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Effect of loquat (*Eriobotrya japonica*) extracts on LDL oxidation

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Abstract

The antioxidative activity of the extracts of loquat fruits, *Eriobotrya japonica* Lindley was examined. Loquat fruit was separated into three parts (peel, flesh and seed), and each part was extracted with either water or ethanol. The extracts were then assessed for their free radical scavenging ability and effects on the oxidation of human low density lipoprotein (LDL) *in vitro*. Results in this study show that the ethanol extracts of all three loquat parts and the water extract of the peels exhibited a strong ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Among extracts of different loquat parts, the ethanol extract of loquat seeds was the most potent one. The ethanol extract of the seed was also effective in suppressing the oxidation of linoleic acid which was demonstrated by a slow discoloration of β-carotene/linoleic acid conjugation system. The ethanol extract of loquat seeds as compared to other extracts could also suppress significantly the 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN)-induced LDL oxidation. When the content of total polyphenolic compounds in different loquat parts (peel, flesh and seed) was examined, a significantly higher level of total polyphenols was found in the seed than the peels and flesh. Using reverse-phase HPLC-ESI EM analysis, significant levels of polyphenolic compounds such as chlorogenic acid, cyanidine glucoside, epicatechin, epigalocatechin gallate and procyanidin B_2 in the ethanol extract of different loquat parts were identified and quantified. The latter two compounds were found mainly in the ethanol extract of loquat seeds, but not in peels and flesh. Therefore, it is suggested that the high ability to scavenge free radicals and suppress the LDL oxidation exerted by the ethanol extract of loquat seeds was at least in part due to the high content of polyphenolic compounds in the seeds.

Keywords: Free radical scavenging activity; Antioxidants; Flavonoids; Polyphenols; EGCG; Procyanidins; Chlorogenic acid; Cyanidine glucoside

1. Introduction

Loquat, *Eriobotrya japonica* (Lindley), is an Asian fruit. It is a member of the Rosaceae family, which includes apples, pears, and quinces. Loquat is believed to have originated from China, where it is called "pipa". Since antiquity, it has been cultivated in Japan and has become an important food crop. In Japan, its fruit is called "biwa", and is also known as the Japanese medlar or Japanese plum. They are also found in northern India, the Mediterranean region, England, Madagascar and North, Central and South America (Morton, 1987). Loquat fruits are consumed largely as fresh fruit, but recently significant amounts of loquat fruits in Japan are being used as the main ingredient in various processed food products, such as jellies and jams. Normally, flesh is the only part used for food processing. However, both loquat fruit and leaves are often included in Chinese herbal remedies for cough and asthma. In traditional Japanese lore, loquat seed is called "good for health". Village farmers soak the loquat seeds in alcoholic drinks. They believe that this type of drink is good for the health. To date, studies examining the beneficial effects of loquat fruit are scarce. Consumption of apples has been shown to exert protective effects against many chronic diseases, such as cardiovascular

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disease, and positive health effects are attributable to their high flavanoid contents (Roger, 1988; Middleton & Kandaswani, 1992; Cook & Samman, 1996). Thus, it is of interest to examine whether loquat of the same pome family could exhibit similar antioxidant activity. In this study, loquat fruit was separated into three parts (peel, flesh, and seed). Aliquot of each part was extracted with either water or ethanol. The extracts were then assessed for their antioxidant activity, their protective effect on *in vitro* LDL oxidation. Tentative identification of active components present is also reported.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-*t*-butyl-4-hydroxyanisole (BHA), linoleic acid, β-carotene, Tween 40, 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) and ascorbic acid were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). 2-(*N*-morpholine)ethanesulphonic acid (MES) was also purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chlorogenic acid was supplied by Sigma (St. Louis, MO, USA). (−)-Epicatechin, cyanidine-3-glucoside chloride (cyanidine glucoside) and (−)-epicatechin-(4β-8)-(−)-epicatechin (procyanidin B_2) were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). (−)-Epigallocatechin gallate (EGCG) was from EMD Biosciences, Inc. (La Jolla, CA, USA).

2.2. Preparation of loquat

Loquat fruit was purchased from the local store. Each fruit was carefully separated into three parts (peel, flesh and seed). Generally, loquat fruit consisted of 65–70% (by wet weight) of flesh, 16–19% of seed, and 8–9% of the peels (unpublished data). Results from our pilot study show that the DPPH radical scavenging activity of the seed (containing the dormant embryo and storage tissues) was approximately 4-fold more potent than that of the seed coat. Taking into consideration of much smaller mass in comparison to the seed, the contribution of seed coat to overall antioxidant activity would be negligible. In this study, the dark coat of the seed was removed prior to extraction. Each part was then extracted with either water or 80% ethanol.

2.3. Water extraction

To avoid the browning reaction, 10 g of each loquat part (peel, flesh and seed) were first incubated with 70 ml of distilled water at 85 °C for 5 min. After cooling down to room temperature, each loquat sample was homogenized and then allowed to settle at room temperature for 30 min. The homogenate was then filtrated through a No. 5B filter paper into a volumetric flask. The final volume of each filtrate was adjusted with distilled water to 100 ml. The water extract preparation (equivalent to 100 mg part sample weight/ml) was used later for the antioxidant study.

