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Proprietary Herbal Extract Downregulates the Gene Expression of IL-1 α in HaCaT Cells: Possible Implications Against Nonscarring Alopecia

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ABSTRACT

Background: Currently while, topical minoxidil and oral finasteride are the only medications approved in androgenetic alopecia (AGA), the cause oriented treatment and immunosuppressive treatment are being performed in telogen effluvium (TE) and alopecia areata (AA) respectively. Considering the inflammatory factors in the pathogenesis of these three nonscarring alopecia forms, we have formulated a mixture for topical usage composed of six different herbal extracts (HE) which have already known antiinflammatory and antioxidant features. **Materials and Methods:** In addition to performing the phytochemical analysis of HE, we detected the gene expression level of IL-1 α , the crucial hair loss mediator, for the putative efficacy in nonscarring alopecia. Cell proliferation assay was performed by XTT reagent. After determination of non-cytotoxic concentration, HaCaT cells were treated with HE. RNA isolations were carried out from both non-treated and treated cell groups by using TRI-reagent. Gene expressions of IL-1 α and as control GAPDH were determined by RT-qPCR analysis. **Results:** Results were represented as "IL-1 α /GAPDH Fold Change". HE solution caused statistically significant downregulation of IL-1 α gene expressions ($p < 0.0001$), compared to untreated control cells. HE treatment ended up with 0.1900 fold change for IL-1 α . **Conclusion:** IL-1 α is a direct growth inhibitory agent in hair follicles and an important actor in the pathogenesis of AGA, TE, and AA. Considering together the vitamins, flavonoids, and trace elements identified in the phytochemical analyses and downregulation of IL-1 α in HaCaT cells, our HE may be an auxiliary agent in the therapy of these three nonscarring alopecia forms.

Keywords: cosmeceuticals, herbal, topical, alopecia.

1. INTRODUCTION

Although hair loss do not deteriorate general health, it may constitute a ruined self confidence. Alopecic individuals generally have more frequent psychiatric disorders such as depression, anxiety or social phobia compared to whole society (1).

Typically men are affected by androgenetic alopecia (AGA), but it is a widespread dermatological problem affecting women also. Androgens have a significant role probably independent from genetic predisposition which is known as the main etiologic factor in AGA (2). 5 α -reductase (5 α -R) transforms testosterone to its more potent form dihydrotestosterone (DHT) in the AGA prone scalp which reveals augmented androgen receptor (AR) expression (3). In addition to the androgenic interaction, inflammatory processes are increasingly being emphasized as an inseparable part in the pathogenesis of

AGA (3-7). In AGA, scalp biopsies taken from both men and women exposed follicular microinflammation and lymphocytic folliculitis, referring an immunological precipitating ground (6,7). This continuous inflammation of follicles and remodeling of the connective tissue lead to permanent hair loss in AGA (3).

Another frequent cause of diffuse hair loss is telogen effluvium (TE). Although it may be one of the manifestations of various chronic diseases; a connection between stress and hair loss is a general approval among clinicians (8,9). It was demonstrated the sensitivity of human hair follicles to key skin stress mediators: Organ cultured hair follicles responded to substance P by premature catagen development and degranulation of mast cells in the connective tissue sheath of hair follicles, indicating a

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neurogenic inflammation (10). Also, it was defined acute TE due to oxidative stress induced by ultraviolet radiation. Photoactivation of porphyrin compounds which were produced by the bacteria in the pilosebaceous duct, led to oxidative tissue injury and follicular microinflammation (11). It was demonstrated the immune status of murine skin changed substantially during hair follicle cycling, with increased immuno responses connected with telogen skin, while anagen skin was relatively immunoinhibited (12).

AA is admitted as an inflammatory disease already (13-17). The serum levels of interleukin-1 alpha (IL-1 α) were found significantly elevated in AA patients with the localized form (15). It is a T cell dominated autoimmune disease of the hair follicle and histologically featured by focal inflammatory lesions with perifollicular T cell infiltrates (16,17).

