ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 14, Issue 8, 2021

Online - 2455-3891
Print - 0974-2441

Research Article

IMPROVEMENT OF ALOPECIA AND DECREASES IN HAIR CORTISOL AND DIHYDROTESTOSTERONE CONCENTRATIONS AFTER THE TOPICAL USE OF PLANT EXTRACTS IN MEN WITH ANDROGENETIC ALOPECIA

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Received: 18 April 2021, Revised and Accepted: 09 June 2021

ABSTRACT

Objectives: Plant extracts possessing specific constituents with anti-inflammatory, antimicrobial, antioxidant, or 5α-reductase inhibitory properties are known to provide benefits against androgenetic alopecia (AGA) in men. A solid shampoo was formulated, and it contained a mixture of six different plant extracts that possess these beneficial properties against AGA. The improvement in AGA and changes in steroid concentrations were assessed after 4 months of formulated shampoo use.

Methods: This study was conducted based on a randomized, placebo-controlled, and single-blind design. Hair-related variables and hair and saliva samples were collected bi-monthly in the treatment (n=48) and placebo (n=52) groups and at a single time point in the hairy controls (n=50).

Results: The formulated shampoo was more effective on AGA than the placebo based on the hair shaft thickness and hair density in the receding hairline. The baseline hair cortisol and dihydrotestosterone (DHT) concentrations were significantly higher in the treatment and placebo groups than in the hairy controls. After 4 months, the hair steroid concentrations in the treatment group were reduced to those observed in the hairy controls, although the main effect of time on hair steroid concentrations was negligible in the placebo group. Salivary cortisol and DHT levels during the post-awakening period were comparable among the groups or assessment time points.

Conclusion: The constituents of plant extracts included in the formulated shampoo would prevent hair loss, increase hair growth effects, and reduce hair cortisol and DHT concentrations without changes in the post-awakening salivary steroid levels in men with AGA.

Keywords: Androgenetic alopecia, Plant extracts, Local stress response system, Hair cortisol, Hair dihydrotestosterone.

 DOI: http://dx.doi.org/10.22159/ajpcr.2021v14i8.41859. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Androgenetic alopecia (AGA) is the most common type of hair loss in adult men, and it is characterized by the progressive loss of terminal hairs with a characteristic pattern distribution [1]. It is generally accepted that dihydrotestosterone (DHT) is the primary androgen associated with the development and progression of AGA in men [2], and finasteride, an inhibitor of 5α-reductase, is commonly used for the treatment of AGA [3]. The majority of dermatologists prescribed finasteride (1 mg/day) for more than one year to improve alopecia, and it slows hair loss and increases hair growth in men with AGA [4]. However, the beneficial effects of finasteride on AGA disappear within 12 months after the withdrawal of therapy [5], indicating that the continued use of finasteride is required to maintain the beneficial effects. Long-term use of finasteride can also induce adverse effects on sexual function (i.e., low libido, erectile dysfunction, and decreased ejaculation) and central nervous function and lead to depression and anxiety due to the concomitant inhibition of 5α-reductase that occurs in the central nervous system and peripheral organs [6].

The adverse effects of finasteride have led to an increasing interest in alternative remedies that can prevent and treat AGA in men. Plant-derived products, such as extracts and essential oils, have been traditionally used to treat alopecia, and recent human studies have observed that hair growth is promoted and hair loss is reduced in men with AGA after the topical use of plant extracts and essential oils [7,8]. Although none of the isolated chemical constituents of plants have been studied for their activity, the constituents of plant extracts and essential oils possessing anti-inflammatory, antioxidant, antimicrobial, or 5α-reductase inhibitory effects are known to have beneficial effects on alopecia [9]. A systematic review of cell culture and animal studies has suggested that the active constituents of plant extracts and essential oils may promote hair growth and inhibit hair loss through physiological changes in hair follicles and scalp skin, including the reduction of DHT formation by inhibition of 5α-reductase activity and modulation of hair growth stimulating and inhibiting factor expression, such as insulin-like growth factor-1, fibroblast growth factor, keratinocyte growth factor, and transforming growth factor β (TGF-β) [10].

Meanwhile, elevated DHT concentrations and DHT-related factors, such as 5α-reductase activity in scalp skin and hair follicles, are known to be associated with AGA development in men. For example, 5α-reductase activity in hair follicles collected from scalp skin is higher in men with AGA than in normal hairy men [11], and DHT concentrations are higher in biopsied scalp skin and hair samples collected from alopecic areas than unaffected hairy areas in the same subjects [12,13]. Another study line has shown that the topical use of finasteride provides positive outcomes of AGA without changes in circulating testosterone and DHT concentrations [14]. AGA improvements have also been observed after the topical use of anti-inflammatory chemical agents, such as roxithromycin [15], and antimicrobial and antifungal agents, such as piroctone olamine and triclosan [16], suggesting that topical treatment with agents possessing antimicrobial and anti-inflammatory properties mimics the beneficial effect of finasteride on AGA.

However, the physiological mechanism underlying the improvement of AGA after the topical use of plant products remains poorly understood. Based on the expression of mRNAs for 5α-reductase and other steroidogenic enzymes in hair follicles and sebaceous glands and the inhibitory effects of proinflammatory cytokines on hair growth, it has been postulated that cross-talk may occur between proinflammatory cytokines and steroidogenic activity in peripheral organs that disrupts the normal hair cycle [17]. Indeed, recent studies have confirmed that skin and hair follicles have their own proteins, enzymes, and self-regulatory feedback systems by which steroid hormones, including cortisol, are synthesized de novo in response to corticotropin-releasing hormone (CRH) stimulation and homeostasis-disrupting stimuli, such as ultarviolet radiation and proinflammatory cytokines, independent of central hypothalamus-pituitary-adrenal (HPA) axis function [18-20].

Given the presence of the stress response system and the variety of enzymes required for steroid metabolism in skin and hair follicles, the present study hypothesized that topical treatment with plant extracts possessing anti-inflammatory, antimicrobial, and 5α-reductase inhibitory properties might reduce the stress burden imposed on the local stress response system and improve AGA through a decrease in local steroidogenic activities, including DHT and cortisol formation. To examine this hypothesis, we formulated a solid shampoo that contains a mixture of plant extracts that possess these beneficial properties against AGA. Then, hair-related variables and steroid concentrations in the hair and saliva samples were determined in men with AGA after using the formulated shampoo.

