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Protective Effect against Hydroxyl-induced DNA Damage and Antioxidant Activity of Radix *Glycyrrhizae* (Liquorice Root)

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ABSTRACT

***Purpose*:** As a typical Chinese herbal medicine, Radix *Glycyrrhizae* (RG) possesses various pharmacological effects involved in antioxidant ability. However, its antioxidant has not been explored so far. The aim of the study was to investigate its antioxidant ability, then further discuss the antioxidant mechanism. ***Methods*:** RG was extracted by ethanol to obtain ethanolic extract of Radix *Glycyrrhizae* (ERG). ERG was then determined by various antioxidant methods, including DNA damage assay, DPPH assay, ABTS assay, Fe^{3+}-reducing assay and Cu^{2+}-reducing assay. Finally, the contents of total phenolics and total flavonoids were analyzed by spectrophotometric methods. ***Results*:** Our results revealed that ERG could effectively protect against hydroxyl-induced DNA damage (IC_{50} 517.28±26.61μg/mL). In addition, ERG could scavenge DPPH· radical (IC_{50}165.18±6.48μg/mL) and $ABTS^{+}$• radical (IC_{50}7.46±0.07μg/mL), reduce Fe^{3+} (IC_{50} 97.23±2.88 μg/mL) and Cu^{2+} (IC_{50} 59.21±0.18 μg/mL). Chemical analysis demonstrated that the contents of total phenolics and flavonoids in ERG were 111.48±0.88 and 218.26±8.57 mg quercetin/g, respectively. ***Conclusion*:** Radix *Glycyrrhizae* 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 flavonoids or total phenolics.

Introduction

It is well known that reactive oxygen species (ROS) are various forms of activated oxygen including free radicals and non-free-radical species. As an important form of ROS, hydroxyl radical (·OH), for example, can cause oxidative damage to DNA, which leads 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.

As a typical Chinese herbal medicine, Radix *Glycyrrhizae* (RG) or liquorice root (甘草 in Chinese, Figure 1A) has been used in traditional Chinese medicine (TCM) for about 2000 years.[2,3] RG is the dried radixs of *Glycyrrhiza uralensis* Fisch. (Figure 1B), *Glycyrrhiza inflata* Bat., or *Glycyrrhiza glabra* L. From the viewpoint of traditional Chinese medicine (TCM), RG can invigorate *spleen*, replenish *qi*, clear *heat* and remove toxic substance.[3]

Modern medicine indicated that RG possessed various pharmacological effects. It was reported that RG could be used as an effective detoxifying agent and it exerted its detoxifying activity maybe via effects on the function and expression of P-glycoprotein in Caco-2 Cells;[4] Fu suggested that RG and its bioactive components presented neuroprotective effect;[5] Wang pointed out that RG possessed antiviral activity.[6] In addition, RG also showed anti-inflammatory,[7,8] antitumor and antibiosis effects.[9] Based on free radical biology & medicine,[10] we assumed that these pharmacological effects may be associated with antioxidant ability. However, its antioxidant ability has not been explored so far.

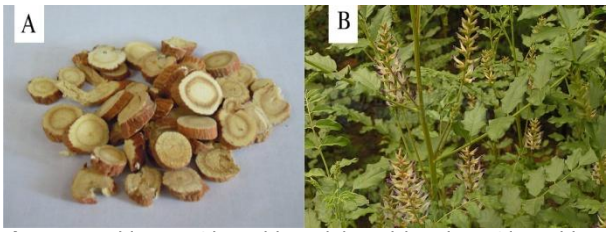

Figure 1. *Rhizoma Glycyrrhizae* (A) and its plant *Glycyrrhiza uralensis* Fisch (B).
Figure 1A was contributed by Weikang Chen, Figure 1B was contributed by www.plantphoto.cn.

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

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Materials and Methods

Plant material

Radix *Glycyrrhizae* was purchased from Caizhilin Pharmacy lacoted in 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 analytic grade.

Preparation of extracts from Radix Glycyrrhizae

Radix *Glycyrrhizae* 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 ERG (ethanol extract of Radix *Glycyrrhizae*). It was stored at 4°C for analysis.

