Antioxidant Activity and Mechanism of Protocatechuic Acid *in vitro*

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Abstract:

Background: Protocatechuic acid (PCA) is a natural phenolic acid widely distributed in plants and is considered as an active component of some traditional Chinese herbal medicines such as *Cibotium barometz* (L.) J.Sm, *Stenoloma chusanum* (L.) Ching, *Ilex chinensis* Sims. PCA was reported to possess various pharmacological effects which may be closely correlated with its antioxidant activities. However, the antioxidant of PCA has not been investigated systematically yet.

Methods: In the study, the antioxidant activities of protocatechuic acid were measured *in vitro* using various antioxidant assays including 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$•), superoxide anion radicals ($•O_2^-$) and hydroxyl radical (•OH) scavenging activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing power, ferrous ions (Fe^{2+}) and cupric ions (Cu^{2+}) chelating activity, compared with the positive controls Trolox or BHT.

Results: In all assays, PCA along with positive controls exhibited dose-dependently antioxidant ability. Comparing to a standard antioxidant Trolox, the relative antioxidant activity of PCA (i.e. the ratio of $IC_{50(Trolox)}/IC_{50(PCA)}$) was calculated as 2.8, 2.3, 3.7, 6.1, 4.2, 1.0, 2.7, 1.5, respectively, for DPPH, ABTS, reducing power (Fe^{3+}), reducing power (Cu^{2+}), superoxide anion radical-scavenging, hydroxyl radical-scavenging, chelating ability (Fe^{2+}) and chelating ability (Cu^{2+}).

Conclusion: Comparing to Trolox, PCA shows much more effective antioxidant activity *in vitro* in both lipid and aqueous media. Hence, it could therefore be used in pharmacological or food industry as a natural antioxidant. It may exhibit antioxidant activity by both chelating metal transition ions as well as by scavenging free radicals via donating hydrogen atom (H•) or electron (e).

Keywords: Protocatechuic acid, antioxidant, reducing power, free radical-scavenging, chelating ability.

Background:

It has been well recognized that, many biochemical reactions involve the generation of ROS (reactive oxygen species) in our body. However, under normal conditions, the balance between the generation and diminution of ROS is controlled by the antioxidant defense system. Under certain pathological conditions, when ROS are not effectively eliminated by the antioxidant defense system, the dynamic balance between the generation and diminution of ROS is broken. Excessive ROS attack lipids, carbohydrates, proteins, DNA, and result in oxidative stress, that leads to various disorders and diseases [1]. In order to protect biomolecules against the attack of ROS and/or to suppress the resultant damage, synthetic antioxidants have been used for industrial processing in recent years. However, these synthetic antioxidants have been suspected of being harmful to liver [2]. Thus, considerable focus has been given to searching for natural antioxidants from plants especially traditional Chinese herbal medicines in recent years.

As a natural phenolic acid, protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid) is widely distributed in many traditional Chinese herbal medicines, such as *Stenoloma chusanum* (L.) Ching, *Ilex chinensis* Sims, *Cibotium barometz* (L.) J.Sm, and is regarede as an active component of these Chinese herbal medicines. Morever, PCA was reported to possess various pharmacological effects which may be closely correlated with its antioxidant activities [3] [4] [5] [6]. However, the antioxidant of PCA has not been investigated systematically yet.

Therefore, in the present work, the antioxidant activity and antioxidant mechanism of protocatechuic acid was clarified using various *in vitro* antioxidant assays including 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and superoxide anion radicals ($•O_2^-$) and hydroxyl radicals scavenging activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing power, ferrous ions (Fe^{2+}) and cupric ions (Cu^{2+}) chelating activity.