Among the cytokines studied for inflammation in alopecia, IL-1 α has been demonstrated as a direct growth inhibitory agent in hair follicles (4, 13,14,16). Currently while, topical minoxidil and oral finasteride are the only medications approved in AGA, the cause oriented treatment and immunosuppressive treatment is being performed in TE and AA respectively. Considering the inflammatory factors in the pathogenesis explained above, we have formulated a mixture for topical usage composed of six different herbal extracts (HE) which have already known antiinflammatory and antioxidant features. Our hypothesis was if the mixture significantly decreases the activity of the major actor in the site of action in hair loss, it might also be effective in clinical manner. Therefore we detected the gene expression level of IL-1 α , the crucial hair loss mediator, for the activity in the three nonscarring alopecia forms that are AGA, TE and AA. Here we studied with human keratinocyte cell line (HaCaT cells) both because the ectodermal keratinocytes are among the functional components of the hair follicle and they are also the major producers of IL-1 α (4, 18, 19).

2. MATERIALS AND METHODS

Preparation of Herbal Extract

HE was prepared from six plants: *Urtica Dioica Root Extract*, *Urtica Urens Leaf Extract*, *Equisetum Arvense Leaf Extract*, *Achillea Millefolium Aerial Part Extract*, *Matricaria Chamomilla Flower Extract* and *Ceratonina Siliqua Fruit Extract*. These plants were bought from Martin Bauer. Dried plants were fine-cut. 40 g of plant mixture was extracted with 500 mL distilled water for 3 hours at 100°C using soxhlet extraction. The extract was filtered through a filter paper into a sterile bottle.

Phytochemical Analyses of Herbal Extract

Vitamin and flavonoid analyses were performed with 'high pressure liquid chromatography' (HPLC) in Phytolab, Vestenbergsgreuth, Germany. Trace elements analyses were performed with 'inductively coupled plasma optical emission spectrometry' (ICP-OES) in Saniter Lab., Istanbul, Turkey.

Cell Culture

HaCaT cells used in this study were in vitro spontaneously transformed keratinocytes from histologically

normal skin. The cells were cultured in Dulbecco's Modified Eagle's medium with high glucose, supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml gentamicin. The cells were maintained at 37°C in a humidified atmosphere at 5% CO₂ in Newbrunswick incubator. All supplements and media were purchased from Sigma Aldrich.

Cell Proliferation Assay

HaCaT cells were seeded into 96-well plates (1x10⁴cells/well) and were subjected to different concentrations (100%, 10%, 5%, 3%, 1%, 0.2% and 0%) of HE solution to assess the cell proliferation. XTT and activator reagents (Roche) were added to the plates after 72 hour incubation period according to the manufacturer's instructions. Then, cells were incubated at 37°C for 4 hours in order that XTT reagent was reduced to orange formazan compound. The optical density of soluble formazan compound was measured at 495 nm by microplate reader (Bio-Rad).

Ribonucleic Acid (RNA) Isolation and Reverse Transcription

Total RNA was extracted from cells treated with HE solution and from untreated cells by using TRI-reagent (an RNA, DNA and protein isolation reagent) according to manufacturer's (Sigma Aldrich) instructions. The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using BioSpec-nano. "Transcriptor First Strand cDNA Synthesis Kit (Roche)" was used for reverse transcription. Complementary deoxyribonucleic acid (cDNA) synthesis was performed with 500 ng total RNA; 2 μ M each final concentration of gene specific primers of IL-1 α and GAPDH (Integrated DNA Technologies); 10 U of "Transcriptor Reverse Transcriptase"; 20 U of "Protector RNase Inhibitor"; 1mM each of "dNTP mix" and "Transcriptor Reverse Transcription Buffer (5X)" according to the manufacturer's (Roche) instructions. Primer sequences (5'-3') are given in Table 1.

Primers	Forward primer	Reverse primer
IL-1 α	ACCAGTGCTGCTGAAGGAGAT	GTGCCGTGAGTTTCCCAGAA
GAPDH [§]	ATGGGTGTGAACCATGAGAA	GTGCTAAGCAGTTGGTGGTG

Table 1. Primers (5'-3') of the genes studied § Glycerinaldehyde-3-phosphate dehydrogenase used as control.

