The fruit of mume, Japanese apricot (Prunus mume Sieb. et Zucc.), was evaluated for its phenolics content, high performance liquid chromatography (HPLC) profile and antioxidative activities. The phenolics content of mume fruit was relatively high, the flesh of fully matured fruit containing up to 1% of phenolics on a dry weight basis. Reflecting such a high content of phenolics, the ORAC (oxygen radical absorbance capacity) value for mume fruit flesh showed high values, ranging from 150 to 320 µmol/g Trolox equivalent, depending upon the stage of maturation. 5-O-Caffeoylquinic acid (chlorogenic acid), 3-O-caffeoylquinic acid and tetra-O-acylated sucrose-related compounds were isolated from the flesh of mume fruit, although many unknown peaks were also apparent in the HPLC chromatogram. An alkali hydrolysate comprised four main phenolic acids, caffeic acid, cis/trans-p-coumaric acid and ferulic acid. No flavonoids were observed in the analysis. These results suggest that the majority of phenolics in mume fruit were hydroxycinnamic acid derivatives.

Key words: Prunus mume; phenolics; oxygen radical absorbance capacity (ORAC); hydroxycinnamic acid; chlorogenic acid

Mume, Japanese apricot (Prunus mume Sieb. et Zucc.), belongs to the Rosaceae family and is one of the most popular fruit trees in Japan. Unlike many other fruits, most of the harvested fruits are first processed and then consumed, due to the presence of cyanoglycoside, prunasin and amygdalin1,2) and the extreme sourness of these properties, the pickled mume fruit (umeboshi) is the most popular fruit trees in Japan. Unlike many other Rosaceae, Zucc.), belongs to the

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Abbreviations: ORAC, oxygen radical absorbance capacity; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance
during fruit maturation, this being the most popular cultivar grown in Wakayama Japan where over 50% of national production is originated. We also evaluated the antioxidative properties of the mume fruit phenolics by the oxygen radical absorbance capacity (ORAC) method and elucidated the chemical structures of some of the phenolics.

Materials and Methods

Fruit samples. Fruit samples of P. mume cv. nanko were randomly collected from a single fixed tree, which was grown in the experimental orchard of the Laboratory of the Japanese Apricot at the Fruit Tree Experiment Station of Wakayama prefectural government, at three different growth stages from 2004–2009. The harvesting of the samples was at the stone-hardening stage (i), premature stage with a deep green peel color approximately 105–110 d after flowering (ii), and mature stage with a yellow peel color approximately 130 d after flowering (iii). The samples were washed in tap water, dried, and then manually separated into the flesh and stone parts. The stone part was further divided into the endocarp, internal seed coat and kernel. The freeze-dried powder was sieved into a wire screen (425 μm aperture) and was stored in light-shielded bottles at 20°C. Any flesh that remained on the surface of the endocarp was removed by brushing, the endocarp was dried in a vacuum desiccator for two days, and the defatted with 20 mL of n-hexane for albumen, prior to being extracted with 90% ethanol. An Agilent 1100 system was used for HPLC analyses. A portion of each phenolic fraction collected from the phenolic fraction was injected into the Hydrosphere C18 column (5 μm pore size, 250 mm × 25 mm; YMC). The column was separated into the flesh and stone parts. The stone part was further divided into the flesh and stone parts. The stone part was further divided into the endocarp, internal seed coat and kernel. The freeze-dried and powdered flesh was sieved into a wire screen (425 μm aperture) and was stored in light-shielded bottles at 20°C. Any flesh that remained on the surface of the endocarp was removed by brushing, the endocarp was dried in a vacuum desiccator for two days, and the freeze-dried powder was stored in a light-shielded bottle at 20°C until needed for further extraction. The kernel was cut and dried in the vacuum desiccator to constant weight. The dried kernel was further shredded prior to subsequent extraction.