Materials:

Protocatechuic acid (IUPAC name: 3,4-dihydroxybenzoic acid, CAS number: 99-50-3, >99%)

was obtained from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products, China); DPPH• (1,1-Diphenyl-2-picrylhydrazyl radical), pyrogallol, linoleic acid, Trolox (± -6-hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid), Ferrozine [3-(2-pyridyl)-5,6-bis (4-phenylsulfonicacid)-1,2,4-triazine], neocuproine (2,9-dimethyl-1,10-phenanthroline), BHA (butylated hydroxyanisole), BHT (butylatedhydroxy-toluene) and murexide (5,5′-nitrilodibarbituric acid monoammonium salt) were purchased from Sigma Co.; ABTS diammonium salt [2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)] , and D-2-deoxyribose were obtained from Amresco Co.; All other chemicals were of analytic grade.

Methods:

DPPH• scavenging activity

DPPH radical-scavenging activity was determined as described [3]. Briefly, 1 mL of DPPH• solution (0.1 mmol/L) was mixed with 0.5 mL of various concentrations of samples dissolved in 95% ethanol. The mixture was kept at room temperature for 30 min, and then the absorbance at 519 nm was measured on spectrophotometer (Unico 2100, Shanghai, China), using 95% ethanol as the blank. Trolox and BHT were used as the positive controls, and the percentage DPPH• inhibition of the test samples was calculated:

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

Where A_S is the absorbance in the presence of the sample protocatechuic acid or positive controls, while A_0 is the absorbance in the absence of the sample protocatechuic acid and positive controls.

ABTS$^+$• scavenging activity

The scavenging activity of ABTS$^+$• was measured by the method [3]. The ABTS$^+$• was produced by mixing 0.35 mL of ABTS diammonium salt (7.4 mmol/L) with 0.35 mL of potassium persulfate (2.6 mmol/L). The mixture was kept in the dark at room temperature for 12 h to allow completion of radical generation, and then diluted with 95% ethanol (about 1:50) so that its absorbance at 734 nm was 0.70±0.02 measured on a spectrophotometer (Unico 2100, Shanghai, China). To determine the scavenging activity, 1.2 mL of ABTS$^+$• reagent was mixed with 0.3 mL of sample or negative control (95% ethanol), and the absorbance at 734 nm was measured at 6 min after the initial mixing, using 95% ethanol as the blank. The percentage inhibition of the samples was calculated as:

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

Where A_0 is the absorbance at 734 nm of the negative control, A is the absorbance at 734 nm of the mixture with sample. Trolox and BHT, with a final concentration range of 0.67-2.67 µg/mL, were prepared as positive controls.

Ferric ions (Fe^{3+}) reducing power

Ferric cyanide (Fe^{3+}) reducing power was determined by the method of Oyaizu et al. [14], as described by Li et al [3]. Samples (x µL) at various concentrations were mixed with Na_2HPO_4/KH_2PO_4 buffer (350-x µL, 0.2 mol/L, pH 6.6) and $K_3Fe(CN)_6$ (250 µL, 1 g/100 mL). The mixture was incubated at 50 ℃ for 20 min, 250 µL of trichloroacetic acid (10 g/100 mL) was added, and the mixture was centrifuged at 3500g for 10 min. The supernatant (400 µL) was recovered, mixed with distilled water (400 µL) and $FeCl_3$ (400 µL, 0.1 g/100 mL) and placed immediately into the spectrophotometer (Unico 2100, Shanghai, China), and the timer was started. The absorbance at 700 nm was measured at 90 s. 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. Trolox and BHT were used as the positive controls, and an increased absorbance reading indicated increased reducing power. The percentage reducing power of the sample as compared to the maximum absorbance tested which appeared in protocatechuic acid at 10 µg/mL was calculated by using the formula: (A_S /A_m) ×100. Here, A_m = absorbance of maximum absorbance tested and A_S= absorbance of sample.