Protective effect against DNA damage

The experiment was conducted as described in previous report.[11] However, deoxyribose was replaced by DNA sodium. Briefly, sample was dissolved in methanol at 6 mg/mL. Various amounts (20-100 μL) of sample methanolic solutions were then separately taken into mini tubes. After evaporating the sample solutions in tubes to dryness, 400 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), 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 adding 50 μL of 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 adding 250 μL of trichloroacetic acid (10g/100mL 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 heating 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:

$$\text{Protective effect \%} = (1 - A/A_0)\times 100\%$$

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 previously described by Li.[12] Briefly, 1 mL DPPH• ethanolic solution (0.1 mmol/L) was mixed with 0.5 mL sample alcoholic solution (4 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:

$$\text{Inhibition \%} = (1 - A/A_0)\times 100\%$$

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

$ABTS^{+\bullet}$ radical-scavenging assay

The $ABTS^{+\bullet}$ -scavenging activity was measured as described[13] with some modifications. The $ABTS^{+}$• was produced by mixing 0.35 mL ABTS diammonium salt (7.4 mmol/L) with potassium 0.35 mL 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 $ABTS^{+}$• reagent was mixed with 0.3 mL of sample ethanolic solution (0.08-0.4 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:

$$\text{Inhibition \%} = (1 - A/A_0)\times 100\%$$

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

Reducing power (Fe^{3+}) assay

Ferric (Fe^{3+}) reducing power was determined by the method of Oyaizu.[14] In brief, sample solution x μL (2 mg/mL, x = 20, 40, 60, 80, 100 and 120) 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). After incubation at 50 °C for 20 min, the mixture was added by 250 μL of trichloroacetic acid (10 g/100 mL), then centrifuged at 3500 r/min for 10 min. As soon as 400 μL supernatant was aliquoted into 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 was aliquoted into $FeCl_3$ to avoid oxidization by air. The relative reducing ability of the sample was calculated by using the formula:

$$\text{Relative reducing effect \%} = (A-A_{min})/(A_{max}-A_{min})\times 100\%$$

Here, 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 Cu^{2+}-reducing capacity was determined by the method,[15] 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 (2 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 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:

$$\text{Relative reducing effect \%} = (A-A_{min})/(A_{max}-A_{min})\times 100\%$$

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.

Determination of total phenolics

The total phenolics content of ERG was determined using the Folin-Ciocalteu method[16] with a little modifications. In brief, 0.5 mL sample methanolic solution (0.4 mg/mL) was mixed with 0.5 mL Folin-Ciocalteu reagent (2 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 determinations were performed in triplicate, and the calculations were based on a calibration curve obtained with quercetin. The result was expressed as quercetin equivalents in milligrams per gram of extract.

Determination of total flavonoids

The total flavonoid content was measured using the $NaNO_2$ -Al $(NO_3)_3$ method.[17] In brief, 1 mL sample methanolic solution (1 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 at ambient temperature for 6 min, 2 mL NaOH aqueous solution (4%, w/w) was added to the mixture which was then adjusted to 5 mL with distilled water. The absorbance was read at 508 nm on a spectrophotometer (Unico 2100, Shanghai, China). The determinations were performed in triplicate, and the calculations were also based on a calibration curve obtained with quercetin. The result was also expressed as quercetin equivalents in milligrams per gram of extract.

Statistical analysis

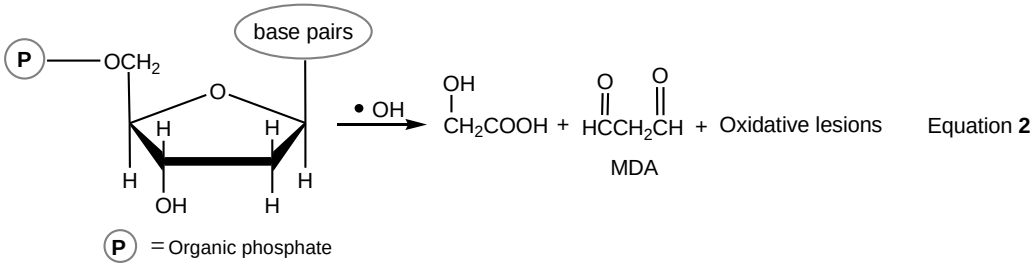
Data are given as the mean ± SD of three measurements. The IC_{50} values were calculated by linear regression analysis. All linear regression in this paper was analyzed by Origin 6.0 professional software. Significant differences were performed using the T-test ($p < 0.05$). The analysis was performed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

