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Improvement of Melasma With Glabridin-Containing Skin Brightening Product: Clinical and *In Vitro* Evaluation

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Synopsis

Melasma is a common skin disorder characterized by alterations in normal skin pigmentation. Glabridin is confirmed to have anti-melanogenesis activity in skin. However, the clinical whitening effects of glabridin still remain to be investigated. This study is aimed to elucidate the clinical whitening performance in melasma and nonmelasma areas via a whitening serum containing glabridin. The inhibitory mechanisms on melanogenesis of the whitening serum containing glabridin was also evaluated by a 3D skin model. The whitening serum effectively improved apparent chromaticity of the melanin model, increased the L* value, and regulated the content and distribution of melanin. A 56-day clinical experiment showed that glabridin effectively improved the skin glossiness and individual typology angle (ITA) value in both melasma and nonmelasma areas. Remarkably reduced melasma area proportion and melanin content were also observed in the melasma areas and nonmelasma areas, respectively. This work demonstrates that a formula containing glabridin could effectively improve pigmentation through 3D skin model and clinical results.

INTRODUCTION

Melasma is a disorder of skin pigmentation characterized by the development of asymmetrical, hyperpigmented macules in sun-exposed areas, especially the upper lip, the cheeks, the forehead, and the neck.[1] The development of this disorder may be attributed to sun exposure, genetic predisposition, pregnancy, oral contraceptives, and certain antiepileptic drugs. Treatment can be difficult, as long-term therapy is often required, and recurrence is common. The first line of treatment is the elimination of risk factors through topical treatment based on sun protection and products aimed at inhibiting melanocyte activity, melanin synthesis, disrupting melanin granules, and removing melanin. The second and third lines of treatment include chemical peels, lasers, and lights.[2] Patients with melasma hyperpigmentation are often enrolled in clinical studies to evaluate whitening or depigmenting products.

Whitening agents such as hydroquinone, corticosteroids, and tretinoin are medically applied to effectively lighten the skin tone of hyperpigmented lesions.[3] However, a variety of side effects cannot be ignored when synthetic agents are used in the cosmetic field.[4] There is a

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growing interest today in cosmetic products that come from naturally derived components. Studies have screened abundant components from natural products, represented by arbutin, tetrahydrocurcumin, and pterostilbene, all of which show favorable effects on pigmentation reduction.[5–7]

Naturally whitening active ingredients can inhibit the formation and transfer of melanin through multiple pathways.[8] Many of these actives are reported to inhibit the transferring of melanin from melanocytes to keratinocytes.[9] For example, glabridin, a prenylated isoflavonoid of *G. glabra L.* roots, has been associated with a wide range of biological properties, including regulation of estrogenic, energy metabolism, neuroprotective, and skin whitening in previous studies.[10] The inhibition activity against tyrosinase and melanin synthesis of glabridin may be the main whitening mechanisms.[11–13] While the present studies have mainly focused on the whitening effects of glabridin at the cell level (melanocytes), few clinical reports on the effects of glabridin on melasma have been published.

In this study, a skin 3D model was established and the effects of a whitening serum containing glabridin on the apparent chromaticity, L* value, along with the content and distribution of melanin were studied. Meanwhile, the whitening effect of the serum on the melasma area and nonmelasma area was studied through clinical experiments.

MATERIALS AND METHODS

MATERIALS

Glabridin (98.5%) was bought from Push Bio-Technology (Push Bio-Technology, Ltd., Chengdu, China). Dimethyl sulfoxide and kojic acid were bought from Sigma-Aldrich (Sigma, Massachusetts, USA). The 3D skin model provided was MelaKutis® (Guangdong Boxi Biological Technology Co., Ltd., Guangdong, China). All other chemicals used were of analytical grade.

