Regular Article

Protective Effect of Sinapine against Hydroxyl Radical-Induced Damage to Mesenchymal Stem Cells and Possible Mechanisms

Xican Li,*a Lu Han,a Yunrong Li,a Jing Zhang,a Jiemin Chen,a Wenbiao Lu,a Xiaojun Zhao,a Yingtao Lai, b Dongfeng Chen, c and Gang Wei*,a

a School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine; Guangzhou 510006, China; b The First Affiliated Hospital, Guangzhou University of Chinese Medicine; Guangzhou 510006, China; and c School of Basic Medical Science, Guangzhou University of Chinese Medicine; Guangzhou 510006, China.

Received October 30, 2015; accepted January 15, 2016; advance publication released online February 3, 2016

As a phenolic alkaloid occurring in Cruciferous plants, sinapine was observed to protect mesenchymal stem cells (MSCs) against ·OH-induced damage in this study. It was also found to prevent DNA from damage, to scavenge various free radicals (·OH, ·O2, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS)+, and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·)), and to reduce Cu2+ to Cu+. To further explore the mechanism, the end-product of sinapine reaction with DPPH· was determined using HPLC-electrospray ionization (ESI)-MS/MS and HPLC-diode array detector (DAD). Four molecular ion peaks (m/z 701, 702, 703, and 351) in HPLC-ESI-MS/MS analysis indicated a radical adduct formation (RAF) pathway; while a bathochromic shift (λmax 334 → 475 nm) in HPLC-DAD indicated the formation of quinone as the oxidized product of the phenolic –OH group. Based on these results, it may be concluded that, (i) sinapine can effectively protect against ·OH-induced damage to DNA and MSCs; such protective effect may provide evidence for a potential role for sinapine in MSC transplantation therapy, and be responsible for the beneficial effects of Cruciferous plants. (ii) The possible mechanism for sinapine to protect against ·OH-induced oxidative damage is radical-scavenging, which is thought to be via hydrogen atom (H·) transfer (HAT) (or sequential electron (e) proton transfer (SEPT))→RAF pathways.

Key words sinapine; mesenchymal stem cell; antioxidant mechanism; Cruciferous plant; ·OH-induced damage; hydrogen atom (H·) transfer (HAT) (or sequential electron (e) proton transfer (SEPT))→radical adduct formation (RAF)

Recently, some phytophenols have been reported to suppress reactive oxygen species (ROS)-induced oxidative stress in mesenchymal stem cells (MSCs).13 MSCs are known as multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. Therefore, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. However, their clinical applications are limited in connective tissue including osteoblasts, chondrocytes, and multipotent fibroblast cells that give rise to cells of the skeletal connective tissue including osteoblasts, chondrocytes, and adipocytes. Therefore, the present study hence tries to firstly investigate its protection of MSCs from ·OH-induced damage using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. On this basis, the possible mechanism will be further investigated using HPLC-electrospray ionization (ESI)-MS/MS and HPLC-diode array detector (DAD) analyses. It will provide basic evidence for the clinical application of sinapine in MSC transplantation therapy. In addition, since sinapine (thiocyanate) as main bioactive compound occurs in some medicinal or edible Cruciferous plants, such as Semen Raphani (Laifuzi), Semen Sinapis Albae (Huangjiezi), White Mustard Seed (Baijizi), and Semen Lepidii (Tinglizi),12,13) thus the study will be helpful to understand their beneficial effects as Chinese herbal medicine, or nutritional value as vegetables.

Experimental

Chemicals and Animals

Sinapine thiocyanate

![Fig. 1. The Structure of Sinapine Thiocyanate](image)

As a special phytophenol, sinapine however contains an N atom in the molecule (Fig. 1). Therefore, it can be also considered as a phenolic alkaloid.11) To our knowledge, there is no similar study concerning phenolic alkaloid interacting with MSCs until now.

