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Antioxidant and Antiulcerative Properties of Phenolics from Chinese Quince, Quince, and Apple Fruits

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To evaluate the health benefits of Chinese quince and quince phenolics, their antioxidant properties and antiulcerative activity were investigated in comparison with apple phenolics as a reference. The strength of antioxidant activity and DPPH radical scavenging activity of these fruit phenolics varied according to different in vitro evaluation systems, whereas the antioxidative property of rat blood increased in all rats orally administered phenolics. Ferulic acid and isoferulic acid were detected as major metabolites in rats given apple phenolics, quince phenolics, and 5-caffeoylquinic acid standard. (−)-Epicatechin and its 3′-*O*-methyl ether could be detected in rats administered apple phenolics and (−)-epicatechin standard. In the ethanol-induced gastric ulcer, pre-administration of Chinese quince and quince phenolics suppressed the occurrence of gastric lesions in rats, whereas apple phenolics seemed to promote ulceration. The trend of myeloperoxidase activity was similar to that of the ulcer index. The results showed that Chinese quince and quince phenolics might have health benefits by acting both in blood vessels and on the gastrointestinal tract.

KEYWORDS: Chinese quince; quince; apple; fruit; polyphenol; flavan-3-ols; procyanidins; hydroxycinnamic acids; antioxidant activity; antiulcerative activity; gastric lesion

INTRODUCTION

Phenolics are regarded as functional components related to the health benefits of fruit. Flavonoids, such as flavan-3-ols (catechins and proanthocyanidins), quercetin derivatives, and anthocyanins are important phenolics often observed in fruit as well as hydroxycinnamic derivatives such as chlorogenic acid. Red wine contains a range of phenolics derived from the skin and seeds of the grape, which is thought to be the main factor related to prevention of cardiovascular disease through moderate consumption of red wine (*1*). Flavonoid intake from fruits or vegetables may be important in reducing the risk of death from coronary heart disease or from cancer (*2*−*4*). Knekt et al. (*4*) found a significant inverse relationship between the intake of apples, the main source of flavonoids, and the incidence of lung cancer. Thus, fruit or other foods rich in phenolics are expected to have some beneficial effects on health.

Chinese quince and quince fruits have been shown to have higher amounts of phenolics than apple fruit (*5*). This high phenolics content can be expected to act as a factor for improvement in health. However, Chinese quince and quince fruits are minor crops in the world and are not edible raw because of their flesh hardness and strong astringency. Thus they are often processed and consumed as fruit liquor, glutinous starch syrup, jam, jelly, candy, etc. In our previous research, we showed that Chinese quince, quince, and apples differed not only as to total phenolic content but also the phenolic profile (*5*). The phenolic profile of Chinese quince was characterized by an extremely high proportion of polymeric procyanidins (a kind of proanthocyanidin) to total phenolics, whereas the main compounds in quince phenolics were procyanidin polymers and hydroxycinnamic derivatives. These differences in the phenolic profile might affect biological activity related to the health benefits of the fruit or fruit products.

Apple phenolics have been widely studied and were shown to have various possible health benefits, such as antioxidant activity in vitro (*6*−*8*) or in vivo (*9*), antiproliferative activity (*10*, *11*), antiallergic effects (*12*), etc. However, few reports of phenolics from Chinese quince or quince fruit are available. Osawa et al. (*13*) found that phenolics from dried Chinese quince showed antiinflammatory activity, and that a high molecular weight phenolic was the most effective compound. Silva et al. (*14*) reported that antioxidant activity of quince pulp, peel, and jam phenolic extracts were strongly correlated with caffeoylquinic acids (chlorogenic acid and its isomers) and total phenolic content. We reported that Chinese quince phenolics were more effective in decreasing the hemagglutination ability of influenza virus than quince phenolics (*5*). We also observed that the antioxidant capacity of phenolics from Chinese quince, quince,

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and apples differed in different evaluation systems and that the order of antioxidant activity also varied.

In vitro evaluation systems to determine the antioxidant capacity of phenolics are useful to estimate antioxidant effects on the surface of organs such as the inner wall of the gastrointestinal tract, but such systems cannot reflect antioxidant effects in vivo. If we expect phenolics to prevent, for example, cardiovascular disease, they must act as antioxidants in blood vessels after absorption and metabolic modification. Procyanidins have been shown to have strong antioxidant and/or radical scavenging functions (*15*−*18*); however, procyanidin polymers longer than trimers are unlikely to be absorbed from the small intestine (*19*) and would have difficulty entering blood vessels in their native forms. If this is so, most phenolics from Chinese quince might not be absorbed from the small intestine because the major phenolic components of Chinese quince are polymeric procyanidins. However, procyanidin oligomers from cocoa extract could be absorbed and were associated with an increase in plasma antioxidant capacity in rats given cocoa extract (*18*). Additionally, recent research on the bioavailability of procyanidins indicates that procyanidins largely reach the colon and will be degraded by microflora, then be absorbed as small phenolic acids (*20*, *21*). Thus, the biological effects of procyanidin-rich extracts are still unclear and need to be investigated.

On the other hand, phenolics will, if not absorbed, act as effective antioxidants or provide other functions in the gastrointestinal tract. Some phenolics have been shown to have a protective effect on gastric injury induced by ethanol or by stress in the rat water restraint model. Phenolics from grape seed (*22*), cacao liquor (*23*), and prickly pear (*24*) have been shown to have antiulcer activity. The antioxidant activity of phenolics may be an important factor because reactive oxygen and/or free radicals are related to the occurrence of ulcers (*25*−*27*).

