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"Exploring the Synergistic Anti-Pigment Efficacy and Mechanisms of a Crithmum Maritimum Ferment-Phenylethyl Resorcinol Complex"

Yuxi Yuan [1], Jie Huang [1], Qiuchan Kong [1], Jingyu He [1], Mengya Lin [1], Qingsheng Chen [1], Zhi Pan [1,*]

[1] Guangzhou Uniasia Cosmetic Technology Co. Ltd, Guangzhou, China
*Presenter and corresponding author.

1. Introduction

Melanin, synthesized within melanocytes and packaged into melanosomes, not only as a main factor determines our skin colour, but also shields the epidermis from ultraviolet radiation, and oxidative stress. Excess melanin deposition underlies hyperpigmented disorders such as melasma, freckles, solar lentigo and post-inflammatory hyperpigmentation, making precise control of melanogenesis a therapeutic priority [1]. Melanin biosynthesis is ratelimited by tyrosinase, DHICA oxidase and dopachrome tautomerase, whose transcription is governed by the microphthalmia-associated transcription factor (MITF) [2].

Macroautophagy—a lysosome-dependent mechanism that engulfs cytoplasmic cargo within LC3-decorated autophagosomes—has emerged as a key modulator of pigment metabolism. Increased LC3-II and concomitant degradation of the autophagy adaptor p62 are hallmarks of autophagy induction, whereas genetic silencing of core autophagy proteins (e.g., LC3 or ATG7) suppresses MITF expression and alters melanogenesis. Several established depigmenting agents, including tranexamic acid, coenzyme Q0 and theophylline [3], exploit this pathway by up-regulating LC3-II and/or ATG proteins to dampen melanin synthesis.

Phenylethyl resorcinol (4-(1-phenylethyl)-1,3-benzenediol; commonly known as "377") , a potent tyrosinase inhibitor has been successfully registered in China as a whitening agent since 2012 for cosmetic topical appliations [4]. However, only limited doses can be used in final products due to their formulation challenges and regulation requirements. With the increasing demand for safe and effective products of skin tone management on the Asian market, the development of novel anti-pigment solusions has become a priority.

Cithmum maritimum ferment (CMF) is a lactobacillus-fermented sea-fennel extract with anti-oxidant, anti-inflammatory and anti-melanogenic activities [5]. By combining CMF with 377 (referred to as CMF377), we observed synergistic tyrosinase inhibition and anti-melanogenic efficacy as well as superior free radical-scavenging and anti-glycation capacities. Moreover, we futher demonstrated a robust conversion of LC3-I to LC3-II and p62 depletion in MNT-1 cells with CMF377 treatment, indicating elevated autophagic flux, while

locking autophagy reversed CMF377's anti-melanogenic effect. We also oberserved fewer mature melanosomes and the appearance of autophagosomes/autolysosomes after 48 hours treatment of CMF377 using transmission-electron microscopy (TEM). This synergistic complex provides a novel and appractical approach to enhance the clinical efficacy for skin lightening and pigmentation disorders through multiple pathways.

2. Materials and Methods

Cell culture

The MNT-1 human melanoma cell line was procured from MeisenCTCC (China). Cells were maintained in DMEM medium (Gibco, USA) containing 20% (v/v) FBS (Sigma, USA), 10% AIMV™ Medium (Gibco, USA), 1% MEM Non-essential Amino Acids (MCE, China), and penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco, USA).

DPPH scavenging rate assay

The DPPH radical scavenging assay was conducted following the methodology previously outlined [5]. CMF377 was mixed with a 0.5 g/L DPPH (Sigma-Aldrich, USA). Absorbance at 519 nm was measured. The scavenging rate was normalized with blank control without DPPH addition.

In vitro glycation of BSA

Glycated BSA was prepared according to the methodology described by Anchondo-Trejo, C et al [6]. BSA (20 mg/mL, Yeasen, China) and glucose (90 mg/mL, Yeasen, China) in 0.1 M phosphate buffer (pH 7.4, Gibco, USA) were incubated with or without CMF377 at 50°C for 48 h. Aminoguanidine (AG, Sigma-Aldrich, USA) was served as positive control. The fluorescence of Advanced glycation end-products (AGEs) was measured with Synergy H1 microplate reader (Biotek, USA) at 370 (ex) and 440 (em) nm.

