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Antioxidant Activity of Prune (*Prunus domestica* L.) Constituents and a New Synergist

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Ethanol extract of prune was separated into hexane-soluble and H_2O-soluble fractions, and the H_2O-soluble fraction was further separated into a methanol (MeOH) eluate and an H_2O eluate by Diaion HP-20 column chromatography. The MeOH eluate exhibited the strongest antioxidant activity among the separated fractions evaluated by oxygen radical absorbance capacity (ORAC). Further purification of the MeOH eluate led to isolation of a novel compound, 4-amino-4-carboxychroman-2-one, together with four known compounds (*p*-coumaric acid, vanillic acid β-glucoside, protocatechuic acid, and caffeic acid), structures of which were identified by NMR and MS analyses. The ORAC values of these isolated compounds showed 0.15−1.43 units (μmol of Trolox equiv)/μmol, and the new compound showed a remarkable synergistic effect on caffeoylquinic acid isomers. The antioxidant activity of the MeOH eluate was highly dependent on the major prune components, caffeoylquinic acid isomers, with a contribution from the new synergist.

KEYWORDS: Prune (*Prunus domestica* L.); oxygen radical absorbance capacity (ORAC); HPLC; caffeoylquinic acid isomers; 4-amino-4-carboxychroman-2-one; synergist

INTRODUCTION

Generally, many kinds of plants include a high amount of antioxidant constituents. Antioxidants in fruits and vegetables are very important in human health because of the large amounts consumed. It is known that a high intake of fruits and vegetables contributes to a significant increase of antioxidant activity in human plasma (*1*). It was also reported that serum antioxidant capacity was enhanced by consumption of strawberry and spinach in elderly women (*2*). Antioxidant activities of fruits correlate with total contents of phenolic compounds (*3*, *4*), and the activity of fruits such as strawberry, plum, orange, and grapefruit is high (*5*). In addition, the antioxidant activity of prune was the highest, followed by raisin, blueberry, blackberry, strawberry, raspberry, and other fruits and vegetables on the basis of oxygen radical absorbance capacity (ORAC) (*6*).

Prunes are the dried fruit of some cultivars of *Prunus domestica* L., which originated from the Caucasus region. Prunes are a healthy food and have been used medicinally in India in combination with other drugs for the treatment of leukorrhea, irregular menstruation, and debility following miscarriage (*7*). High consumption of the dietary fiber in prunes lowered low-density lipoprotein (LDL) cholesterol in human plasma (*8*) as well as plasma and liver lipids in rats (*9*). It was also reported that prune intake prevented and improved ovariectomy-induced hypercholesterolemia (*10*) and bone mineral density loss (*11*, *12*) in rats, and an ethanol extract of prune showed antiemetic action against emesis induced by apomorphine in dogs (*13*).

Prunes contain large amounts of antioxidant constituents such as neochlorogenic acid (3-*O*-caffeoylquinic acid; **Figure 1**) and chlorogenic acid (5-*O*-caffeoylquinic acid; **Figure 1**) (*14*, *15*). In addition, recently we showed that a fairly high amount of cryptochlorogenic acid (4-*O*-caffeoylquinic acid; **Figure 1**) was also present in prunes (*16*). Chlorogenic acid is widely recognized to be an antioxidant for human LDL (*17*, *18*), a scavenger for reactive oxygen and nitrogen species (*19*), and an inhibitor against the formation of conjugated diene from linoleic acid oxidation (*20*). The antioxidant activity of chlorogenic acid is higher than those of vitamin C and vitamin E, based on the Trolox equivalent antioxidant activity (TEAC) (*21*).

It appears that the high antioxidant activity of prunes is dependent on the high content of phenolic compounds such as caffeoylquinic acid isomers. However, the specific antioxidant components in prune have not been well established. We have now isolated some antioxidants from prune and identified them by NMR and MS analyses and assessed their antioxidant activity based on the ORAC assay. In addition, the synergistic effect of a new chromanone on caffeoylquinic acid isomers is also described.

