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"Helichrysum italicum essential oil promotes hair growth by enhancing IGF-1 signalling ex vivo"

Géraldine LEMAIRE[1,*], Cecilia COLOMBERO[2], Karin PAPPELBAUM[2], Marta BERTOLINI[2], Janin EDELKAMP[2], Valérie CENIZO[1]

[1] L'Occitane en Provence, Groupe L'Occitane, Manosque, France
[2] QIMA Life Sciences, QIMA Monasterium GmbH, Münster, Germany

1. Introduction

Hair loss and reduced hair shaft thickness are hallmarks of hair disorders, such as telogen effluvium and male or female pattern hair loss (M/FPHL) and are also a frequent concern during aging.

Under physiological conditions, human hair follicles (HF) undergo a natural cycle including the anagen (growth) phase, during which the hair shaft is produced by proliferation and differentiation of hair matrix (HM) keratinocytes. This is followed by the catagen (regression) phase, in which HM keratinocytes undergo apoptosis, and ends with the telogen (resting) phase. During the telogen-to-anagen transition, when a new hair cycle begins, the newly formed hair shaft pushes out the old hairs, causing them to fall out (exogen) [1].

The hair cycle is tightly regulated by reciprocal interaction between mesenchymal, i.e. dermal papilla (DP) cells and epithelial cells within the HF. At the molecular level, each hair cycle phase is characterised by the spatiotemporal expression and activity of specific growth factors that regulate the length of each phase and the transition from one phase to another. At the end of anagen, the abundance of hair growth promoting factors such as insulin-like growth factor-1 (IGF-1), Hepatocyte growth factor (HGF), and fibroblast growth factor 7 (FGF-7) declines, while the concentrations of catagen promoting factors such as Transforming Growth Factor beta-1 and -2 (TGFβ1 and –2), and Prostaglandin D2 (PGD2), increase. Furthermore, fibroblast start to emigrate from the DP into the DP stalk and dermal cup (DC) and the HF regresses. During telogen the DP releases factors that induce epithelial HF stem cells (eHF-SCs) proliferation and progeny generation as well as HM keratinocyte proliferation and differentiation, ultimately resulting in the beginning of a new anagen phase [1-6].

Disruption of these highly sensitive molecular interplays cause pathological hair cycle changes, which can cause hair loss disorders such as MPHL, FPHL, telogen effluvium and hair loss associated with aging [7-9]. In addition, it can result in progressive HF miniaturization, where thick, highly pigmented hair shafts transform into thin, lighter hair, a condition often associated with hair loss disorders.

Current pharmacological treatment options for mitigating hair loss and promoting hair growth are limited and often associated with adverse effects. Consequently, there is a high demand for natural adjuvants or non-drug agents as alternative solutions. These alternatives should aim to maintain or prolong anagen by increasing the expression of anagen-promoting factors or enhancing DP inductivity, which in turn regulate HM keratinocyte proliferation, differentiation, and hair shaft production. The *Helichrysum Italicum* essential oil (IEO) is recognised for its anti-inflammatory, antibacterial and antioxidant properties [10, 11] and we previously reported that IEO has benefits on epidermal keratinocyte differentiation and barrier formation [12]. Given this re-epithelization effect and the fact that HM keratinocytes proliferation in the HF epithelium is necessary for hair growth, we here investigated the effect of IEO on hair growth promotion.

2. Materials and Methods

a. Plant material and oil distillation

Aerial parts of *Helichrysum italicum italicum* were collected at flowering time in July from a crop cultivation located on the Corsican coast. Fresh aerial parts were hydrodistilled for five hours using a Clevenger-type apparatus.

b. Donor material

Human HF samples were obtained after informed written patient consent and ethics committee approval (under the ML Biobank 2019-297-f-S; study plan 2020-954-f-S and under the University of Muenster 2015-602-f-S) and the Comité de Bioética de la Universidad Fernando Pessoa Canarias (03 (2020-06-22)). This study was conducted according to the Declaration of Helsinki principles.

