RESEARCH ARTICLE

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2017, 8, 353Synthesis, characterization, solubilization,
cytotoxicity and antioxidant activity of
aminomethylated dihydroquercetin†Jianxia Li,^a Jieqiong Dong,^b Jie Ouyang,^b Jie Cui,^c Yuan Chen,^b
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A dihydroquercetin derivative (DHQA) was prepared through aminomethylation to overcome the low water solubility and bioavailability of dihydroquercetin (DHQ). DHQA was characterized through HPLC, nuclear magnetic resonance, scanning electron microscopy, X-ray diffraction, and thermogravimetric analyses. DHQA was converted into the amorphous form, but the major structure of DHQ remained unchanged. Solubilization and dissolution tests were also performed. Results showed that the solubility and dissolution rates of DHQA were approximately 16.28 and 6.31 times higher than those of DHQ, respectively. The MTT assay of DHQA showed a non-toxic effect against non-cancerous HEK-293T cells ($EC_{50} = 820.00 \mu\text{M}$), and potent inhibitory activity against cancerous Hela cells ($EC_{50} = 138.17 \mu\text{M}$). Finally, the antioxidant activity of DHQA was confirmed *in vitro* through DPPH and ABTS radical scavenging activity assays. DHQA displayed high antioxidant activities with low IC_{50} values (0.043 and 0.042 mM, respectively). Reducing Fe^{3+} power assay indicated that DHQA exhibited higher reducing power than DHQ and ascorbic acid.

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1. Introduction

Dihydroquercetin (DHQ; also known as 3,5,7,3,4-pentahydroxy flavanone or taxifolin; Fig. 1) is a flavonoid and one of the most effective natural antioxidants that are important for human health.¹ DHQ is widely distributed in the barks of the genus *Pinus* or *Larix* and in the seeds of the genus *Silybum*.^{2,3} DHQ exhibits antineoplastic, antimicrobial,⁴ anti-inflammatory, analgesic,⁵ and anti-adipogenic properties,⁶ as well as radical scavenging activity.⁷ DHQ can improve blood circulation in the brain and effectively inhibit cellular melanogenesis while increasing the tyrosinase protein level.^{2,8,9} This compound exhibits antiviral activity and induces resistance to cardiovascular diseases and platelet aggregation.¹⁰ DHQ also protects plants against pathogens.^{11,12} Therefore, DHQ has been widely used in medical, food, cosmetic, agricultural, and technical fields. However, DHQ is difficult to absorb and metabolize because of its low water solubility, thereby limiting its bioavailability and efficacy.^{10,13,14} Therefore, an efficient,

water-soluble, and nontoxic synthetic derivative of DHQ must be developed to expand its application.

The aminomethylation of DHQ has been recently investigated. Nifant'ev *et al.* used secondary amines, a primary amine, and amylamine as the amine components in the Mannich (aminomethylation) reaction to synthesize a large number of monosubstituted and disubstituted DHQ derivatives.¹⁵ Kosheleva *et al.* synthesized close structural analogs of DHQ with an equimolar ratio of reagents from Mannich reactions.¹⁶ Kaziev *et al.* reported heteropoly-compounds of DHQ based on a modified flavonoid, 2,3-dihydroquercetin, from Mannich reactions.¹⁷ These results indicated that all these DHQ derivatives have the major structure of DHQ,

Fig. 1 The chemical structure of dihydroquercetin.

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which contains five hydroxyl groups. Therefore, these DHQ derivatives can preserve the antioxidant properties of DHQ because of several hydroxyl groups.¹⁸ DHQ modification is achieved *via* the introduction of amino groups that produce new polyfunctional compounds with broken structural balance to enhance solubility. However, previous studies focused on the synthesis and structure of aminomethylated DHQ derivatives. To the best of our knowledge, the characterization, solubilization, dissolution rate, and antioxidant activity of aminomethylated DHQ derivatives have not been reported to date.

