Reactive Oxygen Species Scavenging Activity of Du-zhong (*Eucommia ulmoides* Oliv.) and Its Active Compounds

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The biologically active compounds and free radical-/ or reactive oxygen species (ROS)-/ scavenging effect of water extract from Du-zhong (WEDZ) were investigated. The WEDZ used included leaves, raw cortex, and roasted cortex. The hot water extract of Du-zhong leaves showed marked activity as a ROS scavenger, and the scavenging effect was concentration dependent. The extract of roasted cortex exhibited a modest scavenging effect on ROS, while the extract of raw cortex had the weakest scavenging effect. The scavenging activity of WEDZ on ROS was correlated to its protocatechuic acid (PCA) content. The content of PCA in Du-zhong determined by HPLC followed the order of leaves (17.17 mg/g) > roasted cortex (2.99 mg/g) > raw cortex (1.16 mg/g). The inhibitory activity of leaf extract of Du-zhong was stronger than that of PCA on the peroxidation of linoleic acid at the same concentration of 0.1 mg/mL. The results presented herein indicated that extract of Du-zhong could possibly act as a prophylactic agent to prevent free radical-related diseases.

Keywords: *Du-zhong; water extract; reactive oxygen species; scavenger; protocatechuic acid*

INTRODUCTION

A growing interest in biology and medicine has been devoted to reactive oxygen species (ROS). ROS indicates active oxygen molecules which are generated as a consequence of normal metabolic events or exposure to oxidizing agents such as xenobiotics, environmental polluting agent, or ionizing radiation. Some of the relevant ROS are as follows: hydroxyl ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydrogen peroxide (H_2O_2), and hypochloride (HOCl). Halliwell and Gutteridge (1989) and Ames et al. (1993) have pointed out that ROS plays an important role in certain clinical diseases, in the process of aging, and in oxygen-mediated food deterioration (King et al., 1993).

Oxidative stress derives from free radicals as well as ROS and causes damage to biological macromolecules such as DNA, lipids and proteins, thus influencing food quality and biological tissues (Halliwell, 1996). Moreover, oxidative stress is one of the mechanisms postulated to play an important role in mutagenesis and carcinogenesis (Sahu, 1991). Unrepaired and/or misrepaired endogenous oxidative DNA damage can lead to cancer induction. Almost all organisms are well protected against free-radical damage by either enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase or compounds such as lipoic acid, uric acid, ascorbic acid, α-tocopherol, and glutathione. When the mechanism of antioxidant protection becomes unbalanced by factors such as aging, deterioration of physiological functions can be triggered, resulting in diseases or accelerated aging. Research has pointed out that the most effective method to reduce oxidative stress is antioxidant supplement. However, the possible toxicity of synthetic chemicals used as antioxidants has long been questioned (Imaida et al., 1983; Namiki, 1990). Natural antioxidants have recently attracted special interest because they can protect the human body from free radicals, which may cause various diseases, carcinogenesis, and aging (Cutler, 1992). Natural antioxidants are found in herbs, spices and in various kinds of plant products such as seeds, leaves, fruits, and seedlings (Namiki, 1990). Antioxidant compounds derived from plants, especially phenols such as quercetin, carnosol, thymol, catechin, and morin etc., are of considerable interest from the viewpoint of dietary antioxidant supplementation and food preservation (Halliwell et al., 1995).

Du-zhong (*Eucommia ulmoides* Oliv.) tea (the aqueous extract of leaves) is commonly used in Japan for treatment of hypertension and is thought to be a functional health food (Nakazawa et al., 1997). Investigation also has shown that leaf extract of Du-zhong may have recuperative effects for hypercholesterolemia and fatty liver (Nakasa et al., 1995). Sasaki et al. (1996) and Nakamura et al. (1997) found that Du-zhong (leaf) tea had a suppressing effect on mutagenicity and chromosome aberration following mutagen treatment. Yen and Hsieh (1998) reported that water extract from Du-zhong (WEDZ) possessed antioxidant activity toward various lipid peroxidation models. These results showed that the inhibitory activity of WEDZ was following the order of leaves > roasted cortex > raw cortex. However, the scavenging ability of ROS by the extract of Du-zhong and its active compounds have not yet been studied. The objectives of this study were to investigate the ROS scavenging activity of the WEDZ, in comparison with known antioxidants such as gallic acid and ascorbic acid, and to elucidate its active antioxidant compounds.