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was carried out in "Light Cycler 96 (Roche)". Amplification of products were detected via intercalation of the fluorescent dye SYBR green (Fast Start DNA Green Master Kit, Roche). Briefly, total volume of reaction mix was 20 μ l; containing 10 μ l "SYBR Green Master Mix (2X)"; 0.5 μ M of reverse and forward primers, 2.5 ng cDNA and appropriate amount of nuclease free water. All samples were run as triplicates in each run including a non-template control and four standards (1:1, 1:10, 1:100, 1:1000). The PCR parameters were determined separately for each target according to melting and annealing temperatures of primers. Each parameter included a pre-incubation step for 10 minutes at 95°C and

followed by 45 cycles of three amplification and melting step. Melting curve analysis was performed to verify specificity. For quantitation of RT-qPCR results, $\Delta\Delta Ct$ method was used ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

All data are representative of three repeats (n = 3) and expressed as mean \pm standard error of the means (SEM). Statistical evaluation was performed by unpaired t-test, using "Graph Pad Prism 5 Software (USA)" and the results with p value less than 0.05 were accepted as significant.

3. RESULTS

Phytochemical Analyses (HPLC and ICP-OES)

The vitamins, flavonoids and trace elements found in phytochemical analyses of the herbal extract are depicted in Table 2. The quantities of all are expressed as mg/100g dry extract.

	mg/100g dry extract
VITAMINS	
Thiamine (B1)	7.4
Riboflavin (B2)	1.4
Pyridoxine (B6)	1.2
Ascorbic acid (C)	<0.5
FLAVONOIDS	
Myricetin	1450
Quercetin	400
Kaempferol	600
TRACE ELEMENTS	
Iron	53.06
Copper	0.645
Zinc	2.048

Table 2. The vitamins, flavonoids and trace elements found in phytochemical analyses of HE

Cytotoxicity Analysis (Cell Proliferation Assay)

Based on cell proliferation ratios of treated cells with respect to the control cells, cytotoxicity levels of the HE solution were determined. Higher concentrations were found to be cytotoxic for HaCaT cells. For the subsequent analysis, the possible highest concentration was determined as 1% and HaCaT cells were incubated with 1% concentration of HE solution before total RNA isolation (Figure 1)

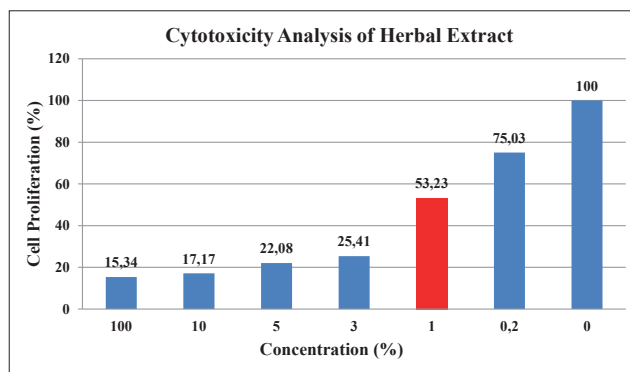


Figure 1. Cytotoxicity analysis result of HE solution. The values of bars represent the proliferation ratio of HaCaT cells in the relevant concentration of HE. Red bar is the concentration chosen for incubation.

Gene Expression Analysis (RT- qPCR)

Results were represented as "Target/GAPDH Fold Change". Results of gene expression analysis via RT-qPCR showed that HE solution caused statistically significant downregulation of IL-1 α gene expressions (p<0.0001), compared to untreated control cells. HE treatment ended up with 0.1900 fold change for IL-1 α (Figure 2).

