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Hydroxycinnamoylmalic Acids and Their Methyl Esters from Pear (*Pyrus pyrifolia* Nakai) Fruit Peel

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ABSTRACT: Two novel caffeoylmalic acid methyl esters, 2-*O*-(*trans*-caffeoyl)malic acid 1-methyl ester (**6**) and 2-*O*-(*trans*-caffeoyl)malic acid 4-methyl ester (**7**), were isolated from pear (*Pyrus pyrifolia* Nakai cv. Chuhwangbae) fruit peels. In addition, 5 known hydroxycinnamoylmalic acids and their methyl esters were identified: 2-*O*-(*trans*-coumaroyl)malic acid (**1**), 2-*O*-(*cis*-coumaroyl)malic acid (**2**), 2-*O*-(*cis*-coumaroyl)malic acid 1-methyl ester (**3**), 2-*O*-(*trans*-coumaroyl)malic acid 1-methyl ester (**4**), and 2-*O*-(*trans*-caffeoyl)malic acid (phaselic acid, **5**). The chemical structures of these compounds were determined by spectroscopic data from ESI MS and NMR. Of all the isolated compounds, five hydroxycinnamoylmalic acids and their methyl esters (**2**−**4**, **6**, **7**) were identified in the pear for the first time.

KEYWORDS: *Pyrus pyrifolia* Nakai, pear fruit, hydroxycinnamoylmalic acid methyl ester, caffeoyl malate, coumaroyl malate

INTRODUCTION

Pear (*Pyrus* spp.) fruit is one of the most widely consumed fruits in the world. The fruit is usually eaten fresh and in processed foods such as juice, puree, jellies, and jams. Pears have also been used as a traditional folk remedy in China and Korea due to their antitussive, anticonstipative, anti-inflammatory, and diuretic activities.[1,2] However, despite the widespread consumption of pear fruits, information on the biological effects of pear fruits is very limited. Recently, the antiulcer,[3] anti-inflammatory,[4] and antioxidative[5,6] effects of the pear fruit have been reported.

Previous studies on pear fruit have focused on its chemical composition such as sugars, organic and fatty acids, minerals, amino acids, volatiles, vitamins, and phenolics.[7−12] The content of these constituents may be responsible for the overall high quality of pear fruit. In particular, many phenolic compounds such as arbutin, chlorogenic acid, hyroxycinnamoyl malates, catechins, and procyanidins have been identified in pear fruits.[2,12−16] These phenolic compounds may be responsible for the antioxidant effects or coloring of pear fruits and their products.[5,6,11,17] Of these phenolic compounds, arbutin and chlorogenic acid are the most common phenolic compounds in all pear fruit cultivars.[2,13] Therefore, these compounds have also been used as a specific marker for the authenticity of pear products.[17−19] More studies on the identification of phenolic compounds have been performed on European pears than on Asian pears, and most studies on the constituents in Asian pears have been restricted to the identification of only some phenolic compounds such as arbutin and chlorogenic acid. More recently, Lin and Harnly[20] examined the phenolic compounds in Asian pears as well as European pears. Nevertheless, information on the composition and constituents in Asian pears including Korean pears is much lower than that for European pears. It is plausible that the composition of Asian pears may be different from that of European pears. Understanding the constituents of fruits is very important in regard to acquiring basic information on plant physiology as well as biological activity. However, systematic studies on the chemical constituents of Asian pear fruits have not yet been performed.

The present study describes the isolation and structural elucidation of two novel and five known hydroxycinnamoylmalic acids and their methyl esters from the fruit peels of *Pyrus pyrifolia* Nakai cv. Chuhwangbae, which is one of the most highly consumed pear fruits in Korea.

MATERIALS AND METHODS

General Methods. Column chromatography was performed using Sephadex LH-20 (25−100 mesh; Pharmacia Fine Chemicals, Uppsala, Sweden) resins. High-pressure liquid chromatography (HPLC) analysis was carried out using Shim-pack Prep-ODS (H) Kit (5 μm, i.d. 20 × 250 mm; Shimadzu, Kyoto, Japan).

