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Research article

Green tea [*Camellia sinensis* (L.) Kuntze] leaves extract and hibiscus (*Hibiscus tilliaceus* L.) leaves extract as topical hair growth promoter in microemulsion

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Abstract

Green tea [*Camellia sinensis* (L.) Kuntze] leaves and *Hibiscus tilliaceus* leaves have been used empirically for hair treatment. However, scientific evidence of their use in herbal preparations for treating hair loss is still limited. This study investigated the hair growth activity, safety and stability of green tea leaves ethanolic extract (GTE) and hibiscus leaves ethanolic extract (HTE) in topical microemulsion preparations. Varied concentrations of each GTE and HTE formulation (2.5%, 5% and 7.5%) were evaluated. The activity of hair growth was investigated using an *in vivo* method on rats; hair lengths were measured on days 7, 14 and 21 and hair weight on day 21. A safety test was carried out using the upper hand area on 18 volunteers. Physical stability testing involved cycling test along with storage at high temperature (40 ± 2°C), room temperature (25 ± 2°C), and low temperature (4 ± 2°C). The formulated GTE and HTE was successful in producing significant hair growth activity ($p < 0.05$) compared to the no treatment and vehicle control groups on days 7, 14 and 21. The optimum formula for GTE was at the concentration of 7.5% and for the HTE microemulsion at 7.5% which showed superior hair growth activity compared to 2.5% minoxidil microemulsion after 21 d. None of the formulas showed irritation and all were stable in storage. GTE and HTE microemulsion could be used as potential natural hair growth promoter.

Introduction

Hair loss which eventually leads to baldness is growing as a prevalent concern in the modern era (Bergfeld and Mulinari-Brenner, 2001; Schmitt et al., 2012). It can occur in men or women at various ages and is caused by emotional/physical stress, physical condition, nutrient deficiency, hormonal disturbances or particular drugs (Harrison and Bergfeld, 2009). As hair loss increases, it intimidates patient's psychologically and psychosocially: women have reported to lose self-confidence and self-esteem, while men become more aggressive and children are more anxious, withdrawn or aggressive (Ganguly and Karnik, 2012). It is a broad concern as it affects the quality of life for every sufferer.

There continues to be industrial research into products to alleviate hair loss issue using either synthetic or natural products. Though minoxidil has been used since 1984 as an officially proven drug to treat hair loss by U.S. Food and Drug Administration (USFDA), it presents potential side effects such as hypertrichosis, pruritus, local

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irritation, contact dermatitis, skin allergy, flushing, headache, vertigo, fatigue and edema (McEvoy, 2002; Lucky et al., 2004; Olsen et al., 2005; Rogers and Avram, 2008). Furthermore, minoxidil needs to be administered regularly over 6−12 mth to gain significant hair growth, yet failure to comply will result in the hair falling out as per prior to the treatment conditions (Price, 1999). In light of this situation, natural herbal products are one of the potential alternative solutions, providing a lower risk of side effects.

Green tea [*Camellia sinensis,* (L.) Kuntze] leaves are one of the potential natural products for hair growth. Green tea has been consumed since early ages as a beverage and food, for which no side effects have been reported despite consumption for long periods (Barrett, 2004). Green tea has been used empirically as a hair growth enhancer (Dalimartha, 1999) by consuming it as a beverage or by topical administration as a traditional cream bath or shampoo preparation. Previous research of green tea formulated in a hair tonic dosage form for topical administration showed significant activity of hair growth with a green tea concentration of 2.5% (Amin et al., 2014).

In addition, hibiscus (*Hibiscus tilliaceus* L.) leaves also have potential as natural ingredients for hair growth. The leaves are abundantly available in Indonesia and their utilization has not been maximized; their main use is as a food preparation material in West Java, Indonesia, yet it has more benefits for hair fertility and hair loss prevention as its empirical function (Dalimartha, 2000). Only a few hair preparations of hibiscus leaves are commercially available in Indonesia such as shampoo.

The scientific evidence of the use of green tea leaves and hibiscus leaves in herbal preparation for treating hair loss is still limited. Therefore, this research investigated green tea leaves ethanolic extract (GTE) and hibiscus leaves ethanolic extract (HTE) formulated in microemulsion dosage forms with dosage variation of 2.5%, 5% and 7.5% to achieve the optimum dose, which was evaluated for its activity regarding hair growth promotion, safety and physical stability.

Materials and Methods

Preparation of plant extract

Camellia sinensis (L.) Kuntze leaves (Bogor, Indonesia) and *Hibiscus tilliaceus* L. leaves were purchased in Bogor City, West Java province, Indonesia in 2013. Both specimens were identified and compared to herbarium voucher specimens (951/IPH.1.02/If.B/V/2013) by the Indonesian Institute of Science, Bogor, Indonesia, and voucher specimens deposited at the Faculty of Pharmacy, University of Indonesia, Depok, Indonesia.

