

ORIGINAL ARTICLE

Dendrobium officinale flowers' topical extracts improve skin oxidative stress and aging

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Funding information

National Key Research and Development Program of China, Grant/Award Number: 2017YFC1702200 and 2017YFC1702202; Foundation for Innovative Research Groups of the National Natural Science Foundation of China, Grant/Award Number: 82274134

Abstract

Background: *Dendrobium officinale* flowers (DOF) have the effects of antiaging and nourishing yin, but it lacks pharmacological research on skin aging.

Objective: Confirming the role of DOF in delaying skin aging based on the “in vitro animal–human” model.

Methods: In this experiment, three kinds of free radical scavenging experiments in vitro, D-galactose-induced aging mouse model, and human antiaging efficacy test were used to test whether DOF can improve skin aging through anti-oxidation.

Results: In vitro experiment shows that DOF has certain scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, hydroxyl free radical, and superoxide free radical, and its IC₅₀ is 0.2090 μg/mL, 15.020, and 1.217 mg/mL respectively. DOF can enhance the activities of T-AOC, SOD, CAT, and GSH Px in the serum of aging mice, increase the content of GSH, and reduce the content of MDA when administered with DOF of 1.0, 2.0, and 4.0 g/kg for 6 weeks. In addition, it can enhance the activity of SOD in the skin of aging mice, increase the content of Hyp, and decrease the content of MDA, activated Keap1/Nrf2 pathway in the skin of aging mice. Applying DOF with a concentration of 0.2 g/mL on the face for 8 weeks can significantly improve the skin water score and elasticity value, reduce facial wrinkles, pores, acne, and UV spots, and improve the facial brown spots and roughness.

Abbreviations: BL, baseline; CAT, catalase; DOF, *Dendrobium officinale* flowers; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GSH, glutathione; GSH-PX, glutathione peroxidase; H&E, hematoxylin and eosin; Hyp, hydroxyproline; Keap1, kelch-like ECH-associated protein 1; MDA, malondialdehyde; Nrf2, nuclear factor-erythroid-2-related factor 2; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

Xing-Li-Shang He, Ning Wang and Xi Teng these three authors contributed equally to this work.

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Conclusion: DOF can significantly improve skin aging caused by oxidative stress, and its mechanism may be related to scavenging free radicals in the body and improving skin quality.

KEYWORDS

antioxidant capacity, *Dendrobium officinale* flower, in vitro animal-human model, skin aging

1 | INTRODUCTION

Dendrobium candidum is the dry stem of orchid plant *Dendrobium officinale* et Migo, which possesses the effect of benefiting stomach and facilitating fluid production, nourishing yin, and clearing heat.¹ *D. candidum* is listed as the first of the "Nine Fairy Grasses" in Taoist collection, and it is a large variety of traditional Chinese medicine in Zhejiang Province. Contemporary pharmacological researches have found that *D. candidum* has the effects of moisturizing, anti-oxidation, antiaging, enhancing immunity, and improving metabolic diseases.²⁻⁴ The Chinese Pharmacopoeia stipulates that *D. candidum* is applied as medicine with dry stems. At present, *D. candidum* is widely used in medicine, health food, and daily chemical industry due to its rich pharmacological effects. Compared with the traditional medicinal part of *D. officinale*-stem, *Dendrobium officinale* flowers (DOF) are commonly directly discarded or exclusively processed into food as by-products in the process of drug collection, resulting in a waste of resources. Studies have shown that DOF have certain antioxidant activity,⁵⁻⁷ but there are few reports on the application of DOF in anti-skin aging. Therefore, based on the rational and diversified utilization of *D. officinale* resources, this experiment studies the antioxidant functional advantages of DOF, and provides scientific basis for the rational utilization of *D. officinale* resources and the enrichment of scientific connotation of *D. officinale*.

The team participated in the formulation of the Zhejiang Local Food Safety Standard Dry *D. officinale* flowers (DB33/3011-2020). The team's previous research demonstrated that both water-soluble and alcohol-soluble components of DOF have certain antioxidant activity. However, it is unclear whether DOF can regulate oxidative stress in the body to combat aging when the human body absorbs the drug ingredients. We need to figure out whether it can play its anti-oxidation and antiaging effect to enhance skin condition and relieve skin wrinkles, and pore spots.

Therefore, we desire to investigate the anti-skin aging effect and mechanism of *D. candidum* in vitro, animals and human body. In this experiment, first, the scavenging rates of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, superoxide free radicals and hydroxyl free radicals of water-soluble components of DOF were compared. We selected and applied the extracts to the aging model mice induced by D-galactose and we tested the MDA, SOD, T-AOC, CAT, GSH, GSH-Px, Keap1, Nrf2, and other indicators associated to oxidative stress in animal serum and skin tissue to evaluate the pharmacodynamic effect on aging mice of DOF. In addition, when the volunteers applied the extract of *D. officinale* on their facial skin, we use a five spectrum skin detector to further verify the antioxidant effect of *D.*

officinale with monitoring indicators of wrinkles, pores, UV spots, and brown spots of human facial skin.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

Dendrobium officinale flowers were provided by the Zhejiang Senyu Co., Ltd., and the batch numbers are 201704 and 202206. Test kits including malondialdehyde (MDA), catalase (CAT), total antioxidant capacity (T-AOC), Coomassie brilliant blue, hydroxyproline (Hyp), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and trace-reduced glutathione (GSH) kits were purchased from Nanjing Jiancheng Institute of Biotechnology. Keap1, Nrf2 antibody was purchased from HUABIO. Secondary antibody HRP-conjugated goat antirabbit IgG was purchased from Zhongshan Goldenbridge Biotechnology Co. Ltd. Analytical-grade acetic acid was purchased from Jiangsu Enox Chemical Co., Ltd. Liquid LS-MS-HPLC-grade acetonitrile was purchased from Sinopharm Chemical Reagent Co., Ltd. The reference standards naringin, and farrerol were purchased from National Institutes for Food and Drug Control, the reference standards naringenin were purchased from Shanghai Yuanye Biotechnology Co., Ltd, The reference standards isoschaftoside were purchased from Chengdu Manchester Biotechnology Co., Ltd. Anhydrous ammonium sulfate, ferrous sulfate, and tri (hydroxymethyl) aminomethane were purchased from Shanghai Linen Technology Development Co., Ltd. 1, 1-Diphenyl-2-2-picrylhydrazine, HE staining kit, Masson staining kit was supplied by Shanghai Yuanye Biotechnology Co., Ltd. Absolute ethanol was provided by Tianjin Yongda Chemical Reagent Co., Ltd. Salicylic acid was provided by Aladdin Reagent Co., Ltd. Thirty percent hydrogen peroxide was provided by Shanghai Lingfeng Chemical Reagent Co., Ltd. Phloroglucinol was supplied by Shanghai Meirui Co., Ltd. Hydrochloric acid was provided by Xilong Science Co., Ltd. Vitamin E is provided by Xinchang Pharmaceutical Factory of Zhejiang Pharmaceutical Co., Ltd. D-galactose were purchased from Acros Organizations.

2.2 | Preparation of DOF extract and HPLC-UV analysis

Put DOF into the oven for drying, then crush them with a high-speed multi-function pulverizer and pass them through an 80 mesh sieve.

Take DOF and extract it three times with purified water at a ratio of 1:50 of material to liquid, each time for 1 h. During the extraction process, the solution remains slightly boiling. After extraction, combine the filtrate, decrease the pressure and concentrate, pour it into an evaporating dish, place it in a 100°C constant temperature water bath, and evaporate it to dryness to acquire the water extract of DOF.