PIGMENTATION IN 3D SKIN EQUIVALENT MODEL

The reconstructed human 3D epidermis (MelaKutis®) consisted of normal human-derived epidermal keratinocytes and normal human epidermal melanocytes that had been cultured to form a multilayered, highly differentiated model, similar to real skin structure. The blank control (BC) group changed the culture medium every day without any other treatment; the negative control (NC) group, the positive control (PC) group with kojic acid (500 μg/mL), and the whitening serum group were required to change the culture medium every day and undergo ultraviolet-B (UVB) stimulation every day (50 mJ/cm^2). The whitening serum and PC groups used samples on the model surface on day 3 and day 5.The test samples were applied topically in a volume of 10 μL. Controls were not treated. The change in media and application of test material was repeated every 2 days for 7 days. The chemical was added in the lower well of the reconstructed epidermis system then penetrated through a membrane to reach the basal cells. The epidermis was then fixed with 4% formalin in phosphate-buffered saline, followed by Fontana-Masson staining to visualize melanin pigments. All tissues were photographed using a digital camera for a visual assessment of depigmentation. It was then processed further for determining skin lightening as a measure

of the tissue luminance values (L* unit) using a CM-2600d Spectrophotometer® (Konica Minolta, Tokyo, Japan), and the melanin reduction was quantified spectrophotometrically by solubilizing the tissue using solvable.

MELANIN CONTENT MEASUREMENT

Briefly, the cell pellets were solubilized in 1 mol/L NaOH (80°C) for 40 minutes. The melanin was evaluated at 405 nm using a spectrophotometer. Melanin was determined by the protein's absorbance from the cell extract.

MELANIN DISTRIBUTION

All samples were fixed with 4% formaldehyde solution for 24 hours after 8 days of cell culture. The sliced samples were stained according to the silver staining kit directions, and then photographed under the microscope. Intel® Integrated Performance Primitives (Intel, California, USA) software was used to determine the relative integral optical density (IOD).

CLINICAL STUDY

Subjects. 33 Chinese urban women between the ages of 33 and 51 were enrolled in the study. These selected volunteers showed the following characteristics: lackluster complexion, color spot density on the cheek of $\geq$2, at least one 2 mm independent color spot on the face, no obvious redness, and skin lesions or scars on the face. During the study period, strong sun exposure was to be avoided. All subjects participating in this study signed an informed consent form. Patients were excluded from the study if they were pregnant, breastfeeding, in hormone or corticosteroid therapy, or had a history of endocrine disorders or allergies. Additionally, potential volunteers who used depigmenting or whitening products (oral or topical) in the past 6 weeks were excluded.

Test materials and procedure. The test formula contained glabridin was in a vehicle base. A neutral cream without glabridin was used as a placebo. Additionally, the whitening essence passed the safety test. Both study formulas were identical in appearance and supplied with two identical pump dispensers. One test formula was applied twice a day on the subjects' right face and the other one on the left face for 56 days, and the subjects were also asked to not use any cosmetics containing ingredients that could potentially interfere with their skin color status during the study period. The skin color (Colorimeter® CL400, EnviroDerm Services, Longhope, UK), melanin index value (Mexameter® MX18, EnviroDerm Services, Longhope, UK), the gloss of skin (Glossymeter® GL200, EnviroDerm Services, Longhope, UK) and photographic documentation (VISIA-CR, Canfield Scientific Inc., New Jersey, USA) were evaluated at baseline, then at 14 days, 28 days, and 56 days after treatment. During evaluation, the entire study was conducted under controlled environmental conditions of temperature and relative humidity. Participants were asked to rest for at least

15 minutes in a temperature of 20–24°C and humidity of 40–60% (relative humidity) before instrumental measurements.

Statistical analysis. All statistical analyses of data were performed using OriginPro® 9.0 (OriginLab Corporation, Massachusetts, USA). The significance analysis was performed using SPSS 13.0 software (IBM, New York, USA). In the analysis results, * indicates comparison with baseline, $p \leq 0.05$; ** indicates comparison with baseline, $p \leq 0.01$; # indicates comparison with the control group, $p \leq 0.05$; ## indicates comparison with the control group, $p \leq 0.01$.