2.4. Ethanol extraction

Ten grams of each part (peel, flesh and seed) from different pieces of fruits were homogenized with approximately 70 ml of ethanol (final ethanol concentration was adjusted to 80%). The homogenate was then incubated at 50 °C for 5 min. After cooling down to room temperature, the homogenate was filtrated through an No. 5B filter paper into a volumetric flask. The volume of the filtrate was adjusted to 100 ml with 80% ethanol. The ethanol extraction (equivalent to 100 mg sample weight/ml) was then used for evaluating antioxidant activity.

To study the dose response, both water extracts and ethanol extracts were diluted with water or 80% ethanol, respectively, or concentrated by rotary evaporator, and then re-suspended to proper concentration with water and 80% ethanol, respectively.

2.5. Determination of free radical scavenging activity of loquat extracts

The free radical scavenging ability was determined using DPPH (Blois, 1958). Briefly, an aliquot (100 μl) of the loquat extract (either water or ethanol extraction at various concentrations) was pipetted into a test tube, and was mixed with 1.9 ml of 50 mM MES–NaOH buffer (pH 6.0) dissolved in ethanol/water (50:50, v/v) containing 0.1 mM DPPH. For peels, the aliquot of the water extract contained approximately 0–10 mg of the starting material, and for flesh and seed, each aliquot of water extract contained 0–50 mg of sample weight. For all ethanol extracts of different loquat parts, each aliquot contains 0–10 mg. Following 30 min of incubation at room temperature, the absorbance at 517 nm was then measured using a Hitachi U3010 spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). Free radical scavenging activity was expressed as the weight (mg) of each loquat sample required to scavenge 50% of the DPPH radicals. BHA was used as a positive control.

2.6. Effect of loquat extract on oxidation of emulsified linoleic acid

Effect of extracts of loquat samples on oxidation of emulsified linoleic acid was determined by monitoring the discoloration of β-carotene induced by the peroxides which were generated by oxidation of linoleic acid at elevated temperatures (Tsushida, Suzuki, & Kurogi, 1994). The method was based on that reported by Miller (1971). Briefly, 12 mg of linoleic acid, 0.2 mg of β-carotene and 100 mg of Tween 40 (surfactant) were suspended in a flask containing 10 ml of the oxygenated distilled water to form the β-carotene–linoleic acid conjugation system. The test

mixture was prepared freshly and used immediately. To each flask, 1 ml of the extract of loquat sample was added, and the mixture was then incubated at 40 °C for 4 h. During incubation, the discoloration was monitored by measuring every 30 min the absorbance at 470 nm. For controls, 1 ml of either distilled water or 80% ethanol in place of loquat extract was added to the system. BHA (0.1 and 0.5 mg/ml) was used as a positive control.

2.7. Isolation and preparation of LDL

LDL was isolated from human plasma with EDTA (2.5 mM) prepared from the fasting normolipidemic volunteers. Ethylenediaminetetraacetic acid (EDTA)-containing LDL (1.019–1.063 g/ml) fraction was prepared by stepwise ultracentrifugation as described by Harvel, Eder, and Bragdon (1955). The EDTA-containing LDL fraction was then stored at 4 °C in the dark under a nitrogen atmosphere until use. Prior to oxidation experiments, the EDTA-containing LDL fraction was pipetted into a centrifuge tube lined with the filter which has a cut off point for molecules smaller than 30,000 Da (Amicon Ultra-15, 30,000MWCO, Millipore Co., Bedford MA). EDTA was removed from the LDL solution by centrifugation at 5000*g* at 4 °C for 10 min. The LDL residue was suspended with phosphate buffered saline (PBS, pH 7.4). After the process was repeated twice, the LDL residue was re-suspended with PBS to give a final LDL protein concentration of 70 μg/mL. The protein concentration was determined by Lowry's method (Lowry, Rosenbough, Farr, & Randall 1951).

2.8. Effect of loquat extract on oxidation of LDL

LDL oxidation was measured using the method described by Hirano, Kondo, Iwamoto, Igarashi, and Itakura (1997). Briefly, 10 ml of the LDL solution (containing 700 μg of protein) were incubated with freshly prepared MeO-AMVN with or without the addition of the water extract or 80% ethanol extract of the loquat sample. The MeO-AMVN was used as an initiator of radical formation. In this study, the concentration of MeO-AMVN was 200 μM for the water extract, and 500 μM for the ethanol extract. LDL oxidation was determined by monitoring the change in absorbance (234 nm) due to conjugated diene formation using a Hitachi U-3010 spectrophotometer.

2.9. Effect of loquat ethanol extract on the level of thiobarbituric acid reactive substances (TBARS) in LDL

LDL solution (10 ml; containing 700 μg of protein) was incubated with freshly prepared MeO-AMVN with or without the ethanol extract of loquat sample. Final concentration of AMVN-CH_3O was 500 μM. LDL oxidation was determined by measuring the level of TBARS using commercial assay kits (Lipid peroxide-test Wako, Wako Pure Chemical Industries, Osaka, Japan).

2.10. Contents of chlorophyll, carotenoids, ascorbic acid and polyphenols in loquat extract

The concentrations (mg/100 g) of chlorophyll and carotenoids in loquat extracts were determined by spectrometric method described by Nagata and Yamashita (1992). Briefly, the absorbance of each loquat extract was measured at 663, 645, 505 and 453 nm by a Hitachi U-3010 spectrophotometer (Hitachi Co. Ltd., Tokyo Japan). The content of chlorophyll (*a*) and (*b*), lycopene, and β-carotene was estimated using the equations reported by Nagata and Yamashita (1992). The level of ascorbic acid in loquat extracts was measured by HPLC according to the method reported by Otsuka, Kurata, and Arakawa (1986). The HPLC system was composed of a Hitachi LaChrom chromatograph (Hitachi Co. Ltd., Tokyo Japan) with a YMC J'spher ODS H80 column (150 mm × 4.6 mm i.d., 4 μm, YMC, Kyoto, Japan) coupled with a Hitachi LaChrom L-7420 UV/visible spectrophotometer. The column was eluted with 30 mM KH_2PO_4 containing 0.1 mM Na_2EDTA. The flow rate was 0.5 ml/min. The eluants were detected at 254 nm at 25 °C. The concentration of total polyphenols in loquat extracts was measured by the method reported by Dcendit and Merillon (1996).

