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Inhibitory effects of Du-zhong (*Eucommia ulmoides* Oliv.) against low-density lipoprotein oxidative modification

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Abstract

This study presents the effects of water extracts from leaves, raw cortex, and roasted cortex of Du-zhong (*Eucommia ulmoides* Oliv.) on the oxidative modification of human low-density lipoprotein (LDL) induced by copper ion (Cu^{2+}). Through determination of the formation of thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD), as well as relative electrophoretic mobility (REM), all extracts of Du-zhong were found to possess inhibitory effects on the oxidative modification of LDL induced by Cu^{2+}. The leaf extract had the most significant inhibitory effect. The ethyl acetate extract from leaf extract of Du-zhong was separated into six bands by preparative TLC. Band 2 (Rf = 0.40), which was most inhibitory against LDL oxidative modification, was analyzed by HPLC; a single, main peak was collected and further examined using NMR and MS. The antioxidant compound thus identified was protocatechuic acid (PCA). At a concentration of 10 μg/ml, PCA showed a stronger inhibitory effect against LDL oxidative modification induced by Cu^{2+} than did Du-zhong extracts or ascorbic acid at the same concentration. The results suggest that, in leaf extract of Du-zhong, PCA could be a major source of the inhibitory effect against LDL oxidative modification induced by Cu^{2+}.

Keywords: *Eucommia ulmoides* Oliv.; Low-density lipoprotein; Protocatechuic acid

1. Introduction

Researchers have shown that atherosclerosis has an intimate relationship with oxidative modification of LDL (Esterbauer, Gebicki, Peul, & Jurgens, 1992; Steinberg, Parthasarathy, Carew, Khoo, & Witzum, 1989). When oxidized LDL is formed, macrophage receptor scavenges the oxidized LDL and then forms foam cells. Overtime, cholesterol, lipoprotein, hemeatoblasts, connective tissues, and calcium may deposit and form plaque in arteries. Plaque makes arteries thicker and narrower, which may lead to the indus of atherosclerosis (Berliner & Heinecke, 1996; Jialal, Facn, & Fuller, 1996). The best way to prevent the formation of a fatty streak of atherosclerosis at the initial stage, is to suppress LDL oxidation and use of drugs to decrease hyperlipidermia (Tangney, 1997).

LDL oxidative modification may be suppressed by supplementing antioxidants, such as vitamin E, BHT and probucol (Nagano et al., 1992). The antioxidant may primarily inhibit LDL peroxidation by scavenging free radicals and chelating metal ions. Epidemiological studies show that diets rich in vegetables and fruits containing antioxidants are associated with lower risk of cardiovascular diseases (Ness & Powles, 1997). Cardiovascular diseases are believed to be diminished by antioxidants in diets which can reduce LDL oxidative modification (Diaz, Frei, Vita, & Keaney, 1997; Steinberg, 1997). Phenolic compounds, including flavonoids and phenolic acids, are considered the main antioxidants in vegetables and fruits. Phenolics possess conjugate and hydroxyl groups which scavenge free radicals such as $O_2^{\bullet -}$, $^1O_2$, and $ROO^{\bullet}$, and chelate metal ions. The French people, who often consume red wine, are at less risk of cardiovascular diseases. The reason could be the rich phenolic compounds in red wines (Frankel, Kanner, German, Parks, & Kinsella, 1993; Renaud & de Lorgeril, 1992). Therefore, an important research goal is to study how natural antioxidants suppress LDL peroxidation, thus decreasing atherosclerosis.

