A Comparative Study to Test a Plant Extract against Minoxidil in HaCaT Cells

Murat Türkoğlu[*], **Cihat Dündar, Hakan Sevinç, Songül Kılıç**

Biota Laboratories R&D Center, Sancaktepe, Istanbul, Turkey

Abstract   Topical 5% minoxidil solution twice a day was reported to be effective in many types of hair loss. However, some plant extracts showed a similar anagen prolongation effect in clinical studies. In this study, anti-hair loss drug minoxidil was compared to a botanical extract using HaCaT cells to investigate the cellular response to both treatments. HaCaT cells were incubated at 1% concentration of both minoxidil solution and the botanical extract. Total RNA isolation, cDNA synthesis and the gene expression analysis (RT-qPCR) were performed. The plant mixture of the botanical extract was as follows: *Chamomilla recutita*, *Urtica urens, Urtica dioica, Equisetum arvense*, *Achillea millefolium*, and C*eratonia siliqua*. Results of the gene expression analysis showed that the botanical extract and minoxidil solution caused significant downregulation of interleukin-1α (IL-1α) gene expression. The botanical extract treatment resulted in five-fold decrease in the IL-1α gene expression and minoxidil treatment resulted in a three-fold decrease. Stereoid-5α-reductase type 2 (SRD5α2) gene expressions was not significantly affected by the botanical extract. However, it was also downregulated after minoxidil treatment. It was hypothesized that anti-hair loss effect of minoxidil could be due to the suppression of IL-1α and on SRD5α gene.

Keywords   Hair Loss, Minoxidil, Interleukin 1-α, 5-α reductase type 2, Real-time PCR, Herbal Extract

1. Introduction

Interleukin (IL-1) was shown to be a potent inhibitor of hair growth. Furthermore, it was reported to be a significant factor in alopecia areata (AA) which is a disease of the anagen stage hair follicles, considered to be autoimmune in origin [1, 2]. In isolated hair follicles IL-1β decreased hair growth approximately 60-80%. This hair growth inhibition was mediated by cAMP [2]. IL-1α and IL-1β share common receptors. Rückert, et al. [3] reported that high dose proinflammatory cytokines such as IL-1β induced apoptosis of hair bulb keratinocytes *in vivo*. The hair loss that is seen in transgenic mice overexpressing IL-1α in the epidermis and outer root sheath was reported and compatible with the view of inflammatory cytokines may induce apoptosis within the hair follicle epithelium [4]. In AA a more serious disease was encountered in patients who show gene polymorphism and have insufficient amount of IL-1 receptor antagonists [2]. There were many publications on the mechanism of action of minoxidil. Topical 5% minoxidil solution twice a day on 47 patients was reported to be effective in AA [5]. Minoxidil's effect on stimulating hair growth does not depend on either a

hormonal factor or on the inhibitor action on 5α-reductase. It was also reported that minoxidil increased the mRNA expression for the VEGF at the dermal papilla level [6]. In cell cultures, minoxidil extend the survival and slowed the aging of human keratinocytes and it acts on follicular matrix cells extending the anagen phase [7, 8]. When considering the well documented effects of minoxidil on hair growth it is logical to use it as a reference for comparison purposes of any other formulation to promote hair growth. We developed a botanical extract and carried out a clinical study to show its hair growth promoting effects [9]. *Chamomilla recutita* was reported to contain *apigenin* as an active ingredient [10] whereas *Urtica urens* and *Urtica dioica* were a source of *kaempferol, myricetin, linoleic acid* [11]*, oleic acid* [12]*, iron* [13] *and beta-sitosterol* [14]. *Equisetum arvense* was reported to contain *apigenin, kaempferol and beta-sitosterol* [15]. Together with *apigenin and beta-sitosterol, Achillea millefolium* contained *folic acid and several minerals* [16] as active ingredients. *Ceratonia siliqua*, on the other hand, contained, *tocopherol, thiamine, riboflavin, pantothenic acid, folic acid, zinc, copper, iron* as some of the ingredients [17]. Among these active ingredients *apigenin, kaempferol, myricetin, linoleic acid, oleic acid, iron and zinc* were reported to stimulate hair growth [18]. In this study, we compared minoxidil and the experimental extract in the human keratinocyte cell line (HaCaT).