2.11. Reversed-phased HPLC-ESI MS analyses of loquat extracts

Analyses of the ethanol extracts of loquat samples were carried out using a Shimadzu LCMS-2010 system (*m*/*z* 0–1000), equipped with an electrospray ion (ESI) source (Shimadzu Ltd., Kyoto, Japan). Identification and quantification of different components in the ethanol extract of loquat were performed using the reversed-phase HPLC on a Hypersil ODS column (200 × 2.1 mm i.d., 5 μm, Hewlett Packard Co. Ltd., Palo Alto, CA, USA) at 25 °C. Two mobile phases were used: 0.5% formic acid in water (solvent A) and 0.5% formic acid in methanol (solvent B). The mobile phases were delivered at flow rate of 0.2 ml/min by a Shimadzu LC-10ADvp pump. The elution was carried out with a mobile phase consisting of a gradient from 100% solvent A to 100% solvent B during the initial 35 min interval. The elution was then maintained isocratic (100% solvent B) for another 10 min. The wavelength of the diode array detector (Shimadzu SPD-M10Avp, Shimadzu Ltd., Kyoto Japan) was set at 280 nm for polyphenols.

Positive ion mode electrospray ionization mass spectrometry (ESI-MS) (Shimadzu Ltd., Kyoto, Japan) was employed to identify major polyphenolic compounds in loquat extracts based on retention time and selective ion mass in comparison with the authentic standards. Positive ions were acquired in full scan mode (*m*/*z* 100–1000 in 1 s). Ion spray voltage was set at 4500 V. Quantitative MS data for polyphenolic compounds were acquired by setting the specific precursor ion mass-to-charge ratio (*m*/*z*), for example, chlorogenic acid (*m*/*z* 355), cyanidin glucoside (*m*/*z*

449), epicatechin (*m*/*z* 291), EGCG (*m*/*z* 459) and procyanidin B_2 (*m*/*z* 579).

3. Results

3.1. Radical scavenging ability of loquat extracts

The radical scavenging ability of loquat extract fractions was tested with DPPH. Results in Fig. 1 depict the amount of sample required to scavenge 50% of DPPH radical. The water extract of the peel and the ethanol extracts of all three parts of loquat exhibited a strong ability to scavenge the DPPH radicals. In this study, 10 mg of the water extract of peel was required to scavenge 50% of DPPH radicals (0.2 μmol/2 ml). To achieve the same levels of activity, it required approximately 2.0 mg of authentic antioxidant BHA. The ethanol extracts of the peels and flesh exhibited similar level of effects on free radicals. The ethanol extract of loquat seed was most potent: 6 mg of the seed extract exhibited the same level of activity as 3.1 mg of BHA.

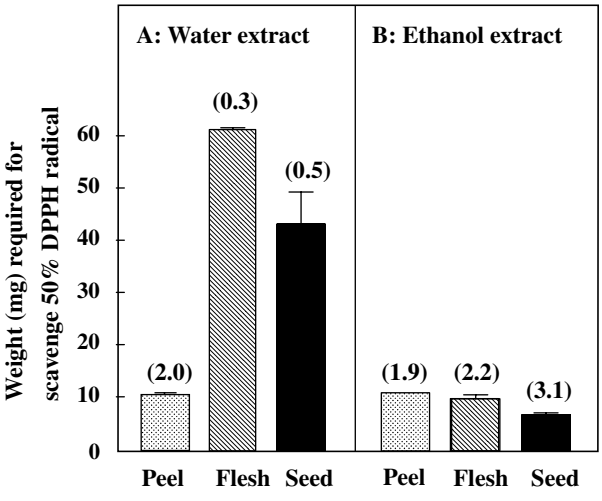

Fig. 1. DPPH radical scavenging ability of loquat extracts (A: water extracts, B: ethanol extracts). Each data point (mean ± SE of three determinations) represents the wet weight (mg) of loquat extracts required to scavenge 50% of DPPH radical. Value in parentheses represents the amount of BHA (mg) that expresses same level of the activity.

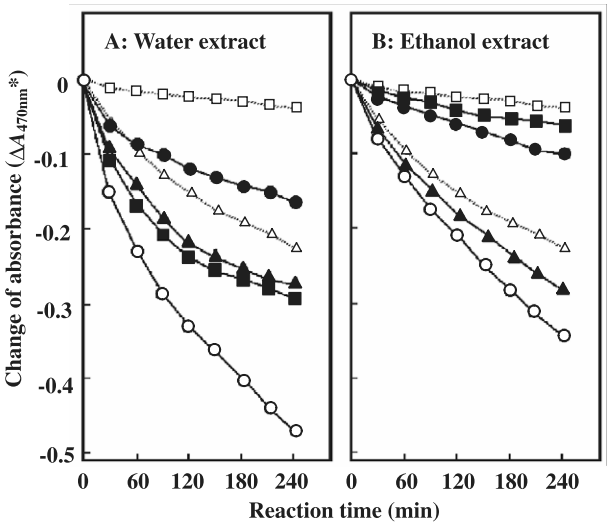

Fig. 2. Effect of loquat extracts (A; water extracts, B; ethanol extracts) on discoloration of β-carotene resulted of oxidation of linoleic acid. (○) Control; (●) peel; (▲) flesh; (■) seed; (△) BHA (0.1 mg/mL); (□) BHA (0.5 mg/mL) $^{*}\Delta A_{470nm}$ = change in absorbance after the reaction.

