Antioxidant activity and mechanism of Rhizoma Cimicifugae

Xican Li1†, Jing Lin1†, Yaoxiang Gao1, Weijuan Han1 and Dongfeng Chen2*

Abstract

Background: As a typical Chinese herbal medicine, rhizoma Cimicifugae (RC, 升麻 in Chinese) possesses various pharmacological effects involved in antioxidant activity. However, its antioxidant activity has not been reported so far. The aim of the present study was to systematically evaluate the antioxidant ability of RC in vitro, then discuss the mechanism.

Methods: Firstly, five RC extracts (i.e. petroleum ether extract PERC, ethyl acetate extract EARC, absolute ethanol extract AERC, 95% ethanol extract 95ERC, and water extract WRC) were prepared and determined by various antioxidant methods, including anti-lipid peroxidation, protection against DNA damage, •OH scavenging, •O2− scavenging, DPPH• (1,1-diphenyl-2-picryl-hydrazl radical) scavenging, ABTS+• (2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid radical ion) scavenging, Cu2+-chelating, and Fe3+ reducing assays. Subsequently, we measured the chemical contents of five RC extracts, including total phenolics, total saponins, total sugars, caffeic acid, ferulic acid and isoferulic acid. Finally, we quantitatively analyzed the correlations between antioxidant levels (1/IC50 values) and chemical contents.

Results: In the study, the antioxidant levels and chemical contents (including total phenolics, total saponins, total sugars, caffeic acid, ferulic acid and isoferulic acid) of five RC extracts were determined by various methods. In all antioxidant assays, five RC extracts increased the antioxidant levels in a dose-dependent manner. However, their antioxidant levels (IC50 values) and chemical contents significantly differed from each other. Quantitative analysis of the correlation showed that total phenolic was of significant positive correlations (average R value was 0.56) with antioxidant levels; In contrast, total sugars and total saponins had no positive correlation with antioxidant (the average R values were −0.20 and −0.26, for total sugars and total saponins, respectively); Among total phenolics, three phenolic acids (caffeic acid, ferulic acid and isoferulic acid) also displayed positive correlations (the average R values were 0.51, 0.50, and 0.51, for caffeic acid, ferulic acid and isoferulic acid, respectively).

Conclusions: As an effective antioxidant, Rhizoma Cimicifugae can protect DNA and lipids against oxidative damage. Its antioxidant ability can be responsible for its various pharmacological effects and may be mainly attributed to the existence of total phenolics, among which caffeic acid, ferulic acid and isoferulic acid are regarded as main bioactive components. Rhizoma Cimicifugae exerts its antioxidant effect through metal-chelating, and radical-scavenging which is via donating hydrogen atom (H⋅) and donating electron (e).

Keywords: Rhizoma Cimicifugae, 升麻, Antioxidant activity, Metal chelating, Radical-scavenging, Phenolic acid
Background
As we know, reactive oxygen species (ROS) are various forms of activated oxygen including free radicals and non-free-radical species. ROS can oxidatively damage vital cellular structures such as lipids and DNA [1,2], then lead to severe biological consequences including mutation, cell death, carcinogenesis, and aging [3].

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

As a typical Chinese herbal medicine, rhizoma Cimicifugae (RC, 升麻 in Chinese, the photo is shown in Additional file 1) has been used for over 2000 years [4]. From the viewpoint of tradition Chinese medicine (TCM), RC can elevate yang, lift qi, clear heat, remove toxic, induce sweats to dispel exopathogens, and promote eruption [5].

Modern medicine indicated that RC possessed various pharmacological effects. Ye reported that RC possessed antidepressant-like properties in rodents [6]; Kim pointed out that RC can treat pain and inflammation [7]; An isopropanolic extract of RC, however, was proved to be able to diminish the urinary content of PYR and DPY and the morphometric correlates of bone loss associated with ovariosis in rats [8]; The supply of RC can therefore prevent OVX-induced bone loss in mice [9]. In addition, the extract of RC was found to have protective effect against gastric injury [10]. According to free radical biology & medicine [11], these pharmacological effects are related to antioxidant ability. However, its antioxidant ability has not been explored so far.

Therefore, the aim of the study was to investigate the antioxidant ability of RC in vitro, then further discuss the antioxidant mechanism.

Results and discussion
As an important biomolecule, lipid can be easily attacked by ROS to generate lipid peroxidation which is harmful to cell. For example, the highly reactive •OH radical can attack to lipid to produce lipid peroxidation (Equations 1–3):

\[
\text{Lipid} - H + •OH \rightarrow \text{Lipid}• + H_2O \quad (1)
\]

\[
\text{Lipid}• + O_2 \rightarrow \text{Lipid} - O_2• \quad (2)
\]

\[
\text{Lipid} - H + \text{Lipid} - O_2• \rightarrow \text{Lipid} - O_2H + \text{Lipid}• \quad (3)
\]

In our study, five RC extracts increased the anti-lipidperoxidation percentages in a dose-dependent manner (Additional file 2). It means that five RC extracts can effectively protect lipid against oxidative damage. Among them, EARC possessed the highest anti-lipidperoxidation activity (Table 1).