Thus, the function of the health benefits of phenolics should be taken into account from two aspects: first, the possible action of a functional component after gut absorption, and, second, possible action in the gut without absorption. The objective of this research was to investigate the properties of the health benefits of Chinese quince and quince phenolics, such as antioxidant properties and antiulcerative activity, in comparison with apple phenolics as a reference.

MATERIALS AND METHODS

Plant Materials. Three Rosaceae fruits were used in the experiment. The full ripe fruits of Chinese quince (*Pseudocydonia sinensis* Schneid. var. Toukarin), quince (*Cydonia oblonga* Mill. cv. Smyrna), and apple (*Malus domestica* Borkh. cv. Fuji) were obtained from a local market in Nagano prefecture or at the Education and Research Center of Alpine Field Science in Shinshu University, Japan.

Chemicals and Reagents. (−)-Epicatechin, ferulic (4-hydroxy-3-methoxycinnamic acid), isoferulic (3-hydroxy-4-methoxycinnamic acid) and *m*-coumaric (*trans*-3-hydroxycinnamic acid) acids, and β-glucuronidase type H-2 (including sulfatase) were purchased from Sigma-Aldrich, Ltd. (St. Louis, MO). 3-Hydroxyphenylpropionic acid was obtained from Lancaster Synthesis, Ltd. (Morecambe, Lancashire, U.K.). 5-Caffeoylquinic acid, (+)-catechin, 3′-*O*-methyl-epicatechin, salicylic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-(N-morpholino)-ethanesulfonic acid (MES) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Solvents were purchased from Nacalai Tesque, and 3,3′,5,5′-tetramethyl-benzidine (TMB) solution (TMBE-500S) was from Moss, Inc. (Pasadena, MD). Thirty percent hydrogen peroxide solution was purchased from Santoku Chemical Industry Co., Ltd. (Tokyo, Japan). Folin-Ciocalteu reagent, sodium dodecyl sulfate (SDS), linoleic acid, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), and toluene-α-thiol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals. Male Wistar rats (Jcl:Wistar) weighing 230∼262 g were obtained from CLEA Japan, Inc. (Tokyo), kept in a controlled environment (temperature 22 ± 4 °C; humidity 55 ± 10%; 12 h light−dark cycle), and maintained on a standard diet (CE-2, CLEA Japan, Inc.) for 3 d. The animals were fasted 24 h before the experiment and allowed free access to water. The experiments were approved by the ethics committee of Shinshu University.

Preparation of Phenolic Extracts. The flesh part of the fruit (from 5 to 25 fruits) was cut into small pieces, frozen with liquid nitrogen, freeze-dried using FD-5N (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and then homogenized. Before the extraction of phenolics, the powdered flesh (30 g) was mixed with petroleum ether in a beaker, stirred and filtered to remove lipids (300 mL × 5 times). Then the phenolics were extracted from the residue with 60% aqueous acetone (300 mL × 3 times) in the same manner. The 60% acetone extracts were evaporated under reduced pressure at 40 °C until all organic solvent was removed, redissolved into acidified water (0.1% trifluoroacetic acid), then applied to a Sep-Pak Vac 20 cm^3 (5 g) C18 cartridge (Waters Co., Milford, MA) which was preconditioned with methanol and acidified water. The column was washed with 30 mL of acidified water, and phenolics were recovered with 20 mL of methanol. The phenolics were redissolved into an aliquot of water, frozen, and then freeze-dried to obtain dried phenolic powder (fruit phenolic extracts). The overall yield of the dried phenolic extracts from 30 g flesh powder was 3.02 g (containing 2022 mg phenolics as (−)-epicatechin equivalent) for Chinese quince, 1.0 g (containing 546 mg phenolics) for quince, and 0.20 g (containing 123 mg phenolics) for apple.

Total Phenolic Assay. The experimental procedure was adapted from that of Hamauzu et al. (*5*). The sample solution (2 mL) was mixed with 2 mL of diluted Folin−Ciocalteu reagent (1 N) in a test tube. After 3 min of reaction, 2 mL of Na_2CO_3 (10 g/100 mL) was added and the mixture was incubated for 60 min at room temperature. The absorbance was measured at 700 nm with a Shimadzu UV-1200 spectrophotometer (Tokyo, Japan) against a blank (2 mL of deionized water, plus reagents) in the reference cell. (−)-Epicatechin was used as the standard ($r = 0.99975$).

HPLC Conditions. Chromatographic separation was carried out on a Luna 5 μm C18 column (150 × 4.6 mm, Phenomenex, Inc., Torrance, CA) at 40 °C. Solvents used for analysis of fruit phenolics were 0.1% trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). Gradient program was started with 5% B and changed to obtain 15% B at 30 min, 32% B at 35 min, 40% B at 45 min, and 75% B at 50 min. The 75% B was maintained until 65 min. For the analysis of phenolic metabolites, 0.1% aqueous phosphoric acid (A) and 0.1% phosphoric acid in acetonitrile (B) were used under the same gradient condition. The flow rate was 1.0 mL/min and the injection volume was 20 μL. Detection was performed at 280 nm for flavan-3-ols and 325 nm for hydroxycinnamic derivatives on a Shimadzu SPD-M10Avp photodiode array detector. For detection and quantification of phenolic metabolites, 320 nm for ferulic and isoferulic acids, 277 nm for *m*-coumaric acid, and 216 nm for 3-hydroxyphenylpropionic acid were also used.

