An Active Ingredient Derived from *Picrorhiza scrophulariiflora* Roots for Prevention and Limitation of Hair Greying: What's about Self-esteem and Mood?

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abstract

Hair greying is one of the phenotypic changes during ageing, cosmetic research focusing on preventing or limiting this process. The aim of this investigation was to evaluate 1) using an *in vitro* model, the effect of *Picrorhiza scrophulariiflora* roots (*PsR*) extract versus placebo on oxidative stress and melanogenesis pathway 2) using a clinical study, the efficacy of *PsR* extract to restore white/greyed hair to its natural color. Two parallel groups of 44 volunteers (29 females and 15 men) with 20% to 50% of white hair (Mean age: 49 years) either applied *PsR* extract 1% or placebo on hair and scalp twice a day during 154 days. Evaluation of *PsR* extract efficacy was analyzed after 77 days and 154 days by 1) a measure of the density (number·cm^{-2}) of white hair 2) a self-assessment questionnaire. Self-esteem and emotion were investigated using the Rosenberg Self-Esteem Scale and the Brief Mood Introspection Scale. *In vitro* results showed that *PsR* extract increases significantly melanin synthesis, enhances the expression of antioxidant genes and reduces oxidative stress. *PsR* extract treatment restores the natural hair color (measuring by grey hair density), increases significantly self-esteem and modulates emotional valence, by significantly decreasing unpleasant emotions.

Introduction

The appearance of hair plays an important role in people's overall physical appearance and self-perception. With today's increasing life expectation, the desire to look youthful plays a bigger role than ever [1]. The discovery of pharmacological targets and the development of safe and effective drugs indicate strategies of the drug industry for maintenance of healthy and beautiful hair, specifically when hair age. Hair aging comprises weathering of the hair shaft and aging of the hair follicle. The latter manifests as greying and androgenetic and senescent alopecia. Hair greying (or canitie) is understood as a progressive loss of pigment from the growing hair shaft. According to the 50-50-50 rule about 50% of the population experiences about 50% of grey hair at the age of 50 years [2]. Several hypotheses have been put forward to explain the biological process of hair follicle pigmentation and its senescence. The decrease of hair pigmentation is always associated to a decrease in functional melanocytes number and melanocytes activity in the hair follicle matrix [3].

Besides, in grey hair follicles, melanocytes are frequently highly vacuolated which is a common mark of increased cellular oxidative stress and associated with cellular loss of function [4]. This oxidative stress is generated by endogenous and environmental processes, including UV exposure, inflammation and even emotional stress. Reactive Oxygen Species (ROS) damage biomolecules and induce mutations in mitochondrial and nuclear DNA. In melanocytes, in addition to chronological aging and (to a lesser extent) UV-induced damage, ROS are also generated during melanogenesis, increasing the level of oxidative stress. Catalase is the key enzyme responsible for hydrogen peroxide degradation in melanocytes. *In vitro*, the levels of catalase expression and activity correlate with the total cellular melanin content, and *ex vivo* they correlate with skin color. *Kauser et al.* [5] showed that whereas superoxide dismustase (SOD) expression levels did not markedly change with age in both matched cell populations, the expression and activity levels of catalase were reduced to higher degrees in older follicular melanocytes vs. epidermal melanocytes. *Schallreuter et al.* [6] also provided detailed documentation on the deregulation of the innate antioxidant system of grey hair bulbar melanocytes and the subsequent accumulation of hydrogen peroxide at millimolar concentrations in the grey and white hair shafts . Interestingly, a significant reduction in catalase expression, in the expression of methionine sulfoxide reductases and in the functional repair activity was demonstrated throughout the entire hair follicles, not only in the melanocytes. The ROS-induced greying concept therefore was expanded by these studies to include an oxidative insult to the whole affected follicle, not only to its bulbar melanocytes. The repigmenting potential of grey hair, combined with the vitiligo-pseudocatalase experience of repigmenting eyelashes and the new findings of catalase depletion in grey hair follicles, led to the development of numerous 'anti-greying' strategies and products. The dietary consumption of catalase-rich foods such as spinach and avocado were also suggested as a natural solution for reducing hair greying, and catalase-based nutritional supplements, containing both pure catalase and tyrosine, or comprising plant extracts that claim to boost catalase activity, are now commercially available.

