

REDENSYL®

THE HAIR GROWTH GALVANIZER

REACTIVATES HAIR FOLLICLE STEM CELLS FOR AN OUTSTANDING HAIR GROWTH



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TABLE OF CONTENTS

1. Introduction.....	4
2. Human body hair	4
2.1.Hair types	4
2.2.Hair follicle structure	4
3. The hair cycle	5
3.1.The hair cycle and hair follicle stem cells	5
3.2.Hair cycle in non balding scalp and in balding scalp (androgenic alopecia).....	7
4. Hair loss.....	7
4.1.Classification of hair loss types	7
4.2.Hair loss in numbers:.....	7
5. Redensyl® composition.....	8
6. Redensyl® mode of action	9
7. Redensyl® technical description	9
8. <i>In vitro</i> assessment of Redensyl® and its components.....	10
8.1.Introduction.....	10
8.2.Materials and methods	10
8.2.1. <i>In vitro</i> tests based on the study of DHQG active ingredient.....	10
8.2.1.1. Cells culture	10
8.2.1.2. Viability assessment.....	10
8.2.1.3. Proliferation assessment.....	11
8.2.1.4. Assessment of mRNA expression profile of ORSc treated with DHQG.	11
8.2.2. <i>In vitro</i> tests based on the study of EGCG2 active ingredient on normal human keratinocytes	13
8.2.2.1. Cells culture and treatment	13
8.2.2.2. Quantification of IL-8 released by NHEK	13
8.2.3. <i>In vitro</i> tests based on the study of the Redensyl®	14
8.2.3.1. Cells culture and treatment	14
8.2.3.2. Western blot analysis: Active β -catenin assay	14
8.2.3.3. Apoptosis Annexin V assay:.....	14
8.3.Results and discussion on <i>in vitro</i> experiments.....	15
8.3.1. Viability/metabolism assessment of HFDP cells treated with DHQG	15
8.3.2. Proliferation assessment of ORSc treated with DHQG	15
8.3.3. Assessment of mRNA expression profile of ORSc treated with DHQG..	16
8.3.4. Anti-inflammatory properties of EGCG2: IL-8 release studies	18
8.3.5. Assessment of beta catenin activation in AGA ORSc treated by Redensyl®	18
8.3.6. Assessment of anti-apoptotic effect of Redensyl®	19
8.4.Conclusions on <i>in vitro</i> experiments.....	20
9. <i>Ex vivo</i> assessment of Redensyl®	21
9.1.Introduction.....	21
9.2.Materials and methods	21
9.2.1. Products tested	21

REDENSYL[®]

9.2.2. Hair follicles culture and treatment	21
9.3. Results and discussion on <i>ex vivo</i> assessments	22
9.4. Conclusions on <i>ex vivo</i> experiments	22
10. Clinical investigation of Redensyl [®]	23
10.1. Introduction	23
10.2. Materials and methods of clinical tests	23
10.2.1. Description of the lotion used	23
10.2.2. Description of the panel and study conditions	23
10.2.3. Clinical assessments methods	24
10.2.3.1. Phototrichograms analysis (PTG) (Efficacy criteria)	24
10.2.3.2. Scalp pictures	24
10.2.3.3. Self evaluation of Redensyl [®] by the volunteers	24
10.2.3.4. Data management	25
10.3. Results and discussion	25
10.3.1. Phototrichograms analysis (PTG) (Efficacy criteria)	25
10.3.2. Clinical pictures before and after treatment	30
10.3.3. Self evaluation of Redensyl [®]	31
10.4. Conclusions on clinical investigations	32
11. General Conclusions	32
12. Bibliographic references	34

1. Introduction

Redensyl® is a hair care ingredient developed by Induchem Companies which acts as a hair growth galvanizer by reactivating hair follicles stem cells and dermal papilla fibroblasts. The present technical report contains the assessment results of Redensyl® *in vitro*, *ex vivo* and *in vivo* (clinical investigation on human volunteers).

2. Human body hair

2.1. Hair types

The character of human hair is constantly changing from the prenatal period to old maturity. Under given physiological conditions, the same hair follicle can successively form different types of hair[1]. Lanugo, the first-generation of hair appears during intra-uterine life and is silky and glossy and contains no pigment or medulla. Near the end of pregnancy, lanugo is replaced by the second-generation hair which is already pigmented[2]. Fine vellus hairs begin to change to terminal hairs before the onset of puberty[3]; and with advancing age, the terminal hairs develop and thicken on all parts of the body[4]. Eyelashes and eyebrows become fully formed before puberty. They grow steadily thicker during childhood but remain relatively unaltered throughout adulthood. Eyelashes are the most highly pigmented of the terminal hairs. Despite differences among individuals, follicle structure and development are the same for all types of hair.

2.2. Hair follicle structure

Studying the histological structures indicate that the outermost aspect of the follicle are the outer root sheath (ORS) consisting of several cell layers, and the inner root sheath (IRS). Henle's, Huxley's, and cuticle layers compose the IRS. The IRS cuticle layer adjoins the cuticle of the hair fiber. Adjoining the ORS on the dermal side is the dermal sheath. The hair shaft comprises an outer layer of overlapping cuticle cells surrounding a cellular cortex and sometimes a central medulla. The region in the bulb where keratinocytes proliferate rapidly is called the hair matrix zone: it surrounds the dermal papilla separated by a basement membrane (Fig 1). Dermal papilla provides essential stimuli for both follicle induction and hair growth.

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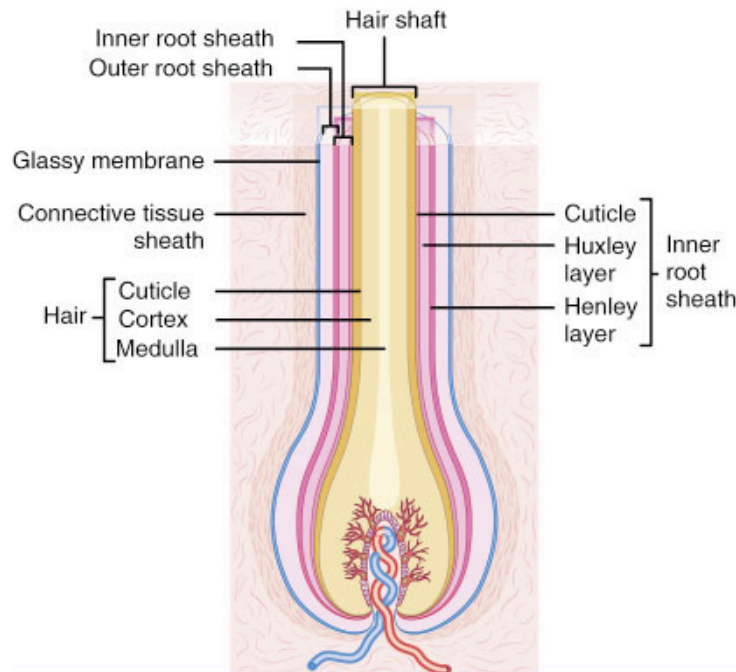


Fig 1: Hair follicle structure (according to Medical Dictionary 2011)

3. The hair cycle

3.1. The hair cycle and hair follicle stem cells

The hair follicle undergoes cycles of degeneration and regeneration throughout life due to stem cells involvement. Cyclical changes in hair follicle growth are divided into different stages, referred to as anagen, catagen, telogen, and exogen[1, 6, 7] (Fig 2).

At the onset of each new **anagen phase**, the cycling portion of the follicle regenerates, a process that necessitates a reservoir of follicle stem cells. Stem cells are able to self-renew as well as give rise to differentiating cells. Hair follicle stem cells are found in the bulge regions below the sebaceous glands in the lowest permanent portion of the follicle, within the ORS[8]. These stem cells are slow cycling and express the cell surface molecules CD34 (Cluster of differentiation 34), keratin K15[9] and VdR (Vitamin D receptor)[10]. At the start of each new hair cycle, a cluster of bulge stem cells becomes activated to proliferate by activation of Wnt (Wingless-related integration site) signaling pathway[11].

The onset of **catagen phase** is marked by cessation of proliferation and apoptosis of the epithelial cells below the bulge. The mesenchymally derived dermal papilla survives the catagen phase and moves upward to about the lowermost portion of the bulge, which then forms the secondary germ at its base, during the telogen phase.

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In **telogen phase**, most bulge cells are in a dormant state (Wnt inhibition, with no detectable nuclear β -catenin). Defined mesenchymal-epithelial interactions likely involving BMPs (Bone morphogenetic proteins) and Wnt signaling are thought to signal anagen onset[12, 13]. Once follicle stem cells become activated, they migrate along the ORS to the base of the follicle, where they produce a new hair shaft from the matrix cells area composed of progenitor cells[14]. Matrix cells have been referred to as tissue amplifying cells because they proliferate rapidly during the growth anagen phase. After proliferating, matrix cells differentiate to form the hair channel, the inner root sheath and the hair shaft. The bulge area stem cells generate cells of the outer root sheath, which in turn drives the highly proliferative matrix cells next to the dermal papilla.

As the new hair grows in, the old hair is shed during the **exogen** phase. The duration of each stage varies depending on the type, site, and genetic programming of the follicle (eyebrow, eyelash, hair scalp...).