Cupric ions (Cu^{2+}) reducing power

In order to further measure the reducing ability of protocatechuic acid, the cupric ions (Cu^{2+}) reducing power capacity was also used with slight modification [15]. Briefly, 250 µL $CuSO_4$ aqueous solution (10 mmol/L), 250 µL neocuproine ethanolic solution (7.5 mmol/L) and 250 µL CH_3COONH_4 buffer solution (100 mmol/L, pH 7.0) were added to a test tube containing a protocatechuic acid sample. Then, the total volume was adjusted with the buffer to 2 mL and mixed vigorously. Absorbance against a buffer blank was measured at 450 nm after 30 min. Increased absorbance of neocuproine-Cu^+ complex in the reaction mixture indicates increased reduction capability. Trolox and BHA were used as the positive controls. The percentage reducing power of the sample as compared to the maximum absorbance tested which appeared in protocatechuic acid at 6 µg/mL was calculated by using the formula: (A_S /A_m) ×100. Here, A_m = absorbance of maximum absorbance tested and A_S = absorbance of sample.

Superoxide anion ($•O_2^-$) scavenging activity

The scavenging ability at pH 8.2 of all test samples was determined by the method of Marklund and Marklund [16], as described by Li et al [3]. Briefly, samples were dissolved in suitable solvents (absolute or 95% ethanol) at a concentration of 5 mg/mL. The sample solution (x µL, where x = 0, 30, 60, 90, 120, 150, or 180 µL) was mixed with Tris-HCl buffer (2920 - x µL, 0.05 mol/L, pH 8.2) containing EDTA (1 mmol/L) and pyrogallol (80 µL, 6 mmol/L), then shaken rapidly at room temperature. The absorbance at 325 nm of the mixture was measured (Unico

2100, Shanghai, China) against the Tris-HCl buffer every 30 s for 5 min. The slope of the correlation of absorbance with time was calculated. The reaction mixture without added sample was used as the control. The $\bullet O_2^-$ scavenging ability was calculated as:

$$(1 - \text{Slope of sample/Slope of control}) \times 100\ \%$$

Hydroxyl radical ($\bullet OH$) scavenging activity

The scavenging activity on the hydroxyl radical ($\bullet OH$) was investigated by the deoxyribose method [17] modified with some modification. Our preliminary experiments demonstrated that almost organic solvents except for petroleum ether can promote the inhibition percentage value, including ethanol, methanol, acetone, ethyl acetate, acetonitrile, chloroform, diethyl ether, DMF, DMSO, and THF. Hence, the inhibition of hydroxyl radical was evaluated as the following procedure: all test samples were firstly dissolved in ethanol (1 mg/mL), and 5-40 μL sample solution was taken into mini tubes, the ethanol solvent was then removed at 80 ℃ to eliminate its interference. The reactions were performed in 0.2 mol/L phosphate buffer (Ph 7.4), containing 2.8 mmol/L   deoxyribose, 2.8 mmol/L H_2O_2, 25 μmol/L $FeCl_3$, 80 μmol/L Na_2EDTA, and the test sample (5-40 μg). The reaction was started by adding ascorbic acid to a final concentration of 100μmol/L and the reaction mixture (600 μL in total) was incubated for 20 min at 50 ℃ in a water bath. After incubation, the color was developed by addition of 0.5 mL 2-thiobarbituric acid (1 g/100 mL) followed by 0.5 mL trichloroacetic acid (5 g/100 mL) and heating in a boiling water bath for 15 min. The sample was cooled and diluted two-fold with 95% ethanol, and the absorbance was measured at 532 nm against buffer (as blank). The reaction mixture without sample was used as control. The scavenging activity on hydroxyl radicals was expressed as:

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

Where A_0 is the absorbance at 532 nm of control (without sample), and A is the absorbance at 532 nm of the reaction mixture containing sample. Trolox and BHT were taken as positive controls.