The quality of DOF was determined by HPLC equipped with Ultimate LP-C18 column (4.6 mm × 250 mm, 5 μm). Detection wavelength was set at 273 nm. The mobile phase was consisted of acetonitrile (A) and 0.2% acetic acid solution (B) and the gradient elution procedure was as follows: 0–30 min, 14% B; 30–40 min, 14%–20% B; 40–60 min, 20%–30% B; 60–80 min, 30%–65% B; 80–90 min, 65%–80% B; flow rate: 1 mL/min; injection volume: 10 μL. Isoschaftoside, naringin, naringenin, and farrerol were set as the quality control to make the chemical components of DOF of different batches nearly the same.

2.3 | Free radical scavenging test in vitro

2.3.1 | Determination of DPPH free radical clearance

The aqueous extract of DOF was prepared into 2.5, 5, 10, 15, and 20 mg/mL solutions. Accurately suck 100 sample solution and 100 absolute ethanol solution with concentration of 0.1 mmol/L DPPH μL was positioned in 96 well plate, and reacted in the dark at room temperature for 30 min. The absorbance measured at 517 nm was recorded as A. Three parallel experiments were conducted. When we calculate the DPPH radical scavenging rate, we can use half inhibition concentration (IC₅₀) to express the DPPH radical scavenging ability.^{8,9}

$$\text{DPPH radical clearance rate} / \% = [1 - (A - A_1) / A_0] \times 100 \%$$

A₀ is the absorbance of the solvent reacting with DPPH; A₁ is the absorbance of sample and absolute ethanol; A is the absorbance of the sample after reaction with DPPH.

2.3.2 | Determination of hydroxyl radical scavenging rate

The aqueous extract of DOF was prepared into 2.5, 5, 7.5, 10, and 12.5 mg/mL solutions. Prepare 6 mmol/L FeSO₄, 0.3% H₂O₂, and 6 mmol/L salicylic acid ethanol solution, add 1 mL FeSO₄, 1 mL sample solution, 1 mL 0.3% H₂O₂, and 1 mL salicylic acid ethanol solution in turn, mix evenly and put them in a 37°C water bath to react for 30 min. Determine the absorbance value of the solution at 510 nm and record it as A for three parallel experiments. When we calculate the hydroxyl radical scavenging rate, we can use half inhibition concentration (IC₅₀) to express the hydroxyl radical scavenging capacity.^{9–11}

$$\text{Hydroxyl radical clearance rate} / \% = [1 - (A - A_1) / A_0] \times 100 \%$$

A₀ is the absorbance of the reaction system of solvent and hydroxyl radical; A₁ is the absorbance of the reaction system of sample and hydroxyl radical with purified water replacing 0.3% H₂O₂; A is the absorbance of the reaction system between the sample and hydroxyl radical.

2.3.3 | Determination of superoxide radical scavenging rate

The aqueous extract of DOF was prepared into 2.5, 5, 7.5, 10, and 12.5 mg/mL solutions. Prepare 50 mmol/L Tris HCl solution (PH=8.2), 25 mmol/L pyrogallol solution, and 8 mmol/L hydrochloric acid solution. Add 4.5 mL Tris HCl solution, 1 mL sample solution, and 0.4 mL pyrogallol solution in turn, stir to blend and heat them in a water bath at 25°C for 4 min. Then add 1 mL hydrochloric acid solution to terminate the reaction, measure the absorbance value at 299 nm and record it as A. Carry out three parallel experiments. When we calculate the scavenging rate of superoxide free radicals, we can use half inhibition concentration (IC₅₀) to express the scavenging capacity of superoxide free radicals.^{12–14}

$$\text{Superoxide radical scavenging rate} / \% = [1 - (A - A_1) / A_0] \times 100 \%$$

A₀ is the absorbance of the reaction system of solvent and superoxide radical; A₁ is the absorbance of the reaction system between the sample and the hydroxyl radical in which purified water is applied to substitute pyrogallol; A is the absorbance of the reaction system of sample and superoxide revolutionary.

2.4 | Animal handling

70 maintained ICR male mice, weighing 18 ± 2 g, were delivered by Zhejiang Experimental Animal Center (SCXK (Zhejiang) 2014-0001 Zhejiang, China). All animal experiments conform to ethical and legal requirements. All animals were supplied at 25°C room temperature and 45%–55% relative humidity. The mice were fed a standard diet and water. All studies were conducted in accordance with the Guidelines for the Use and Care of Experimental Animals issued by the Zhejiang Province. The operation was sanctioned by the Ethics Committee of the Zhejiang University of Technology (SYXK (Zhe) 2017-0001).

2.5 | Animal grouping

ICR mice were randomly divided into seven groups: normal control group (NC), model control group (MC), vitamin E group (100 mg/kg), low-dose group of DOF (1.0 g/kg), medium-dose group of DOF (2.0 g/kg), and high-dose group of DOF (4.0 g/kg), with 10 mice in each group. The DOF concentration was 0.1, 0.2, and 0.4 g/mL at

the time of administration. The drug was administered orally once a day for 6 weeks. At the same time, the normal control group and the model control group were given 1 mL/100 g of water. In addition to the normal control group, the additional six groups of mice were subcutaneously injected with 200 mg/kg D-galactose 0.5 h after administration, and the normal control group was subcutaneously injected with normal saline of the same volume every day for 6 weeks.

2.6 | Collection of serum samples and detection of oxidative stress indicators

After the experiment, the mice fasted overnight, took blood from the ophthalmic vein plexus, bathed at 37°C for 30 min, centrifuged at 3500 rpm for 10 min, and took serum to detect the content of total antioxidant capacity (T-AOC), malondialdehyde (MDA), catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GSH Px). All procedures are tested according to the instructions of the kit. SOD and MDA shall be detected according to the instructions of the biochemical method kit. GSH Px and CAT were detected using ELISA test kit instructions. The process is described as follows: Dilute the sample in 96-well plate with 0.1% BSA/Tris buffer saline, followed by incubation at 37°C for 30 min, then wash the plate with 0.05% Tween20/PBS, incubate it with the corresponding secondary resistance for 30 min, then wash the plate again, and add color developer. OD value is obtained at 450 nm.

2.7 | Collection of skin samples and detection of oxidative stress indicators

After the experiment, use depilatory cream to remove the skin hair on the back of mice, cut the back skin, clean it, and dry it with filter paper. Weigh 0.1 g of skin into a centrifuge tube filled with physiological saline to prepare 10% homogenate, which is centrifuged at 3500 r/min for 15 min. Take the supernatant, and measure the protein content in the skin homogenate with Coomassie brilliant blue method. The contents of superoxide dismutase (SOD), malondialdehyde (MDA), and hydroxyproline (Hyp) in skin were measured. All procedures are tested according to the instructions of the kit.

2.8 | Hematoxylin and eosin (H&E) staining and Masson staining

Tissues (skin) were collected from each mouse, and gross lesions were recorded. The collected tissues were then fixed with a 4% formalin solution, dehydrated using different concentrations of alcohol, and embedded in paraffin. Subsequently, 4 μ m paraffin sections were prepared from the embedded tissues. Finally, the histopathological examination of the tissue was assessed using hematoxylin and eosin (H&E) staining, Masson staining. All H&E and Masson staining

were photographed with the biological microscope (BX43, Olympus, Japan) and analyzed with the Image-Pro Plus software.

2.9 | Immunohistochemical staining

The expression and localization of Nrf2, Keap1 proteins in the skin was evaluated using immunohistochemical staining. The paraffin tissue sections were incubated with Nrf2, Keap1 antibodies. A secondary antibody HRP-conjugated goat antirabbit IgG was added. The signals were visualized using DAB solution and the nuclei were counterstained with hematoxylin. Under the microscope, positive expression appeared as a yellow color and sections incubated without primary antibody was used as negative control. The protein levels were quantified by measuring the integrated optical density (IOD) within the positive area.