Preparation of the phenolic fractions. Five hundred mg of freeze-dried powder of the flesh and of vacuum-dried powder of the kernel and endocarp were sonicated in 25 mL of 90% ethanol for 2 min by a Branson 450 sonifier (20 kHz, 200 W). Each sonicated suspension was collected. Whenever centrifugal separation was insufficient, the supernatant was further filtered through Millipore-LG (0.2 μm pore size, Millipore Corporation). A 1 g amount of the shredded sample was defatted with 20 mL of n-hexane for albumen, prior to being extracted with 90% ethanol.

HPLC and LC/MS analyses. An Agilent 1100 system was used for the HPLC analyses. A portion of each phenolic fraction collected from the flesh, endocarp and kernel of the fruit was evaporated to dryness, and the residue was dissolved in a minimal volume of dimethyl sulfoxide (DMSO) before being injected into a HPLC C18 column (4.6 mm × 250 mm; YMC, Kyoto, Japan). The column was eluted with mixed solvent A (0.1% TFA):B (methanol) at a flow rate of 1.0 mL/min. The eluent was A:B = 80:20 for first 10 min, and then a linear gradient ending with 25:75 after 80 min. All analyses were carried out at 30°C and monitored by UV absorption at 280 nm. The LC/MS analyses of compounds 1 and 2 replaced solvent A with 0.5% formic acid and solvent B as it matured. The mean wet weight of one fruit was 16.0 g, after which the phenolics in the flesh decreased with increasing maturation. The weight of the fruit increased as it matured. The mean wet weight of one fruit was 19.5 g at the stone-hardening stage, 33.9 g at the fully mature stage. The mean wet flesh weight of one fruit was 16.0 g, 22.9 g and 42.3 g at each stage. It has been reported that organic acids and sorbitol were accumulated, but that the phenolics content on a wet weight basis was almost 22.9 g and 42.3 g at each stage. The mean wet weight of one fruit was 16.0 g, 22.9 g and 42.3 g at each stage. It has been reported that organic acids and sorbitol were accumulated, but that the phenolics content on a wet weight basis was almost constant during the maturation process of mume fruit.

Separation was performed at a flow rate of 1.5 mL/min and 4°C with C:B gradient steps of 75:15 to 45:35 in 220 min, and then 0:100 in 40 min. Aliquots of the eluate corresponding to each peak were collected, evaporated to dryness and dissolved in 1 mL of methanol.

Determination of total phenolics. The total phenolic content was determined by the Folin-Ciocalteu method, using gallic acid as a standard. The value is expressed as gallic acid (mg) per gram dry weight.

ORAC assay. The antioxidative activity was determined by an oxygen radical absorbance capacity (ORAC) assay as described by Ou et al. Each sample was diluted to the appropriate concentration, and the fluorescence was monitored by a 96-well fluorescent microplate reader (TEKAN SpectraFlour Plus, Austria). Each ORAC value is expressed as micromole Trolox equivalent per gram dry weight.

Alkaline hydrolysis. An ester or ether linkage was hydrolyzed by following the procedure described by Krygier. Two hundred and fifty mg of freeze-dried flesh powder was suspended in 10 mL of 1 N NaOH that had been deoxygenized in advance by bubbling nitrogen gas. After 4 h of incubation at 37°C, the suspension was acidified by adding phosphoric acid to pH 4.5 and then extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness, and the residue was dissolved in a minimal volume of methanol for analysis under the above-mentioned HPLC conditions.

Results and Discussion

Phenolic content of mume fruit

Changes in the phenolic concentrations of the mume fruit are summarized in Table 1. The flesh demonstrated the highest concentrations throughout the three stages of maturation. At the stone-hardening stage, the flesh contained over 1% of phenolics on a dry weight basis, after which the phenolics in the flesh decreased with increasing maturation. The weight of the fruit increased as it matured. The mean wet weight of one fruit was 19.5 g at the stone-hardening stage, 33.9 g at the premature stage and 46.6 g at the fully mature stage. The mean wet flesh weight of one fruit was 16.0 g, 22.9 g and 42.3 g at each stage. It has been reported that organic acids and sorbitol were accumulated, but that the phenolics content on a wet weight basis was almost constant during the maturation process of mume fruit.