c. Culture and treatment

Human amputated HFs were microdissected and individually cultured in William's E media as previously described [13,14]. At 24h after isolation, culture medium was replaced, and HFs were treated with the vehicle (DMSO 0.5%) or IEO at 0.0005% until Day 7 of culture. At the end of Day 7, HFs were then embedded in a cryomatrix (Fisher Scientific) and stored at −80°C.

d. Melanin clumping in the hair follicle

Ectopic melanin clumps were counted in Masson-Fontana-stained sections of microdissected amputated HFs at the end of the culture in a defined region of interest starting at the Auber‘s line and ending approximately 10 cell lines above the DP.

e. Ex vivo microscopic hair follicle elongation

To determine HF length, each HF was measured from the end of the connective tissue sheath to the end of the distal outer root sheath at different time points (Day 0, Day 3, and Day7), using a digital light microscope at 50X magnification (VHX900; Keyence Corporation, Osaka, Japan) and affiliated software. Measurements were obtained at baseline, after the 24-hour rest period and subsequently every 48 hours. Hair shaft elongation was measured per individual HF.

f. Hair cycle staging and scoring

Hair cycle staging was performed at the end of the culture, using the Masson–Fontana histochemistry and Ki-67/TUNEL immunostaining, and determined according to established parameters from Langan et al [13]. A standardized score was applied to the method developed by Kloepper et al. [15], attributing a score of 100 to anagen, 200 to early catagen, 300 to mid-catagen, 400 to late catagen and 500 to dystrophic HFs. Thus, the lower the score, the more established the HFs were in anagen, and the higher the score, the more they progressed into catagen.

g. Immunofluorescence

Snap frozen hair follicle samples embedded in OCT matrix were sectioned with a cryostat (Leica), and 6-µm sections were collected. Consecutive sections of each amputated HF were collected, and slides were stored at −80°C.

To stain apoptotic and proliferating cells in the hair matrix, a Ki-67/TUNEL double staining was performed using the ApopTag® Fluorescein in Situ Apoptosis Detection Kit (Merck Millipore) in combination with a Ki-67 staining. Cryosections were fixed with 4% paraformaldehyde (PFA) in PBS and incubated overnight at 4°C with a mouse anti-Ki-67 antibody (1:800 in PBS; Cell Signalling Technology) after the TdT enzyme step. Ki-67 primary antibody was detected with a secondary antibody, goat anti-mouse IgG rhodamine (1:200; Jackson ImmunoResearch), incubated for 45min at RT after the fluorescent-labelled anti-digoxigenin step of the ApopTag® kit.

For IGF-1 [16], cryosections were fixed in acetone at −20°C and pre-incubated with 2% normal goat serum in TNB buffer (Tris-HCl + NaCl + Casein) or 10% NGS for 30 min at room temperature (RT) prior to overnight incubation at 4°C with a rabbit anti-human IGF-1 primary antibody

(1/200 in 2% NGS, Abcam). Secondary antibody incubation was performed at RT for 45 min using a goat anti-rabbit Alexa Fluor 488 (1/400 in 2% NGS (IGF-1), Invitrogen). Counterstaining with DAPI was performed to visualize nuclei. Images were taken using a Keyence fluorescence microscope (BZ9100; Osaka, Japan), maintaining a constant set exposure time throughout imaging for further analysis.

h. Data Management

Statistical analyses were performed using Graphpad Prism 10 (GraphPad Software Inc.). Data were tested for normal distribution using the D'Agostino & Pearson omnibus normality test. When data did not follow normal distribution the Mann-Whitney *U* test was used. When data were normally distributed an unpaired student's *t*-test was used. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results

a. IEO induces hair shaft production in HFs

First, it was validated that IEO (0.0005%) application induced no cytotoxicity in human HF organ cultures, in that no excessive melanin clumping was observed (data not shown). In addition, there is no significant increase in the LDH release of IEO-treated HFs compared with control HFs. This indicates no toxicity after seven days of treatment with IEO (0.0005%) (data not shown).

To investigate the impact of IEO on HF growth, organ-cultured HFs were incubated in serum-free supplemented medium containing 0.0005% IEO. At day 7, HFs maintained with IEO showed a statistically significant increase (+25%) in hair follicle elongation due to increased hair shaft formation over control (Figure 1).