RESULTS AND DISCUSSION

EFFECT OF WHITENING SERUM ON APPARENT CHROMATICITY OF MELANIN MODEL

The 3D reconstructed pigmented skin model composed of keratinocytes and melanocytes was employed for skin whitening efficacy testing. The 3D melanin skin model is highly similar to human skin structure and function. A multi-dimensional scientific detection of whitening products was carried out with respects to the apparent chromaticity, L* value, and melanin content.[14] The chromaticity appearance of the melanin model is shown in Figure 1. Compared to the BC group, the more darkened apparent chromaticity of the NC group was significantly, while the apparent chromaticity of the PC group was significantly white relative to NC group. The color of the model in the whitening serum group was obviously lighter, indicating that the whitening serum had a significant improvement effect on the apparent chromaticity of the melanin model.

CHANGES OF L* VALUE AND MELANIN CONTENT

The color change of skin model was measured by a L*a*b* chromaticity system spectrophotometer. The L* value refers to brightness. The higher the value, the more the color tends to be white, and vice versa. In Figure 2A, compared with the NC group, the whitening serum group significantly improved the L* value of the melanin model ($P < 0.01$). Changes in melanin content are also shown in Figure 2B. The whitening serum group displayed the lowest melanin content among the groups, indicating that treated groups containing glabridin had a significant inhibitory effect on melanin ($P < 0.01$).

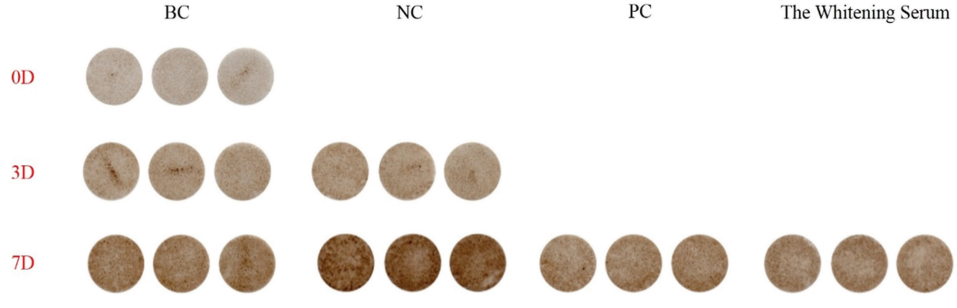

Figure 1. Chromaticity appearance of the melanin model.

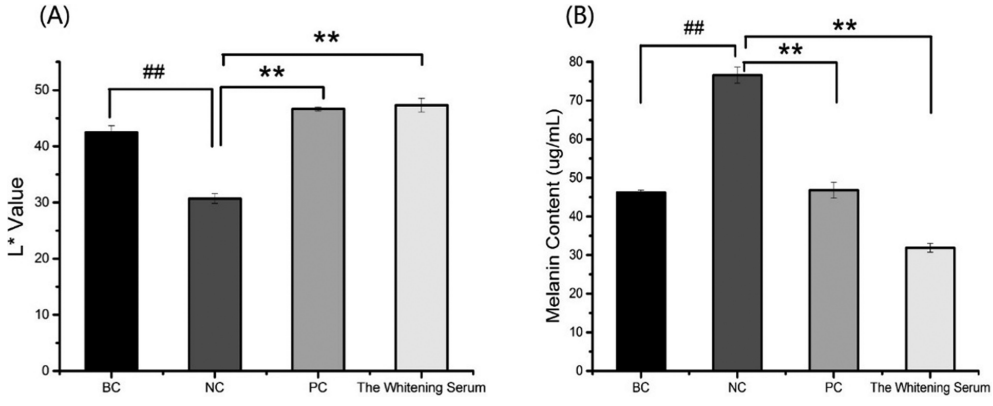

Figure 2. Changes of L* value (A) and melanin content (B) in melanin model. Data are expressed as the mean ± SD (n = 3). #$P < 0.05$ and ##$P < 0.01$ indicate significant differences between the BC group and the NC group. *$P < 0.05$ and **$P < 0.01$ indicate significant differences between the NC group and the other group.