Identification and Quantification of Phenolics. Identification was achieved by comparing retention times and UV spectra with those of standards or previously purified and identified procyanidins recorded under the same chromatographic conditions. The HPLC-MS system (Agilent 1100 series LC-MS, Agilent Technologies, Palo Alto, CA) was also used for qualitative confirmation of some phenolics as described in a previous report (*5*).

Quantitative determinations were made from calibration curves obtained by injection of different volumes of commercial standards (3−25 mg/100 mL for fruit extracts and 25−250 μg/100 mL for phenolic metabolites). Procyanidins were determined as total flavan-3-ols after thioacidolytic degradation using toluene-α-thiol reagent (5% v/v in methanol) as described by Guyot et al. (*28*). Experimental conditions and the calculation method were the same as described in our previous report (*5*). Procyanidin dimers (B1 and B2) and 3′-*O*-methyl-epicatechin were quantified as (−)-epicatechin.

DPPH Radical Scavenging Assay. The DPPH radical scavenging activity of phenolics was measured using the method of Brand−Williams et al. (*29*) with some modification. In a test tube, 1 mL of 0.2 mM DPPH ethanol solution was diluted by the addition of 1 mL of 200 mM MES buffer. To the diluted DPPH solution, 0.1 mL of sample was added at different concentrations, then mixed vigorously. After 20 min, the decrease in absorbance at 517 nm was measured. A control was added with 0.1 mL of distilled water instead of the sample solution. The result was expressed as the EC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%.

In Vitro Antioxidant Activity Assay Using Linoleic Acid Peroxidation System. The linoleic acid peroxidation system (SDS/LH system), which contained 10 mM phosphate buffer (pH 7.4), 100 mM SDS, 2.6 mM linoleic acid, and 2% AAPH as the radical generator, was used to estimate antioxidant activity against lipid oxidation. The experimental condition was the same as described in our previous report (*5*). Conjugated diene formation was measured at 234 nm, and the activity was expressed as the IC_{50} value, defined as the amount of antioxidant necessary to inhibit the formation of conjugated diene by 50%.

In Vitro Antioxidant Assay Using Rabbit Erythrocyte−AAPH System. The antioxidant effect using radical-induced hemolysis was determined using the method described by Zhu et al. (*18*) with some modifications. Preserved rabbit blood purchased from Nippon Biotest Laboratory, Inc. (Tokyo, Japan) was centrifuged at 3000 rpm for 10 min to separate the erythrocytes from the preservative solution then washed 3 times with phosphate buffered saline (PBS) containing 0.1 M phosphate and 8.5 g/L of sodium chloride (pH 7.4). The washed erythrocytes were diluted 4 times with PBS for further use. In a test tube, 1 mL of diluted erythrocytes was mixed with 1 mL of PBS and 100 μL of sample solution. One milliliter of 200 mM AAPH solution was then added to the mixture. The reaction mixture was shaken gently while being incubated at 37 °C for 3 h. After incubation, the reaction mixture was diluted with an equal volume of PBS and was centrifuged at 3000 rpm for 5 min. The absorbance (A) of the supernatant fraction at 540 nm was recorded in a Shimadzu UV-1200 spectrophotometer. As a control, 100 μL of water was added to the mixture instead of the sample solution. Blank was made with supernatant obtained by centrifugation of 24× diluted erythrocytes. The percentage of inhibition was calculated by the following equation:

$$\% \text{ inhibition} = [A_{cont} - A_{test}] \times 100/A_{cont}$$

where A_{test} is the absorbance of the sample containing antioxidants and A_{cont} is the absorbance of the control sample that contained no antioxidants. The activity was expressed as the IC_{50} value, defined as the amount of antioxidant necessary to inhibit hemolysis by 50%.

In Vivo Study of Antioxidant Effect in Rat Blood Using AAPH−Hemolysis. Rats ($n = 5-7$) were given 4 mL of water with or without 100 mg of fruit phenolic extracts or phenolic standards by gastric intubation. After 90 min, the rats were anaesthetized with pentobarbital, the abdominal wall was opened, and blood was collected from the right atrium into heparinized tubes. Erythrocytes from each rat were separated from the plasma by centrifugation at 3000 rpm for 10 min at 4 °C. Plasma was removed from the erythrocytes. The remaining erythrocytes were gently washed with PBS (6 vol × 3 times) then resuspended in the plasma. The reconstituted blood (erythrocytes plus plasma) was subjected to the hemolysis assay. Reconstituted blood (1 mL) was added to 1 mL of PBS and 1 mL of 200 mM AAPH solution followed by incubation at 37 °C for 3 h. The incubation mixture was centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant fraction at 540 nm was measured. Blank was made by centrifugation of 3× diluted reconstituted blood without incubation. The percentage of inhibition was calculated by the following equation:

$$\% \text{ inhibition} = [A_{water} - A_{ph}] \times 100/A_{water}$$

where A_{ph} is the absorbance of the reconstituted blood obtained from rats given a solution containing 100 mg of phenolics in 4 mL of distilled water, and A_{water} is the absorbance of the reconstituted blood obtained from rats given only distilled water.