Results and Discussion

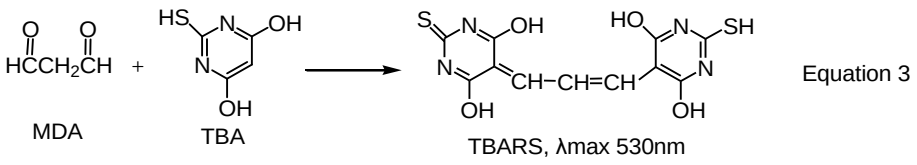
Hydroxyl radical (•OH) is known to be generated in human body via Fenton reaction (Equation 1).

$$Fe^{2+} + H_2O_2 \longrightarrow \bullet OH + OH^- + Fe^{3+} \quad \text{Equation 1}$$

Since •OH radical possesses extreme reactivity, it can easily damage DNA to produce malondialdehyde (MDA) and various oxidative lesions[18] (Equation 2 and Figure 2).



MDA combines TBA (2-thiobarbituric acid) to yield TBARS (thiobarbituric acid reactive substances) which present a maximum absorbance at 530 nm (Equation 3).[19]



On the other hand, as the oxidative lesions mentioned above have not conjugative system in the molecules (Figure 2), they cannot be detected by a spectrophotometer at 530 nm. It means that these oxidative lesions cannot disturb the determination of MDA.

Hence, the value of A_{530nm} can reflect the amount of MDA, and ultimately reflect the extent of DNA

damage. In terms of the formula "protective effect % = (1 - A/A_0)×100%", it can be deduced that the decrease of A_{530nm}value indicates a protective effect against DNA damage. As seen in Figure 3A, ERG showed a protective effect against DNA damage in a dose dependent manner and its IC_{50} value was 517.28±26.61μg/mL (Table 1).