MELANIN DISTRIBUTION

In Figure 3A, compared with the BC, the distribution of melanin granules in the NC group increased, while the distribution of melanin granules of the model in the PC group decreased significantly. Compared with the NC group, the distribution of melanin granules in the whitening serum group clearly decreased, indicating that the whitening serum had a significant improvement on the reduction of melanin granules in the melanin model. The relative IOD value represents relative stain fluorescent strength. As can be seen quantitatively in Figure 3B, the NC group, 50mJ/cm^2 UVB, produced a relative IOD of 3.2. It shows the induction of melanin accumulation significantly ($P < 0.01$). Compared with the NC group, the relative IOD value of whitening essence group was significantly decreased 78.1% ($P < 0.01$).

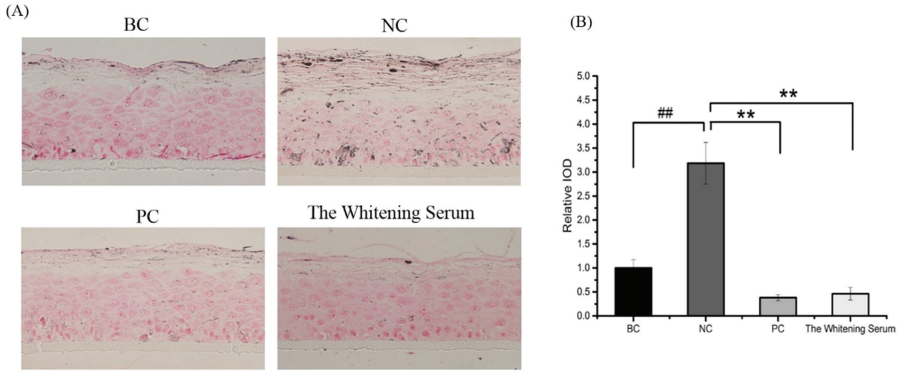

Figure 3. Detection of melanin distribution in the 3D reconstructed pigmented skin model (A) and the relative IOD value (B). Data are expressed as the mean ± SD (n = 3). #P < 0.05 and ##P < 0.01 indicate significant differences between the BC group and the NC group. *P < 0.05 and **P < 0.01 indicate significant differences between the NC group and the other group.

CLINICAL ASSESSMENT

Melasma area. As shown in Figure 4A, an increasing skin glossiness was observed for the control and treated groups. Specially, a significant increase was found for treated groups until the day 28 treatments, compared to the initial treatment (day 0), while the control group only displayed a slight increase with significant difference. The differences between the control and treated groups were clearly observed after a 14-day experiment, where treated groups showed higher glossiness than the control group. The ITA values (Figure 4B) of the experiment group and the control group increased significantly on day 28 and day 56 compared with day 0 ($P < 0.01$). There was significant difference between the experiment