Dried leaves of camelia leaves and hibiscus leaves were grounded into powder, then extracted using a maceration process with 70% ethanol as the solvent. A ratio about 1:10 leaves to 70% ethanol was used for extraction over 10 cycles. The solvent was removed using a rotary evaporator producing fine extracts afterward, resulting in 991 g of camelia dried leaves powder producing 367 g extract or an extraction yield of 37.03% (weight per weight; w/w). On the other hand, hibiscus leaves were extracted using 6 L of 70% ethanol for six cycles, after which the solvent was removed using a rotary evaporator. The dried leaves of hibiscus (1,238 g) produced 138 g of extract or an extraction of 11.15% (w/w).

Animals and ethics statements

The animals used were healthy Spraque-Dawley male rats (*Rattus norvegicus*), aged 7–8 wk and weighing 150–200 g (obtained from Bogor Agricultural University, Bogor, Indonesia). The experimental protocol was approved by the Health Research Ethics Committee Faculty of Medicine University of Indonesia, Cipto Mangunkusumo Hospital (Approval no. 638/H2.F1/ETIK/2013).

Formulation, preparation and evaluation of microemulsion

The formula of each microemulsion is presented in Table 1. Preparation of microemulsions started by dissolving methyl paraben and propyl paraben into propylene glycol. In a separate beaker, sodium metabisulfite was dissolved in aqua dest, while GTE or HTE was dissolved in 96% ethanol and butyl hydroxyl toluene in 96% ethanol. Afterward, the oil phase, water phase and Tween 80 were mixed and stirred constantly using a homogenizer at 1,000 revolutions per minute (rpm) for 5 min at room temperature while adding co-solvent (propylene glycol) gradually. The formed microemulsions were left for 2–3 hr to produce the final products. The evaluations in this research were organoleptic, pH (Eutech Instruments; Singapore), viscosity (RVF model Brookfield viscometer, Middleborough, MA, USA), surface tension (tensiometer Du Nuoy, Cole-Parmer® Surface Tensiomat® 21, Vernon Hills, IL, USA), particle size distribution (Zeta sizer Nano S; Malvern, UK), and centrifugal test (Kubota 5100; Fujioka, Japan).

Hair growth activity test

The rats were allowed to acclimatize for 2 wk in advance of each new environment. In this evaluation, each microemulsion of green tea extracts and hibiscus extracts was conducted in two separate tests. Each test was performed using 24 rats that were randomly divided into 6 treatment groups containing 4 rats each, as follows:

- Group I: shaved with no formula applied, acting as no treatment group (No Treat)
- Group II: shaved and applied only basis/excipients of the microemulsion, acting as vehicle control group (VC)
- Group III: shaved and applied formula 1, extract concentration of 2.5% (GTE1/HTE1)
- Group IV: shaved and applied formula 2, extract concentration of 5.0% (GTE2/HTE2)
- Group V: shaved and applied formula 3, extract concentration of 7.5% (GTE3/HTE3)
- Group VI: shaved and applied 2.5% minoxidil microemulsion, acting as positive control group (PC)

Each rat had an area shaved of approximately 4 cm × 4 cm on the dorsal area, then depilatory cream was applied (Yoon et al., 2010) using hair removal cream Veet® (obtained from PT. Reckitt Benckiser, Indonesia). The test area was specified as 2 cm × 2 cm for each application by topical administration, twice a day, in the morning and evening.

Hair length determination was performed on days 7, 14 and 21 after treatments, for which the hair was pulled randomly and 10 longest hairs were measured and the average length was calculated and expressed the mean length ± SD of 10 hairs (Mitsui, 1996; Yoon et al., 2010).

Table 1 Formulation of microemulsions of green tea leaves ethanolic extract (GTE) and hibiscus leaves ethanolic extract (HTE), including vehicle control and positive control

Ingredients	Quantity (%) (weight per weight)							
	Vehicle control	Green tea extract micro-emulsion 2.5% (GTE1)	Green tea extract micro-emulsion 5% (GTE2)	Green tea extract micro-emulsion 7.5% (GTE3)	hibiscus extract micro-emulsion 2.5% (HTE1)	hibiscus extract micro-emulsion 5% (HTE2)	hibiscus extract micro-emulsion 7.5% (HTE3)	Positive control
Green Tea Extract	-	2.50	5.00	7.50	-	-	-	-
Hibiscus Extract	-	-	-	-	2.50	5.00	7.50	-
Minoxidil	-	-	-	-	-	-	-	2.50
Propylene glicol	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Isopropyl miristat	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Tween 80	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00
Butyl hydroxytoluen	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Sodium metabisulfite	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Metyl paraben	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Propyl paraben	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Ethanol 95%	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Aqua dest	41.09	38.59	36.09	33.59	38.59	36.09	33.59	38.59

Hair weight determination was performed on day 21 by weighing all the hairs produced on the test area on day 21 and expressed as hair weight ± SD from four rats (Mitsui, 1996; Adhirajan et al., 2003; Yoon et al., 2010).

