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ORIGINAL ARTICLE

Flos *Chrysanthemi Indici* protects against hydroxyl-induced damages to DNA and MSCs via antioxidant mechanism

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Total phenolics

Abstract Flos *Chrysanthemi Indici* (FCI) is a Chinese herbal medicine used in China for over 2000 years. In this study, its ethanol extract (EFCL) was found to protect against hydroxyl radical (·OH) induced oxidative damages to mesenchymal stem cells (MSCs) and DNA. To explore the mechanism, EFCL was further determined by chemical and antioxidant assays. Folin–Ciocalteu colorimetric assay suggested that content of total phenolics was 93.85 ± 3.56 mg catechin/g and HPLC analysis indicated a content of 10.05 ± 1.44 mg/g as chlorogenic acid in EFCI. Antioxidant assays revealed that EFCI could also scavenge $\cdot O_2^-$ radical (IC_{50} 58.74 ± 1.30 μg/mL), DPPH· radical (IC_{50} 46.46 ± 1.35 μg/mL) and $ABTS^{+\cdot}$ radical (IC_{50} 20.59 ± 0.52 μg/mL), bind Fe^{2+} (IC_{50} 364.27 ± 19.57 μg/mL) and Cu^{2+} (IC_{50} 734.77 ± 34.69 μg/mL), reduce Fe^{3+} (IC_{50} 147.11 ± 11.09 μg/mL) and Cu^{2+} (IC_{50} 35.69 ± 3.14 μg/mL). On the basis of mechanistic analysis, we concluded that FCI can effectively protect against hydroxyl-induced damages to DNA and MSCs, therefore FCI shows promise as a possible therapeutic reagent for many diseases. The effect may be mainly attributed to phenolics especially chlorogenic acid, which exerts the antioxidant action possibly through metal-chelating, and radical-scavenging which is via hydrogen atom transfer (HAT) and/or sequential electron proton transfer (SEPT) mechanisms.

1. Introduction

It is well known that mesenchymal stem cells (MSCs) are capable of self-renewal and differentiation into all mesodermal cell types and neuro-ectodermal cells. These abilities make MSCs an excellent seed cell for cell transplantation, tissue engineering, and gene therapy [1]. However, these clinical applications are usually limited by the oxidative damages induced by reactive oxygen species (ROS) especially hydroxyl radical (·OH)

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[2]. The hydroxyl radical (·OH) is well-known as the most powerful reactive oxygen species (ROS) and can damage DNA to bring about various oxidative lesions (Supplemental file 1). These oxidative lesions, however, can result in genomic instability and lead to cell death [3,4].

Therefore, it is critical to search for potential therapeutic agents for oxidative damage. In recent years, medicinal plants especially Chinese medicinal herbals have attracted much attention.

As a typical Chinese herbal medicine, Flos *Chrysanthemi Indici* (FCI, 野菊花 in Chinese, Supplemental file 2) has been used in traditional Chinese medicine (TCM) for over 2000 years [5,6]. From the perspective of TCM, FCI can clear away *heat* and *toxicity* [5]. Therefore, FCI is widely consumed as a healthcare tea which is generally called Chrysanthemum Tea (菊花茶 in Chinese) in China. The so-called *heat* and *toxicity* in TCM, however can be regarded as oxidative stress in free radical biology and medicine, and excessive ROS can be a resource of *heat* and *toxicity* [7].

Phytochemical analyses have demonstrated that there are total flavones, chlorogenic acid, and some volatile compounds (such as 1,7,7-trimethyl-bicyclo[2,2,1]heptan-2-one and isoborneol) [8–10]. Obviously, flavones and chlorogenic acid belong to the family of total phenolics.

In the present study, we employed a Soxhlet extractor to prepare its ethanolic extract (EFCI). The extract (EFCI) was then evaluated for the protective effect against oxidative damages to MSCs and DNA, and the relevant chemical contents especially total phenolics and chlorogenic acid. On this basis, the antioxidant mechanism of CFI was further discussed using an *in vitro* model. Undoubtedly, it will be helpful to understand the pharmacological effects or functions in TCM of CFI, and to discover a new therapeutic agent against oxidative damage in MSC transplantation or tissue engineering.