METHODS

Study subjects

Men aged 20–65 with patterned hair loss and those with little or no hair loss were recruited from Gwangju Women's University (GWU) through advertisements and posters. Subjects were excluded if they met any of the following criteria: (1) Diagnosis of any skin disorders of the scalp; (2) history of finasteride administration or current administration of finasteride; (3) topical minoxidil solution use, either currently or within the last month; (4) use of cosmetic hair dye, and (5) hair length <2 cm at the vertex region of the head. Subjects were initially screened in a telephone interview to exclude those with conditions known to influence central HPA axis function according to the prespecified exclusion criteria [21].

A text message was sent to each participant to notify them of their scheduled date. During their visit to the laboratory, their weight and height were measured, and each participant was asked to complete self-report questionnaires on their hair loss duration from the scalp and history of hair loss in their parents and grandparents. Hair loss regions, including the vertex, frontal, temporal, and occipital areas, were observed, and hair loss area images were captured for further analysis. The type of AGA was evaluated according to a previous study [22]. This study conformed to the declaration of Helsinki, and all of the participants provided written informed consent. This study was approved by the Institutional Review Board of GWU (1041848-201805-HR-001-16) and Seoul National University Bundang Hospital (B-1911-577-302).

Preparation of a solid shampoo containing plant extracts

Six different edible or medicinal plants were chosen based on previous studies that reported on the antioxidant and anti-inflammatory features of extracts from the tuber of Jerusalem artichoke (*Helianthus tuberosus*) and seeds of quinoa (*Chenopodium quinoa*) [23-25] and the antioxidant and antimicrobial features of extracts from the kernel of hazelnut (*Corylus avellana* or *Corylus heterophylla*), and fruit of goldenberry (*Physalis peruviana*) [26,27]. In addition, *Paeonia radix* and *Pleuropterus multiflorus* have been traditionally used for the treatment of alopecia in China and Korea, and phytochemical studies have reported that extracts from the roots of these medicinal plants have inhibitory activity against 5α-reductase [28,29].

For the present study, LK Biotech (Seongnam, Korea) prepared a solid shampoo that contained approximately 50% crude extracts from the plants using the following procedures. Plant extracts were prepared using the pressurized hot water extraction method (temperature: 120–125°C, pressure: 1.5–1.8 bar, time: 8 h). Extract solution A was prepared from 50 g of the dried kernel of hazelnut, 100 g of the dried and chopped root of Jerusalem artichoke, and 750 ml of water. Extract solution B was prepared from 100 g of the dried fruit of goldenberry, 150 g of the dried seed of quinoa (without dehusking), 150 g of the dried and chopped root of *P. radix*, 150 g of *P. multiflorus*, and 2750 ml of water. Solution A was mixed 1:1 (v/v) with solution B, and the mixture was evaporated at 85°C using a rotary evaporator (N-4000, Eyela, Japan) until the initial volume was decreased by approximately 75%. The mixture was then filtered through sterile stainless-steel 300-μm wire mesh (NBC Meshtec Inc., Japan). The concentrated and filtered plant extract mixture was named JU7505. Two hundred fifty grams of JU7505 was added to a freshly prepared mixture containing 79.5 ml of 50% NaOH solution, 100 ml of coconut oil, and 150 ml of palm oil. The mixture was then poured into molds and allowed to solidify for 48 h at RT. After 15 days of drying at RT, a bar of solid shampoo (185 g each) was provided every month to the participants of the present study.

Collection of hair samples and assessment of hair-related variables

After applying the exclusion criteria, the eligible men with AGA were randomly assigned into two groups (placebo group: n=52, treatment group: n=48) using a research randomizer (https://www.randomizer.org). Men with AGA in the treatment group used the formulated shampoo containing JU7505 over four consecutive months, and those in the placebo group used an unmedicated liquid cleansing shampoo over the same period. Participants in the treatment and placebo groups were not informed about the specific nature of the shampoo, and they were asked to report any adverse events during shampoo use, such as stinging, burning, and itching. Fifty men with little or no hair loss were also included as hairy controls.

Hair samples and hair-related variables were collected bimonthly for four months from men with AGA. The hair samples were collected from the receding hairline, a transition zone between the normal hair area and balding area (e.g. temples, vertex scalp, and/or mid-frontal area) in men with AGA before (t0) and at 2 (t1) and 4 (t2) months after using the provided solid shampoo. Hair samples were also collected at a single time point (t0) in the corresponding areas in the hairy controls. Approximately 70–100 mg of hair collected 2 cm from the scalp was obtained from the target areas using scissors, and the areas were photographed. An Aramo TS phototrichogram system equipped with magnification lenses (Aram HUVIS Co., Korea) was used to evaluate hair-related variables (hair shaft diameter and hair density). The hair-related variables at the target site were evaluated by three independent investigators 3 times, each in a double-blind fashion.

Collection of saliva samples

The concentrations of steroids in saliva are known to reflect those in the blood [30], and the cortisol awakening response (CAR) is used as a reliable index for central HPA axis function [31]. To determine the CAR and testosterone, DHT, epitestosterone (Epi-T), and dehydroepiandrosterone (DHEA) levels during the post-awakening period, a set of saliva samples (immediately on awakening and 30 and 60 min after awakening) was consecutively collected from men with AGA (t0, t1, and t2) and at a single time point (t0) from hairy controls. Each participant was instructed on the procedures and precautions for saliva collection described in previous studies [32,33]. Each participant was also asked to submit their saliva samples at each scheduled visit for hair collection and collect their saliva samples on one of the working days, with a minimum volume of 1.5 ml of saliva provided at each time point.