*To whom correspondence should be addressed. e-mail: lixican@126.com; Weigang021@163.com

© 2016 The Pharmaceutical Society of Japan
Flow Cytometry Analysis for Annexin V and PI

In the control group, MSCs were incubated in DMEM; in the model and sample groups, MSCs were added by FeCl$_2$ ($100\mu$g/mL) followed by H$_2$O$_2$ ($50\mu$L). After incubation for 25 min, the mixture of FeCl$_2$+$H_2O_2$ was removed. MSCs in the model group were incubated for 24 h in DMEM, while MSCs in the sample group were incubated for 24 h in DMEM with various sinapine concentrations. After culture, cells were harvested by trypsin (0.05%) digestion in phosphate-buffered saline (PBS). The cells were washed and re-suspended in PBS, fixed in 70% ethanol and washed with PBS. In the next step, they were labeled with Annexin V and 50ng/mL PI and ribonuclease (RNase) (10ug/mL) and then incubated at 37°C for 45 min. Fluorescence was measured using flow cytometry (Coulter EPICS XL) with standard software. Experiments were performed with 2 different batches of cells and each batch was tested in duplicate.

ROS-Scavenging Assay

The ROS-scavenging herein refers to hydroxyl radical (·OH) scavenging and superoxide anion (·O$_2^-$). Experiment of ·OH radical-scavenging was conducted in terms of our method.$^{[5]}$ In brief, the sample ethanol solution (4mg/mL, 10–50µL) was separately added into tubes. After evaporating the sample solutions in the tubes to dryness, 300µL of phosphate buffer (0.2m, pH 7.4) was added to the sample residue. Subsequently, 50µL DNA sodium (10.0mg/mL), 75µL H$_2$O$_2$ (33.6mm), 50µL FeCl$_3$ (3.1mm), and 100µL sodium tetrahydroxylaminoacetate (Na$_4$EDTA) (0.5mm) were added. The reaction was initiated by adding 75µL of ascorbic acid (12mm). After incubation in a water bath at 50°C for 20min, the reaction was terminated by adding 250µL of trichloroacetic acid (10%, w/w). The color was then developed by addition of 5% thiobarbituric acid (TBA) (0.5mM) and then incubated at 37°C for 15 min. Absorbance of the cooled solution was then measured spectrophotometrically at 530nm. The percent of protection against DNA damage is expressed as follows:

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

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

Measurement of ·O$_2^-$ scavenging activity was based on our method.$^{[6]}$ Briefly, the sample was dissolved in ethanol. The sample solution ($x\mu$L, where $x=0, 70, 140, 210, 280$, and $350\mu$L) was mixed with 2950–$x\mu$L Tris–HCl buffer (0.05M, pH 7.4) containing Na$_4$EDTA (1mm). When $50\mu$L pyrogallol (60mm in 1mM HCl) was added, the mixture was shaken at
room temperature immediately. The absorbance was read at 325 nm in a spectrophotometer (Unico 2100, Shanghai, China) against the Tris–HCl buffer as blank every 30 s for 5 min. The calculation of ·O_2^- scavenging percentages was described in our previous study.

**ABTS**^+^ and **DPPH**^−^ Radical-Scavenging Assay** The ABTS^+^-scavenging activity was determined as described.\(^{(7)}\) The ABTS^+^ was produced by mixing 0.2 mL ABTS diammonium salt (7.4 mM) with 0.2 mL K_2S_2O_8 (2.6 mM). 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 ABTS^+^-scavenging activity, 1.2 mL aliquot of diluted ABTS^+^ reagent was mixed with 0.3 mL of sample ethanolic solution. 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 “DNA Protection Assay.”

**DPPH**^−^ radical-scavenging activity was determined as described.\(^{(7)}\) Briefly, 1 mL DPPH: ethanolic solution (0.1 mM) was mixed with 0.5 mL sample alcoholic solution at various concentrations. The mixture was kept at room temperature for 30 min, and then measured with a spectrophotometer (Unico 2100, Shanghai, China) at 519 nm. The DPPH^−^ inhibition percentage was calculated as “DNA Protection Assay.”

