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Antioxidant properties of Cortex Fraxini and its simple coumarins

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Abstract

We present the antioxidant properties of crude extract, fractions and ingredients of Cortex Fraxini (CF) and compare the antioxidant capacities of coumarin ingredients of CF and known antioxidants, including catechin, quercetin, ascorbic acid, trolox and BHT. The IC_{50} values for CF in the DPPH and TEAC methods were 406 and 39.3 μg/ml, respectively. Among all fractions the chloroform fraction is the most active fraction in scavenging DPPH, ABTS and hydroxyl radicals, and there is a significant relationship between the antioxidant activities and the contents of the antioxidant phenolic ingredients. The contents of esculetin and fraxetin in the chloroform fraction were 8.44% and 11.1%, respectively. Esculetin and fraxetin also had good radical-scavenging capacities, and esculetin was the best, among all test compounds, against the DPPH radical. Moreover, esculetin and fraxetin had selective scavenging activity upon hydroxyl radicals and hydrogen peroxide, and this potency was better than known antioxidants and equal to quercetin in scavenging hydrogen peroxide. These results show that CF, partitioned with chloroform, gave a phenolic coumarin-enriched fraction, and that this fraction had the best free radical-scavenging activity and inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation, mainly due to its reducing power.

Keywords: Cortex Fraxini; Coumarins; Antioxidant activity; DPPH method; TEAC assay; Active oxygen scavenging capacity

1. Introduction

Reactive oxygen species (ROS), major free radicals generated in many redox processes (McDermott, 2000), often induce oxidative damage to biomolecules, such as carbohydrates, proteins, lipids and DNA. Biomolecule degeneration, followed by the initiation and propagation of oxidative chain reactions, causes accelerated aging and many chronic diseases, including neurodegenerative diseases, cancer, cardiovascular diseases and inflammation (Castro & Freeman, 2001). Thus, some researchers suggest that antioxidant compounds prevent neurodegenerative disorders by scavenging free radicals and delaying or preventing oxidation of the above biomolecules (Jackson et al., 2002). Plants are potential sources of natural antioxidants because they contain phenolic ingredients, such as phenolic acids, flavonoids, tannins and phenolic diterpenes (Shahidi, 1997). In addition, some natural coumarins also affect the formation and scavenging of ROS and influence free radical-mediated oxidative damage (Fylaktakidou, Hadjipavlou-Litina, Litinas, & Nicolaides, 2004). Numerous studies reveal that these natural antioxidants possess multiple pharmacological activities, including neuroprotective, anticancer, antimutagenic and anti-inflammatory activities, and that these activities may be related to their antioxidant properties (Borges, Roleira, Milhazes, Santana, & Uriarte, 2005; Stanczyk, Gromadzinska, & Wasowicz, 2005).

Fraxinus species have been used as anti-inflammatory drugs, possibly due to several phenolic acids and

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coumarins that possess free radical-scavenging activities (von Kruedener, Schneider, & Elstner, 1996). Cortex Fraxini (CF) is a widely and traditionally used herb in Taiwan. This herb also has anti-inflammatory properties and its ingredients include coumarins, such as esculin, esculetin, fraxin and fraxetin (Liu, Sun, Sun, & Cui, 2005). Therefore, the present investigation evaluates the antioxidant properties of CF crude extract and fractions by free radical-scavenging assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical and hydroxyl radical, generated in Fenton reactions. Then, we further assayed the relationship between the antioxidant capacity and ingredients, including total phenolics, flavonoids and coumarins. Finally, we compared the antioxidant capacity of these coumarins with known antioxidants, such as catechin, quercetin and trolox.

2. Materials and methods

2.1. Preparation of herb extracts and fractionation

CF (6 kg), authenticated by Dr. Chi-Rei Wu, was purchased from a herb supplier in Taiwan and extracted five times with 50% ethanol (5:1). The resultant extract was combined and concentrated under reduced pressure to obtain 1400 g of the residue (23.3%). The crude extract was suspended in water and then partitioned successively with four volumes of chloroform, ethyl acetate and *n*-butanol (1:1). Each fraction was evaporated in vacuum to obtain chloroform (96 g, 6.86%), ethyl acetate (129 g, 9.21%), *n*-butanol (255 g, 18.21%) and water fractions (772.2 g, 55.16%), respectively.