MATERIALS AND METHODS

Materials. Du-zhong (*Eucommia ulmoides* Oliv.), including leaves, raw cortex, and roasted cortex, was purchased at a local market in Taichung, Taiwan. Gallic acid, ascorbic acid, linoleic

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acid, ferrous chloride, nitro blue tetrazolium (NBT), α,α-diphenyl-β-picrylhydrazyl (DPPH), 5,5-dimethyl pyrroline-*N*-oxide (DMPO), mannitol, phenol red, horseradish peroxidase (HRPase), and histidine were purchased from Sigma Chemical Co. (St. Louis, MO). Dihydronicotinamide-adenine-dinucleotide (NADH), phenazine methosulfate (PMS), and ammonium thiocyanate were purchased from E. Merck Co. (Darmstadt, Germany). Hydrogen peroxide, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Shimakyu Co. (Japan). Trolox (Hoffman-La Roche) was purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*,*N*-Dimethyl-4-nitrosoaniline (RNO) and rose bengal B were purchased from Fluka Bio Chemical (Switzerland).

Preparation of Water Extract of Du-zhong. Each sample of leaf, raw cortex, and roasted cortex of Du-zhong (20 g) was extracted with boiling water (200 mL) for 60 min, respectively. The extracts were filtered through Whatman no. 2 filter paper, and the filtrates were freeze-dried to a powder form. The yields of extracts from leaves, raw cortex, and roasted cortex of Du-zhong were 1.92, 1.41, and 1.60 g, respectively.

Scavenging of Hydroxyl Radical. The hydroxyl radical reacts rapidly with nitrone spin trap DMPO, and the resultant DMPO−OH adduct is detectable with an electron paramagnetic resonance (EPR) spetrometer (Rosen and Rauckman, 1984). The EPR spectrum was recorded 2.5 min after WEDZ (0−12.5 mg/mL) mixed with DMPO (0.3 M, 0.2 mL), Fe^{2+} (10 mM, 0.2 ML), and H_2O_2 (10 mM, 0.2 mL) in a phosphate buffer solution (pH 7.2) using an EPR spectrometer (Bruker ER 200D 10/12) set at the following conditions: receiver gain, 2×10^5; modulation amplitude, 1.0 G; scan time, 200 s; field, 3461.3 ± 50 G; time constant, 0.5 s (Shi et al., 1991).

Scavenging of Superoxide Anion. The influence of WEDZ on the inhibition of superoxide was determined by spectrophotometric measurement of the product on reduction of nitro blue tetrazolium (Nishikimi et al., 1972). Superoxide was generated in a nonenzymatic system. The reaction mixture, which contained 1 mL of WEDZ (0−0.25 mg/mL), 1 mL of PMS (60 μM) in phosphate buffer (0.1 M, pH 7.4), 1 mL of NADH (468 μM) in phosphate buffer, and 1 mL of NBT (150 μM) in phosphate buffer, was incubated at ambient temperature for 5 min, and the color was read at 560 nm against blank samples.

Scavenging of Singlet Oxygen. Singlet oxygen production was estimated by the bleaching of *N*,*N*-dimethyl-4-nitrosoaniline (RNO) and quantitatively determined by the oxidation of added histidine. This assay was carried out using the method of Kraljic and Mohsni (1978) with slight modifications. The reaction mixture, which contained 1 mL of WEDZ (0−1.4 mg/mL) or histidine (0−10 μM), 1 mL of rose bengal B (20 μM) in phosphate buffer (0.1 M, pH 7.4), 1 mL of RNO (50 μM), was irradiated with a 100 W lamp (Philips AS 100, 115V) in ice bath for 60 min. The color was read at 440 nm against blank samples.

Scavenging of Hydrogen Peroxide. Methods for detecting hydrogen peroxide are based on the peroxidase assay systems. The most commonly used is horseradish peroxidase (HRPase) which can react with hydrogen peroxide to oxidize phenol red into a purplish-red product, as described by Rinkus and Taylor (1990). The assay was started by adding 0.6 mL of the HRPase/phenol red solution to the reaction system. Thus, the final assay mixture had a total volume of 2.0 mL with a pH of about 6 and contained 0.1 M phosphate buffer, 1.5 mM phenol red, 50 μg HRPase /mL, and WEDZ (0−2 mg/mL). The tubes were vortexed and then allowed to sit for 5 min, at which time the reaction was retarded by ice bath and the absorbance at 610 nm was read immediately.