Enzymatic Deconjugation of Phenolic Metabolites in Plasma. Phenolic metabolites in plasma were determined by HPLC after samples were deconjugated by use of combined enzymatic hydrolysis according to Zhao et al. (*30*), Baba et al. (*31*), and Azuma et al. (*32*) with some modifications. Plasma (1 mL) was added to 1 mL of 0.1 M acetate buffer (pH 5.0), to which was added 200 μL of 2 mM salicylic acid aqueous solution as the internal standard. To the mixture was added 50 μL of β-glucuronidase type H-2 solution (with 5000 U of β-glucuronidase and 250 U of sulfatase) to hydrolyze all conjugates into nonconjugated forms. The reaction mixture was then saturated with nitrogen gas and incubated at 37 °C for 2.5 h. The phenolic metabolites in nonconjugated forms were extracted with 6 mL of 0.06 M HCl−ethanol, and the supernatant was separated by centrifugation at 5000*g* for 10 min at 4 °C. The supernatant was evaporated to dryness, dissolved in 500 μL of distilled water, and filtered, and a 20 μL sample was used for HPLC analysis.

Antiulcerative Activity. *Treatment.* Rats were administered doses of 1.5 mL/ rat (containing 20 mg of phenolics) of test solution intragastrically and then given 1.5 mL/rat of acidified ethanol solution (150 mM HCl/ethanol = 40:60, v/v) after 30 min. For control rats, 1.5 mL of water was given instead of the phenolic solution. Animals were sacrificed under anesthesia 60 min after HCl/ethanol administration. Stomachs were removed, opened along the greater curvature and rinsed with physiological saline solution. They were stretched on balsa boards and pinned with the mucosal side up. After taking digital pictures of the mucosal surface of each stomach for morphometrical analysis as described below, stomach samples were frozen with liquid nitrogen and kept at −20 °C under nitrogen gas for further evaluation of myeloperoxidase (MPO) activity in the mucosa.

Morphometrical Analysis. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system (Zeiss, KS400, Göttingen, Germany). Percentage of the total lesion area (hemorrhagic sites) to the total surface area of the stomach except the forestomach was defined as the ulcer index (UI).

Measurement of Myeloperoxidase Activity. *Preparation of Crude Enzyme from Stomach Mucosa.* From the frozen samples, small pieces of mucosa were taken using a razor blade to obtain a random sample of approximately 50 mg of mucosa. Those 50 mg were then homogenized in 5 mL of 0.2 M acetate buffer (pH 5) using a potter homogenizer. The homogenates were used to determine protein concentration and MPO activity.

Protein Concentration in Crude Enzyme Solution. The concentration of protein was determined by the Bradford method (*33*) using bovine serum albumin (BSA) as a standard. Bradford reagent (1 mL) was added to 100 μL of the diluted sample. After 15 min, absorbance was measured at 595 nm. Protein concentrations were expressed in mg/L (A).

Myeloperoxidase Activity. Activity of MPO, a marker enzyme of leukocytes, is thought to represent leukocyte migration to injured tissues. In a glass cell, 1 mL of crude enzyme solution was mixed with 1 mL of TMB solution and 755 μL of 0.2 M acetate buffer (pH 5). To the mixture was added 245 μL of 3.67% H_2O_2 solution. The reaction mixture consisted of 117 mM acetate buffer, 0.52 mM TMB, 0.3% H_2O_2, and crude enzyme solution. Absorbance at 655 nm (A_{655}) of the mixture was immediately recorded for 5 min. Activity (U) was calculated from the increase in optical density per min (ΔA_{655}/min) (B) and finally expressed as activity per milligram of protein as follows:

$$\text{MPO activity (U/mg protein)} = (B) \times 1000/(A)$$

Statistics. Results are expressed as means ± SE. The statistical difference in data for inhibition of hemolysis was determined by the two-sided Student's t test. A difference with $P < 0.05$ was considered significant.

RESULTS

Phenolic Profile of Chinese Quince, Quince, and Apple Fruit Extracts. Extracted and semi-purified fruit phenolics used in this experiment were analyzed by HPLC. Hydroxycinnamic

Table 1. Main Phenolic Components in a 100 mg[a] Dose of Fruit Phenolic Extract and Corresponding Tissue Weight Providing the Indicated Amounts of Each Compounds

	3-CA[b]	4-CA	5-CA	total HCA[b]	EC[b]	B2[b]	total F3OL[b]	mDP[b]	tissue[c]
Chinese quince	0.33 (0.01)	0.04 (0.001)	0.32 (0.01)	0.76 (0.01)	nd	nd	73.5 (10.1)	18.6 (1.7)	7.8
quince	4.25 (0.03)	0.46 (0.01)	5.43 (0.05)	11.3 (0.11)	nd	nd	36.8 (2.4)	28.7 (2.8)	33
apple	nd	nd	22.1 (0.33)	24.4 (0.37)	12.1 (0.28)	8.80 (0.14)	31.2 (2.6)	3.0 (0.6)	164

[a] (–)-Epicatechin equivalent by Folin–Ciocalteu assay. [b] CA, caffeoylquinic acid; HCA, hydroxycinnamic acid; EC, (–)-epicatechin; B2, procyanidinB2; F3OL, flavan-3-ols (catechins + procyanidin); nd, not detected. Values except for those of mDP and tissue are expressed in mg (SE) determined by HPLC. mDP, mean degree of polymerization in total F3OL. [c] Flesh weight (g) providing 100 mg of total phenolics.