Materials and Chemicals. The fresh fruits were harvested from *P. pyrifolia* Nakai cv. Chuhwangbae, which were grown in Naju city, South Korea, in September 2008 and identified by W.-S. Kim (Laboratory of Pomology, College of Agriculture and Life Science, Chonnam National University). A voucher sample (No. JNU PE 20050831-2) was deposited in the herbarium of the laboratory. The fruits were hand-peeled with a peal thickness of about 3 mm. The fruit peels (15 kg fresh wt) were immediately stored at −70 °C until used. Methanol-d_4 (CD_3OD) was obtained from Merck (Darmstadt, Germany). Solvents used for analyses

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were of HPLC grade and purchased from Fisher Scientific Korea (Seoul, Korea). Spectrophotometric grade trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) and ethyl acetate (EtOAc), which were used for extraction and solvent fractionation, were of extra pure quality and were obtained from Duksan (Ansan, Korea). All other chemicals used in this study were of reagent grade and were obtained from commercial sources.

Extraction and Solvent Fractionation. The fresh peels (15 kg fresh wt) were homogenized using a homogenizer (BM-2 Nissei biomixer, Nihonseiki Kaiseiki LTD, Japan) with MeOH (24 L). After extraction for 3 days at room temperature, the mixture was filtered under vacuum through No. 2 filter paper (Whatman, Maidstone, England). The residue was repeatedly extracted with MeOH (11 L). The solutions extracted with MeOH were combined and concentrated in vacuum at 38 °C. The MeOH extracts (3,709 g) were suspended in acidic buffer (0.2 M glycine−0.2 M HCl, pH 3.0, 6 L) and partitioned with EtOAc (6 L, 3 times). The EtOAc layer was partitioned with phosphate buffer (0.2 M NaH_2PO_4−0.2 M Na_2HPO_4, pH 8.0, 6 L, 3 times) to produce the EtOAc-soluble neutral fraction. The aqueous layer was adjusted to pH 3.0 using 1.0 N HCl and then partitioned with EtOAc (6 L, 3 times) to yield the EtOAc-soluble acidic fraction.

Isolation. The EtOAc-soluble acidic fraction (5.17 g) was fractionated on a Sephadex LH-20 column (3.3 × 82 cm) with a mobile phase that consisted of 80% MeOH (2.6 L). This fractionation process resulted in 18 groups (A−R). Fraction H [91.1 mg, elution volume/total volume (V_e/V_t) 0.95−1.05] was injected onto an HPLC equipped with a prep-ODS column using a linear gradient of H_2O (pH 2.65 by TFA, eluent A) to 60% MeOH (pH 2.65 by TFA, eluent B), starting with 100% A, increasing to 100% B for 40 min, and then holding at 100% B for 60 min to afford 7 fractions (H1−H7). Of these fractions, fractions H1 (t_R 36.9 min, 33 mg) and H2 (t_R 43.0 min, 4.3 mg) were further purified because they were the main fractions of the seven peaks (H1−H7). Compounds **1** and **2** (t_R 21.7 min, 16.1 mg) as a mixture were purified from fraction H1 (t_R 36.9 min, 33 mg) by HPLC on a prep-ODS column using a linear gradient of 30% MeOH (MeOH, pH 2.65 by TFA, eluent C) to 60% MeOH (pH 2.65 by TFA, eluent D), starting with 100% C, increasing to 100% D for 30 min, and holding at 100% D for 40 min. In addition, fractions H2 and I were further purified using the same HPLC conditions as described for the purification of fraction H. Compounds **3** (t_R 41.9 min, 1.1 mg) and **4** (t_R 42.5 min, 1.2 mg) from fraction H2 (4.3 mg) and compound **5** (t_R 33.8 min, 8.1 mg) and a mixture of compounds **6** and **7** (t_R 39.1 min, 1.1 mg) from fraction I (66.8 mg, V_e/V_t 1.06−1.26) were obtained.

