NPC Natural Product Communications

2011
Vol. 6
No. 9
1285 - 1288

Evaluation of Antioxidant Activity of Isoferulic Acid *in vitro*

Xiaozhen Wang[a], Xican Li[a,*] and Dongfeng Chen[b]

[a]*School of Chinese Herbal Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China*
[b]*School of Basic Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China*

lixican@126.com

Received: April 1st, 2011; Accepted: June 4th, 2011

Isoferulic acid (3-hydroxy-4-methoxycinnamic acid, IFA), the isomer of ferulic acid (4-hydroxy-3-methoxycinnamic acid), is a rare phenolic acid occurring in *Rhizoma Cimicifugae*. Unlike ferulic acid, which has been well investigated, the antioxidant activity of IFA has not been measured. In this study, IFA was systematically evaluated for its *in vitro* antioxidant activity for the first time. IC_{50} values were calculated of 7.30±0.57, 4.58±0.17, 1.08±0.01, 8.84±0.43, 7.69±0.39, 1.57±0.2, 13.33±0.49 μg/mL, respectively, for lipid peroxidation, DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and ABTS (3-ethylbenzthiazoline-6-sulfonic acid diammonium salt) radical scavenging, reducing power on Fe^{3+} and Cu^{2+} ions, and hydroxyl and superoxide anion radical scavenging. Comparison with the IC_{50} values with those of the positive controls, Trolox and butylated hydroxyanisole (BHA), it can be concluded that isoferulic acid is an effective natural antioxidant in both lipid and aqueous media.

Keywords: Isoferulic acid, antioxidant activity, lipid peroxidation, radical scavenging, reducing power, *in vitro*.

Isoferulic acid (3-hydroxy-4-methoxycinnamic acid, IFA), the isomer of ferulic acid (4-hydroxy-3-methoxycinnamic acid), is a rare plant constituent. IFA was found in *Rhizoma Cimicifugae* and regarded as one of its active components. It is reported that IFA is active for the treatment of diabetes [1], coronary heart disease, angina pectoris, heart-stroke and cardiovascular disease [2]. However, according to free radical biology & medicine, all the diseases mentioned above have been related to reactive oxygen species (ROS) [3]. Most phenolic acids possess marked antioxidant activity [4], including the isomer of IFA, ferulic acid [5]. Hence, there is reason to believe that IFA may be an excellent antioxidant. However, unlike ferulic acid, which has been well investigated, the antioxidant activity of IFA has not yet been measured. Therefore, the aim of this paper was to evaluate the antioxidant effects of IFA *in vitro* in comparison with the standard antioxidants Trolox and BHA, including lipid peroxidation, DPPH• and $ABTS^{\bullet+}$ radical scavenging, reducing power on ferric and cupric ions, and hydroxyl and superoxide anion radical scavenging.

Lipid peroxidation consists of a series of free radical-mediated chain reaction processes and is associated with several types of biological damage. Our data revealed that IFA exhibited its antioxidant activity in the linoleic acid emulsion system in a concentration-dependent manner within the range 2.5-12.5 μg/mL (data not shown). In line with the IC_{50} values (IC_{50} value is the concentration of sample required to inhibit 50% of peroxidation) listed in Table 1, the anti-lipid peroxidation of IFA and positive controls decreased in the order BHA > Trolox > IFA.

Table 1 IC_{50} values (μg/mL)

Assays	IFA	Trolox	BHA
Anti-lipid peroxidation	7.30±0.57[a b]	2.09±0.09	1.95±0.06
DPPH	4.58±0.17[a b]	2.78±0.14	5.02±0.14
ABTS	1.08±0.01[a b]	1.62±0.03	0.93±0.03
Reducing power (Fe^{3+})	8.84±0.43[b]	8.51±0.41	6.21±0.35
Reducing power (Cu^{2+})	7.69±0.39[a b]	11.05±0.54	5.34±0.14
•OH	1.57±0.20[a b]	3.07±0.86	4.43±0.33
$•O_2^-$	13.33±0.49[a b]	153.9±10.41	58.19±1.03 *

* The positive control was GSH, instead of BHA.
All values were mean ± SD (n=3). Results were analyzed by ANOVA. Value with [a] significantly different from Trolox ($P < 0.01$); Value with [b] significantly different from BHA or GSH ($P < 0.01$).