Hydrolysis and Separation of Phenolics. WEDZ (2 g) was hydrolyzed with 2.5 N HCl at 100 °C for 2 h to break glycosidic bonds, and the released aglycones were extracted into ethyl acetate (Abu-Amsha et al., 1996) until they became colorless. The extracts were combined, evaporated to dryness on a rotary evaporator, and then dissolved in 5 mL of methanol. The crude extracts were filtered through a 0.45 μm filter before HPLC analysis. The filtrates were analyzed by HPLC (Hitachi, Japan), using the LiChrosphere RP-18 column (150 mm × 4 mm, 5 μm) and UV detector (measured at 280 nm). Elution was carried out at room temperature and utilized 2% acetic acid as solvent A and acetonitrile as solvent B. The elution program was 0−20 min, 0−13% B; 20−60 min, 13−40% B; 60−70 min; 40−0% B (linear gradient) at a flow rate of 1 mL/min. An authentic sample of protocatechuic acid (PCA) was used as an external standard for calculation of its content in the WEDZ. A standard PCA curve was prepared by injecting different volumes of standard solutions into the HPLC system. Linear regression was fitted to the data to obtain regression coefficients > 0.99 for PCA curve. BHA was used as the internal standard to calculate the loss of PCA. According to the loss of PCA and the PCA standard curve, the amount of PCA in WEDZ was calculated.

Table 1. Effect of Water Extract from Du-zhong (WEDZ) on EPR Spectrum Signal Intensity of DMPO−OH Spin Adducts

sample	concn (mg/mL)	rel EPR signal intensity[a]	scavenging effects[b]
control	0	100	
leaves	2.50	90.60	9.40
	6.25	45.32	54.68
	12.5	33.59	66.41
raw cortex	2.50	94.58	5.42
	6.25	90.37	9.63
	12.5	75.08	24.92
roasted cortex	2.50	95.20	4.80
	6.25	94.95	5.05
	12.5	68.35	31.65

[a] Relative EPR signal intensity (%) = {[$h\ \Delta H^2$(sample)/$h\ \Delta H^2$(dpph)]/[$h\ \Delta H^2$(control)/$h\ \Delta H^2$(dpph)]} × 100. h = the height of the peak. ΔH = the width of the peak. [b] Scavenging effects (%) = 100 − relative EPR signal intensity (%).

Antioxidant Activity of PCA in Linoleic Acid Emulsion. An antioxidant activity of Du-zhong was determined according to the thiocyanate method (Mitsuda et al., 1966). Each sample (500 μg) in 0.5 mL of distilled water was mixed with linoleic acid emulsion (2.5 mL, 0.02 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2840 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier, and 50 mL of phosphate buffer; and then the mixture was homogenized. The reaction mixture was incubated at 37 °C. Aliquots of 0.1 mL were taken at different intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding ethanol (4.7 mL, 75%), and ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL), and ferrous chloride (0.1 mL, 0.02 M in 3.5% HCl). After the mixture stood for 3 min, the peroxide value was determined by reading the absorbance at 500 nm using a spectrophotometer (Hitachi U-2000). A control was performed with linoleic acid but without samples.

Statistical Analysis. All analyses were run in three replicates and averaged. Data were analyzed using the Statistical Analysis System software package (SAS Institute Inc., 1985). Analyses of variance were performed by ANOVA procedures. Significant difference ($p < 0.05$) between means were determined by Duncan's multiple-range tests.

RESULTS AND DISCUSSION

Scavenging of Hydroxyl Radical. The hydroxyl radical ($OH^•$) is the most reactive free radicals in biological tissues and is believed to have significance as an initiator for peroxidation of lipids. To test the reaction of $OH^•$ with WEDZ, we used the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^•$) as a source of $OH^•$. The reaction of Fe^{2+} with H_2O_2 in the presence of spin-trapping agent DMPO generated a 1:2:2:1 quartet with hyperfine coupling parameters (a^n and a^h = 14.9 G) (data not shown). Among WEDZ, leaf extract markedly scavenged the DMPO−OH spin adduct. Table 1 shows the effects of WEDZ on the EPR signal intensity