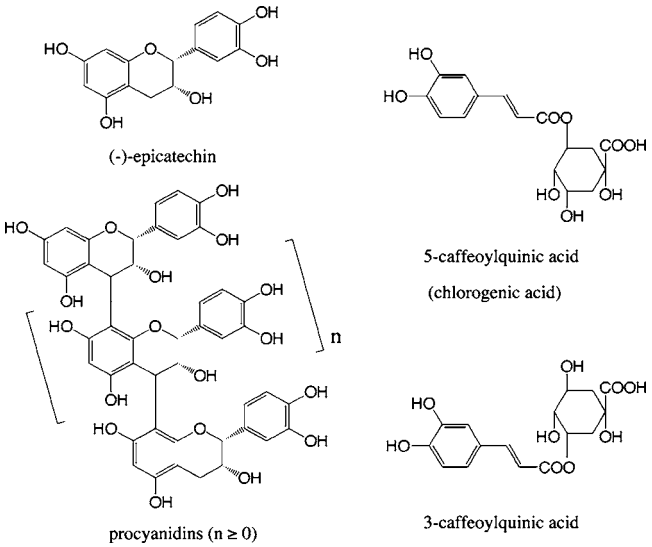

Figure 1. Flavan-3-ols and hydroxycinnamic compounds as major components of flesh phenolics in Chinese quince, quince, and apple.

acid derivatives and flavan-3-ols content in 100 mg total phenolics estimated by the Folin−Ciocalteu method are shown in **Table 1**. A high proportion of flavan-3-ols to total phenolics was characteristic of Chinese quince phenolic extract. Phenolics from quince fruit were characterized by two major chlorogenic acid isomers (3-caffeoylquinic acid and 5-caffeoylquinic acid) and flavan-3-ols in which the mean degree of polymerization was high. Apple phenolics were composed of mainly low molecular weight compounds such as 5-caffeoylquinic acid (chlorogenic acid) and monomeric and dimeric flavan-3-ols as well as oligomeric flavan-3-ols having few oligomers as described in a previous report (*5*). The structures of major flavan-3-ols and hydroxycinnamic derivatives observed in the fruit are shown in **Figure 1**.

Antioxidant Properties Measured in the In Vitro System. In the in vitro assay, the DPPH radical scavenging activity and the antihemolytic activity of Chinese quince and quince phenolics were superior to that of apple phenolics, whereas in the linoleic acid peroxidation system, apple phenolics were most effective in preventing oxidation (**Table 2**). In the DPPH system, EC_{50} of phenolics from Chinese quince, quince, and apples were 6.46, 4.65, and 7.56, respectively, which were superior to those of ferulic acid (12.3), L-ascorbic acid (17.7), 5-caffeoylquinic acid (16.1), and α-tocopherol (24.5), but were less effective than caffeic acid (2.71) and gallic acid (3.44). In the linoleic acid peroxidation system, IC_{50} of apple phenolics was 4.05 and had the highest activity among antioxidant standards tested in the experiment with the exceptions of α-tocopherol (2.47) and (−)-epicatechin (3.55). Chinese quince phenolics and quince phenolics had moderate activity (IC_{50} = 10.5 and 8.14, respectively), but ferulic acid (30.5) and ascorbic acid (57.0) had relatively weak activity in the linoleic acid peroxidation system.

Table 2. In Vitro Antioxidant Properties of Phenolic Extracts and Antioxidant Standards

	DPPH system EC_{50}[a]	SDS/LH system IC_{50}[b]	hemolysis system IC_{50}[c]
Chinese quince	6.46 (0.08)	10.5 (0.55)	33.8 (4.10)
quince	4.65 (0.18)	8.14 (0.85)	32.0 (1.28)
apple	7.56 (0.30)	4.05 (0.22)	49.0 (0.66)
(-)-epicatechin	6.26 (0.24)	3.55 (0.21)	24.1 (4.91)
procyanidin B2	8.44 (0.17)	5.68 (0.67)	33.3 (9.49)
caffeic acid	2.71 (0.05)	4.64 (0.20)	15.5 (0.53)
ferulic acid	12.3 (0.22)	30.5 (2.24)	41.4 (1.54)
5-caffeoylquinic acid	16.1 (0.39)	11.9 (0.40)	32.2 (1.15)
gallic acid	3.44 (0.10)	14.3 (1.04)	40.9 (9.09)
L-ascorbic acid	17.7 (0.26)	57.0 (2.46)	161.1 (7.86)
α-tocopherol	24.5 (0.51)	2.47 (0.10)	1.33 (0.03)

[a] EC_{50}, concentration (mg/100 mL) at 50% scavenging of DPPH radical. [b] IC_{50}, concentration (mg/100 mL) at 50% inhibition of conjugated diene formation induced with AAPH. [c] IC_{50}, concentration (mg/100 mL) at 50% inhibition of hemolysis of erythrocyte induced with AAPH.

