

# Protective Effect against Hydroxyl-induced DNA Damage and Antioxidant Activity of *Citri reticulatae* Pericarpium

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## ABSTRACT

**Purpose:** As a typical Chinese herbal medicine, *Citri reticulatae* pericarpium (CRP) possesses various pharmacological effects involved in antioxidant ability. However, its antioxidant effects have not been reported yet. The objective of this work was to investigate its antioxidant ability, then further discuss the antioxidant mechanism. **Methods:** CRP was extracted by ethanol to obtain ethanol extract of *Citri reticulatae* pericarpium (ECRP). ECRP was then measured by various antioxidant methods, including DNA damage assay, DPPH assay, ABTS assay, Fe<sup>3+</sup>-reducing assay and Cu<sup>2+</sup>-reducing assay. Finally, the content of total flavonoids was analyzed by spectrophotometric method. **Results:** Our results revealed that ECRP could effectively protect against hydroxyl-induced DNA damage (IC<sub>50</sub> 944.47±147.74 µg/mL). In addition, it could also scavenge DPPH· radical (IC<sub>50</sub> 349.67±1.91 µg/mL) and ABTS<sup>•+</sup> radical (IC<sub>50</sub> 11.33±0.10 µg/mL), reduce Fe<sup>3+</sup> (IC<sub>50</sub> 140.95±2.15 µg/mL) and Cu<sup>2+</sup> (IC<sub>50</sub> 70.46±1.77 µg/mL). Chemical analysis demonstrated that the content of total flavonoids in ECRP was 198.29±12.24 mg quercetin/g. **Conclusion:** *Citri reticulatae* pericarpium 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, especially hesperidin and narirutin.

## Introduction

Reactive oxygen species (ROS) include hydroxyl radical (·OH), superoxide anion (·O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and nitric oxide (NO), with the ·OH being the most harmful. All macromolecules are sensitive to free radical damage. For example, DNA can be easily damaged by ·OH then lead to severe biological consequences including mutation, cell death, carcinogenesis, and aging.<sup>1</sup>

Therefore, it is vital to search for potential therapeutic agents for DNA oxidative damage. Over the last two decades, much attention has been focused on the antioxidant of medicinal plants especially Chinese medicinal herbs.

As a typical Chinese herbal medicine, *Citri reticulatae* pericarpium (CRP, 陈皮 in Chinese, Figure 1A) has been used in traditional Chinese medicine (TCM) for about 2000 years<sup>2,3</sup> CRP is the dried and ripe pericarpium of *Citri reticulatae* Blanco (Figure 1B). From the viewpoint of TCM, CRP can invigorate spleen, replenish qi, eliminate dampness and phlegm.<sup>3</sup> Modern medicine has demonstrated that CRP possessed various pharmacological effects. For

example, the extract from CRP could induce the apoptosis on SNU-C4 (human colon cancer cells) via Bax-related caspase-3 activation.<sup>4</sup> Ou reported that the extract from CRP could protect rats against myocardial ischemia.<sup>5</sup> Fang pointed out that CRP had antibacterial action.<sup>6</sup> Fan indicated an insecticidal effect of CRP.<sup>7</sup> In addition, CRP was also proved to be of anti-ulcer and anti-inflammatory.<sup>8</sup> However, according to free radical biology & medicine,<sup>9</sup> all these pharmacological effects may be associated with antioxidant ability.



**Figure 1.** Dried *Citri reticulatae* pericarpium (A) and its plant *Citri reticulatae* Blanco (B)

Figure 1A was contributed by Weikang Chen, Figure 1B was contributed by [www.plantphoto.cn](http://www.plantphoto.cn)

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Until now, its antioxidant ability has not been reported. Therefore, the purpose of the study was to investigate the antioxidant ability, then further discuss the antioxidant mechanism.

## Materials and Methods

### Plant Material

*Citri reticulatae* pericarpium was purchased from Caizhilin pharmacy located 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-hydrazyl radical), ABTS [2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid diammonium salt)], BHA (butylated hydroxyanisole), Trolox [(±)-6-hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic acid], DNA sodium salt (fish sperm), and neocuproine (2,9-dimethyl-1,10-phenanthroline) 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 *Citri reticulatae* Pericarpium

*Citri reticulatae* pericarpium was powdered then extracted by absolute ethanol using a Soxhlet extractor for 6 hr. The extract was filtered using a Buckner funnel and Whatman No 1 filter paper. The filtrate was then concentrated to dryness under reduced pressure to yield ECRP (ethanol extract of *Citri reticulatae* pericarpium). It was stored at 4°C for analysis.