Data obtained were analyzed statistically using the IBM software SPSS Statistic version 21 (SPSS Inc., Chicago, IL, USA). A one-way ANOVA test, followed by least significant difference analysis was used for normal and homogenous data distribution. For irregular and homogenous/not homogenous data distribution, nonparametric statistics (Kruskal Wallis) testing was used, followed by the Mann-Whitney test with a confidence level of 95%. A value of $p < 0.05$ was considered statistically significant.

Safety test

A skin irritation test was conducted on 18 volunteers in Depok, Indonesia. Each microemulsion formula was applied to three volunteers. This test was ethically approved (638/H2.F1/ETIK/2013) and the prior informed consent of all subjects was obtained. The tested formulas were GTE1, GTE2 and GTE3 with concentrations of 2.5%, 5.0% and 7.5% green tea extract, respectively, and HTE1, HTE2 and HTE3 containing hibiscus leaves extract at 2.5%, 5.0% and 7.5%, respectively. Following each test, cleansed volar upper arm skin of each volunteer was applied with 2 mL of microemulsion (2.5 cm × 2.5 cm). Each test area was covered with transparent plastic and wrapped in a gauze pad for 24 hr, then unwrapped, washed, and observed for any redness, edema, or itching. Observations were repeated after 48 hr.

The obtained data were evaluated to calculate a primary irritation index Equation 1 (Euasathien et al., 2005):

$$PII = \frac{(\Sigma erythema + \Sigma edema\ grade) at\ 24\ hr and\ 48\ hr}{total\ volunteers\ \times number\ of\ observations} \quad (1)$$

Physical stability test

The physical stability tests performed were a cycling test and storage at high temperature (40 ± 2°C), room temperature (25 ± 2°C) and low temperature (4 ± 2°C). The cycling test was performed by changing the storage temperature at 24 hr intervals, from a low temperature (4° ± 2°C) to a high temperature (40 ± 2°C), and repeating for six cycles. The physical evaluations performed were organoleptic observation, pH and homogeneity.

Results and Discussion

Extraction of leaves and formulation of microemulsion

In extracting the green tea leaves and hibiscus leaves, ethanol was used to capture the active constituents because the green tea leaves are rich in polyphenols (catechin, flavonol, flavandiol, and phenolic acid) in the form of a glycoside (Ebadi, 2001; Daniel, 2006), while the hibiscus leaves are rich in polyphenolic compounds, flavonoid and saponins (Salem et al., 2014). The presence of phytochemical constituents using qualitative testing with *Camellia sinensis* (L.) Kuntze extract was positive for flavonoid, phenolic, alkaloid, saponin, tannin, triterpenoid and glycoside. The *Hibiscus tilliaceus* L. extract was positive for flavonoid, phenolic, saponin, alkaloid, tannin, steroid and glycoside. Specifically, the assay content of total catechin in GTE was 75.10% using spectophotometry. These extracted constituents are polar; hence, there is low absorption on the skin due to the nature of polar molecules that have high molecular sizes and so are unable to cross lipid membranes which leads to loss of bioavailability and efficacy. In this respect, the microemulsion preparation should overcome the absorption difficulty because it can encapsulate the active ingredients and using it as a surfactant to form small particle sizes (10–100 nm).

In formulating the microemulsion, prior optimization had been performed to determine the final formulations described in Table 1. The formulation consisted of oil, water, surfactant and co-surfactant. The surfactant was used to stabilize the microemulsion system; the co-surfactant was used to reduce the surface tension between oil and water (Madhav and Gupta, 2011). In these formulations, isopropyl myristate was used as an oil phase, Tween 80 as a surfactant and 96% ethanol as a co-surfactant and a dissolving agent for the extracts. Additionally, 96%

ethanol might also act as an enhancer (Rowe et al., 2009). The formulated microemulsions had excellent solubility properties in completely solubilizing the extracts, giving clear solutions without any precipitation.

Microemulsion properties

The initial evaluation of GTE microemulsions and HTE microemulsions is presented in Table 2. Organoleptic observation showed that each microemulsion was transparent and homogenous, having increasing color intensity with higher concentration**.** The higher the extract concentration, the lower the pH because the ethanolic GTE extract and the ethanolic HTE extract contributed acidity. Nonetheless, all the GTE and HTE formulations were acceptable based on the skin pH balance (pH 4.5–6.5). Density testing for product characterization, showed a similarity to water (Table 2). The viscosity of each formula based on the rheogram results showed Newtonian movement due the its small particle sizes (less than 100 nm). The distribution of particle size initial results for GTE1, GTE2 and GTE3 were 17.97 nm, 15.90 nm and 16.26 nm, respectively, whereas HTE1, HTE2 and HTE3 had smaller particle sizes of 14.34 nm, 14.38 nm and 14.72 nm, respectively. All microemulsions met the microemulsion particle size specification (10–100 nm). The centrifugal test was used to determine microemulsion stability to gravitational force in storage based on Stokes law. After being centrifuged at 3,750 rpm speed at room temperature for 5 hr, all formulas remained homogenous without phase separation. These results proved that the surfactant layer was sufficiently robust to cover the contents of the oil drops in the microemulsion. Additionally, it proved that the microemulsions were stable over 1 yr of storage.