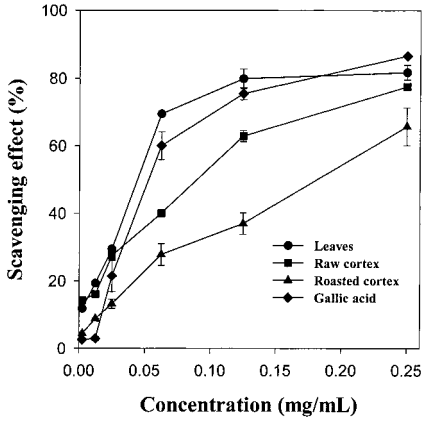

Figure 1. Superoxide anion radical scavenging effect of water extract from Du-zhong (WEDZ). Superoxide anion radicals generated by PMS/NADH/NBT. Scavenging effects % (capacity to scavenging the superoxide anion) = [(absorbance of control at 560 nm) − (absorbance of sample at 560 nm)/(absorbance of control at 560 nm)] × 100.

of DMPO−OH adducts. All WEDZ showed inhibitory effect with a concentration dependent manner. The inhibitory effect of WEDZ on $OH^{\bullet}$ was in the order of leaves (66.4%) > roasted cortex (31.7%) > raw cortex (24.9%) at a concentration of 12.5 mg/mL ($p < 0.05$).

The $OH^{\bullet}$ is an extremely reactive free radical formed in biological systems. It can act on and damage almost every molecule found in living cells, such as sugars, amino acids, lipids, and DNA base (Namiki, 1990). Du-zhong leaves contain specific phytochemicals such as phenolic derivatives, flavonoids, iridoids, and triterpenoid (Nakazawa, 1997). Phenolic derivative-pyrogallol, protocatechuic acid, coumaric acid, and chlorogenic acid as well as flavonoids quercetin, kaempherol, and astragarin have been isolated from Du-zhong tea (Nakamura et al., 1997). The antioxidant activities of phenolic derivatives and flavonoids have been well reported. Ueda et al. (1996) showed that protocatechuic acid greatly suppressed the DNA strand scission by $OH^{\bullet}$ produced from the reaction of Cu^{2+} with H_2O_2. Chlorogenic acid could prevent the formation of the $OH^{\bullet}$ by forming a chelation with iron whose complex cannot catalyze the Fenton type reaction (Kono et al., 1998). Thus, leaf extract of WEDZ possesses clear scavenging effect on $OH^{\bullet}$. The ability of WEDZ to scavenge $OH^{\bullet}$ seems to relate directly to the prevention of lipid peroxidation (Yen and Hsieh, 1998).

Scavenging of Superoxide Anion. Superoxide and hydrogen peroxide have been implicated in regulation of cell proliferation (Burdon and Rice-Evans, 1989). It has been estimated that about 1−3% of the O_2 is converted to $O_2^{\bullet-}$ (Halliwell, 1996). Though SOD can dismutate $O_2^{\bullet-}$ in vivo, SOD would be destroyed in the stomach after intake and could therefore not be absorbed in the intestine. Therefore, studies began focusing on seeking $O_2^{\bullet-}$ scavengers, generally referred to as SOD-like or activators of SOD.

Superoxide anions generated by the PMS/NADH system were monitored by the reduction of NBT. Figure 1 shows the effects of scavenging superoxide anions ($O_2^{\bullet-}$) by WEDZ. The scavenging effect of WEDZ and gallic acid was enhanced with increasing concentration. $O_2^{\bullet-}$ (65−87%) was inhibited by WEDZ at a concentration of 0.25 mg/mL. The scavenging effect of WEDZ and other positive controls were in the order of leaf (79.9%) > gallic acid (75.5%) > roasted cortex (62.9%) > raw cortex (37.0%) at a concentration of 125 μg/mL ($p < 0.05$). The half-inhibition concentrations (IC_{50}) of WEDZ were calculated from the concentration-activity curves (Figure 1). The leaf extract had very strong scavenging effect with IC_{50} values 0.043 mg/mL. The IC_{50} values of gallic acid, raw cortex, and roasted cortex were 0.049, 0.091, and 0.179 mg/mL, respectively. In the presence of SOD, the NBT reduction was reduced by more than 80%, to an activity of 10 units/mL. From these data, it can be concluded that the leaf extract showed the strongest scavenging effect on $O_2^{\bullet-}$ compared to other antioxidants in this study. This trend is similar to the scavenging of $OH^{\bullet}$. Thus, WEDZ might be a good SOD-like material. Decker (1995) demonstrated that phenolic compounds could be nonessentially dietary antioxidants. The compounds are mainly flavonoids, polyphenols, tannin, and α-tocopherol. Sato et al. (1996) reported that there had been a positive correlation between $O_2^{\bullet-}$ scavenging ability and polyphenol content. The fact that the leaf extract contained the highest content of polyphenol may be the contributing factor to its superior ability of scavenging $O_2^{\bullet-}$.