2.10 | Human skin efficacy test

Thirty subjects aged 20–35 years were randomly selected for the human skin efficiency test. Volunteer conditions include: No severe systemic diseases, no immune deficiency or autoimmune diseases, no skin treatment, cosmetic, or other tests that may affect the results of the test site; individuals without active allergic diseases; individuals without physical high sensitivity; those who have not used hormone drugs, anti-inflammatory drugs, or immunosuppressants within the past month; those who have not participated in other clinical trials on the test site currently or in the past 3 months. Avoid strong sun or artificial ultraviolet radiation during the test; all subjects voluntarily participated in the study and signed the informed consent form.¹⁵

2.10.1 | Short-term skin moisturizing test

Before the test, the subjects cleaned and dried the inside of their forearms, and waited for more than 30 min in an environment with a temperature of $(25 \pm 2)^{\circ}\text{C}$ and a relative humidity of $(55 \pm 5)\%$. Mark in the forearm of the subject's hands in the area of 2 cm^2 to be tested, took the sample to be tested and the reference sample according to the dosage of $(2.0 \pm 0.1)\text{ mg/cm}^2$, and used latex gloves to uniformly smear DOF until it is absorbed. Used the skin detector to measure the skin moisture content and skin elasticity of the sample and the control before and 4 h after application in the test area. Replicated the measurement three times, and took the mean value as the test value.¹⁶

2.10.2 | Skin antiaging test

The test area for the volunteers consisted of both cheeks and forehead, where they applied moisturizing essence water every

night for 8 consecutive weeks until the end of the test. The volunteer's face was photographed with a five-spectrum skin detector before use and on the day of the end of the test, and the data of skin wrinkles, roughness, UV spots, and brown spots were collected for statistical analysis. The data collected before use served as the baseline (BL), and the improvement rate at each time point was calculated as follows: (test value at each time point-BL value)/BL value, to observe the improvement of each indicator.^{17,18}

2.11 | Statistical analysis

All data were expressed by mean $\pm$ standard deviation. SPSS19.0 software was applied to analyze the data. The *t*-test for comparing the mean of two samples was performed for the differences between groups. $p < 0.05$ indicated a significant difference, and $p < 0.01$ indicated a highly significant difference. Use Graph Prism software for plotting.

3 | RESULTS

3.1 | HPLC-UV analysis of DOF

There are studies indicating that the total flavonoids in DOF have good antioxidant activity.⁵ Therefore, our study conducted quality control on the flavonoids in the extract of DOF and searched for the major active components with higher content.

HPLC-UV analysis of the DOF water extract (Figure 1) identified four flavonoids (isoschaftoside, naringin, naringenin, and farrerol) by comparing retention times against verified authentic references. The contents of these flavonoids, isoschaftoside, naringin, naringenin, and farrerol, were 9.13 ± 1.91 , 28.46 ± 1.22 , 2.51 ± 0.05 , and 17.99 ± 2.44 mg/mL, respectively (Table 1).

3.2 | Comparison of antioxidant activity of DOF in vitro

At present, in vitro free radical scavenging test is one of the commonly used methods to evaluate cosmetics for delaying skin aging. The evaluation indicators predominantly include DPPH free radical, superoxide free radical and hydroxyl free radical scavenging test.

DPPH is a stable nitrogen center free radical, which possesses a single electron and can receive an electron or hydrogen ion. When there is a free radical scavenger, the single electron of DPPH is captured to create its color lighter. The level of decreased absorbance indicates the strength of antioxidant capability. The DPPH free radical scavenging test was applied to detect the DPPH free radical scavenging rate of water extract of DOF.

Superoxide anion is active in nature and the longest active free radical in the body. It has potent oxidizing and reducing properties. Excessive accumulation of superoxide anion free radicals in the body will destroy macromolecular substances in cells and damage the health of the body. We used pyrogallol autoxidation method to determine the scavenging rate of water extract of DOF on superoxide anion free radicals.

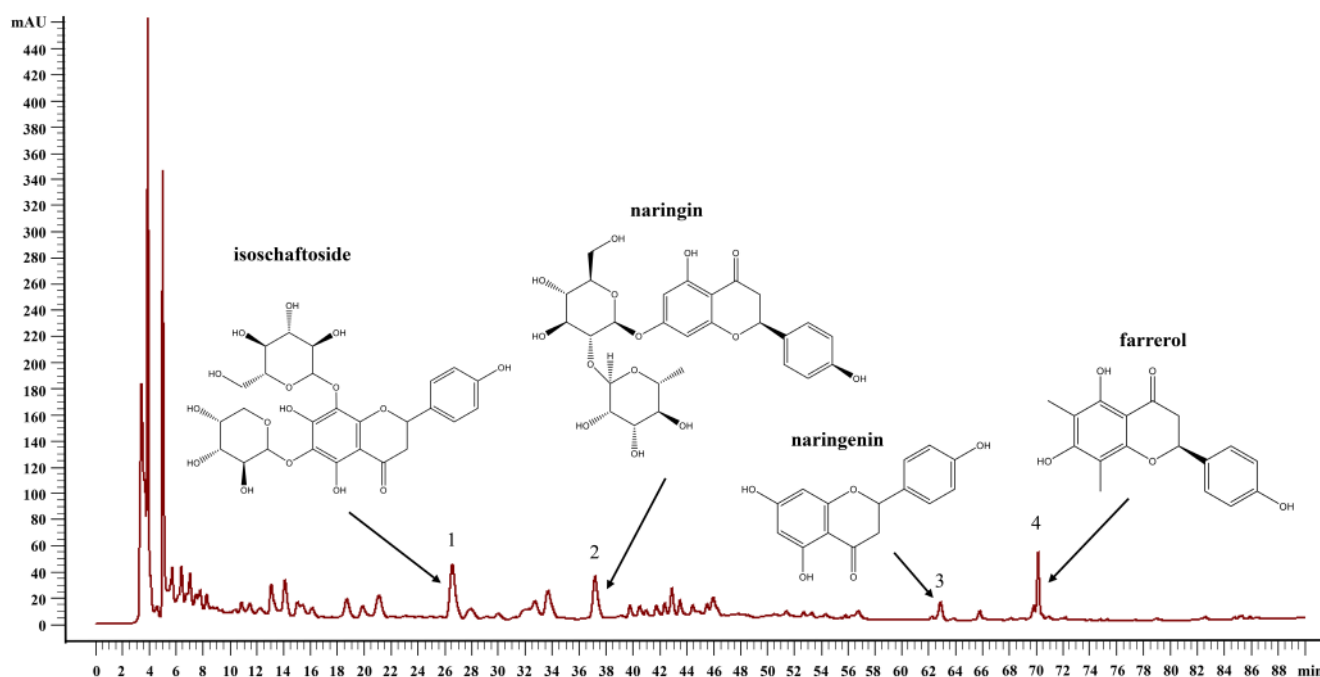


FIGURE 1 DOF water extract. 1=isoschaftoside, 2=naringin, 3=naringenin, and 4=farrerol with retention time 26.591 min, 37.215 min, 62.913 min, and 70.190 min, respectively.

Hydroxyl radical is one of the most active and toxic free radicals. When excessive hydroxyl radicals are generated in the body, it will lead to pathological changes in cell tissues. The Fenton system was used to detect the hydroxyl radical scavenging ability of water extract of DOF.

The results of DPPH free radical, hydroxyl free radical, and superoxide free radical scavenging rate of water extract of DOF are shown in Table 2. The aqueous extract of DOF can effectively scavenge DPPH free radicals, hydroxyl free radicals, and superoxide free radicals, and the scavenging rate increases with the increase of sample concentration, exhibiting a substantial dose effect relationship. The calculated IC₅₀ of the aqueous extract of DOF on DPPH free radicals, hydroxyl free radicals, and superoxide free radicals were 0.209 µg/mL, 15.020, and 1.217 mg/mL, respectively. It can be seen that the aqueous extract of DOF possesses good antioxidant activity in vitro.