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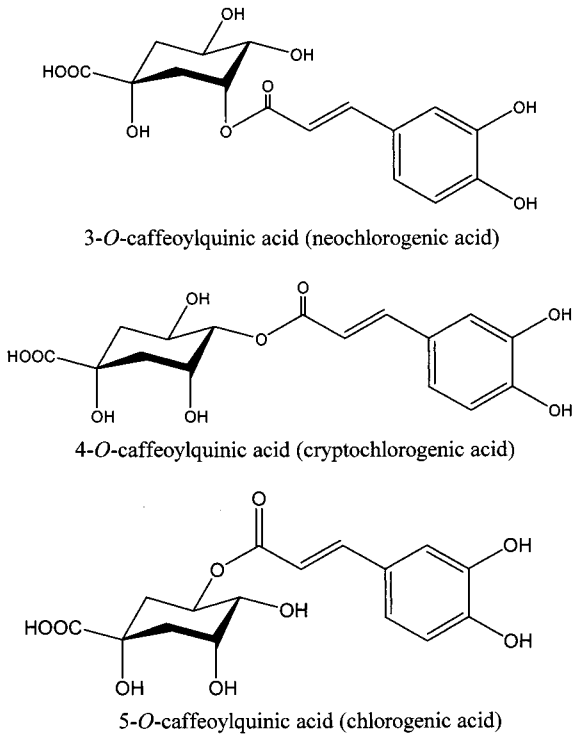

Figure 1. Structures of caffeoylquinic acid isomers.

MATERIALS AND METHODS

General Procedures. ^{1}H NMR, ^{13}C NMR, and 2D-NMR spectra (H−H COSY, ^{1}H−^{1}H correlation spectroscopy; HMQC, ^{1}H-detected multiple quantum coherence spectrum; HMBC, ^{1}H-detected multiple-bond heteronuclear multiple quantum coherrence spectrum) were obtained on a Varian Unity Plus 500 instrument (Varian Inc., Palo Alto, CA) at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively, at 25 °C and referenced to the residual proton solvent resonance (CD_3OD at 3.30 ppm for ^{1}H and 49.0 ppm for ^{13}C NMR). MS analysis was performed on a Hitachi M-1200AP mass spectrometer (Hitachi Ltd., Tokyo, Japan) with atmospheric pressure chemical ionization (APCI) interface, and HR-MS analysis was carried out on a JMS 700T mass spectrometer (JEOL Ltd., Tokyo, Japan) with a fast atom bombardment (FAB) ionization interface using a methanol (MeOH) solvent. Optical rotations were measured using a Jasco P-1030 automatic digital polarimeter (Jasco Co., Tokyo, Japan). IR spectra were run on a Perkin-Elmer 1800 instrument (Perkin-Elmer Inc., Wellesley, MA). HPLC analysis was carried out on a Waters 600E multisolvent delivery system with a 717plus autosampler and a 996 photodiode array detector (Waters Co., Milford, MA). An Arvo 1420sx (Wallac Berthold Japan Co., Tokyo, Japan), a type of microplate reader, was used for measurement of ORAC. Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), Chromatorex ODS DM1020T (100−200 mesh, Fuji Silysia Chemical, Tokyo, Japan), and silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany) were used for column chromatography. Silica gel 60 F_{254} plates (Merck) and ODS plates (Merck) were used for thin-layer chromatography (TLC).

Plant Material. Prunes, cv. d'Agen, were supplied by Miki Foods Co., Ltd. (Hyogo, Japan), which were imported from the United States as the material for commercial prune extract (concentrated prune juice). It is called "natural condition prune" (NC prune) with moisture levels adjusted to 21% (*22*).

Chemicals. β-Phycoerythrin from *Porphyridium cruentum* was obtained from Molecular Probes, Inc. (Eugene, OR), and 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chlorogenic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All commercial chemicals were of the highest analytical grade. Neochlorogenic acid and cryptochlorogenic acid were prepared from chlorogenic acid using the method described previously (*23*). The purities of these compounds were >99% for neochlorogenic acid and >96.5% for cryptochlorogenic acid as determined by HPLC analysis.