3.2. Effect of loquat extract on autoxidation of emulsified linoleic acid

Protective effect of loquat extracts (equivalent to 100 mg wet weight) on oxidation of 12 mg of linoleic acid was measured by monitoring the discoloration of β-carotene/linoleic acid conjugation. As shown in Fig. 2, the colour of β-carotene faded away during incubation with time. Addition of either water or ethanol extract of loquat parts delayed the discoloration, indicating that these extracts decreased the autoxidation of linoleic acid. This effect was more evident using the ethanol extracts than the water extract of loquat peels, and more so the loquat seed. The oxidation-protective effect of the ethanol extract of loquat seed (equivalent to 100 mg wet weight) was comparable to that of 0.5 mg of BHA.

3.3. Effect of loquat extracts on MeO-AMVN-induced LDL oxidation

The LDL oxidation was induced by MeO-AMVN. As shown in Fig. 3A, when LDL (700 μg protein/10 ml) was incubated with 200 μM of MeO-AMVN, the absorbance at 234 nm increased with time, and reached the plateau at 3 h. Supplementation of 10 mg of loquat water extract (peel, flesh and seed) to the system delayed the oxidation. The reaction time required for 50% oxidation of LDL (LC_{50}) was estimated to be 78 min for the control, 183, 168, and 213 min for the water extracts of loquat peels, flesh and seed, respectively. When comparing with the control progression phase, the slope of the LDL oxidation rate (Abs./min) for the water extracts of the peels, flesh and seed was 0.60, 0.60, and 0.53, respectively.

A similar study was carried out using the ethanol extracts. When LDL (700 μg protein/10 ml) was incubated with 500 μM MeO-AMVN in the presence of 0.1 ml of ethanol, absorbance at 234 nm increased in relation to time, and reached a plateau at 5 h (Fig. 3B). Supplementation with 10 mg of the ethanol extracts of the peels and seed but not flesh into the system delayed the oxidation. The LC_{50} was estimated to be 108 min for the control, 163 min, 106 min and 552 min for the ethanol extracts of the skin, flesh and seed, respectively. When comparing with the control progression phase, the slope of the LDL oxidation rate (Abs./min) for the ethanol extracts of the skin, flesh and seed was 0.71, 1.01, and 0.20, respectively. The antioxidative effect was much more evident in the seed extract than the skin extract.

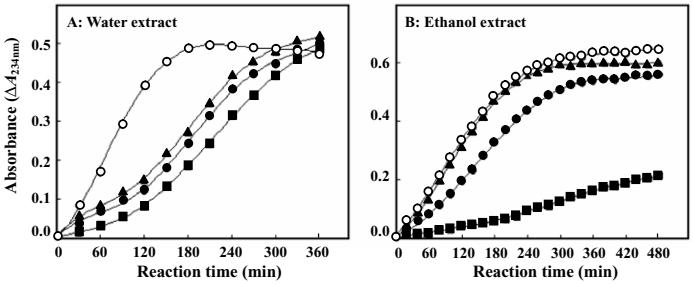

Fig. 3. Effect of loquat extracts (A: water extracts, B: ethanol extracts) on formation of the conjugated dienes in LDL induced by MeO-AMVN. LDL (70 μg protein/mL) was incubated with 200 μM (for water extracts) or 500 μM (for ethanol extracts) MeO-AMVN in the presence of different loquat extracts (10 mg). (○) Control; (●) peel; (▲) flesh; (■) seed.

The effect of supplementation of different levels of loquat extract on LDL oxidation was also studied. Results in Fig. 4 show that all loquat water or ethanol extracts inhibited the oxidation of LDL dose-dependently. Supplementation with the water extract (Fig. 4A–C), the wet weight (mg) required for 50% oxidation of LDL (IC_{50}) was calculated to be 11.2, 12.9 and 10.0 mg for the peel, flesh and seed of loquat, respectively. Supplementation with the ethanol extracts (Fig. 4D–F), the IC_{50} was calculated to be 23.9, 36.7, and 2.47 mg for the peel, flesh and seed of loquat, respectively.

3.4. Levels of TBARS in the LDL oxidation system

The effect of the ethanol extract of loquat parts on the levels of TBARS formed in the LDL oxidation system (approximately 10 ml) were also measured at 0, 240 and 360 min after initiation of oxidation. Results in Fig. 5 show that the level of TBARS increased with time. Supplementation of ethanol extracts of the peels and flesh exerted no effect on the level of TBARS. However, incubation with the ethanol extract of loquat seed significantly lowered the TBARS level by 74.5% at 240 min and 52.3% at 360 min in comparison with the control.