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethy-chroman-2-carboxylic acid (trolox), ascorbic acid, butylated hydroxytoluene (BHT), (+)-catechin, esculetin standard, esculin standard, quercetin and trichloroacetic acid (TCA) were purchased from Sigma–Aldrich Chem. Corp. (St. Louis, MO). Fraxetin standard was purchased from ChromDex Inc (St. Santa Ana, CA). Fraxin standard was purchased from Extrasynthese (France). All solvents were of HPLC grade and purchased from Merck.

2.3. Determination of antioxidant and radical scavenging activities

2.3.1. Radical-scavenging activity

The scavenging capacity of DPPH free radical was monitored according to the methods of Wu et al. (2005), modifying this to a 96-well microtitre spectrophotometric method. Twenty-five microlitres of different sample concentrations were pipetted into each well and then 175 μl of 300 μM DPPH methanolic solution were added. The mixture was shaken for 20 s and then left to stand at room temperature for 30 min. The absorbance of the resulting solution was read spectrophotometrically at 517 nm. The inhibition percentage (%) of radical-scavenging capacity was calculated as

$$\text{Inhibition percentage (\%)} = ((A_{\text{DPPH}} - A_{\text{blank}}) - (A_{\text{s-DPPH}} - A_{\text{s-blank}}))/(A_{\text{DPPH}} - A_{\text{blank}}) \times 100,$$

where A_{DPPH} is the absorbance of only DPPH solution, A_{blank} is the absorbance of methanol instead of DPPH solution, $A_{\text{s-DPPH}}$ is the absorbance of DPPH solution in the presence of sample and $A_{\text{s-blank}}$ is the absorbance of methanol in the presence of sample. The results are expressed as mmol catechin equivalents/g of sample.

2.3.2. Trolox equivalent antioxidant capacity (TEAC)

TEAC was measured by using ABTS radical-scavenging assay, following a modified method of Re et al. (1999). ABTS radical was prepared by reaction of 8 mM aqueous ABTS solution and 8.4 mM potassium persulfate solution at a ratio of 2:1. After storage in the dark at RT for 12–16 h, the radical solution was further diluted in ethanol until the initial absorbance value of 0.70 ± 0.05 at 734 nm was reached. One hundred and seventy-five microlitres of diluted ABTS solution was mixed with 25 μl of different sample concentrations. The inhibition percentage (%) of radical-scavenging capacity was calculated as

$$\text{Inhibition percentage (\%)} = ((A_{\text{ABTS}} - A_{\text{blank}}) - (A_{\text{s-ABTS}} - A_{\text{s-blank}}))/(A_{\text{ABTS}} - A_{\text{blank}}) \times 100,$$

where A_{ABTS} is the absorbance of only ABTS solution, A_{blank} is the absorbance of ethanol instead of ABTS solution, $A_{\text{s-ABTS}}$ is the absorbance of ABTS solution in the presence of sample and $A_{\text{s-blank}}$ is the absorbance of ethanol in the presence of sample. The results were expressed as TEAC values.

2.3.3. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activity was monitored using the 2-deoxyribose method of Halliwell, Gutteridge, and Aruoma (1987). The sugar deoxyribose is degraded by exposure to hydroxyl radicals generated by Fenton systems when mixed with ascorbic acid, hydrogen peroxide and Fe^{2+}–EDTA. Fragments of deoxyribose may be detected by their reaction with TBA to form a pink chromogen under heated acid conditions. Briefly, the assay mixture contained 2.8 mM 2-deoxyribose, 20 μM ferrous ion solution, 100 μM EDTA and different sample concentrations in a total volume of 1 ml of 10 mM potassium phosphate buffer (pH 7.4). All components were dissolved in 10 mM phosphate buffer (pH 7.4). The ferrous iron solution and EDTA were premixed before they were added to the assay mixture. The reaction was initiated by the addition of a mixture of 1.42 μM H_2O_2 and 100 μM ascorbate. The mixture was incubated at 37 °C for 30 min. At the end of the incubation period, 1 ml of 1% (w/v) TBA in 50 mM

sodium hydroxide and 1 ml of 2.8% (w/v) TCA were added and the mixture was heated for 30 min in a boiling water bath, cooled and the absorbance at 532 nm was measured, which corresponds to the deoxyribose damage. The reciprocal absorption values obtained for different concentrations were plotted against the concentrations of all samples and, from the graph, the second order rate constants were calculated as described by Halliwell et al. (1987) assuming that 2-deoxyribose reacts with hydroxyl radicals at a constant rate of $3 \times 10^9\ M^{-1}\ s^{-1}$.