### Protective Effect against Hydroxyl-Induced DNA Damage

The experiment was conducted as described in previous report.<sup>10</sup> However, deoxyribose was replaced by DNA sodium. Briefly, sample was dissolved in methanol at 8 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<sub>2</sub>O<sub>2</sub> (50 mmol/L), 50 µL FeCl<sub>3</sub> (3.2 mmol/L) and 50 µL Na<sub>2</sub>EDTA (1 mmol/L) were added. The reaction was initiated by adding 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 adding 250 µL 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 mixture without sample, and  $A$  is the absorbance of the mixture with sample.

### DPPH• Radical-Scavenging Assay

DPPH• radical-scavenging activity was determined as previously described by Li.<sup>11</sup> Briefly, 1 mL DPPH• ethanolic solution (0.1 mM) was mixed with 0.5 mL sample alcoholic solution (4.0 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 sample, while  $A_0$  is the absorbance without sample.

### ABTS<sup>+</sup> Radical-Scavenging Assay

The ABTS<sup>+</sup> -scavenging activity was measured as described<sup>12</sup> with some modifications. The ABTS<sup>+</sup> 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  $0.70 \pm 0.02$ . To determine the scavenging activity, 1.2 mL aliquot of diluted ABTS<sup>+</sup> 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<sup>3+</sup>) Assay

Ferric (Fe<sup>3+</sup>) reducing power was determined according to the method of Oyaizu.<sup>13</sup> In brief, sample solution  $x$  µL (2 mg/mL,  $x = 30, 60, 90, 120, \text{ and } 150$ ) was mixed with  $(350-x)$  µL Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer (0.2 mol/L, pH 6.6) and 250 µL K<sub>3</sub>Fe(CN)<sub>6</sub> 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<sub>3</sub> (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<sub>3</sub> 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<sup>2+</sup>-Reducing Power Assay**

The Cu<sup>2+</sup>-reducing capacity was determined by the method,<sup>14</sup> with minor modifications. Briefly, 125  $\mu$ L CuSO<sub>4</sub> aqueous solution (0.01 mol/L), 125  $\mu$ L neocuproine ethanolic solution (7.5 mmol/L) and (750-x)  $\mu$ L CH<sub>3</sub>COONH<sub>4</sub> buffer solution (0.1 mol/L, pH 7.5) were brought to test tubes. Then, different volumes of samples (2mg/mL, x = 40-120  $\mu$ L) were added to the tubes. Then, the total volume was adjusted to 1000  $\mu$ L 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:

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 Flavonoids**

The content of total flavonoids was measured using the NaNO<sub>2</sub>-Al(NO<sub>3</sub>)<sub>3</sub> method.<sup>15</sup> In brief, 0.05mL sample methanolic solution (20 mg/mL) was mixed with 0.15 mL NaNO<sub>2</sub> aqueous solution (5%, w/w). The mixture stood for 6 min, followed by the addition of 0.15 mL Al(NO<sub>3</sub>)<sub>3</sub> 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