Preparation of hair and saliva samples for steroid assay

Steroids were extracted directly from the finely cut hair following previously reported methods [34,35]. Briefly, approximately 50 mg of

hair was cut into small pieces (2–3 mm in length) with small surgical scissors and then placed into a disposable glass scintillation vial to extract hair steroids. Then, 2 ml of methanol was added to each vial. Each tightly capped vial was incubated for 24 h at RT, with gentle shaking using an orbital shaker (Jeio Tech, Korea). The next day, the tubes were briefly spun in a vortex, and 1.5 ml of supernatant was transferred into a clean tube and evaporated at RT in a centrifuge-type vacuum drier (Thermo Fisher Scientific, MA, USA). The samples were resuspended in 1 ml of 0.1% gelatin containing 50 mmol/l phosphate-buffered saline (pH=7.2), and an aliquot of 500 µl was taken for the DHT assay. All samples were stored at −80°C until the assay was performed. The collected saliva samples were centrifuged (10,000 g for 15 min at 4°C) to remove debris. The supernatant was mixed with the same volume of GPBS, and then an aliquot of 500 µl was taken for DHT assay. The prepared saliva samples were stored at −80°C until the assay was performed.

Exclusion of saliva samples collected from non-adherent participants

Saliva samples that had insufficient volume and showed visible blood or sputum contamination were excluded. After exclusion, a cortisol assay was performed to evaluate whether the saliva samples were collected as described above. Typical CAR is defined as an increase in cortisol levels to at least 2.5 nmol/L above an individual's baseline in healthy subjects [36]. However, the first sample collection after a delay of more than 10 min post waking is known as the leading cause of failure to capture a typical CAR in healthy subjects (that is, non-adherent subjects) [37]. We did not observe the typical CAR, and either a <2.5 nmol/L increase or the complete absence of a post-awakening increase was observed in some participants' saliva samples. For example, at t0, the cortisol concentrations immediately on waking and at 30 and 60 min after waking in some of the men with AGA (n=31) were 20.9±2.2, 17.3±2.1, and 11.5±1.5 nmol/L (mean±SEM), respectively, while those in some of the hairy men (n=14) were 19.0±3.4, 13.4±1.7, and 9.6±1.3 nmol/L (mean±SEM), respectively. The subjects without a typical CAR were considered non-adherents, and data obtained from the non-adherents were excluded from further analyses. Finally, data were obtained from 36, 18, and 18 adherents in the treatment group at t0, t1, and t2, respectively; 26, 15, and 16 adherents in the placebo group at t0, t1, and t2, respectively; and 33 adherents in the hairy controls.

Steroid RIA

The cortisol, testosterone, DHT, Epi-T, and DHEA concentrations in the collected hair and saliva samples were determined using radioimmunoassay by modifying the method used in previous studies [32,33]. DHT assays were conducted after the selective inactivation of testosterone with an aqueous potassium permanganate solution [38]. The reference standards for the steroids were obtained from Sigma-Aldrich (MO, USA). Iodine-125-labeled cortisol, testosterone, DHT, Epi-T, and DHEA were prepared by modifying a previously described protocol for steroid radioiodination [39]. Cortisol antiserum was purchased from USbiological Life Sciences (MA, USA). Testosterone and DHT antiserum were purchased from Absolue antibody (MA, USA) and Fitzgerald Industries (MA, USA), respectively. DHEA antiserum was purchased from LSBio (WA, USA), and Epi-T antiserum was purchased from Creative Diagnostics (NY, USA). As described by the suppliers, the cortisol antiserum cross-reacted with 11-deoxycortisol, prednisolone, corticosterone, cortisone, and 11-deoxycorticosterone, with cross-reaction levels of 0.9%, 5.6%, 0.6%, 0.6%, and <0.1%, respectively. Testosterone antiserum cross-reacted with 11β-hydroxy testosterone, 17α-methyltestosterone, DHT, estradiol, and progesterone, with cross-reaction levels of 3.3%, <0.1%, 0.8%, <0.1%, and <0.1%, respectively. DHT antiserum cross-reacted with testosterone, 5α-androstane-3β, 17β-diol, 5α-androstane-3α, 17β-diol, epitestosterone, and other steroids, with cross-reaction levels of 75%, 2.0%, 1.0%, 1.1%, and <0.1%, respectively. DHEA antiserum cross-reacted with DHEAS, androsterone, androstenedione, 20-dihydroprogesterone or 11-hydroxyprogesterone, with cross-reaction levels of <0.1%.

The standards, quality control materials, and samples were assayed in duplicate. The interassay coefficients of variation (CVs) assessed from quality controls with mean cortisol concentrations of 3.6 and 10.9 nmol/L were 7.6% and 8.7%, respectively (n=18). The analytical sensitivity for cortisol was 0.4 nmol/L. The interassay CVs assessed from the quality controls with mean testosterone concentrations of 34.7 and 173.3 pmol/L were 8.9% and 7.6%, respectively (n=22). The analytical sensitivity for testosterone was 0.3 pmol/L. The interassay CVs assessed from the quality controls with mean DHT concentrations of 172.0 and 516.4 pmol/L were 9.6% and 8.7%, respectively (n=19). The analytical sensitivity for DHT was 0.3 pmol/L. Epi-T antiserum cross-reacted with testosterone, 5-androsten-3β,17 β-dione, DHT, aldosterone, cortisol, and progesterone, with cross-reaction levels of <1%, <0.3%, <15%, 0.015%, and <0.1%, respectively. The interassay CVs assessed from the quality controls with mean Epi-T concentrations of 34.7 and 173.3 pmol/L were 9.4% and 8.3%, respectively (n=20). The analytical sensitivity for epi-testosterone was 0.3 pmol/L. The interassay CVs assessed from the quality controls with mean DHEA concentrations of 28.8 and 144.2 nmol/L were 8.7% and 8.0%, respectively (n=20). The analytical sensitivity for DHEA was 0.4 nmol/L

Data analyses

Student's t-test or Mann–Whitney's U-test was applied according to the data distribution (Shapiro-Wilk test) to evaluate the differences between the two examined groups. The Chi-squared test was used to analyze the differences in the categorical demographic data. The differences between three or more groups were determined with a parametric or nonparametric one-way ANOVA. The differences in the levels and patterns of cortisol, testosterone, DHT, Epi-T, and DHEA secretions during the post-awakening period between t0 and t1, and t2 were analyzed using a two-way ANOVA. The total cortisol, testosterone, DHT, Epi-T, or DHEA secretions during the post-awakening period were calculated as the area under the curve (AUC) with respect to the ground from the time point immediately after awakening to 60 min after awakening (CARauc, $Tauc_{awk}$, $DHTauc_{awk}$, $EpiTauc_{awk}$, or $Dauc_{awk}$, respectively). The relationships between the steroid concentrations were analyzed using Pearson's correlation test. The results are expressed as the mean±SEM, and a $p<0.05$ was considered statistically significant. NCSS 11 statistical software (NCSS, UT, USA) was used for the data analysis.