Besides lipid, another biomolecule DNA can also be oxidatively damaged by ROS (especially •OH). It is well known that DNA consists of deoxyribose, organic phosphate and various base pairs. When DNA is attacked by •OH radical, MDA (malondialdehyde) and a number of oxidative lesions are generated [12]. MDA combines with 2-thiobarbituric acid (TBA) to produce thiobarbituric acid-reactive substances (TBARS) with \( \lambda_{\text{max}} \) at 530 nm (Scheme 1).

\[
\text{Fe}^{2+} + H_2O_2 \rightarrow •OH + OH^- + Fe^{3+} \quad (4)
\]

Besides •OH radical, superoxide anion radical (•O_2) is also regarded as one important form of ROS in living cell. It can directly attack DNA or lipid [13], and can transferred into •OH via Haber-Weiss reaction (Equation 5) to indirectly damage DNA or lipid as well [2]. The results in Table 1 and Additional file 2 showed that five RC extracts could also scavenge •O_2 radicals.

\[
•O_2^- + H_2O_2 \stackrel{\text{Fe}^{3+}}{\rightarrow} •OH + OH^- + O_2 \quad (5)
\]

Taken together, the protective effect of RC against hydroxyl-induced DNA oxidative damage can be assumed to be relevant to the •OH or •O_2 radical-scavenging ability.

However, as illustrated in Equations 4 and 5, the generations of •OH and •O_2 radicals rely usually on the catalysis of transition metals (especially Fe and Cu). So we further explored the metal chelating abilities of five RC extracts. The dose–response curves showed that five RC extracts possessed effective metal chelating abilities (Additional file 2). Our previous reports have shown that...
the chelating ability might be mainly attributed to ortho dihydroxy groups in phenolic compounds [14]. For example, phenolic acids can bind Fe$^{2+}$ as the following mechanism (Scheme 2) [15].

The fact that five RC extracts can effectively bind Fe$^{2+}$ ion, suggests that metal-chelating may be one of mechanisms for scavenging $\cdot$OH or $\cdot$O$_2$.

In order to verify whether five RC extracts can directly scavenge radicals. We determined their DPPH$^\bullet$ and ABTS$^{•+}$ radical-scavenging abilities.

As seen in Table 1 and Additional file 2, five RC extracts scavenged both DPPH$^\bullet$ and ABTS$^{•+}$ radicals in a dose-dependent manner. The previous studies have demonstrated that DPPH$^\bullet$ may be scavenged by an antioxidant through donation of hydrogen atom (H-) to form a stable DPPH-H molecule [16-18]. Caffeic acid, a phenolic acid occurring in RC, for example, could scavenge DPPH$^\bullet$ via the proposed reaction (Scheme 3) [19].

Radical (A) can be further withdrawn hydrogen atom (H$^\bullet$) to form stable quinone (B) (Scheme 4).

Unlike DPPH$^\bullet$ scavenging, ABTS$^{•+}$ scavenging however is considered as an electron (e) transfer reaction [20]. For example, the proposed reaction for caffeic acid to scavenge ABTS$^{•+}$ can be briefly illustrated using Scheme 5. Similarly, if ABTS$^{•+}$ is excessive, radical (A) can also further change to quinone (B).

As illustrated in Scheme 3, 4 and 5, the generations of both DPPH$^\bullet$ and ABTS$^{•+}$ radicals did not rely on the transition metal catalysis.

Based on the discussion above, it can be deduced that direct radical-scavenging was regarded as another mechanism for five RC extract to scavenge $\cdot$OH or $\cdot$O$_2$, and they exerted radical-scavenging action by donating hydrogen atom (H-) and electron (e).