Research has shown that polyphenolic compounds in tea and grape juice can suppress LDL peroxidation (Frankel, Bosanek, Meyer, Silliman, & Kirk,

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1998; Vinson, Dabbafh, Serry, & Jang, 1995). In addition, flavonoids and phenolic acids in plants are capable of scavenging free radicals, chelating metal ions and retarding peroxidation of LDL. Hagerman et al. (1998) pointed out that tannin, a macromolecule of plant polyphenolic compounds, can be used as a good bioantioxidant to scavenge peroxyl radical. In our previous studies, Du-zhong extracts exhibited inhibitory effects on lipid peroxidation and oxidative damage in biomolecules (Hsieh & Yen, 2000; Yen & Hsieh, 1998). Good correlation between the contents of polyphenolics in Du-zhong extracts and their antioxidant activity was observed. Du-zhong tea has been found to reduce cholesterol and fatty liver (Nakasa, Yamaguchi, Okinaka, Metori, & Takashi, 1995). Nakazawa (1997) also reported that Du-zhong tea could lower blood pressure. High cholesterol and high blood pressure are related to atherosclerosis. Atherosclerosis also has a close relationship with LDL oxidative modification. Although Du-zhong extracts (tea) have been reported to lower cholesterol and hypertension, and to suppress lipid peroxidation, it is not clear whether Du-zhong can suppress LDL peroxidation. Therefore, the purpose of this study was to investigate the inhibitory effects of Du-zhong extracts on LDL oxidative modification induced by copper ion and to identify the main antioxidant compounds.

2. Materials and methods

2.1. Materials

Du-zhong (*Eucommia ulmoides* Oliv.), including leaves, raw cortex, and roasted cortex, was purchased at a local store in Taichung, Taiwan. Protocatechuic acid, ascorbic acid, butylated hydroxytoluene (BHT), phosphotungstic acid, sodium dodecyl sulfate (SDS) and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Sodium dihydrogen-phosphate, disodium hydrogen phosphate, potassium bromide and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were obtained from Wako Pure Chemical Co. (Osaka, Japan). Agarose gel, barbital buffer and Sudan black were obtained from Biomidi Co. (Toulouse, France). Copper sulfate pentahydrate ($CuSO_45H_2O$), sodium chloride (NaCl), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). *n*-Hexane, methanol, ethyl acetate, chloroform, *n*-butanol, tetrahydrofuran and *o*-phosphoric acid were obtained from J.T. Baker Co. (Phillipsburg, NJ).

2.2. Preparation of Du-zhong extracts

Leaves, raw cortex and roasted cortex of Du-zhong (20 g) were extracted with boiled water (200 ml) for 60 min. The extracts were filtered through Whatman No. 2 filter paper, and the filtrates were freeze-dried into 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.

2.3. LDL preparation

Fasting plasma, for LDL isolation, was collected from normal human volunteers in tubes containing ethylenediaminetetraacetic acid (EDTA; 1 mg/ml). LDL (d 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation using a Hitachi ultracentrifuge (Himac CS 120GX, Hitachi) as described by Yamanaka, Oda, and Nagao (1997) with a minor modification. LDL solution was flushed with N_2, stored at 4 °C, and used within 1 week after preparation. Protein was measured using a Bio-Red kit, with bovine serum albumin as a standard. For oxidation experiments, LDL was dialyzed three times against 1 l (1000-fold volume) of phosphate buffered saline (PBS, containing 0.01 M phosphate-buffer and 0.15 M NaCl, pH 7.4) in the dark at 4 °C for 24 h.

2.4. LDL oxidation

Dialyzed LDL (100 μg protein/ml) was diluted in 10 mM PBS and incubated at 37 °C in the presence or absence of 10 μM $CuSO_4$. Oxidation was performed with or without the water extracts of Du-zhong. After incubation, lipid peroxidation of the LDL was measured as described below. Ascorbic acid oxidation was used for reference.

2.5. Conjugated diene

Conjugated diene formation was measured by determining the absorbance increase at 232 nm of the solution of LDL (100 μg protein/ml) in PBS incubated with 10 μM $CuSO_4$ in the absence or presence of water extracts of Du-zhong (10 μg/ml). The absorbance was measured every 15 min for 195 min using a Hitachi U-2000 spectrophotometer, and the results were expressed as relative absorbance at 232 nm. The duration of the lag phase was calculated by extrapolating from the propagation phase.

