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## Molecular and Human In Vivo Study of an Innovative Plant-Derived Multifunctional Peptide Signaling the Collagen and Elastin Pathways and Melanin Production

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**Abstract:** Chrono Control Penta is a novel plant derived multifunctional bioactive peptide, which offer a tailored targeted approach to skin health by addressing both pigmentation and aging. Chrono Control Penta inhibits tyrosinase with an IC<sub>50</sub> value of 202.8  $\mu$ M. Additionally, it significantly increased collagen (+87.53%) and elastin (+61.29%) production and secretion (+66.29% and +69.74%, respectively) and decreased the Matrix metalloproteinase-9 (MMP-9) and MMP-2 secretion in aged human dermal fibroblasts, vs. aging condition. At the clinical level, Chrono Control Penta was demonstrated to be already active after 2 weeks, promoting a 9.3% reduction in pigmentation after 6 weeks of use, showing its efficacy in promoting skin complexion. Furthermore, it exhibited significant moisturizing (13.05%), anti-wrinkle (11.55%), and purifying effects (12.45%), as well as firming effects (6.35%), after 6 weeks. The peptide was also well tolerated, with no adverse effects reported in clinical patch tests. This timely study presents novel research on a plant derived peptide, Chrono Control Penta, a significantly contribution to the burgeoning cosmetic peptide market. Our rigorous findings make it a new powerful ingredient, offering a comprehensive solution for skin health, and establishing a strong foundation for future research and application.

**Keywords:** Chrono Control Penta; anti-aging peptides; skin health; tyrosinase inhibition; ECM stability; collagen production

## 1. Introduction

Peptides are short chains of amino acids (A.A.) (2-20 A.A.), which are chemically linked by an amidic bond known as peptide bond between the carboxylic acid at the C-terminus and the amino group at the N-terminus. They are considered safe, hypoallergenic, and highly biocompatible; in addition, some of them are also endowed with biological activity [1]. Indeed, bioactive peptides are a group of biological molecules that are normally buried in the structure of parent proteins and become active after the cleavage of the proteins. Evidence in the literature suggests that bioactive peptides, of both synthetic and natural origins, have been successfully applied in many healthcare fields, i.e., the pharmaceutical, nutraceutical, and cosmetic sectors, respectively [2]. In this panorama, the cosmetics market, particularly for natural products, is experiencing rapid growth driven by consumer demand for improved appearance [3]. This growth should be supported by the development of innovative formulations containing bioactive compounds (e.g., antioxidants, proteins, peptides, growth factors) that offer therapeutic and protective benefits, such as skin whitening, moisturizing, and rejuvenation [4,5]. Notably, in the cosmetics field, peptides can be applied for anti-ageing propose and their most common classification is based on their mechanism of action as follows: signal peptides, carrier



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Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/ licenses/by/4.0/). peptides, neurotransmitter inhibitor peptides, and enzyme inhibitor peptides [6,7]. For instance, palmitoyl pentapeptide-3 was one of the first synthetic bioactive peptides used to stimulate collagen synthesis for anti-aging and wound-healing treatments [8]. The copper Gly–His–Lys (Cu-GHK) was developed and incorporated into cosmetic products to heal skin, promote collagen synthesis, and repair DNA damage [8,9]. Another popular commercialized synthetic peptide with potential anti-wrinkle and moisturizing properties is Acetyl hexapeptide-3 (Argireline<sup>®</sup>) [8,10]. Despite the growing scientific evidence supporting their potential application in cosmetics, there is only a handful of evidence in the literature describing findings regarding promising applications. Indeed, most of these peptides are not so well characterized and no scientific evidence describing their efficacy and safety is robustly provided. However, the increased demand for effective cosmetic products has led to an interest in developing new-generation products based on bioactive ingredients and in this peculiar context, doubtless, a new generation of bioactive peptides can be considered promising and potential candidates for addressing this issue.

Taking up this challenge and with the aim to overcome the gap in the use of peptide in cosmetics—bringing, therefore, real innovation in this sector—this study provides a multidisciplinary and bottom-up approach for screening and identifying a new generation of multidisciplinary plant-derived bioactive peptide. More in detail, our study focuses on Chrono Control Penta. The commercially available Chrono Control Penta is a hempseed derived peptide of five amino acids in length that was recently patented (https://www.plantechlab.com/home-page/, accessed on 1 April 2025). Specifically, to guarantee a reproducible purity, safety, and efficacy, and for producing it at a large scale, Chrono Control Penta is obtained using solid phase synthesis. Its safety has been assessed in vitro by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for monitoring the peptide effects on human dermal fibroblasts and melanocytes viability, respectively, and on volunteers by performing the patch test. As regards its efficacy, dedicated in vitro experiments have been carried out using a combination of biochemical and cellular techniques. Indeed, using both human fibroblasts and melanocytes, its anti-aging mechanism of action has been assessed, monitoring its ability to modulate both collagen and elastin homeostasis and melanin production. The promising findings allowed us to deepen its efficacy by performing a clinical study in which 20 enrolled female volunteers (40-65 years) tested the ingredients for 2 and 6 weeks, respectively. The results suggested that this precision peptide can boast seven different claims, clearly underlining that it can be considered the innovative choice for formulating new generation of effective and personalized cosmetic products for improving longevity and skin health.