Table 1. ORAC of Prune Fractions

	ORAC[a] (units/mg)	yield[b] (mg)	total ORAC[c] (units)
hexane-soluble fraction	0.12 ± 0.01	77540	9305
H_2O eluate	nd[d]	4770000	
MeOH eluate	2.43 ± 0.23	70400	171072
fraction 1	1.07 ± 0.28	5	5
fraction 2	1.82 ± 0.05	2294	4175
fraction 3	1.95 ± 0.17	3267	6371
fraction 4	1.21 ± 0.12	17701	21425
fraction 5	2.03 ± 0.23	7454	15132
fraction 6	2.91 ± 0.16	17449	50777
fraction 7	2.81 ± 0.19	8063	22657
fraction 8	2.82 ± 0.19	5473	15434
fraction 9	1.90 ± 0.10	153	291

[a] ORAC values are expressed as 1 unit for 1 μmol of Trolox equivalent per mg of fractions. Each sample was measured in triplicate, and data are presented as mean value ± standard deviation. [b] Recovered weight of each fraction from 11.24 kg of prune. [c] Total ORAC values are calculated as ORAC (units/mg) × yields (mg). [d] nd, not detected.

Fractionation of Prune Extract. Prune fruits (11.24 kg) were pitted, cut into small pieces, and homogenized for 5 min with 8 L of 90% aqueous ethanol (EtOH). After filtration of the extract, another 8-L portion of aqueous EtOH was added to the residue. This procedure was repeated four times. The combined extract was evaporated in vacuo to remove EtOH, then 4.4 L of hexane was added to the concentrated extract, and the mixture was partitioned between hexane and H_2O. Another portion of hexane (4.4 L) was added to the H_2O-soluble fraction, and this procedure was repeated five times. The hexane-soluble fractions were combined and evaporated in vacuo to give concentrate (77.54 g). The H_2O-soluble fraction was separated by Diaion HP-20 column chromatography using H_2O as an eluting solution followed by elution with MeOH, and each solution was evaporated in vacuo to give the H_2O eluate (4770 g) and the MeOH eluate (70.40 g). The antioxidant activity of the hexane-soluble fraction, the H_2O eluate, and the MeOH eluate was then evaluated based on ORAC.

An aliquot of the MeOH eluate (65 g), which showed a high ORAC value (**Table 1**), was rechromatographed over a column of Sephadex LH-20 gel using 80% aqueous acetone as the mobile phase to give nine fractions (fractions 1−9) by monitoring with silica gel and ODS TLC analysis. The ORAC of each fraction was evaluated, and the quantity of caffeoylquinic acid isomers in these fractions were measured by HPLC analysis.

Measurement of ORAC. ORAC was measured according to the method described previously (*24*). This assay is based on the principle that antioxidant compounds delay the decrease of β-phycoerythrin fluorescence induced by AAPH, a peroxyl radical generator.

The test mixture was prepared with 170 μL of 19.6 nM β-phycoerythrin in 75 mM phosphate buffer, pH 7.0, and 10 μL of sample in 75 mM phosphate buffer, or acetone at 20 μM for pure compounds or 20 μg/mL for crude extract, in the microwell plate (96/well, black, Corning Costar Co., Cambridge, MA). Phosphate buffer alone was used as a blank, and 20 μM Trolox (**Figure 2**) was used as a control. After 30 min of incubation at 37 °C, 20 μL of 200 mM AAPH solution was added to the mixture to initiate the assay, and the fluorescence of each well was read every 2 min over a 70 min period at 37 °C. The area under the fluorescence curve was calculated, and the ORAC value of each sample was expressed as 1 unit for 1 μmol equivalent of Trolox. Each sample was measured in triplicate, and data were expressed as mean value ± standard deviation. The ORAC values were determined using an Arvo 1420sx, automated microplate reader, at an excitation wavelength of 530 nm and emission wavelength of 570 nm.