This study focused on the preparation and characterization of a water-soluble DHQ derivative formed by aminomethylation without changing the original DHQ molecular structure. We accomplished the selective bisaminomethylation of DHQ with L-proline; the reaction was performed at the DHQ: formaldehyde: L-proline molar ratio of 1:2:2 (Fig. 2). The structure and properties of the aminomethylated DHQ derivative were characterized by high performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), scanning electron microscopy (SEM), X-ray diffraction (XRD) and thermogravimetry (TG). Finally, solubility, dissolution rate, cytotoxicity and antioxidant activity tests were performed to investigate the biological activities of the aminomethylated DHQ derivative. This work can provide valuable information for improving the solubility of DHQ to be easily absorbed and metabolized by the human body.

2. Materials and methods

2.1. Materials

DHQ (96% purity) was isolated from *Larix gmelinii* according to our previous study.¹⁹ HEK-293T (human embryonal kidney 293T) and Hela (human cervical carcinoma) cells were purchased from Union Hospital (Beijing, China). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Penicillin-streptomycin was purchased from Gibco (Grand Island, NY, USA). Cisplatin, aristolochic acid, dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were obtained from Sigma (St. Louis, MO, USA). All other solvents and chemicals were of analytical grade and obtained from Beijing Chemical

Reagents Co., Ltd. (Beijing, China). Water was purified with the NW Ultra-pure water system (Pudong, Shanghai, China).

2.2. Preparation of aminomethylated DHQ

The compound (DHQA: aminomethylation of DHQ) was synthesized according to the method of Nifant'ev *et al.* with slight modifications.²⁰ A mixture of 33% formalin (1.32 mmol), L-proline (0.152 g; 1.32 mmol), and 10 mL of ethanol was stirred at 60 °C until complete homogenization. Finally, a solution of DHQ (0.2 g, 0.66 mmol) in 5 mL of ethanol was slowly added. The reaction mixture was stirred at 60 °C for 1.5 h. The resultant pale yellow powder-like precipitate was separated by filtration, sequentially washed with ethanol, benzene, and hexane, and dried in a vacuum drying oven to constant weight.

DHQA was analyzed with HPLC using a modified method.¹⁶ A Shimadzu LC-2010A HPLC system (Kyoto, Japan) with an LC-2010 detector and a Shimadzu VP-ODS-C18 column (250 mm × 4.6 mm) was employed. The mobile phase was MeOH-CH₃COOH (0.1%) with gradient elution from 10 to 90% MeOH over 30 min. The eluent flowed isocratically at 1.0 mL min⁻¹. The sample was detected at 288 nm, and the injection volume was 20 µL. All samples were filtered through 0.45 µm membrane filters before injection into the HPLC apparatus.

2.3. ¹H and ¹³C NMR spectroscopy

¹H and ¹³C NMR spectra were acquired on a 400 MHz Bruker AV-III spectrometer (Germany) operating at room temperature, with DMSO-d₆ as the solvent and tetramethylsilane (TMS) as the internal standard. Before measurement, 20 and 40 mg of the dried samples were dissolved in 0.5 mL of DMSO-d₆ for ¹H and ¹³C NMR, respectively. The ¹H NMR spectra were obtained with a minimum of 64 scans, whereas the ¹³C NMR spectra were performed in the pulse FT mode (100.6 MHz) with a minimum of 2048 scans.

2.4. SEM

The samples for SEM were lyophilized in a HITACHI ES-2030 apparatus, mounted on metallic stubs with colloidal graphite, and coated with gold in a Hitachi E-1010 sputter coater for 70 s. The materials were observed, and micrographs were obtained with a HITACHI S-3400.

Fig. 2 Synthesis of the DHQ aminomethylated derivative.

2.5. Powder XRD

Powder XRD analyses were conducted on a Shimadzu XRD-7000 X-ray diffractometer with Cu K α radiation. Diffraction patterns were recorded between 5° and 60° with a step size of $2\theta = 0.02^\circ$ at a scanning rate of 5° min^{-1} .