## 2. Materials and Methods

#### 2.1. Chemicals

All reagents used for the research were of analytical grade and commercially available. The complete list of chemicals is available in Supplementary Materials Section S2.1.

#### 2.2. In Vitro Tyrosinase Activity Assay

The tyrosinase inhibitory assay was carried out using the method provided by Liyanaarachchi [11], with L-DOPA as the substrate. The detailed procedure is reported in Supplementary Materials Section S2.2.

#### 2.3. Cell Culture Condition

hTERT-immortalized human skin fibroblasts BJ-5ta cell line was bought from ATCC (ATCC (CRL-4001<sup>TM</sup>), from LGC Standards, Milan, Italy), cultured in DMEM high glucose and Medium 199 (4:1 ratio) with stable L-glutamine supplemented with 10% FBS,

100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin, and incubated at 37 °C under a 5% CO<sub>2</sub> atmosphere. hTERT-immortalized dermal melanocyte cell line was bought from ATCC (ATCC CRL-4059<sup>TM</sup>), from LGC Standards, Milan, Italy), cultured in dermal cell basal medium supplemented with 5  $\mu$ g/mL rh Insulin, 50  $\mu$ g/mL Ascorbic Acid, 6 mM L-Glutamine, 1.0  $\mu$ M Epinephrine, 1.5 mM Calcium Chloride, 1.0 mL of Peptide Growth Factor, 5 mL of M8 Supplement, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin and incubated at 37 °C under a 5% CO<sub>2</sub> atmosphere.

#### 2.4. Cells Viability Assay

For the assessment of cellular viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments were conducted, following a procedure published elsewhere [12].

#### 2.5. Cellular Melanin Extraction and Quantification

Melanocytes were plated and incubated for 24 h then exposed to UV irradiation or treated with  $\alpha$ -MSH to induce melanogenesis. After 24 h, they were treated with Chrono Control Penta or Kojic acid and incubated for another 24 h. For the quantification of cellular melanin, the method of Chan et al. was followed with slight modifications [13]. More details are reported in Supplementary Materials Section S2.5.

#### 2.6. Enzyme-Linked Immunosorbent Assay (ELISA) Assays

The AuthentiKine ELISA test by Proteintech was used to quantify the matrix metalloproteinases (MMP-2 and MPP-9) and collagen and elastin levels in cell culture supernatants. See Supplementary Materials Section S2.6 for further details.

#### 2.7. Western Blot Analysis

For evaluating the collagen and elastin production in fibroblasts, Western blot experiments were conducted following a procedure previously published [14].

#### 2.8. Clinical Study

#### 2.8.1. Chrono Control Penta Formulation

The Chrono Control Penta formulation has been prepared by Etichub s.r.l. (Pavia, Italy) with the following ingredients: Aqua, Di-C12-13 Alkyl Tartrate, Glycerin, Polyglyceryl-10 Myristate, Hydrogenated Hexaisoprene, Helianthus Annuus Seed Oil, Polyglyceryl-2 Diisostearate, Chrono Control Penta, Xanthan Gum, Phenoxyethanol Ethylhexylglycerin Sodium Hydroxide, and Parfum.

The Placebo formulation was used as control. The ingredients of the Placebo formulation are as follows: Aqua, Di-C12-13 Alkyl Tartrate, Glycerin, Polyglyceryl-10 Myristate, Hydrogenated Hexaisoprene, Helianthus Annuus Seed Oil, Polyglyceryl-2 Diisostearate, Xanthan Gum, Phenoxyethanol Ethylhexylglycerin Sodium Hydroxide, and Parfum.

#### 2.8.2. Study Design

The study has been carried out by Etichub s.r.l. (Pavia, Italy) according to the Helsinki declaration (Ethical Principles for Medical Research Involving Human Subjects); the European Regulation No. 1223/2009; the Commission Regulation (EC) No. 655/2013; and the EEMCO group guidelines (European group on efficacy measurement and evaluation of cosmetics and other products): EEMCO Guidance for the assessment of transepidermal water loss in cosmetic sciences (Skin Pharmacol Appl Skin Physiol. Mar-Apr 2001;14(2):117–128.) and the revised EEMCO guidance for the in vivo measurement of water in the skin.

