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Original Research

Protective Effect against Hydroxyl-induced DNA Damage and Antioxidant Activity of Radix *Codonopsis*

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Abstract

Aim: As a typical Chinese herbal medicine, Radix *Codonopsis* has been used in traditional Chinese medicine for about 250 years. The study tried to investigate its antioxidant activity, then to discuss the antioxidant mechanism.

Methods: Radix *Codonopsis* was extracted by ethanol to obtain ethanolic extract of Radix *Codonopsis*. The extract was then determined by various antioxidant methods, including DNA damage assay, DPPH (1,1-diphenyl-2-picryl-hydrazl radical), ABTS [2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid) radical] assay, Fe^{3+}-reducing assay and Cu^{2+}-reducing assay. Finally, the contents of total phenolics and flavonoids in the extract were determined by spectrophotometric method.

Results: The ethanolic extract of Radix *Codonopsis* showed protective effect against hydroxyl-induced DNA damage (IC_{50} 1180.28±137.73 µg/mL) and exhibited DPPH· scavenging, $ABTS^{+}$• scavenging, Fe^{3+} reducing, and Cu^{2+} reducing abilities, and the IC_{50} values were 3857.79±35.51, 271.82±5.66, 759.99±31.65, and 733.02±9.67 µg/mL, respectively. The contents of total phenolics and flavonoids in the extract were calculated as 12.56±0.56 and 11.95±0.52 mg quercetin/g, respectively.

Conclusion: Radix *Codonopsis* can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e). Its antioxidant ability can be mainly attributed to the existences of flavonoids or phenolic acids.

INTRODUCTION

It is well known that reactive oxygen species (ROS) are various forms of activated oxygen including free radicals and non-free-radical species. ROS, particularly hydroxyl radical (•OH) with high reactivity, can oxidatively damage DNA then lead to severe biological consequences including mutation, cell death, carcinogenesis, and aging [1].

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

Radix *Codonopsis* (RC) (党参 in Chinese, Figure 1A) which comes from dried radixs of *Codonopsis pilosula* (Franch.) Nannf. (Figure 1B), *Codonopsis pilosula* Nannf. var. *modesta* (Nannf.) L.T. Shen, or *Codonopsis tangshen* Oliv., has been used as a Chinese herbal medicine for about 250 years [2,3]. In traditional Chinese medicine (TCM), it can tonify spleen to replenish *qi*.

According to free radical biology & medicine [4], its curative effects can partly be attributed to the antioxidant effect. However, its antioxidant effect has not been explored so far.

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

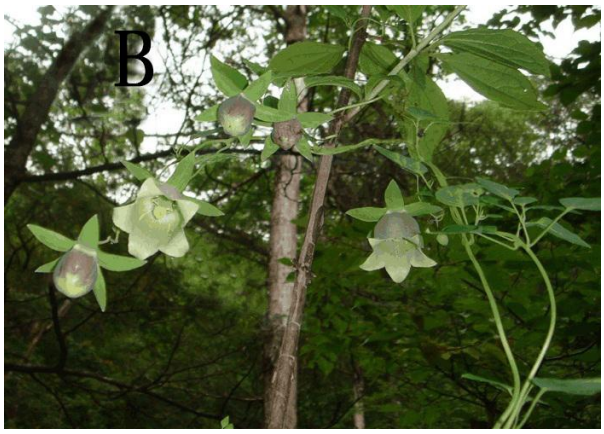


Figure 1. *Rhizoma Codonopsis* (A) and the plant *Codonopsis pilosula* (Franch.) Nannf. (B)

Figure 1A was contributed by Weitao Chen, Oct., 2012; Figure 1B was contributed by Zhijun Guo, Aug., 2011.

MATERIAL AND METHODS

Plant material

Radix *Codonopsis* was purchased from Caizhilin Pharmacy of Guangzhou University of Chinese Medicine (Guangzhou, China), and authenticated by Professor Shuhui Tan. A voucher specimen was deposited in our laboratory.

