

In Vitro Evaluations for a New Topical Anti-Aging Formulation

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Abstract

The study aimed to evaluate the *in vitro* properties of a new gel formulation (P-3086) as anti-aging treatment. Two *in vitro* methods aimed to assess and compare the efficacy of the gel formulation in reducing oxidative damages, artificially induced by UVA on skin-derived keratinocytes and in promoting the synthesis of collagen, compared with other four formulations. P-3086 reduced reactive oxygen species production after UVA stress with the highest effect observed at 0.016 mg/ml and 0.031 mg/ml concentration. P-3086 also promoted the collagen synthesis faster when compared with other formulations. The new gel product, based on hyaluronic acid, Vitamin E and *Humulus lupulus*, showed a good efficacy as anti-aging effect reducing the oxidative damages derived by the action of ROS, moreover stimulating the synthesis of one of the components of the connective tissue, the collagen.

Keywords

Skin, Wrinkles, Anti-Aging, Collagen Synthesis, Anti-Oxidant

1. Introduction

The desire for a long and healthy life is not an invention of modern times. In fact, the dream of eternal youth has accompanied humans since ancient time and in all cultures: from the Byzantine Empress Zoe Porphyrogenita, that tried to keep her youth appearance until a prolonged age, applying on herself cosmetic essences and fragrances made in her personal laboratory in the imperial palace [1] to Indian traditional medicine and the ayurvedic perspectives on theories and management of aging [2]. All of them tried to seek a remedy to rejuvenation. Even today, the demand for an anti-aging therapy is higher day by day. People want to look younger because a youthful appearance reflects also in a physical and mental health until very old age.

The skin aging is mainly genetically determined but also clinically associated with an increased fragility and

loss of elasticity [3]; in addition, oxidative stress is an important contributor to the pathophysiology of various pathological conditions [4]. In fact, reactive oxygen species (ROS) are well-known as a cause of aging. Oxidative stress is one of the main causes of aging process as it is involved in the development of many serious human diseases. Intracellular formation of free radicals can occur by environmental sources, including ultraviolet light, ionizing radiation, and pollutants such as paraquat and ozone [5]; furthermore, diet, drugs intake or pathologies can increase the level of oxidant activity in the skin cells or decrease the effectiveness of endogenous antioxidant systems. Following exposure to oxidative stress, ROS damages the cells membrane and DNA, leading to cell transformation or even death [6].

The skin undergoes aging, genetic determinism and environmental factors, therefore it has to deal with uncounted changes on its structure. The outer layer of skin is the epidermis, made mainly by keratinocytes responsible for the formation of a barrier against environmental damages; the inner one, dermis, consists of connective tissue and structural components, such as collagen (responsible for the skin firmness), elastic fibres (responsible of the skin elasticity) and extracellular matrix (structural component). The skin ageing is characterized by an alteration of structural components of the connective tissue with the consequent formation of skin wrinkles, consisting in a disorganization or damage of these skin structures due to a lack of collagen or to its modification, thinning and/or fractioning and the stretching and repeated extension of some areas of the skin, especially the face [7]. Thus, at the level of epidermis, wrinkles appear as fold, deep lines, ridge or crease of the skin.

A lot of treatments are available for aging wrinkles: pharmaceutical, surgical and cosmetic solutions, which aim to change the nature of aging collagen, stretching the skin, filling in the depressions of the skin or paralyzing the muscles that cause the wrinkle. Retinoids, for example, decrease cohesiveness of follicular epithelial cells, stimulating mitotic activity and increasing the turnover of follicular epithelial cells [8], but they may produce redness, burning and general discomfort. Botulinum toxin treats wrinkles by immobilizing the muscles which cause wrinkles, but it is not indicated for all kind of wrinkles as it is indicated for the treatment of glabellar lines in adults; furthermore, it has high costs. In surgery, there are some techniques that could provide an excellent improvement acting as smoothers, but also produce significant side effects, including scarring and permanent changes in skin color. Or in other cases, the improvements last some months, but then they must be repeated. Moisturizers can make wrinkles look less prominent, keeping the skin hydrated, but their action is temporary. Hyaluronic acid promotes collagen synthesis, repairing and hydrating tissues, and preventing in this way the wrinkle formation. Antioxidants provide a sun protection, neutralizing the ROS. In this way, they could act preventing further worsening of wrinkle formation.