---

2. Materials and Methods

2.1. Cell Culture

The human keratinocyte cell line (HaCaT) was cultured in Dulbecco's Modified Eagle's medium (DMEM) with high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM l-glutamine and 100U/ml gentamicin. Cells were maintained at 37°C in a humidified atmosphere at 5% CO_2 in Newbrunswick incubator. All supplements and media were purchased from Sigma Aldrich.

2.2. Preparation of Minoxidil Solution

522.5 mg minoxidil was dissolved in 25 ml distilled water - 25 ml ethanol mixture to get 5mM minoxidil solution. This solution is used as 100% sample and other concentrations (10%, 5%, 3%, 1% and 0.2%) of the solution were prepared by dilution with distilled water.

2.3. Preparation of Plant Extract

The plants were obtained from a commercial plant supplier (Martin Bauer Group, Germany). *Chamomilla recutita* (Chamomile flowers), *Urtica urens* (Small nettle leaves), *Urtica dioica* (nettle leaves), *Equisetum arvense* (Horsetail leaves), *Achillea millefolium* (Common yarrow flowers), *Ceratonia siliqua* (Carob fruit) were used as the plants for extract. Dried plants were fine-cut. Forty grams of plant mixture was extracted with 500 mL distilled water for 3 hours at 100°C using a soxhlet extractor. The extract was filtered through a 0.22 μm filter.

2.4. XTT Cell Proliferation and Cytotoxicity Assay

The Cell Proliferation Kit II (Roche XTT) was used for cytotoxicity assay. This assay based on the cleavage of XTT by metabolic active cell, resulting in an orange formazan dye. The amount of orange formazan dye is quantitated using microplate reader. Briefly, HaCaT cells were seeded into 96-well plates ($1x10^4$ cells/well) and were subjected to different concentrations (100%, 10%, 5%, 3%, 1% and 0,2%) an of minoxidil solution and botanical extract. After 72 hour incubation period, 50 µl XTT and activator reagents were added to the each plates according to the manufacturer's instructions. Then, cells were incubated at 37°C for 4 hours in order that XTT reagent was reduced to formazan compound. The absorbance was read in BIO-RAD microplate reader (Japan) at 450 nm subtracting the background measurement of 620 nm.

2.5. RNA Isolation

HaCaT cells were incubated with 1% concentration of both minoxidil solution and the botanical extract before total RNA isolation. Total RNA was extracted by using TRI-reagent according to manufacturer's instructions (Sigma Aldrich). The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using BioSpec- nano (Japan).

2.6. Reverse Transcription

Roche Transcriptor First Strand cDNA Synthesis Kit was used for reverse transcription. cDNA synthesis was performed with 500 ng total RNA, 2 µM final concentration of gene specific primers of VEGF, KGF, IL-1α, SRD5α type 2 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 instructions (Roche).

2.7. Real-Time Quantitative Polymerase Chain Reaction

Real-time PCR (RT-qPCR) reaction was carried out in Light Cycler 96 (Roche) and Fast Start DNA Green Master Kit (Roche) was used. Briefly, total volume of reaction mix was 20 µl containing 10 µl SYBR Green Master Mix (2X), 0.5 µM of reverse and forward primers (Table 1), 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 (1:10, 1:100 and 1:1000). The PCR parameters were determined separately for each target according to melting and annealing temperatures of primers. Each parameter includes a pre-incubation step for 10 min at 95°C and followed by 45 cycles of 3-step amplification and melting step. Melting curve analysis was performed to verify specificity. Three repeats of gene expression analysis were done (n=3). For quantitation of RT-qPCR results, $\Delta\Delta C_t$ method was used ($2^{-\Delta\Delta Ct}$).

Table 1. Primer sequences for selected genes (5' to 3')

	Forward Primer	Reverse Primer
IL1A	ACC AGT GCT GCT GAA GGA GAT	GTG CCG TGA GTT TCC CAG AA
SRD5A2	AAA CCC ACT CCC AAA TG	AGA GGG CTC CTG TAT TC
VEGF	ATG CGG ATC AAA CCT CAC CA	CCA CAG GGA CGG GAT TTC TTG
KGF	AGT GGT ACC TGA GGA TCG ATA A	ACT GCC ACT GTC CTG ATT TC
GAPDH*	ATG GGT GTG AAC CAT GAG AA	GTG CTA AGC AGT TGG TGG TG

