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Antioxidant activities of citrus herbal product extracts

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ABSTRACT

Total phenolic content, DPPH free radical-scavenging activity, hydrogen peroxide-scavenging activity, ferrous ion-chelating activity and ferric-reducing antioxidant power (FRAP) of four citrus herbal products, *Citri Reticulatae Pericarpium* (CRP), *Citri Reticulatae Viride Pericarpium* (CRVP), *Aurantii Immaturus Fructus* (AIF) and *Aurantii Fructus* (AF) extracts were determined. EC_{50} values of DPPH radical-scavenging activities ranged from 0.1 mg/ml (AF) to 1.59 mg/ml (AIF). EC_{50} values of hydrogen peroxide-scavenging activities ranged from 0.08 mg/ml (AF) to 0.9 mg/ml (CRP). EC_{50} values of ferrous ion-chelating activities ranged from 0.8 mg/ml (AF) to 2.08 mg/ml (AIF). The differences in DPPH free radical-scavenging activity, hydrogen peroxide-scavenging activity, and ferrous ion-chelating activity of all citrus herbal product extracts were significant. AF had the highest antioxidant activity. In this study, citrus herbal product extracts did not have good reducing power.

1. Introduction

In biological systems, lipid oxidation can produce toxic compounds and initiate other harmful reactions. Phenolic compounds can act as antioxidants by many potential pathways such as free radical-scavenging, oxygen radical absorbance, and chelating of metal ions (Halliwell, Aeschbach, Loliger, & Aruoma, 1995). Interest in traditional Chinese medicines has grown in recent years as they are typically low in toxicity, rarely produce complications and have beneficial pharmacological activities (Endo & Nakamura, 1993). Citrus fruits, such as *Citri Reticulatae Pericarpium* (Chinese name, "Chen-Pi", *Citrus reticulata* Blanco peels), *Citri Reticulatae Viride Pericarpium* ("Qing-Pi", *C. reticulata* Blanco immature peels), *Aurantii Immaturus Fructus* ("Zhi-Shi", *Citrus aurantium* L. and *Citrus sinensis* Osbeck peels), *Aurantii Fructus* ("Zhi-Ke", *C. aurantium* L., *Citrus wilsonii* Tanaka, *C. aurantium* "Tangcheng", *C. aurantium* "Chuluan", *C. aurantium* "daidai" and *C. aurantium* "Huangpi" peels), are an important group in Chinese crude drugs and usually listed in various prescriptions (Dan & Andrew, 1986). According to Dan and Andrew (1986), the liver is responsible for the smooth flow of Qi (energy) throughout the body and smoothing our emotions. Anger, irritability, and frustration are all signs that our Qi is not flowing smoothly. Citrus herbal products, prepared from mature or immature peels of citrus fruits, have been traditionally used to promote the flow of liver Qi (Dan & Andrew, 1986). They were also used to alleviate the pain in chest, breast, and hypochondrial region. Citrus herbal products could also reduce Qi-accumulations, such as food stagnation, with pain and distention symptoms (Dan & Andrew, 1986). Furthermore, as described in traditional Chinese medical literature, they are also utilised to dry dampness and transform phlegm.

Phenolic compounds are secondary metabolites of plants. They are naturally present in fruits and vegetables. These compounds are a part of the everyday diet and also used as medicines or supplements. Research has shown that fruits and vegetables contain other antioxidant nutrients, in addition to vitamins C and E, and carotenoids, which significantly contribute to their total antioxidant capacity (Cao, Sofic, & Prior, 1996; Wang, Cao, & Prior, 1996). The major part of those antioxidant nutrients is polyphenolic compounds, which are components of fruits and vegetables having strong antioxidant capacity (Cao, Sofic, & Prior, 1997; Wang, Cao, & Prior, 1997). Citrus plants are rich in naturally-occurring flavonoids, which are primarily found in peel. Flavonoids have a wide range of biological activities, such as cell-proliferation-inhibiting, apoptosis-inducing, enzyme-inhibiting, antibacterial, and antioxidant effects (Cook & Samman, 1996; Havsteen, 2002; Middleton & Kandaswami, 1993). Moreover, some findings indicate that flavonoids possess various clinical properties, such as antiatherosclerotic, antiinflammatory, antitumour, antithrombogenic, antiosteoporotic, and antiviral effects (Cook & Samman, 1996; Havsteen, 2002). In order to determine the antioxidant activities

Abbreviations: CRP, *Citri Reticulatae Pericarpium*; CRVP, *Citri Reticulatae Viride Pericarpium*; AIF, *Aurantii Immaturus Fructus*; AF, *Aurantii Fructus*.