Table 1. Changes in Phenolics Content and Antioxidative Activity of Mume Fruit through Different Stages of Growth

<table>
<thead>
<tr>
<th>Phenolics content (mg/g gallic acid equivalent, dry weight basis)</th>
<th>Stone-hardening stage</th>
<th>Premature stage</th>
<th>Full mature stage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flesh</strong></td>
<td>11.7 ± 0.2</td>
<td>9.5 ± 0.1</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td><strong>Stone</strong></td>
<td>2.6 ± 0.2</td>
<td>6.4 ± 0.2</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Kernel</strong></td>
<td>5.9 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.9 ± 0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ORAC value (micromole/g, Trolox equivalent, dry weight basis)</th>
<th>Stone-hardening stage</th>
<th>Premature stage</th>
<th>Full mature stage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flesh</strong></td>
<td>320 ± 14</td>
<td>239 ± 14</td>
<td>158 ± 11</td>
</tr>
<tr>
<td><strong>Stone</strong></td>
<td>55 ± 6</td>
<td>96 ± 9</td>
<td>109 ± 10</td>
</tr>
<tr>
<td><strong>Kernel</strong></td>
<td>49 ± 2</td>
<td>18 ± 1</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

a, 75–80 d after flowering, peel color was deep green; b, 105–110 d after flowering, peel color was green; 130 d after flowering, peel color was yellow.
been reported to vary widely among fruit species. The phenolic concentrations of the endocarp throughout the fruit maturation. The overall ORAC values for the level of that of the immature fruits in the last stage of the change in concentration of the phenolics. In contrast, the ORAC value of the flesh decreased with maturation as highest ORAC value among the analyzed samples. The Flesh harvested at the stone-hardening stage showed the fairly steady throughout the harvesting season.

The ORAC values at each stage are shown in Table 1. Flesh harvested at the stone-hardening stage showed the highest ORAC value among the analyzed samples. The ORAC value of the flesh decreased with maturation as the change in concentration of the phenolics. In contrast, the ORAC values of the endocarp reached twice the level of that of the immature fruits in the last stage of fruit maturation. The overall ORAC values for the kernel were not very high and tended to decrease during fruit maturation. It was noted that the change in the phenolic concentrations of the endocarp throughout the fruit maturation process was substantially greater than that of the flesh. The ORAC values of various fruits have been reported to vary widely among fruit species. The ORAC values presented in Table 1 were obtained from the samples extracted with a 90% ethanol solution, so the flesh of mume fruit was extracted with water and then with acetone to compare with other fruits. The total respective ORAC values of the water extract and acetone extract of the flesh were 315, 171 and 139 (Trolox equivalent micromole/g on a dry weight basis) at the stone hardening stage, premature stage, and fully mature stage. The concentration of phenolics remained fairly steady throughout the harvesting season.

The monomer. In contrast, the weight of the kernel remained the stone may be explained by the deposition of a unique HPLC profile at each growth stage. Certain compounds 1 and 2 were isolated from the phenolic fraction of the flesh of mume fruit at the premature stage (not edible) and other stages (edible), although differences were apparent between the stone-hardening stage, premature stage, and fully mature stage. Mume fruit may therefore be regarded as a fruit with high antioxidative activity.

HPLC profiles

Figure 1 shows that the analysis of the flesh presented a unique HPLC profile at each growth stage. Certain differences were apparent between the stone-hardening stage (not edible) and other stages (edible), although there were common peaks across all the stages. The chromatograms of the premature and fully mature samples demonstrated a similar peak profile.

Identification of several compounds

The HPLC analysis of the phenolic fraction of the fruit flesh revealed several common peaks at all stages of the fruit growth (Fig. 1). We isolated three common main peaks from among the various peaks shown. Compounds 1 and 2 were isolated from the phenolic fraction of the flesh of mume fruit at the premature stage, while compound 3 was isolated from the sample at the stone-hardening stage.