3.5. The contents of chlorophylls, carotenoids, ascorbic acid and polyphenols in loquat extracts

Fig. 6 shows concentrations of various antioxidant components in different loquat extracts. Chlorophyll (*a*) and chlorophyll (*b*) were found mainly in the ethanol extract of loquat peels. A low level of chlorophyll (*a*) was also found in the ethanol and water extract of the loquat seeds. Lycopene was found mainly in the ethanol extract of the peels. Beta-carotene was found more in the ethanol extracts than the water extract of different loquat parts, and more in the peels and flesh than the seed. L-Ascorbic acid was found only in the water extract, and more in the peels.

The contents of polyphenols in the water extract of the peels were significantly higher than that in the flesh and

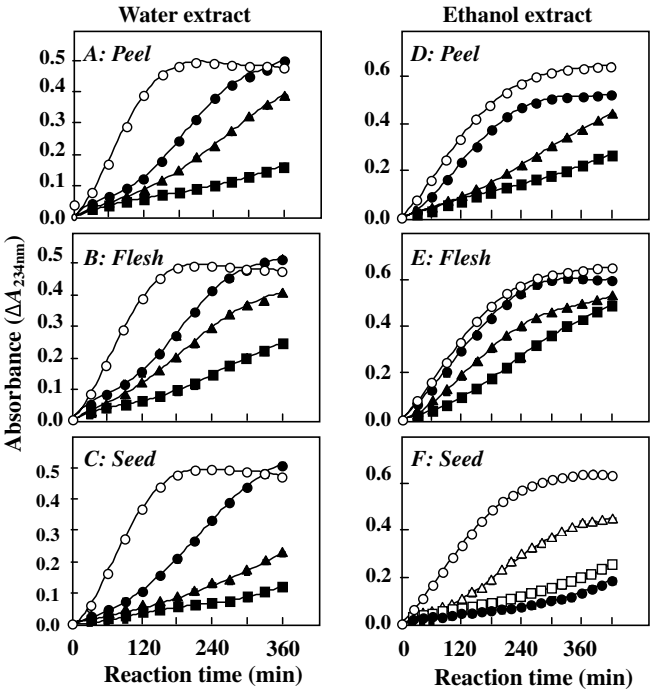

Fig. 4. Dose effect of loquat extract (A, B and C: water extracts, and D, E, and F: ethanol extracts) on LDL oxidation. (○) Control; △ 2 mg; (□) 5 mg; (●) 10 mg; (▲) 20 mg; (■) 50 mg. Value represents the equivalent initial sample weight needed for extraction.

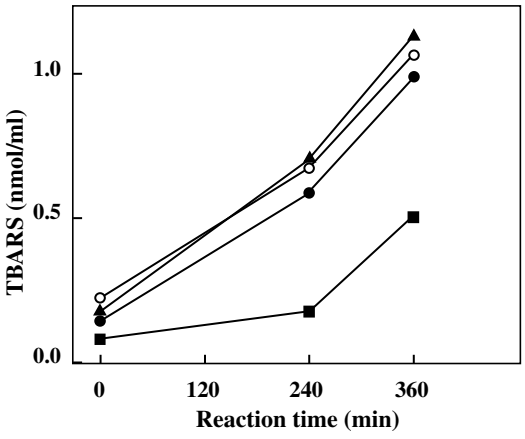

Fig. 5. Effect of loquat extracts on TBARS concentration in LDL (nmol/ml) after LDL oxidation was induced for 0, 240 and 360 min. (○) Control; (●) peel; (▲) flesh; (■) seed. Each data point represents mean of samples from three different fruits.

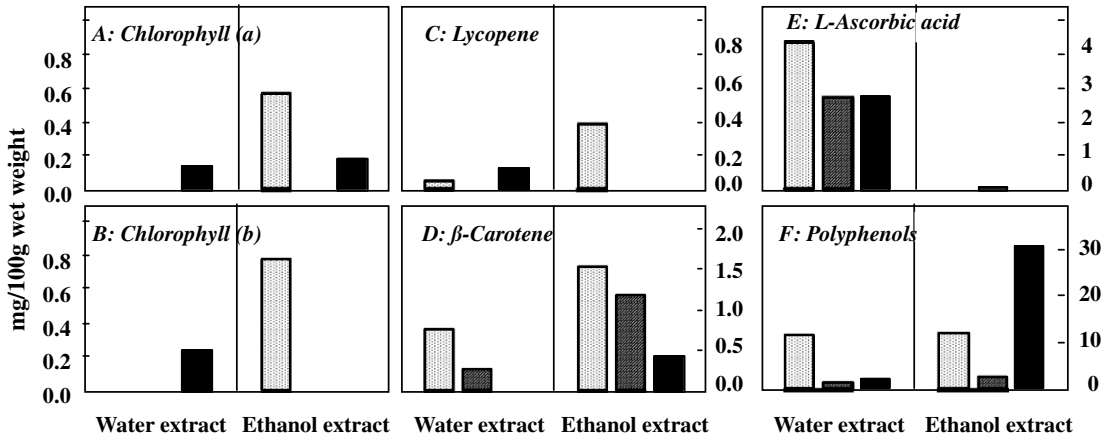

Fig. 6. Concentration of different antioxidants in water and ethanol extracts of loquat parts. A: chlorophyll (*a*), B: chlorophyll (*b*), C: licopene, D: β-carotene, E: L-ascorbic acid, F: polyphenols. (▭) peel; (▨) flesh; (■) seed. Each data point (mean of samples from three different fruits) represents the antioxidant content (mg/100 g initial loquat sample used for the extraction).

seed. However, polyphenols were very rich in the ethanol extract of the loquat seed. Thus, the ethanol extract of all three loquat parts were subjected to further analysis for the polyphenolic contents.