Table 2. Singlet Oxygen Scavenging Effect of Water Extract from Du-zhong (WEDZ)

sample (mg/mL)	RNO bleaching at 440 nm[a]		
	leaves	raw cortex	roasted cortex
0	0.460 ± 0.016[b]		
0.15	0.119 ± 0.005	0.264 ± 0.004	0.190 ± 0.007
0.30	0.055 ± 0.010	0.229 ± 0.004	0.139 ± 0.002
0.60	0.058 ± 0.004	0.204 ± 0.012	0.067 ± 0.004
1.40	0.028 ± 0.008	0.172 ± 0.013	0.059 ± 0.003

[a] Values determined with Rose Bengal-generated singlet oxygen and measured by bleaching of *N*,*N*-dimethyl-4-nitrosoaniline (RNO) at 440 nm. [b] Each value represents mean ± standard deviation ($n = 3$).

Scavenging of Singlet Oxygen. Singlet oxygen ($^1O_2$) is formed from excited-states of various sensitizers, such as rose bengal, and can induce neuron apotosis (Cagnoli et al., 1995). It is a nonradical ROS and thought to be of importance in various biological reports, one important example being photodynamic cancer therapy. It has been observed that when some substrate (A) or $^1O_2$ acceptors (like imidazole deviatives) react with $^1O_2$, the formation of $[AO_2]$ is capable of inducing the bleaching of RNO as followed spectrophotometrically at 440 nm.

As the results in Table 2 show, the absorbances (at 440 nm) of extracts of leaves, roasted cortex, and raw cortex on $^1O_2$ were 0.028, 0.059, and 0.172, respectively, equivalent to the scavenging effect of 93.9, 87.2, and 62.6% at a concentration of 1.4 mg/mL. The concentration of histidine (a known quencher of $^1O_2$) at 1.25, 2.5, 5.0, and 10 μM was equivalent to the scavenging effect of 60.9%, 71.7%, 73.9%, and 78.3%, respectively. The scavenging effect of WEDZ and histidine on $^1O_2$ was also concentration-dependent. $^1O_2$ is a very reactive molecule that can be produced by photosensitization after irradiation and may contribute to cytotoxicity (Steinbeck et al., 1993). It plays an important role in neurodegenerative diseases, such as Parkinson's disease (Perry et al., 1982). According to ancient records, Du-zhong possesses the pharmacological effect of antiaging that may have correlation with its scavenging on $^1O_2$.

Scavenging of Hydrogen Peroxide. Hydrogen peroxide (H_2O_2) is not a radical and is generally relatively unreactive. Its cytotoxicity is partly due to the