2.3.4. Hydrogen peroxide-scavenging activity

The reaction mixture contained 50 μl of 25 mM phosphate buffer (pH 7.4), 50 μl of 0.5 mM H_2O_2 and 50 μl of different sample concentrations which were dissolved in 25 mM phosphate buffer (pH 7.4). After incubation for 5 min at room temperature, 25 μl of 5 mM homovanillic acid and 25 μl of 8 U/ml horseradish peroxidase were added, mixed and incubated for 25 min at room temperature. The fluorescence intensity was measured at an excitation of 315 nm and an emission of 425 nm (Pazdzioch-Czochra & Widenska, 2002). Absorbance values were corrected for radical decay using control solutions and the percentage inhibition was calculated. The results were thus expressed as mmol trolox equivalents/g of sample.

2.3.5. Lipid peroxidation assay

Brain homogenate was used as a source of polyunsaturated fatty acids to determine the extent of lipid peroxidation. Whole rat brains were homogenized (100 mg/ml) in an ice-cold 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was divided into 1 ml aliquots and preserved at −80 °C until use. The reaction mixture consisted of 100 μl of brain homogenate, 50 μl of 1 mM aqueous ferrous sulfate, 100 μl of 5 mM aqueous ascorbic acid, along with 50 μl of different sample concentrations. The reaction solution was incubated in a shaking water bath in an open tube. After incubating at 37 °C for 30 min, a TBA test was performed by adding 250 μl of 1.2% w/v TBA in 50 mM NaOH and 50 μl of 10% TCA. Then, the tubes were incubated at 90 °C for 60 min. After cooling, the solution was centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant was determined at 532 nm (Ohkawa, Ohishi, & Yagi, 1979). The absorbance was recorded against controls prepared in the same way as the experimental samples but without homogenate.

2.4. Ferrous ion-chelating capacity

This method is based on the formation of a ferrozine–Fe^{2+} coloured complex which has a strong absorbance at 562 nm. Reaction mixtures contained 25 μl of different sample concentrations, 100 μl of 50 μM ferrous sulfate and 100 μl of 300 μM ferrozine. After incubation at room temperature for 10 min, the absorbance of resulting solutions was recorded (Tang, Kerry, Sheehan, & Buckley, 2002). The percentage of inhibition of ferrozine–Fe^{+2} complex formation was calculated as

$$\text{Ferrous ion-chelating activity } (\%) = (A_0 - A_s)/A_0 \times 100,$$

where A_0 is absorbance of the control and A_s is absorbance in the presence of sample.

2.5. Reducing power

The reducing power was determined spectrophotometrically from the formation of Perl's Prussian blue-coloured complex (Iqbal, Bhanger, & Anwar, 2005). Twenty five microlitres of different sample concentrations were loaded into each well, and then 50 μl of 50 μM phosphate buffer (pH 6.6) and 50 μl of a 0.1% (w/v) potassium ferricyanide solution were added. After incubation at 50 °C for 20 min, 100 μl of 1% (w/v) TCA solution were added and the mixture was centrifuged at 1500 rpm for 10 min. Finally, 200 μl of the upper layer were transferred into a new 96-well plate and then mixed with 25 μl of 5 mM ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm; increased absorbance of the reaction mixture indicated greater reducing power. The results were expressed as relative reducing equivalents of ascorbate (RRE).

2.6. Determination of antioxidant ingredients contents

2.6.1. Determination of total phenolics and flavonoids

The methods for total phenolic contents and flavonoid concentration were modified from the method of Naczk and Shahidi (1989) and Jia, Tang, and Wu (1999), respectively, with a 96-well microtitre spectrophotometer. These methods are based on the formation of coloured products by redox reaction with Folin–Ciocalteu's reagent or aluminium salts. The absorbance of coloured solutions is proportional to total phenolic or flavonoid concentration. The total phenolic results were expressed as (+)-catechin equivalents in milligrammes per gramme of sample. Total flavonoid concentration was expressed as quercetin equivalents in milligrammes per gramme of sample.