More than 10 mg of each compound was obtained, and the final structural evidence was achieved by 1H-NMR. Compounds 1 and 2 gave the same [M + 1]⁺ ion at m/z 355 in the positive ion mode of their ESI-TOF-MS data. Their 1H-NMR spectra were compared to the spectra previously reported, compounds 1 and 2 being respectively identified as 3-O-cafeoiloquinic acid (neochlorogenic acid) and 5-O-cafeoilquinic acid (chlorogenic acid) by comparing their MS and 1H-NMR spectral data (Fig. 2).

Compound 1 and 3-O-cafeoiloquinic acid (neochlorogenic acid)

1H-NMR (400 MHz, methanol-d₄) δ: 1.95 (1H, dd, J = 7, 11 Hz, H-6ax), 2.13 (2H, m, H-2eq and 6eq), 2.20 (1H, dd, J = 3, 12 Hz, H-2ax), 3.63 (1H, dd, J = 1, 7Hz, H-4), 4.14 (1H, ddd, J = 2, 7, 7Hz, H-5), 5.34 (1H, ddd, J = 2, 2, 3 Hz, H-3), 6.30 (1H, d, J = 13 Hz, H-8), 6.76 (1H, d, J = 6 Hz, H-5), 6.93 (1H, ddd, J = 2, 6Hz, H-6), 7.04 (1H, d, J = 2 Hz, H-2), 7.58 (1H, d, J = 13 Hz, H-7).

Compound 2 and 5-O-cafeoilquinic acid (chlorogenic acid)

1H-NMR (400 MHz, methanol-d₄) δ: 2.02 (1H, dd, J = 7, 11 Hz, H-6ax), 2.16 (2H, m, H-2eq and 6eq), 2.22 (1H, dd, J = 3, 12 Hz, H-2ax), 3.72 (1H, dd, J = 1, 7Hz, H-4), 4.17 (1H, ddd, J = 2, 7, 7Hz, H-5), 5.34 (1H, ddd, J = 2, 2, 3 Hz, H-3), 6.26 (1H, d, J = 16 Hz, H-8), 6.76 (1H, d, J = 8 Hz, H-5), 6.94 (1H, dd, J = 2, 6Hz, H-6), 7.04 (1H, d, J = 4 Hz, H-2), 7.56 (1H, d, J = 13 Hz, H-7).

Compound 3 was obtained as a white powder with the elemental composition of C₂₀H₁₆O₇ established by the high-resolution LC-MS/MS (ESI, positive) data. The LC-MS data of compound 3 showed a molecular ion (M + Na⁺) peak at m/z 679, in addition to a fragment ion (M⁺) peak by LC-MS/MS at m/z 147 derived from the p-coumarate moiety. The 1H-NMR (400 MHz, methanol-d₄) spectrum suggested that compound 3 contained a p-coumaro group [δ: 6.42 (1H, d, J = 15.9 Hz), 7.74 (1H, d, J = 15.9 Hz); 2" and 3"-H, 6.81 (2H, d, J = 8.6 Hz), 7.54 (2H, d, J = 8.6 Hz), 3", 5" and 2", 6"-H] and acetyl groups [δ: 1.77, 1.99,
2.07, 2.09 (3H, s). Compound 3 was thus assigned to the group of polyacylated sucrose, 1,3,4,6'-Tetra-O-acetyl-6-O-p-coumaroyl-sucrose (prunose II; 3-1) and 4,3,4,6'-Tetra-O-acetyl-6-O-p-coumaroyl-sucrose (prunose III; 3-2).

Alkaline hydrolysis

The freeze-dried flesh powder was hydrolyzed under alkaline conditions to elucidate the phenolic composition. Typical HPLC data for the alkali hydrolysate at the premature stage in 2008 are shown in Fig. 3. The chromatograms of the alkali hydrolysate showed similar patterns regardless of the maturity stage or the production year of the fruit. These chromatograms had four common main peaks (A, B, C and D). Compounds A, C and D were tentatively identified from their spectral characteristics and retention times by comparing with the respective commercially available pure compounds, caffeic acid, trans-p-coumaric acid, and ferulic acid. They were respectively identified from an HPLC negative-ion ESI-MS analysis as the ions of caffeic acid, p-coumaric acid, and ferulic acid.