2.6. Thiobarbituric acid reactive substances (TBARS)

TBARS were measured using the method described by Yagi (1989). A 100 μl aliquot of LDL solution (100 μg protein/ml) was mixed with 50 μl 4% (w/v) BHT, 0.5 ml of 0.3% (w/v) sodium dodecyl sulfate (SDS), 2.0 ml of 0.1 N HCl solution, 0.3 ml 10% (w/v) phosphotungstic acid, and 1.0 ml of 0.8% aqueous solution of TBA. The screw cap was closed loosely, and the test tube was set in a water bath at 100 °C for 45 min. After

cooling in an ice bath, 3.0 ml of *n*-butanol were added, and the mixture was shaken vigorously for 30 s. After centrifugation at 3000 rpm for 10 min, the fluorescence of the organic layer was measured at 555 nm (excitation wavelength at 515 nm), using a Hitachi F-3010 spectrofluorometer. In these studies, malondialdehyde (MDA) formation from 1,1,3,3,-tetraethoxypropane was used as a reference standard, and the results were expressed as nmol equivalents of MDA.

2.7. Relative electrophoresis mobility (REM)

LDL electrophoresis was performed at pH 8.6 in 0.05 M barbital buffer on Biomidi gels. The gel was stained with Sudan B black. The increased electrophoretic mobility of LDL was expressed relative to the mobility (REM) of native LDL.

2.8. Extraction and separation of Du-zhong leaf extract

The ethyl acetate-soluble extract of Du-zhong leaf extract was separated on silica gel plates (E. Merck) developed in chloroform:methanol:acetic acid = 80:20:1 (v/v/v). Plates were examined under UV light, and six major absorbing bands were scraped off, redissolved in organic solvent, evaporated and re-dissolved in methanol. The inhibitory activity of each band was assessed against copper-induced LDL oxidation. The most active band was that at Rf 0.4 (Band 2). Band 2 was further purified through preparative HPLC using a Mightysil RP-18 reversed-phase column (5 μm, 250×20 mm i.d., Kanto Chemical Co., Japan) with the same mobile phase system at a flow rate of 5.0 ml/min and an injection volume of 0.5 ml. The HPLC apparatus was a Hitachi liquid chromatograph (Hitachi Ltd., Tokyo, Japan), consisting of a model L-6200 intelligent pump, a Rheodyne model 7125 syringe-loading sample injector, a model D-2000 integrator and a model L-4200 UV-Vis detector set at 280 nm.

2.9. Spectrometry

The UV-vis absorption spectra of the active components in methanol were recorded on a Hitachi U-3000 spectrophotometer. Mass spectra of the active component were recorded using the electron impact (EI) mode at 70 eV with a JEOL JMS-SX/SX 102A mass spectrometer (Tokyo, Japan). The temperature was recorded in steps of 128°C/min from 25 to 300°C. NMR spectra were recorded with a Varian VXR-300S FT-NMR spectrometer (Harbor city, CA) operated at 299.95 MHz for ^{1}H NMR and at 75.43 MHz for ^{13}C NMR with complete proton decoupling. The spectra were observed in CD_3OD. The sweep width, pulse angle, repetition delay and acquisition time for ^{1}H NMR were 4500.0 Hz, 7.0 μs, 0 s and 2.0 s, respectively, and for ^{13}C NMR, were 25 000.0 Hz, 7.0 μs, 2.0 s and 1.0 s, respectively. The chemical shifts are reported in parts per million (ppm) from tetramethylsilane.

2.10. Antioxidant activity of extracts and identified compounds

The antioxidant activities of extracts and identified compounds were determined using the method described above. The concentrations of the samples ranged from 0 to 100 μg/ml. Ascorbic acid was used as a positive control for antioxidant activity test.