3.6. Reversed-phased HPLC-ESI MS analyses of loquat extracts

The ethanol extracts of loquat parts were analyzed by a reversed-phase HPLC and a Shimadzu LCMS-2010 system (*m*/*z* 0–1000), equipped with an electrospray ion (ESI) source (Shimadzu Ltd., Kyoto, Japan) as described in the method section. The retention time (RT) of each peak was compared with that of authentic standard; chlorogenic acid (15.00 min), cyanidin glucoside (22.11 min), epicatechin (16.15 min), EGCG (14.93 min), and procyanidin B_2 (14.35 min). Positive mode ESI-MS was employed to identify major polyphenolic compounds in the extracts, and the intensity of the specific mass (*m*/*z*) was used to estimate the level of the compounds: chlorogenic acid (*m*/*z* 355), cyanidin glucoside (*m*/*z* 449), epicatechin (*m*/*z* 291), EGCG (*m*/*z* 459) and procyanidin B_2 (*m*/*z* 579).

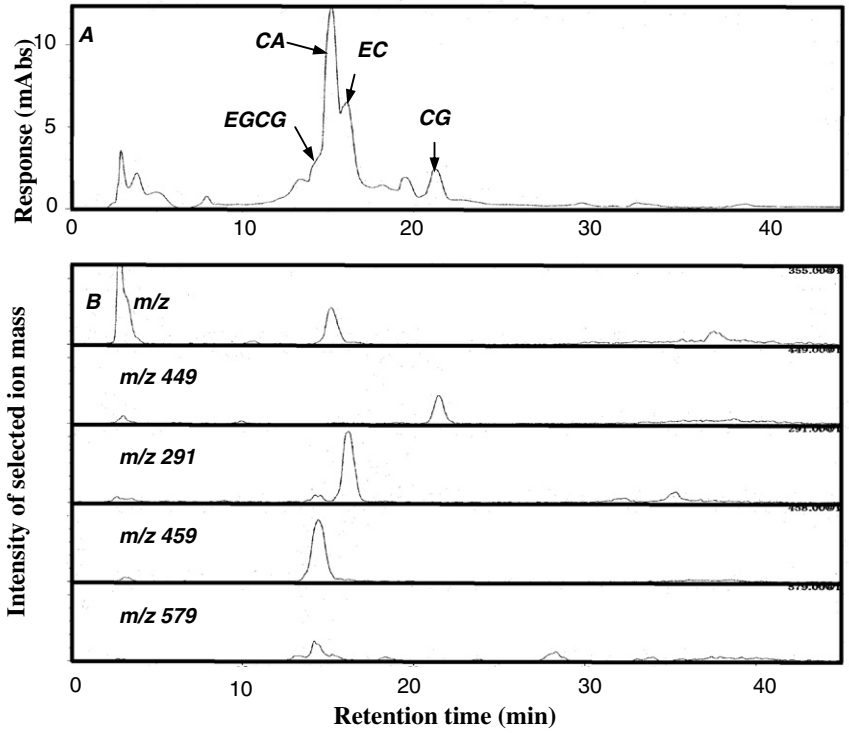

Fig. 7. Reverse-phase liquid chromatography/tandem mass spectrometry (LC/MS) analysis of ethanol extracts of loquat seed. Panel A: UV absorbance at 280 nm; Panel B: Selected ion chromatograph (positive ion at *m*/*z* 355, *m*/*z* 449, *m*/*z* 291, *m*/*z* 459 and *m*/*z* 579). EGCG: epigallocatechin gallate, PB_2: procyanidine B_2, CA: chlorogenic acid, EC: epicatechin, and CG: cyanidine glucoside.

Table 1
Contents of polyphenolic compounds in loquat ethanol extracts determined by LC/MS

	Peel (mg/100 g wet weight)	Flesh (mg/100 g wet weight)	Seed (mg/100 g wet weight)
Chlorogenic acid	36.8	46.4	28.2
Cyanidine glucoside	59.2	21.8	19.6
Epicatechin	nd	nd	58.0
EGCG	nd	nd	59.4
Procyanidin B_2	nd	nd	10.8
Total	96.0	68.2	175.9

EGCG, epigallocatechin gallate; nd, not detected.
Data (expressed as mg/100 g wet weight of loquat part) were average of two determinations from two different fruits.

Fig. 7 shows the HPLC chromatogram and intensity of selected ion mass (*m*/*z* 291, 355, 449, 459 and 579) of the ethanol extract of the loquat seeds. The chromatogram and mass spectra based on selective ion mass suggests the presence of chlorogenic acid (RT: 15.05 min, *m*/*z* 355), cyanidin glucoside (RT: 21.55 min, *m*/*z* 449), epicatechin (RT: 16.07 min, *m*/*z* 291), EGCG (RT: 15.03 min, *m*/*z* 459), and procyanidin B_2 (RT: 14.41 min, *m*/*z* 579). In the ethanol extract of the loquat peels, the HPLC chromatogram shows the presence of chlorogenic acid (RT: 15.3 min) and cyanidin glucoside (RT: 19.2 min). The identities of these two compounds were confirmed by the selective ion mass (*m*/*z*) 355 and 449 (panel B), respectively (data not shown). In the ethanol extract of the loquat flesh, the HPLC chromatogram shows the presence of chlorogenic acid (RT: 15.2 min) and cyanidin glucoside (RT: 19.2 min). The identities of these two compounds were confirmed by the selective ion mass (*m*/*z*) 355 and 449 (panel B), respectively (data not shown).