Chelating activity on Fe^{2+}

The chelating abilities on Fe^{2+} by protocatechuic acid and positive controls were estimated by the method as described by Gülçin [18]. Briefly, 1mg/mL protocatechuic acid solution (200, 400, 600 μL) was added to a solution of 250 μmol/L $FeCl_2$ (100 μL). The reaction was initiated by the addition of 250 μmol/L Ferrozine (150 μL). The total volume of the systems was adjusted to 1000 μL with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm (Unico 2100, Shanghai, China). The percentage of chelating effect was calculated by using the formula given bellow:

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

Where A is the absorbance in the presence of the sample protocatechuic acid or positive controls, while A_0 is the absorbance in the absence of the sample protocatechuic acid and positive controls.

Chelating activity on Cu^{2+}

The chelating activity on Cu^{2+} of protocatechuic acid and positive controls was estimated by a complexometric method using murexide [19]. Briefly, to hexamine HCl buffer (pH 5.0, 30 mmol/L) containing 30 mmol/L KCl and 0.3 μmol murexide, 60 μL $CuSO_4$ (20 mmol/L) was added. After incubation for 1 min at room temperature, the reaction was initiated by the addition of 10 mg/mL protocatechuic acid ethanolic solution (5, 15, 25, 35, 45 μL) .The total volume of the systems was adjusted to 1000 μL with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 485 and 520 nm (Unico 2100, Shanghai, China).The absorbance ratio (A_{485}/A_{520}) reflected the free Cu^{2+} content. Therefore, the percentage of cupric chelating effect was defined as follows:

$$\text{Cupric chelating effect } (\%) = [(A_{485}/A_{520})\ _{max} - (A_{485}/A_{520})] / (A_{485}/A_{520})\ _{max} \times 100$$

Where (A_{485}/A_{520}) is the absorbance ratio in the presence of the samples, while (A_{485}/A_{520}) $_{max}$ is the maximum absorbance ratio without any samples. Sodium citrate and Tolox were used as the positive controls.

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.
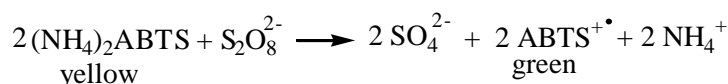
Results and Discussion：

DPPH• and $ABTS^+$• scavenging activity

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. As presented in Fig.1, both the DPPH• and $ABTS^+$• inhibition percentage values were dose dependent where it

increased in the range of the tested concentration, for protocatechuic acid and the positive controls Trolox and BHT. DPPH radical inhibition decreased in the order protocatechuic acid > BHT ≈ Trolox, while ABTS radical ion inhibition in the order protocatechuic acid > BHT > Trolox. The DPPH• and ABTS$^+$• IC$_{50}$ values (IC$_{50}$ value is the concentration of the sample required to inhibit 50% of radical) of protocatechuic acid, Trolox and BHT were calculated and listed in Table 1.

Figure 1. Effect of different concentration of protocatechuic acid in free radical scavenging tests: (A) DPPH assay, (B) ABTS assay. Each value is expressed as mean ± standard deviation, n =3.

It is proposed 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 519nm [7]. According to this hypothesis and previous study [8], the reaction between DPPH• and PCA could be explained by the following mechanism:

Radical (A) can be further withdrawn hydrogen atom (H•) to form stable quinone (B).

However, the scavenging of $ABTS^+\bullet$ is assumed to be an electron transfer process:

$$ABTS^+_\bullet + e \longrightarrow ABTS$$
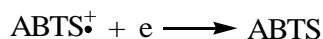
$ABTS^+\bullet$ can be previously produced by the reaction between ABTS diammonium salt and potassium persulfate:

$$2\,(NH_4)_2ABTS + S_2O_8^{2-} \longrightarrow 2\,SO_4^{2-} + 2\,ABTS^{+\bullet} + 2\,NH_4^+$$
$$\quad\text{yellow} \qquad\qquad\qquad\qquad \text{green}$$

Our results indicating that PCA can effectively inhibit $DPPH\bullet$ and $ABTS^+\bullet$, suggested that PCA exerted radical-scavenging action by donating hydrogen atom $(H\bullet)$ and electron (e).