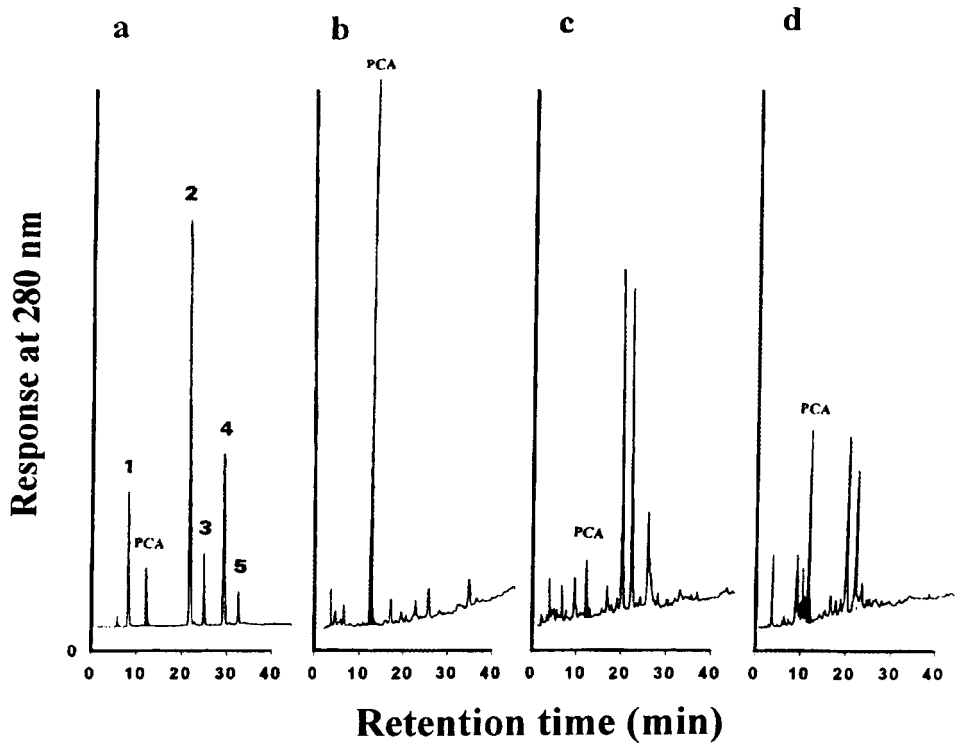

Figure 2. HPLC chromatogram of polyphenol standards and water extract from Du-zhong (WEDZ): (a) polyphenol standards; (peak identification: 1, gallic acid; 2, chlorogenic acid and caffeic acid; 3, catechin; 4, epicatechin; 5, ellagic acid; PCA, protocatechuic acid); (b) leaf extract; (c) raw cortex; (d) roasted cortex.

Table 3. Hydrogen Peroxide Scavenging Effect of Water Extract from Du-zhong (WEDZ)

sample	concn (mg/mL)	scavenging effects[a] (%)
control	0	0
leaves	0.5	15.08 ± 0.02[d b]
	1.0	26.72 ± 0.04[b]
	2.0	69.75 ± 0.01[a]
raw cortex	0.5	3.26 ± 0.07[i]
	1.0	5.82 ± 0.21[h]
	2.0	10.84 ± 0.13[e]
roasted cortex	0.5	7.32 ± 0.09[g]
	1.0	10.58 ± 0.04[f]
	2.0	26.01 ± 0.13[c]

[a] Scavenging effects % (capacity to scavenging the superoxide anion) = [(absorbance of control at 610 nm) − (absorbance of sample at 610 nm)/(absorbance of control at 610 nm)] × 100. [b] Each value is the mean ± standard deviation of three replicate analyses. Values in a column with different superscripts are significantly different ($p < 0.05$).

damage done when H_2O_2 comes to contact with iron or copper in a catalytic form, thus generating hydroxyl radical. Okamoto et al. (1996) pointed out that H_2O_2 caused genetic changes that induced tumorigenic conversion in urothelial cells and that the transformants were stimulated to grow because of their selective response to several cytokines. WEDZ is also capable of scavenging hydrogen peroxide (H_2O_2) in a concentration-dependent manner (Table 3). The scavenging effect was in the order of leaf (69.8%) > roasted cortex (26.0%) > raw cortex (10.8%) at a concentration of 2 mg/mL ($p < 0.05$). The scavenging effect of gallic acid was 67.8 at a concentration of 0.5 mg/mL, which was about equal to the scavenging effect of leaf extract at a concentration of 2 mg/mL. Among the WEDZ extracts, leaf extract showed the strongest scavenging effect on H_2O_2. H_2O_2 can diffuse through membranes and cause DNA damage and lipid peroxidation. Against H_2O_2 induced peroxidation on human erythrocyte membrane, *Ginkgo biloba* extract (EGb 761) can protect the peroxidation from those membranes in a dose-dependent manner (Dogan, 1995). WEDZ could inhibit lipid peroxidation of membrane induced by the Fenton reaction (Yen and Hsieh, 1998), which might be partially due to WEDZ scavenging H_2O_2 and then retarding lipid peroxidation.