Western blotting

The MNT-1 cells were harvested after a 6 h compound treatment for western blot analysis. Proteins (20 µg) were separated by 12% SDS-PAGE electrophoresis and transferred onto PVDF membrane (Millipore, USA). After blocking, the membrane was incubated with primary and secondary antibody for immunolabeling, and visualized by MiniChemi® Mini Chemi/Fluorescence system (Sinsage, China). Following primary antibodies were utilized: LC3 (1: 5000, Cat# 14600-1-AP, Proteintech, USA), P62/SQSTM1 (1: 5000, Cat# 18420-1-AP, Proteintech, USA), and β-Actin (1: 100000, Cat# 66009-1-Ig, Proteintech, USA).

3D melanin skin model

The Skinovo®-Mela models (Regenovo,China) were seeded into 6-well plates and then incubated for 24 h. The tissues were divided into: BC group (blank control), NC group (negative control), PC group (kojic acid, 500 µg/mL) and CMF377 group (1 mg/mL). Each group, except for BC group, was exposed to UVB irradiation at 50 mJ/cm². Different samples (100 µL) were delivered by submersion. After 4 days of treatment, tissues were collected for analysis. Macroscopic photographs were captured by SLR camera (Nikon, Japan). The L* value was measured using Chroma Meter CR400 (Konica Minolta, China). Melanin from the tissue lysate was measured on a microplate reader at 490nm.

Transmission electron microscopy

After treated with 0.05 mg/mL CMF377 for 48 h, MNT-1 cells were fixed and stained with 1% uranyl acetate (Honeywell Fluka, USA). And images were collected by HT7800 transmission electron microscope (Hitachi, Japan).

Human raman spectroscopy penetration assay

Samples (5 mg/cm^2) were applied anterior region of forearm. Raman spectroscopy measurements were conducted at intervals of 1, 2, 4, 6, and 8 h using LabRAM Odyssey (Horiba, Japan). Data analysis was performed using Labspec software (Horiba, Jap-an).

Clinical study

A 4-week, randomized, single-center clinical study was conducted on 32 volunteers (aged between 18 and 26 years old). Ethical approval for the study was obtained from the hospital's ethics committee. All participants met the inclusion and exclusion criteria for volunteer selection. The tested cream (containing 2.1% CMF and 0.3% 377) was applied twice daily. The parameters of facial skin, such as L*, a, b and ITA° were evaluated by Skin-Colorimeter CL400, the melanin index (MI value) by Mexameter MX18, and skin glossiness by Skin-Glossymeter GL200. Facial images and the spot area analysis were conducted with VISIA. All the equipments used in clinical study were from Courage+Khazaka electronic GmbH (CK, Germany).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 9. Data are shown as mean ± SD. Significant differences between groups were assessed using one-way ANOVA or Student's t-test, with P <0.05 indicating statistical significance.

3. Results and Discussion

Recent market analyses highlight a strong demand for skin-lightening actives in Asia. Yet mainstream ingredients such as hydroquinone, kojic acid and arbutin could disrupt the epidermal barrier and heighten cutaneous sensitivity, potentially triggering inflammation and post-inflammatory hyperpigmentation in darker phototypes [7, 8]. Hence, discovering novel, safe, effective depigmenting agents and skin-tone management solutions remains a strategic priority in this region.

Phenylethyl resorcinol (“377”), a powerful hydroquinone-derived tyrosinase inhibitor, is widely used in cosmetics, but its concentration is limited by formulation constraints and regulatory caps. Cithmum maritimum ferment (CMF), a patented plant-fermentation extract, provides complementary antioxidant and anti-inflammatory activities as well as tyrosinase inhibition effect. This study assessed whether combining CMF with 377 (referred to as CMF377) delivers synergistic anti-melanogenic efficacy and also explored the mechanisms underlying this interaction.

