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Characterization of antioxidants present in hawthorn fruits

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Abstract

Hawthorn fruit extract has been shown to have many health benefits including being cardiovascular protective, hypotensive and hypocholesterolemic. The present study was carried out to characterize further the antioxidants of hawthorn fruit and their effect on the oxidation of human low density lipoprotein (LDL) and α-tocopherol. The dry hawthorn fruit was extracted successively with ether, ethyl acetate, butanol and water. The ethyl acetate fraction was only effective in inhibition of Cu^{+2}-mediated LDL oxidation. The column chromatographic separation led to isolation of eight pure compounds; namely, ursolic acid, hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid. All of these phenolic compounds, except ursolic acid, were protective to human LDL from Cu^{+2}-mediated LDL oxidation. They were also effective in preventing the peroxy free radical-induced oxidation of α-tocopherol in human LDL. The inhibitory effect of these compounds on oxidation of LDL and α-tocopherol was dose-dependent at concentrations ranging from 5 to 40 μM. In addition, supplementation of 2% hawthorn fruit powder significantly elevated serum α-tocopherol by 18–20% in rats fed a 30% polyunsaturated canola oil diet, as compared with the control. The present results suggest that part of the mechanism for cardiovascular protective effects of hawthorn fruit might also involve the direct protection to human LDL from oxidation or indirect protection via maintaining the concentration of α-tocopherol in human LDL.

Keywords: Flavonoid; Hawthorn fruit; Low density lipoprotein; α-Tocopherol

1. Introduction

Hawthorn fruit refers to the bright red berries of *Crataegus* species. It has long been used as a folk medicine. Pharmacological and toxicological studies have demonstrated that consumption of hawthorn fruits is associated with long-term medicinal benefits to cardiovascular function with little side effect [1–3]. Hawthorn extract has been used to treat the early stages of congestive heart failure [4,5] and angina pectoris [6]. Hawthorn extract was clinically effective in reducing blood pressure and total plasma cholesterol [7]. Mechanisms of these protective effects of hawthorn extract remain poorly understood, but the antioxidants present in hawthorn are thought to be involved because they reduce the production of free radicals, alleviate subsequent damage to the heart tissue and decrease the arterial deposition of cholesterol. However, the antioxidants present in hawthorn fruit have not been fully characterized.

The oxidative modification of human low density lipoprotein (LDL) may play an important role in contributing to the development of atherosclerosis [8–14]. This hypothesis is supported by the observations that (a) oxidatively modified LDL (ox-LDL) was present in atherosclerosis plaque but absent in normal artery wall [9]; (b) ox-LDL was taken up by macrophage scavenger receptors, promoting cholesterol ester accumulation and foam cell formation [10]; (c) ox-LDL was toxic to endothelial cells and stimulated monocyte adhesion to endothelium [10]. α-Tocopherol (the major form of vitamin E) is a primary antioxidant that protects LDL from oxidation [15,16]. Increased vitamin E intake has been shown to decrease oxidation of LDL, platelet adhesion and thrombosis [17–23]. Epidemiological data also demonstrated that vitamin E supplementation was associated with a lower risk of coronary heart disease [23–25]. Growing evidence for the protective effect of vitamin E in humans also included an inverse association between plasma α-tocopherol and the mortality from cardiovascular heart disease [26–27].