Reducing power assay

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant [9]. The reducing power of a compound may therefore serve as a significant indicator of its potential antioxidant activity [10]. It can be seen that the reducing power percentage values of protocatechuic acid and the positive controls were concentration related and increased with the increasing of sample concentration in the range of the tested concentration (Fig. 2). Their relative reducing powers on Fe^{3+} were as follows: protocatechuic acid > Trolox $\approx$ BHT, while the relative reducing powers on Cu^{2+} were protocatechuic acid > BHA >Trolox. The IC_{50} values of all tested samples were calculated and listed in Table 1.
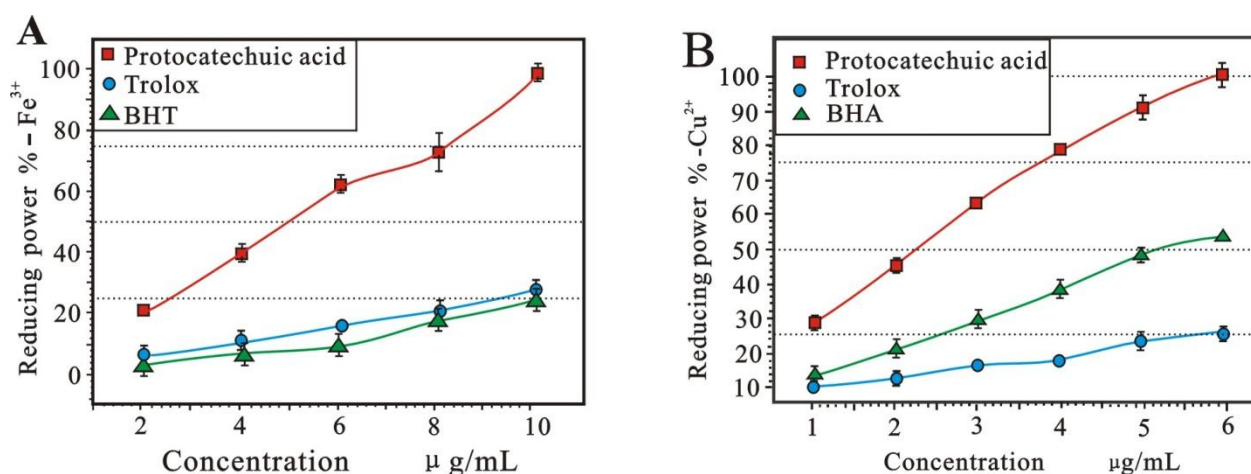

Figure 2. Reducing power assays of protocatechuic acid and positive controls: (A) $Fe^{3+}\rightarrow Fe^{2+}$; (B) $Cu^{2+}\rightarrow Cu^+$. Each value is expressed as mean $\pm$ standard deviation, n =3.

Protocatechuic acid was demonstrated to be a more effective reductant than the standard antioxidants in both reducing power assays ($Fe^{3+}\rightarrow Fe^{2+}$ and $Cu^{2+}\rightarrow Cu^+$). The results are obviously consistent with the total antioxidant activity in DPPH and ABTS assays.

Superoxide anion ($\bullet O_2$ -) and hydroxyl ($\bullet OH$) radical-scavenging activity

ROS are various forms of activated oxygen including free radicals and non-free-radical species. Superoxide anion ($\bullet O_2^-$) and hydroxyl radical ($\bullet OH$) are two of the most important free

radicals in living cells. Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes.   Hydroxyl radical (•OH), however, as the most dangerous form of ROS, has a high reactivity with a very short half-life of approx. 10^{-9} s *in vivo* [11].