Until now, about 200 compounds have been found in RC [21]. Generally, they belong to three different classes, i.e. phenolics, saponins, and polysaccharides. In the study, we used chemical method to measure the contents of total phenolics, total saponins, and total sugars in fiver RC extracts (Table 2). On the other hand, since 1/IC$_{50}$ value showed parallelism with antioxidant level, it was therefore used to evaluate antioxidant level in the study (Additional file 2). On this basis, forty-eight correlation graphs were plotted to quantitative analyze the correlation between antioxidant levels and chemical contents in five RC extracts (Additional file 3). The correlation coefficients (R values) in Table 3 indicated that, total phenolics were of significant positive correlation (R = 0.71 ~ 0.99; average, 0.93) with antioxidant levels, while total sugars and total saponins had no positive correlations (for total sugars, R = 0.17 ~ 0.46; for total saponins, R = -0.88 ~ 0.46, the average was −0.20; for total saponins, R = -0.92 ~ 0.94, the average

---

**Table 1 The IC$_{50}$ values of five RC extracts and the positive controls (µg/mL)**

| Assays                  | PERC          | EARC          | AERC          | 95ERC         | WRC           | GSH           |
|-------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Anti-lipid peroxidation | 13.99 ± 2.11  | 4.55 ± 0.44   | 14.92 ± 4.31  | 10.62 ± 0.97  | 19.18 ± 2.04  | 0.028 ± 0.00  |
| DNA protective effect   | -             | 1905.51 ± 177.54 | 14397.18 ± 1075.01 | 1090.11 ± 21.25 | -             | 114.42 ± 2.66  |
| $\cdot$OH               | 561.19 ± 21.16 | 111.55 ± 1.32 | 132.32 ± 0.15  | 84.30 ± 1.02  | 233.45 ± 15.70 | 37.67 ± 0.67   |
| $\cdot$O$_2$            | 322.81 ± 14.90 | 306.68 ± 0.87 | 284.36 ± 11.43 | 301.08 ± 8.34 | 650.65 ± 59.55 | 81.65 ± 5.21   |
| Chelating               | 191.86 ± 1.84 | 319.20 ± 6.36 | 872.71 ± 30.63 | 553.34 ± 12.59 | 2289.11 ± 100.00 | 107.86 ± 0.31   |
| DPPH$^\bullet$          | 813.00 ± 25.17 | 227.09 ± 10.14 | 260.34 ± 11.37 | 254.73 ± 4.33 | 229.17 ± 5.81  | 5.13 ± 1.19    |
| ABTS$^{•+}$             | 395.65 ± 77.34 | 72.10 ± 1.32  | 96.39 ± 1.12  | 94.38 ± 2.45  | 83.58 ± 0.81   | 4.76 ± 0.28    |
| Fe$^{3+}$ reducing      | 533.12 ± 54.25 | 159.35 ± 1.37 | 317.06 ± 3.47  | 156.19 ± 2.12  | 488.75 ± 18.60 | 51.89 ± 1.29   |

IC$_{50}$ value is defined as the concentration of 50% effect percentage and calculated by linear regression analysis and expressed as mean ± SD (n = 3). The linear regression was analyzed by Origin 6.0 professional software. Means values with different superscripts in the same row are significantly different (p < 0.05), while with same superscripts are not significantly different (p > 0.05). *The positive control was BHA, instead of GSH. ** The positive control was Trolox, instead of GSH. *** The positive control was Sodium citrate. Results were analyzed by independent samples. PERC, petroleum ether extract of rhizoma Cimicifugae; EARC, absolute ethanol extract of rhizoma Cimicifugae; AERC, 95% ethanol extract of rhizoma Cimicifugae; 95ERC, 95% ethanol extract of rhizoma Cimicifugae; WRC, water extract of rhizoma Cimicifugae. -: Cannot be detected.

---

![Scheme 1](http://journal.chemistrycentral.com/content/6/1/140)

**Scheme 1** The reaction of MDA (malondialdehyde) and 2-thiobarbituric acid (TBA).
was −0.26) with antioxidant levels. The data clearly demonstrated the significant contribution of phenolic compounds to the antioxidant of RC.

Among total phenolics, several compounds had been isolated from RC, including salicylic acid, caffeic acid, ferulic acid, isoferrulic acid, flavonoids and tannins. In our study, however, three phenolic acids caffeic acid, ferulic acid and isoferrulic acid were also identified by HPLC assay (Figure 1 and Additional file 4), in which peak areas were used to characterize the relative contents of three phenolic acids.

Then, we used the peak areas to plot the correlation graphs vs 1/IC50 values of five RC extracts, to obtain the correlation coefficients (R values). As shown in Additional file 5 and Table 3, three phenolic acids all presented significant positive correlations (average R value = 0.47, 0.44, and 0.51, respectively for caffeic acid, ferulic acid and isoferrulic acid). On the other hand, previous works have shown that three phenolic acids possess strong antioxidant ability [22-24]. Hence, they are considered as three of main bioactive compounds relevant to antioxidant in RC.