Hawthorn fruits are a rich source of flavonoids [28]. The

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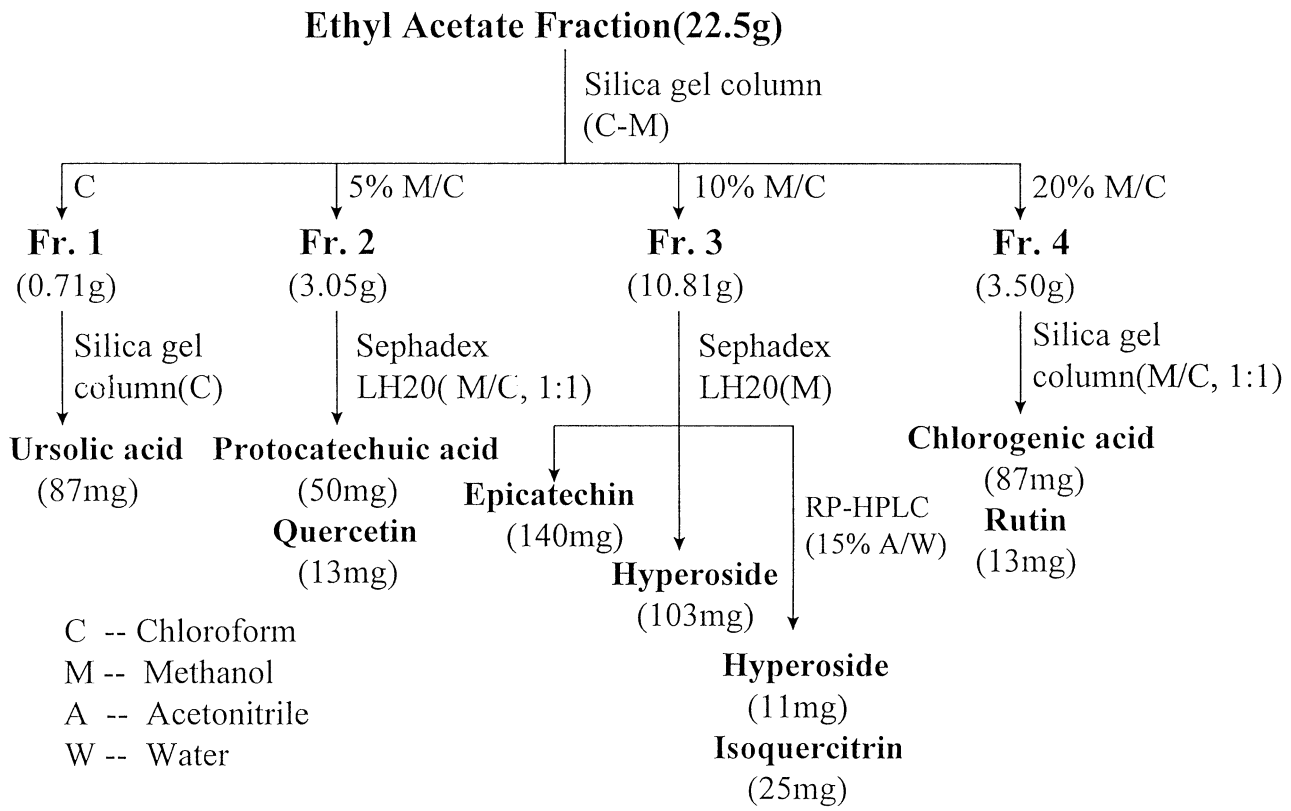

Figure 1. Separation and purification of phenolic compounds from the ethyl acetate fraction of hawthorn fruit.

daily intake of flavonoids in humans is estimated to be as much as 1 g [29–30]. Flavonoid consumption has been documented to be negatively associated with coronary heart disease mortality [31]. Although hawthorn extract has been widely documented to possess cardioprotective, hypotensive and hypocholesterolemic activity, there has been no study to date that examines the effect of hawthorn extract on lipoprotein and α-tocopherol oxidation in human LDL. The present study was designed to quantify the flavonoids present in hawthorn fruits and examine the antioxidant activity of these flavonoids on human LDL and α-tocopherol oxidation.

2. Materials and methods

2.1. Extraction, isolation and identification of hawthorn flavonoids

Hawthorn fruits (*Crateagus pinnatifida*) were collected from Xingling County of Hebei Province, China. The seeds were then removed and the fruit flesh was freeze-dried followed by being ground into powder in a coffee grinder. Ding et al. [32] studied the chemical composition of hawthorn leaves started with extraction using 80% ethanol. In the present study, the fruit powder (1.5 kg) was also extracted three times with 80% ethanol (5 L) for 24 h at room temperature. The pooled ethanol filtrates were concentrated using a vacuum rotary evaporator. The extract was then dissolved in 4 L of water and extracted subsequently using ether (2 L $\times$ 4), ethyl acetate (2 L $\times$ 4) and butanol (2 L $\times$ 4) in an order of increasing solvent polarity. The solvents in three exracts were then evaporated to produce 18 g of ether fraction, 22.5 g ethyl acetate fraction and 94.5 g butanol fraction. The remaining water layer was also freeze-dried to produce a mass of 498 g.

The preliminary LDL-oxidation test described below showed that only the ethyl acetate fraction exhibited strong antioxidant activity. Therefore, only the ethyl acetate fraction was chosen and applied onto a silica-gel column (15.5 $\times$ 8.0 cm i.d.) for further isolation of antioxidants. The column was eluted with 0% (4.0 L), 5% (4.5 L), 10% (5.5 L) and 20% (3.3 L) methanol in chloroform (in an order of increasing polarity) to obtain four fractions as shown in Fig. 1. Fraction 1 was reapplied onto a silica gel column (19 $\times$ 2.0 cm i.d.) and eluted with chloroform to obtain 87 mg of ursolic acid (260–380 ml) at a flow rate of 0.8 ml/min. Fraction 2 was subjected to a Sephadex LH 20 column (32.5 $\times$ 3.5 cm i.d.) and eluted with a mixture of methanol and chloroform (1:1, v/v) at a flow rate of 1 ml/min to obtain 50 mg of protocatechuic acid (630–770 ml) and 13 mg quercetin (770–920 ml). When Fraction 3 was applied onto a Sephadex LH 20 column (32.5 $\times$ 3.6 cm i.d.) and eluted