In our experiments, the superoxide radical was generated by the pyrogallol system at pH 8.2. Fig. 3A showed that PCA and the positive controls (Trolox and BHT) demonstrated an ability to inhibit superoxide anion in a dose-dependent manner. PCA exhibited higher inhibition level than Trolox at the tested concentration, but lower than BHT. Their IC_{50} values were calculated and listed in Table 1. The hydroxyl (•OH) radical-scavenging activity was evaluated by the Fenton reaction using the deoxyribose assay. In this study, a mixture of Fe^{3+}-EDTA, hydrogen peroxide (H_2O_2), and ascorbic acid were used to generate hydroxyl radicals (•OH). The response degrades D-2-deoxyribose into fragments which under heating with thiobarbituric acid at acidic solution, are detected at 540nm because they generate a pink chromogen. The data in Fig. 3B showed that PCA and the positive controls could effectively inhibit the formation of •OH generated in a concentration-dependent manner. According to the IC_{50} values (Table 1), PCA possessed the similar scavenging activity to Trolox, but lower than BHT.
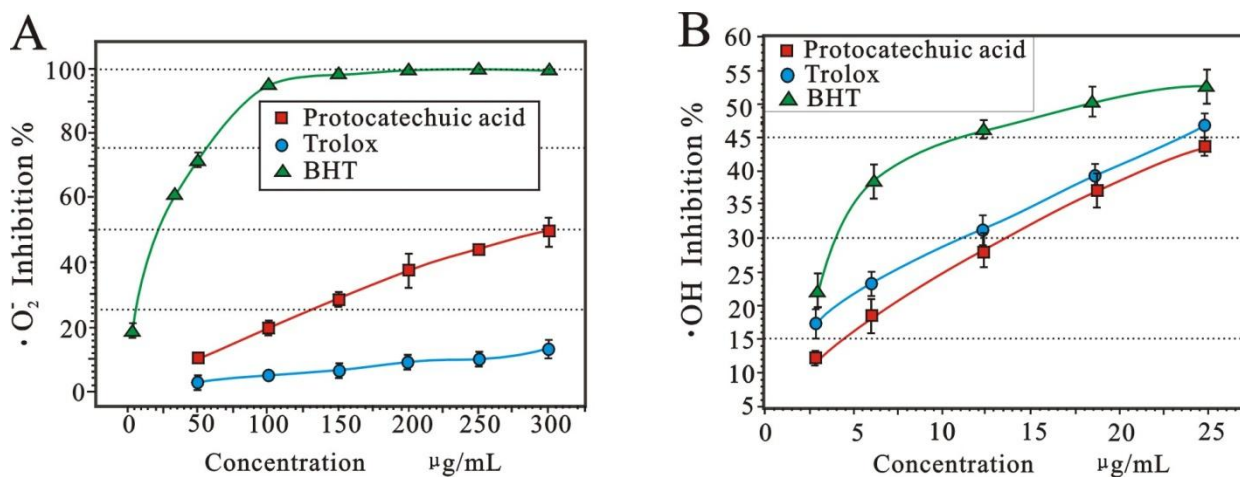

Figure 3. Superoxide anion (A) and hydroxyl (B) radical scavenging activity of protocatechuic acid, Trolox and BHT. Each value is expressed as mean ± standard deviation, n =3.

Metal ions chelating activity

Actually, hydroxyl radical can be generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + •OH + OH^-$), in which iron participates as a catalyst in body. The superoxide radical participates in the Haber–Weiss reaction (•$O_2^- + H_2O_2 \rightarrow O_2 + •OH + OH^-$) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + •O_2^- \rightarrow Fe^{2+}$

+ O_2). As ferrous ion and other transition metal ions (e.g. Cu^{2+}) can catalyze the oxidation in body, it is significant to investigate the metal chelating activity of an antioxidant.