3.3 | Antioxidant activity of DOF on aging model mice induced by D-galactose

3.3.1 | Effect on serum MDA, SOD, T-AOC, CAT, GSH, and GSH-Px of model mice

The essence of the aging process is the imbalance of oxidation and antioxidant functions in the body. When the production rate of free radicals in the body is faster than the clearance rate, it can lead to the accumulation of free radicals in the body and DNA damage, leading to aging.¹⁹ T-AOC is the overall level of various antioxidant macromolecules, antioxidant small molecules, and enzymes in a system. SOD, GSH-Px, and CAT are the primary antioxidant enzymes. GSH can scavenge O²⁻, H₂O₂, and LOOH free radicals and is the substrate of GSH-Px. MDA is the assessment index of lipid peroxide in organisms.²⁰

The results proved that compared with the normal group, the activities of SOD, T-AOC, CAT, and GSH-Px in serum of the aging model mice were considerably decreased, meanwhile, the level of MDA was significantly increased ($p < 0.01$), and the level of GSH had a downward trend. Compared with the model control group, low, medium, and high doses (1.0, 2.0, and 4.0 g/kg) of DOF could markedly increase the activity of SOD, T-AOC, CAT, and GSH-Px in serum of model mice ($p < 0.05$, 0.01), and reduce the level of MDA ($p < 0.05$, 0.01). In addition, 1.0 g/kg of DOF could raise the level of GSH in serum of model mice ($p < 0.05$). It can be seen that take the aqueous extract of DOF orally has better antioxidant activity in vivo in aging model mice (Figure 2).

3.3.2 | Effect on skin MDA, SOD, and Hyp of model mice

The appearance of subacute aging mice induced by D-galactose showed that the skin became thinner with fine wrinkles, the thickness of dermis and epidermis decreased, along with the reduction of collagen and elastic fibers. Moreover, the activity of SOD and the content of hydroxyproline (Hyp) in the skin of the aging model mice decreased, while the content of MDA increased.²¹

The results revealed that compared with the normal control group, the content of MDA in the skin of model mice increased significantly ($p < 0.05$), the content of Hyp decreased, and the activity of SOD had no significant difference. Compared with the model control group, low, medium, and high doses of DOF (1.0, 2.0, 4.0 g/kg) could significantly decrease the content of MDA in the skin of model mice ($p < 0.01$); Low and high doses of DOF (1.0 and 4.0 g/kg) can considerably increase the content of Hyp in skin ($p < 0.05$), as displayed in Figure 3. It can be inferred that DOF has a superior anti-skin aging and oxidative damage activity in vivo on aging model mice.

3.3.3 | Effect on skin pathological morphology and collagen content of model mice

To explore the effects of DOF on aging skin induced by D-galactose, histomorphometric analysis of the skin was conducted (Figure 4A,B). HE staining results showed the interface between the epidermis and dermis becomes flatter, the epidermis and dermis become thinner.

TABLE 1 Amounts of Compounds 1–4 in the DOF.

Standard	Amount of extracts (mg/mL)
1. Isoschaftoside	9.13 ± 1.91
2. Naringin	28.46 ± 1.22
3. Naringenin	2.51 ± 0.05
4. Farrerol	17.99 ± 2.44

Concentration (mg/mL)	DPPH clearance rate/%	O ₂ ⁻ clearance rate/%	OH clearance rate/%
2.5	53.40 ± 4.87	20.07 ± 4.87	13.81 ± 2.51
5	82.02 ± 8.73	33.30 ± 4.87	21.03 ± 2.77
10	88.07 ± 3.39	43.06 ± 2.57	35.55 ± 1.86
15	92.97 ± 3.63	44.95 ± 5.96	43.06 ± 4.43
20	94.11 ± 2.21	49.86 ± 2.43	48.65 ± 2.47

TABLE 2 Free radical scavenging capacity of DOF in vitro.

FIGURE 2 Effect of DOF on serum antioxidant indexes in aging mice induced by D-galactose. (A) Serum T-AOC activity; (B) Serum SOD activity; (C) Serum CAT activity; (D) Serum MDA content; (E) Serum GSH content; (F) Serum GSH-Px content. ## $p < 0.01$, compared with NC group; * $p < 0.05$; ** $p < 0.01$, compared with MC group.

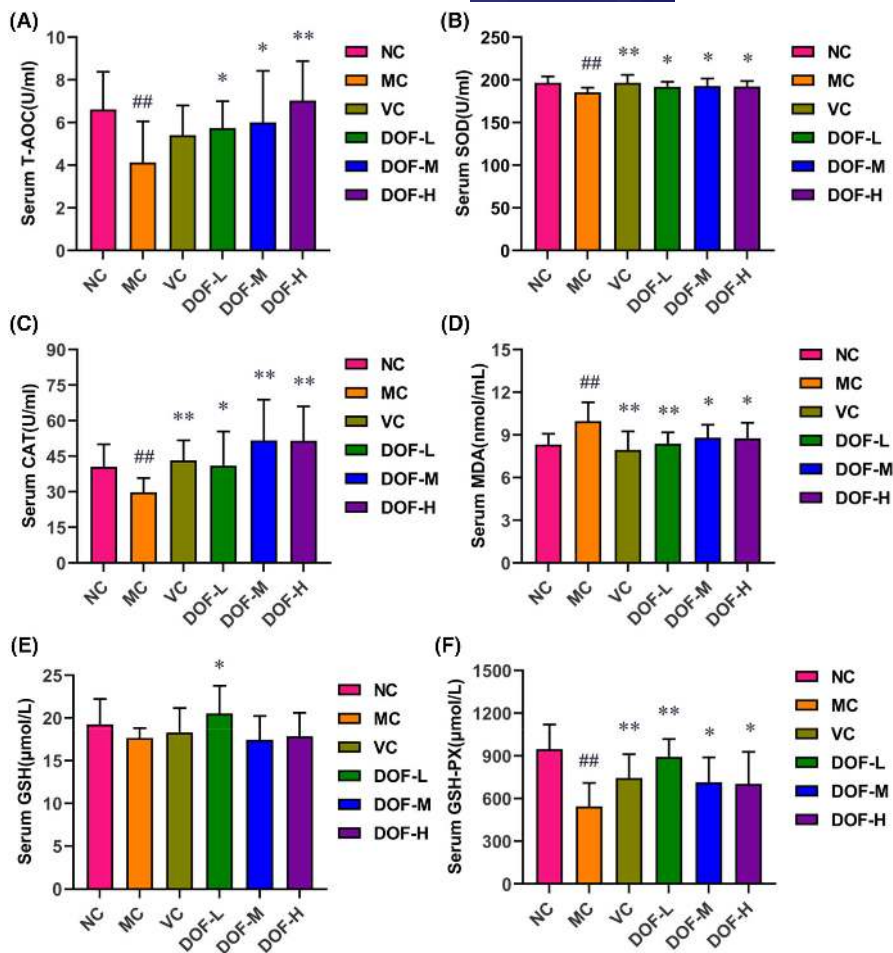
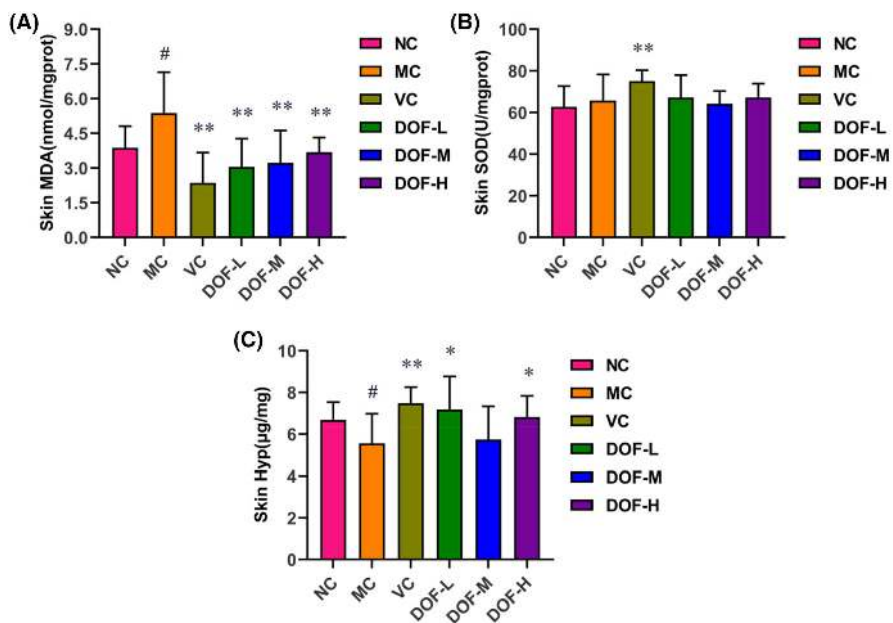