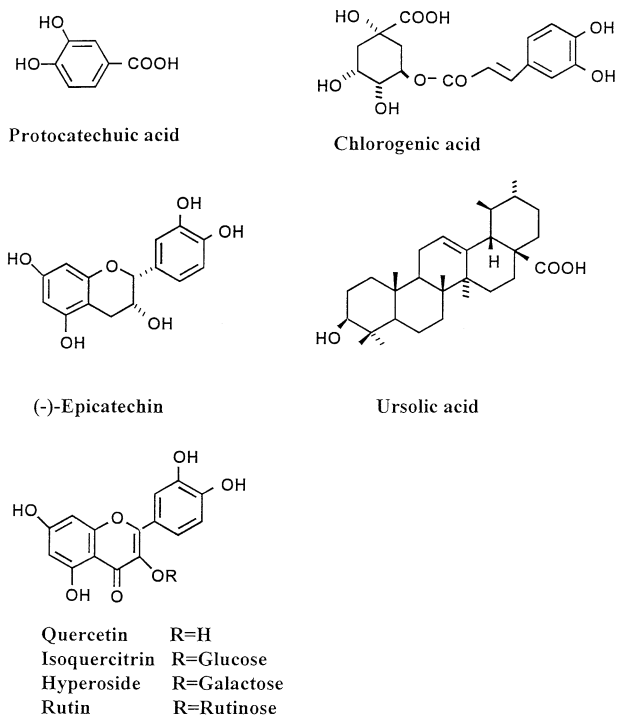

Figure 2. Chemical structures of chlorogenic acid, epicatechin, hyperoside, isoquercitrin, protocatechuic acid, quercetin, rutin and ursolic acid.

with methanol at a flow rate of 3.0 ml/min, 140 mg of epicatechin (1590–2350 ml), 103 mg of hyperoside (2640–3030 ml) and a mixture of hyperoside and isoquercitrin (3030–3750 ml) were obtained. The mixture was further separated on a C-18 high performance liquid chromatography (HPLC) column (Waters Nove-Pek C18 Cartridge, 10 × 0.8 cm i.d., 4 μm) and eluted with a mixture of acetonitrile and water (15:85, v/v) in a flow rate of 3.5 ml/ml to obtain 25 mg of isoquercitrin (6.3 min). When Fraction 4 was applied onto a silica gel column (13 × 4.2 cm i.d.) and eluted with a mixture of methanol and chloroform (1:1, v/v) in a flow rate of 1.2 ml/min, 87 mg of chlorogenic acid (360–540 ml) and 13 mg of rutin (1080–1260 ml) were obtained. Flavonoids partially exist in fruits in forms of their glycosides. It is possible that some of the flavonoids purified were derived from the hydrolysis of their glycosides during the extraction and purification process.

Eight compounds purified from ethyl acetate fraction were subjected to verification of their chemical structures on the basis of their spectra data of UV, Liquid Chromatography-Mass Spectrometer (LC-MS), ^{1}H-NMR and ^{13}C-NMR, and comparison with those of published data [33–37] and reference compounds (Fig. 2).

2.2. LDL isolation

Fresh serum was collected and pooled from healthy subjects (n=20) at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. Ethylenediaminetetraacetic acid (EDTA) (0.1%) and NaN_3 (0.05%) solutions were immediately added to prevent the lipoprotein from oxidative modification. LDL was isolated from plasma according to the method describe elsewhere [38]. In brief, serum was first centrifuged at 1500 g for 15 min to remove cells and cell debris. The density was then increased to 1.019 by addition of a NaCl-KBr solution (dissolve 153 g NaCl, 354 g KBr and 100 μg EDTA in 1 L of H_2O; density 1.33 g/ml) and recentrifuged at 160,000 g at 4°C for 20 h. After removal of the top layer containing chylomicrons and very low density lipoproteins, the density of the remaining serum fractions was increased to 1.064 and recentrifuged at 160,000 g for an additional 24 h. The top LDL fraction was collected and then flushed with nitrogen and stored at −70°C. The protein content of isolated LDL was determined using Lowry's method [39]. The total LDL cholesterol and triacylglycerols were measured using Sigma enzymatic kits. The LDL fraction contained 5.2 mg protein, 2.1 mg cholesterol and 0.8 mg triacylglycerols per milliliter.

2.3. LDL oxidation

The stock LDL fraction (5 mg protein/ml) was dialyzed against 100 volume of the degassed dialysis solution (pH 7.4) containing 0.01 M sodium phosphate, 0.9% NaCI, 10 μM EDTA and 0.05% NaN_3 in the dark for 24 h. The dialysis solution was changed four times. Then, the dialyzed LDL was diluted to 250 μg protein/ml with 0.01 M sodium phosphate buffer (pH 7.4). Oxidation of LDL was conducted as previously described by Puhl et al. [40]. For the control incubation tubes, 0.4 ml LDL (250 μg/ml) was mixed with 50 μl of 50 μM $CuSO_4$ solution and 50 μl of 0.01 M sodium phosphate buffer (pH 7.4), and incubated at 37°C for up to 24 h. For the experimental tubes, 0.4 ml LDL (250 μg protein/ml) was preincubated with 50 μl of varying concentrations of individual hawthorn phenolic compounds for 5 min. Then, 50 μl of 50 μM $CuSO_4$ solution was added to initiate the oxidation, followed by incubation at 37°C for up to 24 hr. The oxidation was then stopped by addition of 25 μl of 1.0% EDTA and cooled at 4°C. The degree of LDL oxidation was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS), as previously described by Buege and Aust [41]. The LDL-incubated tubes were immediately added with 2 ml of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCL solution. The incubation mixture was then heated at 95°C for 1 h, cooled on ice and centrifuged at 1000 g for 20 min. TBARS were then determined by measuring the absorbance at 532 nm. The calibration was done using a malondialdehyde (MDA) standard solution prepared from tetramethoxylpropane. The value of TBARS was expressed as nmol MDA/mg LDL protein.