DPPH and ABTS assays have been widely used to determine the free radical-scavenging activity of various pure compounds and extracts. Our data indicated that the free radical-scavenging activities of IFA and the positive controls were dose-dependent in both the DPPH and ABTS assays (data not shown). According to the IC_{50} values (Table 1), the scavenging ability on DPPH• decreased as follows: Trolox > IFA > BHA, while it decreased in the order of BHA > IFA > Trolox in the ABTS assay.

Although a reductant is not necessarily an antioxidant, an antioxidant is commonly a reductant [6]. The reducing power of a compound may therefore serve as a significant indicator of its potential antioxidant activity [7]. Our results showed that IFA and the positive controls responded in a concentration-dependent manner in both reducing power assays ($Fe^{3+} \rightarrow Fe^{2+}$ and $Cu^{2+} \rightarrow Cu^{+}$) (data not shown). The IC_{50} values listed in Table 1 indicate that

the relative reducing powers on Fe^{3+} were as follows: BHA > Trolox ≈ IFA, while their relative reducing powers on Cu^{2+} were: BHA > IFA > Trolox. Thus, IFA was demonstrated to be an effective reductant.

Hydroxyl radical (•OH) and superoxide anion ($•O_2^-$) are two of the most important free radicals in living cells. Therefore, it was important to investigate the •OH and $•O_2^-$ scavenging ability of IFA. On the basis of our data (not shown), the scavenging effects of IFA and the positive controls on •OH and $•O_2^-$ were concentration-dependent. The hydroxyl radical scavenging ability of IFA and the positive controls decreased as follows: IFA > Trolox > BHA. As for superoxide anion scavenging, the IC_{50} values (Table 1) indicated that IFA (IC_{50} 13.33 μg/mL) was much more effective than the positive controls (IC_{50} 153.90 and 58.19 μg/mL, respectively for Trolox and GSH).

In the seven antioxidant assays mentioned above, lipid peroxidation, DPPH, and ABTS assays were conducted in organic solutions, while reducing power (Fe^{3+} and Cu^{2+}), superoxide anion and hydroxyl radical scavenging assays utilized aqueous solutions. Obviously, IFA is an effective antioxidant in both lipid and aqueous mediums

In conclusion, comparison with the IC_{50} values of the positive controls, Trolox and butylated hydroxyanisole, isoferulic acid is an effective natural antioxidant *in vitro* in both lipid and aqueous mediums. Hence, it may, therefore, be used in the pharmacological and food industry as a natural antioxidant.

Experimental

Chemicals: IFA (CAS number: 537-73-5 > 98%) was obtained from Sigma Co. DPPH• (1,1-diphenyl-2-picrylhydrazyl radical), pyrogallol, linoleic acid, Trolox (±-6-hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid), neocuproine (2,9-dimethyl-1,10-phenanthroline), BHA (butylated hydroxyanisole), linoleic acid, and α-tocopherol were purchased from Sigma Co.; GSH (glutathione), 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 reagents were of analytical grade.

Lipid peroxidation: The effect of IFA on the prevention of peroxidation of linoleic acid emulsion was investigated using the thiocyanate method [8,9], with some modification. 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 30% ethanol (v/v); 0.1 mL of various concentrations of samples (50-250 μg/mL) were added to 1.5 mL of linoleic acid emulsion and 0.4 mL distilled water. The reaction mixtures (2 mL) were incubated at room temperature in glass bottles. The degree of oxidation was measured when the absorbance of the control reached its maximum. To 0.15 mL of sample solution, 3.65 mL of 75% ethanol (v/v), 0.1 mL of ammonium thiocyanate (30%, m/v), and 0.1 mL of ferrous chloride (0.02 mol/L in 3.6% HCl) were added. The peroxide value was measured by monitoring absorbance at 500 nm in a spectrophotometer (Unico 2100, Shanghai, China). The peroxides formed during linoleic acid peroxidation oxidize Fe^{2+} to Fe^{3+}, and Fe^{3+} forms a complex with thiocyanate that has a maximum absorbance at 500 nm. The solution without IFA was used as blank. Linoleic acid mixture without the addition of sample was used as control. The percentage of inhibition of lipid peroxidation in linoleic acid emulsion was calculated from the equation:

$$\text{Inhibition \%} = (1 - A_s/A_c) \times 100$$

Where A_s is the absorbance in the presence of IFA or positive controls, while A_c is the absorbance in the absence of IFA and positive controls.