*GAPDH is used as the control gene

2.8. Statistical Analysis

All data are representative of three experiments and expressed as mean ± 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 and Discussion

3.1. Cytotoxicity Analysis

Minoxidil solution and the botanical extract showed cytotoxic effect for HaCaT cells at high concentrations. For the subsequent analysis, the possible highest concentration was determined to be 1% (Figure 1).
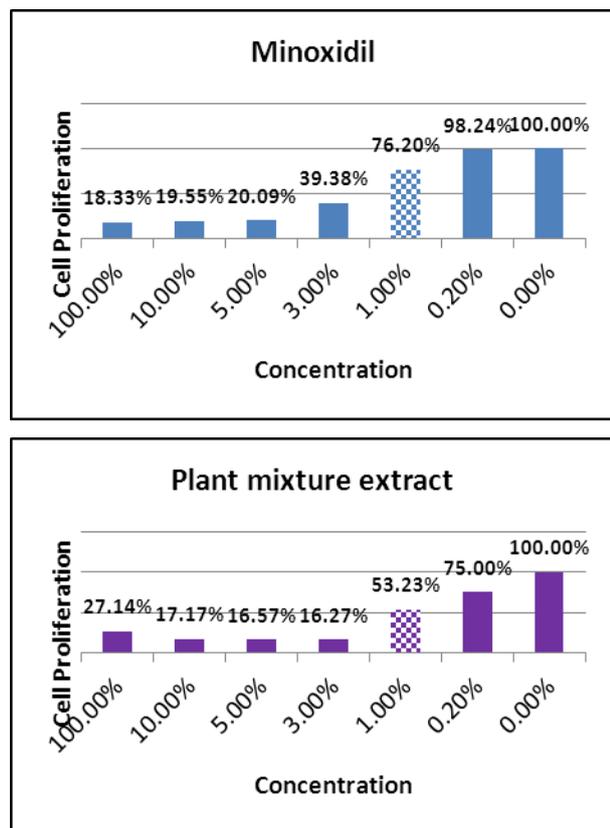
Figure 1. Cytotoxicity analysis results of minoxidil solution and the plant extract (Dashed bars represent the concentrations chosen for incubation.)

Gene Expression Analysis (Real Time qPCR)

Results of gene expression analysis via RT-qPCR showed that both the botanical extract and minoxidil caused a statistically significant downregulation of IL-1α gene expression compared to untreated control cells. The botanical extract resulted in 0.19 fold change in the IL-1α gene expression level whereas minoxidil treatment resulted in 0.34 fold change (Figure 2). SRD5α type 2 gene expression was not significantly affected by the botanical extract treatment. However, it was downregulated upon minoxidil treatment (Figure 2). VEGF and KGF gene expression were significantly affected by minoxidil and botanical extract treatments in a similar way. VEGF was downregulated upon both treatments: Its expression was reduced to one-seventh of the untreated control cells by the botanical extract while minoxidil reduced it to a one-fourth of the controls (Figure 2). Both minoxidil and the botanical extract treatment reduced KGF gene expression to a one-sixth of the controls.

In a recent study [19] it was shown that twice a day minoxidil spray was effective promoting hair density in frontotemporal and vertex regions in men. On the other hand, our clinical study [9] showed that concomitant use of a hair serum and the shampoo formulated with the botanical extract decreased hair loss in subjects suffering from the TE and AGA. Therefore, in this study, minoxidil was compared to our botanical extract at the cellular level. According to the results of our cell culture studies, minoxidil suppressed SRD5α2, VEGF, and KGF expression and the botanical extract suppressed VEGF and KGF in a similar way as minoxidil (Table 2), however, it did not show a significant effect on SRD5α2 expression. Based on these results, clinically approved effects of minoxidil and our botanical extract on the reduction of hair loss cannot be linked to the changes in VEGF and KGF gene expression since both of them was unexpectedly downregulated by both minoxidil and the botanical extract (Figure 2). There can be various mechanisms by which minoxidil and our botanical extract perform their hair-loss-reducing action. Inhibition of 5α-reductase II may be one of those mechanisms for minoxidil but not for our botanical extract (Figure 2). Among the factors that we tested, IL-1α was the only one which was affected by minoxidil and by the botanical extract in the same manner and also the one which was expectedly downregulated by both. The botanical extract reduced IL-1α gene expression to a one-fifth of the control similar to the reduction to a one-third of the control upon minoxidil treatment (Figure 2). Since IL-1α was shown to inhibit human hair follicle growth and the hair fiber production in whole-organ cultures, downregulation of this factor may explain the effect of our botanical extract and of minoxidil on hair loss [20].