2. Materials and methods

2.1. Plant material and animals

Flos *Chrysanthemi Indici* was collected at an elevation of 500 m in the region of Mount Huangshan (Latitudes 30°1′ N and longitudes 118°1′ E, Anhui, China). It was authenticated by Professor Shuhui Fan and sun-dried. A voucher specimen has been deposited in our laboratory. Sprague–Dawley (SD) rats of 4 weeks of age were obtained from the animal center of Guangzhou University of Chinese Medicine.

2.2. Chemicals

DPPH· (1,1-diphenyl-2-picryl-hydrazl radical), pyrogallol, murexide (5,5′-nitrilodibarbituric acid monoammonium salt), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], neocuproine (2,9-dimethyl-1,10-phenanthroline), catechin, and Folin–Ciocalteu reagent were purchased from Sigma Co. (Sigma–Aldrich Shanghai Trading Co., China); ABTS [2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid diammonium salt)] was obtained from Amresco Co. (Solon, OH, USA); Chlorogenic acid and caffeic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA); CD44 was purchased from Wuhan Boster Co., Ltd. (Wuhan, China). All other reagents were of analytical grade.

2.3. Preparation and HPLC characterization of ethanolic extract from Flos Chrysanthemi Indici (EFCL)

Dried Flos *Chrysanthemi Indici* (10 g) was powdered then extracted by 150 mL absolute ethanol using a Soxhlet extractor for 6 h. The extract was filtered using a Buckner funnel and Whatman No. 1 filter paper. The filtrate was then concentrated to dryness under reduced pressure to yield ethanolic extract of Flos *Chrysanthemi Indici* (EFCI, 2.36 g). It was stored at 4 °C for analysis.

The HPLC characterization of EFCI was performed on a Syltech P510 system (Los Angeles, California, USA), equipped with a Diamonsil C_{18} (250 mm × 4.6 mm, 5 μm) column (Dikma Co., Beijing, China). All samples were dissolved in methanol at 10 mg/mL and filtered using 0.45 μm filters. The mobile phase consisted of methanol-0.1% phosphoric acid (85:15, v:v) and the flow rate was 0.5 mL/min, injection volume was 15 μL, detection wavelength was 254 nm. In the study, chlorogenic acid in EFCI was firstly identified using an external standard method based on the retention time (23.18 min), it was then further identified by an internal standard method. The content of chlorogenic acid was calculated based on the linear equation ($y = 715502.78x - 975704.97$, $R = 0.9993$, Supplemental file 3).

2.4. Determination of total phenolics

The total phenolic content of EFCI was determined using Folin–Ciocalteu assay [11] with catechin as a standard. In brief, 0.5 mL sample methanolic solution (3 mg/mL) was mixed with 0.5 mL Folin–Ciocalteu reagent (0.25 mol/L). After incubation for 3 min, 1.0 mL of Na_2CO_3 aqueous solution (15%, w/v) was added. After standing at room temperature for 30 min, the mixture was centrifuged at 3500 r/min for 3 min. The absorbance of the supernatant was measured at 760 nm (Unico 2100, Shanghai, China). The total phenolic content of EFCI was calculated based on a linear regression equation of catechin ($y = 72.322x + 0.1346$, x for catechin content, y for absorbance at 760 nm, $R = 0.994$), and was expressed as catechin equivalents in milligrams per gram of extract.

2.5. Protective effect against ·OH-induced damage to MSCs (MTT assay)

MSC culture was carried out according to our previous report [12] with slight modifications. In brief, bone marrow was obtained from the femur and tibia of rat. The marrow samples were diluted with DMEM (LG: low glucose) containing 10% FBS. MSCs were prepared by gradient centrifugation at 900*g* for 30 min on 1.073 g/mL Percoll. The prepared cells were detached by treatment with 0.25% trypsin and passaged into cultural flasks at $1 \times 10^4/cm^2$. MSCs at passage 3 were evaluated for cultured cell homogeneity using detection of CD44 by flow cytometry and were used for the investigation.