Results in Table 1 summarize the contents of the main polyphenolic compounds in the ethanol extract of different loquat parts. The levels of chlorogenic acid were higher in the loquat peels and flesh than in seeds, and the levels of cyanidin glucoside were higher in the extracts of the peels than those in the flesh and seeds. Flavanoids, such as epicatechin, EGCG and procyanidin B_2 were found only in the ethanol extract of loquat seeds. Overall, the content of total polyphenolic compounds was highest in the seed, followed by the peels and flesh.

4. Discussion

In this study, we examined the antioxidative activity of the water and ethanol extracts of loquat (peel, flesh and seed). The radical scavenging ability of loquat extract was demonstrated using DPPH assay. The results showed that the water extract of the peels and the ethanol extracts of all three parts of loquat exhibited a strong DPPH scavenging ability (Fig. 2). The relative efficiency of the water extract of the peels in comparison with the authentic antioxidant BHA was approximately 1:5 (by weight). Using ethanol in place of water for extraction did not increase the activity of the peels, but significantly enhanced the activity of the flesh, and seeds. The ethanol extracts of the peels and flesh exhibited similar effects on free radicals. The relative efficiency of the ethanol extract of loquat seed in comparison with BHA also significantly increased to about 1:2, suggesting that ethanol was more effective in extraction of the active components from the seeds.

Loquat extracts also suppressed the oxidation of linoleic acid as indicated by slower discoloration of β-carotene (Fig. 3). This effect was more evident using the ethanol extract than the water extract, particularly for loquat seed. However, the oxidation-protective effect of the ethanol extract of loquat seed in comparison with BHA was less efficient (approximately 1:200, by weight).

Using MeO-AMVN to induce LDL oxidation, supplementation of loquat water extract (peel, flesh and seed) to the system delayed the oxidation (Fig. 4A–C). The effect was comparable among different water extracts. When the ethanol extracts were added to the assay system, the oxidation was slowed down only by the peels and seed but not flesh (Fig. 4D–F). The inhibitory effect was most striking when the ethanol extracts of loquat seeds were added.

The protective effect of the ethanol extract of loquat parts has also been demonstrated by the level of TBARS formed in the LDL oxidation system. Supplementation of ethanol extracts of the peels and flesh exerted no significant effect on the level of TBARS. However, incubation with ethanol extract of loquat seeds significantly lowered the TBARS level (Fig. 5). All these findings clearly demonstrated that there are components in the loquat seeds that exhibit antioxidative activity.

The difference of various extracts in exhibiting antioxidant activity could be attributed to several factors. The type of antioxidants present in different parts of loquat fruits varies significantly. For example, flesh contains very little polyphenols and lycopene, whereas the peels and more so the seeds are rich in polyphenols (Fig. 6). The solvent used for extraction could also affect significantly the levels of components recovered. For example, the lipophilic compounds, such as lycopene, β-carotene, and polyphenols were extracted more efficiently with ethanol. On the other hand, the water-soluble compounds, such as ascorbic acid, were easily extracted with water. These differences might play a major role in determining the antioxidative ability of each extract.

In order to identify and quantify active ingredients, various ethanol extracts were subjected to reverse-phase HPLC and LC-mass spectrometry. Positive mode ESI-MS was employed to identify major polyphenolic compounds in the extracts, and the intensity of the selective ion mass (*m*/*z*) was used to estimate the level of the specific compounds. For example, the main fragment (M^+) of chlorogenic acid in mass spectrum was 355, cyanidin glucoside was 449, epicatechin 291, EGCG 459 and procyanidin B_2 579. In this study, we observed the presence of EGCG in

loquat seeds. This polyphenolic compound is found mainly in green tea (Hirano et al., 1997). If this finding holds true, this will be the first time that EGCG in loquat seeds is identified. More sophisticated approaches are needed to confirm the identities of these compounds.

Nonetheless, the preliminary results in Table 1 show that chlorogenic acid and cyanidin glucoside were present in all three loquat parts. Flavonoids, such as epicatechin, EGCG and procyanidin B_2 were found only in the ethanol extract of loquat seeds. Ample evidence has suggested that LDL oxidation plays an important role in the pathogenesis of atherosclerosis (Jessup, Dean, de Whalley, Rankin, & Leak, 1980; Steinberg, Parthasarathy, Khoo, & Witztum, 1986; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Witztum, 1994; Halliwell, 1995). Earlier, EGCG, EGC, ECG and EC among all catechins, were found to be the most important components in terms of antioxidant ability (Vinson, Hao, Su, & Zubik, 1998; Lin, Tsai, & We, 1999). Hirano et al. have shown that EGCG was the most effective free radical scavenger in green tea (Hirano et al., 1997). Consumption of epicatechin-rich cocoa powder has been shown to significantly reduce the susceptibility of LDL to oxidation in human volunteers (Osakabe et al., 2001). Osakabe et al. (2002) have reported that epicatechin and procyanidin B_2 contribute to the activity of cocoa to protect LDL from oxidation. Recently, Tsao, Yang, Xie, Sockovie, and Khanizadeh (2005) have also demonstrated that epicatechin and procyanidin B_2 were the major contributors to the antioxidant activity of apple. Thus, it is reasonable to conclude that the high content of epicatechin, EGCG and procyanidin B_2 found in the loquat seeds was responsible for the antioxidative ability of scavenging the free radicals and reducing the LDL oxidation shown in this study.