HPLC Analysis. The quantities of caffeoylquinic acid isomers in prune fractions were measured by HPLC analysis. Five milligrams of each sample was dissolved in 50 mL of 50% aqueous MeOH. Standards

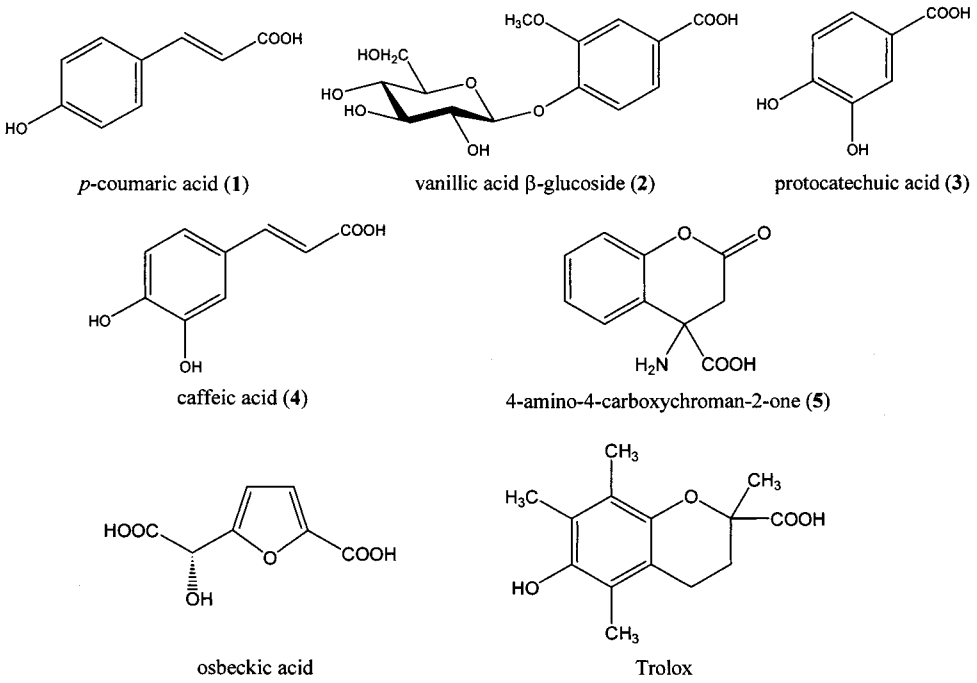

Figure 2. Structures of isolated prune components, osbekic acid, and Trolox.

Table 2. Contribution of Caffeoylquinic Acid Isomers on ORAC of Prune Fractions

	contents of CQA[a] isomers (mg/g)			ORAC of CQA isomers[b] (units/g)				ORAC of fractions[f]	contribution of
	3-CQA[c]	4-CQA[d]	5-CQA[e]	3-CQA	4-CQA	5-CQA	total	(units/g)	CQA isomers[g] (%)
fraction 1[h]									
fraction 2	nd[i]	nd	nd					1820	0.0
fraction 3	nd	nd	nd					1950	0.0
fraction 4	96.0	nd	nd	506			506	1210	41.8
fraction 5	41.3	nd	nd	218			218	2030	10.7
fraction 6	139.0	28.7	nd	733	155		888	2910	30.5
fraction 7	231.9	73.1	18.8	1222	396	86	1704	2810	60.6
fraction 8	253.8	27.9	nd	1338	151		1489	2820	52.8
fraction 9	18.3	nd	nd	96			96	1900	5.1

[a] CQA, caffeoylquinic acid. [b] Calculated as contents of CQA isomers (mg/g) × ORAC of each CQA isomer (units/mg; see **Table 3**). [c] 3-CQA, 3-*O*-caffeoylquinic acid. [d] 4-CQA, 4-*O*-caffeoylquinic acid. [e] 5-CQA, 5-*O*-caffeoylquinic acid. [f] Calculated as ORAC of **Table 1** × 1000. [g] Calculated as total ORAC of CQA isomers × ORAC of fractions^{-1} × 100. [h] Fraction 1 was so small that it was omitted. [i] nd, not detected.

of neochlorogenic acid, cryptochlorogenic acid, and chlorogenic acid were also dissolved in 50% MeOH to make a concentration of 1−6 ppm. The sample and standard solutions were filtered through 0.45 μm olefin polymer syringe-tip filters (Biofield Co., Tokyo, Japan) and analyzed by HPLC according to the method described previously (*16*). Each peak detected in sample solutions was identified by comparing retention time and UV−vis spectra given by a photodiode array detector with the standards. Each caffeoylquinic acid isomer in prune fractions was quantified by calibration with the standards.