Hair growth activity of microemulsion

The growth of hair undergoes cyclic periods: anagen (phase of growth), catagen (phase of involution) and telogen (resting phase). The disruption of these hair cycles is the cause of all hair loss (Harrison and Bergfeld, 2009; Orasan et al., 2016b).

The triggering source which disturbs the hair cycle varies for individuals and the etiology is complex and not well understood. Therefore, increasing hair growth during the continuous hair cycle could act as an indication of drug efficacy. In addition, a hair loss model on rats based on the shaving and depilation method could be used as the model of hair cycle disruption and it has been generally used in several studies (Orasan et al., 2016b; Yoon et al., 2010).

Other than the depilation method, there are several hair loss models being developed for a specific cause of hair loss, such as Chemo-Therapy Induced Alopecia (Hussein et al., 1990), Testosterone Induced Alopecia (Wang et al., 2017) or models still under development such as for stress-induced hair loss (Stalder et al., 2017). Nonetheless, in the current study, a method of depilation was applied to capture how GTE and HTE could impact hair growth and specifically on hair follicles without additional external factors. Further investigation using a specific cause of hair loss could be performed as a separate study.

Basically, the hair follicle cycle in rats (about 7 wk old) follows a precise timeline. It is acknowledged that depilated hair follicles will enter the final stage of growth phase of hair cycle (anagen VI) after 9 d of depilation, followed by regression (catagen) after about 17 d of depilation, and finally enter the resting phase (telogen) around 20 d after depilation (Müller-Röver et al., 2001).

Hair length represents the growth capacity of hair, while hair weight represents the total amount of hair on a specific study area. These measurements could indicate the efficacy of drugs that stimulate hair growth as they could distinguish the effect of hair growth from non-treated with treated subjects (Price and Menefee, 1990; Price et al., 1999).

A study performed by Price and Menefee (1990) showed that calculated quantities of total hair weight could clearly distinguish treated minoxidil-treated subjects from untreated subjects; in addition, these data are easier to obtain and less prone to error during sampling and measurement. Other research by Orasan et al. (2016a) found that a Kerium substance being tested for hair growth activity presented a significant increase in anagen induction compared with the control group and they also used hair weight evaluation as one of the parameters. Based on the study, data from through trichoscopic examination

Table 2 Initial evaluation results from each microemulsion of green tea leaves ethanolic extract (GTE) and hibiscus leaves ethanolic extract (HTE)

	Color	Opacity	Homogeneity	pH	Density (g/mL)	Particle Size Distribution (nm)	Viscosity	Surface Tension ($\mu N/cm^2$)	Centrifugal test
GTE1	Yellowish Green+	Transparent	Homogeneous	5.23	0.988	17.97	Newtonian fluid	341	Homogeneous, no phase separation
GTE2	Brownish Green++	Transparent	Homogeneous	5.18	0.990	15.90	Newtonian fluid	345	Homogeneous, no phase separation
GTE3	Dark Green+++	Transparent	Homogeneous	5.10	1.01	16.26	Newtonian fluid	350	Homogeneous, no phase separation
HTE1	Light green brownish +	Transparent	Homogeneous	5.81	1.04	14.34	Newtonian fluid	363	Homogeneous, no phase separation
HTE2	Green brownish ++	Transparent	Homogeneous	5.68	1.05	14.38	Newtonian fluid	367	Homogeneous, no phase separation
HTE3	Green brownish +++	Transparent	Homogeneous	5.47	1.05	14.72	Newtonian fluid	370	Homogeneous, no phase separation

(GTE1 = 2.5% green tea extract microemulsion; GTE2 = 5.0% green tea extract microemulsion; GTE3 = 7.5% green tea extract microemulsion; HTE1 = 2.5% hibiscus extract microemulsion; HTE2 = 5.0% hibiscus extract microemulsion; HTE3 = 7.5% hibiscus extract microemulsion Light green brownish+ = Pantone 112 PC; Green brownish++ = Pantone 119 PC; Green brownish+++ = Pantone 133 PC; Yellowish Green+ = Pantone 583 C; Brownish Green++ = Pantone 581 C; Dark Green+++ = Pantone 5747 C).

could not determine a minor increase in hair density (as it was not possible to count the number of hairs per unit or determine their diameter); nonetheless, hair weight evaluations were concluded to be one objective and quantitative method which can determine slight increases in hair density.

Green tea extract microemulsion

As shown by the visual observation illustration (Fig. 1), rats treated with green tea extract microemulsions produced hair that appeared to be fully grown compared to no treatment and the vehicle control group on day 21.