2.6. Thermal analyses (TG)

The weight loss of DHQ and DHQA was determined with a TG analyzer (Q500, TGA Instruments). Approximately 10 mg of sample was used for each run. The sample was heated in a dynamic nitrogen atmosphere (flow rate = 70 mL min^{-1}) from room temperature to 600°C , with a heating rate of $10^\circ \text{C min}^{-1}$.

2.7. Solubilization test

The solubility of DHQ and DHQA was determined according to Yang *et al.*¹⁰ An excess amount of the respective compound was placed in 2 mL of water (*ca.* pH 5.7) under nitrogen and sheltered from light. The mixture was stirred for 1 h at $20 \pm 2^\circ \text{C}$. The solution was filtered on a $0.45 \mu\text{m}$ cellulose acetate membrane. The filtrate was evaporated to dryness under reduced pressure, and the residue was dosed by a weighing method.

2.8. Dissolution test

The dissolution rates of DHQ and DHQA were studied on the premise of maintaining the sink condition. The environmental conditions of the process included a speed of 100 rpm and a dissolution temperature of $37.0 \pm 0.5^\circ \text{C}$. Artificial gastric juice (pH 1.6) served as the dissolution medium, which was used according to the procedure described by the National Pharmacopoeia Committee.²¹ The tablets of DHQA (0.12 g) and DHQ (0.12 g) were added to the respective dissolution cups containing 500 mL of the dissolution medium. Subsequently, 5 mL aliquots of the dissolution medium were collected at 3, 8, 13, 18, 23, 28, 38, and 48 min as well as 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, and 6 h; 5 mL of the dissolution medium was added after sample collection to ensure that the volume remained constant. Finally, 200 μL of the dissolution medium was collected and mixed with 3 mL of the solvent (HCl, pH 1.6) in a fresh centrifuge tube. After evenly mixing the sample, the mixture was analyzed by UV spectrophotometry (PGENERAL UV-T6 New Century). The DHQA and DHQ calibration curves were obtained from the equations $Y = 27.441X - 0.0065$ ($R^2 = 0.9999$) and $Y = 55.797X - 0.0225$ ($R^2 = 0.9982$), respectively (Y , absorbance; X , concentration, mg mL^{-1}). The values were calculated from the absorbance at 288 nm of a series of DHQA and DHQ solutions at concentrations of 0.0064–0.0192 mg mL^{-1} .

2.9. Cytotoxicity assay

HEK-293T and Hela cells were cultured in DMEM medium supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin. The cells were incubated at 37°C in a

humidified incubator with an atmosphere of 5% CO_2 . DHQ, DHQA and positive controls (cisplatin and aristolochic acid) were prepared in DMSO, and diluted to various concentrations (2–1280 μM) with the culture medium. The final DMSO concentration in the medium was less than 0.1%.

The cells were inoculated into 96-well plates (1×10^4 cells per well, 200 μL per well) and cultured at 37°C . After 12 h pre-incubation to allow cellular attachment, the supernatant was removed, and blank medium or medium containing various concentrations of test solution was added and incubated for 48 h. The supernatant was removed after incubation and 100 μL of tetrazolium reagent (1 mg mL^{-1}) was added into each well followed by further incubation at 37°C for 4 h. The supernatant was decanted, and DMSO (150 μL per well) was added to allow formazan solubilization. The optical density (OD) of each well was detected using a Microplate reader (BIO-RAD Model 680) at 570 nm. Each treatment was repeated 5 times. The 50% effective concentration (EC_{50}) was determined by curve fitting.