In this scenario, the best product to treat and prevent wrinkle formation should act in the sense of regenerating tissues, mostly collagen and opposing to oxidation. In front of these considerations, a new gel formulation, P-3086, based on hyaluronic acid, Vitamin E (for its antioxidant defence of the skin) and *Humulus lupulus*, has been developed for the treatment and prevention of wrinkle formation.

Humulus lupulus contains myrcene, humulene, xanthohumol, myrcenol, linalool, tannins, 8-prenylnaringenin and resin. Use of lupulus to relieve signs of skin ageing and to lead wrinkles be less evident or disappear has been disclosed in a paper where one of the flavonoids isolated from hop plant resulted efficacious in improving skin structure and firmness [9]. Lupulus is also employed mainly for its soothing, sedative, tonic and calming effect on the body and the mind [10].

P-3086 has been tested in the present study in order to evaluate its *in vitro* properties in the collagen synthesis and antioxidant activity, comparing our test product with other formulations of P-3086 where one or more of the elements have been deprived

2. Materials and Methods

2.1. Determination of Antioxidant Activity

In vitro method was used to evaluate anti-oxidant, regenerating and anti-age properties of cosmetic products. In the present study, we investigated the effectiveness of P-3086 to counteract oxidative damages, artificially induced by UVA on skin-derived keratinocytes, comparing the result with that of other formulations. The general compositions of the test products were as follows:

P-3086: *Humulus lupulus* (1%), hyaluronic acid (0.05%), ethanol (5%) and Vitamin E (0.02%);

Test Product 2: ethanol and Vitamin E;

Test Product 3: *Humulus lupulus*, ethanol and Vitamin E;

Test Product 4: hyaluronic acid, ethanol and Vitamin E;

Test Product 5: *Humulus lupulus*, hyaluronic acid and Vitamin E;

Reference standard (for ROS investigation): Vitamin C.

The concentration of the actives in the different test products reflects those of P-3086, taking into account that the excipients content is unchanged in the different test products.

The cells were exposed to a photo induced oxidative stress (UVA) with and without the tested products at different percentages, with the aim to evaluate their ability to neutralize the oxidative stress-induced damages.

We investigated ROS formation and their inhibition as a direct indicator of the anti-scavenger activity. Human primary keratinocytes come from paediatric foreskins, after ethic committee's permission, from pre-planned routine surgery. The epidermis was separated from dermis by incubation with dispase then trypsinized in order to generate a single cell suspension. Keratinocytes were cultivated in Dulbecco's modified Eagle's and Ham's F12 media (3:1) enriched with 10% foetal calf serum (v/v) and specific enrichments.

These cells multiply in culture until a cell monolayer is reached. In this study, the cells were seeded in 96-well plates and semi-confluency (30.000 cells/well) was reached in 24 hours. Once a confluence of 60% - 70% has been reached, fresh medium was added with scalar dilutions of the tested sample. Non-treated cells were used as negative controls. At this stage the cell cultures were treated with different dilutions of the test compound and of the controls to obtain final concentrations ranging from 0.5 to 0.016 mg/ml. For each dilution, three replicate tests were performed. The product was dissolved in the culture medium. 0.15 mg/ml Vitamin C were added separately as positive control. Part of the cells was checked for their vitality with the NRU assay (Neutral Red Uptake), based on the cell ability to incorporate and bind the Neutral Red (NR), a weak cationic dye that penetrates the cell membrane through a mechanism of non-ionic diffusion and that is accumulated in the lysosomes, on matrix anionic sites. Cell and lysosome membrane alterations cause lysosomes fragility and gradual irreversible changes in the cells. These changes induced by xenobiotics determinate the decrease of NR uptake and of its linkage to lysosomes. This method is able to discriminate alive, damaged or dead cells. Cells were incubated with scalar concentrations of the products and with the Neutral Red solution (NR). If the membrane is damaged, it releases the dye in the medium. After incubation, the medium was replaced with fresh medium + NR medium and cells were incubated for 4 h at 37°C. Then cells were washed more times to eliminate exceeding dye wastes and read at the colorimeter.