All procedures related to the clinical study, along with descriptions of the instruments used, are provided in Supplementary Materials Section S2.8.2.

#### 2.9. Statistical Analysis

The data obtained are processed both as a descriptive statistical analysis and as statistical analysis with comparison tests for parametric and nonparametric data. A significant level of 5% was chosen, so the changes were considered statistically significant for p < 0.05(GraphPad Prism 10, GraphPad Software, La Jolla, CA, USA).

## 3. Results

## 3.1. Chrono Control Penta Description

Chrono Control Penta is a five-amino-acid peptide identified within hempseed proteins; the new INCI name is S-Cannabis Sativa-pentapeptide-1. It has a molecular weight of 600.3 g/mol and it is soluble in both water and organic solvents. After the initial identification, the peptide is then, routinely, obtained using F-moc solid phase synthesis and freeze-dried. In this way, a white freeze-dried powder with purity of 97%, (the peptide chromatogram is available in the Supplementary Materials), with a hydrophobicity of 4.70 Kcal/mol and an isoelectric point of 5.53 can be obtained in large scale production with always the same characteristics and functionality. After the synthesis, the trifluoroacetic acid (TFA) was removed obtaining the HCl salt form of peptide.

# 3.2. Chrono Control Penta: Identification of the Mechanism of Action Using Biochemical and Cellular Techniques

3.2.1. Evaluation of Chrono Control Penta Safety on Human Dermal Melanocytes and Human Dermal Fibroblasts

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments on dermal cells were assessed to verify the possible cytotoxicity exerted by the Chrono Control Penta peptide. The results in Figure 1 clearly show that Chrono Control Penta is safe at all concentrations tested, demonstrating high cell compatibility and the absence of toxic effects in both melanocytes (Figure 1a) and fibroblasts (Figure 1b).





3.2.2. Chrono Control Penta Inhibits Tyrosinase Activity and Reduces Melanin Production in Melanocytes Exposed to UV and  $\alpha$ -MSH

Figure 2a shows the inhibition of tyrosinase activity exerted by Chrono Control Penta and Kojic acid (positive control) at concentrations ranging from 10 to 1000  $\mu$ M. The results indicate that Chrono Control Penta inhibits the enzyme with an IC<sub>50</sub> value of 202.8  $\mu$ M, compared to the standard Kojic acid, which inhibits tyrosinase with an IC<sub>50</sub> value of 450.0  $\mu$ M. Thus, Chrono Control Peptide is almost two times more active than Kojic acid in inhibiting tyrosinase activity in vitro (Figure 2a).



**Figure 2.** Effects of Chrono Control Penta on the inhibition of in vitro tyrosinase activity (**a**) and on modulation of melanin production in UV (**b**) and  $\alpha$ MSH (**c**) stimulated melanocytes. Cells were stimulated with UV or  $\alpha$ -MSH and treated for 24 h with Chrono Control Penta at 1 mM concentration or Kojic acid at 2 mM concentration. Data represent the mean  $\pm$  s.d. of four independent experiments performed in duplicate. Ctrl: not stimulated cells; \*\* (p < 0.001), \* (p < 0.05), \*\*\* (p < 0.001).

Since tyrosinase is involved in melanin production, the inhibitory effect of the peptide on the tyrosinase activity in melanocytes was confirmed by the evaluation of the production of melanin. Kojic acid was used as a standard tyrosinase inhibitor at 2 mM concentration. Thus, melanocytes were stimulated with UV ray at 0.5 J/cm<sup>2</sup> for 30 min or treated with 0.3  $\mu$ M  $\alpha$ -MSH for 24 h, to promote melanogenesis. Upon exposure to UV alone, the melanin production was significantly increased up to 248.7  $\pm$  18.1% (Figure 2b) compared with cells not exposed to UV; after 24 h of incubation with Chrono Control Penta 1 mM, the melanin levels were reduced up to193.6  $\pm$  5.4%, while Kojic acid reduced it up to 208.9  $\pm$  5.5% (Figure 2b). The treatment with  $\alpha$ -MSH alone enhanced the melanin levels up to 138.7  $\pm$  7.29% (Figure 2c) compared with untreated cells, while in presence of Chrono Control Penta 1 mM, the melanin reached 106.4  $\pm$  9.1%, and in presence of Kojic acid, the melanin was decreased up to 101.3  $\pm$  5.5% (Figure 2c). These results demonstrate the ability of the Chrono Control Penta peptide to effectively modulate the melanin production, leading it at basal level, upon UV and  $\alpha$ -MSH exposition, respectively.

