Phenolic Constituents with Antioxidative, Tyrosinase Inhibitory and Anti-aging Activities from *Dendrobium loddigesii* Rolfe

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Abstract
Aqueous ethanol extracts of powdered stems of *Dendrobium loddigesii* afforded three new phenolics including threo-7-O-ethyl-9-O-(4-hydroxyphenyl)propionyl-guaiacylglycerol (1), (R)-4,5,4\(^\prime\)-trihydroxy-3,3\(^\prime\),\(\alpha\)-trimethoxybibenzyl (2) and (S)-5,5\(^\prime\),7-trihydroxy-3,3\(^\prime\),4\(^\prime\)-dimethoxyflavanone (3), together with eleven known analogues. Their structures were determined by extensive spectroscopic analysis. To identify natural antioxidants, whitening, and anti-aging agents, the abilities of these phenolics were assessed to scavenge the 1,2-diphenyl-2-picrylhydrazyl (DPPH) radical, their abilities to inhibit tyrosinase production, and their abilities to stimulate collagen production by human dermal fibroblasts-adult (HDFa) assay. It was found that compounds 1, 4–8, 13 and 14 exhibited significant DPPH radical scavenging activities, compound 10 exhibited tyrosinase inhibitory activity (IC\(_{50}\) 37.904 μg/mL), and compound 9 showed significant collagen production with an EC\(_{50}\) value of 3.182 μg/mL. These results suggest that phenolic constituents from *D. loddigesii* may be candidate antioxidants, skin-whitening and/or anti-aging agents.

Graphic Abstract

Keywords *Dendrobium loddigesii* · Phenolic constituents · Antioxidative · Tyrosinase inhibitory · Anti-aging

1 Introduction
The genus *Dendrobium* (Orchidaceae) contains approximately 1500 species globally, of which about 80 species grow in China [1]. The stems of several plants in this genus...
are known as “Shi-Hu”, which have been used for thousands of years as both traditional Chinese medicine and folk remedies for the treatment chronic atrophic gastritis, skin aging, fever, cardiovascular disease and a tonic for promoting the production of body fluid [2]. Previous studies on this genus led to the isolation of a series of polysaccharides, phenolic compounds, alkaloids, and sesquiterpenoids [1, 3–5], some of which possess various bioactivities including anti-inflammatory [6], antimicrobial [2], antioxidant [7], antitumor [8], antiplatelet aggregation [9], immunomodulatory [10], and against influenza A activities [11].

*Dendrobium loddigesii*, a perennial epiphytic herb, is widely distributed in the southwestern area of China, such as Guangxi, Guizhou, and Yunnan Provinces [12]. Its stem has been applied in folk medicine to treat gastrosis, fever, and dizziness [13]. In continuing the search for structurally diverse and biologically active natural products from this genus [1, 5, 14–17], an in-depth investigation of pharmaco-logically active constituents from this plant species was performed herein. As a result, three new phenolic compounds (1–3) as well as eleven known ones (Fig. 1) were isolated from an 80% ethanolic extract of the stem of *D. loddigesii*. The isolation, structure elucidation, and biological evaluation of these compounds are presented herein.