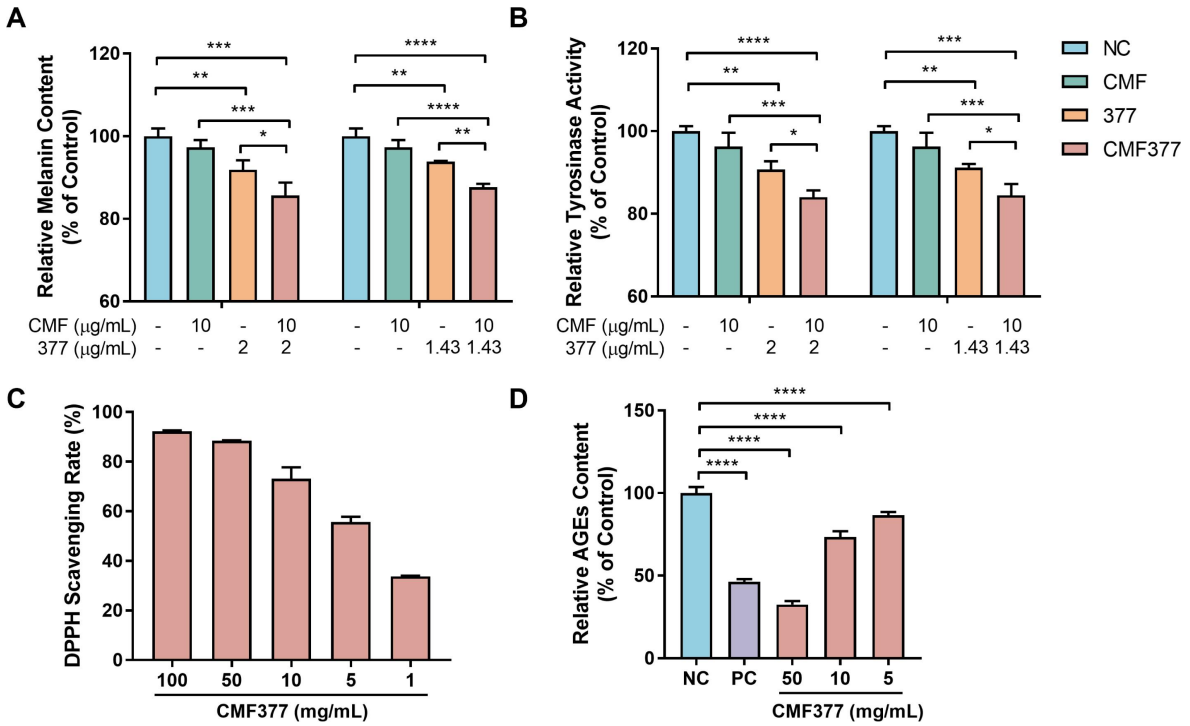

Figure 1. Co-treatment with CMF (Nuowei, China) and 377 (Symrise, Germany) synergistcally enhanced the inhibition of melanogenesis and tyrosinase activity in MNT-1 cells: (A-B) MNT-1 cells were treated with CMF and 377 alone or together for 48h to compare their impacts on in vitro melanogenesis and tyrosinase activity; (C) Results of CMF377 in DPPH scavenging rate assay; (D) Effect of CMF377 on AGEs content. **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001, error bars, S.D.

Synergistic suppression of melanogenesis

In MNT-1 human melanoma cells, CMF377 produced a clear synergistic effect on both anti-melanogenesis and tyrosinase activity inhibition. Cells were exposed for 48 hours to either individual components or CMF377 combinations at mass ratios of 5 : 1 and 7 : 1. Co-treatment with CMF377 suppressed melanin synthesis and tyrosinase activity far more effectively than CMF or 377 alone (Figure 1A,B). On the basis of these results, a CMF : 377 ratio of 7 : 1 was selected for further work, yielding a test formulation containing 2.1 % CMF and 0.3 % 377. This composition was used as the active material in all subsequent MNT-1 and other in-vitro assays.

Antioxidant and anti-glycation capacity

Phenylethyl resorcinol down-regulates the oxidative-stress sensor Nrf2 [9], whereas CMF efficiently scavenges free radicals and suppresses inflammatory mediators [5]. In DPPH assays CMF377 exhibited strong radical-scavenging capacity (Figure 1C), reflecting the combined antioxidant power of its two components. Because UV-induced reactive oxygen species (ROS) trigger DNA damage and melanogenic signalling, this antioxidant activity is directly relevant to photoprotection [10].