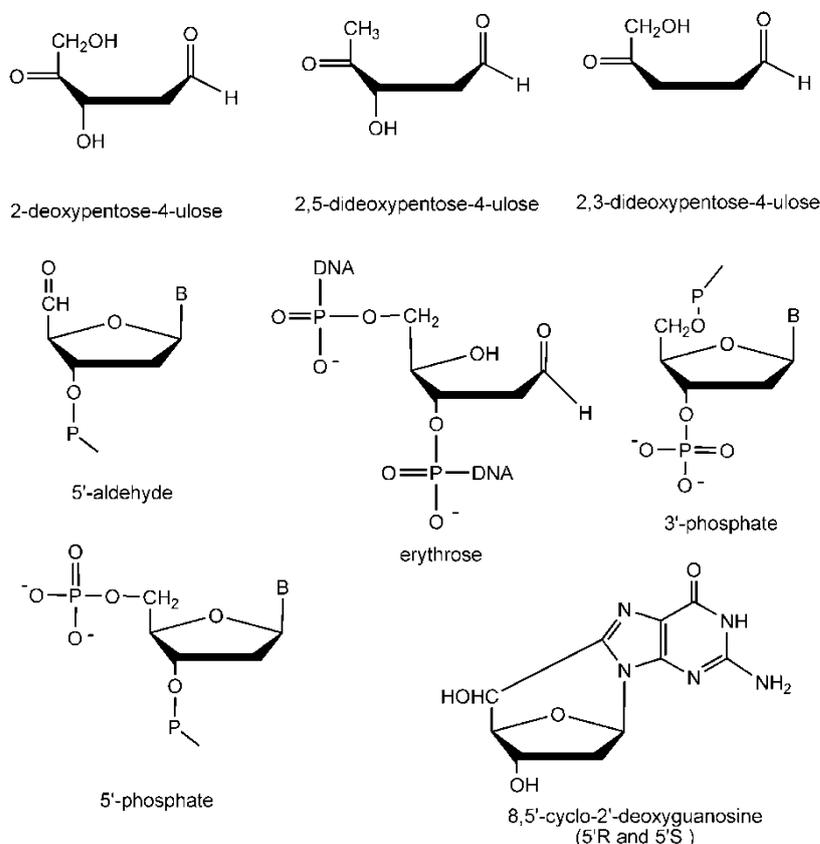
water. The absorbance was read at 508 nm on a spectrophotometer (Unico 2100, Shanghai, China). The standard curve was prepared using different concentrations of quercetin and the results were also expressed as quercetin in milligrams per gram extract.

**Statistical Analysis**

Data are given as the mean  $\pm$  SD of three measurements. The IC<sub>50</sub> 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**

It is well known that hydroxyl radical ( $\bullet$ OH) is generated in human body via Fenton reaction. Since  $\bullet$ OH radical has extreme reactivity, it can easily damage DNA to produce malondialdehyde (MDA) and various oxidative lesions.<sup>16,17</sup> MDA combines TBA (2-thiobarbituric acid) to produce TBARS (thiobarbituric acid reactive substances) which present a maximum absorbance at 530 nm.<sup>18</sup> On the other hand, as the oxidative lesions mentioned above have no conjugative system in the molecules (Figure 2), they cannot be detected by a spectrophotometer at 530 nm. It means that these oxidative lesions can bring about no interference with the determination of MDA.



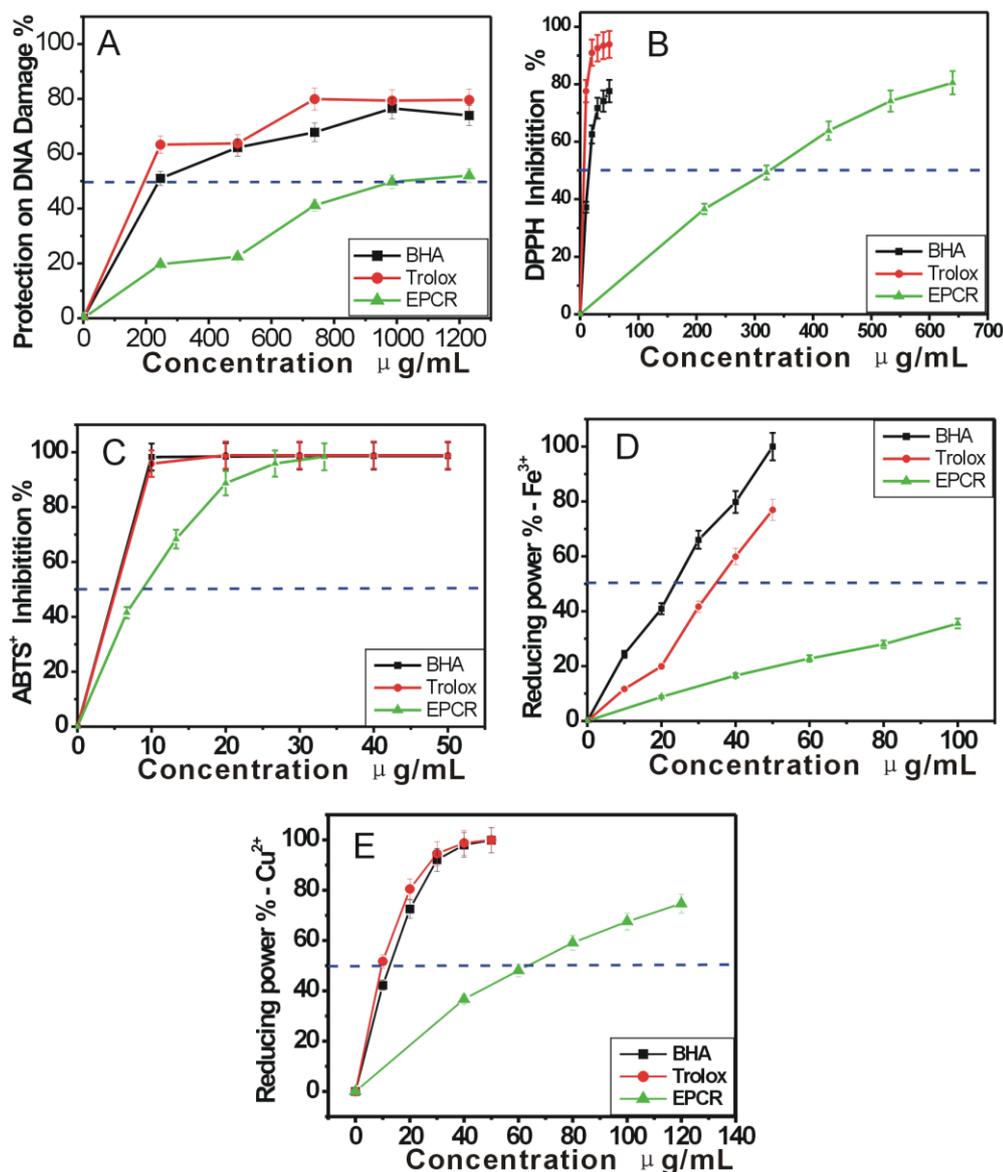
**Figure 2.** The structures of some oxidative lesions.