All fractions obtained using this purification process were spotted on silica gel for thin-layer chromatography analysis (TLC; silica gel 60 F_{254}, 0.25 mm thickness; Merck, Darmstadt, Germany) and developed using a mixture of *n*-BuOH/acetic acid/H_2O (4:1:1, v/v/v). The purity of the fractionated compounds was visualized by UV and 1% cerium sulfate solution spray.

Structural Analysis. Nuclear magnetic resonance (NMR) spectra were obtained with a ^{unit}INOVA 500 and 600 spectrometer (Varian, Walnut Creek, CA, USA) using tetramethylsilane as an internal standard in CD_3OD. All mass spectra were acquired on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu LCMS-IT-TOF, Kyoto, Japan) that was equipped with an electrospray ionization (ESI) source (ESI MS). Accurate masses were corrected by calibration using sodium trifluoroacetate clusters as internal references. Sample solutions were prepared by dissolving each sample in a solution of MeOH to a final concentration of 50 μg/μL. All ions produced were introduced into the TOF instrument for accurate mass determination. Data acquisition and analysis was performed using LC Solution 3.0 software (Shimadzu, Kyoto, Japan).

UPLC ESI TOF MS Analysis of 6 and 7 in EtOH Extract of Pear Fruit Peels. The fresh peels (5.0 g fresh wt) were homogenized using a homogenizer (BM-2 Nissei biomixer) with EtOH (150 mL). After extraction for 24 h at room temperature, the mixture was filtered under vacuum through No. 2 filter paper (Whatman). The EtOH solution was concentrated in vacuum at 38 °C. The EtOH extracts (10.9 mg) were suspended in distilled water (100 mL) and partitioned with $CHCl_3$ (150 mL, 3 times) and EtOAc (150 mL, 3 times), successively. After the EtOAc layer was evaporated in vacuum at 38 °C, the EtOAc fraction (10.9 mg) was dissolved in EtOH. The fraction was analyzed using an Ultra performance liquid chromatograph trap time-of-flight mass spectrometer (UPLC TOF MS, Synapt HDMS TOF Mass, Waters, U.K.). An electrospray ionization (ESI) source (ESI MS; negative ion mode; electron voltage, 30 eV) system was used to identify **6** and **7** under the following UPLC conditions: column, NanoAcquity BEH C18 (i.d. 75 μm × 250 mm, 1.7 μm, Waters); flow rate, 0.4 mL/min; column temperature, 40 °C. The sample was eluted using a gradient system of 100% H_2O (containing 1% formic acid, eluent E) to 100% MeCN (containing 1% formic acid, eluent F), starting with 100% E, increasing to 5% F for 5 min, increasing to 15% F for 15 min, increasing to 25% F for 20 min, increasing to 75% F for 22 min, and holding at 75% F for 24 min.

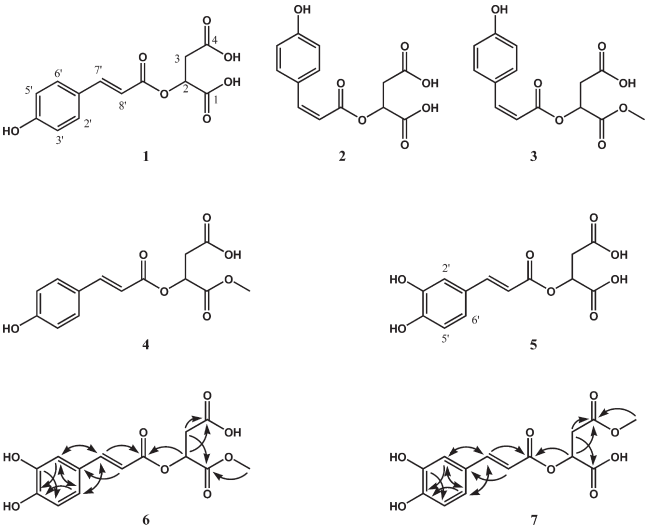

Figure 1. Structure and HMBC correlations (arrows) of the isolated compounds.