CMF377 also showed pronounced anti-glycation activity. In the BSA–glucose model of non-enzymatic protein glycation, CMF377 significantly inhibited AGE formation (Figure 1D). Such advanced glycation end-products generated both by ageing and UV exposure drive oxidative stress, inflammation and MITF-dependent melanogenesis [11].

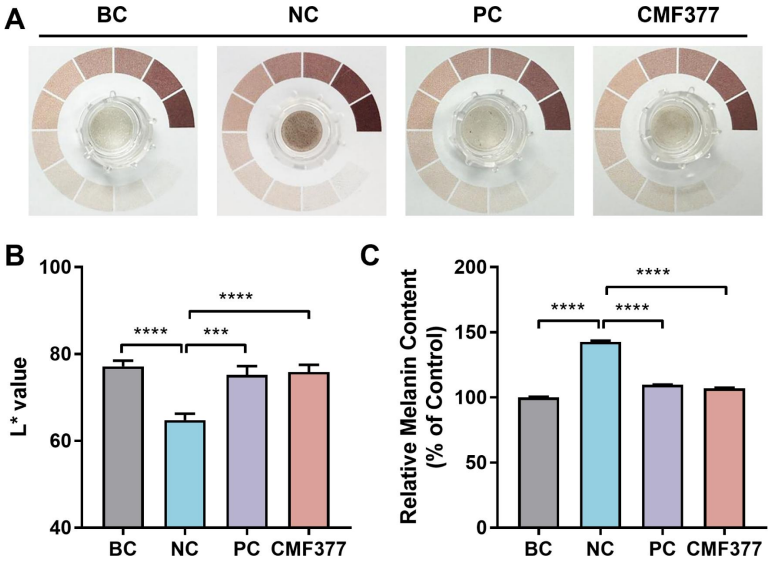

Figure 2. CMF377 decreased melanin production in a 3D skin model exposed to UVB: (A) Apparent chromaticity was measured after 4 days' treatment of CMF377; (B) L* value results; (C) Melanin content results. ***P <0.001, ****P <0.0001, error bars, S.D.

Furthermore, in a UVB-challenged 3-D reconstructed skin model, pretreatment with CMF377 markedly lightened the tissue, as evidenced by higher L* values (Figure 2A,B) and reduced melanin content (Figure 2C). These results indicate that CMF377 mitigates UV-induced hyperpigmentation, through combined anti-melanogenesis, antioxidant, anti-glycation and anti-inflammatory effects of this combination.