Chemicals

DPPH• (1,1-diphenyl-2-picryl-hydrazl radical), ABTS [2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid diammonium salt)], BHA (butylated hydroxyanisole), Trolox [(±)-6- hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid], DNA sodium salt (fish sperm), neocuproine (2,9-dimethyl-1,10-phenanthroline), and Folin-Ciocalteu reagent were purchased from Sigma Co. (Sigma-Aldrich Shanghai Trading Co., China). Other chemicals used in this study were of analytical grade.

Preparation of extracts from Radix Codonopsis

Radix *Codonopsis* was powdered then extracted by absolute ethanol using a Soxhlet extractor for 6 hr. Extract was filtered using a Buckner funnel and Whatman No 1 filter paper. Filtrate was then concentrated to dryness under reduced pressure to yield ERC (ethanol extract of Radix *Codonopsis*). It was stored at 4°C for analysis.

Protective effect against hydroxyl-induced DNA damage

The experiment was conducted as described in previous report [5]. However, deoxyribose was replaced by DNA sodium. Briefly, sample was dissolved in methanol to prepare the sample solution at 8 mg/mL. Various amounts (20-100 μL) of sample solutions were then separately taken into mini tubes. After evaporating the sample solution in tube to dryness, 400 μL phosphate buffer (0.2 mol/L, pH 7.4) was brought to the sample residue. Then, 50 μL DNA sodium (10.0 mg/mL), 50 μL H_2O_2 (50 mmol/L), 50 μL $FeCl_3$ (3.2 mmol/L) and 50 μL Na_2EDTA (1 mmol/L) were added. The reaction was initiated by mixing 50 μL ascorbic acid (18 mmol/L) and the total volume of the reaction mixture was adjusted to 800 μL with buffer. After incubation in a water bath at 55 °C for 20 min, the reaction was terminated by 250 μL trichloroacetic acid (10g/100mL water). The color was then developed by addition of 150 μL 2-thiobarbituric acid (TBA) (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 measured using a spectrophotometer (Unico 2100, Shanghai, China) at 530 nm against the buffer (as blank). The percent of protection of DNA is expressed as follows:

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

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

DPPH• radical-scavenging assay

DPPH• radical-scavenging activity was determined as described [6]. Briefly, 1 mL DPPH• ethanolic solution (0.1 mM) was mixed with 0.5 mL sample alcoholic solution (20 mg/mL). 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:

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

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

$ABTS^+$• radical-scavenging assay

The $ABTS^+$• scavenging activity was measured as described [7]. Briefly, the $ABTS^+$• was produced by mixing ABTS diammonium salt (0.35 mL, 7.4 mmol/L) with potassium persulfate (0.35 mL, 2.6 mmol/L), kept in the dark at room temperature for 12 h to allow completion of radical generation. Before usage, the mixture was diluted with 95% ethanol (about 1:50) so that its absorbance at 734 nm was 0.70 ± 0.02. A 1.2 mL aliquot of diluted $ABTS^+$• reagent was brought to 0.3 mL sample ethanolic solution (8 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:

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

Here, A_0 is the absorbance of the mixture without sample, *A* is the absorbance of the mixture with sample (or positive control).

Fe^{3+}-reducing power assay

Ferric cyanide (Fe^{3+}) reducing power was determined according to the method of [8] as described by Li [9]. In brief, sample solution *x* μL (20 mg/mL, *x* = 10, 30, 50, 70, and 90) was mixed with (350-*x*) μL Na_2HPO_4/KH_2PO_4 buffer (0.2 mol/L, pH 6.6) and 250 μL $K_3Fe(CN)_6$ aqueous solution (1 g/100 mL). The mixture was incubated at 50ºC for 20 min, 250 μL 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 μL supernatant was mixed with 400 μL $FeCl_3$ (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 $FeCl_3$ to avoid oxidization by air. The relative reducing ability of the sample was calculated by using the formula:

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

Here, Error! Reference source not found.A_{max} is the maximum absorbance and A_{min} is the minimum absorbance in the test. *A* is the absorbance of sample.