RESULTS

Differences in demographics and self-reported variables between examined groups

Table 1 summarizes the demographic characteristics of each group. The mean age of the hairy controls was significantly lower than that of the treatment (t=3.6, df=96, p<0.001) and placebo groups (t=2.3, df=100, p<0.05). Height and body weight were significantly different among the examined groups (all $F_{2,147}>4.0$, $p<0.05$) (Table 1). Smoking status differed among the examined groups ($\chi^2=16.5$, $p<0.05$), although a difference was not observed between the treatment and placebo groups ($\chi^2=8.22$, $p<0.05$). No difference was observed in the drinking status (drinking days and amount of drinking) among the examined groups (all $F_{2,147}<0.82$, $p>0.05$). The presence of a family history of hair loss was reported in 69.0% of men with AGA (68.8% and 69.2% in the treatment and placebo groups, respectively) (Table 1), with 80.5% presenting a paternal family history, 20.8% presenting a maternal family history, and 19.5% presenting both paternal and maternal histories. The men with AGA had a significantly higher proportion of positive family history of hair loss than the hairy controls (34.0%) ($\chi^2=20.5$, $p<0.0001$). Type M, MC/MO/CO, and O regressions were common in the men with AGA in the present study (90 cases out of 100). There were no differences in the prevalence rates of the type of AGA and stage of hair loss between the treatment and placebo groups (all $\chi^2<2.17$, $p>0.05$) (Table 1). None of the subjects included in the treatment group reported any deleterious effect on the scalp after using the formulated shampoo.

Table 1: Demographic characteristics

Demographic variables	Treatment group (n=48)	Placebo group (n=52)	Hairy controls (n=50)
Age (year)	49.0 (29–65)*	47.7 (30–61)	41.24 (22–63)
Height (cm)	172.1 (160–192)	172.6 (165–183)	174.8 (161–186)
Body weight (kg)	73.5 (55–117)	75.6 (55–92)	79.3 (63–110)
Smoking status			
Never	21.0%	23.6%	35.0%,
Quit	35.5%	16.4%	15.0%.
Current	43.5%	60.0%	50.0%
Quit period of stop smokingw (year)	16.6 (2–30)	19.1 (8–30)	9.64 (5–20)
Amount of smoking of quit-smoker (cigarettes/day)	13.4 (2–30)	13.9 (10–20)	16.5 (7–30)
Smoking period of current-smoker (year)	22.5 (6–36)	23.9 (10–40)	17.1 (3–40)
Amount of smoking of current-smoker (cigarettes/day)	17.4 (10–40)	16.0 (2–40)	16.4 (2–40)
Drinking days (days/week)	1.7 (0–7)	1.4 (0–6)	1.7 (0–7)
Amount of drinking (cups of bear/week)	6.7 (0–24)	5.7 (0–20)	7.6 (0–30)
Family history of AGA			
Presence	65.6%	65.5%	33.0%
Absence	34.4%	34.5%	67.0%
Who is affected? (multiple marking)	,		
Paternal side	93.1%	92.8%	90.0%
Maternal side	6.9%	14.3%	10.0%
Brother (or sister)	24.1%	32.1%	20.0%
Paternal side grandfather	17.4%	14.3%,	15.0%
Maternal side grandfather	3.4%	2.5%	2.5%
Duration of AGA	10.6 (2–26)	9.2 (1–30)	
Type of AGA	O+MC type: 36.7%, C type: 13.3%, O type: 30.0%, M type: 20%	O+MC type: 36.3%, C type: 7.3%, O type: 30.9%, M type: 12.7%	

*Data are presented as mean (range). AGA: androgenetic alopecia

Differences in hair-related variables between the examined groups

Fig. 1 presents the hair shaft thickness and hair density in the examined groups and shows differences in these hair-related variables between the examined groups at each different sampling time point. The hair shaft thickness and hair density obtained from the placebo and treatment groups at t0, t1, and t2 were significantly different from those obtained from the hairy controls ($F_{6, 343}$=11.89, p<0.0001), with the treatment and placebo groups presenting thinner hair shafts and less hair density compared with the hairy controls (p<0.05 for all post hoc tests) (Fig. 1a and b). The main effect of the time of sampling on the hair shaft thickness and hair density was significant in the treatment group (all $F_{2,47}$>7.9, p<0.001), and both hair-related variables were significantly greater at t2 than t0 (p<0.001 for all post hoc tests) (Fig. 1a and b). However, the main effect of the sampling time on the hair shaft thickness or hair density was negligible in the placebo group (all $F_{2,51}$<1.82, p>0.05) (Fig. 1a and b). The hair shaft thickness measured at t2 in the treatment group was significantly lower than that measured at t0 in the hairy controls (p<0.05 by *post hoc* tests), although the hair density measured at t2 in the treatment group was comparable to that in the hairy controls (p>0.05 by *post hoc* tests) (Fig. 1a and b). In the treatment group, the mean hair shaft thickness and hair density increased by 14.6% and 29.8% at t2, respectively, compared with those at t0.