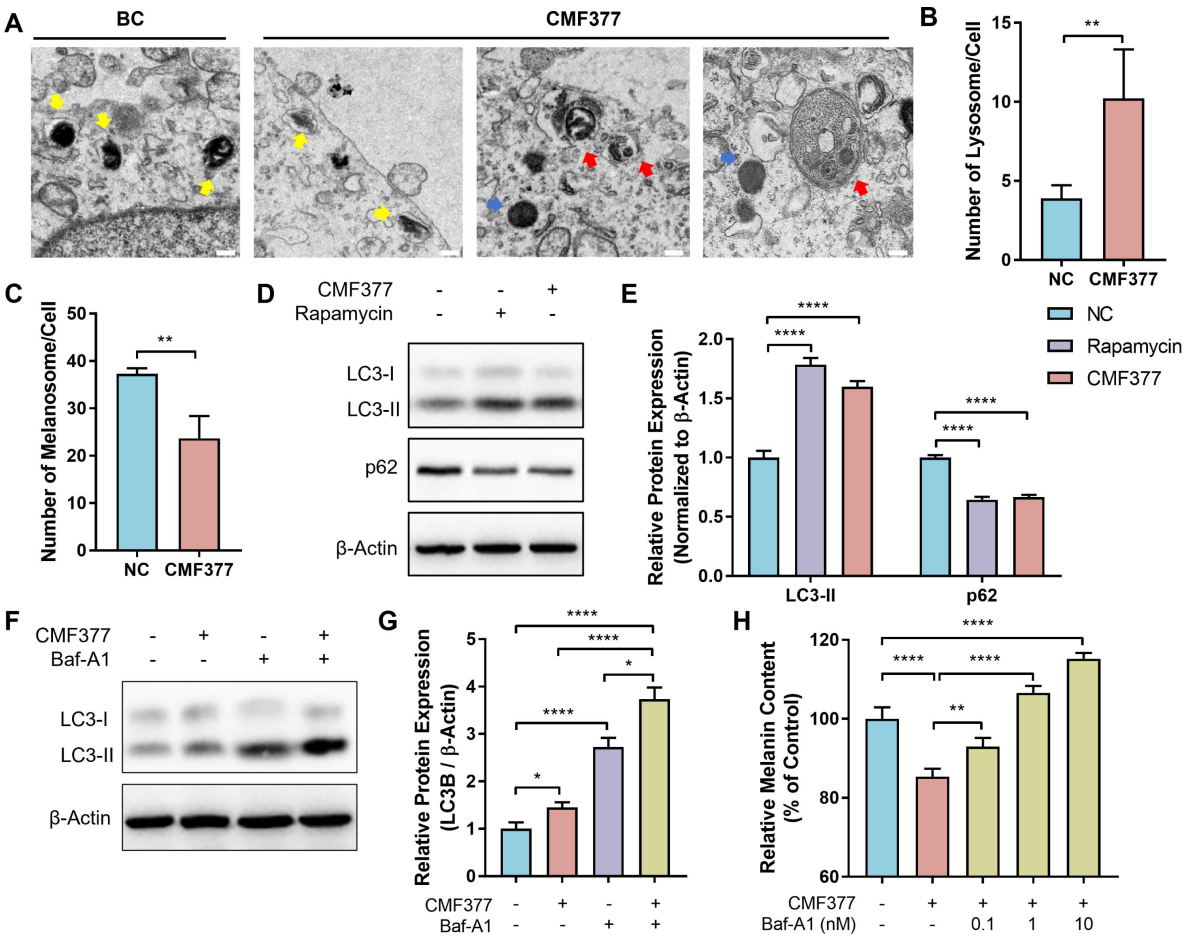

Figure 3. CMF377 inhibited melanogenesis by promoting melanocyte autophagy: (A) MNT-1 cells were treated with CMF377 (0.05 mg/mL) for 48 h, and the melanosomes (yellow arrow), lysosomes (blue arrow) and autophagosomes (red arrow) were determined by TEM; (B-C) Quantification of the lysosome and melanosome number in each cell; (D) Representative immunoblots of LC3B and p62 protein expression in MNT-1 cells treated with CMF377 (0.05 mg/mL) or rapamycin (1 nM) for 6 h; (E) Quantification of LC3 II and p62 pretein levels normalized to β-actin; (F) Representative immunoblots of LC3B in MNT-1 cells treated with CMF377 (0.05 mg/mL) and/or Baf-A1 for 6 h; (G) Quantification of LC3 II normalized to β-actin; (H) MNT-1 cells were treated with CMF377 (0.05 mg/mL) for 48 hours to measure the effect on melanin production in MNT-1 cells, in the presence or absence of Baf-A1. Yellow arrow points to melanosomes; blue arrow points to lysosomes; red arrow points to autophagosomes. **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001, error bars, S.D.

Autophagy-mediated melanin degradation

In addition to tyrosinase inhibition, CMF377 activates macroautophagy as an alternative depigmenting pathway. Autophagy-mediated degradation of melanosomes in keratinocytes and melanocytes is a recognised regulator of skin pigmentation [12]. To determine whether CMF377 exploits this mechanism, we analysed the autophagy markers LC3 and p62 in MNT-1 cells. CMF377 markedly promoted LC3-I → LC3-II conversion and accelerated p62 degradation (Figure 3D,E). Autophagic flux was quantified with bafilomycin A1 (Baf-A1, TargetMol, USA), an inhibitor of autophagosome-lysosome fusion, is known to elevate LC3II

levels. CMF377 further elevated LC3-II in the presence of Baf-A1, confirming enhanced flux (Figure 3F,G). Notably, Baf-A1 restored melanin synthesis in CMF377-treated cells (Figure 3H), establishing autophagy as essential for its anti-melanogenic effect. Transmission electron microscopy corroborated these findings, revealing fewer intact melanosomes and abundant autophagosomes/autolysosomes after CMF377 exposure (Figure 3A–C). Together, these results demonstrate that CMF377 suppresses melanogenesis by triggering autophagy-dependent melanosome degradation in MNT-1 melanocytes.