DPPH• scavenging activity: DPPH• radical-scavenging activity was determined as previously described [10]. Briefly, 1 mL of DPPH• solution (0.1mmol/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 a 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 IFA or positive controls, while A_0 is the absorbance in the absence of IFA and positive controls.

ABTS•$^+$ scavenging activity: The scavenging activity of ABTS•$^+$ was measured by a previously described method [10]. 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 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 BHA.

Ferric ions (Fe^{3+}) reducing power: Ferric cyanide (Fe^{3+}) reducing power was determined by the method of Oyaizu *et al.* [11], as described by Li *et al*.[10]. Samples

(1 mg/mL, *x* mL, *x* = 5, 10, 15, 20, 25 μL) were mixed with Na_2HPO_4 / KH_2PO_4 buffer (350-*x* μL, 0.2 M, pH 6.6) and $K_3Fe(CN)_6$ (250 μL, 1 g/100 mL). The mixture was incubated at 50ºC for 20 min, before 250 μL of trichloroacetic acid (10 g/100 mL) was added, and the mixture centrifuged at 3000 *g* 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 started. The absorbance at 700 nm was measured at 90 s. Samples were analyzed in groups of 3, and when the analysis of one group was finished, the next group of 3 samples were mixed with $FeCl_3$ to avoid oxidization by air. Trolox and BHA were used as the positive controls, and an increased absorbance reading indicated increased reducing power. The percentage reducing power of the sample as compared with the maximum absorbance tested, which appeared in BHA at 13.9 μg/mL, was calculated by using the formula:

$(A_S / A_m) \times 100$. Here, A_m = maximum absorbance tested and A_S = absorbance of sample.

Cupric ions (Cu^{2+}) reducing power: In order to further measure the reducing ability of IFA, the cupric ions (Cu^{2+}) reducing power capacity was also determined [12], with slight modification Briefly, 125 μL $CuSO_4$ aqueous solution (10 mM), 125 μL neocuproine ethanolic solution (7.5 mM) and 500 μL CH_3COONH_4 buffer solution (100 mM, pH 7.0) were added to test tubes with different volumes of IFA (2-12 μL). Then, the total volume was adjusted with the buffer to 1 mL and mixed vigorously. Absorbance against a buffer blank was measured at 450 nm after 30 min. Increased absorbance of the reaction mixture indicates an increase of reduction capability. Trolox and BHA were used as the positive controls. The percentage reducing power of the sample as compared with the maximum absorbance tested, which appeared in BHA at 12 μg/mL, was calculated by using the formula: $(A_S / A_m) \times 100$. Here, A_m = maximum absorbance tested and A_S = absorbance of sample.

***Hydroxyl (•OH) radical-scavenging activity*:** The scavenging activity on the hydroxyl radical (•OH) was investigated by the deoxyribose method [13], with some modification. Our preliminary experiments demonstrated that almost all organic solvents except for light petroleum can promote the inhibition percentage value, including ethanol, methanol, acetone, ethyl acetate, acetonitrile, chloroform, diethyl ether, DMF, DMSO, and THF. Hence, the inhibition of the hydroxyl radical was evaluated as follows: all test samples were first dissolved in methanol (3 mg/mL), and 0.9-5.4 μL sample solution was taken into mini tubes, and the methanol then removed at 80ºC to eliminate its interference. The reactions were performed in 0.2 M phosphate buffer (pH 7.4), containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 μM $FeCl_3$, 80 μM Na_2EDTA, and the test sample (2.7-16.2 μg). The reaction was started by adding ascorbic acid to a final concentration of 100 μM, and the reaction mixture (800 μL in total) was incubated for 20 min at 50ºC 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 an oven at 100ºC for 15 min. The sample was cooled and the absorbance was measured at 532 nm against buffer (as blank). The reaction mixture not containing test sample was used as control. The scavenging activity on hydroxyl radicals was expressed as:

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

Where A_c is the absorbance at 532 nm of control (without sample), and A is the absorbance at 532 nm of the reaction mixture containing sample.