Experimental

**Chemicals and plant material**

Trolox (±-6-hydroxyl-2,5,7,8-tetramethylhromane-2-carboxylic acid), Ferrozin [3-(2-pyridyl)-5,6-bis(4-phenylsulfonylacid)-1,2,4-triazine], DPPH: (1,1-Diphenyl-2-picrylhydrazyl radical), pyrogallol, linoleic acid, BHT (2,6-diteriary butyl-p-cresol) and murexide (5,5'-Nitrilodibarbituric acid monoammonium salt) were purchased from Sigma Co. (Sigmaaldrich Trading Co., Shanghai, China); ABTS diammonium salt [2,2'-Azino-bis(3-ethylbenothiazoline-6-sulfonic acid diammonium salt)], D-2-deoxyribose, and GSH (glutathione) were Amresco Inc. (Solon, OH, USA); DNA sodium salt (fish sperm) was purchased from Aladdin Chemistry Co. (Shanghai, China); Ferulic acid, caffeic acid were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Acetonitrile, methanol and water were of HPLC grade; All other chemicals used were in analytical grade.

*Rhizoma Cimicifugae* was purchased from Guangzhou University of Chinese Medicine Yanghe Interlink Limited Company and identified by Prof. Shuhui Tan. A voucher specimen was deposited in our laboratory.

**Preparation of different extracts of rhizoma Cimicifugae**

The dried rhizoma *Cimicifugae* was coarsely powder then extracted in sequence with petroleum ether (60–90), ethyl acetate, ethanol, 95% ethanol and water by Soxhlet extractor for 12 hours. The extracts were concentrated under reduced pressure to a constant weight. Then the dried extracts were stored at 4°C until used (Figure 2).

**Protective effect against lipid-peroxidation**

The protective effect against lipid-peroxidation was investigated using a linoleic acid emulsion [25]. The linoleic acid emulsion was prepared by mixing and homogenizing 312.6 mg of linoleic acid, 78.2 mg of Tween-20 as emulsifier, and 30 mL of 75% ethanol (v/v), 0.1 mL of various concentrations of samples (0.4–2.8 mg/mL) were added to 1.5 mL of linoleic acid emulsion and 0.4 mL distilled water. The reaction mixture (2 mL) was incubated at room temperature in glass bottles for 72 hours. To 0.15 mL of sample solution, 3.65 mL of 75% ethanol, 0.1 mL of ammonium thiocyanate (30%, m/v), and 0.1 mL of ferrous chloride (0.02 M in 3.6% HCl) were added.
added. The mixture was diluted to two-fold volume with methanol in case the color was too dark, the peroxide value was measured by monitoring absorbance at 500 nm in a spectrophotometer (Unico 2100, Shanghai, China). The percentage of inhibition of lipid-peroxidation in linoleic acid emulsion was calculated by following equation:

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

Where $A$ is the absorbance with samples, while $A_0$ is the absorbance without samples.

**Protective effect against hydroxyl-induced DNA damage**

The experiment was conducted as described in previous report [26]. However, deoxyribose was replaced by DNA sodium salt. Briefly, sample was dissolved in methanol to prepare the sample solution. Various amounts (10–100 μL) of sample solutions (10 mg/mL) were then separately taken into mini tubes. After evaporating the sample solutions in the tubes to dryness, 400 μL phosphate buffer (0.2 M, pH 7.4) was added to the sample residue. Then, 50 μL deoxyribose (2.8 mM), 50 μL H₂O₂ (2.8 mM), 50 μL FeCl₃ (25 mM), and 100 μL Na₂EDTA solutions (0.8 mM) were added. The reaction was initiated by mixing 50 μL ascorbic acid (1.2 mM) and the total volume of the reaction mixture was adjusted to 600 μL with buffer. After incubation in a water bath at 50°C for 20 min, the reaction was terminated by addition of 500 μL trichloroacetic acid (5%, w/w). The color was then developed by addition of 150 μL 2-thiobarbituric acid (TBA) (0.4 M, 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 of DNA is expressed as follows:

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

Where $A$ is the absorbance with samples, while $A_0$ is the absorbance without samples.

**Hydroxyl (•OH) radical-scavenging assay**

The hydroxyl radical-scavenging activity was investigated by the deoxyribose degradation method [26], with some modifications. In brief, the sample was dissolved in methanol, and then the sample solution was aliquoted into mini tubes. After evaporating the sample solutions in the tubes to dryness (48–240 μg), 300 μL of phosphate buffer (0.2 M, pH 7.4) was added to the sample residue. Subsequently, 50 μL deoxyribose (2.8 mM), 50 μL H₂O₂ (2.8 mM), 50 μL FeCl₃ (25 mM), and 100 μL Na₂EDTA (0.8 mM) were added. The reaction was initiated by mixing 50 μL ascorbic acid (1.2 mM) and the total volume of the reaction mixture was adjusted to 600 μL with buffer. After incubation in a water bath at 50°C for 20 min, the reaction was terminated by addition of 500 μL trichloroacetic acid (5%, w/w). The color was then developed by addition of 500 μL TBA (1 g/100 mL, in 1.25% NaOH aqueous solution) and heated in an oven at 105°C for 15 min. The mixture was cooled and the absorbance was measured at 532 nm against the buffer (as a blank control). The inhibition percentage for •OH was expressed as follows:

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

Where $A$ is the absorbance containing samples, while $A_0$ is the absorbance without samples.
Table 2 Chemical contents of five RC extracts

| Extracts | PERC | EARC | AERC | 95ERC | WRC |
|----------|------|------|------|-------|-----|
| Total phenolics (mg Pyr./g) | 13.17 ± 0.43 a | 6.01 ± 0.34 a | 15.73 ± 0.34 b | 31.10 ± 1.30 c | 12.46 ± 1.00 a |
| Total sugars (mg Glucose/g) | 237.16 ± 38.67 a | 175.46 ± 21.50 a | 62.75 ± 17.62 b | 603.48 ± 18.74 b | 804.54 ± 50.53 c |
| Total saponins (mg Ole./g) | 917.52 ± 87.83 c | 379.46 ± 15.46 b | 185.16 ± 4.10 a | 180.44 ± 1.95 a | 198.34 ± 12.30 a |
| Caffeic acid (peak area) | 2.96 ± 0.85 a | 125.22 ± 8.84 c | 6.45 ± 2.93 b | 14.19 ± 10.14 b | 2.79 ± 1.23 a |
| Isoferulic acid (peak area) | 4.92 ± 1.68 a | 33.24 ± 0.44 c | 0.39 ± 0.37 b | 0.68 ± 1.18 c | 0.0040 ± 0.0010 a |
| Total phenolics (mg Pyr./g) | 9.17 ± 0.34 a | 6.01 ± 0.34 a | 15.73 ± 0.34 b | 31.10 ± 1.30 c | 12.46 ± 1.00 a |
| Total sugars (mg Glucose/g) | 237.16 ± 38.67 a | 175.46 ± 21.50 a | 62.75 ± 17.62 b | 603.48 ± 18.74 b | 804.54 ± 50.53 c |
| Total saponins (mg Ole./g) | 917.52 ± 87.83 c | 379.46 ± 15.46 b | 185.16 ± 4.10 a | 180.44 ± 1.95 a | 198.34 ± 12.30 a |
| Caffeic acid (peak area) | 2.96 ± 0.85 a | 125.22 ± 8.84 c | 6.45 ± 2.93 b | 14.19 ± 10.14 b | 2.79 ± 1.23 a |
| Isoferulic acid (peak area) | 4.92 ± 1.68 a | 33.24 ± 0.44 c | 0.39 ± 0.37 b | 0.68 ± 1.18 c | 0.0040 ± 0.0010 a |

Each value is expressed as mean ± SD (n = 3). Means values with different superscripts in the same row are significantly different (p < 0.05), while with same superscripts are not significantly different (p > 0.05). PERC, petroleum ether extract of rhizoma Cimicifugae; EARC, absolute ethanol extract of rhizoma Cimicifugae; AERC, 95% ethanol extract of rhizoma Cimicifugae; 95ERC, 95% ethanol extract of rhizoma Cimicifugae; WRC, water extract of rhizoma Cimicifugae.

Superoxide anion (•O2−) radical-scavenging assay

Measurement of superoxide anion (•O2−) scavenging activity was based on our method [27]. Briefly, samples were dissolved in methanol at 3 mg/mL. The sample solution × μL (x = 67, 117, 167, 217, 267, and 317) was mixed with Tris–HCl buffer (2950–x μL, 0.05 M, pH 8.2) containing EDTA (1 mM) and pyrogallol (50 μL, 6 mM in 10.0 mM HCl), then shaken rapidly at 37°C. The absorbance at 325 nm of the mixture was measured (Unico 2100, Shanghai, China) against the Tris–HCl buffer (pH 5.3, 30 mM) containing 30 mM KCl and 0.20 mM murexide. After incubation for 1 min at room temperature, 80–230 μL sample solutions (4 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 by a spectrophotometer (Unico 2100, Shanghai, China) at 485 nm. The absorbance at 485 nm of the mixture without the sample and the mixture without sample was used as the control. The •O2− scavenging ability was calculated as:

\[
\text{Relative chelating effect} = \frac{A_{520} - A_{485}}{A_{520} - A_{485, \text{max}}} \times 100%
\]

Here, \(A_{520, \text{max}}\) is the increase in absorbance ratio of the sample mixture without the sample and \(A_{520, \text{max}}\) is that for the mixture with the sample; \(T = 5\) min. The experiment temperature was 37°C.