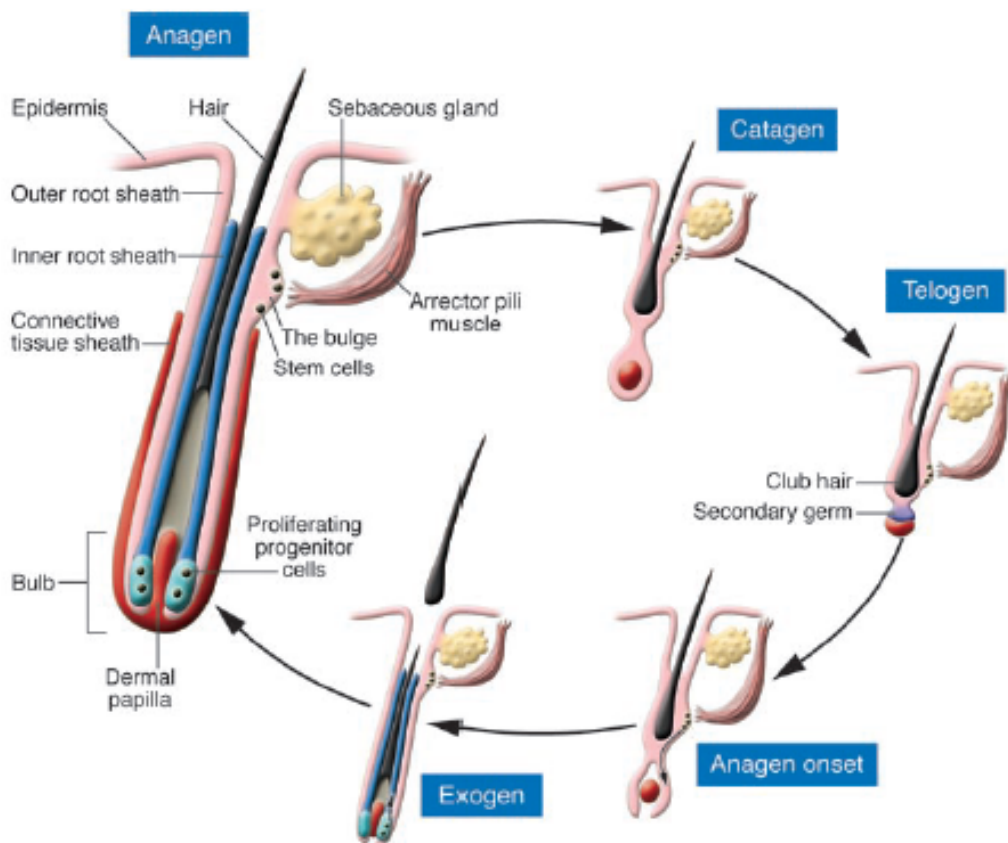


Fig 2: Hair follicle cycle (according to Costarellis G.[6])

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3.2. Hair cycle in non balding scalp and in balding scalp (androgenic alopecia)

The growth of scalp hair is a cyclical process, made up of successive phases of growth (anagen) and rest (telogen)[6]. In non-balding scalp, more than 90% of scalp hair is in anagen phase[15]. However, during androgenetic alopecia for men (male pattern hair loss), the progressive shortening of the anagen phase, as well as the increase in the duration of the lag phase (the interval between the shedding of a telogen hair and the emergence of a replacement anagen hair) and with successive hair cycles, a progressive decrease in the percentage of hair follicles in anagen phase occurs. For men with male pattern hair loss, only 60 to 80% of total hairs are in anagen phase. This shortening of the anagen phase leads to progressive miniaturization of hair follicles, which contributes to a decrease of visible hair over affected areas of the scalp[16].

4. Hair loss

4.1. Classification of hair loss types

Hair loss, a common affliction of humans, occurs in many pathophysiological conditions of the skin as well as in systemic disorders. Classification of hair loss is commonly divided into two categories, cicatricial (scarring) and non-cicatricial alopecia.

Cicatricial alopecia results from hair follicle damage complicated by various pathological changes of the surrounding skin.

Non-cicatricial alopecia is caused by either functional or structural disorders of the hair follicle itself. Non-cicatricial alopecia has many causes: result of chemotherapy or physical (radiation) treatment of cancers, nutritional and hormonal disorders, or stress[1]. The causes and pathogenetic backgrounds of non-cicatricial alopecia are largely unknown; this refractory and mostly irreversible hair loss being a major therapeutic challenge for the dermatologists. Male pattern alopecia (androgenetic alopecia) and alopecia areata are the most common afflictions linked to non-cicatricial hair loss. Alopecia areata is a condition in which hair is lost from some or all areas of the body, usually from the scalp. Because it causes bald spots on the scalp, especially in the first stages, it is sometimes called spot baldness. Alopecia areata is thought to be a systemic autoimmune disorder in which the body attacks its own anagen hair follicles and suppresses or stops hair growth.

4.2. Hair loss in numbers:

The human scalp has an average 110,000 hairs on a global surface of 600cm², which are growing and falling on a daily basis. On average 50-100 scalp hairs are lost each day. When the balance between the growing hairs and the falling ones is altered, then hair loss starts and baldness occurs.

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Hair loss (also called alopecia) can happen at any age, all around the world, mainly targeting men. It is a known fact that 40% of the men will have noticeable hair loss by age 35, this number reaches 65% by 60 years of age. Women are also deeply impacted by such process: 50 to 75% of them suffer noticeable hair loss by age 65. Hair loss can be devastating for one's self image and emotional well-being.

According to the International Society of Hair Restoration Surgery, almost 1 million patients worldwide were treated by surgical and non-surgical hair restoration means in 2012. Ninety three percent (93%) of the hair restoration surgery procedures achieved in 2012 were targeting scalp, and 4.5% eyebrows. Men represent 86% of the patients for hair transplant surgery and 67% for non-surgical hair restoration. They initiate such treatment at the average age of 38.

Each hair surgery enables the transition of 2016 grafts, each containing 4 hairs, representing about 8,100 hairs transplanted on patients' scalp. Patients generally need 3 procedures to restore the appropriate hair density. Data shows that 64% of the patients' post-surgery complaints are about the final density of their hair.

5. Redensyl® composition

Redensyl® is composed of patented molecules (DHQG and EGCG2: two stabilized polyphenols) targeting the ORS bulge stem cells (named in this report ORSc for Outer Root Sheath stem cells) and the fibroblasts located in the dermal papilla (named in this report HFDPc for Human Follicular Dermal Papilla cells). Glycine and zinc are involved in hair metabolism. Glycine is a major constituent of specific hair proteins called keratin associated proteins (KAP)[17]. Zinc is essential for cystin incorporation into keratin[18].

Redensyl® is composed by:

- Dihydroquercetin-glucoside
- Epigallocatechin gallate-glucoside
- Glycine
- Zinc chloride
- Meta-bisulfite
- Glycerin
- Water

INCI name:

WATER, GLYCERIN, SODIUM METABISULFITE, GLYCINE, LARIX EUROPAEA WOOD EXTRACT, ZINC CHLORIDE, CAMELLIA SINENSIS LEAF EXTRACT

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6. Redensyl® mode of action

Graphical summary of Redensyl® mode of Action

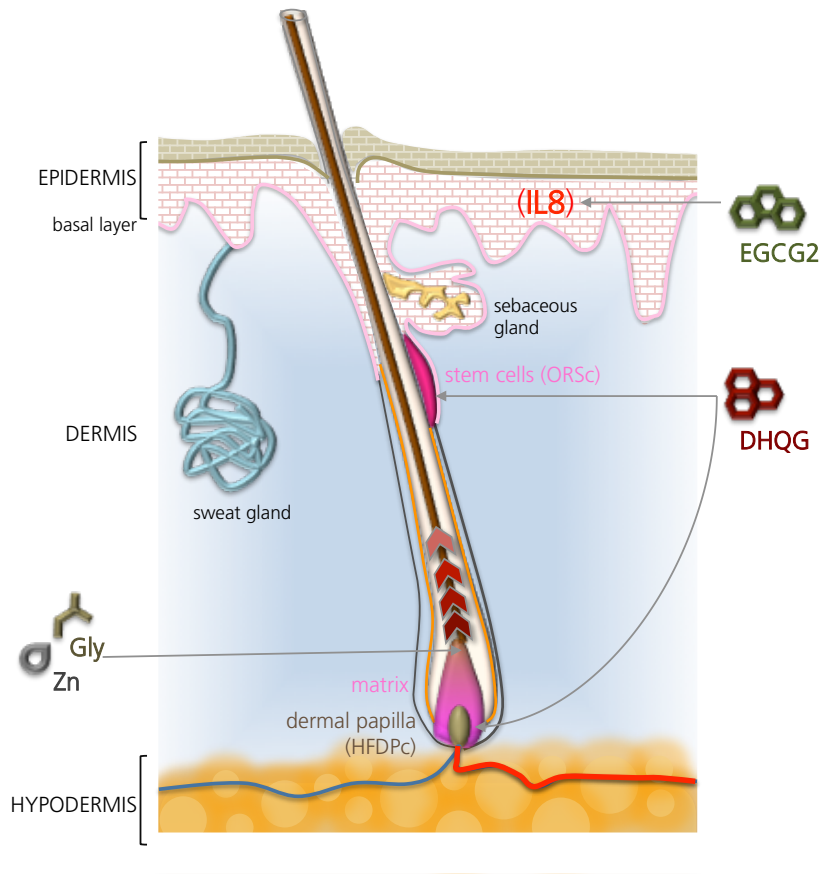


Fig 3: Summary of Redensyl® mode of action

7. Redensyl® technical description

Appearance:	Yellow liquid
Origin:	Plants and Biotechnology
Safety assessment:	Ocular irritation: Human cornea model test; Non-irritant at 100% Skin irritation: Occlusive patch test; Non-irritant at 100% Mutagenicity: Ames assay; Non-mutagenic Sensitization: HRIPT assay; Non-sensitizing at 100%
Dosage:	1-3 %
Storage:	Recommended storage temperature: 4-7°C Do not store at temperatures over: 10°C
Processing:	Can be added at the end of the formulation under stirring or homogenizing or can be heated for a short time with the oil phase of formulation. Formulate at temperature below 50°C.
Shelf life	2 years

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8. *In vitro* assessment of Redensyl[®] and its components

8.1. Introduction

In vitro assessments were performed first on DHQG alone (a major component of Redensyl[®]) to evaluate its effects on viability, proliferation and gene expression of specific cells (ORSc and/or HFDPc) involved in hair growth and initiation of a new hair cycle. EGCG2 was assessed alone on normal human keratinocytes to evaluate its anti-inflammatory property. Redensyl[®] was then tested on AGA ORSc and tested for its capability to induce beta catenin activation and anti-apoptotic effect.

8.2. Materials and methods

8.2.1. *In vitro* tests based on the study of DHQG active ingredient

8.2.1.1. Cells culture

The viability and proliferation tests were performed on human ORS cells (ORSc, Celprogen) and on human HFDP cells (HFDPc, Promocell) seeded at 10000 cells /cm². The qRT-PCR analysis was performed only on ORSc. ORSc were incubated for 24 hours and HFDPc were incubated for 48 hours with increasing amounts of DHQG at 2µM, 10µM and 50µM.

Remark: 2µM, 10µM and 50µM correspond respectively to the amounts contained in Redensyl[®] at 0.04%, 0.2% and 1%.

The following controls were used:

- β-FGF at 10 ng/mL (Invitrogen): positive control for HFDPc
- EGF (Sigma) at 10 ng/ml: positive control for ORSc
- DMSO at 10% (dimethyl sulfoxide, Sigma): negative control for both cell types.

8.2.1.2. Viability assessment

Principle: The XTT test (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) has been used to evaluate the cell viability. XTT (a yellow tetrazolium salt) is cleaved to a soluble orange formazan dye, in the mitochondria of metabolically active cells. The reduction of XTT is dependent upon the presence of NADH and NADPH systems. Then, the formazan can be measured by absorbance at 450 nm in a microplate reader. In actively proliferating cells, an increase in XTT conversion is quantified. Conversely, in cells that are undergoing apoptosis, XTT reduction decreases, reflecting the loss of cell viability.