Compound A: $m/z = 179.0343$ (179.0350 calcd. for $C_9H_7O_4\frac{1}{2}M$)

Compound C: $m/z = 163.0391$ (163.0401 calcd. for $C_9H_7O_3\frac{1}{2}M$)

Compound D: $m/z = 193.0501$ (193.0506 calcd. for $C_{10}H_9O_4\frac{1}{2}M$)

Compounds A to D were isolated and purified by using preparative HPLC. The $^1$H-NMR spectrum of compound B was consistent with the spectrum of cis-p-coumaric acid previously reported, and compounds A, C and D were also confirmed.

Alkali-hydrolysis experiments revealed that aglycones of the hydroxycinnamic acid derivatives were largely made up of trans-p-coumaric acid, caffeic acid, ferulic acid and cis-p-coumaric acid in the order of peak area. The experiments also showed the relative proportions of the aglycones to be constant during the
maturity of mume fruit, although there were some differences in the HPLC profiles between the samples from the stone-hardening stage and other stages. It appears that these hydroxycinnamic acids were present in such forms as ester bound or glycosidic.

**Compound A and caffeic acid**

1H-NMR (400 MHz, MeOD, TMS) δ: 7.52 (1H, d, J = 16 Hz), 7.03 (1H, d, J = 2.0 Hz), 6.93 (1H, d), J = 8.0, 2.0 Hz), 6.77 (1H, d, J = 8.0 Hz), 6.21 (1H, d, J = 16 Hz).

**Compound B and cis-p-coumaric acid**

1H-NMR (400 MHz, MeOD, TMS) δ: 7.59 (2H, d, J = 8.0 Hz), 6.77 (1H, d, J = 12 Hz), 6.73 (2H, d, J = 8.0 Hz), 5.77 (1H, d, J = 16 Hz).

**Compound C and trans-p-coumaric acid**

1H-NMR (400 MHz, MeOD, TMS) δ: 7.59 (1H, 22d, J = 16 Hz), 7.18 (1H, d, J = 2.0 Hz), 7.06 (1H, dd, J = 8.0, 2.0 Hz), 6.81 (1H, d, J = 8.0 Hz), 6.31 (1H, d, J = 16 Hz), 3.89 (3H, s).

It is generally understood that the beneficial effects of phenolic compounds would reduce the risk of major chronic diseases. However, information on their efficacy in humans is limited, although the health benefits of phenolic compounds have been shown in *in vitro* and animal studies. The main obstacles to enhancing their efficacy are the poor absorption of phenolic compounds in the alimentary canal and extensive metabolism in the intestines and liver. Phenolic acids are considered to be anti-inflammatory, anticarcinogenic, and antimicrobial agents, as well as antioxidants. The benefits provided by phenolic acids may lie in their antioxidative properties and their bioavailability. Konishi *et al.* have reported the transport rate of *p*-coumaric acid being 100 times higher than that of gallic acid across Caco-2 cell monolayers and the relative bioavailability of *p*-coumaric acid against gallic acid being about 70 based on calculations from the serum concentration profile in the portal vein of rats. We also confirmed that the bioavailability of *p*-coumaric acid was significantly higher than that of caffeic or ferulic acid in mice and rats (unpublished data). Mume fruit phenolics may have the capacity to reduce the risk of certain chronic diseases.

We clarified in this study that the total content of extractable phenolics in mume fruit flesh was about 1% on a dry weight basis and that the phenolics mostly consisted of hydroxycinnamic acid derivatives. It is well known that there are many economically valuable fruits throughout the world in the group of genus *Prunus*; for example, dried plums (*P. domestica*) contain a large amount of chlorogenic acids which constitute as much as 90% of the entire phenolic content. Nectarines, peaches, and plums contain such phenolics as hydroxycinnamic acids, flavan-3-ols, and flavonols. Chlorogenic acids were the predominant hydroxycinnamic acids, this being different from the present finding for mume fruit. The contents and proportion of phenolics in *Prunus* fruits vary with the species within the genus. The characteristic health benefits that stone fruits provide would consequently also vary to reflect these differences in the fruit constituents.

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**References**