These MSCs were seeded at 1×10^4 cells per well in 96-well plates. After adherence for 24 h, these MSCs were then divided into normal, model, and sample (EFCI and chlorogenic acid) groups. In the normal group, MSCs were incubated for 24 hr in DMEM; In the model and sample groups, MSCs however were added by $FeCl_2$ (100 μM) followed by H_2O_2 (50 μM). After incubation for 20 min, the mixture of $FeCl_2$ and H_2O_2 was removed. MSCs in the model group were incubated in DMEM for 24 hr, while MSCs in the sample group were incubated in DMEM with 3 and 30 μg/mL EFCI (or 3 and 30 μg/mL chlorogenic acid) for 24 hr. All groups had five independent wells. After incubation, 20 μL MTT (5 mg/mL) was added and then incubated for further 3 h. Culture medium was discarded and was replaced with 150 μL DMSO. Absorbance at 490 nm was measured by a Bio-Kinetics reader (PE-1420; Bio-Kinetics Corporation, Sioux Center, IA, USA). In the experiment, culture with serum medium was used for the control group and each sample test was repeated in five independent wells.

2.6. Protective effect against hydroxyl-induced DNA damage

The protective effect against hydroxyl-induced DNA damage of EFCI was estimated by our method [13]. Briefly, sample was dissolved in methanol at 4 mg/mL. Various amounts (9–36 μL) of sample methanolic solutions were then separately taken into tubes. After evaporating the sample solutions in tubes to dryness, 300 μL of phosphate buffer (0.2 mol/L, pH 7.4) was added to the sample residue. Subsequently, 50 μL DNA sodium (10.0 mg/mL), 75 μL H_2O_2 (33.6 mmol/L), 50 μL $FeCl_3$ (3.2 mmol/L), and 100 μL Na_2-EDTA (0.5 mmol/L) were added. The reaction was initiated by adding 75 μL of ascorbic acid (12 mmol/L). After incubation in a water bath at 50 °C for 20 min, the reaction was terminated by adding 250 μL of trichloroacetic acid (10 g/100 mL water). The color was then developed by addition of 150 μL of TBA (2-thiobarbituric acid) (0.4 mol/L, in 1.25% NaOH aqueous solution) and heated in an oven at 105 °C for 15 min. The mixture was cooled and absorbance was measured at 530 nm against the buffer (as blank). The percent of protection against DNA damage is expressed as follows:

$$\text{Protective effect } \% = \frac{A_0 - A}{A_0} \times 100\%$$

where A_0 is the absorbance of the control without sample, and A is the absorbance of the reaction mixture with sample.

2.7. Superoxide anion ($\cdot O_2^-$) radical-scavenging assay

Measurement of superoxide anion ($\cdot O_2^-$) scavenging activity was based on our improved method [14]. Briefly, the sample was dissolved in methanol at 3 mg/mL. The sample solution (x μL, where x = 0, 5, 10, 15, 20, and 25 μL) was mixed with 2950-x μL Tris–HCl buffer (0.05 mol/L, pH 7.4) containing Na_2EDTA (1 mmol/L). When 50 μL pyrogallol (60 mmol/L in 1 mmol/L HCl) was added, the mixture was shaken at room temperature immediately. The absorbance at 325 nm of the mixture was measured (Unico 2100, Shanghai, China) against the Tris–HCl buffer as blank every 30 s for 5 min. The $\cdot O_2^-$ scavenging ability was calculated as:

$$\text{Inhibition } \% = \frac{\left(\frac{\Delta A_{325\ \text{nm,control}}}{T}\right) - \left(\frac{\Delta A_{325\ \text{nm,sample}}}{T}\right)}{\left(\frac{\Delta A_{325\ \text{nm,control}}}{T}\right)} \times 100\%$$

Here, $\Delta A_{325\ \text{nm, control}}$ is the increment in $A_{325\ \text{nm}}$ of the mixture without the sample and $\Delta A_{325\ \text{nm, sample}}$ is that with the sample; T = 5 min. The experiment temperature was 37 °C.

2.8. Fe^{2+}-chelating assay

The Fe^{2+}-chelating capacity was evaluated by the method as described by Li [11]. Briefly, x μL sample solutions (6 mg/mL, x = 0, 20, 40, 60, 80, and 100) were added to 100 μL $FeCl_2$ aqueous solutions (0.25 mmol/L). The reaction was initiated by the addition of 150 μL ferrozine aqueous solutions (0.50 mmol/L) and the total volume of the reaction mixture was adjusted to 1000 μL with methanol. Then, the mixture was shaken vigorously and stood at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm (Unico 2100, Shanghai, China). The percentage of chelating effect on Fe^{2+} was calculated by the following formula:

$$\text{Inhibition } \% = \frac{A_0 - A}{A_0} \times 100\%$$

where A_0 is the absorbance without sample, and A is the absorbance with sample.