### 2 Results and Discussion

Compound 1, obtained as white solid, gave a molecular formula of C_{21}H_{26}O_{7} as determined by the (−)-HRESIMS ion at m/z 389.1602 [M–H]− (calcd for C_{21}H_{25}O_{7}, 389.1606) with nine degrees of unsaturation. The 1H NMR spectrum of 1 contains seven aromatic protons at δ_{H} 6.85 (1H, d, J = 1.7 Hz), 6.75 (1H, d, J = 8.0 Hz), 6.68 (1H, dd, J = 8.0, 1.7 Hz), 7.01 (2H, d, J = 8.5 Hz), and 6.68 (2H, d, J = 8.5 Hz), suggesting the presence of a 1,3,4-trisubstituted benzene ring and a 1,4-disubstituted benzene ring. Its 13C NMR spectrum exhibited 21 carbon resonances including two methyl (one methoxy), four aliphatic methylene, nine methine (two sp3, seven sp2), and six quaternary carbons (one carbonyl, five olefinic including three oxygenated). The HMBC correlations (Fig. 2) of H-7/C-1 (δ_{C} 131.6), C-2 (δ_{C} 111.7), C-6 (δ_{C} 121.4), C-8 (δ_{C} 74.2) and C-10 (δ_{C} 65.3); H-9/C-7 (δ_{C} 83.8) and C-8 (δ_{C} 74.2); H-10/C-11 (δ_{C} 15.6); 3-OMe (δ_{H} 3.81)/C-3 (δ_{C} 149.1), together with correlations of H-7/H-8/H-9 and H-2/H-12 from 1H–1H COSY spectrum (Fig. 2) indicated the presence of 7-O-ethylguaiacylglycerol [18]. It has been reported that J_{7,8} was about 5 Hz for the
erythro isomer and 7 Hz for the threo isomer in the cases of syringoyl-glycerols and guaiacylglycerol derivatives. Thus, compound 1 was considered to be the threo isomer with $J_{7,8}$ (6.5 Hz) [18]. The HMBC correlations of H-7' ($\delta_H = 2.79$, t, $J = 7.5$ Hz)/C-8' ($\delta_C = 37.1$), C-1' ($\delta_C = 132.7$), C-2', 6' ($\delta_C = 130.2$) and C-9' ($\delta_C = 174.6$), H-8' ($\delta_H = 2.59$, t, $J = 7.5$ Hz)/C-7' ($\delta_C = 31.0$), C-1', and C-9', along with COSY cross-peaks of H-8'/H-7' indicated the presence of p-hydroxycoumaric acid [19]. On the basis of the evidences described above, 1 was proposed to have a 7-O-ethylguaiacylglycerol moiety and a p-hydroxycoumaric acid via an ester linkage. The HMBC correlation from H-9 to C-9' suggested the ester linkage was between C-9 and C-9'. Thus, the structure of 1 was determined as shown.

(R)-4,5,4'-Trihydroxy-3,3',α-trimethoxybibenzyl (2) was obtained as white solid. The HRESIMS spectrum of 2 displayed a quasi-molecular ion peak at $m/z$ 319.1180 [M–H]$^-$ (calcd. for $C_{17}H_{16}O_6$, 319.1187) with 8 degrees of unsaturation. The $^1$H NMR spectrum of 2 showed three methoxyl groups at $\delta_H = 3.75$ (3H, s), 3.70 (3H, s) and 3.17 (3H, s); one oxygenated methine proton at $\delta_H = 4.13$ (1H, t, $J = 6.8$ Hz, H-α); two methylene signals at $\delta_H = 2.73$ (1H, dd, $J = 13.5$, 6.9 Hz) and 2.96 (1H, dd, $J = 13.5$, 6.9 Hz); and five aromatic protons, appearing as a 1,3,4,5-tetrasubstituted aromatic ring at $\delta_H = 6.28$ (1H, d, $J = 1.8$ Hz) and 6.34 (1H, d, $J = 1.8$ Hz), and a 1,3,4-trisubstituted aromatic ring at $\delta_H = 6.49$ (1H, d, $J = 2.0$ Hz), 6.62 (1H, d, $J = 8.0$ Hz) and 6.52 (1H, dd, $J = 8.0$, 2.0 Hz). The $^{13}$C NMR and DEPT spectra of 2 showed three oxyethyils, one methylene, one oxygenated methine and 12 aromatic carbons (five oxygenated). Comparison of its NMR data (Table 1) with those of dendrocandin C [20] showed great similarities except for the presence of one more methoxyl group, which was located at C-3' by the HMBC correlations from 3'-OMe and H-5' to C-3' ($\delta_C = 148.4$). In addition, the multiple HMBC interactions (Fig. 2) of 3-OMe and H-2/C-3 ($\delta_C = 149.5$); α-OMe/C-α ($\delta_C = 86.9$) suggested the other methoxyl groups at C-3, and C-α, respectively. The absolute configuration at C-α was determined as R on the basis of the negative optical rotation ([α]D$^{20}$ = −12.46), similar to aphyllal D [α]D$^{20}$ = −20.3, MeOH) [15]. Accordingly, the structure of 2 was determined as shown.