Chelating activity on Cu2+

The Cu2+–chelating activities of five RC extracts were measured by the method [28]. Briefly, 60 μL CuSO4 aqueous solution (20 mM) was added to hexamine HCl buffer (pH 5.3, 30 mM) containing 30 mM KCl and 0.20 mM murexide. After incubation for 1 min at room temperature, 80–230 μL sample solutions (4 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 by a spectrophotometer (Unico 2100, Shanghai, China) at 485 nm and 520 nm. The absorbance ratio (A485/A520) reflected the free Cu2+ content. Therefore, the percentage of cupric chelating effect was calculated by the following formula:

\[
\text{Relative chelating effect} = \frac{A_{485} - A_{520}}{A_{485} - A_{520, \text{max}}} \times 100%
\]

Where \(A_{520, \text{max}}\) is the maximum absorbance ratio and \(A_{485, \text{min}}\) is the minimum absorbance ratio in the test.

DPPH• scavenging assay

DPPH• radical-scavenging activity was determined as previously described by Li [23]. Briefly, 0.8 mL DPPH•

Table 3 The R values between chemical contents and 1/IC50

| Total phenolics | Caffeic acid | Ferulic acid | Isoferulic acid | Total sugars | Total saponins |
|----------------|-------------|--------------|----------------|--------------|----------------|
| DNA            | 0.61        | 0.38         | 0.37           | 0.40         | −0.11          |
| 2-OH           | 0.57        | 0.37         | 0.34           | 0.47         | 0.11           |
| 2⁻OH           | 0.36        | 0.38         | 0.33           | 0.53         | −0.53          |
| Chelating      | 0.17        | 0.31         | 0.31           | 0.21         | −0.88          |
| DPPH•          | 0.43        | 0.39         | 0.35           | 0.35         | 0.46           |
| ABTS•          | 0.57        | 0.54         | 0.61           | 0.49         | 0.30           |
| Reducing       | 0.82        | 0.65         | 0.68           | 0.68         | −0.26          |
| Average        | 0.56        | 0.51         | 0.50           | 0.51         | −0.20          |

R, correlation coefficient; 1/IC50: the unit is mL/μg, all 1/IC50 values are listed in Additional file 2. LPO, lipid peroxidation.
solution (0.1 M) was mixed with 4.2 mL various concentrations (15–300 μg/mL) of samples dissolved in 95% ethanol. 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 of the samples was calculated:

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

Where \( A_0 \) is the absorbance without samples, while \( A \) is the absorbance with samples.

**ABTS•⁺ scavenging assay**

The ABTS•⁺-scavenging activity was measured as described [23] with some modifications. The ABTS•⁺ was produced by mixing 0.35 mL ABTS diammonium salt (7.4 mM) with 0.35 mL potassium persulfate (2.6 mM). The mixture was kept in the dark at room
temperature for 12 hours 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 scavenging activity, 1.2 mL aliquot of ABTS*⁺ reagent was mixed with 0.3 mL of sample ethanolic solutions (40–540 μg/mL). After incubation for 6 min, the absorbance at 734 nm was read on a spectrophotometer (Unico 2100, Shanghai, China). The percentage inhibition of the samples was calculated as:

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

where \( A_0 \) is the absorbance at 734 nm without samples, while \( A \) is the absorbance at 734 nm with samples.

Reduction power (Fe³⁺) assay
Ferric cyanide (Fe³⁺) reducing power was determined according to the method of Oyaizu [23,29]. In brief, sample solutions \( x \mu L \) (4 mg/mL, \( x = 20, 40, 60, 80, \) and 100) were mixed with (350-\( x \)) \( \mu L \) Na₂HPO₄/KH₂PO₄ buffer (0.2 M, pH 6.6) and 250 \( \mu L \) K₃Fe(CN)₆ aqueous solution (1 g/100 mL).

The mixture was incubated at 50°C for 20 min, 250 \( \mu L \) of trichloroacetic acid (10 g/100 mL) was added, and the mixture was centrifuged at 3500 r/min for 10 min. As soon as 400 \( \mu L \) supernatant was mixed with 400 \( \mu L \) FeCl₃ (0.1 g/100 mL in distilled water), the timer was started. At 90 s, absorbance of the mixture was read at 700 nm (Unico 2100, Shanghai, China). Samples were analyzed in groups of three, and when the analysis of one group has finished, the next group of three samples were mixed with FeCl₃ to prevent the mixture from being oxidized by air. The relative reducing ability of the sample was calculated by using the formula:

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

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

HPLC analysis for caffeic acid, ferulic acid, and isoferulic acid
Caffeic acid, ferulic acid, and isoferulic acid were identified by comparing their retention times using HPLC method. HPLC analysis was performed on a Syltech P510 system (Los Angeles, California, USA) equipped with Dikma DiamondSil C₁₈ (250 mm × 4.6 mm, 5 μm) (Beijing, China). The mobile phase consisted of acetonitrile-0.5% acetic acid in water (17:83, v: v), the flow rate was 1.0 mL/min, injection volume was 30 μL and absorption was measured at 316 nm.