2.4. Protection of hawthorn fruit flavonoids to α-tocopherol in LDL

The stock LDL was dialyzed as described above. Oxidation of α-tocopherol in LDL was induced by addition of 1 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), a peroxyl free radical initiator. In brief, the LDL (150 μg protein/ml) was incubated in sodium phosphate buffer (pH = 7.4, 10 mM) with constant stirring at 40°C. The varying amounts of hawthorn phenolic compounds were added together with 1.0 mM AAPH. An aliquot of the incubation solution (1 ml) was periodically taken and chilled in ice. After immediate addition of 1 ml of ethanol containing 0.5 mg of butylated hydroxyltoluene as an antioxidant and 1.0 μg of tocopherol acetate as an internal standard, the mixture was immediately extracted using 2 ml of hexane. The hexane was evaporated under a gentle stream of nitrogen and the resulting extracts were redissolved into 100 μl of ethanol followed by HPLC analysis for determination of the remaining α-tocopherol in LDL.

α-Tocopherol in LDL was determined as described previously [42] using a Shimadzu LC-10AD HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. In brief, 10 μl of the extracts in ethanol derived from 1 ml of the LDL incubation solution was injected onto a C-18 column (Microsorb MV, 250 × 4.6 mm, 5 μm i.d., Rainin, Woburn, MA, USA) via a rheodyne valve (20 μl capacity, Cotati, CA, USA). Methanol was used as an eluting solvent at a flow rate of 2 ml/min. α-Tocopherol was monitored using a diode detector at either 200 nm or 280 nm and quantified according to the amount of α-tocopherol acetate added. The initial concentration of α-tocopherol in the final LDL incubation solution was found to be 1.5 μM (0.7 μg/ml).

2.5. Effect of hawthorn fruit supplementation on serum α-tocopherol in rats

To investigate whether hawthorn fruit is protective to α-tocopherol in vivo, male Spraguge-Dawley rats (130–150 g, $n = 32$) were randomly divided into two groups. The control group was fed a semipurified diet containing (100 g): 29.3 g cornstarch, 10 g sucrose, 20 g casein, 30 g canola oil, 5 g cellulose, 4 g AIN-76 mineral mix, 0.4 g choline bitartrate, 1 g AIN-76A vitamin mix and 0.3 g DL-methionine. The hawthorn group was fed the same diet supplemented with 2% hawthorn fruit powder. The two diets were isocaloric. In view of the fact that rats fed a diet containing 30% polyunsaturated perilla oil had a lower level of plasma α-tocopherol, and that supplementation of green tea catechin led to a onefold increase in plasma α-tocopherol [43], we hypothesized that hawthorn fruit flavonoids were also capable of elevating plasma α-tocopherol in rats fed a 30% polyunsaturated canola oil.

All animals were allowed free access to tap water. Eight animals from each group were killed at the end of 3 and 6 weeks after overnight fasting. The blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1300 *g* for 10 min and serum was then collected. α-Tocopherol was then measured using the HPLC method as described above.

2.6. HPLC analysis of hawthorn fruit antioxidants

To quantify these phenolic compounds present in hawthorn fruits, a HPLC method was developed using a Backman Gold HPLC system (Model 126) equipped with a photodiode array detector (Model 168), an autosampler (Model 507 E) and a Gold Nouvean analytical workstation. The samples of ether, ethyl acetate, butanol and water fractions were injected onto a HPLC column (Waters Nove-Pek C18 cartridge, 8 × 100 mm, 4 μm i.d.) and eluted at a flow rate of 1 ml/min with a gradient mobile phase (pH 2.4) composed of solvent A (25 μM NaH_2PO_4 buffer containing 5% acetonitrile) and B (25 mM NaH_2PO_4 containing 25% acetonitrile). Ratio of A to B was changed from 9:1 to 2:8 during the first 20 min, then held for 35 min and changed back to 9:1 within 5 min. The eluting peaks were monitored at both 278 nm and 360 nm (Fig. 3). The content of each compound in the four fractions was pooled and calculated based on 100 g dry fruit powder. To simplify the presentation, only the HPLC profile from the ethyl acetate fraction is shown (Fig. 3).

2.7. Statistics

Data are expressed as means ± S.D. Where applicable, analysis of variance was used for statistical evaluation of significant differences among the control and hawthorn fruit-supplemented groups using Sigmastat (Jandel Scientific Software, San Rafael, CA, USA). Differences were considered significant when $P < 0.05$.