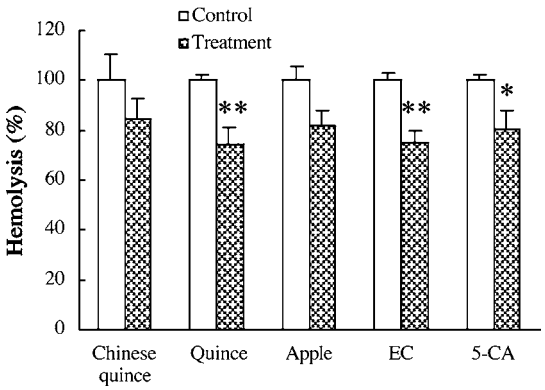

Figure 2. AAPH-induced hemolysis of red blood cells from control rats (given only water) and treated rats given an intragastric dose of 100 mg of fruit phenolic extracts or phenolic standards in 4 mL of water. EC, (–)-epicatechin; 5-CA, 5-caffeoylquinic acid (chlorogenic acid). Bars indicate SE (n = 7 for fruit phenolic group; n = 5 for phenolic standard group). *P < 0.05 vs control, **P < 0.01 vs control.

In the radical-induced hemolysis system, Chinese quince and quince phenolics showed moderate activity (IC_{50} = 33.8, and 32.0, respectively), and apple phenolics were weak (49.0). In this system, α-tocopherol (IC_{50} = 1.33) had the highest activity whereas gallic acid (40.9) and ferulic acid (41.4) had relatively weak activity and ascorbic acid had very weak activity (161.1).

Antioxidant Effect on Rat Blood after Oral Administration. Radical-induced hemolysis in rat blood decreased in all rats given phenolics, indicating that the antioxidant capacity of rat blood increased with oral administration of phenolics (**Figure 2**). The preventive effect (% inhibition 90 min after administration) of quince phenolics was 25.7%, and those of apple and Chinese quince phenolics were 18.4% and 15.5%, respectively. (−)-Epicatechin and 5-caffeoylquinic acid also showed a

Table 3. Phenolic Metabolites Detected in Rat Plasma 90 min after the Oral Administration of a Fruit Phenolic Extract or a Standard (100 mg/rat, with Enzyme Treatment Using β-Glucuronidase/Sulfatase)

	fruit phenolic extracts			phenolic standards	
metabolites	Chinese quince	quince	apple	(-)-epicatechin	5-caffeoylquinic acid
(-)-epicatechin	nd	nd	tr	1.97 (0.87)[a]	nd
3-*O*-methyl-epicatechin	nd	nd	0.39 (0.21)	6.85 (2.22)	nd
5-caffeoylquinic acid	nd	0.37 (0.20)	0.18 (0.10)	nd	0.04 (0.04)
caffeic acid	nd	0.57 (0.08)	0.59 (0.07)	nd	0.45 (0.23)
ferulic acid	nd	1.21 (0.16)	4.94 (0.87)	nd	2.48 (0.13)
isoferulic acid	nd	0.79 (0.14)	1.53 (0.28)	nd	1.93 (0.002)
m-coumaric acid	nd	0.09 (0.09)	0.24 (0.24)	nd	0.40 (0.22)

[a] Values are expressed as μM (SE, $n = 3$). nd, not detected; tr, trace.

preventive effect on hemolysis after oral administration (25.3% and 19.5%, respectively).

Phenolic Metabolites Detected in Rat Plasma after Oral Administration. (−)-Epicatechin, 3′-*O*-methyl-epicatechin, and some phenolic acids, such as caffeic acid, ferulic acid, isoferulic acid, *m*-coumaric acid, and 3-hydroxyphenylpropionic acid, were selected for analysis to estimate the bioavailability of fruit phenolics because they were previously reported as major metabolites (including by microbial activities) of flavan-3-ols and hydroxycinnamic derivatives (*20*, *31*, *32*, *34*). All metabolites were analyzed by HPLC-DAD after deconjugation by glucuronidase/sulfatase.

Only (−)-epicatechin and its 3′-*O*-methyl ether could be detected in rats administered (−)-epicatechin and apple phenolics. However, these two compounds could not be detected in other rats given 5-caffeoylquinic acid or Chinese quince and quince phenolics (**Table 3**). Ferulic acid and isoferulic acid were detected as major metabolites in rats given 5-caffeoylquinic acid, and slight amounts of caffeic acid, 5-caffeoylquinic acid, and *m*-coumaric acid were also detected. A peak that seemed to be 3-hydroxyphenylpropionic acid was detected; however, it was difficult to be certain that it was hydroxyphenylproprionic acid. These phenolic acid metabolites could also be detected in rats given apple phenolics or quince phenolics. No metabolites could be detected in plasma of rats administered Chinese quince phenolics.

Antiulcerative Activity. The effect of fruit phenolics on ethanol-induced gastric ulcers in the rats was compared with the UI and the activity of MPO, which is the enzyme specific to leukocyte and is able to be an indicator for their migration (**Figure 3**). When rats were given only water (as control) before 60% ethanol treatment, intense gastric hyperemia extending with a band-like conformation and thickened lesions as well as many filiform lesions were observed (data not shown). In the control rats, the UI was 12.0. Administration of Chinese quince phenolics was very effective in preventing the formation of gastric ulcer (UI = 0.69). Quince phenolics also showed moderate activity (UI = 6.6). Unfortunately, apple phenolics had an inverse effect in preventing ulcer (UI = 34.5). The trend in MPO activity in mucosa was similar to the UI. Chinese quince phenolics suppressed activity to 13.2 U/mg protein from 19.9 U/mg protein of control, quince phenolics had a moderate effect (15.0), and apple phenolics enhanced MPO activity (26.9).