Protocol: After treatment with the active ingredient or the reference (see §5,2,1), cells were incubated with XTT at 0.25 mg/mL for 3 hours, and then the optical density (OD) is read at 450 nm on Tecan Genios Microplate Reader. A blank was also performed using wells without cells. Each condition was performed in triplicate. Morphological observations of cells were performed under a microscope.

Data management: The results of cell viability have been expressed in percentage in comparison to untreated group.

The statistical analysis was performed using the Student's t-test with the following significant threshold:

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Significant difference at 95% if $p < 0.05^*$, at 99% if $p < 0.01^{**}$, and at 99.9% if $p < 0.001^{***}$

8.2.1.3. Proliferation assessment

Principle: The BrdU (5-bromo-2'-deoxyuridine) Cell Proliferation Assay Kit has been used to assess the cell proliferation. The proliferation test is based on the detection of BrdU incorporated into cellular DNA during cell proliferation using an anti-BrdU antibody. When cells are cultured with labeling medium that contains BrdU, this pyrimidine analog is incorporated in place of thymidine into the newly synthesized DNA of proliferating cells. After removing labeling medium, cells are fixed and the DNA is denatured with our fixing/denaturing solution. Then a BrdU mouse antibody is added to detect the incorporated BrdU (The denaturing of DNA is necessary to improve the accessibility of the incorporated BrdU to the detection antibody). Anti-mouse IgG, Horseradish peroxidase (HRP)-linked antibody is then used to recognize the bound detection antibody. Horseradish peroxidase (HRP) substrate, TMB (3,3',5,5'-Tetramethylbenzidine) is added to develop color. The magnitude of the absorbance for the developed color is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation.

Protocol: BrdU (diluted at 1/100) was incorporated in the culture media during the last 16 hours of active ingredient treatment. After staining, optical density (OD) was read at 450 nm on Tecan genios Microplate Reader.

Data management: The optical density read was corrected by using the blank value. The results of cell proliferation have been expressed in percentage in comparison to untreated group. The statistical analysis was performed using the Student's t-test with the following significant threshold:

Significant difference at 95% if $p < 0.05^*$, at 99% if $p < 0.01^{**}$, and at 99.9% if $p < 0.001^{***}$

8.2.1.4. Assessment of mRNA expression profile of ORSc treated with DHQG.

The effects of DHQG at 2 μ M (Redensyl[®] at 0.04%), 10 μ M (Redensyl[®] at 0.2%) and 50 μ M (Redensyl[®] at 1%) on ORSc gene expression were studied using RT-qPCR technology.

Protocol: At the end of the incubation, ORSc were washed in phosphate buffered saline (PBS; Life Technologies) solution and immediately frozen at -80°C until mRNA extraction. Total RNA was extracted using "TriPure Isolation Reagent" kit (Roche Applied Science). The amount and quality of RNA were evaluated using a lab-on-a-chip Bioanalyzer (Agilent technologies). Potential contaminant traces of genomic DNA were removed using the DNA-free system (Ambion by Life Technologies). The reverse-transcription of mRNA was conducted in presence of oligo (dT) and Superscript[™] II reverse-transcriptase (Life Technologies). Quantification of cDNA was performed using NanoVue Plus (GE Healthcare) and adjustment of cDNA at 5 ng/ μ l.

Extracted mRNA was analyzed on a customized PCR array containing target genes Ki-67, Proliferating cell nuclear antigen (PCNA), B-cell CLL/lymphoma 2 (BCL-2), beta catenin, Keratin 15 (KRT 15), Wingless-type MMTV integration site family, member

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10B (WNT10B), Vitamin D receptor (VDR), and BCL-2 associated X protein (BAX) and including 1 housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase). Primer sequences used were mentioned in the table 1 below:

Gene name	Abbreviation	Gene Bank	Primers forward	Primers reverse	cDNA bp
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046	GGCTCTCCAGAACATCATCCCTGC	GGGTGTCGCTGTTGAAGTCAGAGG	269
Antigen identified by monoclonal antibody Ki-67	MKI67	NM_002417	TGATAGCTTTACAAGCGCTCCAAGC	CTTGTTCCCGTACGCTTCCATC	214
Proliferating cell nuclear antigen	PCNA	NM_002592, NM_182649	CCATATTGGAGATGCTGTTGTAATTTCC	ACATACTGAGTGTACCGTTGAAGAG	233
B-cell CLL/lymphoma 2	BCL2	NM_000633	CCTGTGGATGACTGAGTACCTGAAC	GCAGGCATGTTGACTTCACTTGTG	214
Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB1	X87838, Z19054	GGCCTGTAGAGTTGCTGGAG	ACAAGCAAGGCTAGGGTTTG	229
Keratin 15	KRT15	NM_002275	GAGAACTCACTGGCCGAGAC	CTGAAGAGGCTTCCCTGATG	244
Wingless-type MMTV integration site family, member 10B	WNT10B	NM_003394	AATGCGAATCCACAACAACA	GGGTCTCGTCCACAGAAGTC	288
Vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	J03258	GAGACCTCAGCCATGAGGAG	CGTGAGTAAGGCAGGAGAGG	250
BCL2-associated X protein	BAX	NM_004324, NM_138761, NM_138763, NM_138764, NM_138765	TGCCGCCGTGGACACAGAC	GGAGTCTCACCAACCCACCTG	245

Table 1: Primer sequences

The PCRs (Polymerase Chain Reactions) were performed using the LightCycler® system (Roche Diagnostic, France). The incorporation of fluorescence in amplified DNA was continuously measured during the PCR cycles. This resulted in a “fluorescence intensity” versus “PCR cycle” plot allowing the evaluation of a relative expression (RE) value for each marker.

Data management:

The RE (relative expression) value was expressed in arbitrary units according to the formula:

$$1/2^{\text{number of cycles}} \times 10^6$$

The relative expression calculated was normalized to the housekeeping gene and to untreated cells (control).

For the interpretation of the effect the following table was used:

Relative expression (% of control)	Classification of the effect
> 300%	strong stimulation
>200% and <300%	stimulation
>30% and <50%	inhibition
< 30%	strong inhibition

Table 2: Classification of the effect of the active ingredient

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8.2.2. *In vitro* tests based on the study of EGCG2 active ingredient on normal human keratinocytes

EGCG2 was tested for its ability to reduce IL-8 release, a cytokine involved in scalp irritation[19]. An irritating skin is much willing to be losing hair. This study evaluated the assessment of anti-inflammatory properties of EGCG2 by the quantification of IL-8 release by normal human keratinocytes under cytokine treatment.

8.2.2.1. Cells culture and treatment

Keratinocytes (NHEK) seeded at 20000 cells/well, were cultured in a specific keratinocytes medium KSFM (Gibco Invitrogen) until 80% of confluence at 37°C, and 5% CO₂. After achieving 80% of confluence, keratinocytes (NHEK) were cultured for 24 hours in 96-well plates in a culture medium (KSFM added with CaCl₂ -1,7 mM) containing EGCG2 at 8µM.

Remark: EGCG2 at 8 µM correspond to the amount contained in Redensyl[®] at 1.6%. The medium was then removed and replaced for 24 hours by medium containing the EGCG2 at 8 µM and IL-1β at 1ng/ml (Interleukine-1 β: Recombinant Human IL-1b, 201-LB, R&D Systems). A non-stimulated control condition and a stimulated control condition without active ingredient were also performed in parallel. At the end of the cultured the supernatants containing IL-8 were collected and conserved at -20°C until the assay by Elisa kit. All experimental conditions were performed in triplicate.

8.2.2.2. Quantification of IL-8 released by NHEK

Protocol: At the end of incubation, the quantities of IL-8 in culture supernatants were measured using ELISA kits according to the supplier's instructions (R&D Systems D8000C). The absorbance was read at 450nm (Genios, Tecan).

Data management: Raw data were analyzed with Microsoft Excel software. All reported data are expressed as mean ± sem (pg/ml of IL-8 / µg of protein). The standard error of the mean (sem) is calculated as the standard deviation (sd) divided by the square root of sample size. Standard error of the mean: $sem = Sd/\sqrt{n}$

The inter-group comparisons were performed by Student's t-test (for paired data). The significance was judged as followed.

Significant difference at 95% if $p < 0,05^*$ and at 99% if $p < 0.01^{**}$.

A percentage of inhibition was determined by the comparison of the mean values of control condition and treated conditions:

$$\% \text{ Inhibition} = 100 - (\text{mean value of treated condition} / \text{mean value of untreated condition}) * 100$$

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8.2.3. *In vitro* tests based on the study of the Redensyl[®]

8.2.3.1. Cells culture and treatment

ORSc from 3 AGA donors (occipital hair follicles) were seeded on collagen I-coated 6 well plates (2000 x 105 cells per well) and allowed to adhere for 24-48 h. ORSc were cultured in keratinocyte serum-free medium (KSFM) (GIBCO, Paisley, UK) containing 0.25% v/v DPE (GIBCO, Paisley, UK), 0.2 ng/ml epidermal growth factor (GIBCO, Paisley, UK), 300 μ M calcium chloride (Sigma-Aldrich, Poole, UK), 100 units/ml penicillin (GE Healthcare, Little Chalfont, UK) 100 mg/ml streptomycin (GE Healthcare, Little Chalfont, UK). Once normal keratinocyte morphology was observed treatment with cell culture grade water vehicle control (GIBCO, Paisley, UK) or with the Redensyl[®] diluted at 0.001%, 0.01% and 0.1% containing respectively the following amounts of EGCG2/DHQG: 0.005/0.05 μ M, 0.05/0.5 μ M and 0.5/5 μ M.

8.2.3.2. Western blot analysis: Active β -catenin assay

ORSc were treated with the Redensyl[®] diluted or with the vehicle control for 24 h, then washed in ice cold PBS (Sigma-Aldrich, Poole, UK) and lysed using RIPA lysis buffer containing 50 mM Tris-HCl pH 7.4 (Sigma-Aldrich, Poole, UK), 150 mM NaCl (Sigma-Aldrich, Poole, UK), 0.25% Na-deoxycholate (Sigma-Aldrich, Poole, UK), 1 mM EDTA (Sigma-Aldrich, Poole, UK) protease inhibitor tablet (Roche, Lewes, UK). Lysates were centrifuged at 10,000 RPM for 10 minutes at 4°C and then the supernatant was extracted and quantified for total protein using the DC quantification kit (Biorad, Hemel Hempstead, UK). Lysates were adjusted according to their total protein concentration using RIPA buffer. Western blot analysis was carried out using the Life Technologies NuPage kit. Gels were transferred to PVDF membranes, blocked with 5% BSA (GE Healthcare, Little Chalfont, UK) and stained with 1:1000 dilution of non-phospho beta-catenin (Clone #8814 - Cell Signalling, Lieden, Netherlands) or 1:10,000 dilution of β -tubulin loading control (Clone AA2 -Millipore, Watford, UK).