FIGURE 3 Effect of DOF on skin antioxidant indexes of aging mice induced by D-galactose. (A) Skin MDA content; (B) Skin SOD activity; (C) Skin Hyp content; # $p < 0.05$, compared with NC group; * $p < 0.05$; ** $p < 0.01$, compared with MC group.



Histopathological analysis proved that compared with the normal group, the thickness of dermis and epidermis of the aging model mice were considerably decreased ($p < 0.01$), meanwhile, the content of dermal collagen was significantly decreased ($p < 0.05$). Compared with the model control group, low, medium, and high doses (1.0, 2.0,

and 4.0 g/kg) of DOF dramatically increased the thickness of dermis and epidermis (Figure 4D,E).

Dermal collagen content was quantified by Masson trichrome staining (Figure 4C,F). The dermal collagen content from model mice was reduced significantly compared to normal mice ($p < 0.05$), and

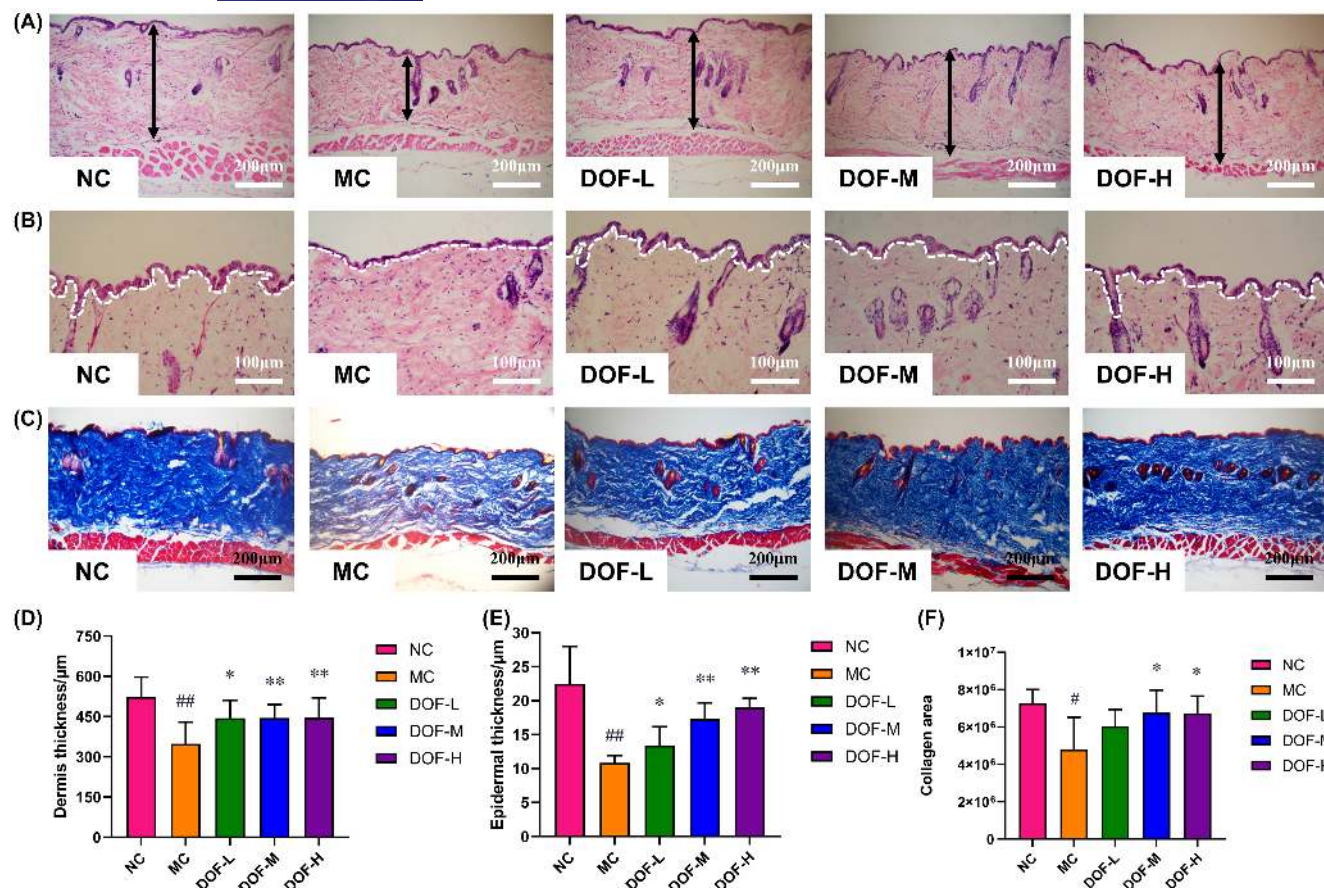


FIGURE 4 Effect of DOF on skin pathological morphology and collagen content of aging mice induced by D-galactose. (A) The skin tissues were stained with H&E staining to observed dermis ($\times 200$); (B) The skin tissues were stained with H&E staining to observed epidermis ($\times 400$); (C) The skin tissues were stained with Masson staining to observed dermal collagen content ($\times 200$); (D) Histopathological analysis of dermal thickness; (E), Histopathological analysis of epidermal thickness; (F), Masson staining analysis of dermal collagen content. [#] $p < 0.05$; ^{##} $p < 0.01$, compared with NC group; ^{*} $p < 0.05$; ^{**} $p < 0.01$, compared with MC group.

the results demonstrated that medium and high doses (2.0 and 4.0 g/kg) of DOF led to an increase in dermal collagen content ($p < 0.05$). Together, DOF increased dermis, epidermis thickness and dermal collagen content, thus improving skin aging.

3.3.4 | Effect on skin Keap1, Nrf2 expression of model mice

Nrf2/Keap1 signaling pathway plays a crucial role in maintaining oxidative balance in the body. On this basis, we further evaluated the effect of DOF on the expressions of Keap1 and Nrf2 in skin tissues of aging mice induced by D-galactose by IHC staining as shown in Figure 5.

The results of immunohistochemical study showed that the expression of Keap1 protein in the skin tissue of the model group was higher than that of the normal group ($p < 0.01$), and the expressions of Nrf2 proteins in skin were significantly reduced ($p < 0.05$). The expression level is high near the epidermal layer and hair follicles. Compared with the model group, the expression of Keap1 protein in the skin tissue of mice in the DOF-M and DOF-H group reduced

($p < 0.01, 0.05$), as well as DOF-H remarkably increased Nrf2 protein expression in skin ($p < 0.05$). It is suggested that the DOF can activated Keap1/Nrf2 pathway in the skin of aging mice, thereby exerting antioxidant stress effects.

3.4 | Evaluation of antiaging effect of DOF on human skin

3.4.1 | Effect of DOF on skin moisture and elasticity before and after use

The human bioelectrical impedance pulse technology can be employed to quantify the moisture content and skin elasticity of the cuticle,²² and indirectly reflect the aging of the skin. The results revealed that compared with the control group (BL baseline), the skin water score and elasticity value after DOF topical application were significantly increased ($p < 0.01$) by 26.03% and 10.29%, respectively. It can be seen that DOF possesses a good moisturizing and tightening effect (Figure 6 and 7).