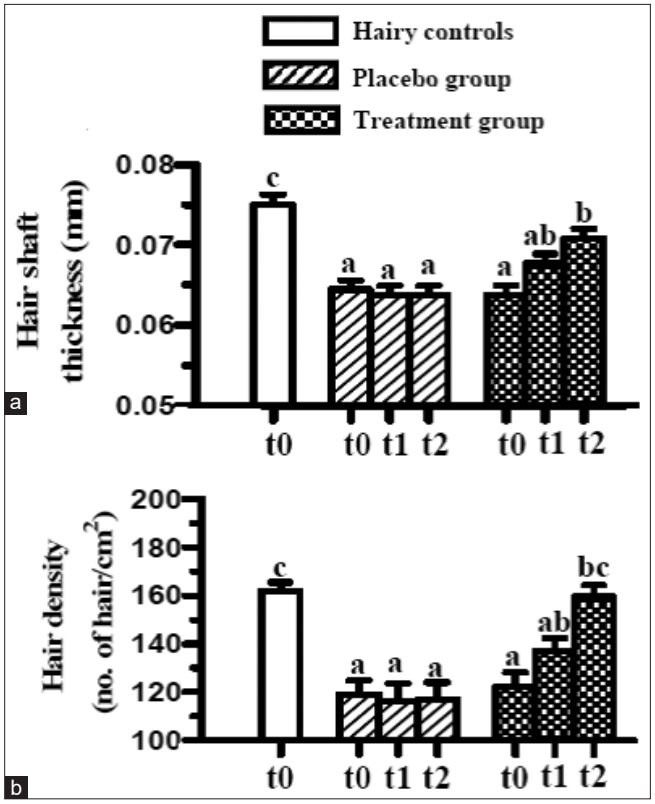

Figure 1: Hair shaft thickness and hair density in the treatment, placebo, and control groups. One-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was used to compare the mean values of hair shaft thickness and hair density (no. of hair/cm^2) at designed time points (t0, t1, and t2). Different letters above bar graphs indicate statistical significance at p<0.05. All of the data are expressed as the mean±standard error of the mean

Differences in hair steroid concentrations between the examined groups

Fig. 2 presents the variations in the concentrations of hair steroids during the examined periods (t0, t1, and t2) in the placebo and treatment groups and the concentrations of hair steroids determined at t0 in the hairy controls. The hair cortisol and DHT concentrations at t0 among the examined groups were significantly different ($F_{2,147}$=9.8, p<0.0001), and the post hoc tests revealed that hair cortisol and DHT concentrations were significantly higher in the treatment and placebo groups than in the hairy controls (p<0.001 for all) (Fig. 2a and c). However, the hair testosterone, Epi-T, and DHEA concentrations at t0 were comparable among the examined groups ($F_{2, 147}$=0.4, p>0.05) (Fig. 2b, d, and e).

The time effects on the concentrations of hair cortisol, DHT, Epi-T, and DHEA were significant (all $F_{2,141}$>3.9, p<0.01) (Fig. 2a, c, d, and e), and the hair cortisol, DHT, Epi-T, and DHEA concentrations in the treatment group were significantly lower at t2 than at t0 (p<0.05 for all post hoc tests) (Fig. 2a, c, d, and e). However, the time effects on the concentrations of hair steroids were negligible in the placebo group (all $F_{2, 153}$<1.1, p>0.05) (Fig. 2a-e). Significantly lower hair cortisol, DHT, Epi-T, and DHEA concentrations were observed at t2 in the treatment group than the placebo group (all t<2.4, df=98, p<0.05) (Fig. 2a-e). In the treatment group, the mean concentrations of hair cortisol, testosterone, DHT, Epi-T, and DHEA at t2 decreased by 31.6%, 0.3%, 30.1%, 28.2%, and 39.8%, respectively, compared with those at t0. Differences were not observed in the concentrations of hair steroids at t2 in the treatment group and at t0 in the hairy controls (all t<1.3, df=96, p>0.05) (Fig. 2a-e).

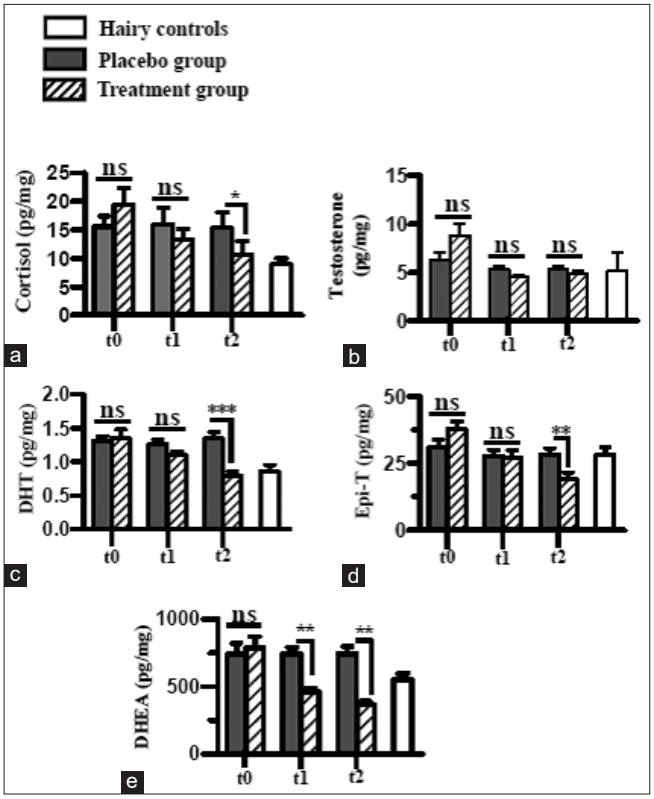

Figure 2: Hair cortisol, testosterone, dihydrotestosterone (DHT), Epi-T, dehydroepiandrosterone (DHEA) concentrations in the examined groups. Cortisol, testosterone, DHT, Epi-T, and DHEA concentrations determined in the hair samples collected from the treatment and placebo groups and hairy controls at the designated time points are presented in Fig. 2a-e, respectively. All of the data are expressed as the mean±standard error of the mean. Note: ns (nonsignificant) indicates p>0.05, and *, **, and * indicate p<0.05, p<0.01, and p<0.001, respectively**

Differences in salivary steroid profiles between the examined groups

Fig. 3 presents the levels and patterns of steroid secretions within the 1st h after awakening in the examined groups and shows differences between the examined groups at each sampling time point. No difference was observed in the levels and patterns of cortisol, testosterone, DHT, Epi-T, and DHEA secretions at t0 between the examined groups (all F<1.4, p>0.05) (Fig. 3a-1-e-1). Moreover, a significant main effect of group (treatment vs. placebo) was not observed on the levels of steroids during the postawakening period at t1 (all $F_{1, 93}$<0.25, p>0.05) (Fig. 3a-2-e-2) and t2 (all $F_{1, 96}$<2.9, p>0.05) (Fig. 3a-3-e-3). In addition, differences were not observed in the patterns of steroid secretions during the postawakening period at t1 (all $F_{2, 93}$<0.3, p>0.05) (Fig. 3a-e-2) and t2 (all $F_{2, 96}$<0.3, p>0.05) (Fig. 3a-3-e-3).