2.11. Statistical analysis

Statistical analyses were performed using the SAS software program. Analysis of variance (ANOVA) was performed. Significance ($P<0.05$) of mean differences was determined using Duncan's multiple range test.

3. Results

3.1. Effects of Du-zhong extracts on the formation of TBARS on LDL oxidation induced by Cu^{2+}

LDL oxidation was induced by Cu^{2+} at a concentration of 0–50 μM. The maximum fluorescence intensity of TBARS increased generally with concentration in the range 0–50 μM copper (data not shown). Therefore, oxidative modification of LDL (100 μg protein/ml) induced by Cu^{2+} (10 μM) was employed to study the effects of water extracts from Du-zhong on LDL

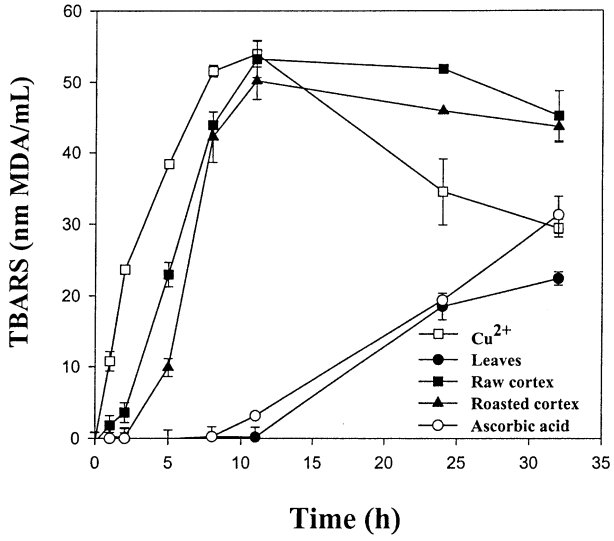

Fig. 1. Effect of water extracts from Du-zhong on Cu^{2+}-mediated TBARS formation in LDL. LDL (100 μg protein/ml) was incubated with 10 μM $CuSO_4$ at 37 °C in the absence or presence of water extracts of Du-zhong (10 μg/ml). Data represent mean±standard deviation from three experiments.

oxidation. Fig. 1 shows the inhibitory effects of Du-zhong extracts on the oxidative modification of LDL induced by Cu^{2+}. Du-zhong leaf extract showed a marked inhibitory effect, which was equal to that of ascorbic acid. In the control group with Cu^{2+} only, TBARS reached the highest concentration (52 nM MDA/ml) at 11 h of incubation. However, only a small amount of TBARS was formed in the presence of Du-zhong leaf extract. The extracts of raw cortex and roasted cortex of Du-zhong also showed inhibitory effects but were less effective than Du-zhong leaf extract.

3.2. Effects of Du-zhong extracts on the formation of conjugated dienes in LDL oxidation induced by Cu^{2+}

Fig. 2 shows the effects of Du-zhong extracts on the formation of conjugated diene from LDL oxidation induced by Cu^{2+}. The lag time was 66 min for the control group with LDL and copper ion. However, oxidative modification of LDL was suppressed when Du-zhong extracts were added, and the lag time was also extended. Du-zhong leaf extract and ascorbic acid had the best inhibitory effect on LDL oxidative modification, extending the lag time to 128 min. Raw cortex and roasted cortex of Du-zhong had smaller inhibitory effects, and the lag times were 87 and 104 min, respectively.

3.3. Effects of Du-zhong extracts on relative electrophoresis mobility (REM) in LDL oxidation induced by Cu^{2+}

When LDL is oxidized, the electronic charges on its surface become more negative. The more oxidation occurs, the more negative are the electronic charges on LDL. Differences in oxidation levels can be distinguished by means of electrophoresis. When LDL oxidation becomes more advanced, the relative electrophoresis mobility (REM) increases. As shown in Fig. 3, REM increased to 3.8 in the presence of Cu^{2+} (10 μM), compared to the REM of native LDL which is 1. However, REM decreased following the addition of Du-zhong extracts (100 μg/ml), indicating that LDL oxidation was inhibited. Based on the LDL electrophoresis pattern, Du-zhong leaf extract and ascorbic acid had the best suppressing effects on LDL oxidation. However, when Du-zhong leaf extract was diluted 10-fold (10 μg/ml), the suppressing effect was decreased, and its REM was equal to that of raw cortex and roasted cortex of Du-zhong (100 μg/ml).