Cu^{2+}-reducing power assay

The cupric ions (Cu^{2+}) reducing capacity was determined by the method [10], 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. Then, different volumes of samples (4 mg/mL, *x* = 50-170 μL) were added to the tubes and mixed vigorously. The total volume of reaction mixture was adjusted to 1000 μL with the buffer. After acubation for 30 min, the mixture was measured at 450 nm (Unico 2100, Shanghai, China). The relative reducing power of the sample as compared with the maximum absorbance, was calculated by the formula:

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

Here, Error! Reference source not found.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.

Determination of total phenolics

Total phenolic content was determined using the Folin-Ciocalteu method [9]. Briefly, 0.5 mL sample methanolic solution (2 mg/mL) was added to 0.5 mL Folin-Ciocalteu reagent (2 mol/L). The mixture was stood for 3 min, followed by addition of 1.0 mL Na_2CO_3 aqueous solution (15 %, w/w). After incubation at ambient temperature for 30 min, the mixture was centrifuged at 3500 r/min for 3 min. The supernatant was measured using a spectrophotometer (Unico 2100, Shanghai, China) at 760 nm. The standard curve was prepared using different concentrations of quercetin and the result was expressed as quercetin equivalents in milligrams per gram extract.

Determination of total flavonoids

Total flavonoid content was measured using the $NaNO_2$ -Al $(NO_3)_3$ method [11]. Briefly, 1 mL sample methanolic solution (25 mg/mL) was mixed with 0.15 mL $NaNO_2$ aqueous solution (5%, w/w). The mixture stood for 6 min, followed by the addition of 0.15 mL Al $(NO_3)_3$ aqueous solution (10%, w/w). After incubation for another 6 min, the mixture was added by 2 mL NaOH aqueous solution (4%, w/w) then adjusted to 5 mL with distilled water. The $A_{508\ nm}$ value was read on a spectrophotometer (Unico 2100, Shanghai, China). The standard curve was obtained using standard quercetin and the result was also expressed as quercetin

in milligrams per gram extract.

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).

Determination of total phenolics

The calculation of total phenolics was based on a calibration curve obtained with quercetin (not shown) and the result was expressed as quercetin equivalents in milligrams per gram of extract. According to the regression equation ($y = 74.23137x + 0.27967$), the content of total phenolics in ERC was calculated as 10.56±0.56 mg quercetin /g.

RESULTS

Protective effect against hydroxyl-induced DNA damage

Our data revealed that ERC along with the positive controls increased the percentages of protection in a dose-dependent manner (Figure 2A) and the IC_{50} value of ERC was 1180.28±137.73 μg/mL (Table 1).

DPPH• and $ABTS^{+}$• radical-scavenging assay

DPPH and ABTS assays have been widely used to determine the free radical-scavenging activity of various pure compounds or extracts. Both DPPH• and $ABTS^{+}$• are stable free radicals which dissolve in methanol or ethanol, and their colors show characteristic absorptions at 519 nm or 734nm, respectively. When an antioxidant scavenges the free radicals by hydrogen donation, the colors in the DPPH and ABTS assay solutions become lighter. The DPPH assay revealed that ERC can effectively inhibit DPPH• (Figure 2B) and its IC_{50} was 3857.79±35.51 μg/mL (Table 1). The ABTS assay indicated that ERC can also scavenge $ABTS^{+}$• in a concentration-dependent manner (Figure 2C) and its IC_{50} was 271.82±5.66 μg/mL (Table 1).