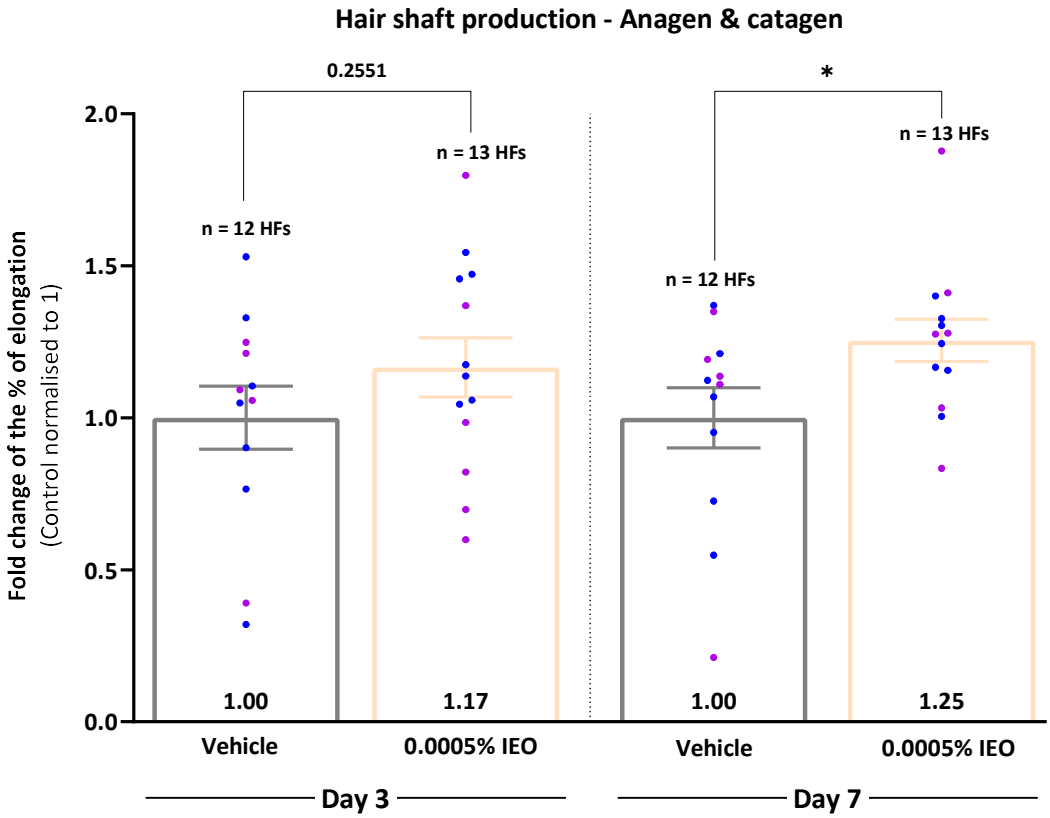

Figure 1. IEO induces hair shaft production in HFs. Hair shaft elongation of human HFs *ex vivo* with IEO treatment and vehicle (control) was measured over 7 days using a digital light microscope at 50X magnification. Mean ± SEM, (n = 12 HFs vehicle, 13 HFs IEO 0.0005% from 2 healthy donors, indicated with dots of different colors: blue represent HFs from the female donor and purple represent HFs from the male donor). The data were compared with an unpaired Student's t test, $*p < 0.05$

b. IEO induces hair matrix keratinocytes proliferation in human HFs

The proliferation of keratinocytes in the germinative matrix is directly correlated with the anagen phase, with induction of the catagen phase leading to a decrease in proliferation [13]. Hair matrix keratinocyte apoptosis is used as an additional indicator of the anagen/catagen transition or of the direct effect of the active ingredient if only anagenic HFs are assessed.

As shown in Figure 2a, application of IEO 0.0005% maintained more HFs in anagen phase when compared to vehicle treated controls. The anagen-prolonging effect of IEO was verified by a decrease in hair cycle score (Figure 2b).