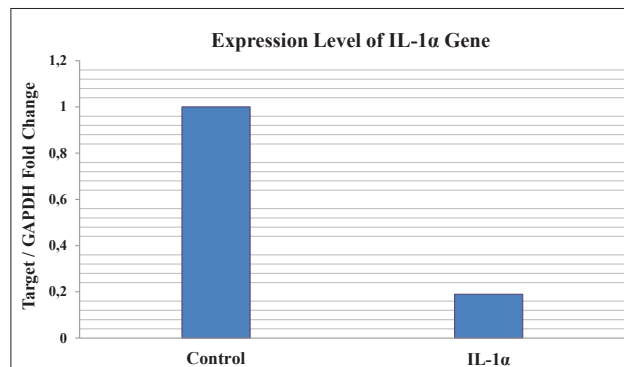


Figure 2. Expression level of IL-1 α gene compared to control

4. DISCUSSION

The pilo-sebaceous unit is a ground of various cell and tissue interactions, involving epithelial cells, sebaceous gland, dermal papilla fibroblasts, melanocytes, endothelial cells and the immune system Langerhans' cells (20). Although the mesenchyme derived papilla regulates the epithelial follicle in many aspects, intrinsic dermal-epidermal interactions are central to the development and growth of hair. Besides the inductive powers of dermal papilla cells, germinative epidermal cells of the lower follicle also can stimulate hair growth (21, 22). Together with the fact that the keratinocytes are the major producers of the inflammatory cytokine IL-1 α (18, 19), HaCaT cells seem the best target to measure the effect of our HE in hair follicle.

Among the plants used in our formulation *Urtica dioica* (*Ud*) is the most widely studied one. Symptomatic benign prostate hyperplasia (BPH) is the best researched indication of this plant which is mainly due to its 5 α -R inhibition activity (23, 24, 25). Inhibition of 5 α -R precludes the conversion of testosterone to dihydrotestosterone (DHT) high levels of which are associated with BPH (26). The same pathogenesis is also valid for AGA (3,4). Although *Ud* leaves have traditionally been used for hair loss, confirmatory clinical trials are still lacking (26). In a study performed with a combination of herbal extracts including *Ud*, the combination was found to increase the proliferation of human dermal papilla cells significantly at concentrations ranging from 1.5% to 4.5% (27). *Ud* contains β sitosterol which stimulates angiogenesis by increasing vascular endothelial growth factor (VEGF) synthesis and supports new hair growth (28).

The leave extract of *Urtica urens* (*Uu*) contains a high amount of total phenolics, flavonoids, tannins, ortho-diphenols and flavonols (29,30). It decreased the paw oedema after carrageenin administration in rats, and enhanced the activities of catalase, superoxide dismutase, glutathione peroxidase, and malondialdehyde exhibiting

a powerful antiinflammatory and antioxidant activity (30).

In a study performed with *Equisetum arvense* (*Ea*) alone and with combination of some other plants, *Ea* suppressed the superoxide anion levels in the xanthine/xanthine oxidase system and abolished the hydroxyl radical. *Ea* also reduced reactive oxygen species (ROS) levels in phorbol myristate acetate stimulated neutrophils (31). *Ea* is one of the highest silicon accumulators among plant species. Silicon penetrates the hair follicles, enters in the hair matrix and makes the hair fibers thicker. Higher silicon content in the hair leads to a decrease in the rate of hair loss (32,33).

Leaves and flowers of *Achillea millefolium* (*Am*) have been used for centuries because of their antiinflammatory properties in some problems such as rheumatism, wound healing and skin inflammation (34). In an in vivo study performed with *Am* oil extract on artificially irritated skin, the tested parameters (i.e pH, capacitance and erythema index) were restored to basal values after 3-7 days treatment (35). *Am* extract showed an improved expression profile of cytokeratin-10, transglutaminase-1 and filaggrin in cultured skin biopsies as well as an increase in epidermal thickness. Also in vivo a two month treatment with 2% *Am* extract significantly improved the appearance of wrinkles and pores compared to placebo (36).

Traditionally *Matricaria chamomilla* (*Mc*, German chamomile) oil has been used for the treatment of inflammatory skin disorders such as eczema (37). In a study performed with 3% *Mc* oil topically on the skin of mice showing its immunoregulatory potential, it alleviated the atopic dermatitis through influencing helper T cell 2 (Th2) activation (38). A pharmacologically active flavonoid apigenin which is contained by both *Mc* and *Am*, supports hair growth by suppressing transforming growth factor- β 1 (TGF- β 1) which stimulates the catagen phase in hair growth cycle (39,40).