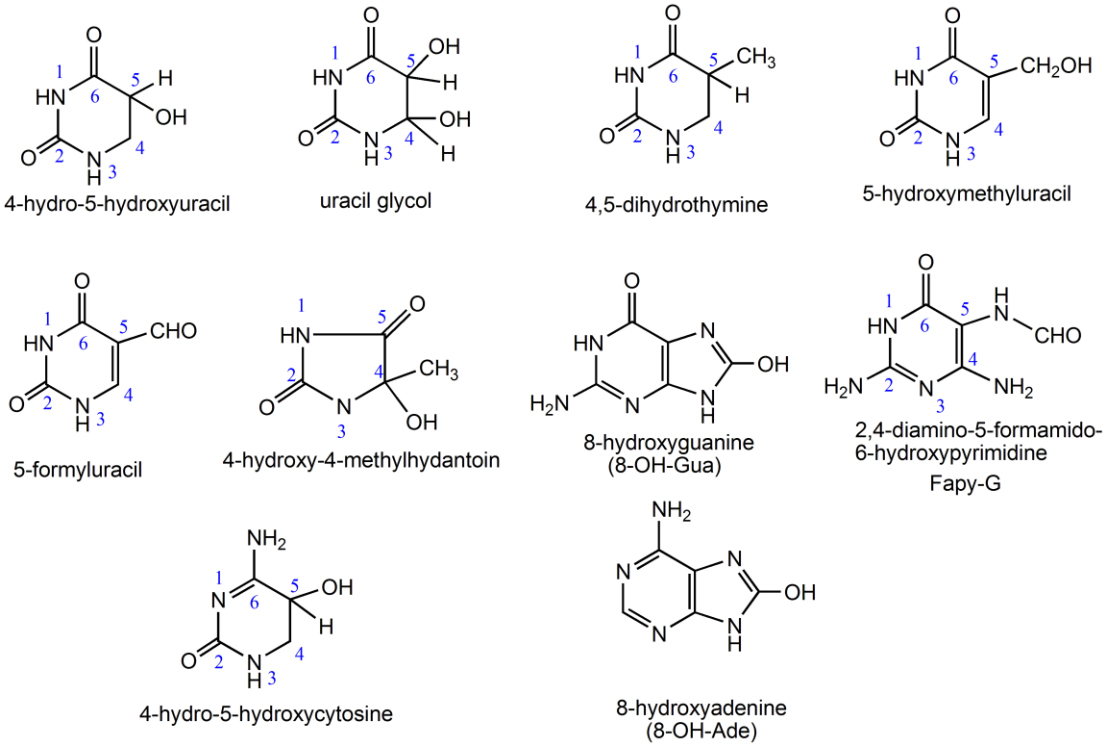


Figure 2. The structures of some oxidative lesions.

Previous reports have shown that there were two approaches for natural phenolic antioxidant to protect DNA oxidative damage: one was to scavenge the •OH radicals then to reduce its attack; one was to fast repair the deoxynucleotide radical cations which were damaged by •OH radicals.[20] In order to further confirm whether the protective effect of ERG against DNA oxidative damage was relevant to its radical-scavenging ability, we then determined the DPPH· and $ABTS^{+}$· radical-scavenging abilities.

The DPPH assay revealed that ERG can effectively eliminate DPPH• radical (Figure 3B) and its IC_{50} was 165.18±6.48 μg/mL (Table 1). The previous studies 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.[21] For example, liquifitigenin which occurred in Radix *Glycyrrhizae*,[22] may scavenge DPPH• via the following proposed mechanism[23,24] (Equation 4 and 5)

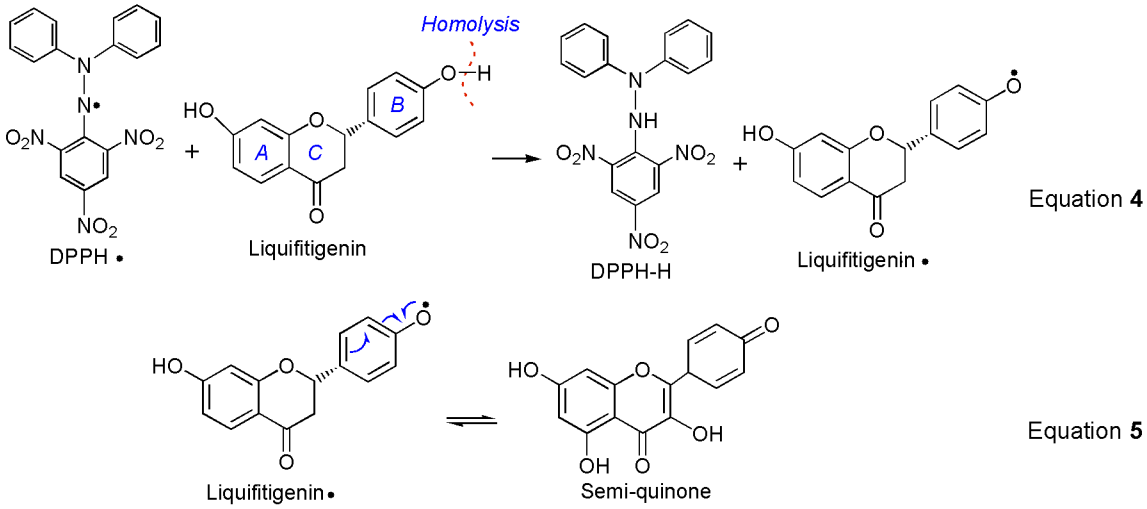


Table 1. The IC_{50} values of ethanol extract from Radix *Glycyrrhizae* (ERG) (μg/mL)

		Positive controls	
	ERG	**Trolox**	**BHA**
Protecting DNAdamage	517.28±26.61	306.13±26.11	344.89±30.28
DPPH· scavenging	165.18±6.49	9.75±0.06	22.35±0.58
$ABTS^{+}$· scavenging	7.46±0.07	5.09±0.02	5.21±0.25
Fe^{3+}-reducing	97.23±2.88	34.58±1.45	22.88±1.03
Cu^{2+}-reducing	59.21±0.18	13.82±0.30	16.09±0.47

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 signifiacntly different (p<0.05). BHA, butylated hydroxyanisole.