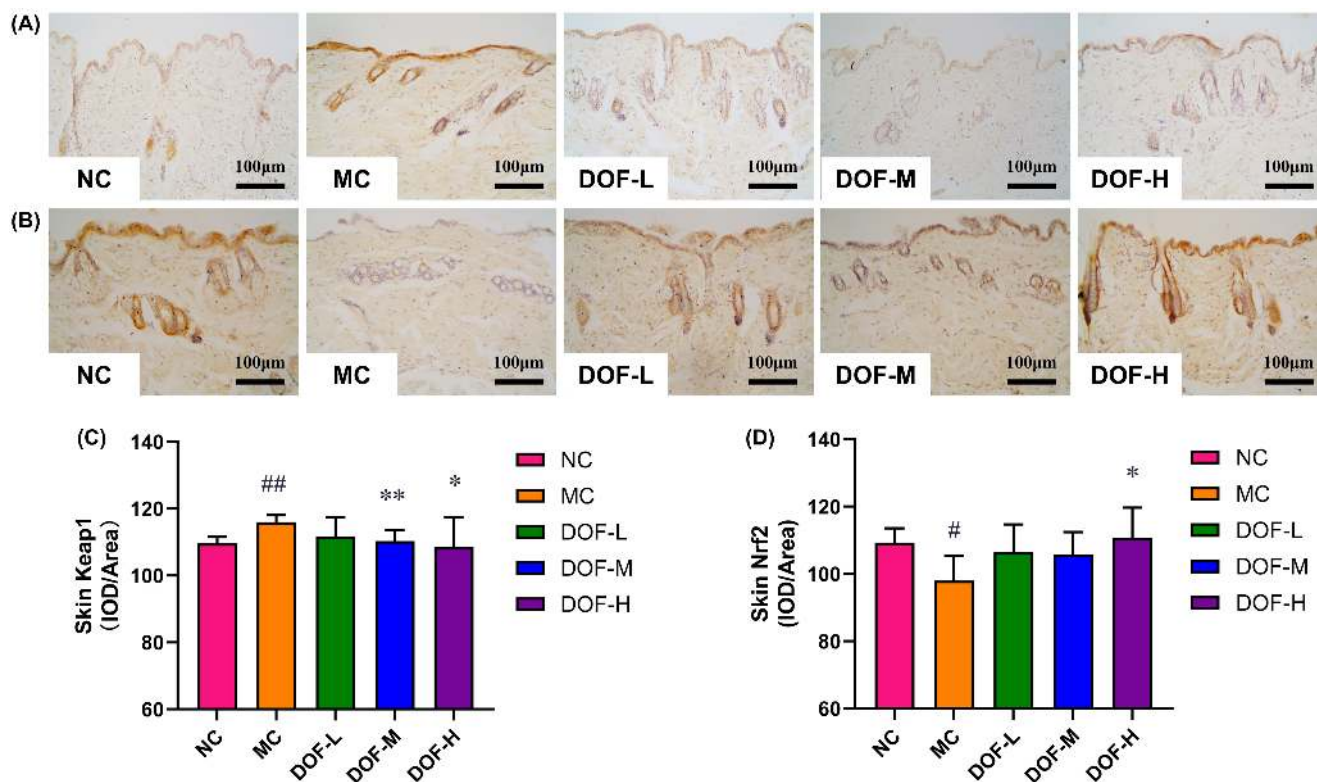


FIGURE 5 Effect of DOF on skin pathological morphology and collagen content of aging mice induced by D-galactose. (A) Skin Keap1 IHC pictures (x400); (B) Skin Nrf2 IHC pictures (x400); (C) Skin Keap1 OD value; (D) Skin Nrf2 OD value. # $p < 0.05$; ## $p < 0.01$, compared with NC group; * $p < 0.05$; ** $p < 0.01$, compared with MC group.



FIGURE 6 Fixation method of arm test area for short-term skin moisturizing test.

3.4.2 | Effect of DOF on skin aging-related indicators before and after use

With the increase of age, the metabolic capacity of human skin tissue gradually decreases, and long-term staying up late will generate a substantial number of free radicals to accelerate skin aging. Therefore, it is imperative to intervene with antiaging ingredients to slow down skin aging. In recent years, when evaluating the anti-aging

effect of cosmetics and other skin care effects, the human body test is one of the most intuitive and efficient means, and the image analysis method has the advantages of convenience, noninvasive, visualization, quantitative analysis of skin characteristics, etc.²³

The five-spectrum skin detector used in this experiment has deep autonomous learning technology, automated face recognition technology, and five-spectrum imaging technology. Through RGB visible light, UV ultraviolet light, PL-positive polarized light, PL-negative polarized light, and blue light 405, the human face is scanned and photographed, and automatically processed to generate data to detect skin wrinkles, pores, UV spots, brown spots, roughness, and other skin aging-related indicators.^{24,25} Among them, feature count is the number of indicators obtained by the instrument. The lower the number value, the better the skin condition. Therefore, by comparing the changes of various skin indicators before and after use, we can comprehensively and objectively evaluate the anti-skin aging effect of DOF.

Wrinkles refer to wrinkles on the skin. The more wrinkles, the more severe the aging of the skin; Pore refers to the opening of hair follicle sebaceous gland visible to the naked eye and the opening of hair follicle sebaceous gland containing angle plug.^{26,27} With the increase of age, the pore wall becomes thicker due to the reduction of elasticity, which is one of the symptoms of skin aging; ultraviolet spot is the result of melanin condensation under the epidermis due to long-term exposure to ultraviolet radiation; Brown spots refer to

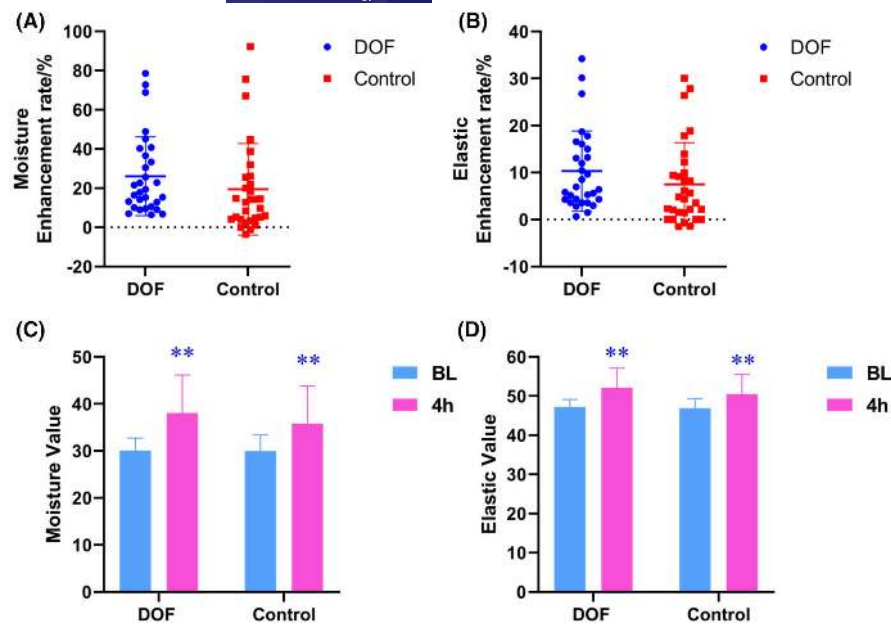


FIGURE 7 Effect of DOF on moisture and elasticity of human skin. (A) moisture increase rate; (B) elastic increase rate; (C) moisture value; (D) elastic value. ** $p < 0.01$, compared with BL.