8.2.3.3. Apoptosis Annexin V assay:

Apoptosis analysis was carried out using the Guava Nexin[®] reagent (Millipore, Watford, UK) in conjunction with a MUSE flow cytometer according to the manufacturers instructions. Briefly, ORSc were treated with Redensyl[®] diluted at 0.001%, 0.01% and 0.1% or vehicle control for 24 h, then trypsinised using versene (Lifetech, Paisley, UK). ORSc were counted using Chemometec Nucleocassettes (Sartorius, Epsom, UK) and centrifuged for 5 min at 900 RPM, then resuspended in DMEM media (GIBCO, Paisley, UK) containing 10% Fetal bovin serum FBS (Biosera, Uckfield, UK) to a volume of 50,000 cells per ml. Cell suspension was then mixed with an equal volume of Guava Nexin[®] reagent and incubated at room temperature in the dark for 20 minutes. Vials of cell suspension were then measured using the MUSE flow cytometry system (Millipore, Watford, UK).

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8.3. Results and discussion on *in vitro* experiments

8.3.1. Viability/metabolism assessment of HFDP cells treated with DHQG

DHQG stimulates the viability/metabolism of HFDPc (Fig 4) significantly. The improvement of HFDPc viability was 12%, 16% and 24% at respectively 2µM, 10µM, and 50µM of DHQG.

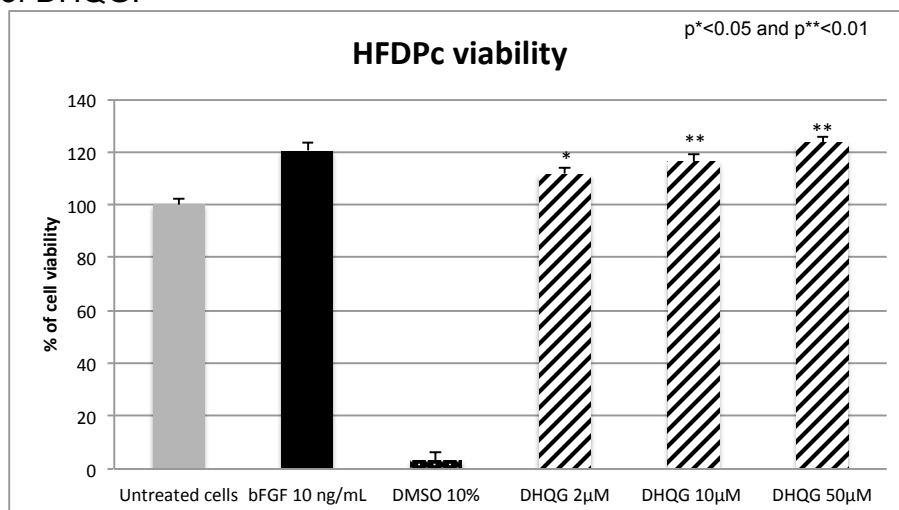


Fig 4: HFDPc viability assessment (XTT test)

8.3.2. Proliferation assessment of ORSc treated with DHQG

DHQG stimulates the proliferation of ORSc (Fig 5) significantly. The improvement of ORSc proliferation was respectively 28%, 40% and 44% at 2µM, 10µM and 50µM of DHQG.

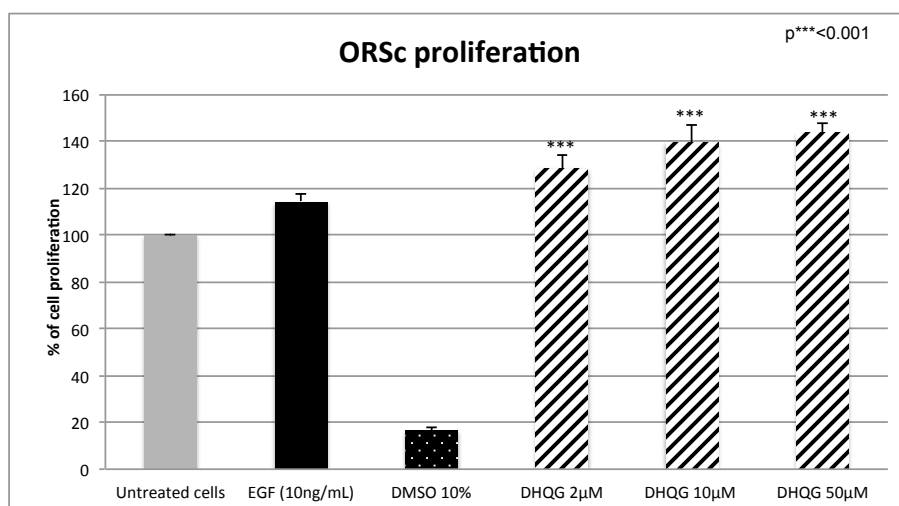


Fig 5: ORSc proliferation assessment (BrdU test)

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8.3.3. Assessment of mRNA expression profile of ORSc treated with DHQG.

Effect of DHQG treatment on gene expression related to cell proliferation:

DHQG induced the stimulation of the expression of two proliferation markers Ki67 and PCNA (Fig 6). For the proliferation marker Ki67, the strong induction (between 400% to 700%) was seen at all concentrations tested. For the proliferation marker PCNA, the induction was seen at 2 and 10 μ M.

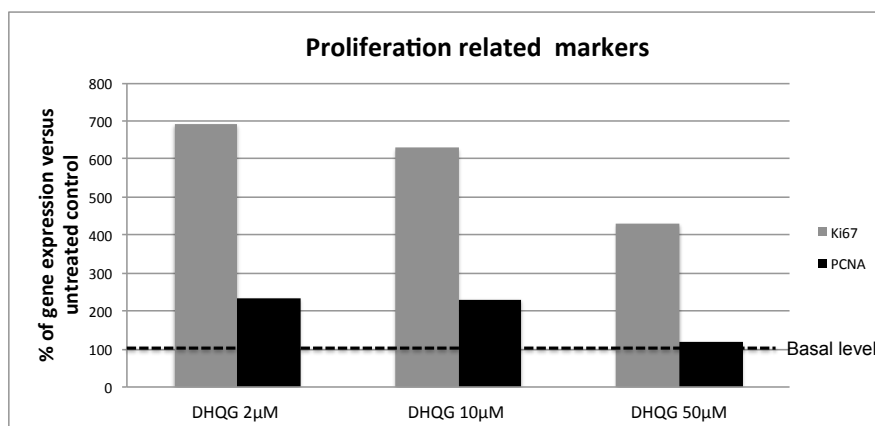


Fig 6: Percentage of gene expression of cells proliferation related markers (RT-qPCR technology)

Effect of DHQG treatment on gene expression related to cell survival functions:

DHQG induced the stimulation of the expression of the anti-apoptotic gene BCL-2 and the total inhibition of expression of the pro-apoptotic gene BAX at the 3 concentrations tested (Fig 7).

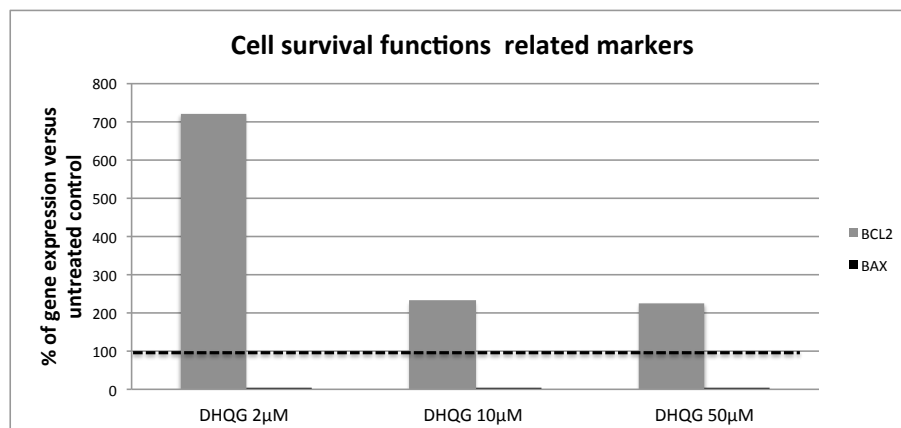


Fig 7: Percentage of gene expression of cells survival functions related markers (RT-qPCR technology)

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Effect of DHQG treatment on gene expression related to cell differentiation:
DHQG induced the stimulation of the expression of two differentiation genes involved in hair follicle morphogenesis: Wnt10B and Beta catenin. The induction of beta catenin was seen at all concentrations tested. The induction of Wnt10B is seen at 2 and 10 μ M (Fig 8).

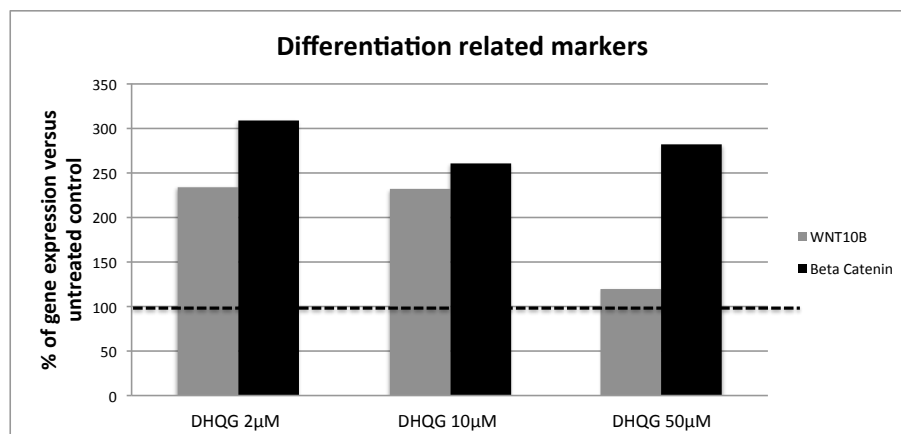


Fig 8: Percentage of gene expression of cell differentiation markers involved in hair follicle morphogenesis (RT-qPCR technology)

Effect of DHQG treatment on gene expression related to hair follicle bulge stem cells phenotype maintenance:

DHQG induced the stimulation of the expression of two stem cells markers K15 and VDR (vitamin D Receptor) (Fig 9). Stimulation of Vitamin D receptor gene expression was seen at all concentrations tested. For Keratin 15 the stimulation was seen only at 10 μ M.