(S)-5,7,5'-Trihydroxy-3',4'-dimethoxyflavanone (3) was obtained as yellow amorphous powder and had the molecular $C_{17}H_{16}O_7$ (10 indices of hydrogen deficiency) according to the (−)-HRESIMS ion at $m/z$ 331.0819 [M – H]$^-$ (calcd. 331.0823). The UV absorption maxima at 206 and 294 nm indicated the presence of a flavanone [21]. The $^1$H NMR spectrum (Table 1) showed three signals in non-aromatic region placed at $\delta_H = 5.29$ (1H, dd, $J = 12.7$, 3.1, H-2), 3.04 (1H, dd, $J = 17.1$, 12.7, H-3ax), and 2.71 (1H, dd, $J = 17.1$, 3.1, H-3 eq); four aromatic protons $\delta_H = 5.87$ (1H, d, $J = 2.2$, H-6), 5.91 (1H, d, $J = 2.2$, H-8), 6.62 (1H, d, $J = 2.0$, H-2') and 6.61 (1H, d, $J = 2.0$, H-6'), two methoxyl groups $\delta_H = 3.83$ (3H, s) and 3.77 (3H, s). The $^{13}$C NMR and DEPT spectra (Table 1) contain resonances for 17 carbons including one two methoxyl, one methene, one carbonyl carbon and 12 aromatic carbons. Comprehensive analysis of its NMR data indicated that its planar structure is closely related with that of dihydrotricin [22], except for the OH-4' and OCH$_3$-5' resonances in dihydrotricin were transposed in 3. This was confirmed by the HMBC cross-peaks (Fig. 2) from H-2', H-6' and OCH$_3$-4' to C-4' ($\delta_C = 137.7$), from H-6' to C-5' ($\delta_C = 151.8$). The absolute configuration at C-2 was postulated as being in the S-form on the basis of a negative specific rotation value (−46.64, MeOH) in its optical rotation [23]. Therefore, the structure of compound 3 was thus unambiguously assigned as shown.

Eleven known compounds were identified to be crepidatin 4 [24], moscatilin 5 [25], 4,5,4' -trihydroxy-3,3'-dimethoxybibenzyl 6 [26], 4',5-dihydroxy-3,3'-dimethoxybibenzyl 7 [27], tristin 8 [28], batatasin III 9 [27], 3,5,3'-hydroxybibenzyl 10 [29], aphyllals C 11 [15], densiflorol A 12 [30], dihydroconiferyl dihydro-p-coumarate 13 [31], p-hydroxyphenethyl trans-ferulate 14 [32] by spectroscopic analysis and comparing their spectral data with literature.

Phenolic compounds, are an essential part of the human diet and are known as powerful antioxidants due to their
potent chain breaking action and they may contribute directly to the anti-oxidative activity [33]. The DPPH radical scavenging assay is one of the most common and relatively quick methods used for evaluating antioxidant activity. Compounds that can donate a hydrogen atom to the DPPH radical, and then gives rise to the reduced form of DPPH which will be considered as potential antioxidant agents. All compounds were evaluated for their DPPH radical scavenging activities. The present results (Table 2) exhibited that the majority of the phenolic compounds (1, 4–8, 13 and 14) showed significant activities with scavenging capacities ranging from 89.411 to 94.278% at 100 μg/mL.

On the other hand, tyrosinase is the copper containing enzyme and plays a critical role in controlling melanin biosynthesis pathway in melanocytes [34]. Therefore, tyrosinase inhibitors became important constituents of cosmetics or as medicinal products for hyperpigmentation and developing skin whitening agents. In the present study, all the isolates were evaluated for their tyrosinase inhibitory activity (Table 2). Kojic acid, a purported skin-lightening agent, was used as a positive control. 3,5,3’-hydroxybibenzyl (10) revealed a significant inhibitory activity with an IC50 value of 37.904 μg/mL. Aphyllals C (11) showed moderate inhibition (IC50, 152.56 μg/mL). All remaining compounds were inactive at concentrations up to 200 μg/mL. In this study, it can be concluded that compounds 10 and 11 can be potential candidate for the treatment of melanin biosynthesis related skin diseases.