It is also known that loquat seed contains trace amounts of amygdalin, a cyano-compound (Wu et al., 2003). In a preliminary study, we used the DPPH-free radical scavenging system to examine whether amygdalin played a role in the antioxidative activity of the loquat seeds. Result shows that incubation with authentic amygdalin (up to the concentration of 1 mg/0.1 ml in 50% ethanol) exerted no radical scavenging activity (data not shown). Since the level of amygdalin (equivalent to 10 mg weight% of the seed) in that study was significantly higher than the normal level of amygdalin in seeds, it is suggested that amygdalin in loquat seeds was not responsible for the radical scavenge activity, and antioxidant activity demonstrated in this report.

In conclusion, the present study showed that the ethanol extract of loquat seeds provides a rich source of polyphenolic antioxidants. A moderate intake of such antioxidant rich seeds may be beneficial to human health.

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References

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature, 4617*, 1199–1200.

Cook, N. C., & Samman, S. (1996). Flavonoids chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry, 7*, 66–76.

Dcendit, A., & Merillon, J. M. (1996). Condensed tannin and anthocyanin production in Vitis vinifera cell suspension cultures. *Plant Cell Reports, 15*, 762–765.

Halliwell, B. (1995). Oxidation of low-density lipoproteins: question of initiation, propagation, and the effect of antioxidants. *The American Journal of Clinical Nutrition, 61*, 670–677.

Harvel, R. J., Eder, H. A., & Bragdon, J. H. (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *The Journal of Clinical Investigation, 34*, 1345–1353.

Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B., & Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet, 342*, 1007–1011.

Hirano, R., Kondo, K., Iwamoto, T., Igarashi, O., & Itakura, H. (1997). Effects of antioxidants on the oxidative susceptibility of low-density lipoprotein. *Journal of Nutritional Science and Vitaminology, 43*, 435–444.

Jessup, W., Dean, R. T., de Whalley, C. V., Rankin, S. M., & Leak, D. S. (1980). The role of oxidative modification and antioxidants in LDL metabolism and atherosclerosis. *Advances in Experimental Medicine and Biology, 264*, 139–142.

Lin, M. C., Tsai, M. J., & We, K. C. (1999). Supercritical fluid extraction of flavonoids from *Scutellariae radix*. *Journal of Chromatography, 830*, 387–395.

Lowry, O. H., Rosenbough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein Measurement with folin phenol reagent. *The Journal of Biological Chemistry, 193*, 265–275.

Middleton, E. J., & Kandaswani, C. (1992). Effects of flavonoids on immune and inflammatory cell functions. *Biochemical Pharmacology, 43*, 1167–1179.

Miller, H. E. (1971). A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists' Society, 48*, 91–95.

Morton, J. (1987). Loquat. In *Fruits of warm climates* (pp. 103–108). Julia F. Morton.

Nagata, M., & Yamashita, I. (1992). Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. *Nippon Shokuhin Kogyo Gakkaishi (in Japanese), 39*, 925–928.

Osakabe, N., Baba, S., Yasuda, A., Iwamoto, T., Kamiyama, M., Takizawa, T., et al. (2001). Effect of cacao liquor polyphenols on susceptibility of low-density lipoproteins to oxidation demonstrated in healthy human volunteers. *Free Radical Research, 34*, 93–99.

Osakabe, N., Yasuda, A., Natsume, M., Takizawa, T., Terao, J., & Kondo, K. (2002). Catechins and their oligomers linked by C4–C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Experimental Biology and Medicine, 227*, 51–56.

Otsuka, M., Kurata, T., & Arakawa, N. (1986). The tissue distribution of L-ascorbic acid and dehydro-L-ascorbic acid in the guinea pigs injected intravenously with dehydro-L-ascorbic acid. *Journal of Nutritional Science and Vitaminology, 3*, 259–266.

Roger, C. R. (1988). The nutritional incidence of flavonoids: some physiological and metabolic considerations. *Experientia, 44*, 725–733.

Steinberg, D., Parthasarathy, S., Khoo, J. C., & Witztum, J. L. (1986). Beyond cholesterol. Modifications of low-density lipoprotein that increases its atherogenicity. *The New England Journal of Medicine, 320*, 915–924.

Tsao, R., Yang, R., Xie, S., Sockovie, E., & Khanizadeh, S. (2005). Which polyphenolic compounds contribute to the total antioxidant activities of apple? *Journal of Agricultural and Food Chemistry, 53*, 4989–4995.

Tsushida, T., Suzuki, M., & Kurogi, M. (1994). Evaluation of antioxidant activity of vegetable extracts and determination of some active compounds. *Nippon Shokuhin Kokyou Gakkaishi (in Japanese), 41*, 611–618.

Vinson, J. A., Hao, Y., Su, X., & Zubik, L. (1998). Phenol antioxidant quantity and quality in foods: vegetables. *Journal of Agricultural and Food Chemistry, 46*, 3630–3634.

Witztum, J. L. (1994). The oxidation hypothesis of atherosclerosis. *Lancet, 344*, 393–395.

Wu, Q. L., Wang, M., Simon, J. E., Yu, S. C., Xiao, S. C., Xiao, P. G., et al. (2003). Studies on the chemical constituents of loquat leaves (*Eriobotrya japonica*). In C. T. Ho, J.-K. Lin, & Q. Y. Zheng (Eds.), *Oriental foods and herbs: chemistry and health effects* (pp. 292–306). Weimar, TX: Culinary and Hospitality Industry Publication Services.