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of citrus herbal products and evaluate their potential use as antioxidant additives in foods or cosmetics, we have examined the antioxidant activities of four common used citrus herbal products, *Citri Reticulatae Pericarpium* (CRP), *Citri Reticulatae Viride Pericarpium* (CRVP), *Aurantii Immaturus Fructus* (AIF) and *Aurantii Fructus* (AF), extracts.

2. Materials and methods

2.1. Preparation of citrus herbal product extract

Powders of citrus herbal products, *Citri Reticulatae Pericarpium* (CRP, No. 6117), *Citri Reticulatae Viride Pericarpium* (CRVP, No. 5812), *Aurantii Immaturus Fructus* (AIF, No. 5929) and *Aurantii Fructus* (AF, No. 5930), were purchased from Sun Ten Pharmaceutical Co. (Taipei, Taiwan). Samples were authenticated by the College of Chinese Medicine, China Medical University. The citrus herbal product extract was prepared according to the method of Rehman (2006). Each citrus herbal product powder (10 g) was extracted with 100 ml of methanol overnight in a shaker at room temperature. The extract was centrifuged at 3500 rpm (DuPont, model Sorvall RC-5C) for 20 min to obtain the supernatant and the residue was re-extracted under the same conditions. The combined filtrate was filtered through 0.45 μm of filter membrane and evaporated with a rotary evaporator (Buchi R-124, Flavil, Sweden) below 50 °C. After evaporation of organic solvent, the extract was used for further analysis.

2.2. Determination total phenolic content

The Folin–Ciocalteu reagent assay was used to determine the total phenolic content (Kujala, Loponen, Klika, & Pihlaja., 2000). A 0.2 ml aliquot of the extract in methanol (1.0 mg/ml) was mixed with 0.5 ml of Folin–Ciocalteu reagent. The solution was allowed to stand for 3 min at 25 °C before adding 0.2 ml of saturated sodium carbonate solution. The mixture was allowed to stand for another 2 h before the absorbance at 725 nm was measured. Gallic acid was used as standard for the calibration curve. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of sample (mg/g).

2.3. Scavenging of DPPH radical

The effect of citrus herbal product extracts on DPPH radicals was studied using the modified method of Shimada, Fujikawa, Yahara, and Nakamura (1992). Briefly, 100 μM of DPPH in methanol was prepared and 1.0 ml of this solution was added to a test sample (4.0 ml). The reaction mixture was shaken well and incubated for 30 min at room temperature. The absorbance of the resulting solution was read at 517 nm against a blank. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging activity}(\%) = \left(1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}\right) \times 100.$$

EC_{50} value (mg/ml) is the concentration at which the scavenging activity was 50%.

2.4. Hydrogen peroxide-scavenging assay

Hydrogen peroxide-scavenging activity was measured using the modified method of Yen and Chung (1999). Briefly, 1 ml of sample was first mixed with 400 μl of 4 mM H_2O_2 solution, incubated for 20 min at room temperature, and then 600 μl of horse radish peroxidase (HRPase) – phenol red solution (HRPase 300 μg/ml and phenol red 4.5 mM in 100 mM phosphate buffer) were added. After another 10 min of incubation and 10 min on ice to stop the reaction, the absorbance was measured at 610 nm, using an automated microplate reader. The scavenging effect was then calculated according to the following equation:

$$\text{Scavenging activity}(\%) = \left(1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}\right) \times 100.$$

EC_{50} value (mg/ml) is the concentration at which the scavenging activity was 50%.

2.5. Metal ion-chelating assay

The ferrous ion-chelating activity of citrus herbal products extracts was measured according to the method of Yen and Chung (1999). The absorbance of the ferrous iron–ferrozine complex at 562 nm was measured to determine the Fe^{2+}-chelating ability of the extract. Briefly, the reaction mixture, containing citrus herbal product extract in methanol (0.1–1.0 mg/ml), $FeCl_2$ (2 mM), and ferrozine (5 mM), was adjusted to 5 ml with water. The mixture was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank. EDTA was used as positive control.

The ability of extracts to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating activity}(\%) = \left(1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}\right) \times 100.$$

EC_{50} value (mg/ml) is the concentration at which the chelating activity was 50%.

2.6. Preparation of citrus herbal product extract

The FRAP assay was carried out, using a modified version of the method of Benzie and Szeto (1996). A 10 μl aliquot of the sample was mixed with 900 μl of FRAP reagent and 90 μl deionised water. The deionised water was used as control. The absorbance reading at 539 nm was taken after standing for 30 min at 37 °C. A higher absorbance reading indicated a higher reducing power. EC_{50} value (mg/ml) is the concentration at which the absorbance was 1.0.