***Superoxide anion ($\bullet O_2^-$) radical-scavenging activity*:** Measurement of superoxide anion scavenging activity of IFA was based on the pyrogallol autoxidation method [14], as described by Li *et al.* [10]. Briefly, a 1 mg/mL sample solution (*x* μL, where *x* = 0, 20, 40, 60, 80, or 100 μL) was mixed with Tris-HCl buffer (2920 - *x* μL, 0.05 M, pH 8.2) containing EDTA (1 mM) and pyrogallol (80 μL, 6 mM), 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 as blank every 30 s for 5 min. Trolox and GSH were used as the positive controls. The slope of the correlation of absorbance with time was calculated. The reaction mixture without sample was used as the control. The $\bullet O_2^-$ scavenging ability was calculated as:

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

Statistical analysis: Results are reported as the mean ±SD of 3 measurements, the IC_{50} values were calculated by linear regression analysis and one-way analysis of variance (ANOVA) were performed for comparison between groups. A *P* value of less than 0.01 was considered significant. All linear regression in this paper was analyzed by Origin 6.0 professional software.

Acknowledgments - This work was supported by National Nature Science Foundation of China (30371837, 30772861).

References

[1] Liu IM, Chen WC, Cheng JT. (**2003**) Mediation of β-endorphin by isoferulic acid to lower plasma glucose in streptozotocin induced diabetic rats. *Journal of Pharmacology and Experimental Therapeutics*, ***307***, 1196–1204.

[2] Guo XR, Chen XH, Cheng WM, Yang KY, Ma YF, Bi KS. (**2008**) RP-LC determination and pharmacokinetic study of ferulic acid and isoferulic acid in rat plasma after taking Traditional Chinese Medicinal-preparation: Guanxinning Lyophilizer. *Chromatographia*, ***67***, 1007–1011.

[3] Zheng RL, Huang ZY. (**2007**) *Free Radical Biology,* 3rd Ed., Higher Education Press, Beijing, China, p.7.
[4] Sroka Z, Cisowski W. (**2003**) Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids, *Food and Chemical Toxicology*, ***41***, 753-758.
[5] Ernst G. (**1992**) Antioxidant potential of ferulic acid. *Free Radical Biology and Medicine*, ***13***, 435-448.
[6] Prior RL, Cao GH. (**1999**) *In vivo* total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology and Medicine*, ***27***, 1173-1181.
[7] Jung MJ, Heo SI, Wang MH. (**2008**) Free radical scavenging and total phenolic contents from methanolic extracts of *Ulmus davidiana*. *Food Chemistry*, ***108***, 482-487.
[8] Mitsuda H, Yasumoto K, Iwami K. (**1966**) Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo to Shokuryo*, ***19***, 210-214.
[9] Yen GC, Hsieh CL. (**1998**) Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models *in vitro*. *Journal of Agricultural and Food Chemistry*, ***46***, 3952-3957.
[10] Li X, Wu X, Huang L. (**2009**) Correlation between antioxidant activities and phenolic contents of Radix Angelica Sinensis (Danggui). *Molecules*, ***14***, 5349-5361.
[11] Oyaizu M. (**1986**) Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition*, ***44***, 307-315.
[12] Gülçin İ. (**2010**) Antioxidant properties of resveratrol: A structure-activity insight. *Innovative Food Science and Emerging Technologies*, ***1***, 210-218.
[13] Kitts DD, Arosha, NW, Chun H. (**2000**) Antioxidant properties of a North American ginseng extract. *Molecular and Cellular Biochemistry*, ***203***, 1-10.
[14] Marklund S, Marklund G. (**1974**) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and convenient assay for superoxide dismutase. *European Journal of Biochemistry*, ***47***, 469-474.