Figure 3C indicated that ERG could also scavenge $ABTS^{+}$· in a dose-dependent manner and its IC_{50} was 7.46±0.07 μg/mL (Table 1). However, $ABTS^{\cdot+}$ scavenging is considered as an electron (e) transfer reaction.[25]

The fact that ERG can effectively scavenge both DPPH· and $ABTS^{+}$· radicals, suggests that: (1) the protective effect of ERG against DNA oxidative damage was relevant to its radical-scavenging ability; (2) ERG exerted radical-scavenging action by donating hydrogen atom (H·) and electron (e).

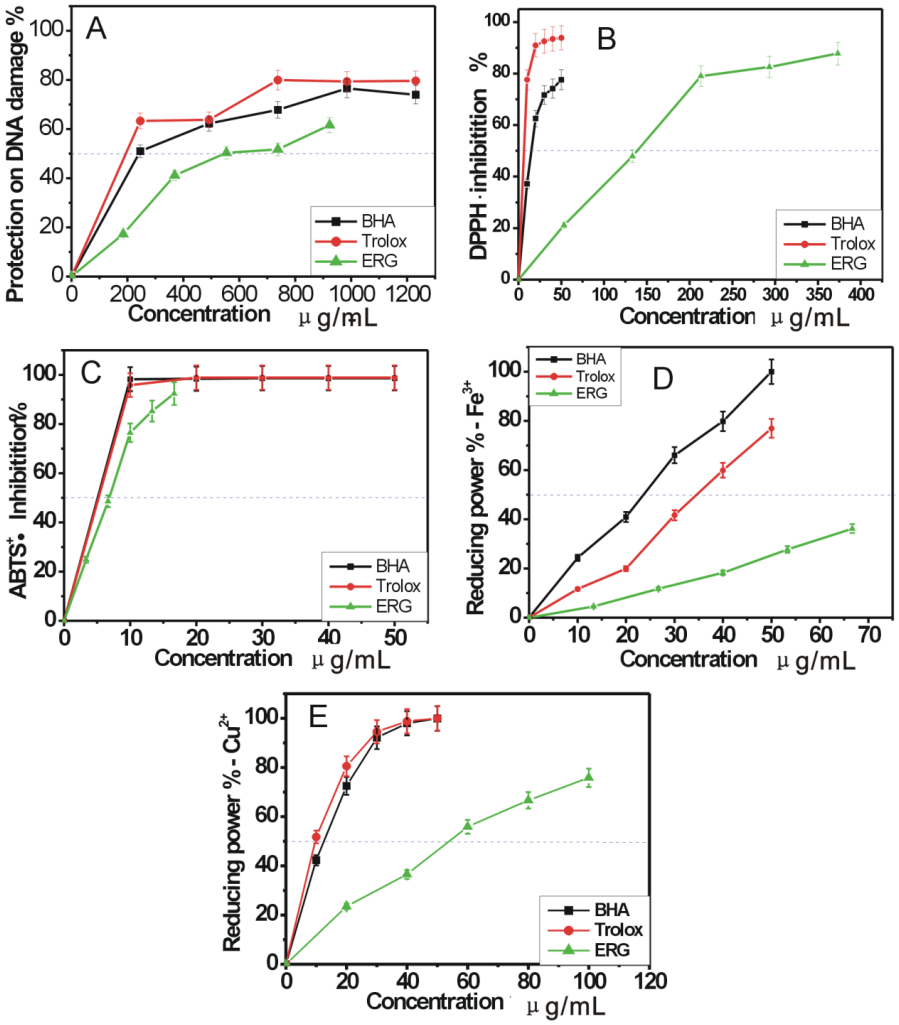


Figure 3. The dose response curves of ERG in the antioxidant assays: (A) protective effect on DNA damage; (B) DPPH· scavenging; (C) $ABTS^{+}$· scavenging (D) Fe^{3+}-reducing; (E) Cu^{2+}-reducing. ERG, absolute ethanol extract of Radix *Glycyrrhizae*. Trolox and BHA (butylated hydroxyanisole) were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

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant.[26] The reducing power of an antioxidant may therefore serve as a significant indicator of its potential antioxidant activity.[27] Figure 3D and 3E showed that ERG exhibited its reducing powers on Fe^{3+} and Cu^{2+} in a concentration dependent manner. The IC_{50} values were 97.23±2.88 μg/mL & 59.21±0.18 μg/mL, respectively for Fe^{3+}-reducing and Cu^{2+}-reducing) (Table 1). Obviously, these data further support the findings mentioned above.