2.9. Cu^{2+}-chelating assay

The Cu^{2+}-chelating capacity was measured by a complexometric method [15]. Briefly, murexide solution (1.2 mL, 0.25 mmol/L) and $CuSO_4$ aqueous solution (60 μL, 20 mmol/L) were added to hexamine HCl buffer (pH 5.0, 30 mmol/L) containing 30 mmol/L KCl. After incubation for 1 min at room temperature, 30–240 μL sample solutions (3 mg/mL in methanol) were added. The final volume was adjusted to 1500 μL with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 485 nm and 520 nm (Unico 2100, Shanghai, China). The absorbance ratio ($A_{485\ \text{nm}}/A_{520\ \text{nm}}$) reflected the free Cu^{2+} content. Therefore, the percentage of cupric chelating effect was calculated by the following formula:

$$\text{Chelating effect } \% = \frac{\left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)_{\max} - \left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)}{\left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)_{\max} - \left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)_{\min}} \times 100\%$$

where $\left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)$ is the absorbance ratio of the sample, while $\left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)_{\max}$ is the maximum absorbance ratio and $\left(\frac{A_{485\ \text{nm}}}{A_{520\ \text{nm}}}\right)_{\min}$ is the minimum absorbance ratio in the test.

2.10. DPPH· radical-scavenging assay

DPPH· radical-scavenging activity was determined as previously described by Li [16]. Briefly, 1 mL DPPH· ethanolic solution (0.1 mmol/L) was mixed with 0.5 mL sample alcoholic solution (0.06–0.18 mg/mL). The mixture was kept at room temperature for 30 min, and then measured with a spectropho-

tometer (Unico 2100, Shanghai, China) at 519 nm. The DPPH· inhibition percentage was calculated as:

$$\text{Inhibition } \% = \frac{A_0 - A}{A_0} \times 100\%$$

where A is the absorbance with samples; while A_0 is the absorbance without samples.

2.11. $ABTS^{+\cdot}$ radical-scavenging assay

The $ABTS^{+\cdot}$-scavenging activity was measured as described [11] with some modifications. The $ABTS^{+\cdot}$ was produced by mixing 0.2 mL ABTS diammonium salt (7.4 mmol/L) with 0.2 mL potassium persulfate (2.6 mmol/L). The mixture was kept in the dark at room temperature for 12 h to allow completion of radical generation, then diluted with 95% ethanol (about 1:50) so that its absorbance at 734 nm was 0.70 ± 0.02. To determine the radical-scavenging activity, 1.2 mL aliquot of diluted $ABTS^{+\cdot}$ reagent was mixed with 0.3 mL of sample ethanolic solution (0.05–0.25 mg/mL). After incubation for 6 min, the absorbance at 734 nm was read on a spectrophotometer (Unico 2100, Shanghai, China). The percentage inhibition was calculated as:

$$\text{Inhibition } \% = \frac{A_0 - A}{A_0} \times 100\%$$

Here, A_0 is the absorbance of the mixture without sample, A is the absorbance of the mixture with sample.

2.12. Cu^{2+}-reducing power assay

Cupric ion (Cu^{2+}) reducing capacity was determined by the method [17], with minor modifications. Briefly, 125 μL $CuSO_4$ aqueous solution (0.01 mol/L), 125 μL neocuproine ethanolic solution (7.5 mmol/L) and $(750 - x)$ μL CH_3COONH_4 buffer solution (0.1 mol/L, pH 7.5) were brought to test tubes with different volumes of samples (3 mg/mL, x = 20–100 μL). Then, the total volume was adjusted to 1000 μL with the buffer and mixed vigorously. Absorbance against a buffer blank was recorded at 450 nm after 30 min (Unico 2100, Shanghai, China). The relative reducing power of the sample as compared with the maximum absorbance, was calculated by the formula:

$$\text{Relative reducing power}\% = \frac{A - A_{min}}{A_{max} - A_{min}} \times 100\%$$

where, A_{max} is the maximum absorbance at 450 nm and A_{min} is the minimum absorbance in the test. A is the absorbance of sample.