The remaining cells were then exposed to 4' (1 J/cm²), 8' (2 J/cm²) and 12' (3 J/cm²). At the end of the exposure period, the ROS formation was investigated in the cell supernatant. The cell vitality was determined after UVA exposure and without UV exposure.

After having exposed the cells to the tested sample, the cell culture medium was removed and the cells washed in PBS. The Dichlorofluorescein acetate (DCA) solution was added to each well. DCA reacts with free radicals in the medium, originating a fluorescent derivative, and the fluorimeter reading allows obtaining a quantitative data related to the ROS content in the cells. After suitable incubation, the DCA solution has been discharged and the cells have then been exposed for different times to UVA irradiation and soon after read in the fluorimeter [11].

The results are expressed in terms of viability:

% cell viability = Optical Density (OD) of treated cells × 100/OD untreated control cells.

2.2. Collagen Synthesis

We investigated the *in vitro* evaluation of the collagen synthesis in human skin fibroblasts (ATCC-CRL-2703) exposed to treatment with P-3086 at three different concentrations. The ex-novo synthesis of the extracellular matrix component collagen was measured by means of colorimetric assays, comparing the result with that of other formulations.

P-3086 was diluted in cell culture medium to achieve the final concentrations chosen for the tests. P-3086 was tested at 20%, 10%, 5%, 2.50%, 1.25%, 0.63% and 0.31% (w/v) for preliminary cytotoxicity test.

In accordance with toxicity data (IC₅₀ = 9.43%, non-cytotoxic), three different non-cytotoxic concentrations were chosen to continue the tests. The concentrations chosen for the efficacy test were 2.50%, 1.25% and 0.63% (w/v).

Cell exposure to P-3086 was prolonged for 24 and 48 hours. At the end of each experimental times cell

proliferation and neo-synthesis of extracellular matrix elements were determined.

P-3086 was added to the wells containing cells in the G₀ phase of cell cycle (the cell number and the treatment to induce G₀ phase allow enough space for cell growing and avoid the contact inhibition phenomenon). Cells were exposed to P-3086 for 24 and 48 hours (medium was replaced every 24 h). At the end of incubation period, MTT test was performed in order to evaluate cell viability and the increasing proliferating rate compared to untreated control cell culture (CTR). For each determination 3 tests were carried out.

MTT assay [12]: MTT-medium was prepared by adding 15 mg of MTT to 30 ml of culture medium. After exposure of cells to the test items, they were washed with 200 µl of PBS. After removal of the washing solution, 200 µl of MTT-medium have been added to each culture well then incubated for 4 hours at 37°C and 5% CO₂. At the end of the incubation period, the MTT-medium was removed and 200 µl of MTT Solubilisation Solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) were added.

The plate was shaken on a rotatory plate for 20 - 30 minutes to ensure that all the crystals have been dissolved from the cells forming a homogeneous solution. The absorbance was measured at 570 nm on a microplate reader, with background reading at 690 nm. The results were expressed as % cell proliferation compared to an untreated control cell culture. Culture media with P-3086 was added to the wells containing cells in confluence (this feature allowed to maintain the cell number constant for the experiment duration time and avoids the influence of cell number in the observed trophism). Cells were exposed to each solution for 24 and 48 hours (medium was replaced every 24 h). At the end of incubation period, medium was collected in order to determine the concentration of collagen produced and released by the cells. For each determination 20 µl of medium was used. For each determination three tests were carried out.

The determination of collagen synthesis was carried out by quantitative dye-binding method. The chromogen agent used in the assay is Sirius Red (Direct red 80). Sirius red is an anionic dye with sulphonic acid side chain groups. These groups react with the side chain groups of the basic amino acids of collagen. The specific affinity of the dye for collagen, under the assay conditions, is due to the elongated dye molecules becoming aligned parallel to the long, rigid structure of native collagen that have intact triple helix organisation (dye affinity is much reduced when collagen is denatured).