3.2.3. Chrono Control Penta Improves the Synthesis of Collagen and Elastin in Aged Human Dermal Fibroblasts via Akt Signaling Pathway Activation

To investigate the capacity of Chrono Control Penta to exert beneficial effects on skin fibroblasts, Western blot experiments were conducted by analyzing the variation in collagen and elastin production. Aging conditions were reproduced by treating cells with  $H_2O_2$  at a 200  $\mu$ M concentration, which is a useful way to do so without compromising cellular viability (MTT assay is available in the Supplementary Materials, Figure S1). The results in Figure 3a indicate that in aged cells, the collagen protein levels significantly decrease by  $60.89 \pm 14.79\%$ , compared with control cells. Chrono Control Penta positively contrasts the  $H_2O_2$  effects by improving the collagen production until 76.08  $\pm$  37.13% at 50  $\mu$ M concentration (Figure 3a). In accordance with this evidence, under the aging condition, elastin levels were reduced by 55.91  $\pm$  11.60% (Figure 3b), with respect to untreated and unstimulated cells, while Chrono Control Penta augmented elastin up to 71.35  $\pm$  7.53%, tested at the same concentration, under aging condition. Strikingly, Chrono Control Penta

can augment collagen and elastin protein levels by 87.53  $\pm$  37.86% and 61.29  $\pm$  39.86%, respectively, compared to the aging condition. Notably, this molecular effect might be attributed to the activation of the Akt signaling pathway, a key regulator of cell survival, proliferation, and protein synthesis [15]. The quantification of the phospho-Akt/Akt ratio presented in Figure 3c further supports this observation. In fact, in aged cells, the phospho-Akt/Akt ratio was 0.78  $\pm$  0.04, compared to control group. Treatment with Chrono Control Penta increased this ratio to 1.24  $\pm$  0.03, indicating a marked enhancement in Akt pathway activation.



**Figure 3.** Effects of Chrono Control Penta on collagen (**a**) and elastin (**b**) production and on phospho-Akt(Ser473)/Akt ratio and phospho-Akt(Ser473) production (**c**) in human dermal fibroblasts. Cells were pre-treated for 24 h with Chrono Control Penta at 50  $\mu$ M concentration; then, aging conditions were induced. Data represent the mean  $\pm$  s.d. of six independent experiments performed in duplicate. Ctrl: not stimulated and not treated cells; aging c.: cells treated with H<sub>2</sub>O<sub>2</sub>; \*\*\*\* (p < 0.0001), \*\*\* (p < 0.0001), \*\* (p < 0.001) \* (p < 0.05).

More specifically, the results in Figure 3c show that in aged cells, the phospho-Akt on ser473 (active form) protein levels decreased to  $54.9 \pm 4.65\%$ . However, treatment with the Chrono Control Penta peptide restored and further increased phospho-Akt (Ser473, active form) levels to  $77.47 \pm 8.69\%$ , indicating a significant reactivation of the Akt signaling pathway. Notably, as suggested by the literature, the Akt pathway activation (phospho-Akt) promotes fibroblast activity and ECM protein production, like collagen and elastin [15,16].

3.2.4. Chrono Control Penta Reduces the Secretion of Collagen, Elastin, and the Matrix Metalloproteinases MMP-2 and MMP-9 in Aged Stimulated Human Dermal Fibroblasts

To further study the potential effects of Chrono Control Penta on the stability of extracellular matrix (ECM), ELISA assays were conducted on aged cells. Specifically, as indicated in Figure 4, the aging condition promoted the reduction in collagen and elastin secretion by  $52.2 \pm 16.20\%$  and  $55.91 \pm 11.60\%$ , respectively (Figure 4a,b), while an increase in MMP-2 and MMP-9 secretion up to  $120.9 \pm 7.95\%$  and  $123.3 \pm 11.06\%$ , respectively, was observed. (Figure 4c,d). Effectively, Chrono Control Penta improves the aging-reduced effects, promoting collagen secretion up to  $79.49 \pm 12.97\%$  and elastin secretion up to  $71.11 \pm 17.57\%$ , in the presence of aging condition (Figure 4a,b). Notably, Chrono Control Penta can augment collagen and elastin secretion by  $66.29 \pm 27.13\%$  and  $69.74 \pm 18.20\%$ , respectively, compared to the aging condition.



**Figure 4.** Chrono Control Penta effects on collagen (**a**), elastin (**b**), MMP-2 (**c**), and MMP-9 (**d**) secretion in human dermal fibroblasts. Cells were pre-treated for 24 h with Chrono Control Penta at 50  $\mu$ M concentration; then, aging conditions were induced. Data represent the mean  $\pm$  s.d. of six independent experiments performed in duplicate. Ctrl: not stimulated and not treated cells; aging c.: cells treated with H<sub>2</sub>O<sub>2</sub>; \*\*\*\* (*p* < 0.0001), \*\*\* (*p* < 0.0001), \*\* (*p* < 0.0001), \* (*p* < 0.05).