2.6.2. Determination of esculin, esculetin, fraxin and fraxetin

CF crude extract and all fractions were dissolved in methanol and then filtered with a 0.22 μM filter. The HPLC system consisted of a Shimadzu VP series LC-10ATvp pump, an SCL-10AVP system controller, an SPD-M10AVP photodiode array detector, and a SIL-10AF autosampler. Data were monitored using a Shimadzu Class-VP™ chromatography data system. An Alltima C18 (100 × 4.6 mm, 3 μm) column (Alltech associates Inc.) was used. The mobile phase was a mixture of acetonitrile, methanol and 0.01% phosphoric acid solution (2:1:12 (v/v)). The flow rate was 0.8 ml/min, and the detection wavelength was 340 nm. The HPLC chromatography is shown in Fig. 1.

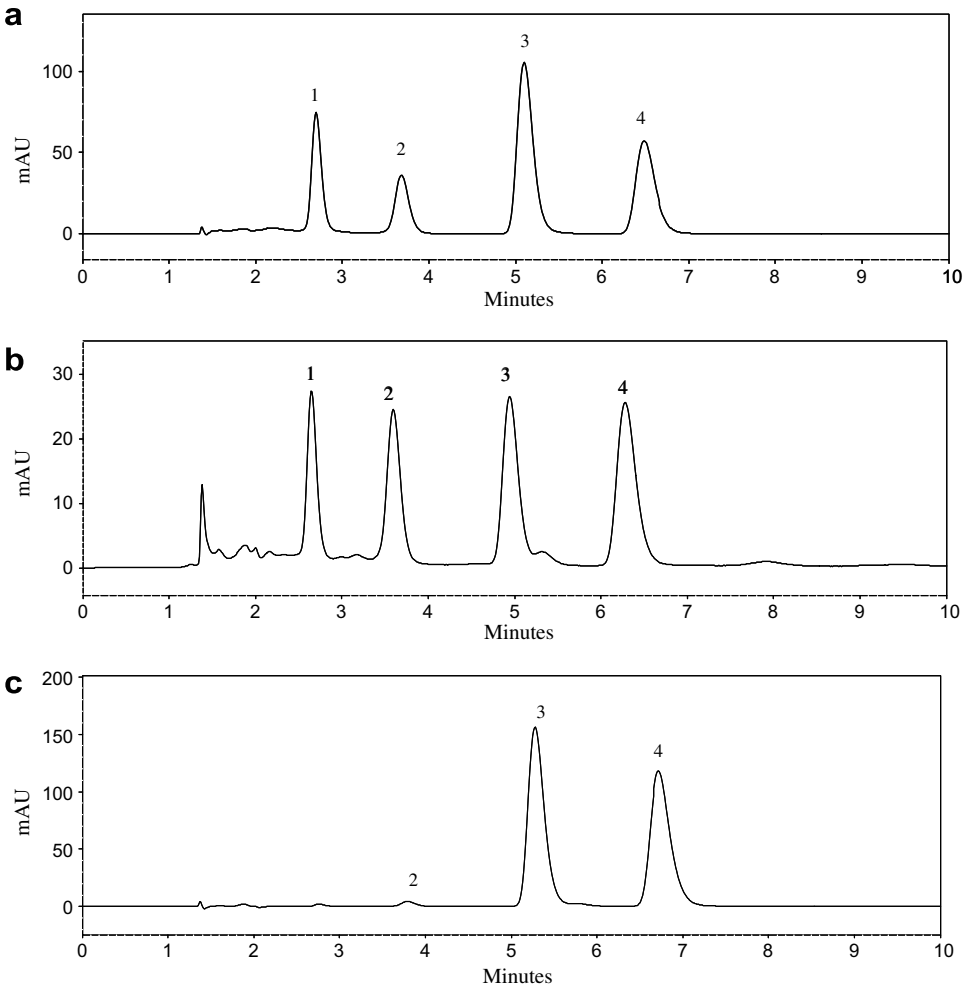

Fig. 1. The HPLC chromatographic profiles, at 340 nm, of (a) standard; (b) crude extract; and (c) chloroform fraction of Cortex Fraxini (CF). Peak 1: esculin, peak 2: fraxin, peak 3: esculetin, peak 4: fraxetin.

2.7. Statistical analysis

All results were expressed as means ± standard deviation (SD). The significance of difference was calculated by SPSS one-way ANOVA, followed by Scheffe's test, and values <0.05 were considered to be significant.