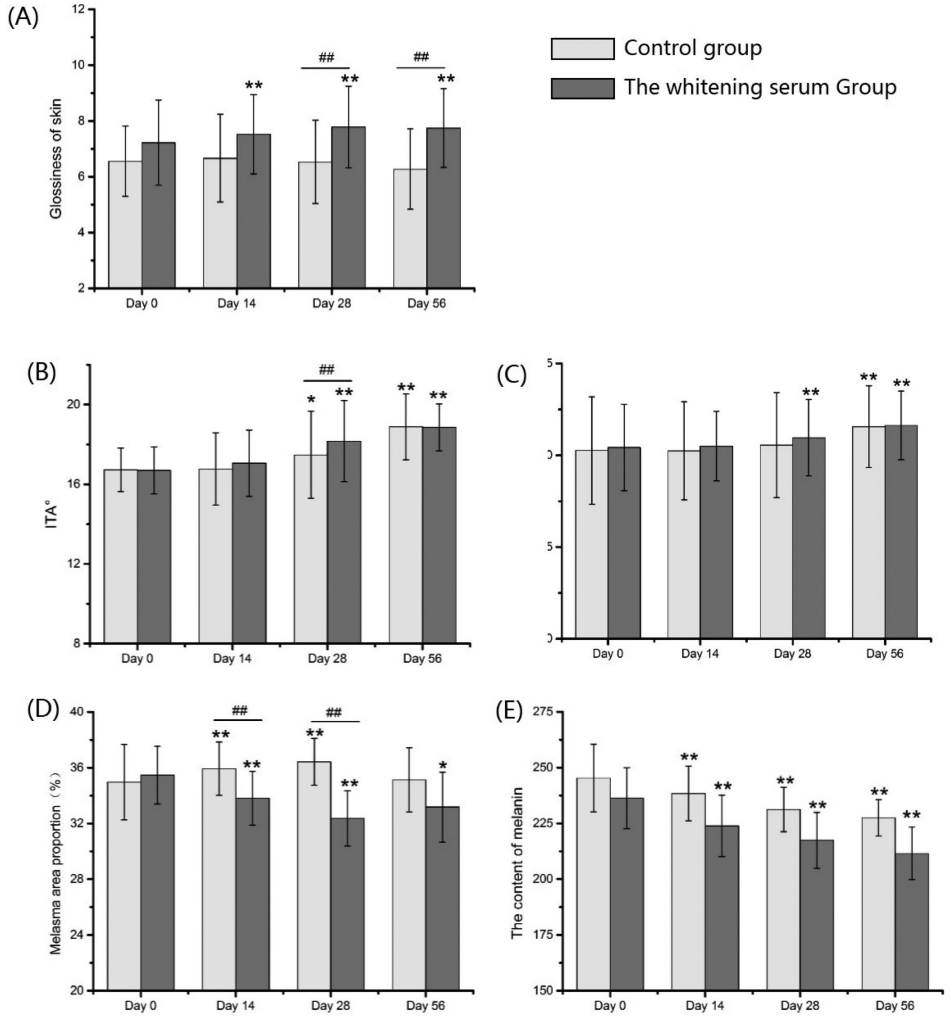

Figure 4. Clinical study of melasma areas. The changes of (A) glossiness, (B) ITA, (C) the value of L*, (D) melanin area proportion, and (E) the content of melanin of these subjects treated by the test sample (the whitening serum group) and placebo (control group) along with the varied periods. *$P < 0.05$ and **$P < 0.01$ indicate significant differences compared with day 0. #$P < 0.05$ and ##$P < 0.01$ indicate significant differences between experiment group and control group.

group and the control group on day 28 ($P < 0.01$). The L* value (Figure 4C) of both the experiment group and the control group increased significantly on day 56 ($P < 0.01$), but there was no significant difference between them. The area proportion of melanin (Figure 4D) in the experiment group was significantly decreased on the day 14, 28 ($P < 0.01$), and 56 ($P < 0.05$), and there was a significant difference between the experiment group and the control group on day 14 and day 28. The content of melanin (Figure 4E) of the experiment group and the control group decreased significantly on days 14, 28, and 56 ($P < 0.01$), but there is no significant difference between the two groups. These results suggested that the whitening essence containing glabridin is effective in the treatment of melasma.