Correlations between measured steroids across the examined time points

As presented in Table 2, significant interrelationships were observed between hair steroids. In the three examined groups, a significant correlation was observed between the baseline (t0) cortisol and testosterone concentrations (all Pearson's ρ>0.28, p<0.01) and between the baseline testosterone and DHT concentrations (all Pearson's ρ>0.30, p<0.05) (Table 2). However, neither the baseline Epi-T nor DHEA concentrations were correlated with the baseline cortisol, testosterone, or DHT concentrations in the examined groups. Significant stability was observed in the hair steroid concentrations across the examined time points in both the placebo and treatment groups. A positive relationship was observed between the hair cortisol and DHT concentrations across the three time points in the placebo group (all Pearson's ρ (t0, t1), (t1, t2), and (t0, t2)>0.28, p<0.05) and the treatment group (all Pearson's ρ (t0, t1), (t1, t2), and (t0, t2)>0.38, p<0.05) (Table 2). The hair testosterone concentration between t0 and t1 also showed a positive relationship in the placebo and treatment groups (all Pearson's ρ>0.35, p<0.05), and the hair Epi-T concentrations between t0 and t1, between t1 and t2, and between t0 and t2 also showed a positive relationship in the treatment group (all Pearson's ρ> 0.35, p<0.05) (Table 2).

Table 3 shows the relationship between auxiliary indices for salivary steroids secretions, that is, CARauc, $Tauc_{awk}$, $DHTauc_{awk}$, $EpiTauc_{awk}$, and $Dauc_{awk}$ at t0. There was no inter-relationship among the indices for steroid secretion during the post-awakening period (all Pearson's ρ<0.30, p>0.05) (Table 3).

DISCUSSION

The present study examined hair-related variables and steroid levels in hair and saliva samples of AGA in men after using a formulated shampoo containing plant extracts (JU7505) to examine the physiological effect of these plant extracts. The findings of the present study were twofold: (1) the hair shaft thickness and hair density increased along receding hairlines, and (2) the concentrations of hair steroids decreased while the levels and patterns of salivary steroids did not show significant changes during the post-awakening period in men with AGA after four months of formulated shampoo use.

We observed that the treatment group had an increased hair shaft thickness and hair density along receding hairlines after four months of formulated shampoo use, indicating the improvement of AGA, with reduced hair loss and increased hair growth, by the active constituents of JU7505. The results were similar to those of previous human studies that reported the improvement of alopecia in men with AGA after the topical use of finasteride [14], roxithromycin [15], a piroctone olamine and triclosan mixture [16], and ketoconazole [40]. Another line of studies reported the improvement of AGA after using a hair tonic containing crude extracts of *Curcuma aeruginosa*, which possesses 5α-reductase inhibitory effects [41], and shampoo containing crude extracts of six different plants, which possess antioxidant, anti-inflammatory, and 5α-reductase inhibitory effects [42]. Therefore, it is likely that the active constituents included in JU7505 may lead to the improvement of AGA through changes in the physiological function of scalp skin and hair follicles.

In the present study, the levels of steroids in the hair and saliva samples were determined to assess the physiological mechanism underlying the improvement of AGA after the topical use of plant extracts, and the presence of steroid metabolic enzymes and the local stress response systems in the skin and hair follicles were considered [18,19]. We observed higher baseline hair DHT concentrations in the AGA group than the hairy controls, although the baseline hair testosterone concentrations were comparable among the examined groups. These results of the present study were consistent with other studies that reported elevated hair DHT concentrations in balding men compared with nonbalding controls [12] and comparable testosterone concentrations between balding and hairy scalp skin [13]. We also observed comparable levels of baseline $Tauc_{awk}$ and $DHTauc_{awk}$ between the examined groups. Previous studies have reported no association between circulating testosterone and DHT levels in men with AGA [43,44]. The feedforward regulation of gene expression of 5α-reductase by testosterone and DHT [45] and elevated 5α-reductase activity in the scalp skin and isolated hair roots of men with AGA compared to nonbalding men [11,46] may explain the higher DHT concentration in the hair of men with AGA.

The baseline hair cortisol concentrations were higher in men with AGA in the treatment and placebo groups than in the hairy controls. Although information is limited regarding hair cortisol in men with AGA, the results were consistent with previous studies that reported higher concentrations of hair cortisol in samples collected from stress-exposed