3. Results and discussion

3.1. Antioxidant and radical-scavenging capacities of CF crude extract and all fractions

3.1.1. DPPH, ABTS and hydroxyl radical-scavenging activities

DPPH and TEAC assays are simple, quick and commonly used to assess the antioxidant activities of natural plants and compounds which act as free radical-scavengers or hydrogen donors *in vitro*. Thus, we evaluated the free radical-scavenging activity of CF crude extract and all fractions using the above two assays. All samples exhibited appreciable scavenging properties against both the radicals, and the inhibition percentage was proportional to the concentration of each sample. The IC_{50} values for CF crude extract, water, *n*-butanol, ethyl acetate and chloroform fractions in the DPPH method were 406, 424, 104, 22.2 and 16.7 μg/ml, respectively. For TEAC assay, the IC_{50} values for CF crude extract, water, *n*-butanol, ethyl acetate and chloroform fractions were 39.3, 145, 36.1, 8.88 and 4.24 μg/ml, respectively. The relative radical-scavenging potency of CF crude extract and all fractions in the DPPH method for catechin and TEAC assay for trolox is shown in Fig. 2a and b. The chloroform fraction has a greater scavenging capacity against both the radicals compared to other fractions.

ROS constitute a major pathological factor causing many serious diseases, including cancer and neurodegenerative disorders (Castro et al., 2001). The most commonly formed ROS are oxygen radicals, such as superoxide and hydroxyl radicals, and non-free radicals, such as hydrogen peroxide and singlet oxygen (Galli et al., 2005). The hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules. Thus, the hydroxyl radical-scavenging activity of CF crude extract and all fractions was investigated by the 2-deoxyribose method. The chloroform fraction had the highest second order rate constants

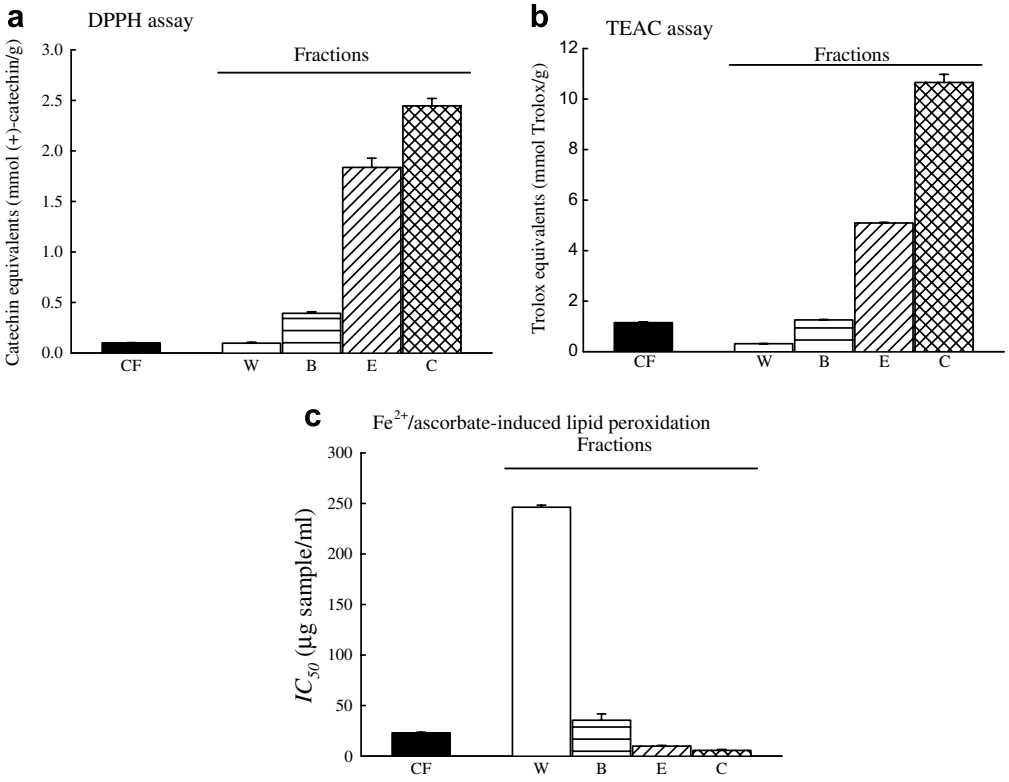

Fig. 2. Antioxidant activity of crude extract and fractions of Cortex Fraxini (CF) on (a) DPPH assay; (b) TEAC assay; (c) Fe^{2+}/ascorbate-induced lipid peroxidation. Data are expressed as means ± SD ($n = 3$). W, B, E and C are represented as the water, *n*-butanol, ethyl acetate and chloroform fractions of CF, respectively.