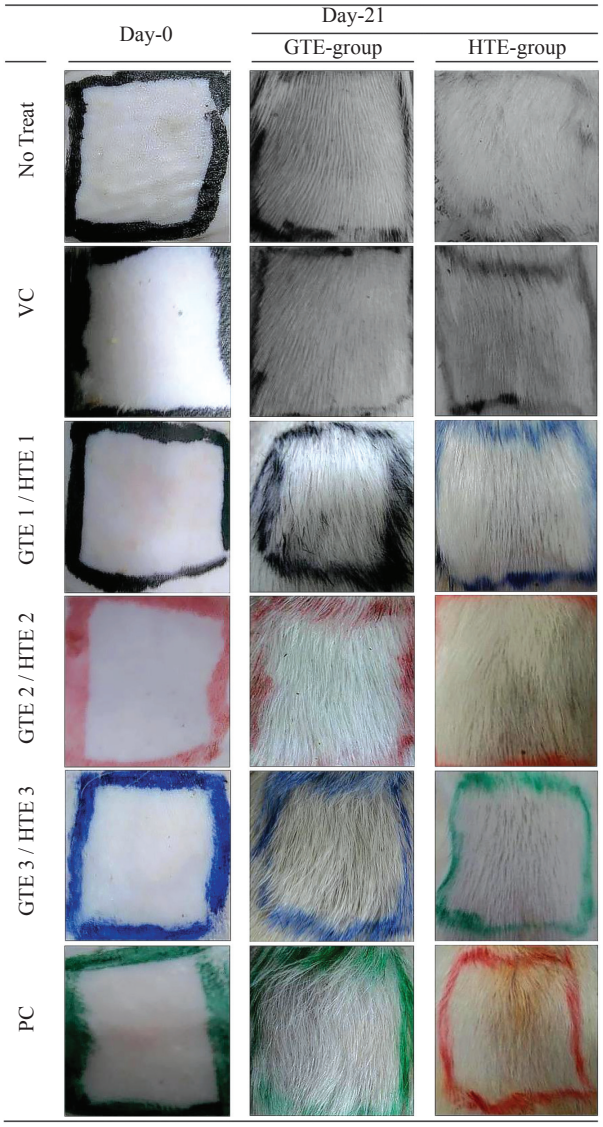

Fig. 1 Visual observation of green tea extracts microemulsion and hibiscus leaves extract microemulsion hair growth activity on days 0 and 21, where No Treat = no treatment group; VC = vehicle control group; GTE1 = 2.5% green tea extract microemulsion group; GTE2 = 5.0% green tea extract microemulsion group; GTE3 = 7.5% green tea extract microemulsion group; HTE1 = 2.5% hibiscus extract microemulsion group; HTE2 = 5.0% hibiscus extract microemulsion group; HTE3 = 7.5% hibiscus extract microemulsion group; PC = positive control group

Evaluation at day 7 indicated that GTE2 and GTE3 had comparable hair growth performance to the positive control, with no significant differences among the groups; GTE2 produced a hair length mean of 4.59 mm, GTE3 of 5.10 mm and the positive control of 5.33 mm. On day 14, GTE3 had superior mean hair length from all groups; nevertheless, least significant difference analysis did not indicate a significant difference between GTE1, GTE2 or GTE3 and the positive control. Therefore, on days 7 and 14, the hair growth activity of GTE1, GTE2 and GTE3 were comparatively similar to the positive control (minoxidil 2.5%).

On day 21, the last day of hair growth observation, the mean hair lengths of GTE1, GTE2 and GTE3 and the positive control were significantly different with GTE3 promoted the longest length compared to the others. The Mann-Whitney test on mean hair length for GTE1 (14.94 mm), GTE2 (15.51 mm) and the positive control (15.55 mm) showed no significant differences, whilst there was a significant difference for GTE3, with longer length of 20.08 mm (Fig. 2). Hair weight evaluation was undertaken only on day 21 (Fig. 3) and showed that the positive control had the greatest weight (66.78 mg/cm^2) compared to the others with GTE3 being second (61.18 mg/cm^2). However, statistical analysis using the Mann-Whitney test found no significant difference between the positive control (minoxidil 2.5%) and GTE3 (p = 0.564) for total hair weight. Hence, on day 21, GTE3 had the greatest hair growth activity compared to the other treatments and showed that green tea leaves microemulsion (7.5%) contributed to significant hair growth.

Esfandiari and Kelly (2005) also showed that 50% of the green tea polyphenol extract fraction administered orally to black rats with spontaneous hair fall resulted in 33% of the rats showing significant hair growth in 6 mth. Although the drug process with oral and topical delivery is different, this may infer the potency of green tea extract. A quintessential case was demonstrated with Minoxidil that was originally developed to treat high blood pressure (Loniten®) orally, yet it stimulated a side effect of hypertrichosis or growth of hair in unwanted places (Sica, 2004; Blumeyer et al., 2011). Minoxidil henceforth was studied for its hair growth activity and approved by the USFDA for promoting hair growth, regardless of the exact's mechanism remaining unclear (Blumeyer et al., 2011). After all, topical administration was selected as it targeted directly hair dermal papilla rather than oral administration which had to pass through the digestive track beforehand.