Table 2. IL-1a /GAPDH, SRD5a /GAPDH, VEGF /GAPDH and KGF /GAPDH fold change values for plant extract or minoxidil-treated HaCaT cells

	IL-1a /GAPDH	**SRD5a /GAPDH**	**VEGF /GAPDH**	**KGF /GAPDH**
Plant extract	0,1900 ± 0,03055 (P<0.0001)	1,450 ± 0,8298 (P=0.6164)	0,1467 ± 0,05925 (P=0.0001)	0,1733 ± 0,0405 (P<0.0001)
Minoxidil	0,3433 ± 0,07688 (P=0.0010)	0,2200 ± 0,01732 (P<0.0001)	0,2300 ± 0,09074 (P=0.0011)	0,1633 ± 0,06333 (P=0.0002)
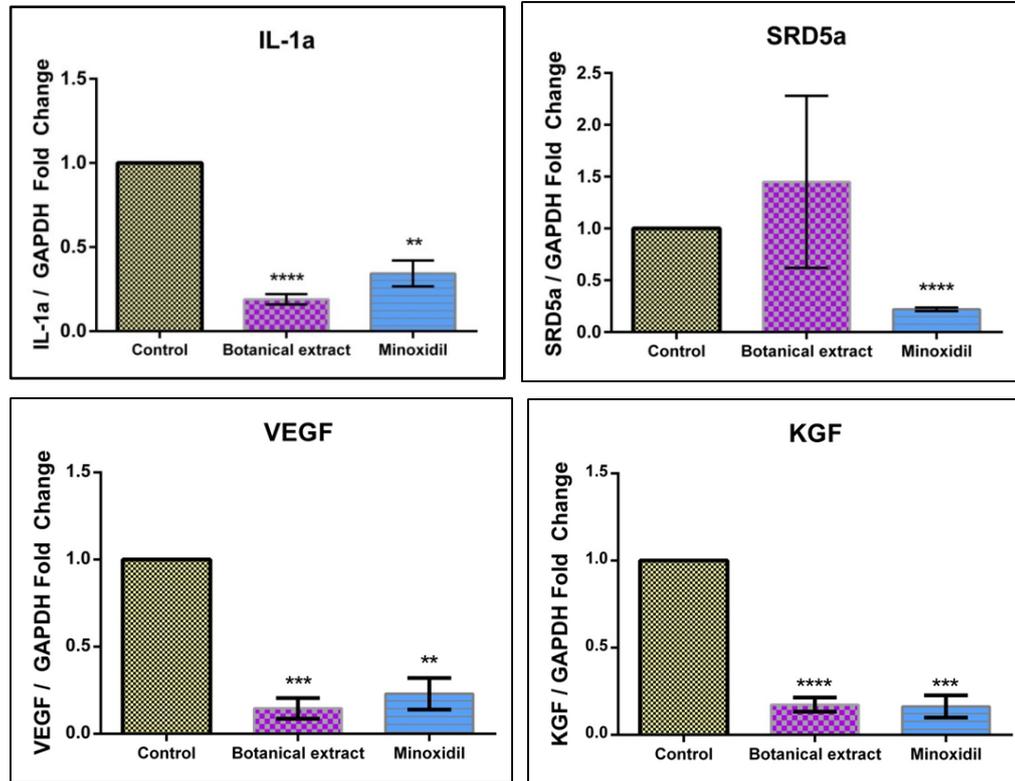
Figure 2. Gene expression levels of IL-1a, SRD5a2, VEGF, and KGF upon plant extract or minoxidil treatment

4. Conclusions

By taking all those into consideration, it was our hypothesis that botanical extracts consisting of different plants can be effective against hair loss. However, their effectiveness must be evaluated by placebo-controlled clinical studies as well as further cell, tissue and organ culture studies.

REFERENCES

[1] Safavi KH, Muller SA, Suman VJ, Moshell AN, and Melton LJ. Incidence of alopecia areata in Olmsted county, Minnesota, 1975-1989. *Mayo Clinic Proceedings,* 70 (1995) 628–633.