2.10. Antioxidant activity assays

2.10.1. DPPH radical scavenging activity. The scavenging activities of DHQ and DHQA against the DPPH radical were evaluated according to a standard method with some modifications.²² The extract (2 mL) was mixed with 2 mL of DPPH solution ($2 \times 10^{-4} \text{ mol L}^{-1}$). The reaction mixture was incubated in the dark at room temperature for 30 min before the absorbance of the mixture was obtained at 517 nm. The DPPH radical of ascorbic acid was used as the positive control. The percentage of scavenging activity against the DPPH radical was calculated with the following equation:

$$\text{Radical scavenging activity (\%)} = [1 - (A - B)/C] \times 100$$

where A is the absorbance of the DPPH radical solution mixed with the sample extract, B is the absorbance of the sample extract mixed with alcohol, and C is the absorbance of DPPH radical solution mixed with water. All measurements were performed in triplicate ($n = 3$).

2.10.2. ABTS radical scavenging activity. The assay was performed according to the procedure described by Li, Wang, Wang, Walid, & Zhang with some modifications.²³ The working solution was prepared by mixing stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate in equal quantities; the mixture was allowed to react for 12 h at room temperature in the dark. The resulting solution (1 mL) was diluted with alcohol to obtain the required absorbance at 734 nm of 0.7 ± 0.02 units with a UV spectrophotometer. A freshly prepared solution of ABTS was used for the assay. Briefly, 0.2 mL of the extract was allowed to react with 0.8 mL of the ABTS solution; the absorbance at 734 nm was recorded after 6 min with a spectrophotometer. The ABTS radicals of ascorbic acid were used as the positive controls. The percentage of scavenging activity against the ABTS radical was calculated with the following equation:

$$\begin{aligned} \text{Radical scavenging activity (\%)} \\ = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \end{aligned}$$

where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS in alcohol and $\text{Abs}_{\text{sample}}$ is the absorbance of ABTS solution mixed with the sample extract. All measurements were performed in triplicate ($n = 3$).

2.10.3. Reducing Fe^{3+} power assay. The reducing Fe^{3+} power of DHQ and DHQA was assessed by a previously described method with slight modifications.²⁴ Different gradient concentrations of the DHQ and DHQA solutions were prepared. First, 2 mL of a potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] solution (1%, w/v), 2 mL of a phosphate (KH_2PO_4 – K_2HPO_4) buffer (0.2 M, pH 6.6), and 1 mL of the sample solution were sequentially added into the same centrifuge tube. The resulting solution was mixed and incubated at 50 °C for 20 min before 2 mL of the trichloroacetic acid solution (10%, w/v) was added. After cooling to room temperature, 2 mL of the abovementioned solution, 0.4 mL of fresh ferric chloride (FeCl_3) solution (0.1%, w/v), and 2 mL of water were added to the reaction mixture, which was thoroughly shaken. At 10 min after the reaction, the absorbance of the reaction mixture was measured at 700 nm against a blank solution with a UV spectrophotometer. The reducing Fe^{3+} power of ascorbic acid was considered as the positive control. All tests were performed in triplicate. The reducing Fe^{3+} power was evaluated by the following equation:

$$\text{Reducing power} = A_1 - A_0$$

where A_1 is the absorbance of a mixture solution of the sample and other reaction reagents; A_0 is the absorbance of the sample under identical conditions when A_1 was mixed with water instead of the FeCl_3 solution.

3. Results and discussion

3.1. HPLC analysis

The identification and quantification of DHQA was based on a combination of retention times and area normalization method using an HPLC system. In Fig. 3A, the results showed that the retention time of DHQA was 10.39 min which was different from that of DHQ (RT = 18.01 min) in Fig. 3B. According to the area normalization method, the peak area of DHQA was 96%. This phenomenon clearly showed that the aminomethylation of DHQ occurred and the DHQA had quite high purity.

3.2. ^1H and ^{13}C NMR analysis

The ^1H NMR spectra of DHQ and DHQA are shown in Fig. 4. In the ^1H NMR spectra of DHQ (Fig. 4A), the peak at 2.49 ppm is related to the protons of the DMSO solvent. The doublet peaks at 4.96 ppm and the doublet of the doublet peaks at 4.49 ppm are attributed to the protons of ring C, namely, H2 and H3, respectively. The two doublet peaks at 5.90 and 5.85 ppm are attributed to the protons of ring A, namely, H6

Fig. 3 HPLC chromatogram of DHQ and DHQA (A: DHQA; B: DHQ).