The pharmacological effect of green tea toward hair growth activity may exist from its major active polyphenol constituent being catechin. Kwon et al. (2007) reported an increase in *ex vivo* and *in vivo* human hair growth activity from green tea epigalocatecin-3-gallat (EGCG). Their research explained the mechanism of hair growth from EGCG as being by stimulating cell proliferation and an anti-apoptosis effect on hair dermal papilla cells. Hiipakka et al. (2002) showed that EGCG has potential to treat androgenic alopecia by inhibition of 5α-reductase.

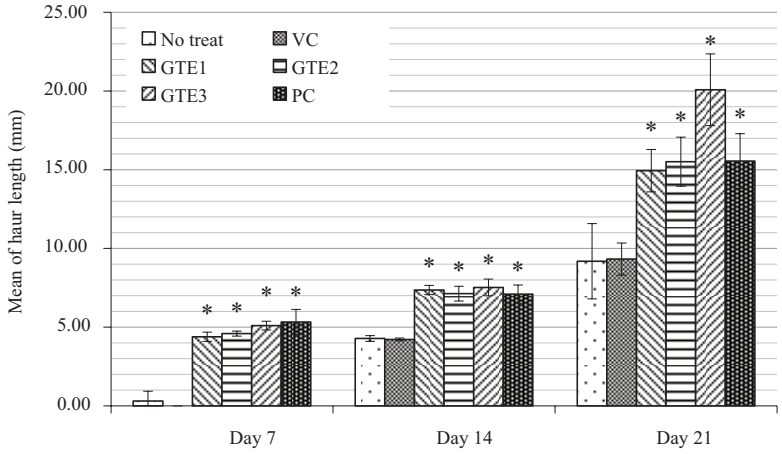

Fig. 2 Mean hair length on days 7, 14 and 21 after treatment of green tea extract microemulsion with various concentrations, where No Treat = no treatment group; VC = vehicle control group; GTE1 = 2.5% green tea extract microemulsion group; GTE2 = 5.0% green tea extract microemulsion group; GTE3 = 7.5% green tea extract microemulsion group; PC = positive control group; * = $p < 0.05$ compared to normal control group and vehicle control group; error bars = ±SD

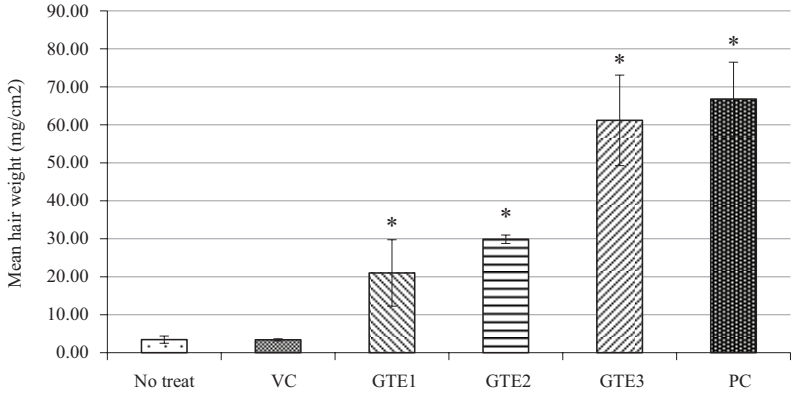

Fig. 3 Mean hair weight determination results on day 21 after treatment of green tea extract microemulsion with various concentrations where No Treat = no treatment group; VC = vehicle control group; GTE1 = 2.5% green tea extract microemulsion group; GTE2 = 5.0% green tea extract microemulsion group; GTE3 = 7.5% green tea extract microemulsion group; PC = positive control group; * = $p < 0.05$ compared to normal control group and vehicle control group; error bars = ±SD

Hibiscus tilliaceus extract microemulsion

The final visual observations of hair growth after day 21 from applying the hibiscus leaves extract microemulsion are illustrated in Fig. 1. Hair on rats subjected to no treatment or the vehicle control group was half grown, while following applications of HTE1, HTE2, HTE3 and the positive control (minoxidil 2.5%) the hairs were fully grown.

Since day 7 and day 14, HTE3 exhibited potential hair growth capacity, with its mean hair length value being 9.59 mm compared to that of the positive control of 8.81 mm on day 7 and 12.44 mm compared to 11.53 mm, respectively, on day 14. At the latter stage, HTE3 indicated similar hair growth capacity to the positive control.