on scavenging hydroxyl radical (compared with other fractions) (Table 1). Due to the fact that some compounds, e.g., hydroxyl radical scavengers, may be correlated with iron-chelating properties or directly with hydrogen peroxide-scavenging activity (Moran, Klucas, Grayer, Abian, & Becana, 1997; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995) and that iron generally acts as a catalyst during lipid peroxidation, further investigation of the hydrogen peroxide-scavenging and ferrous ion-chelating activity of CF crude extract and all fractions was carried out. The highest hydrogen peroxide scavenging activity was observed for the ethyl acetate fraction, followed by the chloroform, *n*-butanol and water fractions (Table 1). These results reveal that the chloroform and ethyl acetate fractions had better ROS-scavenging activities, consistent with the results from the DPPH and TEAC assays. However, CF crude extract and all fractions showed low ion-chelating capacity (data not shown).

Table 1
The hydrogen peroxide, hydroxyl radical-scavenging capacity and reducing power of crude extract and fractions of Cortex Fraxini (CF)

Samples	Hydrogen peroxide scavenging capacity (mmol trolox/g)	Second order rate constant in scavenging hydroxyl radical ($\times 10^9$)	RRE ($\times 10^{-3}$)
CF	1.08 ± 0.02	25.7 ± 2.04	87.3 ± 0.56
Chloroform fraction	3.20 ± 0.15	1302 ± 28.58	420 ± 5.71
Ethyl acetate fraction	4.28 ± 0.20	300 ± 6.41	384 ± 6.85
n-Butanol fraction	1.68 ± 2.76	57.0 ± 2.52	91.5 ± 0.22
Water fraction	0.32 ± 0.01	15.2 ± 2.47	23.1 ± 0.16

Data are expressed as means ± SD ($n = 4$). RRE is relative reducing equivalent of ascorbate.

3.1.2. Inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation in rat brain homogenate

Because ROS attack biomolecules such as lipids to initiate free radical chain reactions and cause lipid peroxidation, we further evaluated the antioxidant capacity of CF crude extract and each fraction using Fe^{2+}/ascorbate-induced lipid peroxidation in rat brain homogenate. All samples showed a linear correlation ($r^2 = 0.964$–0.9969, $p < 0.05$) between their concentration and lipid peroxidation-inhibiting activity within the applied concentrations. Fig. 2c shows the IC_{50}values corresponding to the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation. The

chloroform fraction also possessed the highest activity, among all fractions, for the inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation. It is well known that the protective effects of antioxidants in biological systems are mainly due to their free radical-scavenging capacity, metal-chelating activities and activation of biological antioxidant enzymes (Kulkarni, Aradhya, & Divakar, 2004). Hence, these results suggested that the antioxidant mechanisms of CF crude extract and chloroform fraction are possibly due to an oxidative chain termination by radical-scavenging capacity, but not to iron chelation.

3.1.3. Reducing power of CF crude extract and all fractions

Literature reports suggest that the antioxidant activity of plant herbs is associated with their reducing power, which terminates free radical chain reactions (Singh & Rajini, 2004). Our present results also show that the reducing power of all samples was proportional to their used concentrations. The highest reducing power, among all fractions, was also observed for the chloroform fraction, which was consistent with the free radical-scavenging capacity (Table 1). A positive correlation was observed between the reducing power and the antioxidant ingredients or activity in all CF fractions ($r^2 = 0.977$, $p < 0.05$ for reducing power vs DPPH; $r^2 = 0.997$, $p < 0.001$ for reducing power vs the inhibition capacity of lipid peroxidation). Therefore, our present results, in accordance with other reports, also show that the free radical-scavenging capacities of CF crude extract and the chloroform fraction are concomitant with their reducing power, and termination of free radical chain reactions.