2.13. Statistical analysis

Each experiment was performed for three times, and the results were presented as mean ± standard deviations (SD). Based on the data of dose–response curve, the regression equation for calculating IC_{50} value was established, and the IC_{50} value was defined as the final concentration of 50% radical inhibition (relative reducing power, or chelating effect) in the study. Based on the calibration curve, the regression equation for calculating total contents of phenolics was established, and the correlation coefficient R was quoted. All linear regressions were analyzed using Origin 6.0 professional software (OriginLab Corporation, Northampton, MA, USA). Determination of significant differences between the mean IC_{50} values of the sample and positive controls was performed using one-way ANOVA the T-test. The analysis was performed using SPSS software 13.0 (SPSS Inc., Chicago, IL) for windows. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

It is well known that ROS are various forms of activated oxygen including free radicals and non-free-radical species, especially hydroxyl radical (·OH) and superoxide anion ($\cdot O_2^-$). For example, the most reactive ROS form, hydroxyl radical (·OH), once generated via Fenton reaction *in vivo* (Eq. (1)), can oxidatively damage DNA to yield malondialdehyde (MDA) and a number of oxidative lesions (Supplemental file 4). These oxidative lesions along with ROS lead to apoptosis of cells especially stem cell [3,4].

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (1)$$

In the study, however, ECFI was observed to be able to protect MSCs against oxidative damage (Fig. 1).

Undoubtedly, the protective effect is assumed to be related to its protection on DNA. Therefore, we used our method to estimate its protective effect against hydroxyl-induced DNA damage. The data indicated that EFCI increased dose-dependently the protection percentages (Supplemental file 4). It means that EFCI can effectively protect against hydroxyl-induced DNA damage, and the IC_{50} value was observed to be 405.72 ± 4.08 μg/mL (Table 1). Since cancer and inflammation have been demonstrated to be closely related with the oxidative stress, the protective effect against oxidative damages to DNA and MSCs may be therefore responsible for the anti-cancer and anti-inflammation effects of CFI [18,19]. In addition, the fact that FCI can reduce the oxidative stress and protect against damage induced by ROS in cells, can also account for the effects of clearing away *heat* and *toxicity* in TCM, and may show promise as a new therapeutic reagent against oxidative damage in MSC transplantation or tissue engineering.

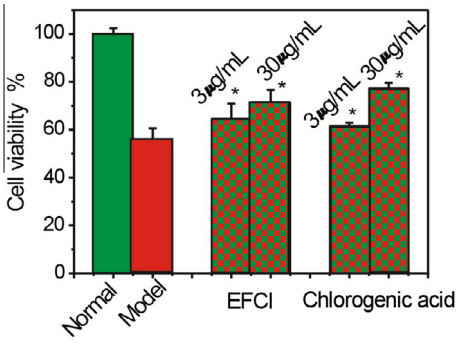

Figure 1 The protective effect of extracts from Flos *Chrysanthemi Indici* (ECFI) and chlorogenic acid against ·OH-induced damage to MSCs (mesenchymal stem cells) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) assay. Each value is expressed as mean ± SD (n = 3). $^*P < 0.05$ vs model.

Table 1 The IC_{50} values of EFCI and positive controls (μg/mL).

Assays	EFCI	Positive controls
		Catechin
DNA protecting	405.72 ± 4.08[b]	192.07 ± 13.74[a]
$\cdot O_2^-$ scavenging	58.74 ± 1.30[b]	7.12 ± 0.49[*,a]
Fe^{2+}-chelating	364.27 ± 19.57[b]	3.21 ± 0.14 [**,a]
Cu^{2+}-chelating	734.77 ± 34.69[b]	191.58 ± 2.10 [**,a]
DPPH· scavenging	46.46 ± 1.35[b]	4.66 ± 0.070[a]
$ABTS^{+\cdot}$ scavenging	20.59 ± 0.52[b]	0.92 ± 0.010[a]
Cu^{2+}-reducing	35.69 ± 3.14[b]	6.22 ± 0.060[a]

IC_{50} value is defined as the concentration of 50% effect percentage and expressed as mean ± SD (n = 3). Means values with different superscripts in the same row are significantly different (one-way ANOVA, $p < 0.05$).

[*] The positive control is caffeic acid.

[**] The positive control is sodium citrate. EFCI, ethanol extract of Flos *Chrysanthemi Indici*.