Hence, the value of  $A_{532nm}$  can evaluate the amount of MDA, and ultimately reflect the extent of DNA damage. Based on the formula “protective effect % =  $(1 - A/A_0) \times 100\%$ ”, it can be deduced that the decrease of  $A_{530nm}$  value indicates a protective effect against DNA damage. As seen in Figure 3A, ECRP dose-dependently increased the protective effect against DNA damage from 0-1240  $\mu\text{g/mL}$  and its  $IC_{50}$  value was  $944.47 \pm 147.74 \mu\text{g/mL}$  (Table 1).

Previous works have shown that there were two approaches for natural antioxidant to protect DNA oxidative damage: one was to scavenge the  $\bullet\text{OH}$  radicals then to reduce its attack; one was to fast repair the deoxynucleotide radical cations which were

damaged by  $\bullet\text{OH}$  radicals.<sup>19</sup> To further confirm whether the protective effect of ECRP was associated with its radical-scavenging ability, we determined the DPPH- and ABTS<sup>+</sup>- radical-scavenging abilities.

The DPPH and ABTS assays have been widely used to determine the free radical-scavenging activity of various plants and pure compounds. Both DPPH $\bullet$  and ABTS<sup>+</sup> are stable free radicals which dissolve in methanol or ethanol, and their colors show characteristic absorptions at 519 nm or 734 nm, respectively. When an antioxidant scavenges the free radicals, the values of  $A_{519nm}$  or  $A_{734nm}$  will decrease. On this basis, the inhibition percentages were defined as: inhibition % =  $(1 - A/A_0) \times 100\%$ .



**Figure 3.** The dose response curves of ECRP in the antioxidant assays: (A) protective effect against DAN damage; (B) DPPH-scavenging; (C) ABTS<sup>+</sup>-scavenging (D)  $\text{Fe}^{3+}$ -reducing; (E)  $\text{Cu}^{2+}$ -reducing. ECRP, absolute ethanol extract of *Citri reticulatae* pericarpium. Trolox and BHA (butylated hydroxyanisole) were used as the positive controls. Each value is expressed as Mean $\pm$ SD ( $n=3$ ).