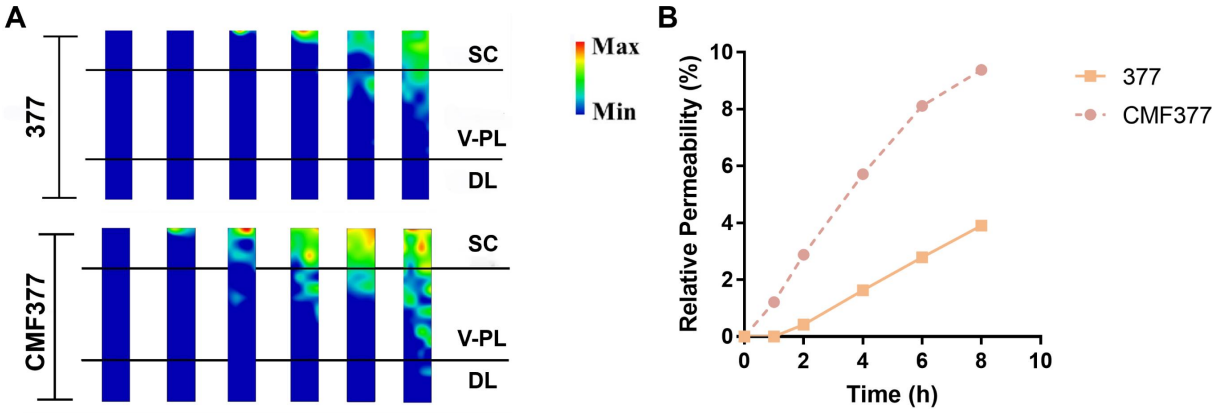

Figure 4. CMF enhanced the penetration of 377 into skin layers: (A) Content distribution map of 377 and CMF377 at different skin depths; (B) Comparison of skin penetration rates of 377 and CMF377 at different time periods.

Enhanced in vivo skin penetration

Real-time in-vivo skin permeation of 377 and CMF377 was evaluated with Raman spectroscopy. The results showed that CMF markedly improvesd the penetration of 377. 377 crossed the stratum corneum within 1 h, diffused through the viable epidermis over 4–6 h, and reached the upper dermis by 8 h (Figure 4). This penetration enables CMF377 to reach basal-layer melanocytes, simultaneously suppressing melanogenesis and accelerating melanin degradation.