Fe^{3+} & Cu^{2+} reducing power assays

The dose-response curves in Figure 2D suggested that ERC exhibited Fe^{3+}-reducing power and IC_{50} value was 759.99±31.65 μg/mL (Table 1); Similar results (Figure 2E) could be observed in Cu^{2+}-reducing power assay, in which ERC also exhibited effective Cu^{2+}-reducing and its IC_{50} value was calculated as 733.02±9.67 μg/mL (Table 1).

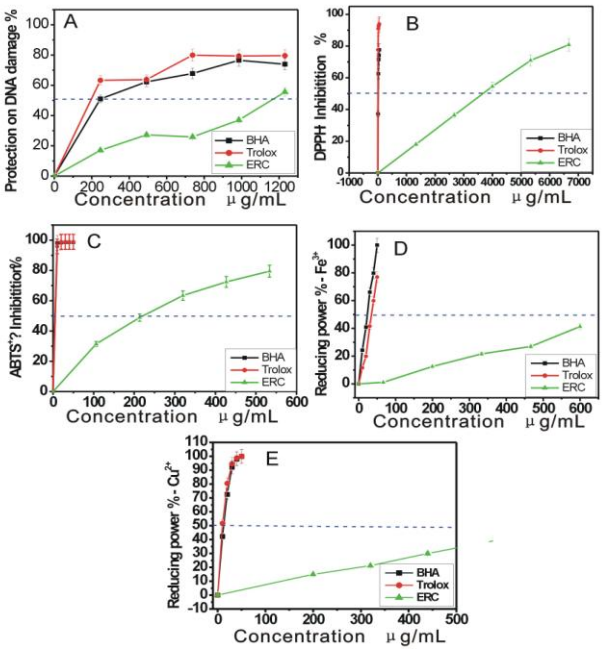


Figure 2. The dose response curves of ERC in the antioxidant assays: (A) protective effect on DNA damage; (B) DPPH· scavenging; (C) $ABTS^{+}$· scavenging (D) Fe^{3+}-reducing; (E) Cu^{2+}-reducing. Each value is expressed as Mean±SD (*n*=3). ERC, absolute ethanol extract of Radix *Codonopsis*. Trolox and BHA (butylated hydroxyanisole) were used as the positive controls

Table 1. The IC_{50} values of ethanol extract from Radix *Codonopsis* (ERC) (μg/mL)

	ERC	Positive controls	
		Trolox	BHA
Protecting DNA damage	1180.28±137.73[c]	306.13±26.11[a]	344.89±30.28[b]
DPPH• scavenging	3857.79±35.51[c]	9.75±0.06[a]	22.35±0.58[b]
$ABTS^{+}$• scavenging	271.82±5.66[b]	5.09±0.02[a]	5.21±0.25[a]
Fe^{3+}-reducing	759.99±31.65[c]	34.58±1.45[b]	22.88±1.03[a]
Cu^{2+}-reducing	733.02±9.67[c]	13.82±0.30[a]	16.09±0.47[b]

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 ($p<0.05$), while with same superscripts are not significantly different ($p<0.05$). BHA, butylated hydroxyanisole.

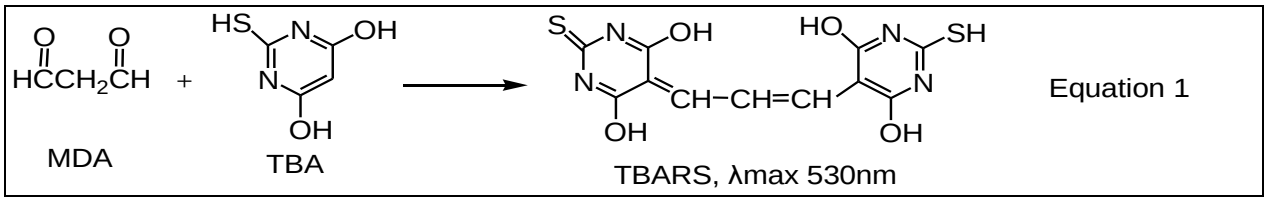
Determination of total flavonoids

The calculation of total flavonoids was also based on a calibration curve obtained with quercetin and the result was expressed as quercetin equivalents in milligrams per gram of extract. According to the regression equation ($y = 1.10239x + 0.02173$), the content of total flavonoids in ERB was calculated as 11.95±0.52 mg quercetin/g.