**Cu^2^+-Reducing Power Assay** The cupric (Cu^2^+) reducing capacity was determined by the method\(^{(27)}\) with minor modifications. Briefly, 125 µL CuSO_4 aqueous solution (10 mM), 125 µL neocuproine ethanolic solution (7.5 mM), and CH_3COONH_4 buffer solution (100 mM, pH 7.5) were brought to test tubes with different volumes of samples (1 mg/mL). Then, the total volume was adjusted to 1000 µL with the buffer and mixed vigorously. Absorbance against a buffer blank was measured 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:

\[
\text{Relative reducing effect} \% = \frac{A_{\text{max}} - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}} \times 100\%
\]

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

**HPLC-DAD and HPLC-ESI-MS/MS Analyses of End-Product of Sinapine Reaction with DPPH**^−^ The analytical methods were based on our previous study.\(^{(10)}\) Sinapine (thiocyanate) methanol solution was mixed with DPPH^−^ radical methanol solution at molar ratio 1:3, and the mixture was incubated for 1 h at room temperature. The product mixture was then filtered using a 0.45 µm filter and analyzed by a HPLC technique combined with DAD (Shimadzu LC20A/SPD-M20A, Japan), equipped with a Diamonsil C_{18} (250 mm×4.6 mm, 5 µm) column (Dikma Co., Beijing, China). A mobile system with methanol (phase A) and water (phase B) was applied for the separation. With a flow rate of 0.6 mL/min, the gradient elution program was set as follows: 0–22 min, linear changed from 75%A to 87%A. Injection volume was 20 µL, and the DAD detection was performed in the range of 200–800 nm at 1 nm/step. Screenshot wavelength: 475 nm. Reactants sinapine thiocyante and DPPH^−^ were also comparatively measured under the same chromatographic conditions. Particularly, the HPLC profile at 275 nm was used to recognize the reactants and products in the product mixture.

The above product mixture was further analyzed using a HPLC-ESI-MS/MS system (TSQ Quantum Access MAX, Thermo Fisher Corp., U.S.A.), equipped with the above Diamonsil C_{18} column. The above mobile system was applied for the separation. Mass spectrometer was equipped with an ESI source and run in negative mode. The scan range was 150–1000 m/z. ESI parameters were optimized with direct infusion of dansylated amine mixture by an external syringe and set as follows: capillary, +2.5kV; nebulizer pressure, 30 psi; dry gas flow, 5 arb; dry gas temperature, 180°C. High purity nitrogen was applied both as a nebulizer gas and a drying gas.

**Statistical Analysis** Each experiment was performed in triplicate and the data were recorded as mean±standard deviation (S.D.). The \(IC_{50}\) value was defined as the final concentration of 50% radical inhibition (relative reducing power or chelating effect). Statistical comparisons were made by one-way ANOVA to detect significant difference using SPSS 13.0 (SPSS Inc., Chicago, IL, U.S.A.) for windows. \(p<0.05\) was considered to be statistically significant.

**Results and Discussion** Several latest studies have revealed that, in the process of proliferation and differentiation, MSCs are particularly susceptible to damage by chemical or physical environments, such as radiation\(^{(18)}\) and iron overload.\(^{(19)}\) Radiation and iron overload however have been shown to be related with the generation of ROS. For example, iron overload can generate ·OH radical, the most harmful radical of ROS, through Fenton reaction.\(^{(20)}\) Thereby, the present study used Fenton’s reagent (H_2O_2+FeCl_2) to damage MSCs. The extent of cellular damage was evaluated using MTT assay. As seen in Fig. 2, the viability of MSCs in the control group presented the highest OD values at 490 nm; By comparison, the model group treated with Fenton’s reagent exhibited the lowest cell viability (40.3%). It means that the MSCs in model group were oxidatively damaged by ·OH radicals. However, the viability of MSCs in the sample groups (2.7–271.4 µm sinapine) was effectively

![Fig. 2. Sinapine Prevents against MSCs Damage Induced by Fenton’s Reagent](image-url)
The anti-apoptotic effect of the sinapine on MSCs was further investigated using flow cytometry. The annexin V staining method can be used to determine the presence of externalized phosphatidylserine, a phospholipid normally found in the inner leaflet of the plasma membrane and externalized during the apoptotic process. As shown in Fig. 3, MSCs apoptosis was quantified by staining them with annexin V-FITC/PI. The number of annexin V-positive cells was 2.0 ± 1.1% in MSCs treated without FeCl₂ and H₂O₂, 40.4 ± 1.8% in MSCs treated with FeCl₂ and H₂O₂, and 25.2 ± 2.2% and 13.1 ± 1.5% in MSCs treated with H₂O₂ and FeCl₂ in the presence of indi-