A number of phytochemical researches suggest that the antioxidant ability in plants can be attributed to the existence of total phenolics. In the Folin–Ciocalteu assay, EFCI exhibited a high amount of total phenolics (93.85 ± 3.56 mg catechin/g). As a phenolic compound, chlorogenic acid has also been found to be of substantial amount (10.05 ± 1.44 mg/g) in EFCI (Fig. 2). The high ratio of chlorogenic acid *versus* total phenolics (10.05:93.85) suggests that chlorogenic acid is one of main phenolic compounds. On the other hand, chlorogenic acid itself has been known as a good antioxidant [20], and presented a protective effect against MSCs oxidative damage in the study (Fig. 1). Thereby, the antioxidant ability of ECFI can be mainly attributed to the existence of chlorogenic acid. The assumption is further supported by the previous studies, in which chlorogenic acid has been considered as the functional component in *Chrysanthemum* [21].

Earlier investigators have pointed out that there are two approaches for natural phenolic antioxidant to protect against oxidative DNA damage: one approach is to scavenge ROS then to reduce their attack; another approach is to repair the deoxynucleotide radicals which are damaged by free radicals [22]. In order to further confirm whether the protective effect of EFCI against oxidative DNA damage was related to its ROS scavenging, we determined its $\cdot O_2^-$ radical scavenging ability.

As we know, superoxide anion ($\cdot O_2^-$) is also regarded as an important form of ROS in living cells. Although $\cdot O_2^-$ is much weaker than ·OH, however, it is able to directly attack DNA and lipids too [23], or transform into ·OH via Haber–Weiss reaction (Eq. (2)) to damage biomolecules (e.g., DNA) [24].

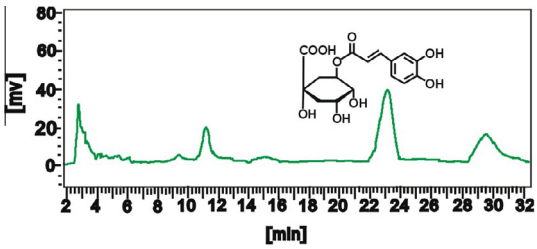

Figure 2 A typical HPLC profile of ethanol extract of Flos *Chrysanthemi Indici* (ECFI).

$$\cdot O_2^- + H_2O_2 \xrightarrow[(\text{or } Cu^{2+})]{\text{Iron ion}} \cdot OH + OH^- + O_2 \quad (2)$$

To obtain more reliable values, the $\cdot O_2^-$-scavenging ability of EFCI was determined at physiological pH 7.4 [14]. As shown in Supplemental file 4, EFCI increased dose-dependently the $\cdot O_2^-$ radical-scavenging percentages, and the IC_{50} value was 58.74 ± 1.30 μg/mL (Table 1). The fact that EFCI can effectively scavenge $\cdot O_2^-$ radical suggests ROS scavenging as one possible approach for EFCI to protect against oxidative DNA damage.

As shown in Eqs. (1) and (2), transition metals (especially Fe and Cu) can catalyze the generation of ROS (especially ·OH and $\cdot O_2^-$ radicals). The metal-chelating ability of EFCI was thus explored in the study. The dose–response curves in Supplemental file 4 demonstrated an effective metal-chelating capacity of EFCI, and the IC_{50} values were calculated as 364.27 ± 19.57 and 734.77 ± 34.69 μg/mL, respectively for Fe^{2+}-chelating and Cu^{2+}-chelating (Table 1). It was previously reported that the chelating ability might mainly result from the existence of the *ortho*- or adjacent hydroxyl group (–OH) and carbonyl group (C═O) [11]. For example, chlorogenic acid occurring in FCI [25], may bind metal ions based on the proposed reaction (Fig. 3).

Undoubtedly, metal-chelating was regarded as one mechanism for EFCI to scavenge ROS.

To verify whether EFCI can directly scavenge free radicals, we further measured its radical-scavenging on DPPH· and $ABTS^{+\cdot}$. The assays revealed that EFCI can efficiently eliminate both DPPH· and $ABTS^{+\cdot}$ radicals (Supplemental file 4). Its IC_{50} values were respectively 46.46 ± 1.35 and 20.59 ± 0.52 μg/mL (Table 1). As we know, both DPPH· and $ABTS^{+\cdot}$ radicals could be yielded without transition metal-catalysis. On this basis, it can be induced that another mechanism for EFCI to scavenge ROS is direct radical-scavenging.

Taken together, one approach for EFCI to protect against oxidative damages to DNA or MSCs may be ROS scavenging, which would be mediated via metal-chelating and direct radical-scavenging.