Nonmelasma area. The clinical evaluation of the nonmelasma area is seen in Figure 5. The changes of skin glossiness (Figure 5A) in the experiment group on day 28 and day 56 were significantly increased when compared with day 0 ($P < 0.01$). There were significant differences ($P < 0.01$) between the experiment group and the control group on day 28 and day 56. The L* value (Figure 5B) of both the experiment group and the control group increased significantly on day 56 ($P < 0.01$), but there is no significance between the two groups. The ITA values (Figure 5C) of the two groups were significantly different

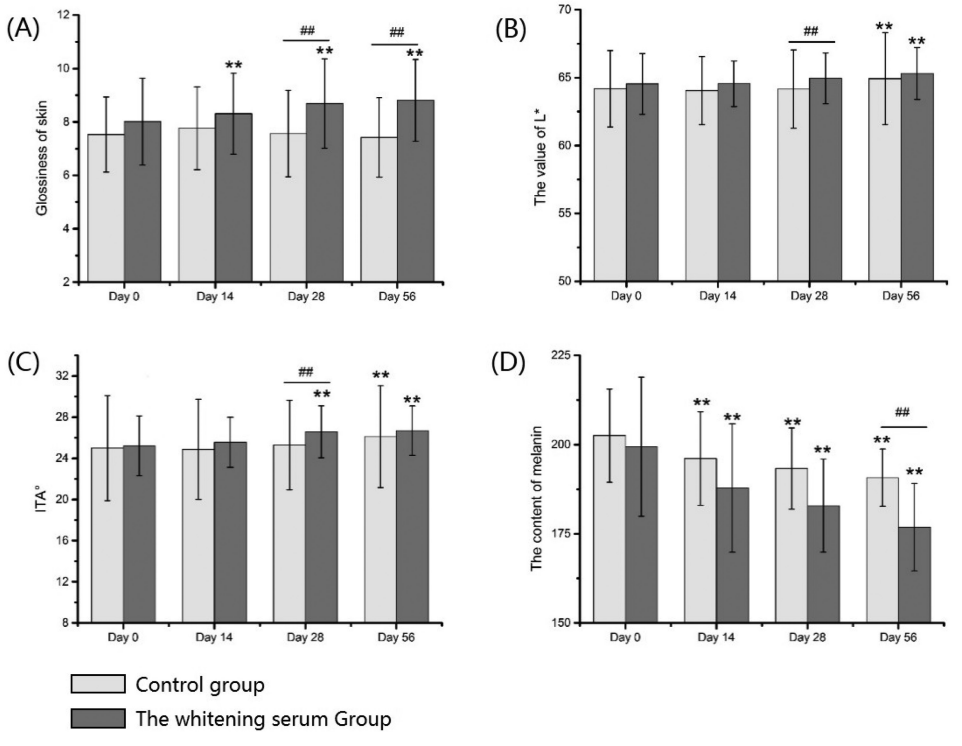

Figure 5. Clinical study of nonmelasma areas. The changes of (A) glossiness, (B) The value of L*, (C) ITA, and (D) the content of melanin of these subjects treated by test sample (the whitening serum group) and placebo (control group) along with the varied periods. *$P < 0.05$ and **$P < 0.01$ indicate significant differences compared with day 0. #$P < 0.05$ and ##$P < 0.01$ indicate significant differences between the experiment group and the control group.

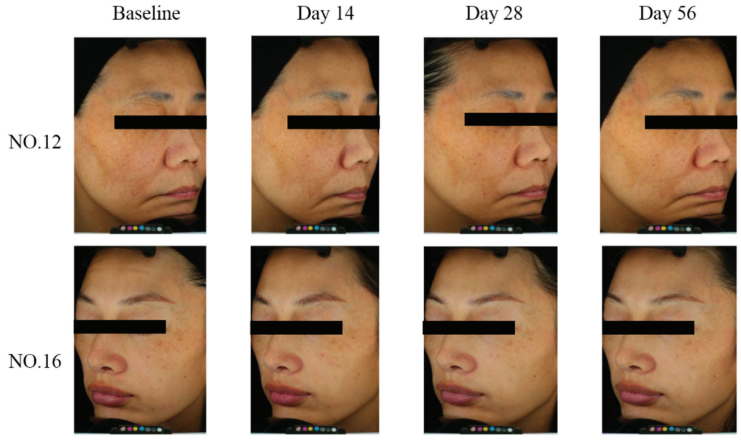

Figure 6. Images obtained by VISIA-CR.