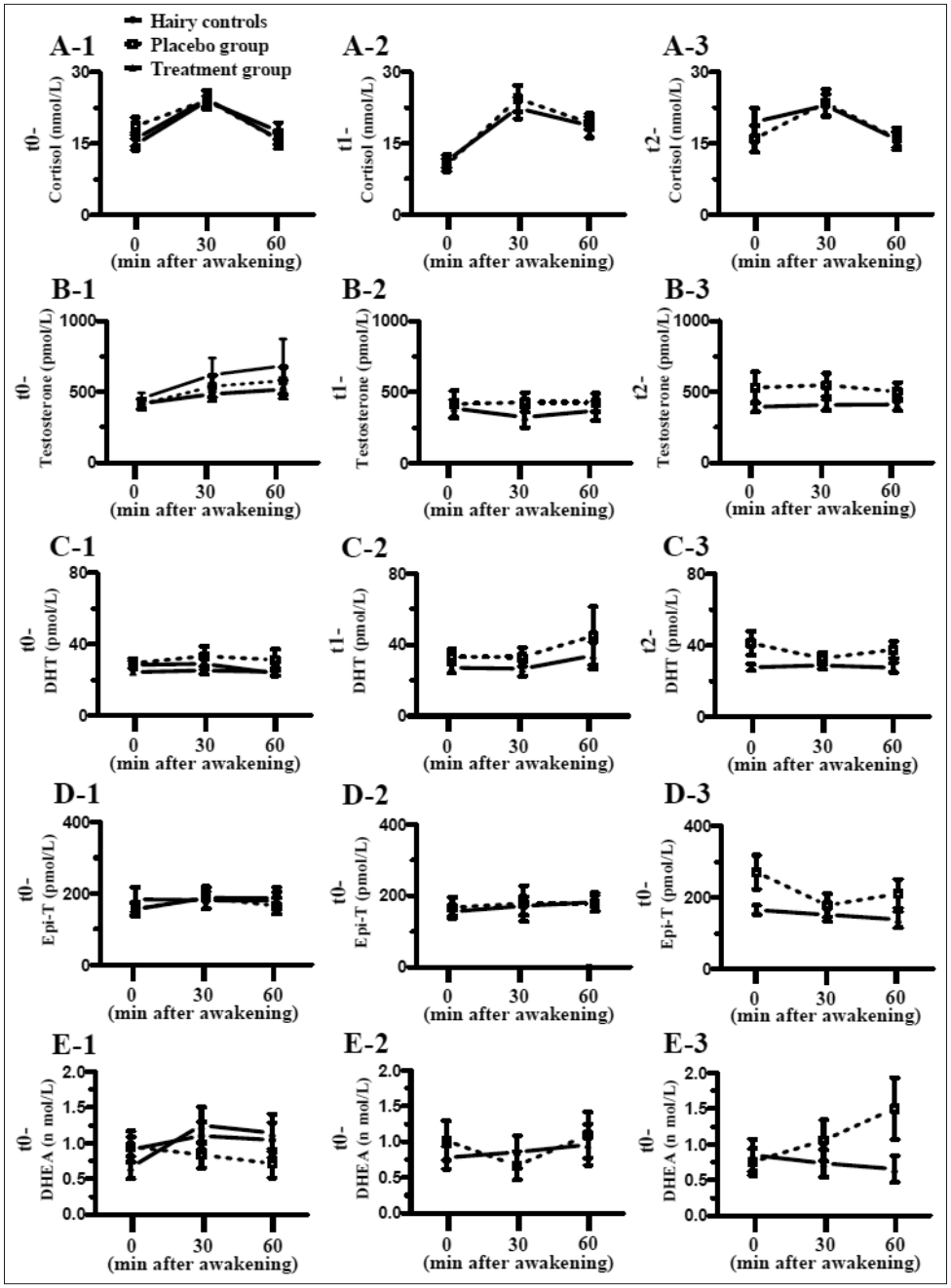

Figure 3: Salivary cortisol, testosterone, dihydrotestosterone (DHT), Epi-T, dehydroepiandrosterone (DHEA) profiles in the treatment, placebo, and control groups. Salivary cortisol profiles of the treatment and placebo groups and hairy controls at t0, t1, and t2 are presented in Fig. 3a-1-a-3, respectively. Salivary testosterone profiles of the examined groups at t0, t1, and t2 are presented in Fig. 3b-1-b-3, respectively. Salivary DHT profiles of the examined groups at t0, t1, and t2 are presented in Fig. 3c-1-c-3, respectively. Salivary Epi-T profiles of the examined groups at t0, t1, and t2 are presented in Fig. 3d-1-d-3, respectively. Salivary DHEA profiles of the examined groups at t0, t1, and t2 are presented in Fig. 3e-1-e-3, respectively. All of the data are expressed as the mean±standard error of the mean

areas of skin than from unaffected areas in the same subject [34,47]. Hair cortisol concentrations are considered a manifestation of the function of the central HPA axis in the immediate past, and elevated central HPA axis functions caused by chronic stress might be associated with elevated hair cortisol concentrations [48]. It is well documented that activation of the central HPA axis by chronic stress also manifests as altered CAR values (i.e., heightened or reduced) [31]. In the present study, we observed no difference in the CAR between men with AGA and the hairy controls, which suggested no difference in the central HPA axis function between men with AGA and the hairy controls. Therefore, the results of the present and previous studies suggest that cortisol produced in the skin and hair follicles under local stress conditions likely contributes to the majority of cortisol concentrations measured in the hair.

Similar to the central HPA axis, CRH triggers a cascade of hormonal pathways that leads to the production of cortisol in the local stress response system in skin and hair follicles [18,19]. However, cortisol alone might not be the cause of hair loss in humans. For example, a hair follicle organ culture study found that treatment with CRH inhibits hair shaft elongation and hair follicle keratinocyte proliferation and stimulates premature catagen development and hair matrix

Table 2: Correlation matrix between hair steroids

Relationship between steroids examined at t0 (Pearson r)						**Relationships of hair steroid concentrations between two examined time points (Pearson r)**			
1. Hairy controls									
	Cortisol	**T**	**DHT**	**Epi-T**	**DHEA**				
Cortisol	1	0.28*	0.21	0.06	0.17				
T		1	0.30*	0.20	0.15				
DHT			1	0.07	0.08				
Epi-T				1	0.19				
DHEA					1				
2. Placebo group									
	Cortisol	**T**	**DHT**	**Epi-T**	**DHEA**		**(t0,t1)**	**(t1,t2)**	**(t0,t2)**
Cortisol	1	0.48*	0.24	0.14	0.22	Cortisol	0.28*	0.30*	0.32*
T		1	0.41*	0.17	0.19	T	0.53**	0.23	0.59**
DHT			1	0.21	0.15	DHT	0.47*	0.66**	0.57**
Epi-T				1	0.07	Epi-T	0.20	0.20	0.17
DHEA					1	DHEA	0.60	0.13	0.17
3. Treatment group									
	Cortisol	**T**	**DHT**	**Epi-T**	**DHEA**		**(t0,t1)**	**(t1,t2)**	**(t0,t2)**
Cortisol	1	0.33*	0.26	0.16	0.14	Cortisol	0.38*	0.54**	0.51**
T		1	0.43*	0.24	0.02	T	0.35*	0.35*	0.19
DHT			1	0.27	0.07	DHT	0.38*	0.48*	0.39*
Epi-T				1	0.15	Epi-T	0.39*	0.62**	0.46**
DHEA					1	DHEA	0.01	0.22	0.03

*,**Represent statistical significance at $p<0.05$ and $p<0.01$, respectively. DHT: Dihydrotestosterone, DHEA: Dehydroepiandrosterone