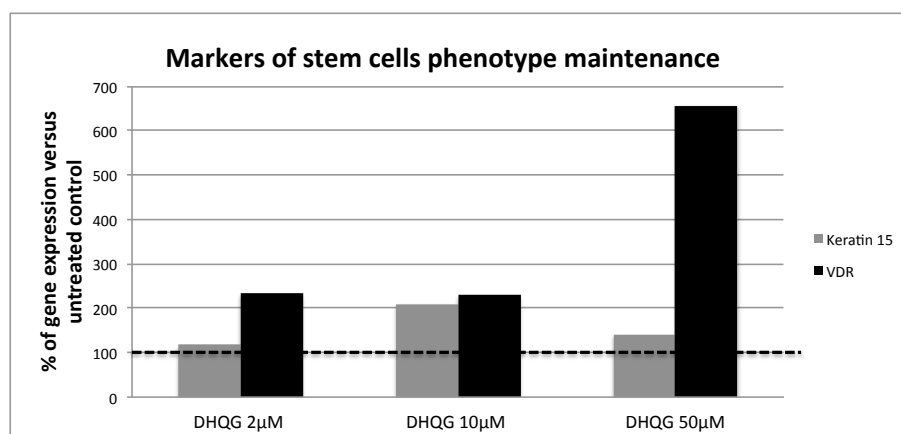


Fig 9: Percentage of gene expression of hair follicle bulge stem cells markers phenotype maintenance (RT-qPCR technology)

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8.3.4. Anti-inflammatory properties of EGCG2: IL-8 release studies

EGCG2 reduced significantly by -21% the release of IL-8 by normal human keratinocytes in inflammatory conditions (Fig 10).

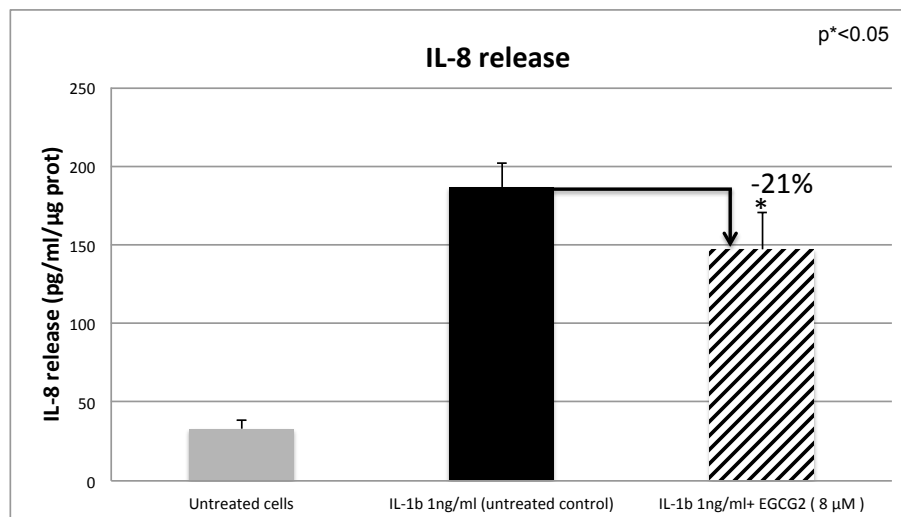


Fig 10: IL-8 release by Human keratinocytes treated by EGCG2 and cytokines (Elisa assay)

Remark: EGCG2 at 8 µM corresponds to the amount contained in Redensyl[®] at 1,6%.

8.3.5. Assessment of beta catenin activation in AGA ORSc treated by Redensyl[®]

Redensyl[®] diluted at 0.1% demonstrated a strong stimulation of activated β-catenin in ORSc (Fig 11). This data confirmed the RT-qPCR results showing a strong stimulation of β-catenin by DHQG, a major component of Redensyl (Fig 8).

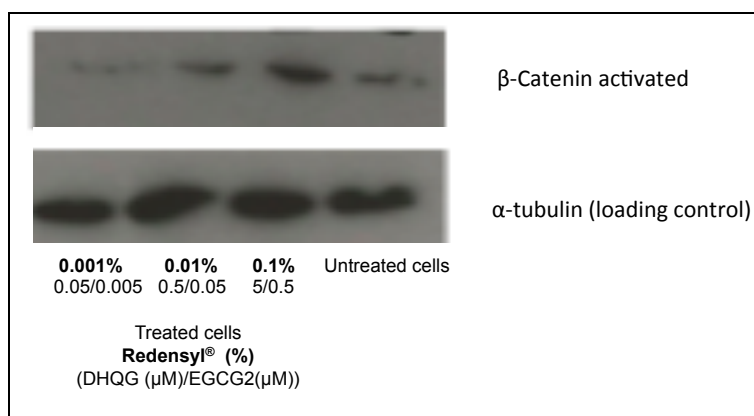


Fig 11: β-catenin activation study (Western blot). Redensyl[®] diluted at 0.1% demonstrates a strong stimulation of activated beta catenin in ORSc. Loading control was α tubulin.

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It was therefore observed that Redensyl® and its main component DHQG are able to activate β -catenin a key component of the Wnt/ β -catenin pathway. As this pathway has been shown to be inactivated in AGA hair follicle stems cells[6] our data indicate that DHQG may be a potent stimulator of these cells and that this appear to happen without the need for cross talk with HFDPc. During hair cycle morphogenesis this cross talk is crucial for the initiation of a new anagen hair follicle[14]. In fact, soluble factors from human hair papilla cells (HFDPc) induce an increase in the clonal growth of outer root sheath cells, and their differentiation into hair matrix cells[15].

8.3.6. Assessment of anti-apoptotic effect of Redensyl®

Redensyl® diluted at 0.1% demonstrated a clear anti-apoptotic effect on ORSc (Fig 12)

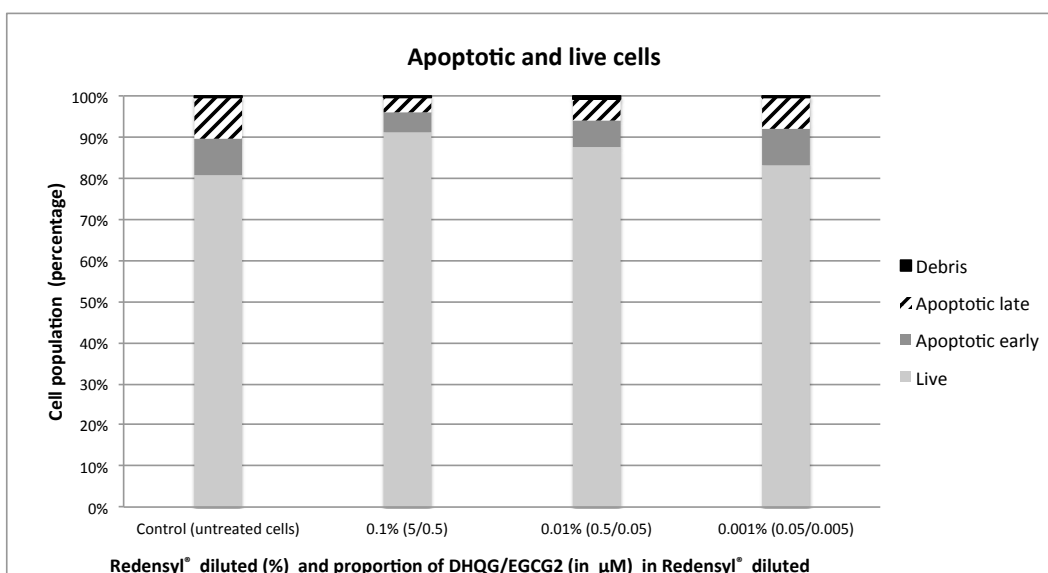


Fig 12: Proportion of apoptotic and live cells under Redensyl® treatments (Anti-apoptotic Annexin V). Less apoptotic cells were counted in Redensyl® diluted at 0.1 % (8% of apoptotic cells) and 0.01% (12% of apoptotic cells) in comparison to untreated cells (20% of apoptotic cells).

This anti-apoptotic effect observed on ORSc cell treated with the mix is most likely due to the action of DHQG molecule. Indeed in figure 7, a strong stimulation of anti-apoptotic gene BCL-2 was seen after cell treatment with 2 μ M DHQG. DHQG induced the total inhibition of expression of the pro-apoptotic gene BAX and the stimulation of the expression of the anti-apoptotic gene BCL-2 at the 3 concentrations tested (Fig 7). The anti-apoptotic effect seen with Redensyl® is also most probably due to a synergic effect of DHQG and EGCG2. Kwon *et al* [16] have shown on HFDPc, that the non-glycosylated form of EGCG2, EGCG (Epigallocatechin gallate) from 0.01 to 0.5 μ M had anti-apoptotic properties. This property was also confirmed by Park *et al*[17] on human dental pulp cells.

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In AGA pathology a premature termination of anagen is associated with premature entry into catagen an apoptotic driven process. Catagen phase of hair cycle occurs as a consequence of decreased expression of anagen maintaining factors such as the growth factors IGF-1, β -FGF and VEGF and an increased expression of cytokines (TGF β 1, IL -1 α and TNF α), which promotes apoptosis[2]. The anti-apoptotic properties of the two molecules DHQG and EGCG2 could therefore delay the entrance of hair follicles in the catagen phase of hair cycle. The anti-inflammatory properties of EGCG2 may also help to delay the entry into catagen phase.

8.4. Conclusions on *in vitro* experiments

- DHQG was able to stimulate the cellular activities of HFDPc and ORSc (two major cells involved in new hair formation).
 - Viability/metabolism of HFDPc was significantly improved from 12% to 24%
 - Proliferation of ORSc was significantly improved from 28% to 44%. ORSc proliferation was also confirmed by mRNA analysis showing a strong stimulation of the expression of proliferative markers (Ki67 and PCNA).

- DHQG was able to induce the maintenance of stem cells phenotypes of ORSc. The ORSc cells treated by DHQG expressed specific bulge stem cells markers: K15 (keratin 15) and VDR (Vitamin D receptor)

- DHQG was able to protect cells from apoptosis by the induction of specific marker of anti-apoptotic pathway (Bcl-2) and the inhibition of specific marker of pro-apoptotic pathway (Bax)

- EGCG2 anti-inflammatory properties were shown by the significant decrease of IL-8 release by keratinocytes under inflammatory induced conditions: IL-8 release was decreased by -21%

- Redensyl® was able to induce beta catenin activation at the protein level in AGA ORSc cells.

- Redensyl® was able to induce anti-apoptotic effect on AGA ORSc.