Isolation and Identification of Prune Components. Fraction 5, which exhibited high ORAC and contained a small amount of caffeoylquinic acid isomers (**Table 2**), was subjected to ODS column chromatography (700 mm × 100 mm ∅) and eluted with H_2O/CH_3CN (95:5) to give 50 fractions (fractions 5-1−5-50). Some of these fractions were further purified by various column chromatographies to afford compound **1** (4 mg) from fraction 5-6, compound **2** (4 mg) from fraction 5-9, and compounds **3** (4 mg) and **4** (12 mg) from fraction 5-16.

Fraction 5-18 (39 mg) was rechromatographed on a column (170 mm × 10 mm ∅) of silica gel with hexane/EtOAc (60:40) to give seven fractions (fractions 5-18-1−5-18-7). Fraction 5-18-4 was subjected to ODS column (370 mm × 10 mm ∅) chromatography with H_2O/CH_3CN (70:30) to give four fractions (fractions 5-18-4-1−5-18-4-4). Fraction 5-18-4-2 was further separated by Sephadex LH-20 column chromatography (100 mm × 6 mm ∅) eluted with 2-propanol to afford three fractions (fractions 5-18−4−2-1−5-18-4-2-3). Compound **5** (4 mg) was isolated from fraction 5-18-4-2-2 by silica gel column chromatography (170 mm × 10 mm ∅) with EtOAc/MeOH/H_2O (70:20:10).

The structure of each isolated compound (compounds **1**−**5**) was elucidated by NMR and MS analyses and identified as follows: compound **1**, *p*-coumaric acid; compound **2**, vanillic acid β-glucoside; compound **3**, protocatechuic acid; compound **4**, caffeic acid; compound **5**, 4-amino-4-carboxychroman-2-one (**Figure 2**).

Compound **5** was a novel compound, and its physicochemical data are as follows: white powder; $[\alpha]^{28}_D$ −9.7° (*c* 0.42, MeOH); HR-FABMS, *m/z* 206.0482 [M − H]$^-$, calculated for 206.0453 [$C_{10}H_9NO_4$ − H]$^-$; UV (MeOH) λ_{max} (log ϵ), 253 nm (3.33), 287 nm (2.75); IR ν_{max}, 3391 cm^{-1} (NH_2), 2925 cm^{-1} (CH_2), 1736 cm^{-1} (δ-lactone), 1652 cm^{-1} (C=O); ^{1}H NMR (500 MHz, CD_3OD) δ 2.64 (1H, d, J = 16 Hz, H-3), 2.79 (1H, d, J = 16 Hz, H-3), 6.86 (1H, dd, J = 1, 7 Hz, H-8), 6.99 (1H, ddd, J = 1, 7, 8 Hz, H-6), 7.21 (1H, ddd, J = 1, 7, 8 Hz, H-7), 7.41 (1H, dd, J = 1, 7 Hz, H-5); ^{13}C NMR (125 MHz, CD_3OD) δ 43.3 (C-3), 75.5 (C-4), 111.2 (C-8), 123.6 (C-6), 125.1 (C-5), 130.5 (C-7), 133.2 (C-10), 142.8 (C-9), 176.8 (C-11), 181.2 (C-2).

RESULTS AND DISCUSSION

Contribution of Caffeoylquinic Acid Isomers to ORAC of Prune Fractions. The EtOH extract of prune was separated into three fractions, the hexane-soluble fraction, the MeOH