Despite a constant advance in understanding the mechanisms underlying the development of canities, the increased awareness and demand for treatment, solutions remain limited. Nevertheless, as hair color is socially important, numerous strategies have been developed to hide, prevent or reverse hair greying. Natural ingredients such as coconut oil, curry leaves, amaranth and gooseberry, as well as amino acids and nutrients are used in certain traditions and societies with claimed effectiveness. Nutritional supplements including various vitamins or minerals such as biotin calcium panthenate, zinc, copper and selenium are also prescribed, but their efficacy remains questionable [7]. More recently, melanocyte grafting used in repigmentation of vitiligo areas has been suggested to repigment senescence grey hair [8]. Efficient molecules used for treating vitiligo could be also transposed to limit underlying mechanisms of human canities. Indeed, hair greying could be paralleled to vitiligo, a disease of depigmented skin lesions [9]. Since ancient time, herbal products of different nature and effects had been used for the treatment of vitiligo. Ayurvedic medicine had also tried to treat vitiligo with herbal products, such as the genus *Picrorhiza* [10]. Several iridoid glucosides have been isolated from *Picrorhiza* species (such as *Picrorhiza scrophulariiflora* roots – *PsR*), we can particularly cite Picroside II, which has an anti-apoptotic effect following different cellular injuries by increasing the anti-apoptotic BCL-2 protein. This anti-apoptotic activity has been associated with a wide range of pharmacological effects, including neuroprotective, hepatoprotective, anti-apoptosis, anti-inflammatory effects [11]. Antioxidant properties have also been described [12].

The aim of this investigation was to evaluate 1) using an *in vitro* model, the effect of *Picrorhiza scrophulariiflora* roots (*PsR*) extract (trade name: Arcolys®) versus control on oxidative stress and melanogenesis pathway 2) using a clinical study, the efficacy of *PsR* extract to restore white/greyed hair to its natural color.

Material and Methods

Preparation of Extract

The dried roots of *Picrorhiza scrophulariiflora* were collected in the Sichuan Province of China, where they were cultivated at an altitude ranging from 2600 to 3200 m. They were harvested in September, after 3 to 4 years of development. Dried cut roots of *P. scrophulariiflora* were extracted by 50% alcohol at room temperature over 12 hours. After 10 µm clarification, the extracted solution was concentrated, under vacuum at 55°C, up to 5% of dry matter in propan-1,3-diol/water 50/50. The decontamination was realized by filtration under 2 µm. *PsR* extract contains a minimum of 4.0% per dry matter of picroside II.

In vitro Test

In order to highlight biological activities of *PsR* extract, we used several cellular models (human primary dermal cells from melanocytes, dermal papilla cells and keratinocytes).
Firstly, the potential of *PsR* extract to limit accumulation of ROS in human follicle dermal papilla cells (HDPC) was evaluated. For that, HDPC were exposed to H_2O_2-induced oxidative stress (250µM H_2O_2 during 30 minutes) and the ability of *PsR* extract (tested at 0.1 and 0.2% by 24h pretreatment before oxidative stress) to counteract the production of reactive oxygen species (ROS) was tested. The intracellular ROS reacted with the fluorogenic probe, resulting in a fluorometric product. The fluorescence emission intensity was measured (λex = 485nm / λem = 538nm) using an EnVision® microplate reader (PerkinElmer). ROS production was expressed in fluorescence units. Relative quantification was calculated and compared to the control with H_2O_2-induced oxidative stress (100%).
Secondly, we focused on the antioxidative activity of *PsR* extract. For that, we studied the effect of *PsR* extract on the expression of actors involved in antioxidative cellular response in melanocyte cells: Nuclear factor erythroid 2-like 2 (NRF2 or NFE2L2), Heme oxygenase 1 (HMOX-1) and the cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11). The up-regulation of these markers has been involved in response to cellular oxidative stress. In this experiment, the effect of *PsR* extract on gene expression of these antioxidant actors was investigated under basal conditions and under H_2O_2-induced oxidative stress. Gene expression analysis was performed by RT-qPCR on RNA samples from melanocytes culture. Variations of mRNA expression are expressed as percentage of control under basal condition. Each experimental condition was performed in triplicates that were pooled before the RNA extraction. Therefore, results represent the mean expression of 3 samples per condition.
Thirdly, the effect on *PsR* extract on tyrosinase activity was evaluated. The enzymatic activity of the various cell extracts was evaluated by measuring the optical density (OD) at 540 nm (Versamax microplate reader, Molecular Devices) and using a standard range of tyrosinase fungus (0.39 to 400 $U \cdot mL^{-1}$).
Finally, melanin production was evaluated in a coculture model of normal human epidermal melanocytes (NHEM) and normal human epidermal keratinocytes (NHEK) under basal and oxidative stress conditions and the stimulating effect of *PsR* extract was evaluated.