![Fig. 3. Sinapine Inhibited the Apoptosis of MSCs Induced H₂O₂ and FeCl₂](image)

MSCs were treated with or without H₂O₂ and FeCl₂ in the absence or presence of sinapine at indicated concentrations. The apoptotic cells were analyzed by means of flow cytometry analysis for annexin V and PI. This assay was performed to distinguish intact cells (annexin V⁻/PI⁻), necrotic (annexin V⁻/PI⁺) cells, early apoptotic (annexin V⁺/PI⁻) cells, and late apoptotic/necrotic (annexin V⁺/PI⁺) cells. Experiments were performed with 2 different batches of cells and each batch was tested in duplicate. Data are shown as the mean ± S.E.M. (n=4). *p<0.05, compared with the H₂O₂ and FeCl₂ group alone.

![Fig. 4. The Dose–Response Curves of Sinapine and Positive Controls in Various Antioxidant Assays](image)

A for DNA damage assay; B for ·OH radical-scavenging; C for ·O₂⁻ radical-scavenging; D for ABTS⁺ radical-scavenging; E for Cu reducing power assay; F for DPPH radical-scavenging. Each value is expressed as the mean ± S.D. (n=3). BHA, butylated hydroxyanisole.
The Proposed Reaction of ABTS$^+$ with Sinapine

cated concentrations of sinapine. There was significant difference among the groups; post hoc comparisons revealed that the apoptotic cell number of the MSCs treated with FeCl$_2$ and H$_2$O$_2$ was increased and such increase can be inhibited by sinapine ($p<0.05$).

The results from MTT and flow cytometry assays strongly indicate sinapine a protective effect. This may partly explain why sinapine could effectively (97.202%) reduce the mutation of radiated Drosophila$^{21}$ because the mutation has been proven to correlate with oxidative DNA damage induced by ·OH radical,$^{22}$ which can be generated by radiation.$^{23}$ In addition, it can also be responsible for the beneficial effects of Cruciferous plants as Chinese herbal medicines, or their nutritional value as vegetables.$^{12,24}$ More importantly, it also provides the basic evidence for potential application of sinapine in MSC transplantation therapy, especially when they are polluted by radiation, iron overload, or other oxidative stress factors.

It has been demonstrated that such protection can be partly attributed to the repair of DNA oxidative damage.$^{25}$ As seen in Fig. 4A, sinapine exhibited the protective effect against DNA damage in a concentration-dependent manner. Its protective effect even was higher than those of the standard antioxidant Trolox and BHA (Table 1).

The protective effect against DNA oxidative damage has been demonstrated to be involved in ROS scavenging.$^{26,27}$ Hence, sinapine was further investigated for its radical-scavenging abilities on ·OH and ·O$_2^-$ based on our two methods.$^{15,16}$ As seen in Figs. 4B, C, sinapine could effectively scavenge both ·OH and ·O$_2^-$ radicals in a dose-dependent manner. According to the IC$_{50}$ values in Table 1, sinapine possessed higher ROS-scavenging capacities than Trolox. Since both ·OH and ·O$_2^-$ radicals are known as the typical form of ROS, ROS-scavenging is thereby considered as one possible approach for sinapine to protect DNA and MSCs against oxidative damage.

The ROS-scavenging mechanisms of phytophenol have recently been reported to include three main types, i.e., sequential electron (e) proton transfer (SEPT), hydrogen atom (H·) transfer (HAT), and radical adduct formation (RAF).$^{28,29}$

To test the possibility of SEPT mechanism, sinapine was investigated by ABTS$^+$ assay. As can be seen in Fig. 5, ABTS$^+$ radical cation contains a $sp^2$ hybridized N atom with planar configuration; it therefore forms a large $p-\pi$ conjugative system and cause an absorption maximum at 734 nm. Hence, ABTS$^+$ solution appears dark green, and the decrease of $A_{734nm}$ value reflects the extent of ABTS$^+$ scavenging. As shown in Fig. 4D, sinapine increased linearly ($r=0.99326$) the scavenging percentages on ABTS$^+$. ABTS$^+$ scavenging however has been previously described as electron (e) transfer process, which is always accompanied by H· (proton) transfer (Eq. 1).$^{30,31}$ Based on the above discussion, the proposed reaction of sinapine with ABTS$^+$ is illustrated in Fig. 5.