Fig. 4 ^1H -NMR spectra of DHQ (spectrum A) and its derivative DHQA (spectrum B).

and H8, respectively, and the broad single peaks from 6.74 and 6.87 ppm were probably contributed by the protons of ring B, namely, H2', H5', and H6', respectively. The hydrogen atoms of the OH groups of DHQ could be observed at 5.72 ppm (3-OH), 8.95 ppm (4'-OH), 9.00 ppm (3'-OH), 10.80 ppm (7-OH) and 11.88 ppm (5-OH), thereby confirming the structure of DHQ.^{25,26} The number and positions of the strong signals in the ^1H -NMR spectra of DHQA (Fig. 4B) considerably changed compared with those in the spectra of DHQ. The intensive peaks at 5.90 and 5.85 ppm (the signals of H6 and H8, respectively) disappeared because of the substitution of L-proline groups by aminomethylation. The multiple peaks at ≈ 1.81 , 1.93–2.16, and 2.89–3.47 ppm correspond to the methylene protons of the NCH_2CH_2 , CH_2CHCOOH , and NCH_2CH_2 groups of the pyrrole ring, respectively.²⁰ The two doublet peaks at 3.79 and 3.88 ppm are attributed to the protons linked to carboxyl (CHCOOH) groups, whereas the single peaks at 3.98 and 4.08 ppm were probably contributed by the methylene protons linked to C₆ (CH_2C^6) and C₈ (CH_2C^8), respectively. In addition, the broad signals at 13.07 are assigned to the protons of the carboxyl (COOH) groups. The presence of these new signals indicated that L-proline groups were successfully grafted onto DHQ. Simultaneously, the doublet peaks at 4.29 and 4.92 ppm and the single peaks at 6.71 and 6.88 ppm are attributed to the protons of H2, H3, H5', H6' and H2', respectively. The single peaks at 6.07 and 12.83 ppm and the broad peaks at 7.35 ppm are attributed to the hydroxyl protons of 3-OH, 5-OH, 7-OH, 3'-OH, and 4'-OH, re-

spectively. All the signals suggested the successful synthesis of DHQA in our studies.

Characterization by ^{13}C -NMR spectroscopy was performed to further confirm the structural features of DHQ and its derivative DHQA. The results are shown in Fig. 5. Based on the reported data for structurally defined DHQ,^{25,26} the DHQ spectrum (Fig. 5A) is obviously characterized by the signals at 71.6, 83.1, and 197.8 ppm, which are attributed to C3, C2, and C4 of ring C, respectively. The signals at 95.0, 96.0, 100.5, 162.6, 163.3, and 166.8 ppm correspond to C8, C6, C10, C9, C5, and C7 of ring A, respectively. The peaks at 115.2, 115.4, 119.4, 128.1, 145.0, and 145.8 ppm are characteristic of C2', C5', C6', C1', C3', and C4' in ring B, respectively. The peak at 39.5 ppm corresponds to the DMSO solvent. In the ^{13}C -NMR spectra of DHQA (Fig. 5B), several new strong signals are present as compared with DHQ. The signals at 23.5, 28.6, 45.2, 53.1, and 48.0 ppm are attributed to methylene carbons of (NCH_2CH_2) groups, (CH_2CHCOOH) groups, (NCH_2CH_2) groups, CH_2C^6 , and CH_2C^8 , respectively.²⁰ The carbons C (CHCOOH) and (COOH) produce signals at 66.90 and 176.70 ppm. The residual peak intensity and positions of DHQA was similar to those of DHQ. This phenomenon clearly showed that the aminomethylation of DHQ occurred and the double L-proline groups were bound to the C6 and C8 of DHQ.