3. Results

The antioxidant activity of the ether extract, ethyl acetate, butanol and water fractions derived from hawthorn fruits was first compared in LDL incubation under the same conditions. It was found that the ethyl acetate fraction possessed strong protection to LDL. The ethyl acetate fraction was therefore targeted for isolation and purification of hawthorn fruit antioxidants. The column chromatographic separations led to the purification of eight pure compounds (Figs. 2 and 3). As shown in Table 1, the HPLC analysis found that epicatechin was most abundant (178 mg/100 g dry fruit), followed by chlorogenic acid (65 mg/100 g dry fruit), hyperoside (25 mg/100 g dry fruit), isoquercitrin (13 mg/100 g dry fruit), protocatechuic acid (3 mg/100 g dry fruit), rutin (3 mg/100 g dry fruit) and quercetin (1 mg/100 g dry fruit).

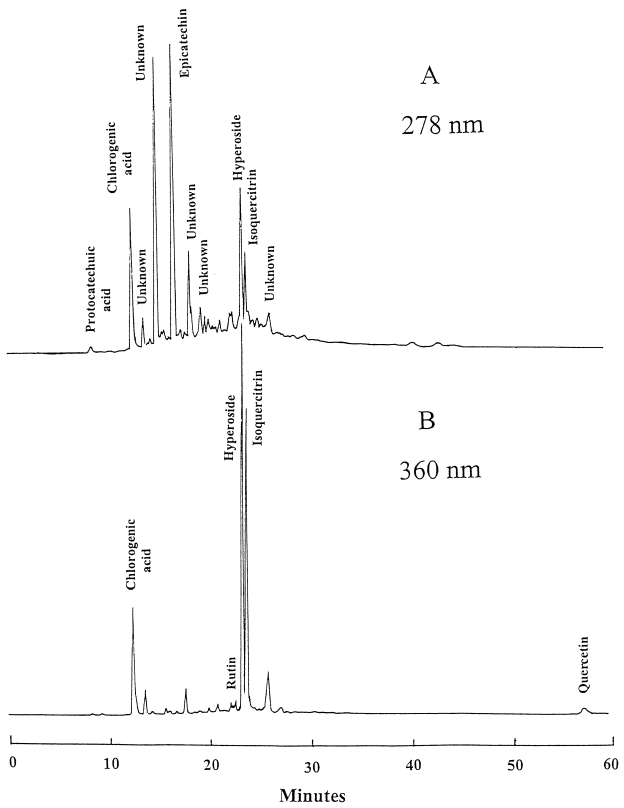

Figure 3. High performance liquid chromatography profile of hawthorn phenolic compounds present in the ethyl acetate extract. See text for the conditions and Table 1 for percentage composition.

The eight compounds purified from the ethyl acetate fraction demonstrated varying antioxidant activity (Fig. 4). Ursolic acid showed no antioxidant activity, whereas hyperoside was most protective to human LDL (lag time = 9.5 h), followed by quercetin (lag time = 6.5 h) and isoquercitrin (lag time = 6.0 h; Fig. 4). Under the same experimental conditions, the antioxdiant activity of epicatechin, chlorogenic acid and rutin (lag times = 4–5 h) was similar, but was much weaker than that of hyperoside, quercetin and isoquercitrin (Fig. 4).

The seven pure compounds (namely, protocatechuic acid, chlorogenic acid, epicatechin, quercetin, rutin, isoquercitrin and hyperoside) isolated from hawthorn fruits demonstrated a dose-dependent antioxidant activity in Cu^{+2}-promoted LDL oxidation (Fig. 5, P for trend = .01). LDL was oxidized significantly within 4 h at the absence of these hawthorn antioxidants. In most cases, addition of 5–10 μM of these hawthorn antioxidants extended the lag-time to more than 8 h. In the presence of 20 and 40 μM of hawthorn antioxidants, LDL showed little or no oxidation throughout the period of 24 h (Fig. 5).

Table 1
Content of some phenolic compounds present in hawthorn fruit

Phenolic compound	Content (mg/100 g dry fruit)
Epicatechin	178.3 ± 6.6
Chlorogenic acid	64.9 ± 2.0
Hyperoside	24.6 ± 1.1
Isoquercitrin	13.4 ± 0.5
Protocatechuic acid	3.2 ± 0.1
Rutin	2.6 ± 0.1
Quercetin	0.9 ± 0.1

Values are the average of three separate extractions.