[2] Hoffmann R, Eicheler W, Wenzel E, and Happle R. Interleukin-1beta-Induced Inhibition of Hair Growth In Vitro Is Mediated by Cyclic AMP. *J Invest Dermatol.,* 10 (1997) 40–42.

[3] Rückert R, Lindner G, Bulfone-Paus S, and Paus R. High-dose proinflammatory cytokines induce apoptosis of hair bulb keratinocytes *in vivo. British J. Dermatol.,* 143 (2000) 1036–1039.

[4] Groves RW, Mizutani H, Kieffer JD, and Kupper TS. Inflammatory skin disease in transgenic mice that express high levels of interleukin 1 alpha in basal epidermis. *Proceedings of the National Academy of Sciences.* 95 (1995) 11874–11878.

[5] Fiedler-Weiss C. Evaluation of oral minoxidil in the treatment of alopecia areata. *Arch Dermatol.,* 123 (1987) 1488–1490.

[6] Lachgar S, Charveron M, Gall Y, and Bonafe JL. Minoxidil upregulates the expression of vascular endothelial growth factor in human hair dermal papilla cells. *British J Dermatol.,* 138 (1998) 407–411.

[7] Cohen RL, Alves MEAF, Weiss VC, West DP, Chambers DA. Direct Effects of Minoxidil on Epidermal Cells in Culture. *J Invest Dermatol.,* 82 (1984) 90–93.

[8] Baden HP, Kubilus J. Effect of Minoxidil on Cultured Keratinocytes. *J Invest Dermatol.* 81(1983) 558–560.

[9] Marzatico F, Nobile V, Oran S, and Turkoglu M. Clinical evaluation of an herbal extract against hair loss in telogen effluvium and androgenetic alopecia. *Annals of Dermatol.,* 26 (2014) 31.

[10] Avallone R, Zanoli P, Puia G, Kleinschnitz M, Schreier P, and Baraldi M. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla. Biochem. Pharmacol.,* 59 (2000) 1387–1394.

[11] Otles S, and Yalcin B. Phenolic compounds analysis of root, stalk, and leaves of nettle. *The Scientific World Journal* 2012 (2012) 1–12.

[12] Guil-Guerrero J, Rebolloso-Fuentes M, and Isasa M. Fatty acids and carotenoids from stinging nettle (Urtica dioica L.). *J. Food Composition and Analysis* 16 (2003) 111–119.

[13] Turan M, Kordali S, Zengin H, Dursun A, and Sezen Y. Macro and micro mineral ontent of some wild edible leaves consumed in eastern Anatolia. *Soil & Plant Science* 53 (2003) 129–137.

[14] Yarnell E. Stinging Nettle: Alternative and Complementary Therapies 4(3) (2009) 180–186.

[15] Mimica-Dukic N, Simin N, Cvejic J, Jovin E, Orcic D, and Bozin B. Phenolic compounds in field horsetail (*Equisetum arvense L.*) as Natural Antioxidants. *Molecules* 13 (2008) 1455–1464.

[16] Chandler RF, Hooper SN, and Harvey MJ. ethnobotany and phytochemistry of yarrow, *Achillea millefolium*, *Economic Botany* 36 (1982) 203–223.

[17] Watson, R., Zibadi, S., and Preedy, V. *Bioactive foods in promoting health fruits and vegetables*. Academic Press, Amsterdam, 2010, pp. 245-247.

[18] Harrison, S., Bergfeld, W., and Andersen, F.A., Cosmeceuticals for Hair and Nail in Cosmeceutical Science in Clinical Practise, Ed. Sadick, N.S, CRC Press, USA, 2010 p. 63.

[19] Hillmann K, Bartels NG, Kottner J, Stroux A, Canfield D, and Blume-Peytavi U. A, Single-centre, randomized, double-blind, placebo-controlled clinical trial to investigate the efficacy and safety of minoxidil topical foam in frontotemporal and vertex androgenetic alopecia in men. *Skin Pharmacol Physiol.,* 28 (2015) 236–244.

[20] Harmon CS and Nevins TD. IL-1 alpha inhibits human hair follicle growth and hair fiber production in whole-organ cultures. *Lymphokine Cytokine Res.,* 12(1993) 197–203.