Considering that this species medicinally used for skin aging, since, collagen is critical for skin strength and elasticity, and its degradation leads to wrinkles that accompany aging [35]. Hence, all the compounds were also purposely evaluated for their effects on collagen production in HDFa. The results (Table 2) showed that compound 9 significant stimulation HDFa collagen production activity (EC50 3.182 μg/mL). Compounds 6 and 7 showed weaker activities, with collagen production of 33.062% and 29.157% at 10 μg/mL, respectively. The present results not only supported the ethnopharmacological usage of D. loddigesii but also provided a reliable structure template

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**Table 1** The 1H and 13C-NMR data of 1–3 (methanol-d4, δ in ppm, J in Hz)

| No. | 1a δH     | δC | No. | 2b δH     | δC | No. | 3b δH | δC |
|-----|-----------|----|-----|-----------|----|-----|-------|----|
| 1   | 131.6 s   | 1  | 2   | 134.6 s   | 1  | 3   | 80.4 d | 1  |
| 2   | 6.85 d (1.7) | 111.7 d | 6.28 d (1.8) | 103.4 d | 2  | 5.29 dd (12.7, 3.1) | 44.2 t |
| 3   | 149.1 s   | 3  | 2   | 149.5 s   | 3  | 3.04 dd (17.1, 12.7) | 2.71 dd (17.1, 3.1) |
| 4   | 147.5 s   | 4  | 6   | 133.4 s   | 4  | 197.4 s |
| 5   | 6.75 d (8.0) | 116.0 d | 6.34 d (1.8) | 108.6 d | 6  | 5.87 d (2.2) | 97.1 d |
| 6   | 4.14 d (6.5) | 83.8 d | 1’  | 131.1 s   | 7  | 168.4 |
| 7   | 3.82 d   | 74.2 d | 2’  | 114.4 d   | 8  | 5.91 d (2.2) | 96.2 d |
| 8   | 3.98 dd (11.5, 3.5) | 66.5 t | 3’  | 148.4 s   | 9  | 164.6 s |
| 9   | 3.37, 3.32 m | 65.3 t | 4’  | 145.8 s   | 10 | 103.3 s |
| 10  | 1.14 t (7.0) | 15.6 q | 5’  | 6.62 d (8.0) | 115.7 d | 1’  | 136.3 s |
| 1’  | 132.7 s   | 6’  | 6.52 dd (8.0, 2.0) | 123.1 d | 2’  | 6.62 d (2.0) | 129.9 d |
| 2’  | 7.01 d (8.5) | 130.2 d | α  | 4.13 t (6.9) | 86.9 d | 3’  | 154.7 s |
| 3’  | 6.68 d (8.5) | 116.2 d | α’ | 2.73 dd (13.5, 6.9) | 45.1 t | 4’  | 137.7 s |
| 4’  | 156.8 s   | 3’-OMe | 3.70 s | 56.4 q | 5’  | 151.8 s |
| 5’  | 6.68 d (8.5) | 116.2 d | 3’-OMe | 3.75 s | 56.5 q | 6’  | 6.61 d (2.0) | 108.2 d |
| 6’  | 7.01 d (8.5) | 130.2 d | α-OMe | 3.17 s | 56.7 q | 3’-OMe | 3.83 s | 56.4 q |
| 7’  | 2.79 t (7.5) | 31.0 t | 4’-OMe | 3.77 s | 61.0 q |
| 8’  | 2.59 t (7.5) | 37.1 t | 5-OMe | 3.81 s | 56.3 q |
| 9’  | 174.6 s   | 3-OMe | 3.81 s | 56.3 q |

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a1H and 13C NMR data were recorded at 600 MHz and 150 MHz, respectively
b1H and 13C NMR data were recorded at 500 MHz and 125 MHz, respectively

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for developing collagen deficiency associated diseases such as burn and ulcer.

3 Experimental

3.1 General Experimental Procedures

Optical rotation was obtained on a JASCO P-1020 digital polarimeter (Horiba, Tokyo, Japan). UV spectra were measured using a Shimadzu UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were obtained on a Bruker Tensor 27 infrared spectrophotometer (Bruker Optics GmbH, Ettlingen, Germany) with KBr pellets. Mass spectra were performed on an API QSTAR time-of-flight spectrometer (MDS Sciqaszex, Concord, Ontario, Canada) and LCMS-IT-TOF (Shimadzu, Kyoto, Japan) spectrometer. NMR spectra were recorded on DRX-500 and Av III-600 instruments with TMS as the internal standard (Bruker, Bremerhaven, Germany). The chemical shifts were given in δ (ppm) with reference to the solvent signal. Column chromatography was performed on silica gel (200–300 and 300–400 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), MCI gel CHP-20P (75–150 μm, Mitsubishi Chemical Corp., Tokyo, Japan), sephadex LH-20 (20–150 μm, Amersham Biosciences, Uppsala, Sweden), and YMC*GEL ODS-A-HG (50 μm, YMC Co. Ltd. Japan). Fractions were monitored by TLC, and spots were visualized by UV light and sprayed with 10% H₂SO₄ in EtOH, followed by heating. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox, mushroom tyrosinase, L-Dopa, and Kojic acid were purchased from Sigma (USA); Transforming growth factor beta (TGF-β) was obtained from Peprotech (USA); Growth media DMEM (high glucose w/L-glut), Hank’s balanced salt solution, fetal bovine serum were purchased from HyClone (USA); Procollagen peptide ELISA kit was obtained from TaKaRa (Japan). All other chemicals and solvents were of analytical grade.