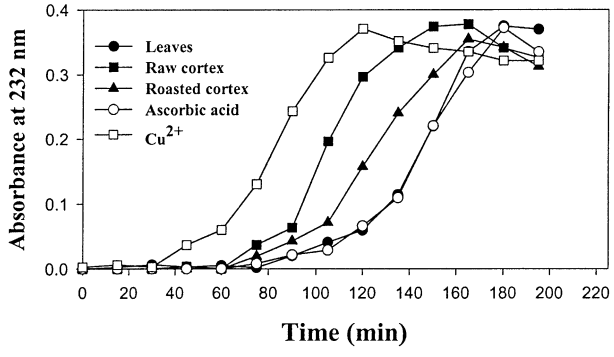

Fig. 2. Effects of water extracts from Du-zhong on Cu^{2+} mediated conjugated diene formation in LDL. LDL (100 μg protein/ml) was incubated with 10 μM $CuSO_4$ at 37 °C in the absence or presence of water extracts of Du-zhong (10 μg/ml). Conjugated diene formation was measured by determining the absorbance at 234 nm every 15 min for 195 min and the results are expressed as relative absorbance at 234 nm.

3.4. Separation of ethyl acetate soluble extract from Du-zhong extracts by preparative TLC

Du-zhong leaf extract, which had the best inhibitory effect on LDL oxidative modification, was further purified to identify its major active compounds. The ethyl acetate soluble extract obtained from Du-zhong leaf extract was separated into six bands by preparative silica gel TLC. The yield, Rf value, and colour after UV-irradiation of the separated bands are shown in Table 1. Band 2 (Rf = 0.40) had the highest yield, 42%, and bands 3–6 had similar yields, about 10%. Since the developing solvent in this system was chloroform: methyl alcohol:acetic acid 80:20:1 (v/v/v), it followed that band 2 should be the compound with higher polarity.

3.5. Effect of ethyl acetate-soluble extract of Du-zhong leaf extract on suppressing LDL oxidation induced by Cu^{2+}

The suppressing effects of Du-zhong leaf extract, the ethyl acetate soluble extract of Du-zhong leaf extract and its TLC fractions on LDL oxidation were compared, and the results are shown in Fig. 4. The suppressing effects of bands 2–4 were not significantly different from that of the ethyl acetate fractionate of Du-zhong leaf extract. Their rates of suppression on LDL oxidation were about 89%. Because of the high yield and purity of band 2, obtained by analytical HPLC, this fractionate (band 2) was further purified by

Table 1
Yield, Rf value and the colour after UV-irradiation of ethyl acetate fraction from Du-zhong leaf extract on the preparative TLC plate

Band	Yield (%)	Rf value	Colour after UV-irradiation[a]
1	8.70±0.05[b]	0.18	Light green
2	41.9±1.17	0.40	Dark purple
3	13.41±0.51	0.45	Dark green
4	10.46±0.36	0.58	Light purple
5	14.38±0.09	0.62	Light purple
6	11.15±0.63	0.70	Light brown

[a] UV-irradiation was at 254 nm.
[b] Each value is the mean of three replicate analyses.

preparative HPLC, and a compound X with higher purity, was obtained as a single peak (compound X occupied 95% of the total integral measures).