Collagen concentration (µg in 20 µl of medium) was calculated by means of data interpolation on a standard curve obtained with known and increasing collagen concentrations.

2.3. Statistical Analysis

Data on ROS are presented with a descriptive statistics only providing the percentages of reduction for those experiments above the threshold (10%). Data on Collagen Synthesis were processed by a two-way ANOVA. Absolute collagen values (named ABS) recorded at 24 and 48 hours were pooled together and analyzed through an General Linear Model with Treatment (P-3086, test Product 2, test Product 3, test Product 4 and test Product 5), Dose (0.00625, 0.0125 and 0.025) and their interaction term (Treatment-by-Dose) as fixed effects (two-way ANOVA). Since the study objective consists in verifying whether the active compound P-3086 shows traits of superiority in comparison with other active compounds, the two-way ANOVA model was integrated with two sets of multiple comparisons. In the first set of comparisons, treatments (main effect regardless of dose) were compared with P-3086 used as control. In the second set of comparisons, treatments were compared with P-3086 used as control for each of the three doses. Statistical analyses were performed using NCSS 9 Software.

3. Results

3.1. Antioxidant Activity

In this test P-3086, at two out of three sub toxic tested concentrations, were able to reduce ROS production after UVA stress (at short time exposure). The results, summarized in **Table 1**, showed that P-3086 was able to reduce ROS production after UVA stress (at short time exposure) with the highest effect observed at 0.016 mg/ml and 0.031 mg/ml concentration showing therefore a superiority compared to the other formulations, without *Humulus lupulus*, hyaluronic acid and/or ethanol, having chosen preliminarily a threshold quote of <10% of ROS inhibition. P-3086 achieved a superiority even above the control (Vitamin C at 0.15 mg/ml, concentration 10- and 5-fold higher than P-3086).

Table 1. % reduction of UVA-induced ROS production after treatment (<under threshold)—a descriptive analysis.

Composition	mg/ml	4' UVA	8' UVA	12' UVA
P-3086	0.031	12.0	10.5	12.7
	0.016	<	14.0	19.4
Test Product 2	0.031	<	<	<
	0.016	<	<	<
Test Product 3	0.031	<	<	<
	0.016	<	13.6	11.7
Test Product 4	0.031	<	<	<
	0.016	12.8	11.2	<
Test Product 5	0.031	<	<	<
	0.016	<	<	<
Vitamin C	0.15	19.6	13.4	14.8

3.2. Collagen Synthesis

All the data are summarized in **Figure 1**. All treatments caused an increase of collagen synthesis at each tested dose. P-3086 was superior to all tested products.

The analysis on the pooled treatment effect significantly demonstrated the superiority of P-3086 on all the other tested formulations (**Table 2**).

The multiple comparisons among treatments at different dosages, demonstrated that the best dose of P-3086 was at 0.0125 which already reached the maximal effect and was superior on the same dose to all the other tested formulations at the same dose ($P < 0.05$). The lowest tested dose of P-3086 resulted inferior to the mid dose, but superior to almost all the other tested products. The highest dose of P-3086 had a similar effect of the mid dose, resulting to almost all the other test products, with the exception of test product 3 which demonstrated an evident dose effect relationship among the three tested dosages. Details of statistical analysis are displayed in **Table 3**.

4. Discussion

According to the data obtained in these *in vitro* studies, we could assess that the treatment of cell culture with P-3086 showed the efficacy of the test product against oxidative damages, artificially induced by UVA on skin-derived keratinocytes.

In fact, our results illustrated that at different time points (4', 8' and 12') of UVA exposure, P-3086 was able to reduce ROS production even at the lowest dose of those tested.

Moreover, comparing the result with that of the other formulations, P-3086 was able to increase collagen synthesis compared with untreated control cell culture, highlighting cell trophism enhancing properties. Hyaluronic acid showed modulate proliferation and collagen synthesis; this activity, combined with the antioxidant properties of hops extracts and their capabilities to soothe, to sedate and to provide a calming effect on the body, had been brought out by the presence of ethanol, which was able to synergise the interaction of hyaluronic acid, hops and Vitamin E, which on the contrary, taken on their own, did not show any relevant effect. Ethanol was therefore able to enhance the effect of these ingredients, boding excellent results in *in vivo* investigation of anti-aging capabilities of P-3086.