Additionally, the peptide treatment reduced the metalloproteinases MMP-2 secretion up to  $101.3 \pm 7.75\%$  and the MMP-9 secretion up to  $102.9 \pm 14.1\%$  (Figure 4c,d).

## 3.3. Clinical Safety and Efficacy Evaluation of Chrono Control Penta

In vivo safety and efficacy evaluations were made by comparing Chrono Control Penta (0.5% w/v) to a placebo treatment. Twenty female volunteers aged between 40 and 65 were selected and divided into two groups. All the participants completed the trial. The clinical evaluation results, based on edema and erythema parameters, suggested that no significant reactions were reported after 96 h treatment. The Patch test confirmed the high tolerability of Chrono Control Penta on human skin, demonstrating the absence of irritation or adverse reactions. After assessing the skin compatibility of Chrono Control Penta on human skin, a cream containing Chrono Control Penta (0.5% w/v) was tested compared to a placebo cream on 20 female volunteers aged between 40 and 65, selected and divided into two groups. All the participants completed the trial. The instrumental evaluation parameters values are reported in Table 1, while Figure 5 shows the pictures taken after 2 weeks and after 6 weeks of Chrono Control Penta application.

Time	Hydrating Effect <sup>1</sup> (ti-t0)	Trans- Epidermal Water Loss (tit0)	Skin Elasticity <sup>2</sup> (ti-t0)	Anti- Wrinkle Effect <sup>3</sup> (ti-t0)	Pigmentation Variation (ti-t0)	Purifying Effect (ti-t0)	Dermal Firmness (ti-t0)
t0	-	-			-	-	-
t2weeks	+9.17% $(p < 0.0001)$	0.38% ( <i>p</i> > 0.05)	-3.71% ( <i>p</i> < 0.05)	-8.38% ( <i>p</i> < 0.01)	-4.98% ( $p < 0.0001$ )	-9.38% ( <i>p</i> < 0.0001)	-3.72% ( <i>p</i> < 0.01)
t6weeks	+13.05% $(p < 0.0001)$	12.33% ( <i>p</i> > 0.05)	-9.86% ( <i>p</i> < 0.0001)	-11.55% ( <i>p</i> < 0.001)	-5.63% ( <i>p</i> < 0.0001)	-12.45% ( <i>p</i> < 0.0001)	-6.35% ( <i>p</i> < 0.0001)

Table 1. Instrumental evaluations.

<sup>1</sup> based on SC water content; <sup>2</sup> based on RO (maximum extension); <sup>3</sup> based on maximum height.



**Figure 5.** Skin wrinkles (**a**) and pigmentation (**b**) images of subject 7 at the beginning (t0), after 15 days (t2weeks), and after 6 weeks (t6weeks) of Chrono Control Penta formulation application acquired by Antera<sup>®</sup> 3D Imaging System (Miravex Limited, Dublin, Ireland). Overall appearance images (**c**) of subject 4 at the beginning (t0), after 15 days (t15days), and after 6 weeks (t6weeks) of Chrono Control Penta formulation application acquired by Vectra H2. Twenty females, 40–65 years old, were exposed to pollutants: 0.5% Chrono Control Penta vs. placebo, half face, twice a day, for 6 weeks. In panel (**a**) the color from red to blue, and in the panel (**b**) the color from red to sand indicate an improvement of the tested parameters.

#### 3.4. Subjective Evaluation

At the end of the study, a questionnaire regarding the subjective efficacy and pleasantness of the Chrono Control Penta formulation was filled from volunteers. Participants reported a high level of effectiveness in various areas. Specifically, 54.55% of participants considered the product effective in reducing wrinkles and 68.19% of participants considered it very effective/effective in firming the skin. Additionally, 40.91% found it effective for improving skin complexion and 13.64% found it very effective, while 45.45% felt it effectively reduced skin impurities. In terms of pleasantness, 54.55% of participants found the skin feeling during application to be very pleasant/pleasant, with 22.73% rating it as very pleasant. After application, 63.64% described the skin feeling as pleasant, and 13.64% found it very pleasant. Regarding overall satisfaction with the cosmetic properties, 59.09% of participants were satisfied, and 4.55% were very satisfied. Furthermore, the product was well tolerated by all participants, with 100% reporting no undesirable effects.