To confirm the effect of IEO on inhibiting catagenic development, we analysed the percentage of proliferative and apoptotic keratinocytes in the hair matrix in all HFs at the end of culture (anagen + catagen). While no change was detected in the number of TUNEL+ cells in the hair matrix (data not shown), IEO-treated hair follicles had +25% proliferating keratinocytes compared with vehicle-treated follicles ($p \leq 0.05$), as assessed by quantitative histomorphometry (immuno) for Ki-67+ cells in the germinative hair matrix (gHM) (Figure 2c and 2b).

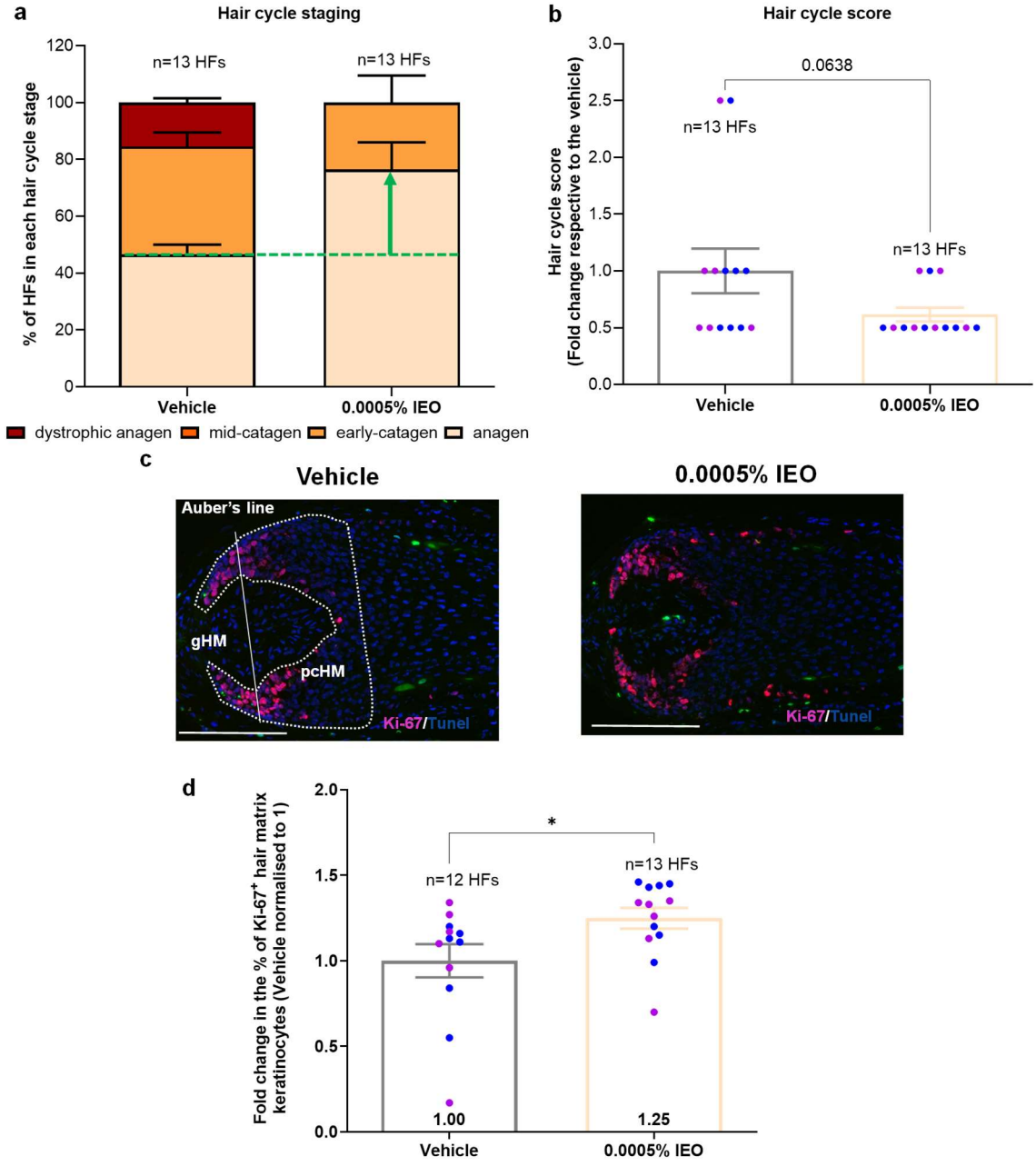

Figure 2. IEO prolongs the anagen phase, decreases hair cycle score and increases hair matrix keratinocytes proliferation in human HFs at Day 7. (a) Quantitative of hair cycle staging of anagen and catagen HFs under vehicle or 0.0005% IEO. (b) Quantitative analysis of hair cycle score of anagen and catagen HFs under vehicle or 0.0005%. Arbitrary units (au) were assigned to anagen (100), early catagen (200), mid-catagen (300), 400 to late catagen and dystrophic (500) HFs. (c) Representative pictures of Ki-67/TUNEL immunostaining to assess hair matrix keratinocyte proliferation and apoptosis. Positive cells in the germinative hair matrix below the Auber's line were counted for proliferation and in the germinative and precortical hair matrix for apoptosis. gHM: germinative hair matrix and pcHM: pre-cortical hair matrix. Scale bar: 100 µm. (d) Quantification of Ki-67+ keratinocytes in the germinative hair matrix of control- and IEO-treated HFs. Mean ± SEM, n = 12-13 HFs from two donors (independent experiments indicated with dots of different colours); The data compared with an unpaired Student's t test, $*p < 0.05$

c. IEO increases IGF-1 expression in DP remaining in anagen

To investigate possible mechanisms of action in IEO, we quantified the expression of IGF-1 which is a potent stimulator of anagen-associated hair follicle growth [17, 18] in all HFs and in HFs remaining in anagen VI at the end of the culture period (D7). As shown in Figure 3a, a slight increase in IGF-1 was detected in the HFs from the two donors analysed, this effect is more evident in the subgroup of anagen HFs (Figure 3b). In contrast, IGF-1 expression in the outer root sheath remained unchanged after IEO treatment (data not shown).