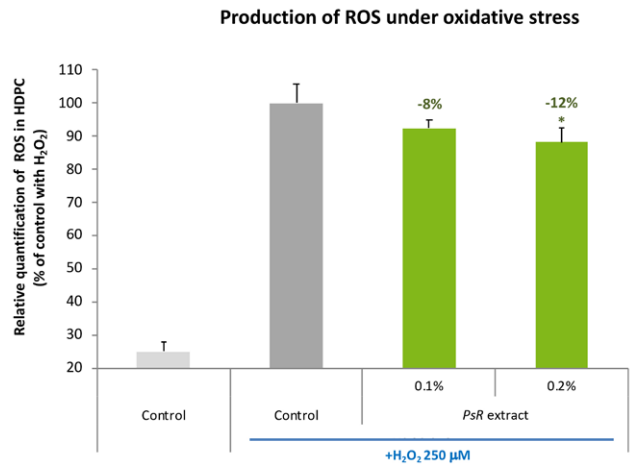

Fig. 1 ROS production in follicle dermal papilla cells after 24-hour treatments with *PsR* extract under oxidative stress conditions (H_2O_2 stress) at different concentrations.
Means ± SD are presented. *p<0.05.

In vivo Clinical Study

Two parallel groups of 44 volunteers (29 females and 15 men) with 20% to 50% of white hair (Mean age: 49 years) either applied *PsR* extract 1% or placebo on hair and scalp twice a day during 154 days (5 months). Evaluation of *PsR* extract efficacy was analyzed after 77 days (2.5 months) and 154 days (5 months) by 1) a measure of the density (number·cm^{-2}) of white hair 2) a self-assessment questionnaire. Before and at the end of the study a psychobiological evaluation of volunteers was performed. For that, individual self-esteem was investigated using the Rosenberg Self-Esteem Scale (RSES) [13]. Mood was evaluated by the Brief Mood Introspection Scale (BMIS) [14]. For this investigation, we only focused on the pleasant-unpleasant mood.

Results

In vitro Results

PsR Extract Limits the ROS Production in Dermal Papilla Cells

H_2O_2 treatment of dermal papilla cells induced an increase in ROS production (**Fig. 1**). A pre-treatment of 24 hours with *PsR* extract at 0.2% before oxidative stress conducted to a significant lower production of ROS compared to the H_2O_2 control ($p<0.05$).

PsR Extract Enhances the Expression of Antioxidant Genes in Melanocytes

Under basal conditions, 48 hours of *PsR* extract treatment at 0.1% and 0.2% applied to melanocyte cells increased *NRF2*

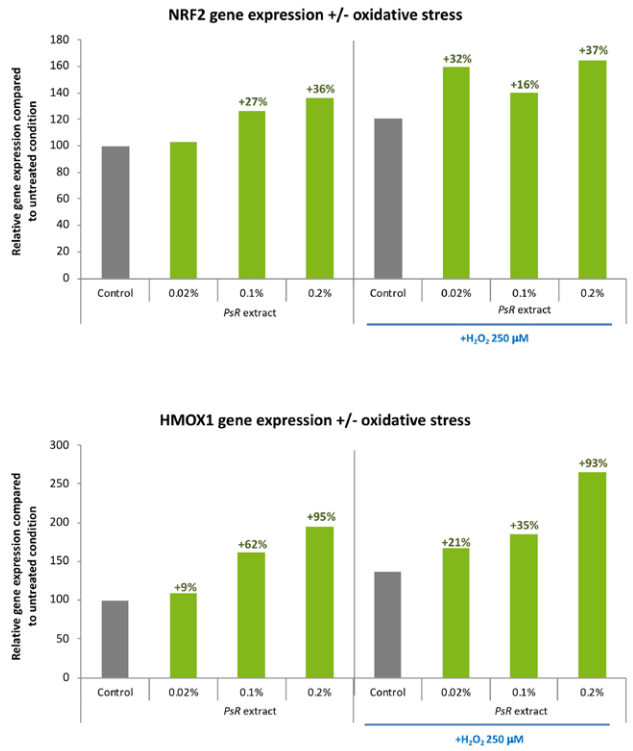

Fig. 2 **a)** NRF2 and **b)** HMOX-1 gene expression in melanocytes after 48-hour treatments with *PsR* extract under basal and oxidative stress conditions (H_2O_2 stress) at different concentrations.