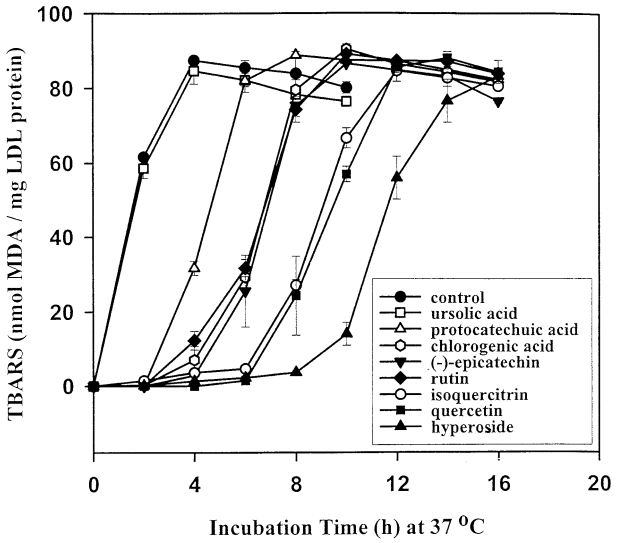

Figure 4. Inhibitory effect of hawthorn phenolic compounds (5 μM) isolated from ethyl acetate extract on production of thiobarbituric acid-reactive substances (TBARS) in Cu^{+2}-mediated oxidation of human low density lipoprotein (LDL). The LDL (100 μg protein/ml) was incubated in sodium phosphate buffer (pH 7.4) containing 5 μM $CuSO_4$. The oxidation was conducted at 37°C. Data are expressed as means ± S.D. of six to eight samples.

Protective effect of the eight hawthorn compounds purified from ethyl acetate on α-tocopherol in human LDL was also examined (Fig. 6). Ursolic acid demonstrated no protection to α-tocopherol. This was in agreement with that observed in the TBARS test (Fig. 4). The remaining seven phenolic compounds demonstrated varying protective activity, with chlorogenic acid being strongest, followed by hyperoside, quercetin and rutin. In contrast, the protective activity of isoquercitrin and protocatechuic acid was very similar, but was much weaker than that of chlorogenic acid, hyperoside, quercetin and rutin. The seven hawthorn antioxidants isolated from ethyl acetate also demonstrated a dose-dependent protecting activity to α-tocopherol in LDL (Fig. 7).

Supplementation of hawthorn fruit (2%) significantly increased serum α-tocopherol in rats (Fig. 8). At the end of 3 weeks, serum α-tocopherol in the hawthorn fruit-supplemented group was increased by 18% compared with that of the control rats. At the end of 6 weeks, serum α-tocopherol in the hawthorn fruit-supplemented group was increased by 20% compared with that of the control rats (Fig. 8). There were no differences in food intake, body and organ weights

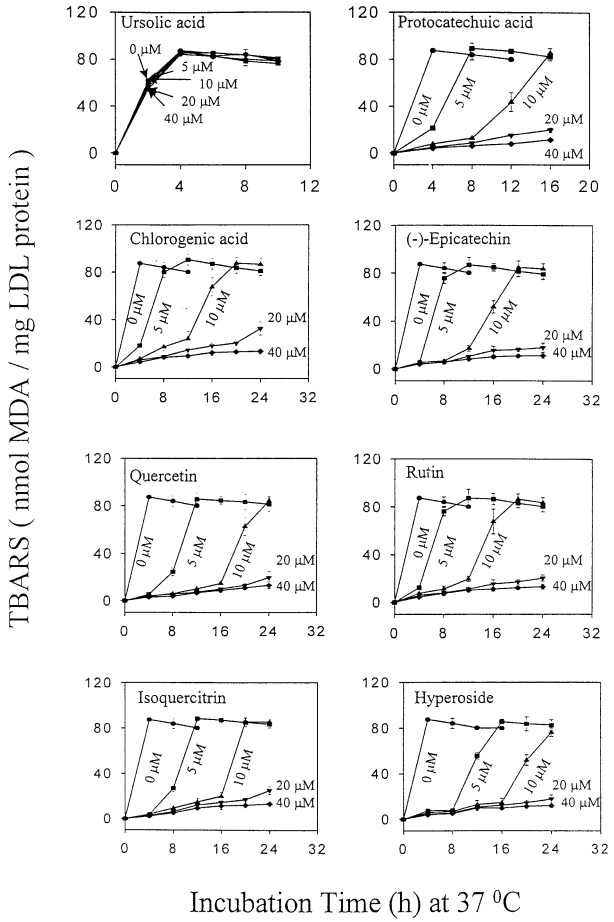

Figure 5. Dose-dependent inhibitory effect of hawthorn phenolic compounds isolated from ethyl acetate extract on production of thiobarbituric acid-reactive substances (TBARS) in Cu^{+2}-mediated oxidation of human low density lipoprotein (LDL). The LDL (100 μg protein/ml) was incubated in sodium phosphate buffer (pH 7.4) containing 5 μM $CuSO_4$. The oxidation was conducted at 37°C. Data are expressed as means ± S.D. of six to eight samples.