3.2 Plant Material

The stems of D. loddigesii were collected in September 2014 from Wenshan City, Yunnan Province, People’s Republic of China and identified by Professor Hong Yu (Yunnan University, Kunming, People’s Republic of China). The voucher specimen (No. 20,140,829) has been deposited at the State key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Sciences.
3.3 Extraction and Isolation

The dried and powdered stems (10.2 kg) of *D. lodgesii* were extracted three times with 80% ethanol under room temperature and concentrated under reduced pressure. Then, the residue was suspended in H₂O and partitioned with EtOAc to obtain the EtOAc fraction (220 g), which was subjected to silica gel column chromatography eluted with a gradient of petroleum ether/acetone (15:1 to 0:1) to afford 22 fractions (Fr.1–22). Fr.11 (6 g) was subjected to silica gel CC eluted with CHCl₃/MeOH (300:1), then followed by MCI column (MeOH/H₂O gradient, 60:40–95:5) and silica gel CC (CHCl₃/MeOH, 200:1) to yield 12 (4 mg). Fr.13 (850 mg) was separated over a column of MCI (MeOH/H₂O, 60:40 to 95:5) to give five fractions (Fr.13.1–Fr.13.5). Fr.13.4 (150 mg) afforded compounds 4 (3 mg) and 5 (5 mg) by HPLC preparation (MeOH/H₂O, 60:40). Fr.16 (19 g) was chromatographed on silica gel column eluted with CHCl₃/MeOH (100:1 to 20:1) to afford 6 subfractions (Fr.16.1–Fr.16.6). Fr.16.4 (2.3 g) was applied MCI column eluted with MeOH/H₂O (50:50–100:0) and then further fractionated over a column of sephadex LH-20 (MeOH/H₂O, 90:10) to yield 7 (716 mg) and 13 (39 mg). By using the same conditions of Fr.16.4, Fr.16.6 (240 mg) afforded compound 9 (7 mg). Fr.18 (10 g) was separated by silica gel CC (CHCl₃/MeOH, 100:1–20:1), then passed through MCI column eluted with MeOH/H₂O (50:50–100:0) and then further fractionated over a column of sephadex LH-20 (MeOH/H₂O, 90:10) to yield 8 (15 mg) and 10 (7 mg). Fr.19 (6.4 g) was fractionated on a column of silica gel (CHCl₃/MeOH, 30:1) to give 5 fractions (Fr.19.6.1–Fr.19.6.5). Fr.19.6.1 (2.2 g) was subjected to silica gel column (CHCl₃/MeOH, 30:1) and after purification by HPLC (MeOH/H₂O, 45:55), providing 1 (15 mg). Fr.19.6.3 (540 mg) was applied to a column of sephadex LH-20 column eluted with MeOH/H₂O (90:10), and then further purified by semipreparative HPLC (MeOH/H₂O, 45:55) to furnish 2 (6 mg) and 6 (5 mg). Fr.20 (12 g) was applied to a MCI gel (MeOH/H₂O gradient, 30:70–100:0) chromatographic step and then was subjected to silica gel CC (CHCl₃/MeOH, 30:1) to give 14 (21 mg).

**three-o-7-O-Ethyl-9-O-(4-hydroxyphenyl)propionyl-guaiacylglycerol (1):** white solid; [α]D²⁰ = −3.72 (c 0.51, MeOH); UV (MeOH) λmax (log ε) 203 (4.53), 225 (4.17), 280 (3.66) nm; IR (KBr) νmax 3426, 1727, 1516, 829 cm⁻¹; ¹H and ¹³C NMR (CD₃OD), see Table 1; ESIMS m/z 389 [M−H]⁻, HRESIMS m/z 389.1602 [M−H]⁻ (calcd. for C₂₁H₂₅O₇, 389.1606).