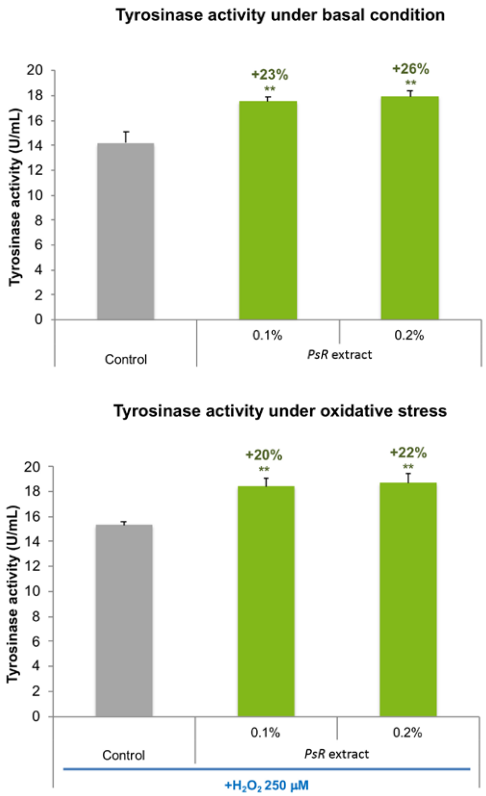

Fig. 3 Tyrosinase activity in melanocytes under basal condition after 72-hour treatments with *PsR* extract at different concentrations. Means ± SD are presented. **p<0.01

gene expression (+27% and +36%, respectively) compared to the non-treated control (**Fig. 2a**). An increase of *HMOX-1* gene expression was also noted (**Fig. 2b**). Under oxidative stress, *NRF2* expression was increased by 32%, 16% and 37% respectively at 0.02%, 0.1% and 0.2% of *PsR* extract compared to the H_2O_2 control. Increase by 21%, 35% and 93% at 0.02%, 0.1% and 0.2% of *PsR* extract, respectively were also noted for *HMOX-1* gene expression.

Finally, under basal and oxidative conditions, 48 hours of *PsR* extract treatment increased *SLC7A11* gene expression compared to the non-treated control.

PsR Extract Stimulates the Gene Expression and Activity of Tyrosinase and Enhances Melanogenesis

Under basal conditions, 48 hours of *PsR* extract treatment at 0.1% and 0.2% applied to melanocyte cells increased tyrosinase (*TYR*) gene expression (+67% and +87%, respectively) compared to the control. Under oxidative stress, an increase of *TYR* gene expression of +11%, +33% and +52% at 0.02%, 0.1% and 0.2% of *PsR* extract, respectively was also noted compared to the H_2O_2 control.

As shown in **Fig. 3**, even after a H_2O_2-induced oxidative stress, pre-treatment of melanocytes during 72 hours with *PsR* extract at 0.1% and 0.2% significantly increased tyrosinase activity as compared to the control ($p<0.01$).

Finally, under basal condition and oxidative stress, *PsR* extract enhances melanogenesis and increases melanin production, inducing pigmentation (**Fig. 4**).