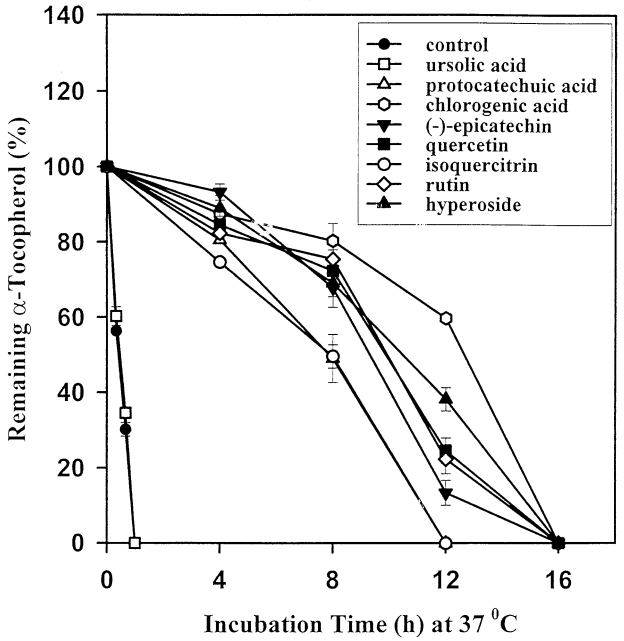

Figure 6. Protective effect of hawthorn phenolic compounds (5 μM) isolated from ethyl acetate extract on α-tocopherol in human low-density lipoprotein (LDL). The LDL (150 μg protein/ml) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol in LDL was induced by 1 mM of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 40°C. Data are expressed as means ± S.D. of six to eight samples.

(liver, heart, kidney, brain and perirenal adipose tissue) between the two groups (data not shown). Serum total cholesterol and triacylglycerols were also analyzed but no significant differences were observed (data not shown). The LDL oxidation in serum of the two groups was not measured due to the lack of serum after the above analyses.

4. Discussion

Many pharmacological studies of hawthorn fruit focus on its cardiovascular protective activity [1–3]. The hawthorn fruit has also been shown to be hypotensive and hypocholesterolemic [4–7]. However, mechanisms of these beneficial effects are poorly understood. Dietary antioxidants may reduce the initiation and propagation of free radicals in vivo and, therefore, minimize the free radical-induced damage to the heart tissue and cardiovascular vessels. Many studies suggest that oxidation of human LDL is one of the risk factors in development of cardiovascular disease [8–14]. The present results demonstrated that hawthorn fruit was abundant in flavonoid antioxidants.

These compounds purified from hawthorn fruits (namely, chlorogenic acid, epicatechin, hyperoside, isoquerecitrin, protocatechuic acid, quercetin and rutin) were protective to human LDL. α-Tocopherol is a primary antioxidant protecting human LDL from oxidative modification [15,16] Several studies suggest that vitamin E supplementation is associated with a lower risk of coronary heart disease in both men and women [23–25]. The population with a high level of plasma α-tocopherol have a low mortality rate of cardiovascular disease [26–27]. The present results clearly demonstrated that there were at least seven antioxidants present in hawthorn fruits. These compounds were effective in protecting α-tocopherol from free radical-induced degradation. If the consumption of hawthorn fruit is associated with a significantly lower risk of cardiovascular disease in humans, part of the mechanism may involve the protective role of these antioxidants to α-tocopherol in human LDL.

The biochemical mechanism by which these seven hawthorn phenolic antioxidants inhibit LDL oxidation could be either one or a combination of the following possibilities. First, these phenolic compounds may function as chelators to inactivate the Cu^{+2} involved in the initiation of free radicals. The spectroscopic study conducted by Brown et al. [44] indicated that most of these phenolic compounds are

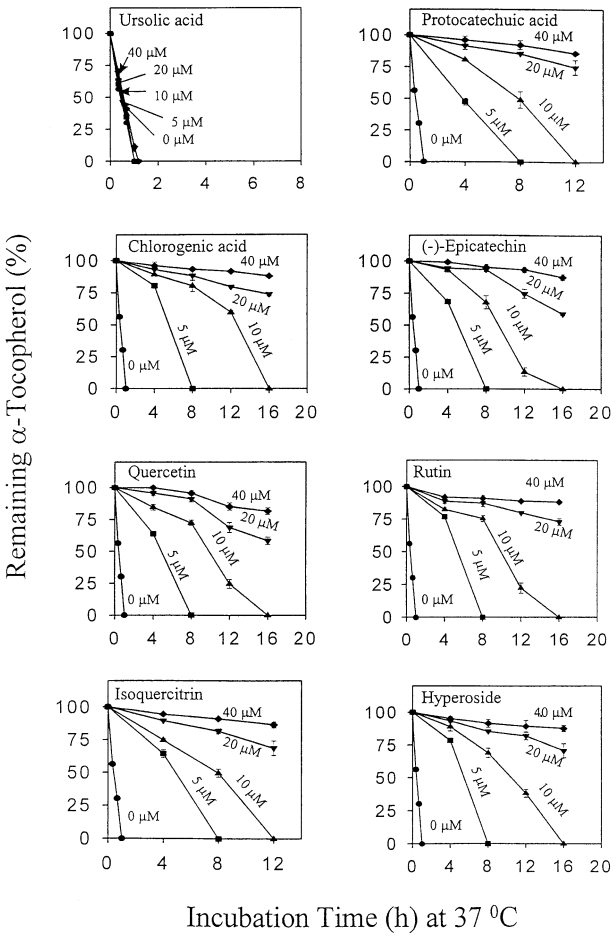

Figure 7. Dose-dependent protective effect of hawthorn phenolic compounds isolated from ethyl acetate extract on α-tocopherol in human low density lipoprotein (LDL). The LDL (150 μg protein/ml) was incubated in sodium phosphate buffer (pH 7.4), and the oxidation of α-tocopherol in LDL was induced by 1 mM of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 40°C. Data are expressed as means ± S.D. of six to eight samples.