On day 21, HTE3 produced the longest hair among all the related groups with a significant difference from no treatment, the vehicle control, HTE1, HTE2 and the positive control. No treatment and the vehicle control group had mean hair lengths of 9.57 mm and 9.68 mm, respectively. HTE1, HTE2 and the positive control (minoxidil 2.5%) had mean hair length values of 15.14 mm, 17.62 mm and 17.53 mm, respectively. However, the mean hair length of HTE3 (25.44 mm) was superior and significantly different compared to the other groups (Fig. 4). HTE3 also had the highest total hair weight (76.39 mg/cm^2) of all groups on day 21 (Fig. 5), compared to no treatment (34.23 mg/cm^2), the vehicle control (36.26 mg/cm^2) HTE1 (51.33 mg/cm^2), HTE2 (60.81 mg/cm^2) and the positive control (67.39 mg/cm^2). To conclude, HTE3 with 7.5% hibiscus leaves extract concentration produced the greatest hair growth activity which was significantly different compared to HTE1 (2.5%), HTE2 (5.0%) and minoxidil (2.5%).

Species other than *Hibiscus tiliaceus* have had their leaves extract analyzed for hair growth potential, with Adhirajan et al. (2003) reporting that 1% petroleum ether leaves extract had greater potential for hair growth than *H. rosa-sinensis* flowers. Additionally, it was reported the *H. rosa-sinensis* leaves extract transformed the hair cycle from the telogen to the anagen phase.

The chemical constituents of *H. rosa-sinensis* include flavonoids, flavonoid glycosides, hibicetin, cyanidine, cyaniding glucosides, stigmasterol and taraxeryl acetate (Maganha et al., 2010). Both *H.*

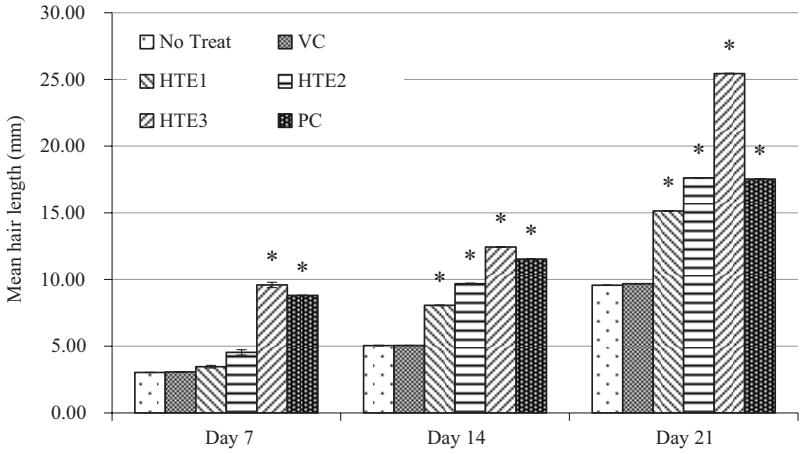

Fig. 4 Mean hair length on days 7, 14, and 21 after treatment of hibiscus extract microemulsion with various concentrations (No Treat = no treatment group; VC = vehicle control group; HTE1 = 2.5% hibiscus extract microemulsion group; HTE2 = 5.0% hibiscus extract microemulsion group; HTE3 = 7.5% hibiscus extract microemulsion group; PC = positive control group; * = $p < 0.05$ compared to normal control group and vehicle control group; error bars = ±SD

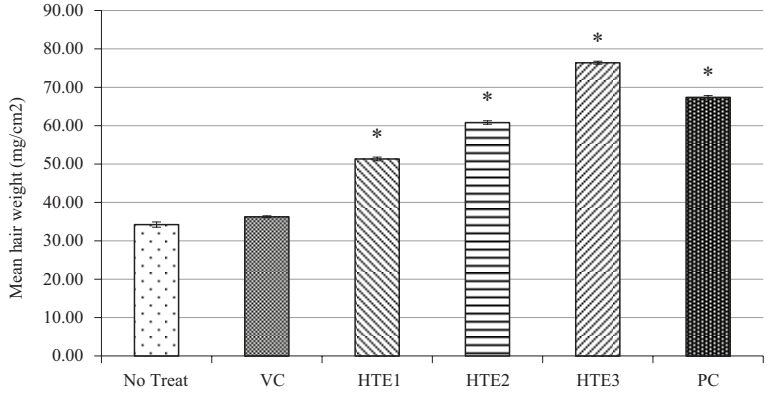

Fig. 5 Mean hair weight determination results on day 21 after treatment of hibiscus extract microemulsion with various concentrations (No Treat = no treatment group; VC = vehicle control group; HTE1 = 2.5% hibiscus extract microemulsion group; HTE2 = 5.0% hibiscus extract microemulsion group; HTE3 = 7.5% hibiscus extract microemulsion group; PC = positive control group; * = $p < 0.05$ compared to normal control group and vehicle control group; error bars = ±SD

rosa-sinensis and *H. tilliaceus* (based on the aforementioned data) are rich in polyphenols, particularly flavonoids. While it may be inferred that flavonoids are involved as the active constituent for hair growth, nonetheless, further study is needed.