DISCUSSION

In the study, hydroxyl radical (•OH) is generated via Fenton reaction. Since •OH radical possesses extreme reactivity, it can easily damage DNA to generate malondialdehyde (MDA) and various oxidative lesions (Figure 3) [12,13].

As can be seen in Figure 3, these oxidative lesions don't contain conjugative system in the molecules and cannot be easily detected by a spectrophotometer. However, another product MDA can be easily detected by a spectrophotometric method. Because MDA can combine TBA (2-thiobarbituric acid) to yield TBARS (thiobarbituric acid reactive substances) which presents a maximum absorbance at 530 nm (Equation 1) [14].



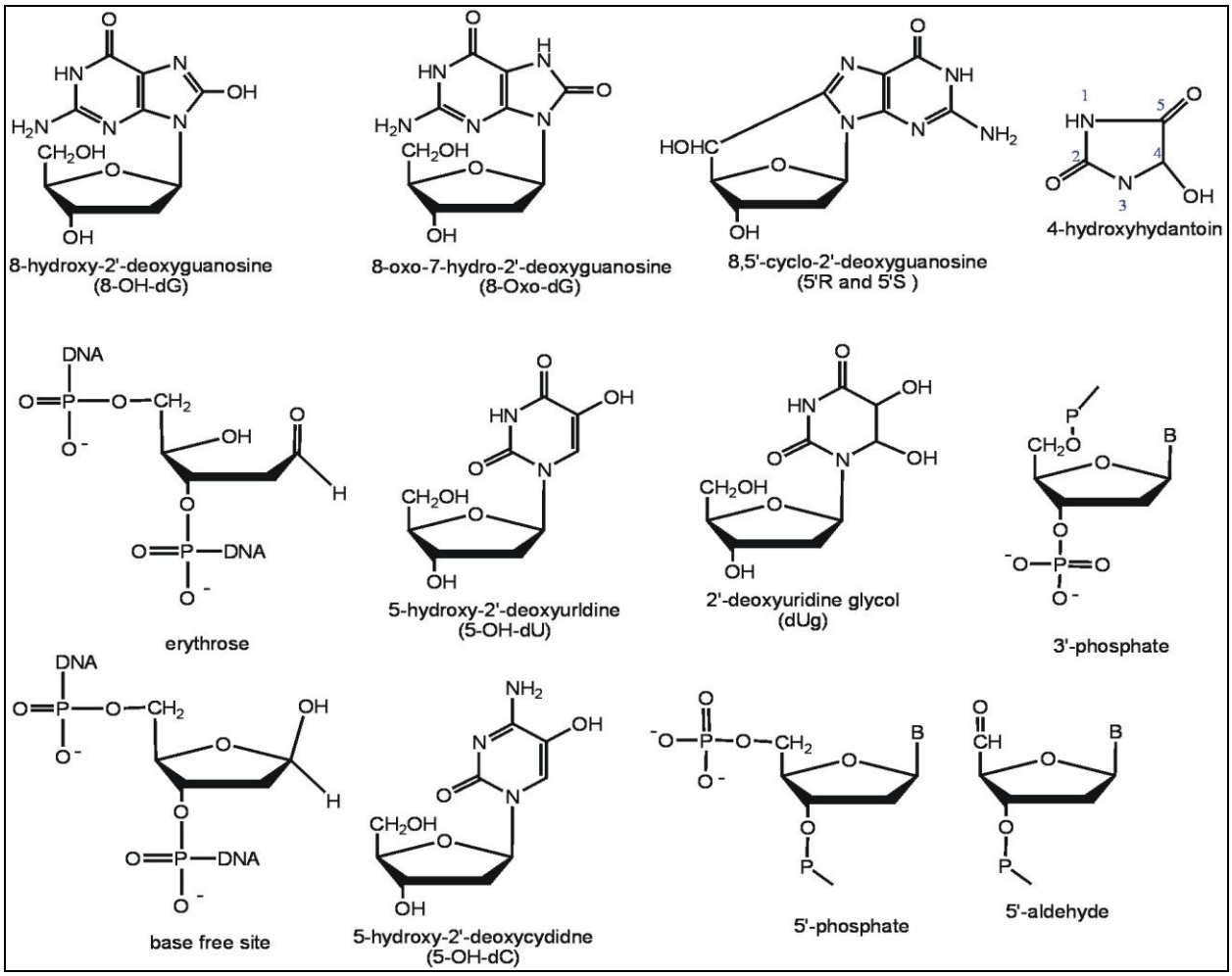


Figure 3. The structures of some oxidative lesions.

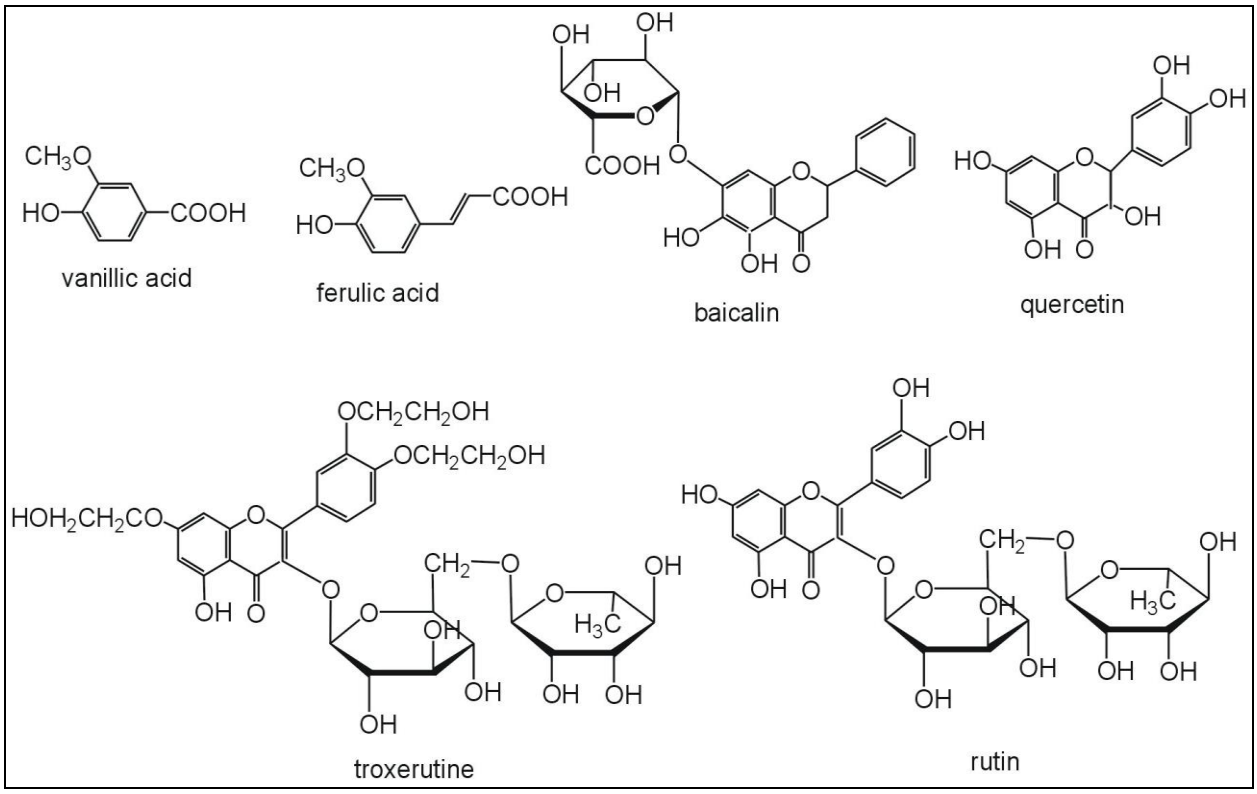


Figure 4. The structures of phenolic acids and flavonoids in Radix *Codonopsis*.