3.3. SEM analysis

The particle morphology of DHQ and DHQA is shown in Fig. 6. DHQ appeared as various irregularly-shaped blocks

Fig. 5 ^{13}C -NMR spectra of DHQ (spectrum A) and its derivative DHQA (spectra B).

with a crystal particle size of 1.55–14.78 μm (Fig. 6A and B). DHQA was in a spherical, loose, and porous state, with a particle size of 0.80–6.44 μm (Fig. 6C and D). Obviously, the shape of DHQA was quite different from that of DHQ, and its particle size was about two times smaller than DHQ, indicating that the formulation technology employed in this study caused effective particle size reduction. In a previous study, the micronized taxifolin produced by a supercritical anti-solvent process showed a particle size distribution of 2–11 μm .³

3.4. XRD analysis

The crystalline states of DHQ and DHQA are presented in Fig. 7. DHQ exhibited intense diffraction peaks at $2\theta = 7.0^\circ$, 7.4° , 14.0° , 15.0° , 16.3° , 17.4° , 20.56° , 25.3° , 27.0° , and 37.8° , indicating its crystalline form (Fig. 7A).¹⁰ However, DHQA showed relatively broad halo patterns and a small sharp peak at 37.8° (Fig. 7B), suggesting that the powder was mainly amorphous. These results indicated that DHQA was no longer present in the crystalline form after reacting with formaldehyde and L-proline during aminomethylation. The similar amorphous state was also observed in other DHQ derivatives by physical or chemical modifications, which dramatically improved the solubility and bioavailability of DHQ.^{10,14,27}

3.5. TG analysis

The thermal stabilities of DHQ and DHQA were investigated by the TG method. The TG profile of DHQ shows two distinct regions of weight loss (Fig. 8A). The initial weight decrease of 10.09% in the temperature range of 30–100 $^\circ\text{C}$ was due to the absorbed water and hydrogen bonded water from DHQ.²⁸ And the second weight decrease of 43.41% in the temperature range of 247–600 $^\circ\text{C}$ was due to the decomposition of DHQ, which is in agreement with the reported thermal behavior.²⁷ In Fig. 8B, the weight of DHQA began to obviously decrease by 64.84% from 217 $^\circ\text{C}$ to 600 $^\circ\text{C}$. These results showed that the stability of DHQA was slightly inferior to that of DHQ and the weight loss of DHQA was higher than that of DHQ. Because the particle size of DHQA was smaller than that of DHQ, which resulted in its higher specific surface area,¹⁴ DHQA was thus more easily evaporated and rapidly decomposed.

3.6. Solubilization

The water solubility of DHQA was remarkably increased to 19.53 mg mL^{-1} , which was 16 times higher than that of native DHQ (*ca.* 1.20 mg mL^{-1}). There were some related documents about the preparation of some water-soluble DHQ derivatives. The solubility of the inclusion complexes of taxifolin and native CDs (α -CD, β -CD, γ -CD, and HP β CD) was

Fig. 6 SEM images of DHQ (A, B) and DHQA (C, D).

8.5, 12.1, 7.0, and 6.8 mg mL⁻¹, respectively.¹⁰ The highest solubility of the inclusion complex of taxifolin/ γ -cyclodextrin (CD) (4:9) was approximately 16.1 mg mL⁻¹ among various proportions of the inclusion complex of taxifolin/ γ -CD.¹⁴ Zolotarev *et al.* synthesized DHQ monosuccinate (DHQMS) and reported that the solubility of DHQMS was 17.3 mg mL⁻¹.²⁹ Taken together, it can be inferred that the amino-

methylation technology would be a potential and effective approach for improving the solubility of DHQ.