3.2. Relationship between antioxidant activities and ingredient contents of CF crude extract and all fractions

Phenolic acids and coumarins are recognized as natural antioxidants (Fylaktakidou et al., 2004), and recent reports indicate that *Fraxinus* species, including CF, contain phenolic acids and coumarins, such as esculetin and fraxetin (Liu et al., 2005; von Kruedener et al., 1996). Hence, the relationships between free radical-scavenging capacity and antioxidant contents, including total phenolics or flavonoids, is assayed are shown in Fig. 3. Our result showed that total phenolic and flavonoid contents of CF crude extract per gramme were equivalent to 172 mg catechin and 14.9 mg quercetin. Among all fractions, the chloroform fraction had the highest phenolic and flavonoid contents (Table 2). We further found that CF crude extract contained 0.7% esculin, 0.449% esculetin, 1.24% fraxin and 0.866% fraxetin. These contents were below other reports, possibly due to different plant materials and extraction processes, especially by the extraction solvent. The chloroform fraction also had highest contents of esculetin and fraxetin; however, highest contents of esculin and fraxin were in the *n*-butanol fraction because esculin and fraxin, glycosides of esculetin and fraxetin, respectively, are more hydrophilic compounds (Table 2). There are linear regressions and a significant relationship between free radical-scavenging capacity and total phenolic or flavonoid content in all CF fractions ($r^2 = 0.999$, $p < 0.001$ for DPPH vs flavonoid; $r^2 = 0.963$, $p < 0.05$ for DPPH vs total phenolic; $r^2 = 0.919$, $p < 0.05$ for ABTS vs flavonoid; $r^2 = 0.909$, $p < 0.05$ for ABTS vs total phenolic; $r^2 = 0.965$, $p < 0.05$ for the inhibition capacity vs flavonoid; $r^2 = 0.910$, $p < 0.05$ for the inhibition capacity vs total phenolic). A positive correlation was further observed between the reducing power and the antioxidant ingredients in all CF fractions ($r^2 = 0.977$, $p < 0.05$ for reducing power vs flavonoids; $r^2 = 0.937$,

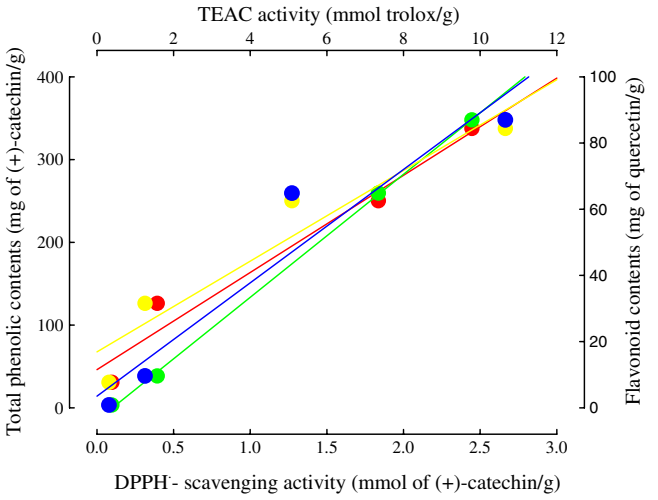

Fig. 3. Relationship between antioxidant compounds and free radical-scavenging capacities of all fractions of Cortex Fraxini. DPPH-scavenging capacity vs total phenolic content (red); DPPH-scavenging capacity vs flavonoid content (green); TEAC activity vs total phenolic content (yellow); TEAC activity vs flavonoid content (blue). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Table 2
The phytochemical compositions of crude extract and fractions of Cortex Fraxini (CF)

Samples	Total phenolics (mg of (+)-catechin/g)	Flavonoids (mg of quercetin/g)	Esculin (mg/g)	Esculetin (mg/g)	Fraxin (mg/g)	Fraxetin (mg/g)
CF	172 ± 8.66	14.9 ± 0.64	7.00 ± 0.01	4.49 ± 0.02	12.4 ± 0.04	8.66 ± 0.03
Chloroform fraction	338 ± 5.05	87.0 ± 2.03	–	84.4 ± 0.20	5.42 ± 0.01	111 ± 0.34
Ethyl acetate fraction	250 ± 4.54	64.9 ± 1.05	12.9 ± 0.23	63.9 ± 0.89	8.29 ± 0.16	18.9 ± 0.32
n-Butanol fraction	126 ± 2.99	9.60 ± 0.70	41.9 ± 0.19	0.75 ± 0.01	44.7 ± 0.20	1.48 ± 0.01
Water fraction	30.8 ± 1.96	0.84 ± 0.22	0.15 ± 0.01	1.20 ± 0.01	1.10 ± 0.04	2.34 ± 0.01

Data are expressed as means ± SD ($n = 4$).