Table 3. ORAC of Prune Components

	ORAC	
	units/mg[a]	units/μmol[a]
3-*O*-caffeoylquinic acid	5.27 ± 0.70	1.87 ± 0.25
4-*O*-caffeoylquinic acid	5.42 ± 0.19	1.92 ± 0.07
5-*O*-caffeoylquinic acid	4.60 ± 0.04	1.63 ± 0.01
p-coumaric acid (**1**)		0.67 ± 0.12
vanillic acid β-glucoside (**2**)		0.35 ± 0.08
protocatechuic acid (**3**)		1.07 ± 0.01
caffeic acid (**4**)		1.43 ± 0.19
4-amino-4-carboxychroman-2-one (**5**)		0.15 ± 0.06
3-CQA[b] + **5** (1 μM + 1 μM)		2.81 ± 0.04
3-CQA + **5** (1 μM + 2 μM)		5.37 ± 1.00
4-CQA[c] + **5** (1 μM + 1 μM)		2.88 ± 0.11
4-CQA + **5** (1 μM + 2 μM)		3.72 ± 0.14
5-CQA[d] + **5** (1 μM + 1 μM)		2.45 ± 0.02
5-CQA + **5** (1 μM + 2 μM)		4.19 ± 0.22

[a] ORAC values are expressed as 1 unit for 1 μmol of Trolox equivalent per mg or μmol of compound. Each sample was measured in triplicate, and data are presented as mean value ± standard deviation. [b] 3-CQA, 3-*O*-caffeoylquinic acid. [c] 4-CQA, 4-*O*-caffeoylquinic acid. [d] 5-CQA, 5-*O*-caffeoylquinic acid.

eluate, and the H_2O eluate. The H_2O eluate was predominant in quantity, and the hexane-soluble fraction as well as the MeOH eluate was quite small. In contrast, the antioxidant activity based on ORAC of the MeOH eluate was ~20 times higher than that of the hexane-soluble fraction, and the activity of the H_2O eluate was too small to be detected (**Table 1**). It was apparent that antioxidant components were almost entirely in the MeOH eluate, and it was presumed that most substances in the H_2O eluate were sugars and organic acids, such as glucose, fructose, sorbitol, malic acid, and quinic acid.

The MeOH eluate, which showed high ORAC, was further separated by column chromatography on Sephadex LH-20 to give nine fractions. The sum of total ORAC of these nine fractions (136267 units, calculated from the data of **Table 1**) is smaller than that of the MeOH eluate, and this discrepancy might be attributable to the yields of each fraction. Fractions 4−8 showed a total ORAC value, as high as 15000−50000 units (**Table 1**), and to estimate the influence of caffeoylquinic acid isomers on antioxidant activity of these fractions, HPLC analysis was carried out. As shown in **Table 2**, the major isomer was neochlorogenic acid followed by cryptochlorogenic acid and chlorogenic acid, and these isomers were detected in fractions 4−9 for neochlorogenic acid, fractions 6−8 for cryptochlorogenic acid, and fraction 7 for chlorogenic acid. In particular, fractions 4, 6, 7, and 8 contained a large amount of caffeoylquinic acid isomers, 100−320 mg/g, and fractions 2 and 3 did not contain any isomers. On the other hand, the ORACs of caffeoylquinic acid isomers were 4.60−5.42 units/mg (**Table 3**), and these isomers contributed significantly to the antioxidant activity of fractions 4, 6, 7, and 8 (30.5−60.6%, **Table 2**). Only fraction 5 indicated high ORAC, but the contribution of caffeoylquinic acid isomers was as low as 10.7% (**Table 2**). It was predicted that other antioxidants might exist in this fraction, and hence further purification of fraction 5 was attempted to isolate unknown antioxidative compounds in prune.

Structural Elucidation of Prune Components. Repeated chromatography of fraction 5 resulted in the isolation of one novel compound along with the four known compounds, *p*-coumaric acid, vanillic acid β-glucoside, protocatechuic acid, and caffeic acid, the structures of which were determined by NMR, including $^1H-^1H$ COSY, HMQC, HMBC, and MS analyses. Vanillic acid β-glucoside and protocatechuic acid were isolated from prune (*P. domestica*) for the first time. A new

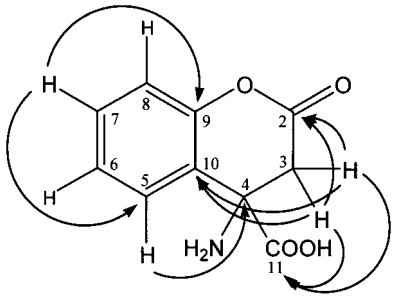

Figure 3. Long-range correlation in the HMBC spectrum of **5**.