Hydrolysis and Separation of Phenolics. Polyphenolic compounds and flavonoids are the most active antioxidants derivative from plants (Aruoma, 1997). The antioxidant activity of WEDZ was correlated to their polyphenol content (Yen and Hsieh, 1998). From this background, phenolics are presumed to be a potential antioxidant in WEDZ. To determine what is the main antioxidant compound in polyphenol, the phenolic composition in WEDZ was assayed and compared to its concentration. The phenolic chromatograms from WEDZ after hydrolysis with acid are shown in Figure 2. Figure 2a shows the chromatograms of polyphenol standard. Figure 2b−d represents the chromatograms of extracts of leaves, raw cortex, and roasted cortex of Du-zhong, respectively. Each polyphenol peak was identified from the retention time of polyphenol standard (Figure 2a). As the results in Figure 2b show, the main compound in leaf extract was protocatechuic acid (PCA) with a retention time of 11.26 min, which was confirmed by co-injection with retention time of polyphenol standard. All WEDZ contained the peak of PCA; however, the PCA content in each sample was varied. Moreover, there are two unknown compounds presented in the raw cortex and roasted cortex with retention times from 20 to 25 min, which did not show in the leaf extract. According to Nakamura et al. (1997), the cortex of Du-zhong contains irridoids of geniposide and geniposidic acid and some other compounds such as ursolic acid. It is worth studying whether these compounds have a scavenging

Table 4. Amounts of Protocatechuic Acid in Water Extract from Du-zhong (WEDZ)

sample	protocatechuic acid (mg/g of WEDZ lyophilized powder)
leaves	17.17 ± 0.01[a] [a]
raw cortex	1.16 ± 0.01[c]
roasted cortex	2.99 ± 0.02[b]

[a] Each value is the mean ± standard deviation of three replicate analyses. Values in a column with different superscripts are significantly different ($p < 0.05$).

Table 5. Antioxidant Effect of Protocatechuic Acid and Relative Compounds on Peroxidation of Linoleic Acid As Measured by the Thiocyanate Method after Incubation for 40 h

sample[c]	inhibition %[a]
control	0
leaf extract	99.8 ± 0.05[a] [b]
protocatechuic acid	58.7 ± 0.35[c]
chlorogenic acid	19.0 ± 0.12[e]
caffeic acid	33.9 ± 0.04[d]
gallic acid	28.0 ± 0.10[ed]
ascorbic acid	73.7 ± 0.61[b]

[a] Inhibition % (capacity to inhibit the peroxide formation in linoleic acid) = [1 − (absorbance of sample at 500 nm)/(absorbance of control at 500 nm)] × 100. A high inhibition % indicated a high antioxidant activity. Each value is the mean ± standard deviation of three replicate analyses. [b] Values in a column with the different superscripts are significantly different ($p < 0.05$). [c] The concentration of samples were 0.1 mg/mL.

effect on ROS. The amount of PCA in WEDZ was calculated and is shown in Table 4. The PCA content in extracts of leaves, roasted cortex, and raw cortex from Du-zhong was 17.17, 2.99, and 1.16 mg/g of lyophilized powder, respectively. From this result, it can be concluded that PCA in polyphenol of leaf extract is the main responsible antioxidant and that the PCA content in WEDZ has a positive correlation to its scavenging ability on ROS.

Antioxidant Activity of PCA in Linoleic Acid Emulsion. Leaf extract also contains ferulic acid, chlorogenic acid, and protocatechuic acid (Nakamura et al., 1997). For evaluation of the antioxidant activity of PCA and its relative phenols in leaf extract, the inhibition effect on the peroxidation of linoleic acid was investigated. As the results in Table 5 show, PCA could inhibit 60% peroxidation of linoleic acid; however, other phenolics only could suppress 20−30% of lipid peroxidation. Moreover, leaf extract could inhibit 99% peroxidation of linoleic acid, among which 60% of the inhibition may be contributed to PCA. Tseng et al. (1996) showed that PCA exhibited protective effects against cytotoxicity and genotoxicity of hepatocytes induced by *tert*-butylhydroperoxide. One of the mechanisms of PCA's protective effect may be associated with its property of scavenging free radicals. Leaf extract had the strongest antioxidant activity in various lipid models (Yen and Hsieh, 1998) and also demonstrated the best scavenging effect on ROS in the present study. These results are in agreement with that the higher antioxidant and scavenging ROS activity, the higher PCA content. In addition, PCA was the main active antioxidant responsible for the antioxidant activity of WEDZ.

CONCLUSION

The phospholipid bilayers of cellular and subcellular membranes are undoubtedly major targets for free radicals. Compounds that inhibit membrane phospholipid peroxidation seem to exert a pharmacological effect in the prevention of radical induced diseases (Icho et al., 1993). The results of the present study show that Du-zhong extract had significant scavenging effect on ROS in vitro, especially leaf extract. PCA is the main active compound responsible for the antioxidant activity of Du-zhong leaf extract. However, further studies are needed to identify the compounds other than PCA in raw cortex and roasted cortex.

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