3.7. Dissolution rate analysis

The dissolution rate of DHQA was much faster than that of DHQ (Fig. 9). DHQA achieved maximum dissolution at 38 min (87.1%); simultaneously, the dissolution of DHQ was approximately 13.8%. The maximum dissolution of DHQ was approximately 83.78% at 360 min. The increased drug release rate observed for DHQA can be attributed to its smaller particle size and higher water solubility than DHQ, due to the presence of L-proline. Zu *et al.* prepared micronized taxifolin with a particle size distribution of 2–11 μ m *via* a supercritical anti-solvent (SAS) process and the solubility was 0.02 mg mL⁻¹ at 60 min (the dissolution medium was PBS).³ By contrast, the solubility of DHQA was approximately 0.21 mg mL⁻¹. It was illustrated that DHQA prepared by the amino-methylation process was amorphous and smaller so the dissolution rate of DHQA increased. According to the Noyes–Whitney equation, with the particle size of materials decreasing, the specific surface area of drugs increased accordingly; thereby the dissolution of the drugs was also improved, which was attributed to the increase of the contact area of the solid drugs and the dissolution medium.

Fig. 7 XRD results of each sample: (A) DHQ; (B) DHQA.

Fig. 8 TG curves of each sample: (A) DHQ; (B) DHQA.

Fig. 9 The dissolution profiles of DHQ and DHQA.

3.8. Cytotoxicity

MTT is a vital dye reduced by the succinate dehydrogenase enzyme of mitochondrial living cells to form purple formazan crystals.³⁰ MTT assay has been widely used to determine the cytotoxic effect of natural compounds.³¹ In the present study, the cytotoxic effects of various concentrations of DHQ, DHQA and positive controls on HEK-293T and Hela cells were assessed using the MTT assay.

To compare the antiproliferative effects of DHQ and DHQA on normal cells, the viability for HEK-293T cells was determined. As shown in Fig. 10A, DHQ more obviously inhibited the growth of HEK-293T cells than DHQA, and the

EC₅₀ values of DHQ and DHQA were 670.66 and 820.00 μ M, respectively. Compared with the positive control, aristolochic acid (EC₅₀ = 118.34 μ M), DHQ and DHQA were much less toxic on HEK-293T cells, by a factor of approximately 5.6 and 7.0, which indicated no cytotoxic effect on HEK-293T cells. Furthermore, DHQ and DHQA displayed a potent cell growth promoting effect on HEK-293T cells after 48 h of treatment in low concentrations (2–360 μ M), and the maximum growth rates of DHQ and DHQA were 22.65% and 54.45% at 200 μ M. The results showed that DHQA exhibited approximately 2.5 times greater cell growth than DHQ. It suggests that the presence of two L-proline side chains in DHQA may significantly increase its activity.

The decrease of Hela cell viability induced by DHQ and DHQA was found to proceed in a dose-dependent manner and their inhibition rates were different from that of the positive compound. DHQ and DHQA showed almost unanimously cytotoxic activity against Hela cells with EC₅₀ values of 125.31 and 138.17 μ M, respectively (Fig. 10B), while both

Fig. 10 *In vitro* cytotoxicity of DHQ and DHQA against HEK-293T (A) and HeLa cells (B).

of them exhibited poor activity against Hela cells, compared with the positive control, cisplatin ($EC_{50} = 29.29 \mu\text{M}$). Despite the significant differences in EC_{50} of DHQA and cisplatin, it is worth mentioning that DHQA showed exactly the same cytotoxicity against Hela cells as procyanidin A2 and hesperetin.^{32,33} Whereas DHQA has no effect on normal cells (HEK-293T cells), it has cytotoxic activity towards Hela cells. This confirmed the reliability of the satisfactory water solubility of DHQA, which will be beneficial for medical utilization.