(R)-4,5,4’-Trihydroxy-3,3’α-trimethoxybibenzyl (2): white amorphous powder; [α]D²⁰ = −12.46 (c 1.07, MeOH);

UV (MeOH) λmax (log e) 204 (4.59), 286 (3.75) nm; IR (KBr) νmax 3418, 1607, 1517, 1455, 1434, 796 cm⁻¹; ¹H and ¹³C NMR (CD₃OD), see Table 1; ESIMS m/z 319 [M−H]⁻, HRESIMS m/z 319.1180 [M−H]⁻ (calcd. for C₁₇H₁₉O₆, 319.1187).

(25)-5,7,3’-Trihydroxy-6,4,5-trimethoxyflavone (3): yellow amorphous powder; [α]D²⁰ = 46.64 (c 0.46, MeOH); UV (MeOH) λmax (log ε) 206 (4.70), 294 (4.17) nm; IR (KBr) νmax 3335, 2940, 1641, 1514, 1462, 1345, 1434, 1182, 1091, 998, 833 cm⁻¹; ¹H and ¹³C NMR (CD₃OD), see Table 1; ESIMS m/z 331 [M−H]⁻, HRESIMS m/z 331.0819 [M−H]⁻ (calcd. for C₁₇H₁₉O₇, 331.0823).

3.4 DPPH Radical Scavenging Activity Assay

The free radical scavenging activity assay was carried out according to previous method [36] with some modifications. Briefly, 30 μL samples (1000 μg/mL, dissolved in ethanol) and Trolox (1 mM) were added to 270 μL DPPH solution (100 μM, dissolved in methanol), respectively. The reaction proceeded for 1 h at 37 °C on a 96-well microplate. The absorbance was then read at 515 nm and percentage of total radical scavenging activity was calculated using the following formula: inhibition % = [(A₀ − A₁)/A₀] × 100%, where A₀ is the absorbance of the DPPH without samples (control reaction) and A₁ is the absorbance of DPPH incubated with the samples. All the tests were conducted in triplicate and Trolox was used as a positive control agent.

3.5 Mushroom Tyrosinase Inhibitory Assay

Tyrosinase activity inhibition was determined spectrophotometrically according to the method described previously [36] with some modifications. Briefly, different concentrations of test compounds were prepared in 10% DMSO. Each of the sample solution (20 μM) was mixed with L-Dopa (1.25 mM), and diluted with 970 μL of 0.05 mM sodium phosphate buffer (PBS, pH 6.8) in the test tubes. The reaction was initiated by adding mushroom tyrosinase (25 U/mL). The reaction mixture was incubated for 5 min at room temperature. The amount of Dopachrome in the mixture was determined by the measurement of the absorbance of each well at 490 nm. Kojic acid was used as a positive control agent. The inhibitory percentage of tyrosinase activity was calculated using the following formula: inhibition % = [(A₀ − A₁)/A₀] × 100%, where A₀ is the absorbance of the DPPH without samples (control reaction) and A₁ is the absorbance of Dopachrome incubated with the test compounds.
3.6 Collagen Production by HDFa Assay

The HDFa cell line was obtained from Cascade Biologics. HDFa cells were seeded in 96-well plates containing DMEM with 10% FBS under a humidified atmosphere of 5% CO2 at 37 °C. After 24 h of incubation, the cells were treated with the test samples for 72 h (37 °C, 5% CO2). TGF-β was used as the positive control. Media (50 μL) was collected from each well, and froze at −80 °C until it was assayed with procollagen peptide ELISA kit. The concentration of procollagen was obtained by measuring the absorbance at 450 nm on the microplate reader. Remove all media from cells and add 100 μL diluted MTS reagent to each well. The reaction was incubated for 40 min at 37 °C. The absorbance was measured at 490 nm with a microplate reader. The increase in collagen production was calculated according to the following equation: cell viability (%) = (Mean OD490 sample/Mean OD490 control); increase of collagen production % = (A1/B/A0 - 1) × 100%. Where A1 is the absorbance with the samples, A0 is the absorbance without samples (control reaction), and B is cell viability.

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Compliance with Ethical Standards

Conflict of interest No potential conflict of interest was reported by the authors in this manuscript.

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