REDENSYL[®]

9. *Ex vivo* assessment of Redensyl[®]

9.1. Introduction

The aim of this *ex vivo* study was to evaluate the capability of Redensyl[®] to induce hair follicle growth of androgenic alopecia patients using Philpott model culture.

9.2. Materials and methods

9.2.1. Products tested

Redensyl[®] at 1% was assessed in comparison to the benchmark reference Minoxidil[®] at 1%.

9.2.2. Hair follicles culture and treatment

Hair follicles were extracted from occipital scalp of patients undergoing hair transplantation surgery due to androgenic alopecia. Thirty (30) follicles per donor were isolated from 2 donors. At this stage, it was not possible to identify hair follicles in anagen phase. As only hair follicles in anagen phase must be included in a growth study, the identification of hair follicle in anagen phase was performed after culture by following only the growing hair follicles. 10, 7 and 7 follicles in anagen phase were therefore respectively selected in the Redensyl[®], Minoxidil[®] and untreated group. These individual hair follicles were cultured according to the Philpott model. Each isolated follicle was immediately placed into a well of a 24-well plate containing a specific medium. This medium consisted of Williams' medium E, L-glutamine, insulin, hydrocortisone, penicillin, streptomycin, and amphotericin B. All cultures were incubated at 37°C in an atmosphere of 5% CO₂. The medium containing or not the tested products was replaced every day.

Protocol: The growth of hair follicles was examined at D7 and D10 using a digital microscope at the magnification x40. Examination of the growth of hair follicles was performed using digital microscope and image analysis software. The length in µm of hair follicles, was measured from digital images at D0, D7 and D10.

Data management: Raw data were analyzed with Microsoft Excel software. All reported data are expressed as mean ± sem (µm). The standard error of the mean (sem) is calculated as the standard deviation (sd) divided by the square root of sample size. Standard error of the mean: $sem = Sd/\sqrt{n}$

The inter-group comparisons and comparisons into a same group according to the time were performed by Student's t-test. The significance was judged as followed. Significant difference at 95% if $p < 0.05^*$ and at 99% if $p < 0.01^{**}$.

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9.3. Results and discussion on *ex vivo* assessments

Androgenetic alopecia hair follicles treated with Redensyl® at 1% grew faster than untreated hair follicle or hair follicles treated with Minoxidil® after 7 or 10 days (Fig 13).
Redensyl® treatment:

In comparison to untreated control the growth rate was increased by:

- + 75% after 7 days of treatment (significant with $p < 0.01^{**}$)
- +214% after 10 days of treatment (significant with $p < 0.01^{**}$)

Minoxidil® treatment:

In comparison to untreated control the growth rate is increased by:

- + 25% after 7 days of treatment (non significant)
- +118% after 10 days of treatment (significant with $p < 0.05^*$)

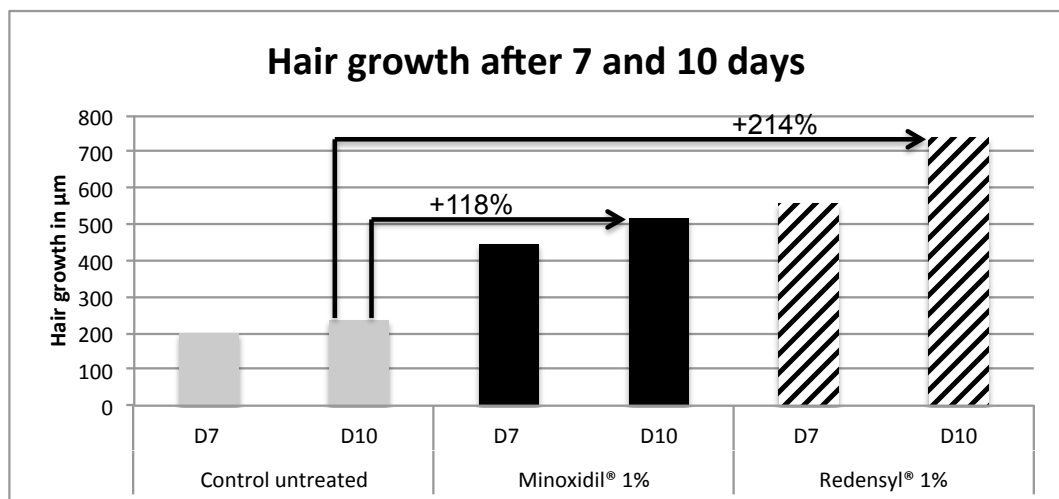


Fig 13: Androgenetic alopecia hair follicles growth studies (microscopically measurements)

9.4. Conclusions on *ex vivo* experiments

Redensyl® is able to induce significantly the growth of alopeic hair follicles known to be very difficult to stimulate.

- The rate of growth achieved is + 214% after 10 days of treatment in comparison to untreated conditions.
- Redensyl® improves the hair follicle growth at D10 in comparison to Minoxidil® by 1.8 times.

REDENSYL®

10. Clinical investigation of Redensyl®

10.1. Introduction

The purpose of the clinical investigation was to evaluate the effects of Redensyl® in a hair lotion at 3% on hair loss parameters on men suffering of androgenic alopecia after 3 months of a daily application. The following hair loss parameters were assessed:

- number of telogen and anagen hair,
- density of anagen and telogen hair,
- ratio of anagen versus telogen hair densities.

These parameters were selected as the hair loss process directly impacts them. In alopecia the percentage of hair in telogen phase increases with time, whereas the percentage of hair in anagen phase continues to decrease.

10.2. Materials and methods of clinical tests

10.2.1. Description of the lotion used

Lotion formula (INCI composition):

AQUA, ALCOHOL DENAT. BUTYLENE GLYCOL, GLYCERIN, XANTHAN GUM, DISODIUM EDTA, CITRIC ACID, (+/-) **REDENSYL® 3%**.

10.2.2. Description of the panel and study conditions

To be included in this study each volunteer had to respect the following criteria:

- Minimum of 40 telogen hairs/ cm²
- Minimum density of hair ≥150 / cm²

Based on these inclusion criteria, 26 males volunteers having a hair loss grade III to IV on the Hamilton scale amended by Norwood were included in the study (between 18 to 70 years old, Caucasian and North African). Two (2) groups were selected: one group testing the active ingredient (14 volunteers), the other testing the placebo (12 volunteers). 3.5 ml of hair lotion (placebo or active ingredient) was applied every day during 84 days on the scalp (whole head) with no rinsing after application. The clinical study was randomized and double blinded. All clinical observations were performed under the control of a dermatologist. The hair parameters were assessed before (D0) and after 1 and 3 months of hair lotion application on a shaved area of 1.5 cm² (for a window measurement corresponding to 0.7 cm²). The methods used are described in detail in the following sections.

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10.2.3. Clinical assessments methods

10.2.3.1. Phototrichograms analysis (PTG) (Efficacy criteria)

An image acquisition of the studied area was carried out at D0, D28 and D84 and 2 days later from each date at D2, D30, and D86. The image acquisition was performed using standardized and reproducible conditions (distance, light, and zoom) with a Reflex camera NIKON associated with a Canfield® Epiflash System and a contact plane (with a graduated straight edge) allowing to press hair on the scalp. Image thus obtained were then saved on computer. PTG were done on one area (1.5 cm X 1.0 cm). Hair were cut with scissors and then shaved from the root with a hair clipper. At least two photos (2.35 cm x 1.35 cm) were taken for each kinetic. For each photo, the reference photo for the position was the one done on the screening day 0.

Image analysis was carried out with specific software Photoshop CS5 extended®. The count of hair was done on a 0.7cm² area (1 cm x 0.7 cm). All hair whose root was in the defined area was counted. Hair was distinguished by their growing phase by different colors, and 3 hair categories were defined:

- Hair in anagen phase (A)
- Hair in telogen phase (T)
- Undetermined hair (I) (hair for which the growing phase was difficult to evaluate were defined as "undetermined")

The pillar formula was studied with the following formula, integrated the undetermined hair (X) in proportion to anagen and telogen hair.

- Density of hair in the anagen phase per cm² (DA),
 $DA = (((I / (A+T)) * A + A) / \text{Surface } (0.7))$,
- Density of hair in the telogen phase per cm² (DT),
 $DT = (((I / (A+T)) * T + T) / \text{Surface } (0.7))$,
- Total density: total number of hairs on the studied zone (per cm²) (DE): $DE = DA + DT$,
- Proportion of hair in the telogen phase (%T). $\%T = DT/DE*100$
- Proportion of hair in the anagen phase (%A). $\%A = DA/DE*100$
- Ratio DA/DT

10.2.3.2. Scalp pictures

Scalp pictures were taken before and after treatment.

10.2.3.3. Self evaluation of Redensyl® by the volunteers

The volunteers completed a self-evaluation questionnaire after 3 months on the following use test parameters (table 3):

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Use test parameters	Volunteer response
After 3 months of treatment, your capillary density is visibly enhanced	Agree. Rather agree. Rather disagree or Disagree
After 3 months of treatment, your hair are thicker	
After 3 months of treatment, your hair grow faster	
After 3 months of treatment, your hair are strengthened	
After 3 months of treatment, your hair loss is diminished	

Table 3: Use test parameters and possible responses

The volunteers were also asked about their satisfaction rate and their intention to purchase the hair lotion containing Redensyl[®].

10.2.3.4. Data management

Raw data were analyzed with Microsoft Excel software. All reported data are expressed as mean ± sem and absolute variations. The standard error of the mean (sem) is calculated as the standard deviation (sd) divided by the square root of sample size. Standard error of the mean: $sem = Sd/\sqrt{n}$

The intra-group comparisons according to the time were performed by Student's t-test. The significance was judged as followed. Significant difference at 95% if $p < 0,05^*$ and at 99% if $p < 0,01^{**}$.

10.3. Results and discussion

10.3.1. Phototrichograms analysis (PTG) (Efficacy criteria)

Analysis on all volunteers from both groups:

A non-significant placebo effect was observed (due probably to mechanical activation of microcirculation), with almost no more evolution after 1 month. None of the results obtained with the placebo were statistically significant.

After 3 months of treatment, Redensyl[®] at 3% significantly increased the percentage of hairs in anagen phase (by about +9%) and decreased the percentage of hairs in telogen phase (by about -17%)(Fig 14 and table 4).