RESULTS AND DISCUSSION

Isolation of Compounds from the EtOAc-Soluble Acidic Fraction and Identification of the Isolated Compounds 1−5. The peels of pear fruit have a much higher and more variable phenolic content than pulp.[2,5] Therefore, the peel was used to investigate the constituents contained in the pear fruit. The EtOAc-soluble acidic fraction obtained by solvent fractionation of the MeOH extract of the fruit peel was purified by Sephadex LH-20 column chromatography and HPLC, which resulted in the purification of seven hydroxycinnamoylmalic acids and their methyl esters.

Five known hydroxycinnamoylmalic acids and their methyl esters were identified to be 2-*O*-(*trans*-coumaroyl)malic acid (**1**), 2-*O*-(*cis*-coumaroyl)malic acid (**2**), 2-*O*-(*trans*-coumaroyl)malic acid 1-methyl ester (**3**), 2-*O*-(*cis*-coumaroyl)malic acid 1-methyl ester (**4**), and 2-*O*-(*trans*-caffeoyl)malic acid (phaselic acid, **5**) (Figure 1).[21−23] These compounds were identified by comparing the ^{1}H and ^{13}C NMR spectral data reported in previous

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of 6 and 7 (CD_3OD, TMS)[a]

	6		7	
position	δ_H (int, mult, J in Hz)	δc	δ_H (int, mult, J in Hz)	δc
1		171.84		171.41
2	5.49 (1H, dd, 3.6, 8.4)	70.01 c	5.48 (1H, dd, 3.6, 8.4)	70.01 c
3a	2.99 (1H, dd, 3.6, 16.5) a	37.13 d	2.99 (1H, dd, 3.6, 16.5) a	37.13 d
3b	2.94 (1H, dd, 8.4, 16.5) b		2.94 (1H, dd, 8.4, 16.5) b	
4		172.80		171.57
$-OCH_3$	3.72 (3H, s)	52.73	3.76 (3H, s)	53.20
1′		127.70		127.68
2′	7.05 (1H, d, 2.4)	115.30 e	7.06 (1H, d, 2.4)	115.30 e
3′		147.01 f		147.01 f
4′		150.02		150.06
5′	6.78 (1H, d, 7.8)	116.65	6.79 (1H, d, 7.8)	116.65
6′	6.96 (1H, dd, 7.8, 2.4)	123.38	6.97 (1H, br.d, 7.8)	123.42
7′	7.58 (1H, d, 16.2)	148.17	7.59 (1H, d, 16.2)	148.31
8′	6.29 (1H, d, 16.2)	114.19	6.30 (1H, d, 16.2)	114.05
9′		168.12		168.09

[a] The chemical shifts of protons or carbons in the same letters were overlapped.

studies to NMR and MS spectroscopic data obtained in this study.