\[
\text{ABTS}^+ + \text{PhOH} \rightarrow \text{ABTS} + \text{PhO}^- + \text{H}^+ \quad (1)
\]

This hypothesis is further supported by the results of Cu$^{2+}$-reducing power assay, in which sinapine also increased linearly ($r=0.99709$) the reducing percentages (Fig. 4E). Such reducing reaction has also been proven to include an electron (e) transfer step.$^{32}$ In short, ABTS assay and Cu$^{2+}$-reducing assay suggest a possibility of SEPT in the interaction of sinapine with free radicals.

Furthermore, DPPH- assay was also used to explore the antioxidant mechanisms of sinapine in the study. As shown in Fig. 4F, sinapine could effectively scavenge DPPH- radical. The end-product of scavenging reaction was further analyzed using HPLC chromatogram, in which three peaks were presented, including peak 1 (r.t.=3.1 min), peak 2 (r.t.=4.9 min), and peak 3 (r.t.=16.9 min) (Fig. 6). Based on their relative retention times, peaks 1 and 3 were respectively assigned as sinapine and DPPH- radical (including its reduced product.

---

**Table 1. The IC$_{50}$ Values of Sinapine (Thiocyanate) and the Positive Controls**

| Compound          | IC$_{50}$ Value (µg/mL) | Trolox µg/mL (µmol) | BHA µg/mL (µmol) | Ratio Value |
|-------------------|------------------------|---------------------|------------------|-------------|
| DNA protection    | 151.2 ± 14.9 (410.3 ± 40.4) | 160.1 ± 7.0 (632.8 ± 27.6) | 204.4 ± 13.2 (1135.5 ± 73.3) | 1.54        |
| ·OH scavenging    | 121.1 ± 9.4 (328.5 ± 25.4) | 296.4 ± 9.1 (1171.5 ± 35.9) | 300.8 ± 10.1 (1671.1 ± 56.1) | 3.57        |
| ·O$_2^-$ scavenging | 37.7 ± 2.1 (102.4 ± 5.8) | 100.4 ± 5.2 (396.8 ± 20.5) | 32.6 ± 2.0 (181.1 ± 11.1) | 9.4         |
| ABTS$^+$ scavenging | 6.1 ± 0.2 (16.6 ± 0.6) | 1.9 ± 0.07 (7.5 ± 0.27) | 1.3 ± 0.1 (7.2 ± 0.56) | 0.45        |
| Cu$^{2+}$-reducing | 20.5 ± 0.2 (55.7 ± 0.7) | 8.8 ± 0.4 (34.8 ± 1.5) | 4.8 ± 0.2 (26.7 ± 1.11) | 0.62        |
| DPPH$^-$ scavenging | 27.6 ± 0.7 (74.8 ± 2.0) | 6.5 ± 0.3 (25.7 ± 1.2) | 6.4 ± 0.3 (35.5 ± 1.7) | 0.34        |

**Average 1.74**

Each IC$_{50}$ value was calculated by linear regression analysis of the dose response curves in Fig. 3. The IC$_{50}$ value in mass unit (µg/mL) was further converted to the value in molar unit and written in the bracket ( ). The linear regression was analyzed by Origin 6.0 professional software. Since each experiment was performed in triplicate, and the IC$_{50}$ values were presented as the mean ± S.D. ($n=3$). Means values in µmol unit with different superscripts in the same row are significantly different ($p<0.05$). BHA, butylated hydroxyanisole. * Caffeic acid (not BHA) as the positive control. Ratio value is defined as IC$_{50}$ Trolox/IC$_{50}$ sinapine, in µmol unit.

---

**Fig. 5. The Proposed Reaction of ABTS$^+$ with Sinapine**
DPPH-H). Peak 2 was thus identified as the oxidized product of sinapine.

The data acquisition of UV spectra was performed using a DAD detector equipped in HPLC system. As seen in Fig. 7, sinapine (peak 1) had an absorption maximum at 338 nm; while oxidized sinapine (peak 2) gave an absorption maximum at 475 nm. This great bathochromic shift (λmax 338 → 475 nm) suggests a quinone-form, which may contain a larger conjugative system than sinapine itself, and can explain the yellowish appearance of the product mixture in our experiment.