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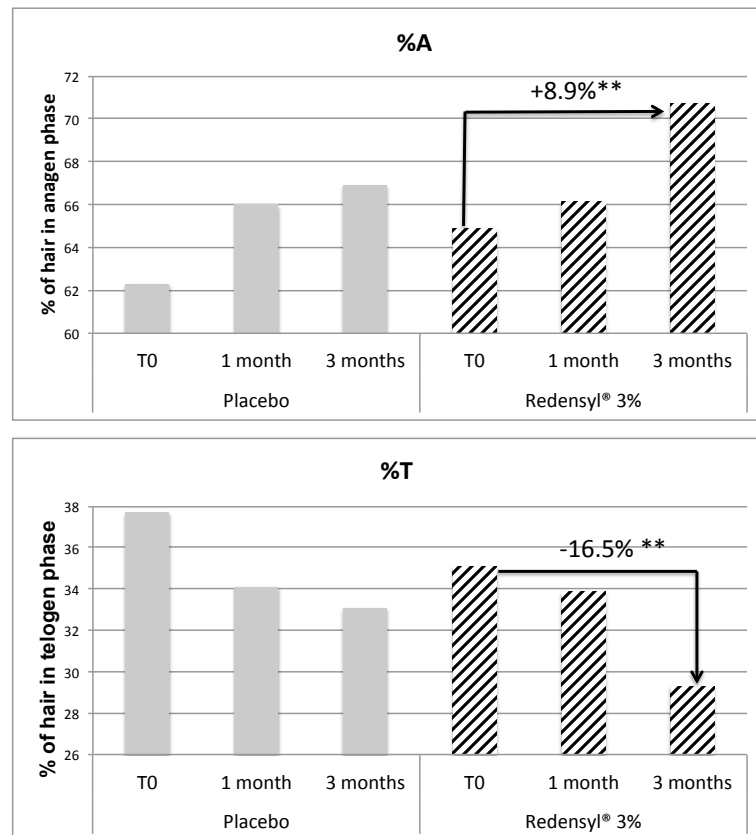


Fig 14: Percentage of hair count in anagen and in telogen phases (phototrichogram analysis)

PARAMETER	TIME	Placebo group				Redensyl® group			
		Mean	Standard deviation	Average variation (%) vs T0	Student t test (T0 vs Tx): p value	Mean	Standard deviation	Average variation (%) vs T0	Student t test (T0 vs Tx): p value
%A	T0	62.3	11.7			64.9	9.9		
	AFTER 1 MONTH	66.0	13.1	5.9%	NS	66.1	8.0	1.9%	NS
	AFTER 3 MONTHS	66.9	16.3	7.4%	NS	70.7	11.0	8.9%	** 0.0038
%T	T0	37.7	11.7			35.1	9.9		
	AFTER 1 MONTH	34.1	13.1	-9.8%	NS	33.9	8.0	-3.5%	NS
	AFTER 3 MONTHS	33.1	16.3	-12.2%	NS	29.3	11.0	-16.5%	** 0.0038

Table 4: Percentage of hair count in anagen and in telogen phases (phototrichogram analysis)

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Redensyl® increased the ratio density of hairs in anagen phase / density of hairs in telogen phase. After 3 months the ratio reached 2,37 (+29%) while the placebo showed no evolution after 1 month (Fig 15 and table 5).

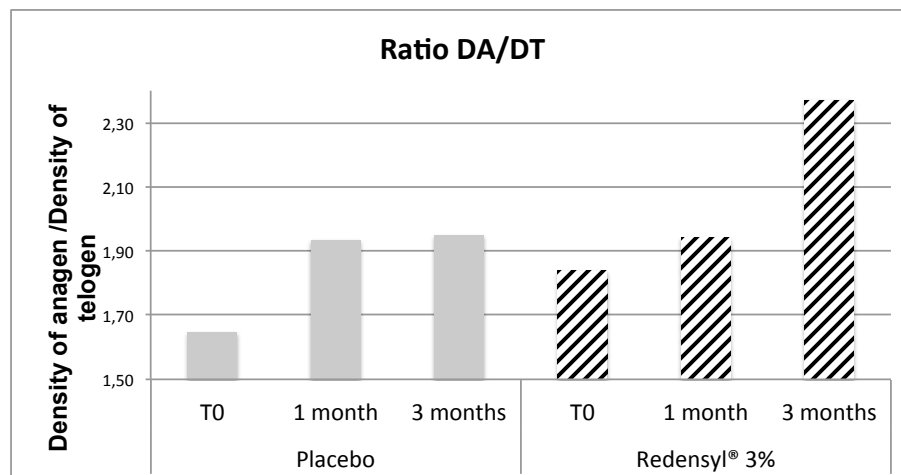


Fig 15: Ratio density of hair in anagen phase/ density of hair in telogen phase (phototrichogram analysis)

TIME	Placebo group		Redensyl® group	
	DA/DT	Average variation (%) vs T0	Mean	Average variation (%) vs T0
T0	1.64		1.84	
AFTER 1 MONTH	1.9	17.5%	1.94	5.7%
AFTER 3 MONTHS	2.00	18.5%	2.36	28.9%

Table 5: Ratio density of hairs in anagen phase/ density of hairs in telogen phase (phototrichogram analysis)

The average density of hair for all volunteers after 3 months of treatment with 3 % of Redensyl® was + 17 hair/cm² (representing +10,200 hair for a 600 cm² scalp surface). An average 10,000 new hairs were observed after 84 days of treatment, with up to 28,200 new hairs.

Analysis based on the selection of responders to Redensyl® treatment:

The responders are the people who showed hair regrowth during the treatment (increase anagen hair, decrease telogen hair and increased hair density).

In the group treated with Redensyl®, 12 out of 14 volunteers were responders to the treatment (85% of the volunteers). As a literature reference, in androgenic alopecia, the rate of responders to Minoxidil treatment at 1% during 12 months is about 17-30%[20].

After 3 months of treatment in the responders group, Redensyl® at 3% significantly increased the percentage of hair in anagen phase by about 11% and decreased the percentage of hair in telogen phase by about 20% (Fig 16 and table 6).

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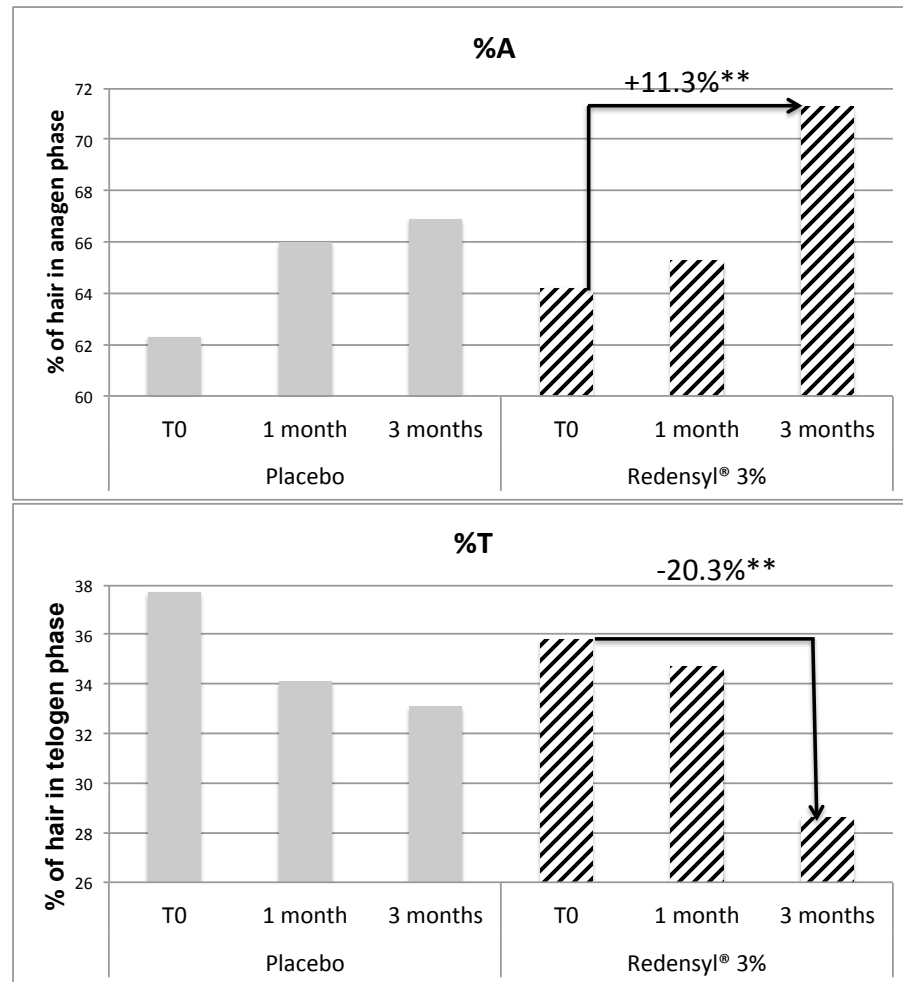


Fig 16: Percentage of hairs count in anagen and in telogen phases in the responders group (phototrichogram analysis)

PARAMETER	TIME	Placebo group				Redensyl® group			
		Mean	Standard deviation	Average variation (%) vs T0	Student t test (T0 vs Tx): p value	Mean	Standard deviation	Average variation (%) vs T0	Student t test (T0 vs Tx): p value
%A	T0	62.3	11.7			64.2	10.2		
	AFTER 1 MONTH	66.0	13.1	5.9%	NS	65.3	8.4	1.7%	NS
	AFTER 3 MONTHS	66.9	16.3	7.4%	NS	71.3	10.9	11.3%	** 0.0005
%T	T0	37.7	11.7			35.8	10.2		
	AFTER 1 MONTH	34.1	13.1	-9.8%	NS	34.7	8.4	-3.1%	NS
	AFTER 3 MONTHS	33.1	16.3	-12.2%	NS	28.6	10.9	-20.3%	** 0.0005

Table 6: Percentage of hairs count in anagen and in telogen phases in the responders group (phototrichogram analysis)

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Redensyl® at 3% increased the ratio density of hair in anagen phase / density of hair in telogen phase in the responders group strongly. After 3 months the ration reached 2.47 (+34%) while the placebo showed no evolution after 1 month. In contrast, this parameter achieved a plateau phase with placebo treatment, consistent with the end of the massage efficacy (Fig 17 and table 7).