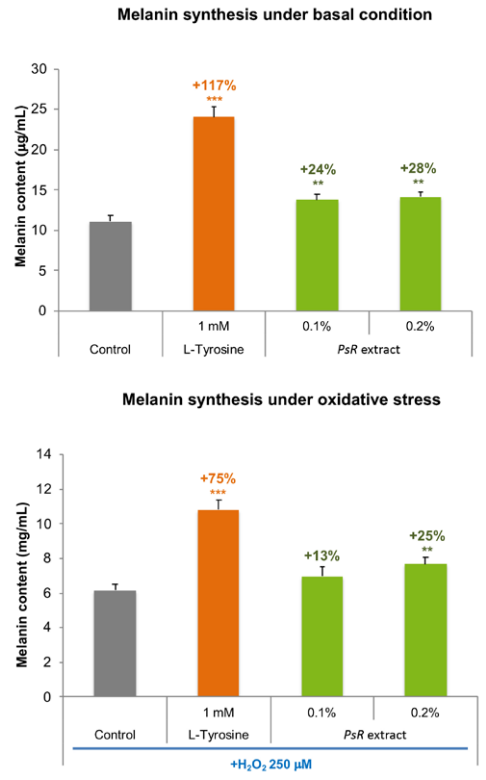

Fig. 4 Melanin content in melanocyte and keratinocyte cocultures after 10-day treatments with *PsR* extract at different concentrations and with repeated H_2O_2-induced oxidative stress. Means ± SD are presented. **p<0.01, ***p<0.001

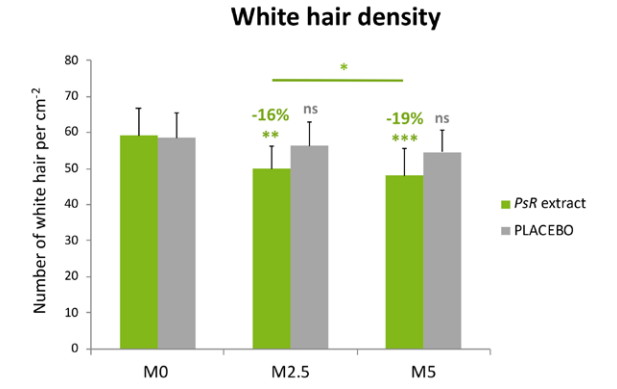

Fig. 5 Evolution of number of white hair per cm^{-2} during the experimentation (Mean ± SD). **p<0.01, ***p<0.001
ns: not significant vs M0, #p<0.05 vs M2.5.

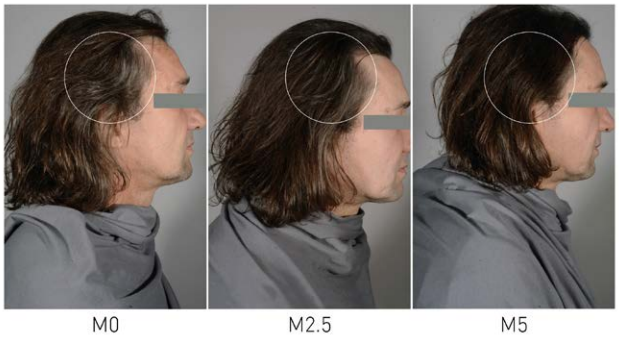

Fig. 6 Visible effects of *PsR* extract.

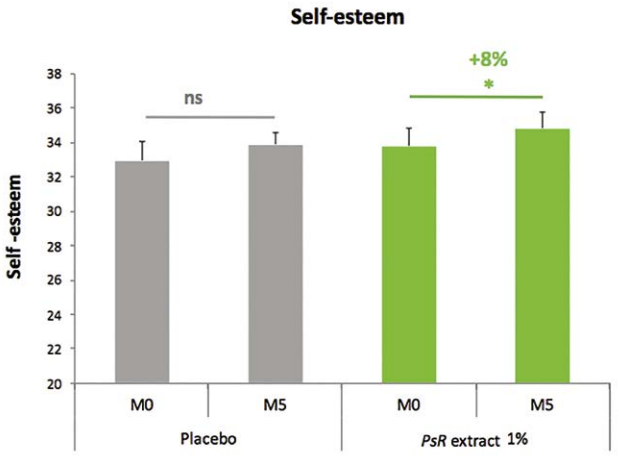

Fig. 7 Evolution of the Self-esteem score during the experimentation (Mean ± SD): *p<0.05 ns: not significant vs M0.