### 4. Discussion

As the body's largest organ, the skin's health and appearance are crucial for overall well-being, acting as a protective barrier and playing a vital role in temperature regulation, sensation, and more. The skin's main layers include the epidermis, dermis, and hypodermis, and it is prone to many problems, including skin cancer, acne, wrinkles, and rashes. Extrinsic aging, which includes ultraviolet exposure, pollution, and lifestyle choices such as smoking, sleeping positions, diet, and daily skincare habits, is estimated to account for 80% of the visible signs of skin aging, which manifests as stratum corneum thickening, mottled pigmentation, dullness, dryness, wrinkles, and laxity [17]. The appearance of youthful, healthy skin is influenced by several key factors in the epidermis and dermis; these include the density and quality of the extracellular matrix, the arrangement of cells within the connective tissue, the condition of the cornified cells on the skin's surface, and the natural fluorescence of the skin [18,19]. Therefore, in this context, cosmetic products, when used correctly, can enhance skin health and appearance by cleansing, moisturizing, and protecting against environmental factors. The efficacy of a certain cosmetic product correlates to the presence of active ingredients to be substances used in a cosmetic formula to achieve certain local biologic effects. Active ingredients can be synthetic in nature (e.g., peptides, ceramides, most vitamins), extracted and more-or-less purified from natural sources (e.g., botanicals, herbal extracts), obtained by biotechnology techniques such as fermentation and cell culture (e.g., enzymes and cofactors, polysaccharides, proteins), or extracted from animal (including marine) sources [20]. In the field of active ingredients, peptides have gained significant attention in the cosmetic industry due to their potential in enhancing skin health and beauty. Indeed, nowadays, they represent the most important trend in the cosmetic market. Overall, they can be classified into four categories: signal, carrier, neurotransmitter-inhibitory, and enzyme-inhibitory peptides [8].

Chrono Control Penta can be classified as both a signal and enzyme-inhibitory peptide. Notably, by using a bottom-up approach, proteins from hempseed press cake were extracted and along with a biotechnological approach exploiting enzymatic hydrolysis, a peptide mixture with different size and physic-chemical properties, was produced. Hence, the hydrolysates' composition is heterogeneous, meaning that while some peptides exhibit biological activity, others do not. Consequently, the biological activity of protein hydrolysates is largely determined by the specific peptide composition. The method of protein hydrolysis, the enzymes used, and the conditions applied can all influence the overall biological activity, which, as a result, may not be consistently reproducible. Indeed, although hydrolysates may have a broad range of biological activities (i.e., antioxidative and anti-inflammatory) thanks to their complex nature, their use may be limited in cosmetics whether a more specific biological function is preferred [21]. Moreover, an additional limitation could be associated with the risk of potential allergens, since most of the preparations of peptide are produced as unpurified mixtures of several components [22]. Plant hydrolysates may contain allergens and potentially toxic contaminating compounds, whose presence must be necessarily certified. Thus, to ensure conformity to the quality standards and answer the safety concerns, the hydrolysates must be subjected to extensive chemical analysis once they are developed as new active ingredients for cosmetic applications. In addition, the protein hydrolysis process at industrial large scale faces some limitation. Indeed, key challenges include the reproducibility of hydrolysis and therefore peptide mixture compositions. Thus, to fill these gaps and overcome this limitation, by performing a deep analysis and screening of total initial hydrolysate, a promising on single peptide sequence, capable of exerting anti-aging effects and improving overall skin heath, was identified. Chrono Control Penta was then synthetized by using the well-known F-moc solid-phase peptide synthesis to guarantee a certain degree of purity (97%) [23], which

can be strongly correlated to its efficacy and safety. This unique peptide, which has a new INCI name, contains five amino acid residues with a molecular weight of 600.3 g/mol. It is soluble in both water and organic solvents, making it versatile for various formulations. The peptide comes in a white freeze-dried powder; its isoelectric point is 5.53 and it has a hydrophobicity of 4.70 Kcal/mol. In addition, Chrono Control Penta does not contain TFA. The potential ban of per- and polyfluoroalkyl substances (PFAS) such as TFA in cosmetic products is a response to growing concern about their impact on environmental sustainability and human health. Most of the commercially available cosmetic peptides are usually delivered as TFA salts. As an integral component in certain cosmetic formulations, TFA has become a concern due to its persistence in the environment and its potential toxic effect [24]. The proposed ban by the European Chemicals Agency (ECHA) reflects the demand for transparent and considerate beauty products from consumers. It marks a pivotal phase in the cosmetic industry, seeking to align product effectiveness with environmental responsibility. Hence, even though the toxicity data for TFA are not well established, the effects of chronic TFA exposure, and their impacts on the wider environments, including on plants, animals, and humans, will represent a critical issue in the future [25].

