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

INDRA SYAHPUTRA ROES LIE*, JOSHITA DJAJADISASTRA, FADLINA CHANY SAPUTRI

Department of Cosmetology, Faculty of Pharmacy, University of Indonesia, Kampus UI Depok, 16424, West Java Indonesia.

Email: indra71090@gmail.com

Received: 21 March 2016, Received and Accepted: 31 March 2017

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.

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 4-8 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. Bratoco (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 Ophthepen 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 metabisulphite 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 Ophthepen 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 product. A stable result at an accelerated temperature over 3 mo indicated the product would be stable for 1 yr 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 Baltnik (Bogor, Indonesia). These eggs were candled to detect the viability and development of the embryos before use. Defective eggs were discarded. 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:

\[
IS = \frac{(301 - \text{Hemorrage time}) \times 5 + (301 - \text{Lysis time}) \times 9}{300} + (301 - \text{Coagulation time}) \times 5
\]

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.

**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 metabisulphite 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 metabisulphite. 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. Ophthepen 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
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 ± 0.0032 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
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.

ACKNOWLEDGMENTS
The authors wish to convey their appreciation to all their colleagues for their encouragement in accomplishing this study.

REFERENCES
1. Hunt N, McHale S. The psychological impact of alopecia. BMJ 2005;331(7522):951-3.
2. Shaikh MY, Bodla AA. Hypertrichosis of the eyelashes from prostaglandin analog use: A blessing or a bother to the patient? J Ocul Pharmacol Ther 2006;22(1):76-7.
3. Cohen JL. Enhancing the growth of natural eyelashes: The mechanism of bimatoprost-induced eyelash growth. Dermatol Surg 2010;36(9):1361-71.
4. Draelos ZD. Cosmetic dermatology products and procedures 2nd ed. United Kingdom: Wiley Blackwell; 2016.
5. Kumar S, Singh N, Archana S. Green tea polyphenols: Versatile cosmetic ingredient. Int J Adv Res Pharm Bio Sci 2012;1:348-62.
6. Asyarie S, Immaculata MI, Triwahyuni E. Hair growth evaluation from combination of green tea extract and celery in a gel formulation. Acta Pharm Indonesia 2007;32:12-7.
7. Kwon OS, Han JH, Yoo HG, Chung JH, Cho KH, Eun HC, et al. Human hair growth enhancement in vitro by green tea epigallocatechin-3-gallate (EGCG). Phytomedicine 2007;14(7-8):551-5.
8. Eskes C. Eye Irritation. Available from: http://www.ec.europa.eu/consumers/sectors/cosmetics/files/doc/antest/(15)_chapter_3/3_eye_irritation_en.pdf. [Last accessed on 2016 Sep 13].
9. Vinardell MP, Mitjans M. Alternative methods for eye and skin irritation tests: An overview. J Pharm Sci 2008;97(1):46-59.
10. Cosmetic Europe. EU Ban on Animal Testing. Available from: https://www.cosmeticsEurope.eu/safety-and-science-cosmetics-europe/alternative-methods/eu-ban-on-animal-testing.html. [Last accessed on 2016 May 14].
11. PETA. Cosmetics and Animal Testing. Available from: http://www.peta.org.uk/issues/animals-not-experiment-on/cosmetics/. [Last accessed on 2016 May 14].
12. Kanter J. E.U. Bans Cosmetics With Animal-tested Ingredients. 11th March, 2013. Available from: http://www.nytimes.com/2013/03/11/business/global/eu-to-ban-cosmetics-with-animal-tested-ingredients.html. [Last accessed on 2016 May 14].
13. Luepke NP. Hen’s egg chorioallantoic membrane test for irritation potential. Food Chem Toxicol 1985;23(2):287-91.
14. Cazedey EC, Carvalho FC, Fiorentino FA, Gremião MP, Salgado HR, Corrosito®, BCOP and HET-CAM as alternative methods to animal experimentation. Braz J Pharm Sci 2009;45:759-66.
15. Gilleron L, Coecke S, Sysmans M, Hansen E, Van Oproy S, Marzin D, et al. Evaluation of a modified HET-CAM assay as a screening test for eye irritancy. Toxicol In Vitro 1996;10(4):431-46.
16. Dajadiasastra J. Cosmetic Stability. Seminar Setengah Hari HIKI. Department of Pharmacy, Faculty of Mathematics and Sciences, University of Indonesia; 2003.
17. Yongho, H. Eyelash measuring device. Patent US 9226537 B1. 5 January; 2016.
18. Rusak G, Komes D, Likic S, Horzic D, Kovac M. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. Food Chem 2008;110:852-8.
19. Hu CJ, Gao Y, Liu Y, Zheng XQ, Ye JH, Liang YR, et al. Studies on the mechanism of efficient extraction of tea components by aqueous ethanol. Food Chem 2016;194:312-8.
20. Rowe RC, Shekcy PJ, Quin ME. Handbook of Pharmaceutical Excipient. USA: Pharmaceutical Press and American Pharmacist Association; 2009.

Table 2: Eyelash length after 2 mo of gel application

| Group        | Eyelash length (m)         |
|--------------|---------------------------|
| GTE gel      | 0.012±632                 |
| Placebo gel  | 0.002±422                 |
| GTE: Green tea extract |                |