3.9. Anti-oxidant activity analysis

The results of the DPPH radical scavenging assay are shown in Fig. 11A, with ascorbic acid as the positive control. The scavenging effect on DPPH $\cdot$ of DHQA was 1.5 times higher than that of ascorbic acid. DHQA exhibited slightly lower antiradical activity (by inhibiting DPPH $\cdot$) than DHQ at a relatively low concentration range of 0.03–0.06 mM. However, when the concentration of DHQA was increased to 0.09 mM, the scavenging capacity reached 85.65%, which was almost consistent with that of DHQ (88.74%). This phenomenon indicated that DHQA had stronger DPPH scavenging ability at high concentrations. The half-maximal (50%) inhibitory concentration of a substance (IC_{50}) is a common indicator of anti-oxidant activity. A low IC_{50} value indicates high DPPH $\cdot$ scavenging activity. Within the tested dosage range, the IC_{50} values of DHQA, DHQ, and ascorbic acid were 0.043, 0.034, and 0.064 mM, respectively. Apparently, ascorbic acid had weaker scavenging activity than the other fractions, whereas DHQA and DHQ had nearly similar scavenging activities. The IC_{50} of another DHQ derivative (micronized dihydroquercetin *via* the SAS process) was found to be approximately 0.5 mg ml^{-1} ,³ which was much higher than that of DHQA ($IC_{50} = 0.022 \text{ mg ml}^{-1}$). These results implied that DHQA contained substances that were hydrogen donors, which could react with free radicals to scavenge DPPH $\cdot$ as good scavenging agents.

The ABTS radical is one of the few stable radical sources. This radical is extensively used to determine total antioxidant capacity.³⁴ The scavenging ABTS activity of DHQA was higher than that of DHQ (Fig. 11B). When the DHQA concentration was 0.1 mM, the scavenging ABTS activity reached 98.33%, which was approximately 1.2 times higher than that of DHQ (84.92%). However, the scavenging ABTS activity of DHQA was almost the same as the antiradical activity of ascorbic acid (by inhibiting the ABTS free radicals) at the same doses. The IC_{50} values of DHQA, DHQ, and ascorbic acid were 0.042, 0.048, and 0.042 mM, respectively.

The reducing Fe^{3+} power is related to the presence of reductones, which are terminators of free radical chain reactions because they donate a hydrogen atom.^{35,36} The reducing Fe^{3+} power values of DHQA, DHQ, and ascorbic acid are indicated in Fig. 11C. Higher absorbance values corresponded with stronger reducing power. The reducing power of DHQA, DHQ, and ascorbic acid increased in a concentration-dependent manner from 0.15 mM to 1.2 mM.

Fig. 11 The antioxidant activities of DHQA measured by reducing scavenging DPPH radical activity (A), scavenging ABTS radical activity (B) and Fe^{3+} power (C). Ascorbic acid was used as positive control. Each value was expressed as the mean $\pm$ s.d. ($n = 3$).

DHQA showed evidently higher ability as compared with ascorbic acid. However, the reducing power of DHQA was almost similar to that of DHQ at the same doses. Therefore, the reducing Fe^{3+} power of all tested samples had the

following order: DHQA $\approx$ DHQ > ascorbic acid. This phenomenon shows that DHQA has a very strong capacity to donate an electron; this ability was comparable with that of DHQ.

4. Conclusion

DHQA was successfully prepared by aminomethylation. The molecular structure and morphology of DHQA was confirmed by HPLC, ^1H and ^{13}C NMR, as well as SEM. The results showed that DHQA preserved the major structure of DHQ, which contained five hydroxyl groups. The results of XRD and TG analyses indicated that DHQA was amorphous, with significantly different characteristics from DHQ. Moreover, the solubility test showed that DHQA has better water solubility than DHQ. The dissolution of DHQA was also significantly improved as compared with that of DHQ. MTT assay revealed that DHQA had a non-toxic effect against non-cancerous HEK-293T cells and potential inhibitory activity against cancerous Hela cells. Finally, the antioxidant activities of DHQA were evaluated by its reducing power and scavenging abilities against DPPH and ABTS radicals. DHQA demonstrated effective antioxidant activity. The antioxidant effect of DHQA was obviously higher than that of ascorbic acid (positive control) and nearly similar to that of DHQ. Therefore, DHQA may have great potential as a novel DHQ formulation in healthcare products.

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