The data in Fig. 4 revealed that the metal chelating activity of PCA and the positive controls were concentration-dependent. As can be seen in Fig. 4A, PCA showed the distinctly higher chelating activity on Fe^{2+} than Trolox and BHT, however, its chelating activity on Cu^{2+} is lower than sodium citrate which is regarded as a strong chelating agent in chemical industry (Fig. 4B). Generally, compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, $-PO_3H_2$, C=O, $-NR_2$, –S– and –O– in a favorable structure–function configuration will have chelating activity [12].
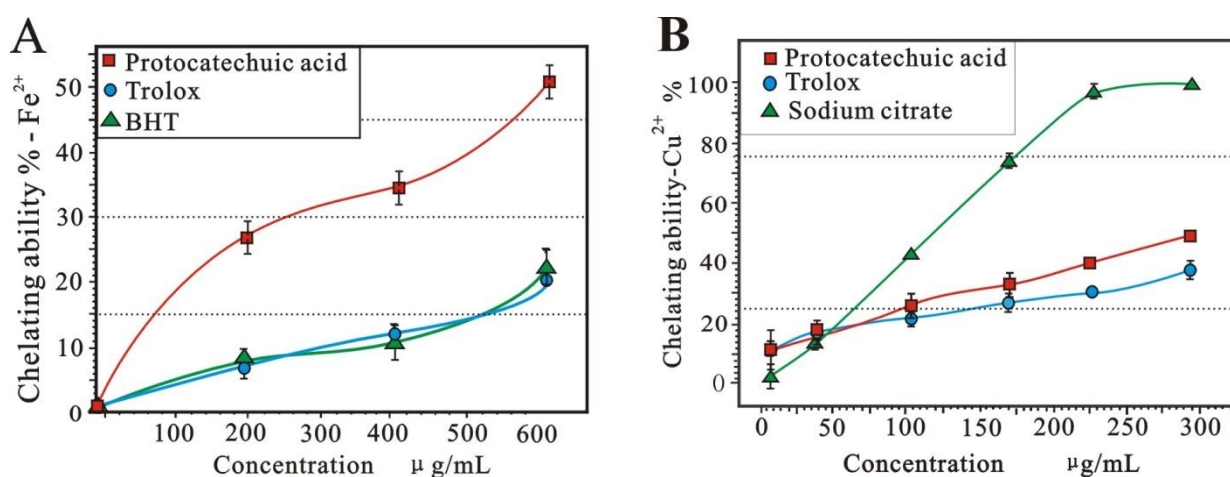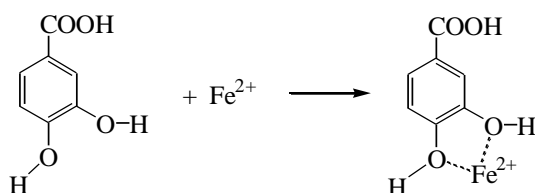

Figure 4. Metal chelating ability of protocatechuic acid, Trolox and BHT: (A) Fe^{2+}, (B) Cu^{2+}. Each value is expressed as mean ± standard deviation, n =3.

Hence, this metal chelating activity may arise from the ortho-dihydroxyl groups in PCA molecule. The proposed reaction of PCA binding for Fe^{2+} is given in the following:

Recently, Torreggiani et al. demonstrated that quercetin-chelated metal ions in the same way [13]. These results also suggested that PCA exhibits antioxidant activity maybe by both chelating metal transition ions as well as by scavenging free radicals. Undoubtedly, PCA's various pharmacological activities mentioned above are closely correlated with its antioxidant activities.

In the present study, DPPH, ABTS and chelating ability (Fe^{2+}) assays were conducted in organic solutions, while reducing power (Fe^{3+} & Cu^{2+}), superoxide anion and hydroxyl radical-scavenging, and chelating ability (Cu^{2+}) assays in aqueous solutions. Our results

showed that PCA exhibited dose-dependently significant antioxidant abilities in both lipid and aqueous mediums. Thus, PCA is regarded as an ideal natural antioxidant in both mediums.