Subsequently, the end-product was further analyzed by HPLC-ESI-MS/MS (Supplementary Materials 2), in which four molecular ion peaks were observed, including m/z 703.6 for [M]+, m/z 702.7 for [M−H]+, m/z 701.74 for [M−2H]−, and m/z 351.5 for [M]+. These peaks clearly indicate a RAF pathway between sinapine· radical (molecular weight (MW) 309), and DPPH· (MW 394). In addition, the largest molecular ion peak (m/z 703.6) was further fragmented to yield several losses (including m/z 59, 31, and so on). Based on the above results and the literature, we hypothesized that the reaction of sinapine (thiocyanate) with DPPH· might be via HAT or SEPT multi-pathways (Fig. 7).

As illustrated in Fig. 8, in the reaction with DPPH·, sinapine was presumed to donate a hydrogen atom (H·) to form phenoxy radical then semi-quinone radical. Both radicals contain a larger conjugative system than sinapine itself, a bathochromic shift was thus observed. This is usually regarded as a HAT pathway. HAT pathway however is usually accompanied by SEPT pathway, and our data in ABTS and Cu-reducing assays also suggest a possibility of SEPT. Thus, the SEPT pathway should not be excluded. The semi-quinone radical then combined with excessive DPPH· radical to produce end-product with four molecular ion peaks. This is undoubtedly a RAF step. Our assumption is further supported by the evidence from theoretical chemistry. In summary, the reaction of sinapine with DPPH· radical is assumed to include HAT (or SEPT) → RAF mechanisms. It obviously differs from our previous study, in which no RAF has been found.

Finally, to quantitatively evaluate the relative antioxidant level of sinapine, the ratio value was defined as IC50, Trolox / IC50, sinapine (molar unit). As shown in Table 1, the ratio values of DNA protection, ·OH scavenging, ·O2− scavenging, ABTS+· scavenging, Cu2+-reducing, and DPPH· scavenging were 1.54, 3.57, 3.90, 0.45, 0.62, and 0.34, respectively. The average ratio value was calculated as 1.74 (Table 1). This implies that sinapine has 1.74 times higher total antioxidant capacity than the standard antioxidant Trolox. Such high anti-
oxidant level and edibility further suggest the potential application of sinapine in MSC transplantation therapy.

Conclusion
As a phenolic alkaloid occurring in Cruciferous plants, sinapine can effectively protect against ·OH-induced damages to DNA and MSCs, thereby it may have a therapeutic potential in MSCs transplantation. Its protective effect can also be responsible for the beneficial effects or nutritional values of Cruciferous plants. A possible mechanism for sinapine to protect against oxidative damages is radical-scavenging, which is thought to be involved in HAT (or SEPT)→RAF mechanisms.

Acknowledgments
This work was supported by the National Nature Science Foundation of China (81273896, 81573558), and the project of Science and Technology Department of Guangdong Province (“Theory on correlation between spleen and kidney in TCM for treatment of osteoporosis,” 2013).

Conflict of Interest
The authors declare no conflict of interest.

Supplementary Materials
The online version of this article contains supplementary materials.