The above results account for the positive effect reported in humans [13] of this composition in terms of wrinkle profilometry, plastoelasticity and skin hydration. These results are consistent with other products that may serve as daily skin care to prevent UVA-induced skin damages by ROS-scavenging, promoting at the same time the collagen synthesis in dermis [14]. Moreover, P-3086 allows achieving comparable efficacious results without producing significant side effects or general discomfort in the subjects, acting in the way of tissue regeneration and not opposite to it, like chemical peels or cutaneous resurfacing [15]. Thanks to its antioxidant effects,

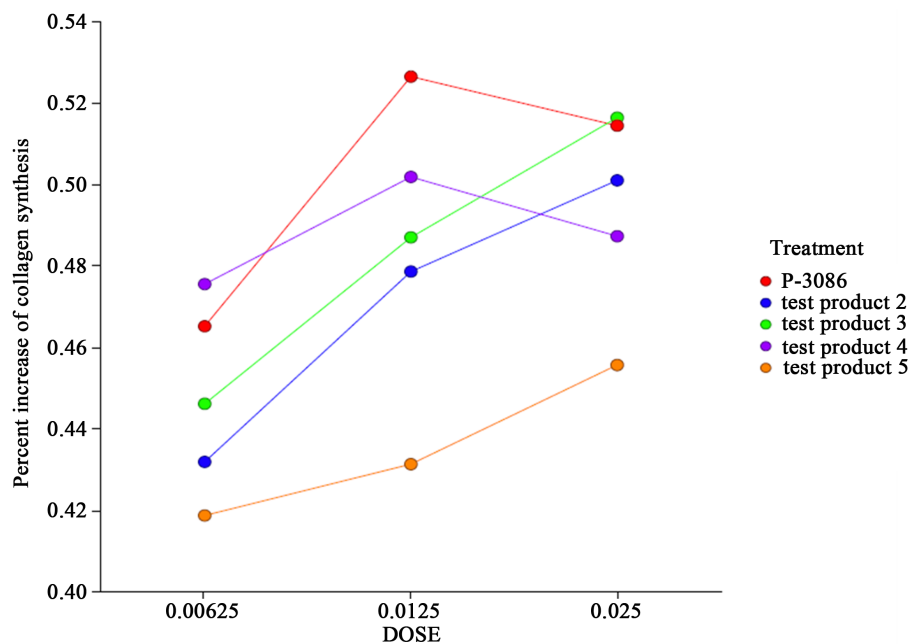


Figure 1. Percent increase of collagen synthesis versus untreated control by treatment and by dose (pooled data).

Table 2. Multiple comparisons of main treatment effects.

Comparison	Significant
Test Product 2 vs. P-3086	P < 0.05
Test Product 3 vs. P-3086	P < 0.05
Test Product 4 vs. P-3086	P < 0.05
Test Product 5 vs. P-3086	P < 0.05

Table 3. Multiple comparisons of treatment effects within dosages.

Dose	Comparison	Significant
0.00625	Test Product 2 vs. P-3086	P < 0.05
	Test Product 3 vs. P-3086	P < 0.05
	Test Product 4 vs. P-3086	N.S.
	Test Product 5 vs. P-3086	P < 0.05
0.0125	Test Product 2 vs. P-3086	P < 0.05
	Test Product 3 vs. P-3086	P < 0.05
	Test Product 4 vs. P-3086	P < 0.05
	Test Product 5 vs. P-3086	P < 0.05
0.025	Test Product 2 vs. P-3086	P < 0.05
	Test Product 3 vs. P-3086	N.S.
	Test Product 4 vs. P-3086	P < 0.05
	Test Product 5 vs. P-3086	P < 0.05

it can provide protection from the sun, neutralizing the free radicals responsible for the collagen breakdown. Finally, thanks to its moisturizing activity, P-3086 can make wrinkles look temporarily less prominent, keeping the skin hydrated. Due to all these properties, the product P-3086 can be definitely of benefit in skin rejuvenation practices.