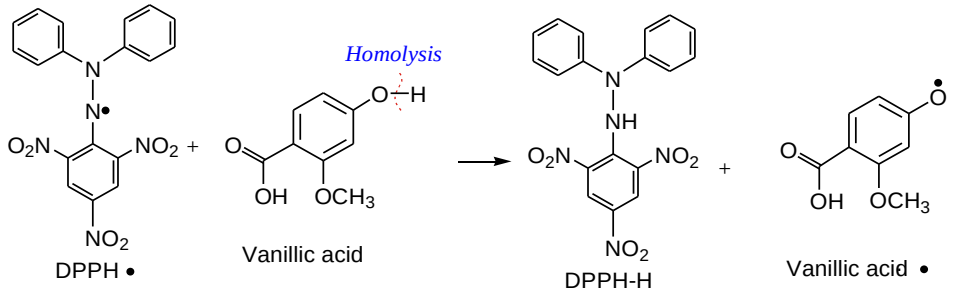
The value of A_{532nm} can therefore reflect the amount of MDA, and ultimately reflect the extent of DNA damage. The decrease of A_{532nm} value indicated a protective effect against DNA oxidative damage. Our results revealed that ERC can effectively protect against hydroxyl-induced DNA damage.

Previous studies showed that there are two approaches for natural antioxidant to protect DNA oxidative damage: one is to scavenge the •OH radicals then to reduce its attack; one is to fast repair the deoxynucleotide radical cations which were damaged by •OH radicals [15]. In order to further confirm whether the protective effect of extracts against DNA oxidative damage was relevant to its scavenging ability, we measured the scavenging abilities of ERC on DPPH• and ABTS+•.

The DPPH assay revealed that ERC can effectively eliminate DPPH•. On the other hand, the previous works suggested that DPPH• may be scavenged by an antioxidant through donation of hydrogen atom (H·) to form a stable DPPH-H molecule which does not absorb at 519 nm [16]. For example, vanillic acid which occurred in Radix *Codonopsis* [17,18], may scavenge DPPH• via the following proposed mechanism [19,20] (Equation 2).

In addition, ERC was proved to be of the ability of ABTS·+-scavenging which is regarded as an electron (e) transfer reaction [21].

The fact that ERC can effectively scavenge both DPPH• and ABTS+• radicals, suggests that ERC exerted radical-scavenging action maybe by donating hydrogen atom (H•) and electron (e).



Equation **2**

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant [22]. The reducing power of an antioxidant may therefore serve as a significant indicator of its potential antioxidant activity [23]. Obviously, the results of Fe3+-reducing and Cu2+-reducing assays supported the results that ERG has antioxidant ability.

Since total phenolics and flavonoids are usually responsible for the antioxidant ability in plants, we determined the total phenolic and flavonoids contents. As mentioned above, ERC contained high amounts of total phenolics and flavonoids. In fact, at least four flavonoids and two phenolic acids (Figure 4) in Radix *Codonopsis* have been determined by HPLC, such as baicalin (3.9l μg/g), quercetin (2.12 μg/g), troxerutine (1.10 μg/g), and rutin (0.27 μg/g) [24].

CONCLUSION

As a typical Chinese herbal medicine, Radix *Codonopsis* can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e). Its antioxidant ability can be mainly attributed to the existences of flavonoids or total phenolics.

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