2.7. Statistical analysis

In this study, three analyses of each sample were made and each experiment was carried out in triplicate ($n = 3$). The mean value and standard deviation were calculated from the data obtained. These data were then compared by the Duncan's multiple range method using SAS Institute Inc., Cary, NC; (2001).

3. Results and discussion

3.1. Total phenolic content

The total phenolic contents of CRVP, CRP, AF, and AIF were 44.6 ± 3.12, 38.8 ± 3.32, 28.8 ± 1.11, and 9.62 ± 0.84, respectively (Table 1). The differences in total phenolic content of all samples were significant ($p \leqslant 0.05$). The CRVP had the highest total phenolic content, followed by CRP, AF, and AIF. The difference between CRVP and AIF was almost five-fold.

3.2. Scavenging of DPPH radical

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples

Table 1
Total phenolic contents and EC_{50} values of CRP, CRVP, AF, AIF, BHT, ascorbic acid, and EDTA in antioxidant properties

Activity Sample	DPPH scavenging (mg/ml)	Hydrogen peroxide (mg/ml)	Ferrous ion-chelating (mg/ml)	FRAP (mg/ml)	Total phenolic (mg of GAE/g)
CRP	0.78 ± 0.04^{b}	0.90 ± 0.02^{a}	1.89 ± 0.10^{b}	0.25 ± 0.02^{a}	38.80 ± 3.35^{b}
CRVP	0.46 ± 0.01^{c}	0.52 ± 0.04^{c}	1.24 ± 0.09^{c}	0.24 ± 0.02^{a}	44.60 ± 3.15^{a}
AF	0.10 ± 0.01^{d}	0.08 ± 0.02^{e}	0.80 ± 0.07^{d}	0.12 ± 0.01^{b}	28.90 ± 1.20^{c}
AIF	1.59 ± 0.39^{a}	0.79 ± 0.06^{b}	2.08 ± 0.08^{a}	0.24 ± 0.02^{a}	9.60 ± 1.84^{d}
BHT	0.09 ± 0.01^{d}	–	–	–	–
Ascorbic acid	–	0.15 ± 0.03^{d}	–	–	–
EDTA	–	–	0.10 ± 0.01^{e}	–	–
Gallic acid	–	–	–	n.a.[A]	–

Within the same column, means followed by different letters are significantly different at $P < 0.05$.
[A] n.a., data is not available.

(Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Hatano, Kagawa, Yasuhara, & Okuda, 1988). DPPH decreases significantly upon exposure to proton radical scavengers (Yamaguchi, Takamura, Matoba, & Terao, 1998). The DPPH free radical-scavenging activities of citrus herbal product extracts and BHT are presented in Fig. 1. For each extract, different concentrations (0.1–1.0 mg/ml) were prepared. The activities of sample extracts were between 30% and 49% at 0.1 mg/ml and between 43% and 78% at 1.0 mg/ml. In this study, BHT showed a high radical-scavenging ability. At 0.1–1.0 mg/ml, the radical-scavenging activities of BHT were between 49% and 87%. With regard to EC_{50} values, BHT ($EC_{50} = 0.09 \pm 0.01$) and AF ($EC_{50} = 0.10 \pm 0.01$) had the highest radical-scavenging abilities, whereas AIF ($EC_{50} = 1.59 \pm 0.09$) had the lowest radical-scavenging ability. The difference was almost sixteen-fold. The differences in EC_{50} values of all four sample extracts were significant ($p \leqslant 0.05$). However, BHT and AF were not significantly different (Table 1). Correlation between EC_{50} values of radical-scavenging ability and total phenolic contents of all samples in this study was good and significant ($r = -0.70$, $p \leqslant 0.05$). According to some studies, free radical-scavenging activity depends on the structural conformation of phenolic compounds (Bors, Heller, Michel, & Saran, 1990; Bors, Michel, & Stettmaier, 1997; Larrauri, Ruperez, & Calixto, 1996). Thus, free radical-scavenging activity is greatly influenced by the phenolic composition of the sample.