References
1) Yagi H., Tan J., Tuan R. S., J. Cell. Biochem., 114, 1163–1173 (2013).
2) Slukvin I. I., Vodyanik M., Cell Cycle, 10, 1370–1373 (2011).
3) Srivastava S., Bankar R., Roy P., Phytomedicine, 20, 683–690 (2013).
4) Li W., Wang Q., Su Q., Ma D., An C., Ma L., Liang H., Mol. Cells, 37, 383–388 (2014).
5) Liu S., Shao Y., Lin Q., Liu H., Zhang D., J. Int. Med. Res., 41, 82–96 (2013).
6) Yang F., Yuan P. W., Hao Y. Q., Lu Z. M., BMC Complement. Altern. Med., 24, 14–74 (2014).
7) Wang H., Liu T. Q., Guan S., Zhu Y. X., Cui Z. F., Eur. J. Pharmacol., 599, 24–31 (2008).
8) Lyublinskaya O. G., Borisov Y. G., Pugovkina N. A., Smirnova I. S., Odihina J. V., Ivanova J. S., Zenin V. V., Shatrova A. N., Bordanina A. V., Aksenov N. D., Zemelko V. I., Burova E. B., Puzanov M. V., Nikol'sky N. N., Oxid. Med. Cell Longev., 2015, 502105 (2015).
9) Li X. C., Hu Q. P., Jiang S., Li F., Lin J., Han L., Hong Y., Lu W., Gao Y., Chen D., J. Saudi Chem. Soc., 19, 454–460 (2015).
10) Li X., Gao Y., Li F., Liang A., Xu Z., Bai Y., Mai W., Han L., Chen D., Chem. Biol. Interact., 219, 221–228 (2014).
11) Zhou H., Jiang H., Yao T., Zeng S., Rapid Commun. Mass Spectrom., 21, 2120–2128 (2007).
12) Liu L., Wang Y., Li H., Ji Y., Chin. J. Chromatogr., 24, 49–51 (2006).
13) China Pharmacopoeia Committee, “Pharmacopoeia of the People’s Republic of China, Part 1,” China Chemical Industry Press, Beijing, 2005, p. 192.
14) Li X., Mai W., Wang L., Han W., Anal. Biochem., 438, 29–31 (2013).
15) Li X., Food Chem., 141, 2083–2088 (2013).
16) Li X., J. Agric. Food Chem., 60, 6418–6424 (2012).
17) Li X., Mai W. Q., Chen D. F., J. Chin. Chem. Soc., 61, 383–390 (2014).
18) Nikolay N. H., Lopez Perez R., Saffrich R., Huber P. E., Oncotarget, 6, 19366–19380 (2015).
19) Zhang Y., Zhai W., Zhao M., Li D., Chai X., Cao X., Meng J., Chen J., Xiao X., Li Q., Mu J., Shen J., Meng A., PLoS ONE, 10, e0120219 (2015).
20) Yang S. R., Park J. R., Kang K. S., Oxid. Med. Cell Longev., 2015, 486263 (2015).
21) Huang D. J., Xu W. Y., Huang D. C. H., Acta Nutr. Sin., 31, 588–590 (2009).
22) Cooke M. S., Evans M. D., Dizdaroglu M., Lunec J., FASEB J., 17, 1195–1214 (2003).
23) Osipov A. N., Smetanina N. M., Pastovalova M. V., Arkhangel'skaya E., Klokov D., Free Radic. Biol. Med., 73, 34–40 (2014).
24) Josefsson E., Uppström B., J. Sci. Food Agric., 27, 438–442 (1976).
25) Li W., Li Q., Guo F. Q., Gu R. Q., Acta Phytoest. Sin., 23, 319–323 (1997).
26) Caputo F., Vegliante R., Ghibelli L., Biochem. Pharmacol., 84, 1292–1306 (2012).
27) Zheng R., Shi Y., Jia Z., Zhao C., Zhang Q., Tan X., Chem. Soc. Rev., 39, 2827–2834 (2010).
28) Foti M. C., Daquino C., Mackie I. D., DiLabio G. A., Ingold K. U., J. Org. Chem., 73, 9270–9282 (2008).
29) Litwinienko G., Ingold K. U., J. Org. Chem., 69, 5888–5896 (2004).
30) Martínez A., Stino C. M., Meléndez-Martínez A. J., J. Phys. Chem. B, 118, 9819–9825 (2014).
31) Aliaga C., Lissi E. A., Can. J. Chem., 78, 1052–1059 (2000).
32) Farver O., Lu Y., Ang M. C., Pecht I., Proc. Natl. Acad. Sci. U.S.A., 96, 896–902 (1999).
33) Xie C., Zhong D., Chen X., Drug Metab. Dispos., 40, 1628–1640 (2012).
34) Martínez A., Galano A., Vargas R., Phys. Chem. B, 115, 12591–12598 (2011).
35) Galano A., Alvarez-I遭bajo J. R., Org. Lett., 11, 5114–5117 (2009).
36) Galano A., Francisco-Márquez M., Alvarez-I遭bajo J. R., Phys. Chem. Chem. Phys., 13, 11199–11205 (2011).