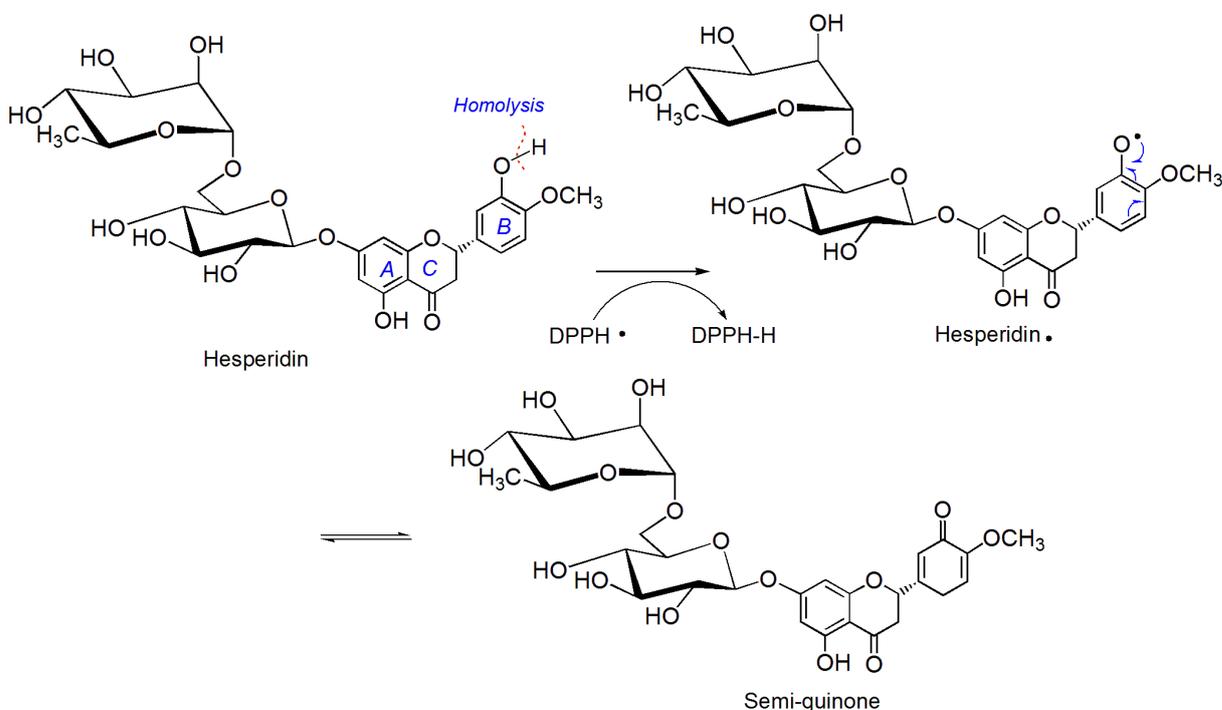
**Table 1.** The IC<sub>50</sub> values of ethanol extract from *Citri reticulatae* pericarpium (ECRP) (μg/mL)

	ECRP	Positive controls	
		Trolox	BHA
Protecting DNA damage	944.47±147.74 <sup>c</sup>	306.13±26.11 <sup>a</sup>	344.89±30.28 <sup>b</sup>
DPPH• scavenging	349.67±1.91 <sup>c</sup>	9.75±0.06 <sup>a</sup>	22.35±0.58 <sup>b</sup>
ABTS <sup>+</sup> scavenging	11.33±0.10 <sup>b</sup>	5.09±0.02 <sup>a</sup>	5.21±0.25 <sup>a</sup>
Fe <sup>3+</sup> -reducing	140.95±2.15 <sup>c</sup>	34.58±1.45 <sup>b</sup>	22.88±1.03 <sup>a</sup>
Cu <sup>2+</sup> -reducing	70.46±1.77 <sup>c</sup>	13.82±0.30 <sup>a</sup>	16.09±0.47 <sup>b</sup>

IC<sub>50</sub> 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.

As can be seen in Figure 3B, ECRP can effectively inhibit DPPH• radical from 0-648 μg/mL and its IC<sub>50</sub> was 349.67±1.91 μg/mL (Table 1). The previous study suggested that DPPH• may be scavenged by an antioxidant through donation of hydrogen atom (H•) to

form a stable DPPH-H molecule.<sup>20</sup> For example, hesperidin which occurred in *Citri reticulatae* pericarpium,<sup>21</sup> may scavenge DPPH• via the following proposed reaction<sup>22,23</sup> (Figure 4).

**Figure 4.** The proposed reaction of hesperidin with DPPH•.

The data in Figure 3C indicated that ECRP could also scavenge ABTS<sup>+</sup> in a dose-dependent manner (0-35 μg/mL) and its IC<sub>50</sub> was 11.33±0.10 μg/mL (Table 1). However, ABTS<sup>+</sup> scavenging is regarded as an electron (e) transfer reaction.<sup>24</sup>

The fact that ECRP can effectively scavenge both DPPH• and ABTS<sup>+</sup> radicals, suggests that: (1) the protective effect of ECRP against DNA oxidative damage was associated with its radical-scavenging ability; (2) ECRP exerted its radical-scavenging action by donating hydrogen atom (H•) and electron (e).