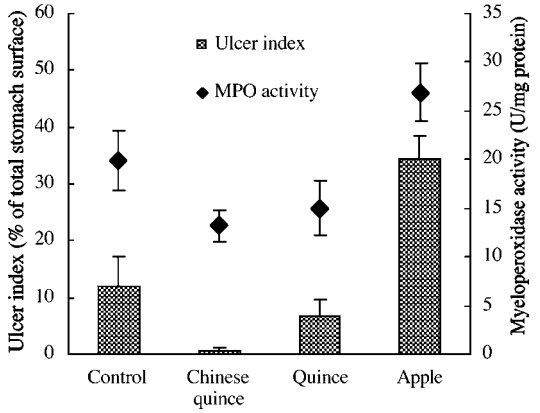

Figure 3. Ulcer index for ethanol-induced gastric lesion and myeloperoxidase activity of mucosa from control rats (given only water before 60% ethanol treatment) and rats given an intragastric dose of 20 mg of fruit phenolic in 1.5 mL of water before the 60% ethanol treatment. Bars indicate SE ($n = 5$).

DISCUSSION

The aim of this study was to evaluate the properties of Chinese quince and quince phenolics that would have health benefits in comparison with those of apple phenolics from two different aspects. The first was the antioxidant activity in blood, which requires their absorption and metabolism, and the second was gastrointestinal protection without absorption.

Confirmation of the quality of phenolic extracts by HPLC analysis clearly showed that a characteristic of Chinese quince and quince phenolics was a high proportion of procyanidins with a high mean degree of polymerization. Proanthocyanidins are said to be poorly absorbed in the gut due to their high molecular weight (*20*). Therefore, we supposed that the effect of phenolics from Chinese quince or quince fruits on enhancement of antioxidant resistance of blood might be inferior to apple phenolics because of their high proportion of procyanidin polymers to total phenolics. However, the result obtained showed that orally administered fruit phenolics all enhanced oxidative resistance of rat blood estimated by radical-induced erythrocyte hemolysis as did (−)-epicatechin and 5-caffeoylquinic acid.

We tried to detect some phenolic metabolites in plasma as biomarkers of bioavailability of fruit phenolics. It is well-known that polyphenolics absorbed are subjected to three main types of conjugation: methylation, sulfation, and glucuronidation (*19*). Catechol-*O*-methyl transferase, present in a wide range of tissues, catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionin to polyphenols having an *o*-diphenolic (catechol) moiety. Because methylation is known to occur predominantly in the 3′ position of polyphenolics, (−)-epicatechin-3′-*O*-methyl ether may be formed as a major metabolite in rats administered (−)-epicatechin. Additionally, ferulic acid (4-hydroxy-3-methoxycinnamic acid) and isoferulic acid (3-hydroxy-4-methoxycinnamic acid) are also known to be methylated metabolites formed from 5-caffeoylquinic acid and/or caffeic acid (*35*).

Although these methylated metabolites are also subjected to glucuronidation and/or sulfation, we treated rat plasma with glucuronidase/sulfatase and deconjugated the metabolites before analysis.

In the present study, (−)-epicatechin and its 3′-*O*-methylated derivative, which seemed to be formed in the small intestine and liver (*36*), could be detected in rats given (−)-epicatechin as reported in a previous study (*31*). Ferulic acid, isoferulic acid, caffeic acid, *m*-coumaric acid, and likely 3-hydroxyphenylpropionic acid were detected in rats given 5-caffeoylquinic acid. It has been shown that 5-caffeoylquinic acid is poorly absorbed through the small intestine and largely reaches the cecum of rats where it is extensively degraded by microflora, then further metabolized as liberated caffeic acid and quinic acid (*34*, *37*). Therefore, a very low concentration of 5-caffeoylquinic acid in plasma from rats given this compound was understandable. However, the actual concentration of this compound in the plasma is unclear because β-glucuronidase type H-2 enzyme used in this experiment contained secondary esterase activity which could convert 5-caffeoylquinic acid into caffeic acid and quinic acid. This should be paid attention in future research dealing with absorption of hydroxycinnamic esters. *m*-Coumaric acid and 3-hydroxyphenylpropionic acid are also formed with microbial modification of caffeic acid. Buchanan et al. (*38*) showed that the intake of phenolic acids liberated by microflora into organs occurred within 2 h.