3.3. Hydrogen peroxide-scavenging assay

Biological systems can produce hydrogen peroxide. Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1988). The effects of citrus herbal product extracts and ascorbic acid on hydrogen peroxide-scavenging activities are presented in Fig. 2. For each extract, different concentrations (0.1–1.0 mg/ml) were prepared. At a concentration of 1.0 mg/ml, ascorbic acid, CRP, CRVP, AF and AIF exhibited 83.8 ± 2.01%, 51.3 ± 2.27%, 63.6 ± 2.42%, 77.3 ± 0.92% and 54.0 ± 3.0% scavenging activities toward hydrogen peroxide, respectively. With regard to EC_{50} values of hydrogen peroxide-scavenging ability, AF ($EC_{50} = 0.08 \pm 0.01$) had the highest radical-scavenging ability, whereas CRP ($EC_{50} = 0.90 \pm 0.02$) had the lowest radical-scavenging ability (Table 1). The difference was almost twelve-fold. The differences in EC_{50} values of ascorbic acid and all four sample extracts were significant ($p \leqslant 0.05$). Correlation between EC_{50} values of hydrogen peroxide-scavenging ability and total phenolic contents of all samples in this study was low and insignificant ($r = -0.11$). According to the results, CRP had the second highest amount of total phenolic content. However, it had the lowest hydrogen peroxide-scavenging effect. This indicates that the components with high hydrogen peroxide-scavenging ability were not present in CRP extract.

3.4. Metal ion-chelating assay

EDTA exhibited an excellent ferrous ion-chelating capacity of approximately 88.7% at a concentration of 1.0 mg/ml. For each extract, different concentrations (0.1–1.0 mg/ml) were prepared. At a concentration of 1.0 mg/ml, CRP, CRVP, AF and AIF had 37.9 ± 1.64%, 44.2 ± 3.37%, 52.3 ± 1.53%, and 40.3 ± 1.53%

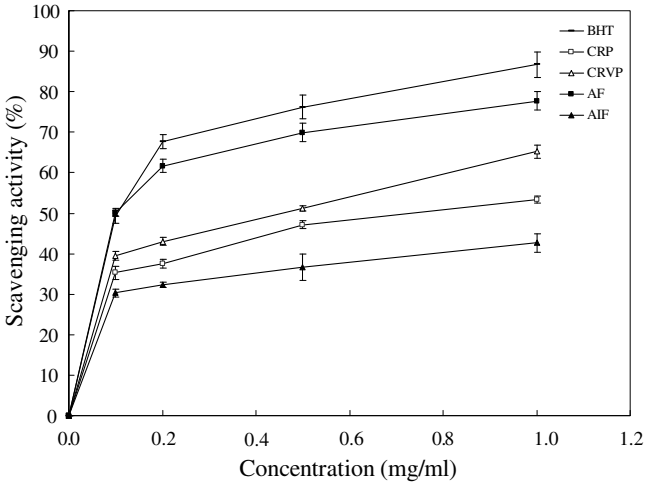

Fig. 1. DPPH free radical-scavenging activities of BHT and citrus herbal product extracts.

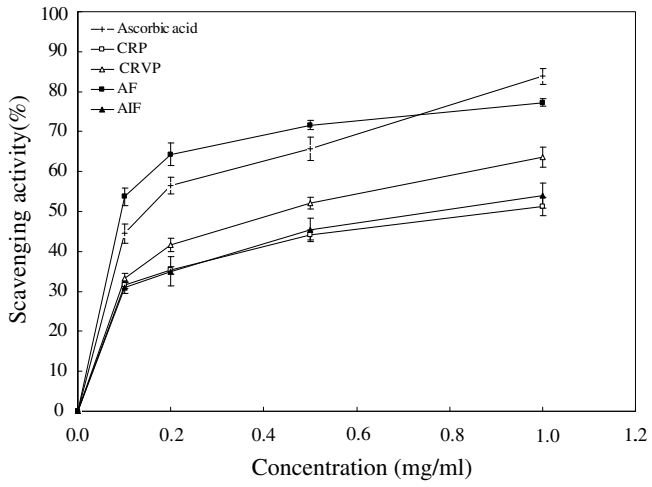

Fig. 2. Hydrogen peroxide-scavenging activities of ascorbic acid and citrus herbal product extracts.

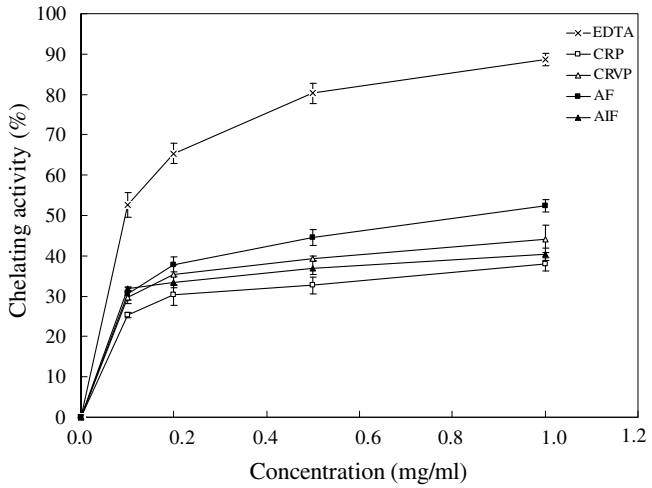

Fig. 3. Ferrous ion-chelating activities of EDTA and citrus herbal product extracts.