Ceratonia siliqua (*Cs*) pod extract exhibits antioxidant properties due to the presence of catechin, epicatechin, epigallocatechin, epigallocatechin gallate, and epicatechin gallate, along with simpler phenolics, such as phloroglucinol, pyrogallol, catechol, and gallic acid. The high antioxidant level of *Cs* pod extract suggesting its potential development as a pharmaceutical product (41).

The phenolic compounds, vitamins and trace elements we identified in the phytochemical analysis of our formulation, may also give more specific support to the maintenance of healthy hair. Myricetin, quercetin, kaempferol and copper keep the hair follicle longer in anagen phase by inhibiting 5 α -R and preventing DHT formation (42,43). Vitamin C (ascorbic acid), improves blood vessel formation and increases blood flow in the scalp by stimulating the synthesis of VEGF (44). Vitamin C might also improve the efficacy of therapeutic angiogenesis by cell transplantation (45). Also the derivatives of vitamin C are found hopeful in the treatment and prevention of DHT induced balding (46). It was demonstrated that high doses of environmental cigarette smoke induce alopecia in mice and this effect was prevented by administration of

a mixture of l-cystine with vitamin B6 (pyridoxine) (47). Combinations of l-cystine and B vitamins are traditionally used in OTC products for the treatment of hair loss. Supplementation with l-cystine, pantothenic acid and vitamin B1 (thiamin) has been shown to increase the anagen rate in apparently healthy women with TE in a placebo-controlled study (48). Vitamin B2 (riboflavin) is known to enhance the metabolism of vitamin B6 (49). Zinc is found in the enzyme systems affecting hair formation and local zinc ions stimulate scalp cellular formation (44). Zinc metabolism disturbances play a key role in hair loss, especially in AGA and TE (50). Iron is a vital co-factor for proteins and enzymes involved in energy metabolism. Historically, it has long been known that iron is essential for healthy skin, mucous membranes, hair and nails. Iron deficiency causes dry, thin and fragile scalp hair clinically (51).

Apart from the phytochemical features explained above, the most significant result of this study is the inhibition of IL-1 α which is a direct growth inhibitory agent in hair follicles and an important actor in the pathogenesis of AGA, TE, and AA (4,14-16,52). Besides the obvious role of androgens in AGA (2,3,16), the three clinical entities AGA, TE, and AA may gather in a common ground as they all involve hair follicles, and consist inflammatory mechanisms in their pathogenesis (3,6,7,10,11,16). Normal human epidermis is a rich source of biologically active IL-1 α . Keratinocytes both synthesize this cytokine and respond to it via cell surface receptors, suggesting that the IL-1 system may play an important role in epidermal physiology and inflammation (18,19). Among various cytokines, IL-1 family and especially IL-1 α is stated as the main hair growth inhibitor. It is reported that IL-1 α exerts antiproliferative effect on hair follicles and causes inhibition of follicle growth as a secondary response (4, 13-16). Previously, due to the inhibitory role of IL-1 α on human hair growth, it was suggested that identifying the "inflammatory alopecic individual" might be of clinical importance to determine the individuals for whom anti-IL-1 strategies might be effective in AGA (4). Elements of the IL-1 signaling system show hair cycle dependent gene expression in murine skin. In murine skin IL-1 α and IL-1 β levels increased dramatically with the onset of spontaneous catagen and peaked during telogen (52). It is also reported the downregulation of IL-1 α during anagen phase of hair cycle (53,54). Therefore we suggest this IL-1 α suppression feature of our formulation may be beneficial for all three nonscarring alopecia forms because of the inflammatory components discussed in their pathogenesis.

5. CONCLUSION

Regarding together; the benefits cited in the literature; vitamins, flavonoids, and trace elements identified in the phytochemical analyses we performed, and downregulation of IL-1 α in the gene expression analysis in HaCaT cells, our HE may be considered as a promising auxiliary agent in the therapy of AGA, TE and AA.

- **Conflict of interest:** none declared.

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