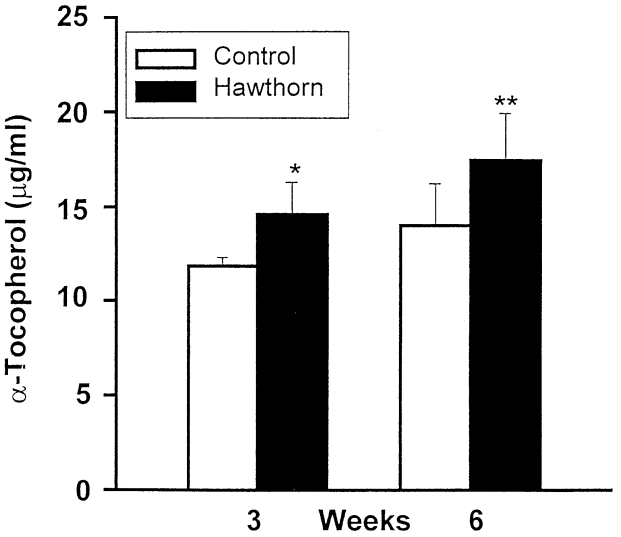

Figure 8. Effect of hawthorn fruit powder supplementation (2%) on serum α-tocopherol in rats. Values are means ± S.D., $n = 8$. Means at a given time point differ significantly, * $P < .05$; ** $P < .01$.

capable of chelating Cu^{+2} ion. Second, they function as a primary antioxidant by directly quenching and reducing the formation of free radicals by Cu^{+2} ion or by maintaining and sparing α-tocopherol. When AAPH was used to replace Cu^{+2} in generating peroxyl free radicals, these phenolic compounds still demonstrated strong protection to α-tocopherol in human LDL incubation, suggesting the validation of the second possibility. To demonstrate this, we have been able to show that supplementation of hawthorn fruit powder significantly elevated serum α-tocopherol, implying that hawthorn fruit possesses the protective activity to α-tocopherol both in vitro and in vivo.

The present study supplemented 2% hawthorn fruit powder in the diet. The amount of flavonoids in the diet was comparable to that commonly consumed by humans. The Zutphen Elderly Study [31] showed that the flavonoid intake in humans could reach 30 mg/day/person; that is, 0.012 mg flavonoids/kcal, provided that a total 2500 kcal is ingested per person (30 mg/2500 kcal = 0.012). In the present study, the rats consumed approximately 20 g diet/day, which is equivalent to 92.4 kcal/day. The total flavonoid intake would be 1.12 mg/day/rat based on the data shown in Table 1 (2% × 20 g diet × 2.8 mg/g = 1.12). Thus, 2% hawthorn fruit supplementation would achieve the flavonoid intake of 0.012 mg/kcal (1.12 mg ÷ 92.4 kcal = 0.012).

The seven antioxidants identified and purified from hawthorn fruit are typical phenolic compounds (Fig. 2). These aromatic compounds are synthesized in plants from the aromatic amino acids phenylalanine and tyrosine, and acetate unit [45]. They are primarily part of pigments in hawthorn and other fruits. The content and profile of phenolic compounds present in different fruits and vegetables have been reviewed thoroughly by Beecher [46]. The structure of these hawthorn phenolic compounds is characterized by having two adjacent hydroxyl groups. An antioxidant, in general, should be an excellent donor of electrons or protons, and the resulting free radical should be relatively stable. In this regard, the two adjacent hydroxyl groups of hawthorn fruits are theoretically more vulnerable to loss of a proton, and the resulting free radical is stable due to resonance delocalization.

It is known that anthocyanidins are natural pigments abundant in fruits and possess antioxidant activity [47]. The present work focused mainly on the isolation of antioxidants from the ethyl acetate fraction because it had stronger antioxidant activity than other fractions. Anthocyanidins cannot be extracted by using the ethyl acetate but they are water soluble. They may be present in water and butanol fractions. It is possible that anthocyanidins are another type of antioxidant present in hawthorn fruit, and deserve further study.

In conclusion, the present study supported the view that

hawthorn fruit contains active antioxidants of the phenolic type. The column chromatographic separation led to isolation of seven antioxidants (namely, hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid). They not only protected LDL and α-tocopherol from oxidation but also increased the level of serum α-tocopherol in rats fed a 2% hawthorn fruit diet, as compared with the control.

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