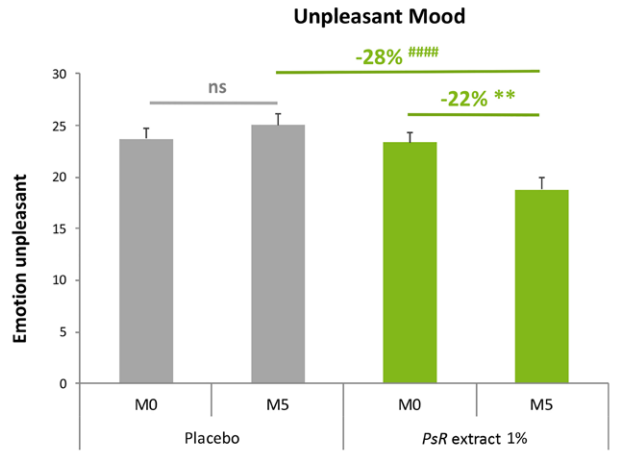

Fig. 8 Evolution of the Unpleasant mood score during the experimentation (Mean ± SD). **p <0.01, ns: not significant vs M0, ####p<0.0001 vs placebo

In vivo results

PsR Extract Restores the Natural Hair Color

At baseline, no difference in the proportion of white hair was observed between groups (**Fig. 5**). *PsR* extract induces a significant decrease in the white hair density through the experimentation.

The efficacy of *PsR* extract is illustrated in these photos (**Fig. 6**). Moreover, 80% of the women reported a good effect of the *PsR* extract on their hair repigmentation.

Psychological Measures

Self-esteem was evaluated at M0 and M5 using the Rosenberg scale. An improvement of self-esteem can be observed at M5, as shown in **Fig. 7**.

Emotional valence is a dimension continuum of pleasant and unpleasant emotions, which can be measured using the Brief Mood Introspection Scale [14]. **Fig. 8** shows the efficacy of *PsR* extract on this parameter, a decrease in unpleasant emotion being noted during the hair treatment.

Discussion

Greying of hair or canitie is an inevitable phenomenon that occurs commonly with age. Theories for the gradual loss of pigmentation include exhaustion of enzymes involved in melanogenesis, impaired DNA repair, loss of telomerase, antioxidant mechanisms, and antiapoptotic signals. By analogy with the free radical theory of ageing, a 'free radical theory of greying' has also been proposed [15]. Besides, having healthy hair is a sign of health, youth and vitality. As hair greying is perceived as a sign of progressing old age, hair greying can affect self-esteem and confidence [7], thus can induce alteration of positive emotion.

Considering the major role of oxidative stress in the occurrence of grey hair, we developed an active ingredient from *Picrorhiza scrophulariiflora* roots (*PsR* extract), titrated in Picroside II, used in Ayurvedic medicine for the treatment of vitiligo.

Our *in vitro* and *in vivo* investigations revealed a great number of biological activities of this *PsR* extract, which contribute to the process of hair pigmentation. Indeed, the results of *in vitro* studies showed its capacity to increase significantly melanin synthesis in a co-culture model of normal human epidermal melanocytes (NHEM) and normal human epidermal keratinocytes (NHEK) under basal and oxidative stress condi-

tions. The physiological hair greying that occurs with ageing is also known to result, in part, from accumulation of oxidative damage generated during normal metabolism [16]. It damages cellular structures via the formation of ROS such as H_2O_2 and superoxide. Moreover, endogenous oxidative stress is high in hair follicles and it is important to note that melanogenesis produces itself a lot of ROS via the hydroxylation of tyrosine and the oxidation of DOPA to melanin. Growing number of data provided the clear evidence of primordial role of antioxidant deficiencies and consequent hydrogen peroxide accumulation in the phenomenon of hair greying. Our data show that *PsR* extract had enhanced the expression of antioxidant genes in melanocytes, consolidating its potential to reduce the incidence and the severity of hair greying.

The relationship between hair and self-image is universal, transcending race, culture and socioeconomic standing. Emotional stress and anxiety have been noted in line with the occurrence of hair greying [17]. Moreover, it has been shown that anxiety and depression may play roles in the etiopathogenesis of hair greying by increasing exogenous oxidative stress. To decrease the high emotional stress and anxiety may be useful for prevention and treatment of hair greying [17]. Our clinical study shows that *PsR* extract treatment restores the natural hair color (measuring by grey hair density), increases significantly self-esteem and modulate emotional valence, by significantly decreasing unpleasant emotions, using the Brief Mood Introspection Scale [14].

Conclusion

Hair greying is one of the phenotypic changes during ageing, cosmetic research focusing on preventing or limiting this process. Our data show that the natural active ingredient we propose, titrated in picroside II, has high potential cosmetic applications, biological efficacy and clinical study results supporting this claim. is an active ingredient and constitutes a new natural solution for hair greying. By acting on hair pigmentation, the hair regains health and beauty. Self-esteem and positive emotions are found again.

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