Table 3
The radical-scavenging activity of simple coumarins and known antioxidants

Samples	IC_{50} in DPPH radical (μM)	IC_{50} in ABTS radical (μM)	IC_{50} in hydrogen peroxide (μM)	Second order rate constant in hydroxy radical ($\times 10^{12}$)
Esculin	3763 ± 71.0	406 ± 15.3 (0.11)	89.1 ± 2.89	0.94 ± 0.24
Esculetin	17.8 ± 0.11	17.2 ± 0.28 (3.66)	46.9 ± 0.16	3.18 ± 0.18
Fraxin	496 ± 3.62	93.0 ± 2.39 (0.49)	218 ± 5.05	0.96 ± 0.07
Fraxetin	44.1 ± 1.24	37.4 ± 0.23 (1.21)	40.5 ± 0.69	1.02 ± 0.03
Catechin	40.7 ± 1.26	10.4 ± 0.19 (4.36)	91.4 ± 0.88	3.77 ± 0.42
Quercetin	35.0 ± 0.07	20.4 ± 0.27 (2.22)	42.3 ± 1.70	10.1 ± 0.25
Trolox	61.7 ± 1.88	45.2 ± 0.73 (1)	95.0 ± 2.29	46.4 ± 3.64
Ascorbic acid	138.4 ± 1.78	41.5 ± 1.77 (1.09)	101 ± 0.6	1.78 ± 0.07
BHT	121 ± 1.13	106 ± 2.91 (0.43)	1780 ± 33.8	1.37 ± 0.01

Data are expressed as means ± SD ($n = 3$).

$p < 0.05$ for reducing power vs total phenolic). Some researchers have indicated that a positive correlation between free radical-scavenging activity and total phenolic compounds (Cai, Luo, Sun, & Corke, 2004) and the reducing power of natural plants is dependent on the contents of their phenolic compounds (Siddhuraju, Mohan, & Becker, 2002). Therefore, the fraction of CF crude extract partitioned with chloroform possessed the best free radical-scavenging, reducing power and lipid peroxidation inhibiting properties because it had highest phenolic content, especially of flavonoids.

3.3. Comparison with antioxidant capacity of simple coumarins and unknown antioxidants

Finally, we assayed four simple coumarins of CF for DPPH, ABTS, hydroxyl radical- and hydrogen peroxide-scavenging capacity and also compared their antioxidant capacities with known antioxidants, including catechin, quercetin, trolox, vitamin C and BHT. These results are shown in Table 3. The TEAC values of quercetin and ascorbic acid were similar to those of Re et al. (1999) and the tendency in scavenging DPPH radical of catechin and quercetin is similar to another report (Butkovic, Klasinc, & Bors, 2004). Among all test antioxidants, esculetin had the highest radical-scavenging capacity for DPPH and ABTS radicals. Many reseachers have suggested that the antioxidant capacities of flavonoids are mainly governed by the number and location of hydroxyl groups in the B ring, hydroxyl substitutions in the A ring, and 3-OH substitution and 2,3-double bond in the C ring (Burda & Oleszek, 2001; Heim, Tagliaferro, & Bobilya, 2002; Lien, Ren, Bui, & Wang, 1999). On the other hand, some researchers have indicated that glycosidic substitution decreases TEAC values of flavonoids (Heim et al., 2002). We also found similar results, in that there is lower free scavenging capacity in the two glycosidic coumarins, esculin and fraxin, than in their aglycones. Furthermore, esculetin and fraxetin also possess the better active oxygen-scavenging activity than do ascorbic acid and BHT, and the different potencies in scavenging various oxygen radicals, between esculetin and fraxetin, are similar to those reported by Hoult and Paya (1996). The active oxygen-scavenging activities of two glycosidic coumarins, esculin and fraxin, were also lower than their aglycones. From these present results and other reports (Fylaktakidou et al., 2004), we suggest that *ortho*-dihydroxy substitution and the 3,4-double bond play an important role in the antioxidant property of simple coumarins that lack the B-ring of flavonoids. A hydroxyl group in the 7-position of simple coumarins is necessary for its antioxidant activity and any C_6–C_8 substitution, including glycosidic or methoxy groups, might decrease their free radical-scavenging capacity.

4. Conclusion

CF is rich in *ortho*-dihydroxy simple coumarins which have better (or equal) radical-scavenging capacities than have flavonoids and known antioxidants, including ascorbic acid, trolox and BHT. Hence, CF exhibited high radical-scavenging potency, and its antioxidant activity can be correlated with its reducing power, but not iron chelation. Furthermore, we suggest that CF partitioned with chloroform has a higher coumarin content and a higher antioxidant activity, associated with better reducing power. There are significant relationships among the contents of phenolic antioxidant ingredients, reducing power and free radical-scavenging capacity.

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