3.6. Identification of antioxidant compound

Compound X was a white powder after drying and was used for further identification. Based on UV-vis, MS, ^{1}H-NMR and ^{13}C-NMR data, compound X was identified as protocatechuic acid (PCA). The spectra of this identified compound were compared with those of the PCA standard (Sigma Chemical Co., MO) and found to be identical. The spectral characteristics of this compound were also identical to those reported by Pouchert (1978) and Zhang, Nagatsu, Okuyama, Mizukami, and Sakaibra (1998).

3.7. Effects of PCA and Du-zhong extracts on LDL oxidation induced by Cu^{2+}

A comparison of the effects of the Du-zhong extracts and of the purified compound PCA on LDL oxidative modification is shown in Table 2. LDL showed remarkable oxidative modification when a solution of 10 μM copper ions was added. The lag time for conjugated diene formation was 64.25 min; however, TBARS and REM were 51.6 nM MDA/ml and 3.8, respectively, in the incubation for 8 h. When incubated with Du-zhong leaf extract, the lag time for conjugated diene formation was 121.5, while TBARS and REM were reduced to 1.7 nmol MDA/ml and 2.0, respectively. The results also indicated that inhibitory effects of the tested samples on LDL oxidation were in the order PCA > Du-zhong leaves > ascorbic acid > roasted Du-zhong > raw Du-zhong.

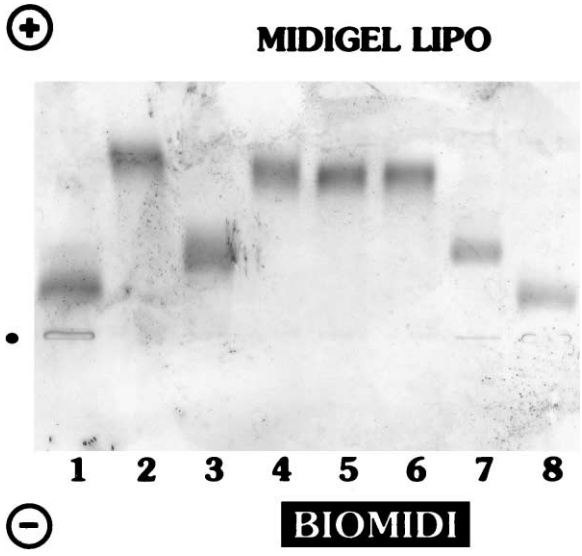

Fig. 3. The eletrophoretic pattern of human LDL incubated with Cu^{2+} with or without water extracts from Du-zhong. LDL (100 μg protein/ml) was oxidized with Cu^{2+} (10 μM) in the presence of Du-zhong extract for 24 h. Lanes 1, 8: native LDL; lane 2: LDL and Cu^{2+}; lane 3: LDL and Cu^{2+} and 100 μg/ml leaf extract; lane 4: LDL and Cu^{2+} and 10 μg/ml leaf extract; lane 5: LDL and Cu^{2+} and 100 μg/ml raw cortex extract; lane 6: LDL and Cu^{2+} and 100 μg/ml roasted cortex extract; lane 7: LDL and Cu^{2+} and 100 μg/ml ascorbic acid.

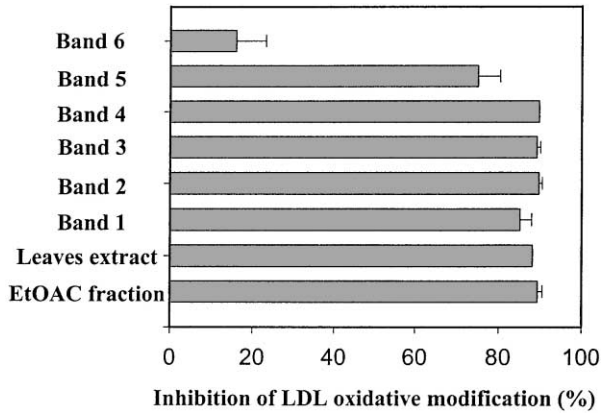

Fig. 4. Effect of Du-zhong leaf extract, ethyl acetate fraction from leaf extract and its TLC fractions on the Cu^{2+}-mediated LDL oxidation. LDL (100 μg protein/ml) was incubated with Cu^{2+} (10 μM), Du-zhong leaf extract, ethyl acetate fraction from leaf extract and its TLC fractions (10 μg/ml) for 12 h at 37 °C.