In results for fruit phenolics, metabolites detected in rats given apple phenolics were similar to those in animals given 5-caffeoylquinic acid. This was conceivable because 5-caffeoylquinic acid is a major component of apple phenolics. Additionally, (−)-epicatechin is also a major component of apple phenolics, and it could be detected as a trace amount from the original compound in rats given apple phenolics. Also, small amounts of 3′-*O*-methyl-epicatechin were detected in such animals. In rats given quince phenolics, metabolites that could be detected in plasma were ferulic acid, isoferulic acid, caffeic acid, 5-caffeoylquinic acid, *m*-coumaric acid, and likely 3-hydroxyphenylpropionic acid. The profile was similar to that of rats given apple phenolics, but concentrations of the metabolites were lower than with apple phenolics, and neither (−)-epicatechin nor its 3′-*O*-methylated derivatives could be detected. The lower concentration of phenolic acid metabolites in rats given quince phenolics than that of apple phenolics reflected a lower proportion of hydroxycinnamic derivatives in quince phenolics than in apple phenolics. However, the effect of other phenolics and impurities on the rate of absorption and metabolism including microbial decomposition should be taken into account. In rats given Chinese quince phenolics, neither phenolic acid metabolites nor epicatechin metabolites could be detected in plasma. This could be predicted because mostly metabolites from hydroxycinnamic derivatives could be detected in rats given quince or apple phenolics, and, moreover, Chinese quince phenolics are composed of almost only procyanidins. Gonthier et al. (*20*) reported that neither the parent compound nor catechin derivatives could be detected in rats fed procyanidins in contrast to animals fed catechin monomers, in which large amounts of catechin and its 3′-*O*-methylated form were excreted. They also found that 16 metabolites of microbial origin were detected in rats fed catechin and procyanidins and that their total yields significantly decreased with increases in the degree of polymerization of the original compounds. The reason for the absence of metabolites in rats given Chinese quince phenolics may be mainly due to the lower ability of the diode array detector, which is not suitable for detection of minor components, but also to the lower bioavailability of Chinese quince phenolics, which are composed of mainly polymeric procyanidins.

Although there was no significant difference, administration of Chinese quince phenolics tended to increase resistance of erythrocytes against radical-induced hemolysis in rat blood as did apple phenolics or 5-caffeoylquinic acid. There was no relation between the metabolite concentration and the oxidative resistance of blood from rats tested. Because the effect of administration of quince phenolics on the increase in antioxidant capacity of blood was superior to that of apple phenolics despite their lower detectable concentration of metabolites in plasma, procyanidins or its metabolites must be related to the increased antioxidant capacity for some reason. However, it seems difficult to explain the antioxidant capacity of blood only from the concentration of various metabolites existing in the blood because other factors affect plasma antioxidant capacity. For example, Lotito and Frei (*39*) reported that the increase in plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids.

As shown by recent research, the major portion of phenolics cannot pass through the gut barrier and thus reaches the colon. Therefore, it is important to evaluate the function of phenolics on the gastrointestinal tract. From this point of view, we investigated the effect of fruit phenolics on prevention of ethanol-induced gastric ulcer in rats and observed that Chinese quince phenolics were quite effective, quince phenolics were moderately effective, but that apple phenolics had a negative effect. There was a similar tendency in the levels of the UI and MPO activity, which is an enzyme marker for leukocytes. MPO activity increased as the lesion surface increased, indicating migration of leukocytes into the inflammation zone and that reactive oxygen generated by leukocytes damaged mucosa (*27*). In rats administered apple phenolics, MPO activity was higher than in control rats, suggesting that apple phenolics might accelerate the migration of leukocytes, resulting in greater damage to the mucosa. We have observed that 5-caffeoylquinic acid tended to increase the ethanol-induced gastric ulcer accompanied by increases in MPO activity (*40*). Therefore, 5-caffeoylquinic acid in apple phenolics might contribute to the ulcer-promoting effect. Nevertheless, 5-caffeoylquinic acid should act as an antioxidant and should have a protective effect from injury by reactive oxygen. Graziani et al. (*41*) reported that catechin and chlorogenic acid (5-caffeoylquinic acid) were equally effective as apple extracts in preventing oxidative injury to human gastric epithelial cells. From this finding, it may be considered that 5-caffeoylquinic acid may possess the efficiency to protect against gastric injury but some interaction with ethanol might stimulate leukocyte migration and reactive oxygen generation, which would surpass the antioxidant effect of 5-caffeoylquinic acid.

On the other hand, Chinese quince phenolics, rich in polymeric procyanidins with a negligible amount of hydroxycinnamics, showed a remarkable effect on prevention from gastric injury induced with ethanol. The MPO activity was also lower in rats given Chinese quince phenolics, indicating that Chinese quince phenolics prevented stimulation of leukocyte migration by protecting against initial mucosal damage. Procyanidins, especially with a high degree of polymerization, have a high affinity for proteins and naturally bind strongly to mucosa (*22*). Hence, the protective effect of procyanidins may be explained by its remaining on mucosa as a protective layer possessing strong antioxidant activity. Besides, phenolic extracts from Chinese quince have been shown to have an inhibitory

effect on histamine release from rat mast cells and on activation of hyaluronidase, indicating that Chinese quince phenolics possess a strong antiinflammatory effect (*13*). Thus, the healthy effects of Chinese quince and quince phenolics in the gastrointestinal tract should be considered from various aspects of their function. Additionally, the ulcer-promoting effect observed in apple phenolic extracts seems to indicate that the application of excess purified compounds might have unforeseeable dangerous aspects. Therefore, care must be taken about the usage of purified supplements.

In conclusion, phenolics from Chinese quince and quince fruits were superior to apple phenolics as protectors against the occurrence of gastric ulcer induced by ethanol. In addition, Chinese quince and quince phenolics could enhance the antioxidant capacity of blood after oral administration as did apple phenolics in vivo. These findings suggest that phenolics in Chinese quince and quince fruit might have health benefits by acting both in blood vessels and on the gastrointestinal tract.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; MES, 2-(*N*-morpholino)ethanesulfonic acid; TMB, 3,3′5,5′-tetramethyl-benzidine; SDS, sodium dodecyl sulfate; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; PBS, phosphate buffered saline; MPO, myeloperoxidase; UI, ulcer index.

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