($P < 0.01$) on day 28. The content of melanin (Figure 5D) in the experiment group was significantly decreased on days 14, 28, and 56 ($P < 0.01$), and there was a significant difference ($P < 0.01$) between the experiment group and the control group on day 56. These results suggested that the whitening serum containing glabridin is also effective for brightening nonmelasma areas.

VISIA-CR ANALYSIS

The visual changes of two subjects (NO.12 and NO.16) treated with the whitening serum for different periods are observed in Figure 6. The application of whitening serum caused a remarkable improvement of skin color for both subjects. A significantly reduced area of skin stain and increased skin lightening were observed after day 56 treatments. At the same time, the area of melasma in the facial skin was significantly reduced and the color became lighter.

DISCUSSION

Melasma is a pigmentary disorder that typically appears on the face, forehead, and cheeks. Although the effectiveness of depigmenting agents (containing hydroquinone) or chemical peels (lactic acid, glycolic acid, ascorbic acid, etc.) was confirmed, the potential side effects caused the rising concerns over their safety.[15,16] Glabridin, as the main hydrophobic component of the flavonoid extract, uralensis, was confirmed to prevent the formation of melanogenesis by decreasing tyrosinase activity (IC 50: 0.43 μmol/L).[17,11]

In this study, the efficacy of this formula containing glabridin was evaluated in the treatment of epidermal melasma and nonmelasma areas in Chinese women utilizing a bilateral (split-face) model. After 56 days, the clinical experiment results show that glabridin can effectively improve the skin glossiness and ITA value in melasma and nonmelasma areas. Meanwhile, there are significant reductions of melasma area proportion in melasma areas

and the melanin content in nonmelasma areas. However, no clear differences in the melanin content were found between the two groups. The uneven color of melasma may contribute to this finding, as subtle differences in the test values may not be accurately reflected in the treatment outcome. At the same time, it was relatively difficult to remove melanin, due to its deep location in the epidermis. It seemed that only addition of glabridin displayed limited effects. The combination with other whitening ingredients was recommend to achieve better whitening effects.

The skin color modulated by the transfer of melanin synthesized by melanocytes was embedded by the melanosome to neighboring keratinocytes. The 3D reconstructed pigmented skin model composed of human melanocytes in the basal layer of multilayered epidermal keratinocytes was used to detect the changes of melanin content. Hence, the distribution of melanin in the different skin layers can be well recognized by its brown-colored pigmentation for the 3D pigmented skin model. The inhibitory effects on melanogenesis of the whitening serum containing glabridin was also evaluated by 3D skin model. The whitening serum effectively improved the apparent chromaticity of the melanin model, increased the L* value, and regulated the content and distribution of melanin.

CONCLUSION

The whitening serum containing glabridin effectively improved the apparent chromaticity of the melanin model, increased the L* value, and regulated the content and distribution of melanin. A 56-day clinical experiment showed that glabridin effectively improved the skin glossiness and ITA value in both melasma and nonmelasma areas. Meanwhile, a remarkably reduced melasma area proportion and melanin content was observed in melasma areas and nonmelasma areas, respectively. In summary, these results suggest that a formula containing glabridin could effectively improve pigmentation in 3D skin models and volunteers with epidermal melasma.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Gang Huo, Liping Du, and Ping Ma designed the experiments and wrote the paper. Ying Zhou performed the experiments. Xinfen Cai analyzed the data.

ETHICAL APPROVAL

The safety test was carried out in Osmum Biological Co., Ltd, Huzhou, China. Before the study, each of the participants signed a copy of the informed consent. The protocol of the 24-hour patch test passed review by the Ethical Commission of Osmum Biological Co., Ltd. The 56-day clinical research was carried out in SGS Testing Center, Shanghai, China. The research protocol was examined and approved by the SGS Ethics Committee for Clinical Research. Benefits, risks, and potential complications were explained to the subjects, and informed written consent was obtained from participants.

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