Table 3: Correlation table between salivary steroid concentrations

Relationship between steroids examined at t0 (Pearson r)					
Hairy controls					
	CARauc	**Tauc$_{awk}$**	**DHTauc$_{awk}$**	**EipTauc$_{awk}$**	**Dauc$_{awk}$**
CARauc	1	0.21	0.15	0.10	0.18
Taucawk		1	0.29	0.18	0.14
DHTauc$_{awk}$			1	0.16	0.05
EipTauc$_{awk}$				1	0.22
Dauc$_{awk}$					1
Placebo group					
	CARauc	**Tauc$_{awk}$**	**DHTauc$_{awk}$**	**EipTauc$_{awk}$**	**Dauc$_{awk}$**
CARauc	1	-0.16	0.11	-0.04	0.18
Taucawk		1	0.30	-0.12	0.11
DHTauc$_{awk}$			1	0.11	0.31
EipTauc$_{awk}$				1	0.06
Dauc$_{awk}$					1
Treatment group					
	CARauc	**Tauc$_{awk}$**	**DHTauc$_{awk}$**	**EipTauc$_{awk}$**	**Dauc$_{awk}$**
CARauc	1	0.02	0.07	0.11	0.16
Tauc$_{awk}$		1	0.27	0.16	−0.15
DHTauc$_{awk}$			1	0.22	−0.07
EipTauc$_{awk}$				1	−0.10
Dauc$_{awk}$					1

DHT: Dihydrotestosterone

keratinocyte apoptosis, whereas treatment with cortisol alone did not demonstrate these effects [18]. In clinical practice, hair loss or changes in hair growth rates are not included in the chief complaints of patients with Cushing's syndrome [49]. Thus, elevated hair cortisol concentrations in men with AGA may indicate stress-induced activation of the local stress response system of scalp skin and hair follicles, which may participate in AGA by providing precursor steroids for DHT formation. This assumption is supported by the presence of enzymes required for the metabolic conversion of progestins and androgens in the peripheral organs [50].

In addition to the improvement of AGA, we also observed a concomitant reduction in the hair cortisol and DHT concentrations but did not observe changes in the levels of salivary steroids in men with AGA after 4 months using the formulated shampoo. These results implied that topical use of the plant extracts reduced the stress burden imposed on scalp skin and hair follicles. Until now, information on the factors that activate the local stress response system has been limited. A possible explanation for our observation is that the expression levels of CRH, CRH-R, and POMC mRNAs in epidermal keratinocytes, melanocytes, and sebaceous glands are stronger in inflammatory lesional skin than in normal skin [51], indicating that inflammatory cytokines upregulate the de novo synthesis of cortisol in the local stress response system. Indeed, cortisol biosynthesis in human epidermal keratinocytes is reported to be enhanced by interleukin-1β (IL-1β) [20]. A recent study showed that exogenously added CRH enhances the synthesis of CRH-receptor 1 and 2, ACTH, melanocortin receptor 2, and TGF-β2 but reduces hair shaft elongation in biopsied hair follicles from balding vertex scalp areas of men with AGA [52]. The expression of TGF-β2, a local mediator of cellular responses to steroids, is known to be upregulated in the hair follicles by testosterone and DHT [53,54]. Therefore, it is speculated that the active constituents included in JU7505 may play a role in reducing the stress burden, such as inflammation in scalp skin and hair follicles, which results in a concomitant decrease in the concentrations of hair steroids, including cortisol and DHT, in men with AGA.

Previous studies showed that the topical use of a lotion containing 1% *Lindera strychnifolia* root extract, which has anti-inflammatory and antimicrobial properties, leads to significant changes in microbiome abundance, a decrease in the relative abundance of *Cutibacterium acnes*, and increases in the proportions of *Malassezia restricta* and *Malassezia globosa* [55]. *C. acnes* is known to upregulate the secretion of proinflammatory cytokines by keratinocytes, sebocytes, and macrophages [56]. Miniaturized hair follicles of men with AGA are known to have an increased abundance of *C. acnes* [57]. We did not assess the microenvironmental status of the scalp skin and hair follicles in men with AGA before and after using the formulated shampoo

in the present study. However, previous findings indicated that the burden of stress, such as microbial-induced inflammation in scalp skin and hair follicles, might be reduced by the actions of the constituents included in JU7505, which manifested as a decrease in the hair cortisol concentrations in the treatment group.

The present study has some limitations that should be acknowledged. First, the number of participants was relatively small to more easily evaluate the beneficial effects of the plant extracts. A statistically significant difference was observed between the treatment and placebo groups in each category of examination. However, the number of participants in each subgroup analysis was relatively small, which may have limited the power of the significant difference in this study. Therefore, the findings of this study need to be confirmed in more extensive studies with a high number of subjects. Second, we observed the beneficial effects of plant extracts on AGA in the present study, which represents an essential preliminary step toward preparing more effective plant extracts for AGA treatment.

CONCLUSION

Hair-related variables are commonly used as a primary outcome measure in studies on AGA in men. To our knowledge, this is the first trial examining the physiological mechanisms underlying the improvement of AGA after the topical use of plant extracts. The present study observed that AGA in men was associated with elevated hair cortisol and DHT concentrations rather than post-awakening salivary cortisol and DHT levels, suggesting that AGA may be associated with the activation of the local stress response system of scalp skin and hair follicles, which provides precursor steroids for DHT formation. In addition, the results showed that men with AGA presented increased hair shaft thickness and hair density after the treatment; moreover, hair loss was reduced, hair growth was increased, and hair steroid concentrations, including cortisol and DHT concentrations, were decreased after using the formulated shampoo containing JU7505, a mixture of extracts of six different plants that were reported to have anti-inflammatory, antimicrobial, antioxidant, cortisol, or 5α-reductase inhibitory properties in other previous studies. However, changes in the levels and patterns of salivary steroids were not observed after the treatment. The present study was not focused on identifying the active constituents present in the plant extract JU7505. However, the results indicated that the active constituents in JU7505 might have beneficial effects on AGA, which led to improvements in AGA symptoms, hair loss prevention, and hair growth effects. In addition, the reduced concentrations of hair steroids, including cortisol, DHT, testosterone, Epi-T, and DHEA, indicated that the extracts had a reducing effect on steroidogenic activities in scalp skin and hair follicles. Therefore, topical applications of such extracts, such as shampoo and hair tonic, containing anti-inflammatory, antioxidant, antimicrobial, cortisol, and 5α-reductase inhibitory properties would be helpful for men with AGA.

ACKNOWLEDGMENTS

We would like to express our deep appreciation to all our participants.

AUTHORS' CONTRIBUTIONS

All authors contributed equally.

CONFLICTS OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

AUTHORS' FUNDING

This work was supported by LK Biotech Inc.

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