compound, 4-amino-4-carboxychroman-2-one (**5**) (**Figure 2**), was obtained as a white powder with a negative optical rotation ($[\alpha]_D^{28}$ −9.7°). Various NMR experiments revealed a 1,2-disubstituted benzene ring, one methylene group, one quaternary carbon, and two carboxyl groups. The downfield shift of the aromatic carbon at the 9-position indicated substitution with an oxygen atom. In an HMBC experiment (**Figure 3**), long-range correlations were observed between an aromatic proton (H-5) and a quaternary carbon (C-4), between methylene protons (H-3) and two carboxyl carbons (C-2 and C-11), and between a methylene proton (H-3) and an aromatic carbon (C-10). From these results, it was determined that the quaternary carbon at C-4, attached to C-10, was substituted with methylene (C-3) and carboxyl carbons (C-11). The IR spectrum of **5** showed absorption at 1736 cm^{-1} (δ-lactone), and the negative HR-FABMS spectrum showed an $[M - H]^-$ peak at *m*/*z* 206.0482 corresponding to $C_{10}H_9NO_4$. On the basis of these IR and MS analyses, it was established that the carboxyl group at C-2 is esterified with the hydroxyl group at the 9-position to form a δ-lactone, and an amino group is attached to a quaternary carbon (C-4). In addition, this compound was positive in the ninhydrin reaction. Hence, the planar structure of **5** was characterized as shown in **Figure 2**.

ORAC of Prune Components. Antioxidant activities of prune components were evaluated on the basis of ORAC (**Table 3**). It was predicted that there was no difference among the ORAC values of caffeoylquinic acid isomers, showing 1.63−1.92 units/μmol. This result is similar to those of scavenging activity on O_2^- and inhibitory effect against oxidation of methyl linoleate reported previously (*16*). It is suggested that the position of esterification by caffeic acid on quinic acid has no effect on its antioxidant activity.

Other phenolic components isolated from fraction 5 showed ORAC values in the range of 0.15−1.43 units/μmol (**Table 3**). The activity of caffeic acid was the highest among them, followed by protocatechuic acid, *p*-coumaric acid, vanillic acid β-glucoside, and 4-amino-4-carboxychroman-2-one (**5**). As well as caffeoylquinic acid isomers, *o*-diphenol compounds such as caffeic acid and protocatechuic acid showed higher ORAC, 1.07−1.43 units/μmol (**Table 3**). Among the *o*-diphenol compounds, the activity of caffeoylquinic acid isomers was the highest, followed by caffeic acid and protocatechuic acid, which was a similar result to that reported recently (*25*). On the other hand, monophenolic compounds such as *p*-coumaric acid and vanillic acid β-glucoside showed relatively lower activity, 0.35−0.67 unit/μmol (**Table 3**). It seemed that the ORAC of the MeOH eluate depended on the compounds bearing an *o*-diphenol group in the molecule. 4-Amino-4-carboxychroman-2-one (**5**) showed a lower ORAC, 0.15 unit/μmol. However, when 1 μM of **5** was added to 1 μM of 3-*O*-caffeoylquinic acid, the ORAC raised to 2.81 units/μmol, which was higher than the expected value of 2.02 units/μmol (sum of the data of 3-*O*-caffeoylquinic acid and **5** in **Table 3**). This compound (**5**) showed a remarkable

synergistic effect on each caffeoylquinic acid isomer, and the ORAC values were enhanced 1.5−2.9 times (**Table 3**). It is known that osbekic acid (**Figure 2**) isolated from *Osbeckia chinensis* L. is an antioxidative synergist to α-tocopherol, methyl gallate, and punicacortein (*26*), and ascorbic acid is also well-known as a synergist to α-tocopherol. 4-Amino-4-carboxychroman-2-one (**5**) may contribute to the antioxidative activity of prune by the synergistic effect on caffeoylquinic acid isomers.

In conclusion, the antioxidant activity of prune evaluated by ORAC highly depends on phenolic compounds such as caffeoylquinic acid isomers. However, the overall ORAC of prune seems not to be dependent on only caffeoylquinic acid isomers, and it is predicted that unknown antioxidant or synergistic compounds exit and contribute to the antioxidant activity of prune.

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