Firstly, this work demonstrated that this innovative plant derived peptide is not cytotoxic in both human melanocytes and human dermal fibroblast. In accordance, the patch test on volunteers revealed that this peptide is not irritating 96 h after application. Furthermore, in this study, it was demonstrated that Chrono Control Penta can inhibit the activity of tyrosinase, the key enzyme responsible for the melanin production, and positively modulate the collagen and elastin homeostasis. More in detail, by performing in vitro tyrosinase inhibition assay, Chrono Control Penta can inhibit tyrosinase in vitro with IC<sub>50</sub> equal to 202.8  $\mu$ M, suggesting it being two-fold more active than Kojic acid (IC<sub>50</sub>: 450  $\mu$ M). Consequently, it was demonstrated that Chrono Control Penta reduces the melanin levels in both UV and  $\alpha$ -MSH stimulated melanocyte at 1 mM, showing similar effects to those exerted by Kojic acid tested at 2 mM concentration, and confirming, therefore, that Chrono Control Penta is more active than Kojic acid, making it a valid alternative to the use of this commercial product.

The results of our study also indicate that Chrono Control Penta, tested at 50 µM, promotes the production of collagen and elastin in aged human dermal fibroblasts; moreover, it positively regulates the secretion of collagen and elastin and the secretion of metalloproteinases MMP-2 and MMP-9, thus contributing to the stability of the extracellular matrix (ECM), supporting its function. In more detail, the results presented in Figure 3c demonstrate that Chrono Control Penta promotes reactivation of the Akt signaling pathway, which plays a crucial role in ECM proteins synthesis [15,16]. Akt, also known as protein kinase B, is activated by phosphorylation at Ser473, and this modification is essential for its full enzymatic activity [26]. Specifically, in dermal fibroblasts, the active phospho-Akt enhances transcriptional and translational processes that lead to the increased production of structural proteins such as collagen and elastin. Moreover, studies have shown that the suppression of PI3K/Akt signaling contributes to the senescent phenotype in skin-derived precursor cells. This suggests that the PI3K/Akt pathway is crucial for preventing the onset of cellular aging [27].

Notably, in our study, the restoration of Akt phosphorylation (phospho-Akt(Ser473)), together with the significant increase in the phospho-Akt/Akt protein ratio, strongly supports the molecular reactivation of this pathway. This enhanced phosphorylation status suggests that the peptide may help reverse signs of aging by restoring intracellular signaling dynamics that support ECM protein production, such as collagen and elastin. Given Akt's pivotal function in supporting fibroblast activity and protein biosynthesis [15,16], its activation is consistent with the observed upregulation of collagen and elastin production

(Figure 3a,b). Thus, our findings suggest that Chrono Control Penta could contribute to skin rejuvenation by modulating intracellular pathways compromised during aging.

In addition, Table S1 indicates that Chrono Control Penta produces effects comparable to those of collagen peptide treatment, as demonstrated by our assessment of collagen peptides activity in the same aged human dermal fibroblasts (Table S1). Indeed, the behavior of Chrono Control Penta closely resembles that of collagen peptides in terms of its beneficial effects on cells, particularly in enhancing collagen and elastin production, while also supporting the overall stability of the ECM. To validate the peptide efficacy, a clinical study was realized on volunteers and confirmed what we obtained at the molecular level, since we observed that, after 2 and 6 weeks of Chrono Control Penta application, the pigmentation variation was 4.9% and 5.63%, respectively, and 9.3% vs. placebo, at the end point of the study. Thus, targeting pigmentation, it promotes a gradual lightening of the skin with a final evening effect. When comparing Chrono Control Penta to other peptides used for skin lightening, we can notice several differences in their mechanisms of action and efficacy. For example, the synthetic Oligopeptide-68 prevents the production of melanin by inhibiting the activity of tyrosinase, thus brightening the skin and reducing pigmentation, and it is typically used until a concentration around 5% (35.5 mM); compared with it, Chrono Control Penta requires a much lower concentration to achieve similar or even better effects on decreasing melanin production or skin pigmentation [28]. Additionally, in the clinical study, Chrono Control Penta demonstrated a moisturizing effect (9.17% after 15 days; 13.05% after 6 weeks), maintained the skin barrier, and exhibited a firming effect (maximum extension -3.71% after 15 days and -9.86% after 6 weeks). It also showed an anti-wrinkle effect (8.38% after 15 days; 11.55% after 6 weeks), an evening effect (4.98% after 15 days; 9% after 6 weeks), a purifying effect (size: 9.38% after 15 days and 12.45% after 6 weeks), and a dermal firmness effect (3.72% after 15 days and 6.35% after 6 weeks). Among the most common commercialized cosmetic peptides, Acetyl Hexapeptide-8 (Argireline) is widely recognized for its ability to reduce the appearance of fine lines and wrinkles by inhibiting the release of neurotransmitters that trigger muscle contractions, effectively providing a temporary "Botox-like" effect [29]. However, unlike Chrono Control Penta, which targets the root causes of skin aging, such as collagen degradation and the loss of elastin, Acetyl Hexapeptide-8 focuses primarily on reducing muscle activity, thus leaving deeper structural issues unaddressed. Considering this observation, Chrono Control Penta stands out as a new generation of precision plant-derived peptide as new concept for personalized skin care. Another peptide, Tripeptide-3 (Syn<sup>®</sup>-Ake), which provides antiaging effects, is often used in higher concentrations (e.g., around 4% in some formulations), which may not be suitable for sensitive skin types or long-term use [30]. On the other hand, Chrono Control Penta is used in lower concentrations (0.5%), which makes it less likely to cause irritation or sensitivity, as proven in this study, which has demonstrated that it is not irritating, making it a more suitable option for those with sensitive skin, offering a safer, long-term solution without compromising efficacy.