ion-chelating activities, respectively (Fig. 3). With regard to EC_{50} values of ferrous ion-chelating capacity, they were the same as DPPH free radical-scavenging and hydrogen peroxide-scavenging assays; AF (EC_{50} = 0.80 ± 0.07) had the highest ferrous ion-chelating ability. However, AIF (EC_{50} = 2.08 ± 0.08) had the lowest ferrous ion-chelating ability (Table 1). The differences in EC_{50} values of EDTA and all four sample extracts were significant ($p \leqslant 0.05$). Correlation between EC_{50} values of ferrous ion-chelating ability and total phenolic contents of all samples in this study was moderate to low, but significant ($r = -0.42$, $p \leqslant 0.05$). According to the results, the total phenolic content of AF was lower than that of CRP. However, the ferrous ion-chelating effect of AF was significantly higher than that of CRP. Factors affecting the ion-chelating ability of citrus herbal product extracts are complex. The main mechanism of ion-chelating activity is the ability to deactivate and/or chelate transition metals which can promote the Fenton reaction and hydroperoxide decomposition.

3.5. Ferric-reducing antioxidant power (FRAP) assay

The antioxidant activities of herb extracts using FRAP assay are shown in Fig. 4. For each extract, different concentrations (0.01–0.3 mg/ml) were prepared. The reducing power of herb extracts increased with concentration (0–0.3 mg/ml). At a concentration of 0.3 mg/ml, reducing powers of CRP, CRVP, AF and AIF were 1.11, 1.09, 2.12, and 1.01, respectively (Fig. 4). AF had the lowest EC_{50} value of reducing power (EC_{50} = 0.12 ± 0.01) among the four herb extracts tested whereas CRP (EC_{50} = 0.25 ± 0.02), CRVP (EC_{50} = 0.24 ± 0.02), and AIF (EC_{50} = 0.24 ± 0.02) had higher EC_{50} values. The differences in EC_{50} values of CRP, CRVP, and AIF extracts were not significant (Table 1). The difference between EC_{50} values of AF extract and any other sample extract was two-fold. In this study, correlation between EC_{50} values of reducing power and total phenolic contents of all samples tested in this study was low and not significant (r = 0.04). This indicates that citrus herbal product extracts and their main phenolic components might not have good reducing power.

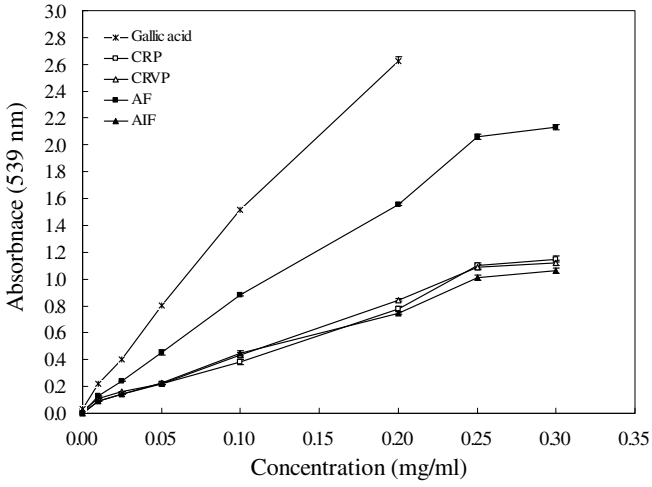

Fig. 4. Ferric-reducing antioxidant power (FRAP) of gallic acid and citrus herbal product extracts.

4. Conclusion

In this study, antioxidant activities of four citrus herbal products were medium to high. However, they did not have good reducing power. EC_{50} values of DPPH radical-scavenging activities ranged from 0.10 mg/ml (AF) to 1.59 mg/ml (AIF). The difference was almost sixteen-folds. EC_{50} values of hydrogen peroxide-scavenging activities ranged from 0.08 mg/ml (AF) to 0.90 mg/ml (CRP). The difference was almost twelve-fold. The differences in three antioxidant activities of all citrus herbal product extracts were significant. The AF had the highest radical-scavenging activity, hydrogen peroxide-scavenging activity, and ferrous ion-chelating activity. However, it did not have the highest total phenolic content. Therefore, other antioxidant compounds in the citrus herbal product extracts should be studied further.

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