TABLE 3 Changes of acne, pores, wrinkles, roughness, brown spots, and UV spots in volunteers before and after using DOF topically.

Evaluation parameter	BL	8 W	Invalid	Effective	Efficient
Acne score	34.54 ± 5.32	35.14 ± 5.40	17	11	39.29%
Pores score	37.68 ± 8.01	$42.29 \pm 10.37^*$	11	17	60.71%
Brown spot score	29.07 ± 3.60	29.04 ± 4.21	19	9	32.14%
Wrinkle score	44.46 ± 10.86	46.68 ± 7.91	12	16	57.14%
Roughness score	47.50 ± 2.60	48.64 ± 3.19	12	16	57.14%
UV spots score	26.79 ± 3.26	27.64 ± 3.03	11	17	60.71%
Acne Count	81.39 ± 131.08	54.07 ± 84.02	11	17	60.71%
Pores Count	1219.46 ± 521.52	$920.54 \pm 622.37^*$	4	24	85.71%
Brown spot Count	14.75 ± 5.72	14.46 ± 5.80	14	14	50.00%
Wrinkle Count	1038.39 ± 441.33	$545.46 \pm 345.03^{**}$	0	28	100.00%
UV spots Count	198.29 ± 73.81	$153.36 \pm 65.86^{**}$	9	19	67.86%

Note: * $p < 0.05$, ** $p < 0.01$, compared with BL.

deep pigmentation of skin, such as frequent chlosma and freckles; roughness refers to the smoothness of the skin surface and is one of the significant indicators to objectively reflect the aging degree of the skin.

Table 3 and Figures 8 and 9 show the changes of characteristic counts and scores of skin aging associated indicators before and after the topical use of DOF. The results demonstrated that after 8 weeks of continuous use of DOF once a day, the clinical indicators related to skin aging were significantly enhanced ($p < 0.05$, 0.01), the pore fraction increased by 4.61 points, the number of pores decreased by nearly 300, the number of wrinkles decreased by 47.5%, and the number of ultraviolet spots decreased by 45. The effective rate of improving wrinkles, pores, UV spots, and acne was as high as 100.00%, 85.71%, 67.86%, and 60.71%. The trend of improvement in skin roughness and texture was further observed, but due to the small sample size, these changes were not statistically significant.²⁸ In the entire research, DOF was well tolerated, without skin

irritation and other adverse reactions, and was safe for the duration of the study.

4 | DISCUSSION

Genetic factors can influence the aging, which is a gradual process that occurs with the aging of organisms, and the deterioration process of the total body's morphological structure and physiological function. The skin is the largest organ of the human body and the initial line of defense of the body. It plays a role in protecting, feeling, regulating body temperature, secreting, excreting, and immunity.²⁹ The evolutionary process of skin aging is extremely intricate. At present, the etiology of skin aging has not been thoroughly studied. Studies believe that skin aging is the result of the interaction of endogenous factors and exogenous factors.³⁰ Endogenous factors refer to the fact that, in the process of natural aging, the body generates

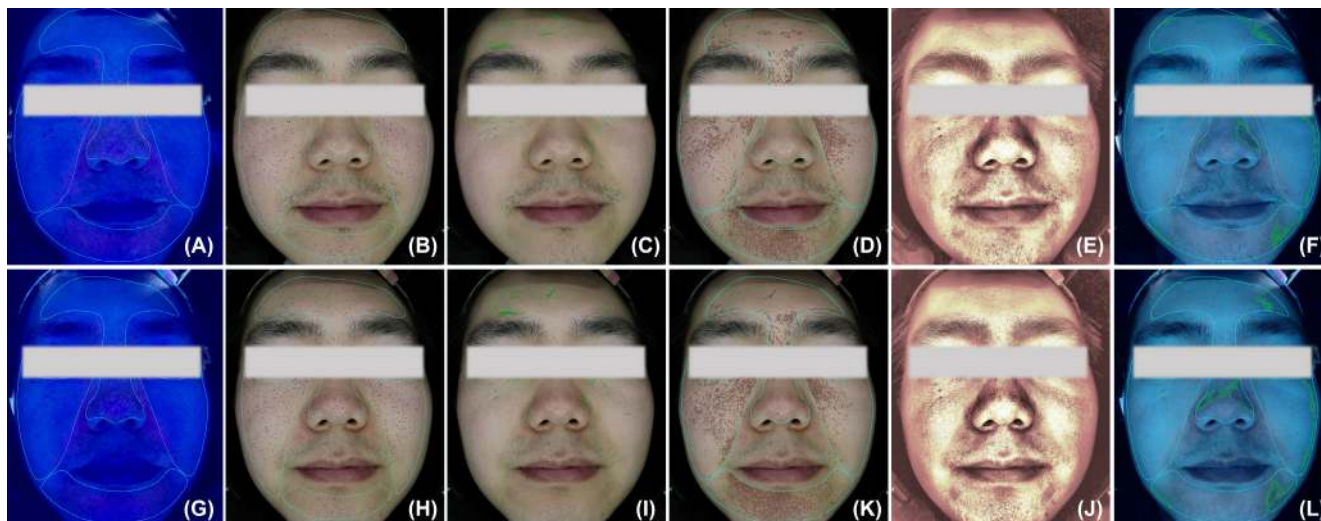


FIGURE 8 Image Acquisition of Human Facial Skin by DOF. (A) Acne in BL (B) Pore in BL (C) Wrinkle in BL (D) Roughness in BL (E) Brown spot in BL (F) UV spot in BL (G) Acne after 8 week treatment (H) Pore after 8 week treatment (I) Wrinkle after 8 week treatment (J) Roughness after 8 week treatment (K) Brown spot after 8 week treatment (L) UV spot after 8 week treatment.

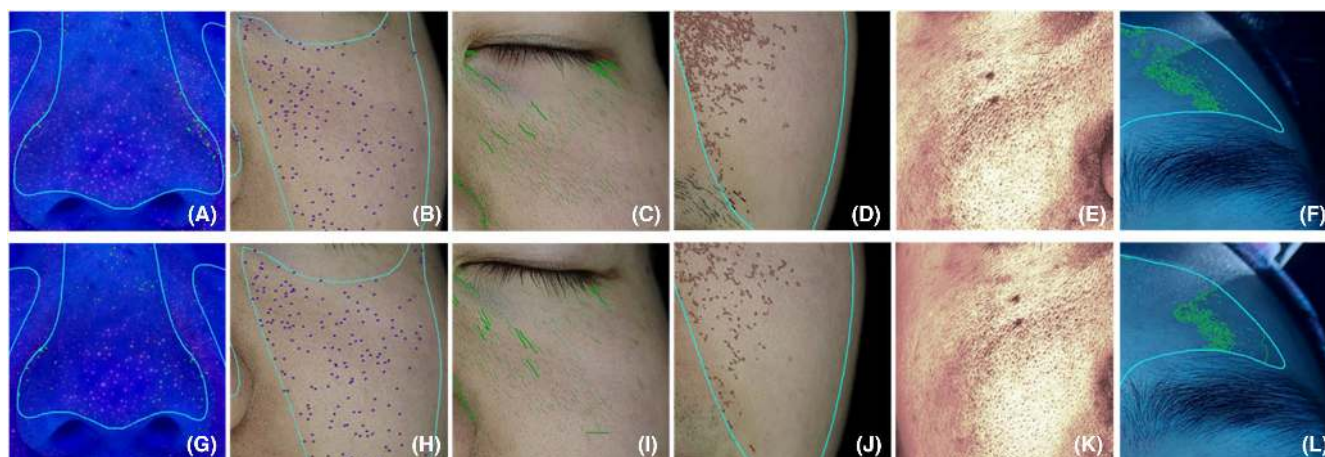


FIGURE 9 Image acquisition of human facial skin by DOF (300 × 300 pixel area). (A) Acne in BL (B) Pore in BL (C) Wrinkle in BL (D) Roughness in BL (E) Brown spot in BL (F) UV spot in BL (G) Acne after 8 week treatment (H) Pore after 8 week treatment (I) Wrinkle after 8 week treatment (J) Roughness after 8 week treatment (K) Brown spot after 8 week treatment (L) UV spot after 8 week treatment.

excessive free radicals due to the decline of free radical scavenging capacity, the decline of immune function, and the imbalance of body endocrine, which makes the free radicals in the body unbalanced.³¹ Excessive free radicals induce body damage. When the damage cannot be repaired by the body, it will lead to the change and loss of cell differentiation status and induce aging. Exogenous factors contain physical and chemical environmental pollution (ozone, exhaust gas from gasoline and diesel, cigarettes, halogens, heavy metals, etc.), which will generate oxidative stress on skin, form ROS, and damage skin cells.³² To sum up, lowering the damage of free radicals is essential for delaying skin aging. Therefore, upgrading the antioxidant ability and protecting the skin barrier function are of great importance to the quality of life of contemporary people.