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References

- [1] Panas, M., *et al.* (2012) The Byzantine Empress Zoe Porphyrogenita and the Quest for Eternal Youth. *Journal of Cosmetic Dermatology*, **11**, 245-248. <http://dx.doi.org/10.1111/j.1473-2165.2012.00629.x>
- [2] Datta, H.S., *et al.* (2011) Theories and Management of Aging: Modern and Ayurveda Perspectives. *Evidence-Based Complementary and Alternative Medicine*, **2011**, Article ID 528527, 6 p. <http://dx.doi.org/10.1093/ecam/nep005>
- [3] Park, H.M., *et al.* (2012) Royal Jelly Increases Collagen Production in Rat Skin after Ovariectomy. *Journal of Medicinal Food*, **15**, 568-575. <http://dx.doi.org/10.1089/jmf.2011.1888>
- [4] Lee, N.J., *et al.* (2011) *In Vitro* Antioxidant Properties of a Ginseng Intestinal Metabolite IH-901. *Laboratory Animal Research*, **27**, 227-234. <http://dx.doi.org/10.5625/lar.2011.27.3.227>
- [5] Khansari, N., Shakiba, Y. and Mahmoudi, M. (2009) Chronic Inflammation and Oxidative Stress as a Major Cause of Age-Related Diseases and Cancer. *Recent Patents on Inflammation & Allergy Drug Discovery*, **3**, 73-80. <http://dx.doi.org/10.2174/187221309787158371>
- [6] Orrenius, S. (2007) Reactive Oxygen Species in Mitochondria-Mediated Cell Death. *Drug Metabolism Reviews*, **39**, 443-455. <http://dx.doi.org/10.1080/03602530701468516>
- [7] Fisher, G.J. (2005) The Pathophysiology of Photoaging of the Skin. *Cutis*, **75**, 5-9.
- [8] Stefanaki, C., *et al.* (2005) Topical Retinoids in the Treatment of Photoaging. *Journal of Cosmetic Dermatology*, **4**, 130-134. <http://dx.doi.org/10.1111/j.1473-2165.2005.40215.x>
- [9] Philips, N., *et al.* (2010) Direct Inhibition of Elastase and Matrixmetalloproteinases and Stimulation of Biosynthesis of Fibrillar Collagens, Elastin, and Fibrillins by Xanthohumol. *Journal of Cosmetic Science*, **61**, 125-132.
- [10] Chevallier, A. (1996) *The Encyclopedia of Medicinal Plants: An Excellent Guide to over 500 of the More Well-Known Medicinal Herbs from around the World.* Dorling Kindersley, London.
- [11] Kanthasamy, A., *et al.* (1997) Reactive Oxygen Species Generated by Cyanide Mediate Toxicity in Rat Pheochromocytoma Cells. *Toxicology Letters*, **93**, 47-54. [http://dx.doi.org/10.1016/S0378-4274\(97\)00068-4](http://dx.doi.org/10.1016/S0378-4274(97)00068-4)
- [12] Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**, 55-63.
- [13] Sparavigna, A., *et al.* (2014) An Innovative Concept Gel to Prevent Skin Aging. *Journal of Cosmetics, Dermatological Sciences and Applications*, **3**, 4.
- [14] Kato, S., *et al.* (2012) Hydrogen-Rich Electrolyzed Warm Water Represses Wrinkle Formation Against UVA Ray Together with Type-I Collagen Production and Oxidative-Stress Diminishment in Fibroblasts and Cell-Injury Prevention in Keratinocytes. *Journal of Photochemistry and Photobiology B: Biology*, **5**, 24-33. <http://dx.doi.org/10.1016/j.jphotobiol.2011.09.006>
- [15] Vedamurthy, M. (2006) Antiaging Therapies. *Indian Journal of Dermatology, Venereology and Leprology*, **72**, 183-186. <http://dx.doi.org/10.4103/0378-6323.25776>

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