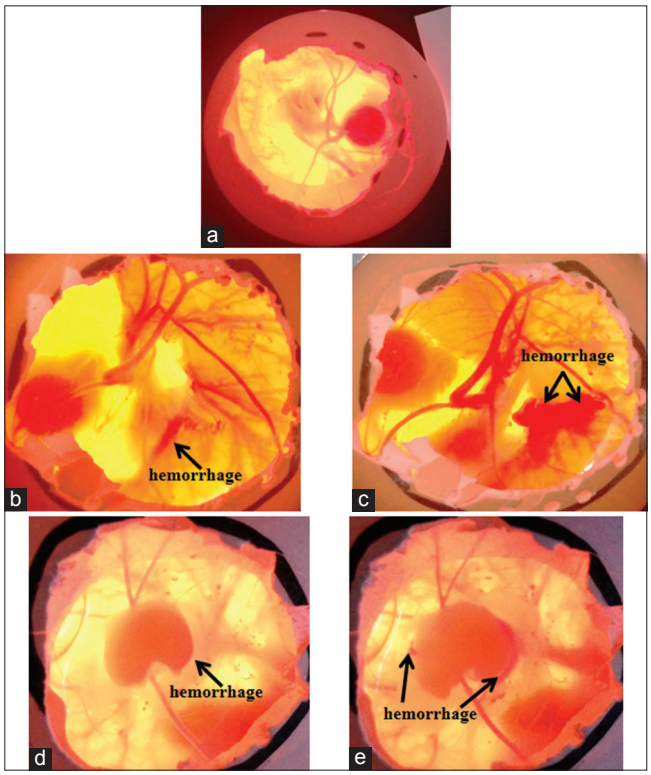

Fig. 3: Results of the hen's egg test-chorioallantoic membrane (CAM) test. (a) Normal CAM, (b) CAM treated with 1% sodium dodecyl sulfate (SDS) after 22 seconds, (c) CAM treated with 1% SDS after 5 minutes, (D) CAM treated with green tea extract (GTE) gel, (E) CAM treated with GTE gel after 5 minutes

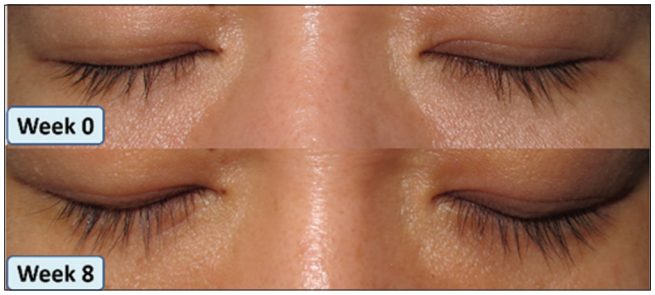

Fig. 4: Eyelash growth before and after 2 mo of green tea extract gel application

Table 2: Eyelash length after 2 mo of gel application

Group	Eyelash length (m)
GTE gel	0.0012±632
Placebo gel	0.0002±422

GTE: Green tea extract

CONCLUSION

Application of GTE in gel form caused eyelash growth activity after 2 mo of application. In addition, the gel exhibited good stability and had no potential for eye irritation. This study was limited by human error in measuring small changes in eyelash length. In addition, changes in eyelash color or thickness were not evaluated, which could contribute to the overall appearance of the eyelashes and cause the perception of positive changes. To the best of our knowledge, this is the first study to evaluate eyelash growth using an active herbal ingredient. Further investigation should continue with other herbal ingredients and technology to determine their effects on eyelash growth.

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