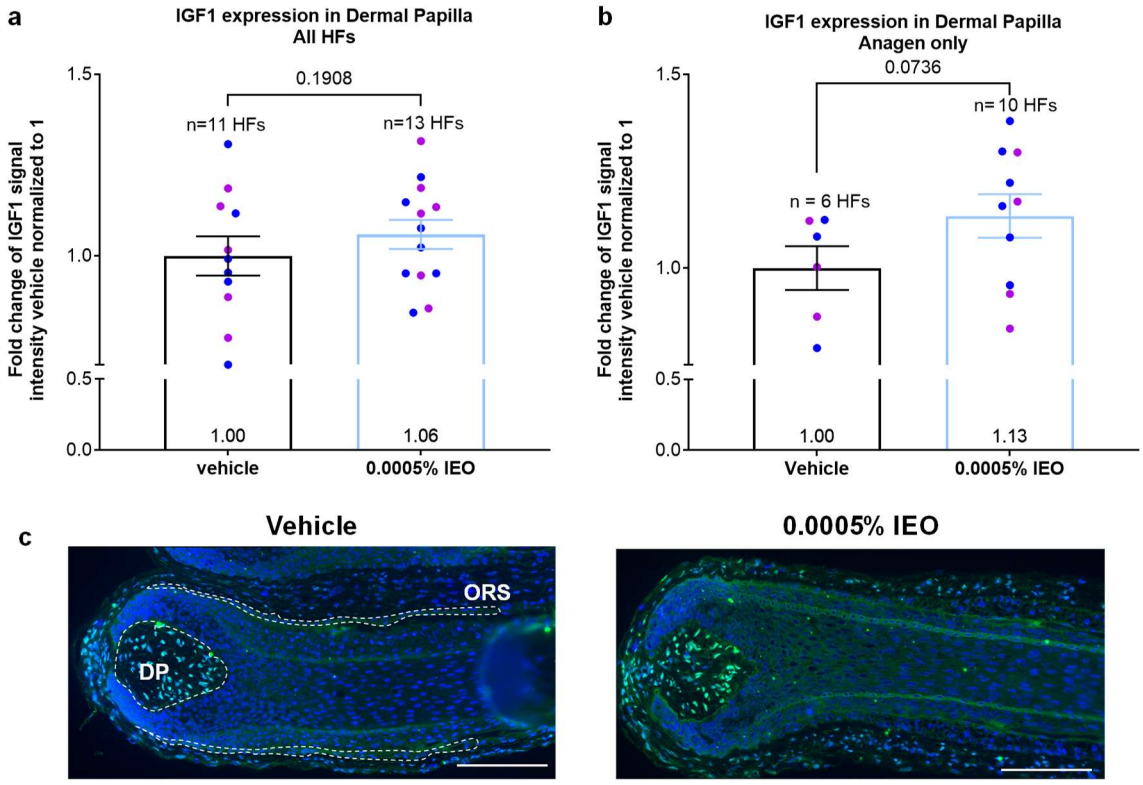

Figure 3. IEO increases IGF1 expression in dermal papilla inductivity on all hair follicles at Day 7. IGF-1 expression was measured in dermal papilla (a) and ORS (b) of vehicle-and IEO-treated HFs on all hair follicles and in dermal papilla (d) and outer root sheath (c) of vehicle-and IEO-treated HFs remaining in anagen after the culture. Mean ± SEM, n = 12-13 HFs from two donors (independent experiment). Mean ± SEM, n = 12-13 all HFs and n =6-10 anagen HFs from two donors (independent experiments indicated with dots of different colours); The data were compared with an unpaired Student's t test or a Mann-Witney test, ns. (e) Representative pictures of IGF-1 immunostaining in vehicle and IEO-treated HFs. Immunoreactivity was quantified in dermal papilla and ORS (demarcated area). DP: Dermal Papilla, ORS: Outer Root Sheat, Scale bar: 100 µm

4. Discussion

Hair loss is a common sign of ageing in both men and women and can cause psychological distress. Today, hair loss sufferers are looking for new, gentler but equally effective natural ingredients. IEO is known for its anti-inflammatory, antibacterial and antioxidant properties and we have previously reported that IEO has beneficial effects on epidermal differentiation and barrier formation. Because of these re-epithelializing and antioxidant properties [11, 12], IEO could have a beneficial effect on hair growth. We therefore tested the potential effect of IEO on the hair follicle and its ability to inhibit hair loss by acting on the proliferation of HF HM keratinocytes and anagen maintaining.

Our data suggests that IEO increases hair shaft production and inhibits the induction of premature catagen, probably by stimulating the proliferation of HM keratinocytes via IGF-1 signaling from the DP. Indeed, DP cells are essential to support keratinocyte proliferation as a source of nutrients and growth factors