Phytochemical studies suggested that total phenolics and total flavonoids can be responsible for the antioxidant ability in plants, we then determined the total phenolics and total flavonoids contents in ERG. The data showed that ERG contained high amounts of total phenolics and flavonoids (111.48±0.88 mg quercetin/g and 218.26±8.57 mg quercetin/g, respetively). In fact, at least 5 flavonoids have been isolated from Radix *Glycyrrhizae*, including liquiritigenin, isoliquiritigenin, liquiritin, isoliquiritin, neoliquiritin, and so on[22] (Figure 4).

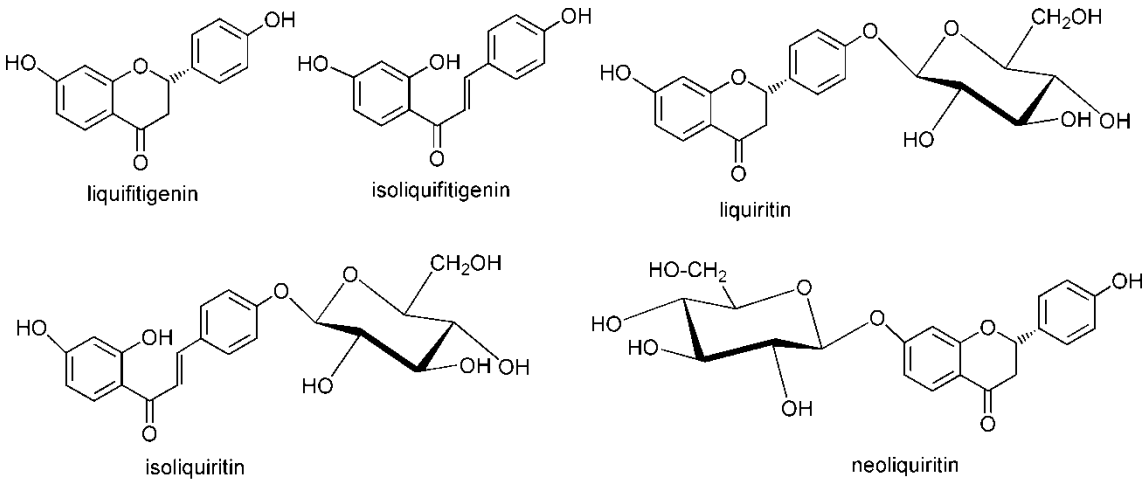


Figure 4. The structures of flavonoids in Radix *Glycyrrhizae.*

Conclusion

As a typical Chinese herbal medicine, Radix *Glycyrrhizae* 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 existence of flavonoids or total phenolics.

Conflict of interest

The authors report no conflicts of interest.