Furthermore, the previous works suggested that DPPH· scavenging has been demonstrated to be a hydrogen atom (H·) transfer process (HAT). In the process, DPPH· was thought to convert to DPPH-H molecule, and the antioxidant was assumed to donate a H· atom to form semi-quinone even quinone form [26]. For instance, chlorogenic acid, an antioxidant occurring in FCI [22,25], may scavenge DPPH· via the following proposed mechanism [27,28] (Fig. 4).

Unlike DPPH· radical, $ABTS^{+\cdot}$ radical cation however needs an electron (e) to neutralize the positive charge, and $ABTS^{+\cdot}$ scavenging is considered an electron (e) transfer reaction [29]. For example, the proposed reaction for chlorogenic acid to scavenge $ABTS^{+\cdot}$ can be briefly illustrated by Fig. 5,

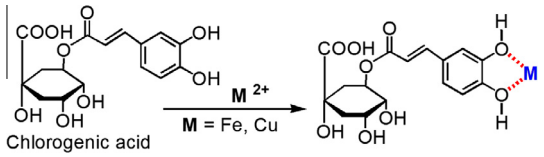

Figure 3 The proposed reaction of chlorogenic acid binding metal ions.

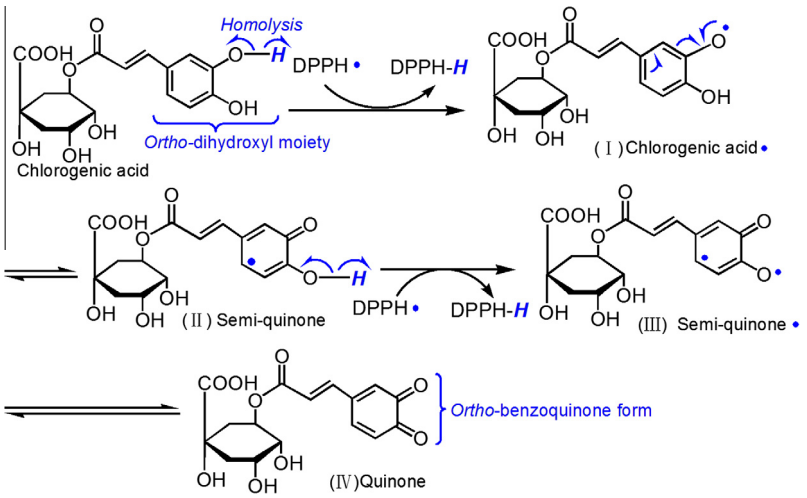

Figure 4 The proposed reaction for chlorogenic acid to scavenge DPPH· (1,1-diphenyl-2-picrylhydrazyl radical).

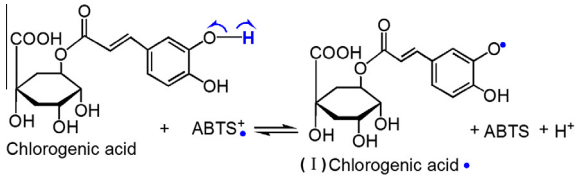

Figure 5 The proposed reaction of chlorogenic acid and $ABTS^{+\cdot}$ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical anion).

in which (I) can be further converted into semi-quinone (II) or quinone (IV).

The fact that EFCI can effectively scavenge $ABTS^{+\cdot}$ radical, suggests that donating electron (*e*) may be another approach for EFCI to directly scavenge radicals. The donating electron (*e*) mechanism is further supported by the Cu^{2+}-reducing power assay. The results in Supplemental file 4 showed that EFCI could dose-dependently reduce Fe^{3+} and Cu^{2+}, and the IC_{50} values were 147.11 ± 11.09 and 35.69 ± 3.14 μg/mL, respectively (Table 1). Obviously, the data indicate an effective reducing power of FCI. As we know, the reducing reaction is actually an electron (*e*) transfer process. Since *e* transfer is always accompanied by deprotonation, it is called sequential electron proton transfer (SEPT) [30].

4. Conclusions

In conclusion, Flos *Chrysanthemi Indici* can effectively protect against hydroxyl-induced damages to DNA and MSCs, therefore FCI shows promise as a possible therapeutic reagent for many diseases. The effect may be mainly attributed to phenolics (including chlorogenic acid), which exert the antioxidant action possibly through metal-chelating, and radical-scavenging which is via hydrogen atom transfer (HAT) and/or sequential electron proton transfer (SEPT) mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jscs.2014.06.004.

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