Table 1 The values of IC_{50} (μg/mL)

| | DPPH | ABTS | Reducing power | | $\cdot O_2{}^-$ | $\cdot$ OH | Chelating | |
			Fe^{3+}	Cu^{2+}			Fe^{2+}	Cu^{2+}
PCA	1.88	0.89	5.15	2.25	310.28	74.11	566	312.34
Trolox	5.28	2.08	19.04	13.78	1289.30	70.51	1520	467.40
BHT	5.75	1.66	20.62	5.46 [a]	28.21	46.67	1440	118.53 [b]

[a] BHA   [b] Sodium citrate

To further evaluate the antioxidant property of PCA, the IC_{50} values were assessed in Table 1. The IC_{50} values of PCA were generally lower than of the positive controls, suggesting that PCA is a better antioxidant than the positive controls, as the lower IC_{50} value reflects the higher antioxidant activity. Among the positive controls, Trolox is considered as the most common standard antioxidant, and the relative antioxidant ability of PCA comparing to Trolox has therefore been defined as the ratio of $IC_{50\,(Trolox)}/IC_{50(PCA)}$. The ratio values were calculated as 2.8, 2.3, 3.7, 6.1, 4.2, 1.0, 2.7, 1.5, respectively for DPPH, ABTS, reducing power (Fe^{3+}), reducing power (Cu^{2+}), superoxide anion radical-scavenging, hydroxyl radical-scavenging, chelating ability (Fe^{2+}) and chelating ability (Cu^{2+}) assays (Fig. 5)
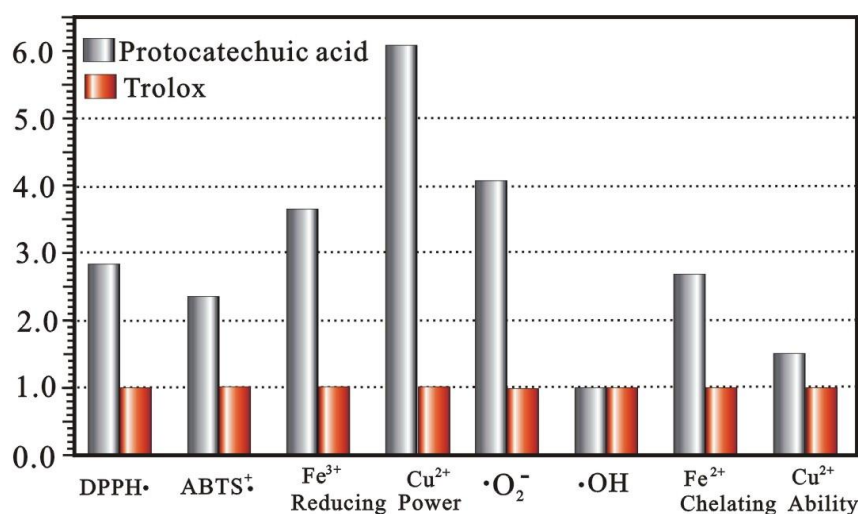

Figure 5.   Relative antioxidant abilities of PCA comparing to Trolox

Conclusion:
In conclusion, protocatechuic acid was proved to be much more effective than Trolox in the comprehensive evaluation of antioxidant activity *in vitro* in both lipid and aqueous mediums,

hence, it may therefore be used in pharmacological or food industry as natural antioxidant. It may exert antioxidant action *in vitro* by both chelating metal ions, and scavenging free radicals via donating hydrogen atom (H•) or electron (e).

Competing interests:

The authors declare that there are no competing interests.

Author's Contributions:

Xican Li is the principle investigator for this study providing oversight and contributed fundamental conceptualization for the research, writing a grant proposal and manuscript.

Xiaozhen Wang performed the experiments and provided statistical analysis.

Dongfeng Chen is the research coordinator for the study and assisted in designing the study.

Shuzhi Chen coordinated the research and assisted in performing the experiments.

All authors read and approved the final manuscript.

Abbreviations:

PCA : Protocatechuic acid; ABTS: 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); DPPH: 1,1-diphenyl-2-picryl-hydrazyl; ROS: reactive oxygen species; Trolox: (±)-6 -hydroxyl -2,5, 7, 8-tetramethlychromane-2-carboxylic acid; BHT: butylatedhydroxy-toluene; BHA: butylated hydroxyanisole.

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