such as IGF-1. Kwack et al [19] have shown that IGF-1 produced by dermal papilla cells promotes hair growth by stimulating keratinocyte proliferation. In addition, IGF-1 prolongs the anagen phase in human hair follicles and has been associated with stimulating the synthesis of specific growth factors in hair follicles [20].

Thus, the IEO-induced increase in IGF-1 expression in the dermal papilla could explain the increased proliferation of hair matrix keratinocytes observed and the effect of IEO on hair shaft production. In addition, through its demonstrated antioxidant [11] and anti-inflammatory activities [10], IEO could also prevent entry into the telogen phase of the hair cycle, which is attributed to the attenuation of oxidative stress and inflammatory response [21].

Thus, because IGF-1 has emerged as a promising therapeutic target for hair loss-related conditions [17, 18], IEO could therefore be used as a promising anti-hair loss agent. However, follow-up studies involving a much larger number of female and male donors are required to clarify this hypothesis, and its clinical efficacy will also need to be demonstrated subsequently. Notwithstanding the limited number of donors, the findings of this study demonstrate that an essential oil of *Helichrysum italicum* has the capacity to promote hair follicle growth *ex vivo*.

5. Conclusion

In conclusion, we have shown for the first time that helichrysum italicum essential oil acts on the hair follicle by prolonging its anagen phase through increased proliferation of HM keratinocytes via IGF-1 signalling. Consequently, the topical application of IEO may be a viable option for individuals seeking to enhance the volume of their hair. Furthermore, IEO may be utilised as an adjuvant strategy for hair loss disorders, catering to individuals who favour milder and safer approaches.

6. References

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