Structural Elucidation of the Isolated Compounds 6 and 7. The ^{1}H NMR (600 MHz, CD_3OD) spectrum of **6** showed the presence of caffeoyl moieties based on the observation of trisubstituted aromatic ring protons at δ 7.05 (1H, d, J = 2.4 Hz, H-2′), 6.78 (1H, d, J = 7.8 Hz, H-5′), and 6.94 (1H, dd, J = 7.8, 2.4 Hz, H-6′) and two protons in the *trans*-configuration for the double bonds at δ 7.58 (1H, d, J = 16.2 Hz, H-7′) and 6.29 (1H, d, J = 16.2 Hz, H-8′) (Table 1). In addition, the ^{1}H NMR spectrum of **6** showed the presence of methylene protons [δ 2.99 (1H, dd, J = 16.5, 3.6 Hz, H-3a) and 2.94 (1H, dd, J = 16.5, 8.4 Hz, H-3b)], an oxygenated methine proton [δ 5.49 (1H, dd, J = 8.4, 3.6 Hz, H-2)], and a methoxyl proton [δ 3.72 (1H, s)]. Fourteen carbon signals were observed in the ^{13}C NMR (150 MHz, CD_3OD) spectrum including three carbonyl carbons [δ 171.84 (C-1), 172.80 (C-4), 168.12 (C-9′)], eight sp^2 carbons (δ 147.01−114.19), two sp^3 carbons [δ 70.01 (C-2), 37.13 (C-3)], and a methoxyl group (δ 52.73) (Table 1). In particular, the presence of the malic acid moiety was suggested due to the presence of the two carbonyl carbons [δ 171.84 (C-1), 172.80 (C-4)], an oxygenated sp^3 methine carbon (δ 70.01, C-2), and an sp^3 methylene carbon (δ 37.13, C-3). Therefore, compound **6** was proposed to be caffeoylmalic acid coupled to a methoxyl group. All of the protonated carbons were assigned according to the results of the HSQC analysis (data not shown). The connection of the caffeoyl malate moiety of **6** was confirmed based on correlations between the protons and carbons detected in the HMBC spectrum (Figure 1). In particular, the correlation of the methoxyl proton signal of δ 3.72 and the carbonyl carbon signal of δ 171.84 (C-1) indicated that the methoxyl group was esterified at the C-1 position of the malic acid. Consequently, **6** was determined to be 2-*O*-(*trans*-coumaroyl)malic acid 1-methyl ester, which was a novel compound. However, the ^{1}H and ^{13}C NMR spectra suggested the presence of another minor compound (**7**) in addition to the main compound, 2-*O*-(*trans*-caffeoyl)malic acid 1-methyl ester (**6**). The proton signals of the minor compound (**7**) mostly overlapped with those of **6**. However, the chemical shift values of the protons from the double bond and the methoxyl group of **7** were different from those of **6**. In particular, the methoxyl group of **7** was shifted upfield by 0.04 ppm (δ 3.76) when compared to that (δ 3.72) of **6**. The ratio of the main compound (**6**) and the minor compound (**7**) was determined to be approximately 2:1 from the intensities of the methoxyl groups in the ^{1}H NMR spectrum. In the HMBC spectrum (Figure 1), the correlation of the methoxyl proton signal of δ 3.76 and the carbonyl carbon signal of δ 171.57 indicated that the methoxyl group was esterified at the C-4 position of malic acid. Consequently, the structure of **7** was determined to be 2-*O*-(*trans*-caffeoyl)malic acid 4-methyl ester, which was also a novel compound. The molecular formula of **6** and **7** was determined to be $C_{14}H_{14}O_8$ (MW 308) by negative HR ESI MS analysis (m/z 309.0617 $[M - H]^-$, calculated for $C_{14}H_{15}O_8$, m/z 309.0616, +0.1 mmu). Therefore, the mixture of **6** and **7** was unambiguously determined to be 2-*O*-(*trans*-caffeoyl)malic acid 1-methyl ester (**6**, main) and 2-*O*-(*trans*-caffeoyl)malic acid 4-methyl ester (**7**, minor), respectively (Figure 1).

Detection of 6 and 7 in EtOH Extract of Pear Fruit Peels by UPLC TOF MS Analysis. Of the compounds isolated from the peels of pear fruit, four compounds (**3**, **4**, **6**, **7**) were in the methyl-esterified forms. Compounds **3** and **4** were already identified in *Euscaphis japonica*[22] and *Brassica rapa*,[23] respectively, and **6** and **7** were determined to be novel compounds in this study. However, the esterification of carboxylic acids with MeOH under acidic conditions has been reported.[24] Therefore, it was thought that these compounds might be produced as artifacts by using MeOH during the extraction and purification. Therefore, the fruit peels were extracted with EtOH instead of MeOH to determine if **6** and **7** were artifacts or native compounds. The H_2O suspension of the EtOH extract was partitioned with $CHCl_3$ to remove hydrophobic compounds. The aqueous layer was successively partitioned with EtOAc to obtain the EtOAc fraction containing **6** and **7**. The EtOAc fraction was analyzed by UPLC ESI MS analysis. Two peaks in TLC chromatogram of the EtOAc fraction of the EtOH extract were