DOF can be used to create tea or as a medicinal material. The contents of nutritional ingredients in DOF, including 18 kinds of amino

acids, 7 kinds of minerals, and 6 kinds of vitamins,³³ and the primary active ingredients are polysaccharides, flavonoids, and polyphenols, which are potent antioxidants. Previous studies have proved their antioxidant effect in vitro.^{4,6} Our experimentation shows the major components' contents of flavonoids, isoschaftoside, naringin, naringenin, and farrerol, were 9.13 ± 1.91 , 28.46 ± 1.22 , 2.51 ± 0.05 , and 17.99 ± 2.44 mg/mL. Based on its main chemical composition and confirmed pharmacological activity, we speculate that DOF may have the function of anti-aging and skin protection. In the research, the "in vitro animal human" model of free radical scavenging trial in vitro, D-galactose mice in vivo experiment simulating human aging process and human efficiency test was employed to research its effect on skin aging.

Aging is that the body generates excessive reactive oxygen radicals (hydroxyl radical HO and superoxide anion $O_2^{\cdot-}$), which

breaks the oxidation/antioxidant system of the body. This research demonstrated that the scavenging rates of DPPH, superoxide, and hydroxyl radicals of aqueous extracts of DOF were concentration dependent. IC_{50} were 0.209 $\mu\text{g/mL}$, 15.020, and 1.217 mg/mL . Recent studies have shown that the scavenging capability of extracts from flowers and leaves of *D. officinale* on DPPH free radicals and ABST free radicals was better than that of stems of *D. officinale*, which is a traditional medicinal part.⁵ DOF have a good antioxidant effect.

MDA is an index reflecting the degree of lipid peroxidation and the damage to cell membrane.³⁴ SOD, CAT and GSH-Px are the main antioxidant enzymes. SOD can catalyze O_2^- to generate H_2O_2 , and eventually generate water under the action of CAT or other antioxidant enzymes³⁵; GSH-Px has the functions of scavenging free radicals, inhibiting free radical reaction system and decreasing lipid peroxidation.³⁶ Compared with the normal control group, the level of MDA in the model group significantly increased, and the activity of antioxidant enzymes was significantly diminished. The results of this research are consistent with those of other papers.^{37,38} DOF could increase the levels of serum SOD, T-AOC, CAT, and GSH-Px and decrease the content of MDA in model mice; DOF 1.0 g/kg can increase the serum GSH level of model mice. It proves that take DOF orally can restore the activity of antioxidant enzymes, clear free radicals in the body, enhance lipid metabolism, alleviate the excessive accumulation of free radicals in the body, and have good antioxidant activity.

Hydroxyproline (HYP) is a unique amino acid in collagen, which maintains the stability of collagen fiber. Its content decreases with skin aging.³⁹ DOF 1.0 and 4.0 g/kg can increase the content of Hyp in skin tissue, implying that DOF can delay skin aging and have certain beauty and beauty effects. On the other hand, DOF can decrease the content of MDA in the skin tissue of model mice, indicating that DOF can reduce the excessive accumulation of skin free radicals. Studies have shown that *D. officinale* flavone can increase the content of superoxide dismutase (SOD), malondialdehyde (MDA), HYP, Type I collagen, and Type III collagen in the skin tissue of mice with skin aging, reduce CAT activity, and effectively delay the skin aging of model mice.⁴⁰ Concurrently, H&E and Masson trichrome staining results proved that the three doses of DOF markedly increased the thickness of dermis and epidermis, and DOF 2.0 and 4.0 g/kg increased the content of dermal collagen, thus improving skin aging. The results demonstrated that DOF could balance the oxidation system and antioxidant system to delay skin aging.

Nrf2 is one of the most important transcription factors in regulating antioxidant stress response in the body. It regulates cellular redox homeostasis and protects cells from oxidative damage.⁴¹ Keap1 is specific receptor for Nrf2. Under normal physiological conditions that Nrf2 is in a relatively inactive state, it couples with Keap1 to form a polymer.⁴² However, when activated by oxidative damage, the conformation of Keap1 changes, and Nrf2 separate with Keap1. Then Nrf2 release and transfer to the nucleus, activating downstream gene transcription. Downstream genes produce a

large number of protective enzymes, including enzymes related to the generation of glutathione, reduce the levels of ROS and alleviate cellular lipid damage.^{43,44} Immunohistochemical results showed that DOF 2.0 and 4.0 g/kg obviously reduced the expression of Keap1 protein, at the same time DOF 4.0 g/kg significantly increased Nrf2 protein expression in skin. DOF can resist oxidative stress by activated Keap1/Nrf2 pathway in the skin.

The content of skin natural moisturizing factor will gradually decrease, the metabolism of keratinocytes slows down, the synthesis of skin collagen will decrease, the deterioration will increase, the skin immunity will also weaken, and then the aging performance will appear to grow.³⁰ The characteristics of skin aging are mainly manifested in the reduction of skin water content, collagen content, wrinkles, rough skin, large pores, dark yellow skin, pigmentation spots or irregular pigmentation.^{29,45} After using DOF topically for 8 weeks, the facial moisture and elasticity of the subjects increased significantly, and wrinkles, pores, purple spots, brown spots and roughness were improved to varying degrees. The number of facial wrinkles, pores, purple spots, and brown spots decreased significantly compared with that before use. These results indicate that external use DOF may resist skin aging by replenishing moisture in the cuticle of the skin, enhancing collagen synthesis, enhancing skin immunity, and activating the skin regulatory function.

5 | CONCLUSION

To sum up, DOF possesses a good antioxidant effect. Both oral and external use can delay skin aging. The aqueous extract of DOF can significantly improve skin aging induced by oxidative stress, and its mechanism may be associated to scavenging free radicals and upgrading skin quality.

AUTHOR CONTRIBUTIONS

Data curation and writing—review and editing, X. H.; Data curation and validation, N. W.; Writing—original draft, X. T. and N. W.; Data curation and visualization, Z. X.; Resources, Y. D.; Formal analysis, M. L.; Investigation, Z. Z.; Software, M. R.; Project administration, Y. C.; Supervision, conceptualization, and methodology, B. L.; Funding acquisition, G. L. and S. C. All authors have read and agreed to the published version of the manuscript.

FUNDING INFORMATION

This research was supported by The National Key R&D Plan (2017YFC1702200, 2017YFC1702202) and The National Natural Science Foundation of China (No. 82274134).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study method and treatment procedure were considered and approved by the ethics committee of Zhejiang University of Technology.

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How to cite this article: He X-L-S, Wang N, Teng X, et al. *Dendrobium officinale* flowers' topical extracts improve skin oxidative stress and aging. *J Cosmet Dermatol*. 2024;23:1891-1904. doi:[10.1111/jocd.16210](https://doi.org/10.1111/jocd.16210)