Table 2
Effect of water extracts of Du-zhong and protocatechuic acid (PCA) on the Cu^{2+}-mediated LDL oxidation

Sample	Lag time (min)[a]	TBARS (μM[b]	REM[c]
Control[d]	64.25±2.47	51.6±0.83	3.8±0.08
Leaves	121.5±9.19	1.65±0.02	2.0±0.02
Raw cortex	89.25±3.18	43.9±0.16	3.5±0.07
Roasted cortex	104.5±0.71	42.2±0.19	3.3±0.01
PCA	>200	0.26±0.00	1±0.02
Ascorbic acid	118.5±13.44	1.63±0.05	2.2±0.01

[a] LDL (100 μg protein/ml) was incubated with 10 μM $CuSO_4$ at 37 °C in the absence or presence of 10 μg/ml water extracts of Du-zhong.
[b] LDL (100 μg protein/ ml) was incubated with 10 μM $CuSO_4$ at 37 °C in the absence or presence of 10 μg/ml water extracts of Du-zhong for 8 h.
[c] LDL (100 μg protein/ml) was incubated with Cu^{2+} (10 μM) in the presence of Du-zhong extract (100 μg/ml) for 24 h. Relative electrophoretic mobility (REM) was measured relative to that of native LDL.
[d] LDL was incubated with 10 μM $CuSO_4$ without samples.

4. Discussion

LDL has been found to possess great quantities of lipids and cholesterol that can be easily oxidized by metal ions, especially Cu^{2+}. Oxidized LDL is considered to be intimately related to atherosclerosis in the heart (Esterbauer et al., 1992; Steinberg et al., 1989). In this study, Du-zhong leaf extract showed a good inhibitory effect on LDL oxidation induced by Cu^{2+}. Nakazawa (1997) reported that Du-zhong tea (water extracts of Du-zhong leaves) can lower blood pressure and the blood fat level. It can also reduce cholesterol and prevent fatty liver (Nakasa et al., 1995). Therefore, Du-zhong leaf extract may be able to help prevent atherosclerosis.

Epidemiological studies have revealed that various human diseases, such as cancer, are associated with diet. A diet rich in vegetables and fruits has a beneficial effect in preventing cancer because of the phytochemicals, such as phenolics or flavonoids, present in the vegetables and fruits (Block, Patterson, & Subar, 1992; Hollman and Katan, 1999). Numerous studies have indicated that phenolic compounds, especially phenolic acids, are the major antioxidants in red wine, vegetables and fruits that can suppress LDL oxidation (Frankel et al., 1993; Frankel, Waterhouse & Tiessedre, 1995; Meyer, Yi, Pearson, Waterhouse, & Frankel, 1997). Most of the phenolic acids in plants are hydroxycinnamic acids (e.g. chlorogenic acid and caffeic acid), hydroxybenzoic acids (e.g. protocatechuic acid), and ellagitannins. These compounds possess many physiological functions, such as antimutagenicity, antioxidant, anticancer (Tanaka, Koijim, Kawamori, Wang, Suzui, & Okamoto, 1993; Tanaka, Koijim, Kawamori, Yoshimi, & Mori, 1994).