detected at t_R of 15.97 and 16.19 min. Their ESI MS (negative) spectra showed the presence of pseudomolecular ion ($[M - H]^-$) signals of m/z 309.0378 (t_R 15.97 min) and m/z 309.0490 (t_R 16.19 min). These data were in agreement with the retention times and ESI MS spectra [m/z 309.00298 (t_R 15.97 min) and m/z 309.0329 (t_R 16.19 min)] of the **6** and **7** mixture purified from pear fruit peels in the present study. In this study, the identity of **6** and **7** between the two peaks detected on the TLC chromatogram of the EtOAc fraction of the EtOH extract could not be determined because **6** and **7** were purified as a mixture. However, the occurrence of **6** and **7** in the peels of pear fruit was unambiguously verified.

Hydroxycinnamoylmalic acids and their methyl esters have been found in various plants. In particular, compound **3** was recently isolated from *Euscaphis japonica*.[22] Compound **5** (phaselic acid) has also been identified in legumes,[25] red clover,[26] and *Brassica* rapa.[27] In addition, the presence of **1** and **5** was previously reported in pear fruits (*P. coummunis* L. and *P. bretschneideri* Reld.).[20] To the best of our knowledge, this is the first study where other hydroxycinnamoylmalic acids and their methyl esters (**2**, **3**, **4**, **6**, and **7**) were identified in pear peel. Furthermore, of the compounds isolated in the present study, **6** and **7** were determined to be novel compounds. In the case of coumaroyl malate derivatives, both *trans* and *cis* isomers were present. The *cis* form (**2**, **3**) might have been produced as an artifact, which commonly occurs during the isolation of the *trans* form.

Information regarding the biological effects of hydroxycinnamoylmalic acids and their methyl esters is very limited. Recently, it was reported that **3** [2-*O*-(*trans*-coumaroyl)malic acid 1-methyl ester] isolated from *E. japonica* strongly inhibits lipopolysaccharide-induced nitric oxide production in marine BV2 microglial cell.[23] In addition, it is well-known that coumaric and caffeic acids, which have a partial structure of the hydroxycinnamoylmalic acids and their methyl esters isolated in the present study, have various biological activities such as antioxidant,[28] anti-inflammatory,[29] antitumor,[30] and antihypertension[31] activities. Therefore, the seven hydroxycinnamoylmalic acids and their methyl esters identified in this study may act as biologically active substances. The biological activities of these compounds on a molecular level will be the subject of subsequent studies.

In addition, several studies have reported that some hydroxycinnamoylmalic acids and their methyl esters accumulate in *Brassica rapa* leaves in response to food-borne and pathogenic microorganisms.[32−36] In particular, it was demonstrated that salicylic acid and methyl jasmonate, which are involved in plant defense against biotic and abiotic stresses, induced an increase in the level of some hydroxycinnamoylmalic acids and their methyl esters such as cinnamoyl, sinapoyl, coumaroyl, feruloyl, and caffeoyl malates.[37,38] Therefore, these compounds may play a role in protecting fruit against various stresses.

Although the chemical constituents of European pear fruits have been extensively examined, compounds **2**−**4**, **6**, and **7** isolated in the present study have not been previously detected in European pear fruits. In addition, the presence of these seven compounds in the Asian pear fruits was also demonstrated for the first time in this study. As described above, β-sitosterol, daucosterol, oleanolic acid, and ursolic acid were also identified as anti-inflammational compounds from *P. bretschneideri*, which was found in China.[4] However, studies on other chemical constituents of Asian pears have been limited to only the main compounds such as arbutin and chlorogenic acid. Therefore, the work conducted in this study will greatly expand on our understanding of the constituents of Asian pears. In addition, investigations on the molecular level play a very important role in establishing the chemical profile of the European pear as well as the Asian pear and therefore offer very useful information in regard to fruit growth, preservation, and the biological function of pear fruits.

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