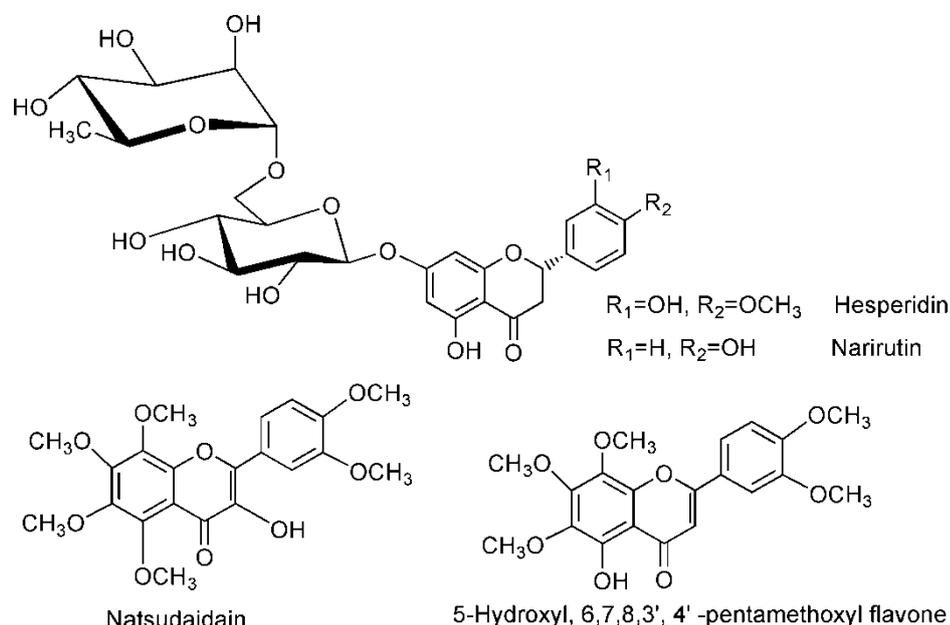
Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant.<sup>25</sup> The reducing power of an antioxidant may therefore serve as a

significant indicator of its potential antioxidant activity.<sup>26</sup> Figure 3D&3E showed that ECRP exhibited its reducing powers on Fe<sup>3+</sup> and Cu<sup>2+</sup> in a concentration dependent manner. The IC<sub>50</sub> values were 140.95±2.15 μg/mL & 70.46±1.77 μg/mL, respectively for Fe<sup>3+</sup>-reducing and Cu<sup>2+</sup>-reducing (Table 1). Obviously, these data further support the findings mentioned above.

Previous studies have shown that flavonoids can be responsible for the antioxidant ability in plants, we then determined the content of total flavonoids in ECRP. Our results indicated a high amount of total flavonoids (198.29±12.24 mg quercetin/g) in ECRP. In fact, at least 9 flavonoids have been isolated from *Citri*

*reticulatae* pericarpium until now, including hesperidin,<sup>27</sup> narirutin,<sup>27</sup> nobiletin,<sup>27</sup> tangeretin,<sup>27</sup> natsudaaidain,<sup>21</sup> 3,5,6,7,8,3',4'-heptamethoxyflavones,<sup>27</sup> 5-hydroxyl-6,7,8,3',4'-pentamethoxyflavone,<sup>28</sup> 5,6,7,8,4'-pentamethoxyflavone,<sup>28</sup> and 5,6,7,8,3',4'-

hexamethoxyflavone (Figure 5).<sup>28</sup> Among them, hesperidin and narirutin presented much higher content than the others in CRP.<sup>27</sup> Therefore, hesperidin and narirutin were regarded as two main active components of antioxidant in CRP.



**Figure 5.** The structures of some flavonoids in *Citri reticulatae* pericarpium.

## Conclusion

As a typical Chinese herbal medicine, *Citri reticulatae* pericarpium can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H<sup>•</sup>), donating electron (e<sup>-</sup>). Its antioxidant ability can be mainly attributed to flavonoids (especially hesperidin and narirutin).

## Conflict of Interest

The authors declare there is no Conflict of interest in the content of this study.

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