In general, we can say that Chrono Control Penta is a plant derived multifunctional cosmetic peptide, never described in the literature, that stands out from other commercial peptides, which typically focus on a specific problem, while Chrono Control Penta addresses both pigmentation regulation by inhibiting tyrosinase and providing anti-wrinkles effects. In addition, it exerts moisturizing effects and improves skin purification, confirming its beneficial activity for the skin. The questionnaire results indicate a generally positive response regarding the efficacy, pleasantness, and overall satisfaction with the formulation.

Referring to peptides applied in the cosmetic field, many commercially available cosmetic products are characterized by the fact that to achieve an overall anti-ageing effect, several peptides are combined in the final formula. This decision is not actually driven by robust and systematic scientific studies that can prove the real advantages in the combination. For instance, the possibility that these peptides may interact, giving origin to dimeric adduct that may affect their ability to target the cells where they should exert the beneficial effect, cannot be excluded.

In conclusion, our study demonstrated that Chrono Control Penta promotes a 9.3% reduction in pigmentation after 6 weeks, favoring skin complexion. Furthermore, it exhibited significant moisturizing (13.05%), anti-wrinkle (11.55%), and purifying effects (12.45%), as well as firming effects (6.35%), after 6 weeks through the molecular inhibition of tyrosinase activity with an IC<sub>50</sub> value of 202.8  $\mu$ M, and improved production of collagen (up to 76.08  $\pm$  37.13% at 50  $\mu$ M) and elastin (up to 71.35  $\pm$  7.53% at 50  $\mu$ M), respectively. In line with all this evidence, the innovative and sustainable multifunctional behavior of seven-claim bioactive Chrono Control Penta peptide can be efficiently used in the cosmetic formula in which fewer active ingredients may be used. Indeed, using highly effective bioactive ingredients allows formulations to achieve optimal results with fewer components, simplifying the product and reducing the risk of interactions or sensitivities. Many active ingredients, especially in large quantities, have not been extensively studied, so focusing on a few well-researched ingredients ensures better safety and efficacy.

## 5. Patents

The authors declare that Patent  $n^{\circ}$  102023000025581, "Metodi per contrastare l'invecchiamento cutaneo", is owned by Università degli Studi di Milano, which is directly related to the content of this publication. It has been licensed to Plantech s.r.l.

**Supplementary Materials:** The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/cosmetics12030100/s1, Materials and Methods; Figure S1: Effect of H<sub>2</sub>O<sub>2</sub> on fibroblasts viability; Chrono Control Penta HPLC analysis and chromatogram; Table S1. Collagen peptides' activities evaluation on aged human dermal fibroblasts.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, the European Regulation No. 1223/2009; the Commission Regulation (EC) No. 655/2013; the EEMCO group guidelines (European group on efficacy measurement and evaluation of cosmetics and other products): EEMCO Guidance for the assessment of transepidermal water loss in cosmetic sciences (Skin Pharmacol Appl Skin Physiol. Mar-Apr 2001;14(2):117-28). The revised EEMCO guidance for the in vivo measurement of water in the skin (Skin Research & Technology, 2018, 24, pp. 351–358).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data will be provided on request.

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**Conflicts of Interest:** The authors declare no conflicts of interest, except for patent n. 102023000025581, "Metodi per contrastare l'invecchiamento cutaneo", owned by Università degli Studi di Milano, which is directly related to the content of this publication. The authors declare that Patent n° 102023000025581, "Metodi per contrastare l'invecchiamento cutaneo", has been licensed to Plantech

s.r.l., a company in which C.L. is a scientific director and co-founder. This potential conflict of interest has been managed in accordance with the ethical guidelines of the journal.

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