Determination of total phenolics, total sugars and total saponins
The total phenolics of five RC extracts were determined by the Folin–Ciocalteu method with a little modifications [30]. Firstly, 0.5 mL extract methanolic solution (0.4 mg/mL) was mixed with 0.5 mL 0.25 M Folin–Ciocalteu reagent. After incubation for 3 min, 1 mL of Na₂CO₃ solution (15%, w/v) was added. After standing at the 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 determinations were performed in triplicate, and the calculations were based on a calibration curve obtained with pyrogallol. The result was expressed as pyrogallol equivalents (Pyr.) in milligrams per gram of extract.

The total sugars were evaluated according to the phenol–sulfuric acid method [30]. A 40-μL aliquot of sample solution (1 mg/mL) was placed in a flask, then 210 μL distilled water, 250 μL phenol solution (5%, w/v) and 250 μL concentrated sulfuric acid were added. After incubation for 20 min at room temperature, the absorbance of reaction mixture was measured at 490 nm (Unico 2100, Shanghai, China). The measurements were performed in triplicate, and the calculations were based on a calibration curve obtained with glucose. The result was expressed as glucose equivalents in milligrams per gram of extract.

The total saponins were measured according to the method [30]. RC extract was dissolved in methanol to prepare the sample solution (4 mg/mL), then a 20-μL aliquot of sample solution was taken into a mini tube. After the sample solution in tube was evaporated to dryness (water bath, 80°C), 0.1 mL vanillin-acetic acid solution (5 mg/mL) and 0.4 mL perchloric acid were added to the sample residue. The reaction mixture was incubated at 70°C for 15 min, then diluted by 1.25 mL acetic acid. The mixture was measured using a spectrophotometer (Unico 2100, Shanghai, China) at 540 nm against a blank control, which contained all reagents except sample. All analyses were run in triplicate. Quantification was based on the standard curve for oleanic acid (10–79 μg/mL) and the results were expressed in milligrams of oleanic acid (Ole.) equivalents per gram of extract.

The correlation graphs and R values
In order to investigate the correlation between antioxidant activities and chemical components of five RC extracts, 48 correlation graphs were plotted between 1/IC₅₀ values (including of LPO, DNA, •OH, •O₂⁻, Cu-chelating, DPPH*, ABTS*⁺, and reducing power assays) and chemical contents (including total phenolics, caffeic acid, ferulic acid, isoferulic acid, total sugars, and total saponins). In the correlation graphs, the correlation coefficients (R values) were calculated by Origin 6.0 professional software.
Statistical analysis

Data are given as the mean ± SD of three measurements. The IC_{50} values were calculated by linear regression analysis. All linear regression in this paper was analyzed by Origin 6.0 professional software. Significant differences were performed using the T-test (p < 0.05). The analysis was performed using SPSS software (v.12, SPSS, USA).

Conclusion

As an effective antioxidant, rhizoma Cimicifugae can protect DNA and lipids against oxidative damage. Its antioxidant ability can be responsible for its various pharmacological effects and may be mainly attributed to the existence of total phenolics, among which caffeic acid, ferulic acid and isoferulic acid are regarded as main bioactive components. Rhizoma Cimicifugae exerts its antioxidant effect through metal-chelating, and radical-scavenging which is via donating hydrogen atom (H*) and donating electron (e).

Acknowledgements

This work was supported by the National Nature Science Foundation of China (81273896) and National Key Technologies R&D Program of China (No. 2008BAI51801). The authors declare that they have no competing interests.