Albeit the specific hair growth activity mechanism of hibiscus leaves is not yet revealed, this research showed that hibiscus leaves microemulsion (7.5%) produced significant hair growth activity. More scientific evidence should be obtained on hibiscus leaves to elucidate it potential as a topical hair growth enhancer.

Safety of microemulsion

The pre-clinical hair growth activity test which was conducted on rats' skin elicited no sign of skin irritation or corrosion, providing confidence to continue the dermal irritancy study on humans. Healthy subjects were used to avoid any external factors arising from previous medication or ongoing treatment on the afflicted area. Additionally, the volar upper arm area was chosen because its skin structure is hairless, comprises fewer sebaceous glands and offers a relatively large skin surface area (Bazin and Fanchon, 2006). Based on previous data gained for the safety of these herbal microemulsions, three subjects per group were designated. The standard test for skin irritation according to OECD guidelines requires a maximum of three subjects for initial and confirmatory testing (Organisation for Economic Co-operation and Development, 2015) based on the Draize test. Nevertheless, further subjects as per Robinson et al. (2002) resulted in approximately 30 subjects for a clinical 4-hour patch test.

The performed safety testing using the 18 volunteers for the GTE formulas (GTE1, GTE2, GTE3) and the HTE formulas (HTE1, HTE2, HTE3) showed no erythema, eschar or edema on the applied skin. Therefore, the primary irritation index of all formulas was 0 (Table 3) which was categorized as no irritation (0–0.4) as shown in Table 4. All formulas of GTE (GTE1, GTE2, GTE3) and HTE (HTE1, HTE2, HTE3) were thus safe to use accordingly.

Table 3 Category of skin condition

Erythema type	Value	Edema type	Value
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight edema (barely perceptible)	1
Well defined erythema	2	Slight edema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate edema (raising approx. 1 mm)	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4	Severe edema (raised more than 1 mm extending beyond the area of exposure)	4

Table 4 Response category and irritation

Category	Primary irritation index
No irritation	0.0−0.4
Light irritation	0.5−1.9
Mid irritation	2.0−4.9
Severe irritation	5.0−8.0

Some publications have expressed concerns regarding the toxicity of green tea. Chengelis et al. (2008) published research regarding the oral toxicity of green tea catechins beverages on rats at 28 d that showed the overall clinical condition of the animals was unaffected by any of the green tea catechin preparations with no-observed-adverse-effect level for systemic toxicity following oral administration at 2,000 mg/kg/day. Hsu et al. (2011) reported that subacute toxicity of green tea extract in mice orally administered at 2,500 mg/kg/day was safe and there were no side effects.

No similar research results were found regarding hibiscus leaves toxicity. Nevertheless, the preliminary safety study reported here should provide some indication of its safety.

Physical stability of microemulsion

Results of the physical stability analysis of GTE1, GTE2, GTE3, HTE1, HTE2 and HTE3 using the cycling test and storage under various conditions for 6 mth at low temperature (4 ± 2°C), room temperature (28 ± 2°C) and high temperature (40 ± 2°C), showed that the tested products were stable. The cycling test was conducted with the aim of observing stability in changing temperature but also showed no change in color, odor, pH or phase separation. Storage under the various conditions showed homogeneity and the organoleptic evaluation indicated no change. At times, storage at low temperature may change the product to a frozen greenish white color due to the isopropyl myristate contents for which the freezing point is 5°C; this returned to the normal condition when allowed to warm at room temperature. Products stored at room temperature and high temperature were stable without any significant changes in color, odor, homogeneity or phase separation. There was no significant change in the pH after 6 mth with the pH for each of GTE1, GTE2, GTE3, HTE1, HTE2 and HTE3 remaining in the pH range for skin (4.5–6.5).

This research successfully evaluated both green tea leaves and hibiscus leaves in microemulsions as herbal preparations and reported efficacy, safety and stability. Both the 7.5% green tea extract microemulsion and the 7.5% hibiscus leaves extract microemulsion promoted significantly higher hair growth activity compared to 2.5% minoxidil microemulsion in general. These formulas produced no irritation to the skin and were generally stable after storage and observation for 6 mth under various temperature conditions.

Although research into solving hair-loss in humans has been conducted for decades and products have been marketed and used worldwide, there remains a myriad of patients with high expectations of various products (hair tonics, balms, masks or other application as anti-hair loss agents) for which there are no proper studies (Bandaranayake and Mirmirani, 2004). In addition, ethnopharmacological study to obtain evidence-based data is limited for green tea leaves extract and hibiscus leaves extract; hence, it has not been possible to date to claim any activity effects. In light of this situation, this study corroborated the traditionally acclaimed hair growth-promotion capabilities of these two plants for topical use in microemulsion.

Therefore, GTE and HTE microemulsions could be used as potential natural hair growth promoters. Ongoing research to resolve the problem of hair loss using naturally derived products should continue. Combinations of herbal extracts would be interesting to explore.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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