Protocatechuic acid (PCA) has been identified as a major antioxidant compound in Du-zhong leaf extract. Its suppressing effect on LDL oxidation is greater than those of Du-zhong leaves and ascorbic acid. The yield of water extracts of Du-zhong leaves is about 10%, and Du-zhong leaf extract contains 20% phenolic compounds (Yen & Hsieh, 1998). Yen and Hsieh (1998) reported that Du-zhong leaf extract contains 1.7% PCA. Therefore, 10 g of water extracts of Du-zhong leaves can be extracted from 100 g of Du-zhong leaves. Two grams of phenolic compounds can be gathered from 10 g of Du-zhong leaf extract, which contains 170 mg of PCA. Frankel et al. (1998) indicated that grape juice contains phenolic compounds (equivalent to 10 μmol/L of gallic acid), and can suppress 62–75% of LDL oxidation. The TBARS results in the present study show that PCA can suppresses 99% of LDL peroxidation at a concentration of 10 μg/ml. Thus, daily drinking of 10 g of tea made from Du-zhong leaves can result in the ingestion of 1 g of Du-zhong leaf extract and 17 mg of PCA (equivalent to 100 μmol/L of PCA). As the results of this study show, this concentration has a good suppressing effect on LDL peroxidation in vitro.

In a study by Nakagawa, Okuda, & Miyazawa (1997), testers were given 3, 5 and 7 cups of green tea, which contained 225, 375, and 525 mg of EGCG, and 7.5, 12.5 and 17.5 mg of ECG, respectively. The EGCG and ECG contents in the plasma were about 0.2–2.0% of the original intake after 90 min. This level in the blood should have a certain antioxidant effect. Therefore, it would be worthwhile to study the antioxidant effect in vivo of Du-zhong tea containing 17 mg PCA from 10 g Du-zhong leaves. PCA, a simple phenolic compound existing in many edible vegetables and fruits, is considered to be a strong antioxidant (Ueno, 1993). Ohnishi et al. (1997) reported that PCA could effectively suppress diverticulum cancer on hamster cheek induced by 7,12-dimethylbenzaanthracence and suppress skin cancer induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Therefore, PCA is considered a good chemoprevention agent (Tseng et al., 1998). In this study, we also proved that PCA in Du-zhong leaf extract plays an important role in suppressing LDL oxidative modification induced by copper ions.

Phenolic compounds in red wine have been thought to be important compounds for suppressing atherosclerosis (Fuhrman, Lavy, & Aviram, 1995). Phenolic compounds in wine have also been used to explain the "French paradox": the fact that the French are extravagant in eating and drinking but have less CHD than people in other North American or European countries. The fact that phenolic compounds in red wine can suppress LDL peroxidation might be due to their chelating ability on metal ions and scavenging ability on free radicals (Abu-Amsha, Croft, Puddey, Proudfoot, & Beilin, 1996). Yoshino and Murakami (1998) also reported those phenolic compounds such as protocatechuic acid and chlorogenic acid can form an inactive Fe^{2+}-polyphenol complex with Fe^{2+}. This complex can be used as a good antioxidant because it does not react with oxygen, while protocatechuic acid and chlorogenic acid can scavenge reactive oxygen species. Tseng, Wang, Kao, and Chu, (1996) reported that PCA can suppresses cell toxicity and genotoxicity of liver cells induced by *t*-butylhydroperoxide, and ascribed this action to its free radical-scavenging activity. In this study, PCA was found to be an important compound in Du-zhong leaf extracts for inhibition of LDL oxidative modification. The mechanisms involved could also be the ability to chelate metal ions and to scavenge free radicals.

Although PCA has good antioxidant activity in vitro, there are no reports about its antioxidant activity in vivo. However, Tsuda, Horio, and Osawa (1999) reported that the aglycone of cyanidin was metabolized to protocatechuic acid in vivo, and claimed that PCA is a principal antioxidant in blood serum. This seems to

prove, indirectly, that PCA possesses antioxidant activity in vivo. Based on the results of this study, Du-zhong leaf extract possesses good ability to inhibit LDL oxidative modification. Moreover, PCA plays an important role in Du-zhong leaf extract in suppressing LDL oxidation. Thus, drinking Du-zhong tea daily should offer some protection against diseases caused by free radicals and reactive oxygen species.

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