References

1. Leichter LL, Gehlke F, Gudishe HV, Blackwell T, Ibert M, Walker AK, Strahler JR, Andrews PC, Jakob U: Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. Proc Natl Acad Sci USA 2008, 105:8197–8202.
2. Halliwell B, Gutteridge JM: Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1989, 261:1–14.
3. Batraacharyee S, Detterding LJ, Chattejee S, Jiang J, Ehrenshaft M, Lardinois O, Ramirez DC, Tomer KB, Mason RP: Site-specific radical formation in DNA induced by Cu(II)-H_{2}O_{2} oxidizing system, using ESR, immuno-spin trapping, LC-MS, and MS/MS. Free Radic Biol Med 2011, 50:1536–1545.
4. Tao HJ: Varioium of Shenong's Classic of Materia Medica. Beijing: People’s Medical Publishing House; 1995:45.
5. Chinese Pharmacopoeia Commission: The Pharmacopoeia of Chinese People’s Republic Beijing China: Chemical Industry Publishing House; 2005:50.
6. Ye L, Hu Z, Du G, Zhang J, Dong Q, Fu F, Tian J: Antidepressant-like effects of the extract from Cimicifuga foetida L. J Ethnopharmacol in press. doi:10.1016/j.jep.2012.10.013.
7. Kim SJ, Kim MS: Inhibitory effects of Cimicifuga rhizoma extracts on histamine, bradykinin and COX-2 mediated inflammatory actions. Phytother Res 2000, 14:596–601.
8. Nishtein T, Freudentein J: Effects of an isopropanolic extract of Cimicifuga racemosa on urinary crosslinks and other parameters of bone quality in an ovariectomized rat model of osteoporosis. J Bone Miner Metab 2003, 21:370–376.
9. Ahn BS, Yang M, Jung H, Lee HJ, Moon C, Kim JC, Jung UJ, Jo SK, Jung JS, Kim SH: Evaluation of the antosteoporotic potential of Cimicifuga heraclefolia in female mice. Phytother Res 2012, 26:663–668.
10. Kim M: Protective effect of Cimicifuga heraclefolia ethanolic extract and its constituents against gastric injury. J Health Sci 2011, 57:289–292.
11. Zheng RL, Huang ZY: The definition and characteristic of free radicals. In Free Radical Biology, Volume 1. Edited by Huang ZY, Zheng RL. Beijing China: Chinese Higher Education Press; 2007:7.
12. Dzdaroglu M, Juraga P, Birinciolgu M, Rodriguez H: Free radical-induced damage to DNA: mechanism and measurement. Free Radic Biol Med 2002, 32:1102–1115.
13. Keyer K, Imlay JA: Superoxide accelerates DNA damage by elevating free-iron levels, Proc Natl Acad Sci USA 1996, 93:3635–3640.
14. Han WL, Li XC: Antioxidant activity of aloeswood tea in vitro. Spatula DO 2012, 24:3–50.
15. Torreggiani A, Tamba M, Trinchero A, Bonora S, Copper (II)-quercetin complexes in aqueous solutions: spectroscopic and kinetic properties. J Mol Struct 2005, 780:759–766.
16. Bondet V, Brand-Williams W, Besret C: Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. LWTFood Sci Technol 1997, 30:659–661.
17. Brand-Williams W, Cuvelier ME, Besret C: Use of free radical method to evaluate antioxidant activity. LWTFood Sci Technol 1995, 28:25–30.
18. Tsimogiannis DI, Oreopoulou V, Rodriguez H: The contribution of flavonoid C-ring on antioxidant effect through metal-chelating, and radical-scavenging reactions of procatechatic acid and its analogues in alcoholic solvents. Tetrahedron 2005, 61:8101–8108.
19. Prior RL, Cao G: In vivo total antioxidant capacity: comparison of different analytical methods. Free Radic Biol Med 1999, 27:1173–1181.
20. Liu BB, Chen SH, Chen SB: The chemical composition and research progress of anti-tumor activity of Cimicifuga. Cent South Pharm 2012, 16:53–58.
21. Gulyin I: Antioxidant activity of caffeine (3,4-dihydroxyxaminic acid). Toxicology 2006, 217:213–220.
22. Wang XZ, Li XC, Chen DF: Evaluation of antioxidant activity of isoferulic acid in vitro. Nat Prod Commun 2011, 6:1285–1288.
23. Li XC, Wu XT, Huang L: Correlation between antioxidant activities and phenolic contents of Radix Angelica Sinensis (Danggui). Molecules 2009, 14:5349–5361.
24. Wang XZ, Li XC, Li HR: Reassessment of antioxidant activity of baikalene in vitro. Asian J Pharm Biol Res 2011, 1:186–194.
25. Li XC, Chen C: Systematic evaluation on antioxidant of magnolol in vitro. Int J Pure Appl Chem 2012, 6:28–76.
26. Li XC: Improved pyrogallol autoxidation method: a reliable and cheap superoxide-scavenging assay suitable for all antioxidants. J Agric Food Chem 2012, 60:6418–6424.
28. Li XC, Wang XZ, Chen DF, Chen SZ. Antioxidant activity and mechanism of protocatechuic acid in vitro. Funct Food Health Dis 2011, 1:232–244.
29. Oyaizu M. Studies on product of browning reaction prepared from glucoseamine. Jpn J Nutr 1986, 44:307–315.
30. Li XC, Chen DF, Mai Y, Wen B, Wang XZ. Concordance between antioxidant activities in vitro and chemical components of Radix Astragali (Huangqi). Nat Prod Res 2012, 26:1050–1053.

doi:10.1186/1752-153X-6-140
Cite this article as: Li et al: Antioxidant activity and mechanism of Rhizoma Cimicifugae. Chemistry Central Journal 2012 6:140.