References

1. Bhattacharjee S, Deterding LJ, Chatterjee S, Jiang J, Ehrenshaft M, Lardinois O, et al. Site-specific radical formation in DNA induced by Cu(II)-H_2O_2 oxidizing system, using ESR, immuno-spin trapping, LC-MS, and MS/MS. *Free Radic Biol Med* 2011;50(11):1536-45.
2. Yang SZ, Bob F. The Divine Farmer's Materia Medica: A Translation of the Shen Nong Ben Cao Jing. Boulder, Co: Blue Poppy Press; 1998.
3. China Pharmacopoeia Committee. Pharmacopoeia of The People′s Republic of China. Beijing: Chemical Industry Press; 2010.
4. He D，Yan M，Li HD，Liu FQ，Yu DY，Yang CC，et al. Effects of Radix Glycyrrhiza and Its Main Components on the Function and Expression of P-glycoprotein in Caco-2 Cells. *Chin pharm j* 2010;45:751-5.
5. Fu YJ, Li ZY. Each Effective Ingredients of Glycyrrhiza on the Research Progress of the Effect of Nerve Protection. *J Int Neurology Neurosurgery* 2012;3:298-300.
6. Wang XQ, Li HY, Liu XY, Zhang FM, Li X, Piao YA, et al. The anti-respiratory syncytial virus effect of active compound of Glycyrrhiza GD4 in vitro. *Zhong Yao Cai* 2006;29(7):692-4.
7. Jin WW, Li YJ. Recent Studies on Anticancer Constituents in Glycyrrhiza Uralensis Fisch. *Journal of Liaoning university of TCM* 2011;4:163-5.
8. Zhang MF, Shen YQ. Advances in studies on Glycyrrhizae Radix et Rhizoma and its active components in anti-inflammation and mechanism. *Drugs & Clinic* 2011;4:261-8.
9. Wang WQ, Wei SL, Li WD. Review of pharmacological effects of Radix *Glycyrrhiza* and its bioactive compounds. *China Journal of Chinese Materia Medica* 2009;21:2695-700.
10. Zheng RL, Huang ZY. Free radical biology. 3rd Ed. China: Higher Education Press; 2007.
11. Wang X, Li X, Chen D. Evaluation of antioxidant activity of isoferulic acid *in vitro*. *Nat Prod Commun* 2011;6(9):1285-8.
12. Li X, Chen C. Systematic evaluation on antioxidant of magnolol *in vitro*. *Int Res J Pure Appl Chem* 2012;2(1):68-76.

13. Gao Y, Hu Q, Li X. Chemical composition and antioxidant activity of essential oil from *Syzygium samarangense* (BL.) Merr.et Perry flower-bud. *Spatula DD* 2012; 2(1): 23-33.
14. Oyaizu M. Studies on product of browning reaction prepared from glucoseamine. *Jpn J Nutr* 1986; 44:307-15.
15. Li XC, Wang XZ, Chen DF, Chen SZ. Antioxidant activity and mechanism of protocatechuic acid *in vitro*. *Functional Foods in Health and Disease* 2011;1(7):232-44.
16. Li XC, Wu XT, Huang L. Correlation between antioxidant activities and phenolic contents of Radix *Angelicae Sinensis* (Danggui). *Molecules* 2009;14:5349-61.
17. Li X, Chen D, Mai Y, Wen B, Wang X. Concordance between antioxidant activities in vitro and chemical components of Radix *Astragali* (Huangqi). *Nat Prod Res* 2012; 26(11):1050-3.
18. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB J* 2003;17(10):1195-214.
19. Cheeseman KH, Beavis A, Esterbauer H. Hydroxyl-radical-induced iron-catalysed degradation of 2-deoxyribose. Quantitative determination of malondialdehyde. *Biochem J* 1988;252(3):649-53.
20. Fang YZ, Zheng RL. Theory and application of free radical biology. China: Science Press; 2002.
21. Bondet V, Brand-Williams W, Berset C. Kinetics and mechanisms of antioxidant activity using the DPPH• free radical method. *LWT-Food Sci Technol* 1997; 30(6):609-15.
22. Tao B, Liu XQ, Qu ZG. Research Progress on the Chemical Constituents of Licorice Root. *J Hebei Agric Sci* 2009;3:77-9.
23. Dimitrios IT, Vassiliki O. The contribution of flavonoid C-ring on the DPPH free radical scavenging efficiency. A kinetic approach for the 3′,4′-hydroxy substituted members. *Innov Food Sci Emerg Technol* 2006;7(1-2):140-6.
24. Khanduja KL, Bhardwaj A. Stable free radical scavenging and antiperoxidative properties of resveratrol compared in vitro with some other bioflavonoids. *Indian J Biochem Biophys* 2003;40(6):416-22.
25. Aliaga C, Lissi EA. Reaction of 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) derived radicals with hydroperoxides: Kinetics and mechanism. *Int J Chem Kinet* 1998;30(8):565-70.
26. Prior RL, Cao G. In vivo total antioxidant capacity: Comparison of different analytical methods. *Free Radic Biol Med* 1999;27(11-12):1173-81.
27. Jung MJ, Heo SI, Wang MH. Free radical scavenging and total phenolic contents from methanolic extracts of Ulmus davidiana. *Food Chem* 2008;108(2):482-7.