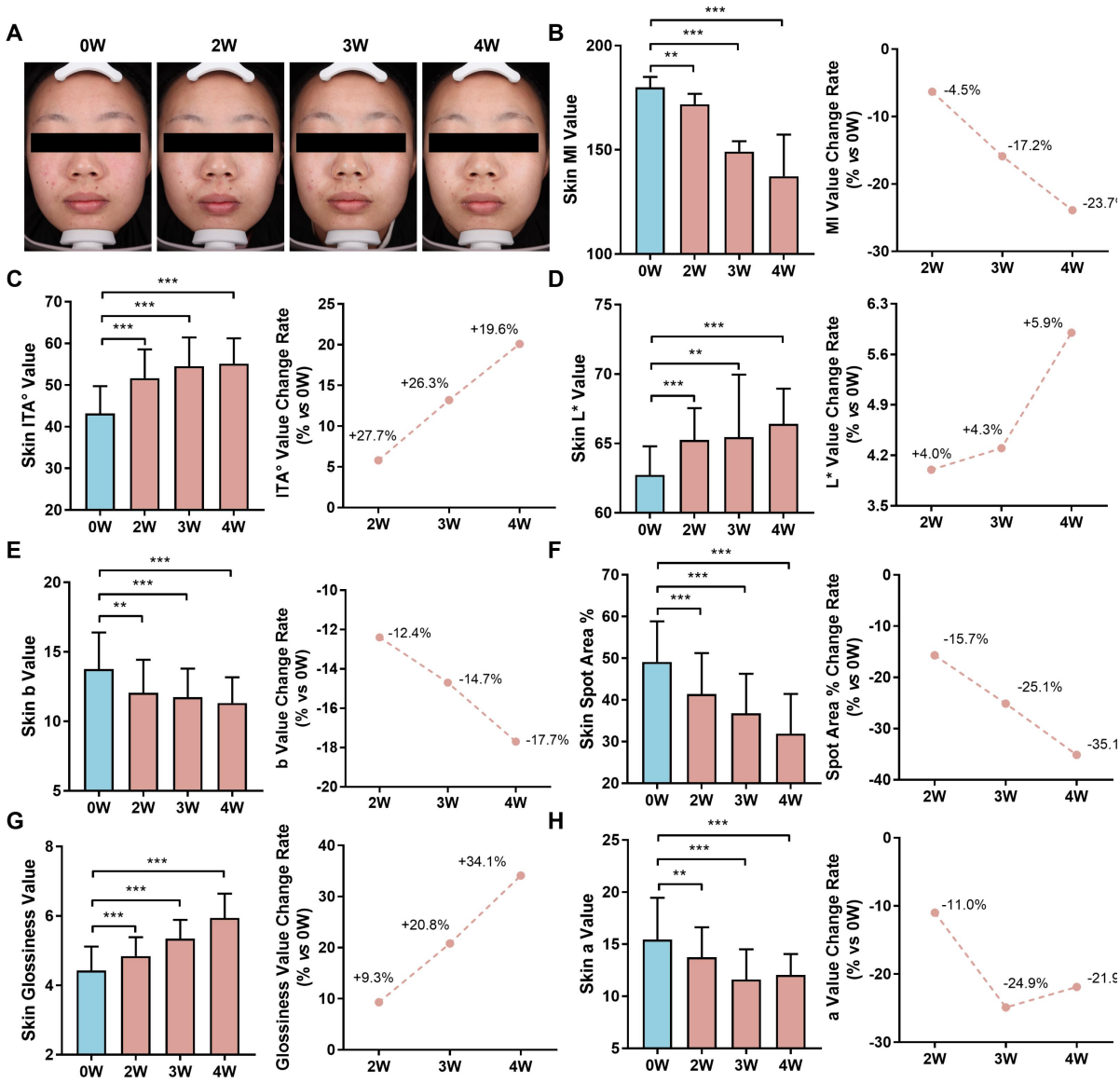

Figure 5. CMF377 reduced skin melanin content and incidence of erythema, and impr oved skin brightness and luster: (A) Alterations in facial erythema observed pre and post-treatment with CMF377; (B-H) Comparison of melanin index, skin ITA° value, skin L* value, skin b value, skin spot area, skin glosiness value and skin a value before and after treatment. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, error bars, S.D.

Accelerated clinical performance

A two-week facial application study showed that CMF377 lowers melanin indices far more rapidly than conventional four-week regimens, confirming its accelerated in-vivo efficacy. After 14 days of treatment, clinical assessments revealed significant increases in L* and ITA° values and in skin gloss (Figure 5C, 5D, 5G). Conversely, the melanin index, a* and b* colour parameters, and total spot area all fell markedly (Figure 5A, 5B, 5E, 5F, 5H). These results demonstrate that CMF377 effectively decreases cutaneous melanin and hyperpigmented spots while enhancing overall skin brightness and luminosity.

4. Conclusion

Collectively, our data show that CMF enhances the performance of phenylethyl resorcinol by supplying complementary antioxidant, anti-glycation, and anti-inflammatory protection, while simultaneously intensifying depigmenting efficacy through synergistic tyrosinase inhibition, improved skin bioavailability, and autophagy-driven melanosome degradation. CMF377 thus represents a well-tolerated, rapidly acting solution for correcting hyperpigmentation within permissible concentration limits, supporting its applications in advanced cosmetic formulations and potential therapeutic interventions.

Conflict of interest statement

The authors declare no conflict of interest.

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