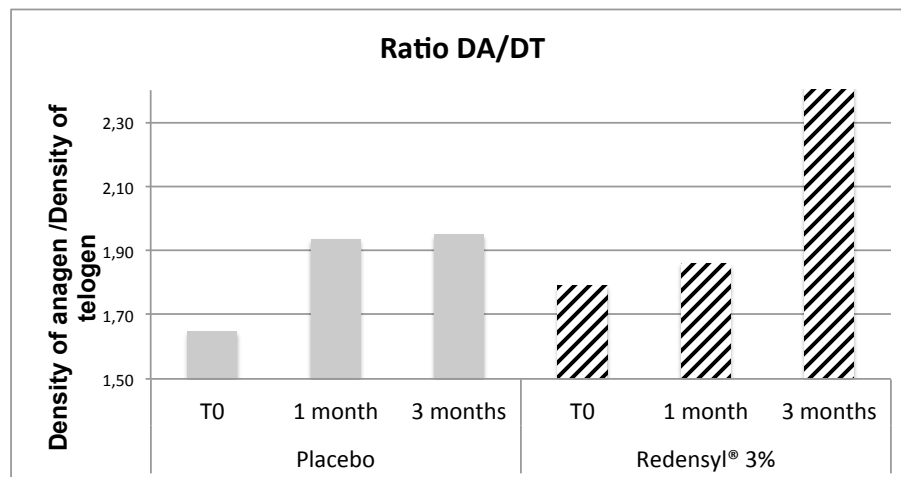


Fig 17: Ratio density of hairs in anagen phase/ density of hairs in telogen phase in the responders group (phototrichogram analysis)

TIME	Placebo group		Redensyl® responders group	
	DA/DT	Average variation (%) vs T0	Mean	Average variation (%) vs T0
T0	1.64		1.84	
AFTER 1 MONTH	1.9	17.5%	1.86	1.0%
AFTER 3 MONTHS	2.00	18.5%	2.47	34.0%

Table 7: Ratio density of hairs in anagen phase/ density of hairs in telogen phase in the responders group (phototrichogram analysis)

The average density of hair for all volunteers after 3 months of treatment with 3 % of Redensyl® was + 17 hair/cm² (representing +10,200 hair for a 600 cm² scalp surface).

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10.3.2. Clinical pictures before and after treatment

Hair loss was stopped after 3 months of Redensyl® daily treatment. A visible increase of hair density was seen after treatment (Fig 18: pictures and table 8: data calculated).



Fig 18: Scalp's pictures and phototrichogram pictures of 3 volunteers treated by Redensyl® at 3% (macrophotography)

Observations of phototrichograms showed after 3 months a thickening effect of Redensyl® treatment on hair shaft (Fig 19).

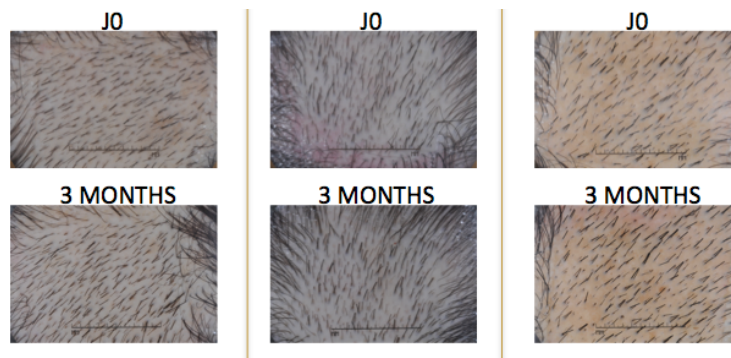


Fig 19: Phototrichograms of 3 volunteers treated by Redensyl® at 3% (macrophotography)

Examples of the clinical results of three volunteers (29 to 52 years old) treated with Redensyl® at 3% during 3 months.

Criteria	Volunteer 3 (52 years old)	Volunteer 6 (42 years old)	Volunteer 26 (29 years old)
% of new anagen hair	+10.8%	+1.2%	+9.2%
% of density of hair increase	+17%	+17%	+17%
Number of new hair/cm2	+47 hair /cm2	+43 hair /cm2	+29 hair /cm2
Total number of new hair on their scalp	+28,200 hair	+25,800 hair	+17,400 hair
Number of new hair per month on their scalp	+9,400 hair	+8,600 hair	+5,800 hair

Table 8: Some representative data seen and calculated for 3 volunteers (phototrichogram analysis)

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10.3.3. Self evaluation of Redensyl®

After 3 months of treatment with Redensyl® at 3%, the majority (71%) of the volunteers reported their satisfaction and their intention to purchase the product based on the following improvements seen (Fig 20 and 21).

- 64% of volunteers reported thicker hairs with a fast growth rate and a visibly enhanced hair density.
- 65% of the volunteers reported a hair loss diminution.
- 71% of volunteers reported to have strengthened hair.

These data suggested that Redensyl® at 3% contributed to improve the quality of hair (thickness, strength, length, growth rate).

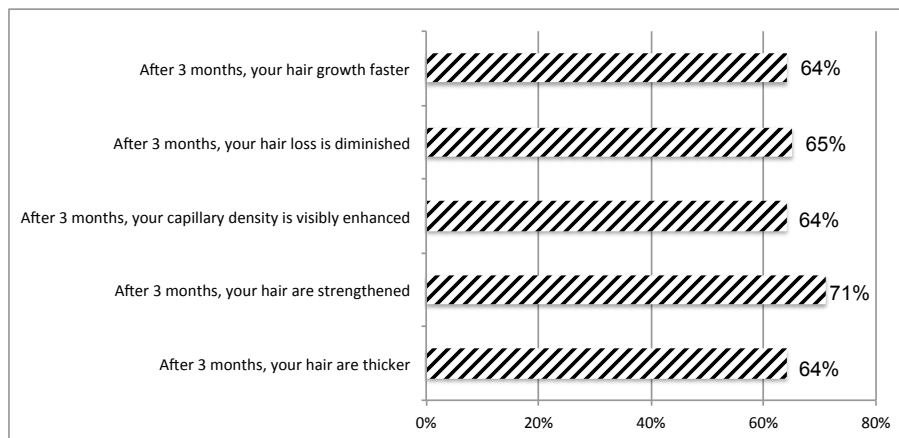


Fig 20: Self-evaluation by volunteers of Redensyl® at 3% after 3 months of use (questionnaire)

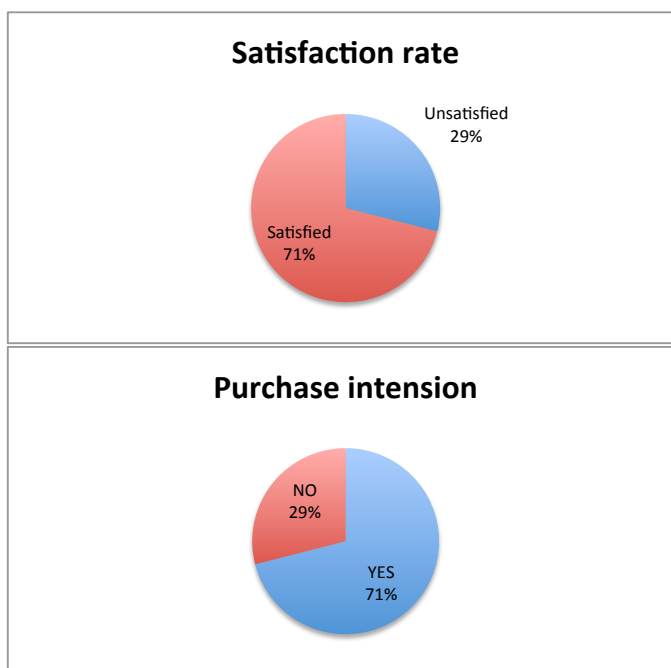


Fig 21: Satisfaction rate and purchase intension of the product Redensyl® at 3% after 3 months of use by the volunteers (questionnaire)

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10.4. Conclusions on clinical investigations

In conclusion, the results of this clinical study (increase in % of anagen hair, decrease in % of telogen hair, and increase of the ratio anagen/telogen) confirmed that treating volunteers suffering from alopecia, with 3% of Redensyl®, improved their hair growth cycle by promoting the conversion of hair follicles from the telogen phase to the anagen phase.

For all volunteers:

After 3 months of daily treatment:

- Anagen hair: +9% of increase
- Telogen hair: -17% of decrease
- Ratio anagen hair/telogen hair: +29% of increase

For the 85% of responders:

After 3 months of daily treatment:

- Anagen hair: +11% of increase
- Telogen hair: -20% of decrease
- Ratio anagen hair/telogen hair: +34% of increase

Self-evaluation of Redensyl® treatment by the volunteers after 3 months of use, confirmed the efficacy seen by phototrichograms analysis. The majority of the volunteers (71%) were satisfied and expressed their intention to purchase the hair lotion containing Redensyl® at 3%. Redensyl® at 3% improved the quality of hair (thickness, strength, length, growth rate) by increasing the number and duration of the hairs in anagen.

11. General Conclusions

Redensyl®, is a mix of four active ingredients: Dihydroquercetin-glucoside (DHQG), Epigallocatechin gallate-glucoside (EGCG2), glycine and zinc.

Glycine is essential for the hair shaft structure as it enters directly in the composition of Keratin associated protein[17]. Zinc is added to the composition to reinforce hair shaft structure. Zinc is also essential for cystin incorporation into keratin[18].

In vitro studies performed have shown the multiple properties of Dihydroquercetin-glucoside:

- Activation of hair follicle stem cells (ORSc) division (expression of proliferation markers: Ki67 and PCNA expression)
- Maintenance of ORSc stem cells properties (expression of stem cells specific markers: VDR and K15)
- Maintenance of ORSc stem cells differentiation capacities (beta catenin pathway induction)
- Protection of ORSc from apoptosis (control of BCL-2 and BAX expression)
- Boosting the metabolism of dermal papilla fibroblasts (HFDPC)

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In vitro studies performed and literature data have shown the multiple properties of Epigallocatechin gallate-glucoside derivatives:

- Anti-inflammatory properties: reduction of IL-8 release by keratinocytes under inflammatory condition
- Stimulation of proliferation of dermal papilla fibroblasts (HFDPc)[21]
- Capture of free radicals[22]
- Promotion of human hair follicle growth[21]

In vitro studies performed on androgenic alopecia ORSc have demonstrated the capability of Redensyl® to induce beta catenin activation at the protein level and preserve cell viability by reduction of apoptotic rate.

Ex vivo studies performed on androgenic alopecia hair follicle explants according to the Philpott model have shown the capacity of Redensyl® at 1% to induce their growth (+210% after 10 days of daily treatment). Results were better than with 1% of Minoxidil.

Clinical investigation has shown that Redensyl® is efficient to treat androgenic alopecia male pattern, and more generally to treat hair loss. Redensyl® is able to re-launch hair growth and to decrease hair loss with visible results in 3 months by promoting the conversion of hair follicles into the anagen phase. Redensyl® is able to make the hair stronger and thicker. An average of 10,000 new hairs were observed after 84 days of treatment, with up to 28,200 new hairs.

The efficiency of Redensyl® as alopecia hair loss treatment was also confirmed by the high user satisfaction rate (+71%) recorded during the clinical investigation.

REDENSYL[®]

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