Gamnamoside, a Phenylpropanoid Glycoside from Persimmon Leaves (*Diospyros kaki*) with an Inhibitory Effect against an Alcohol Metabolizing Enzyme

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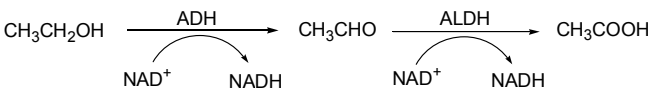
Phytochemical investigation of the methanolic extract of *Diospyros kaki* leaves led to the isolation of osmanthuside H (**1**) and a new phenol glycoside, named gamnamoside [4-(3-hydroxypropyl)-2-methoxyphenol β-D-apiofuranosyl(1 → 6)β-D-glucopyranoside] (**2**) along with (-) catechin (**3**) through a series of reversed phase column chromatography and preparative C_{18} HPLC. The structures of the isolates were determined by spectroscopic methods including IR, UV, HRTOFMS, and 2D NMR. Compounds **1**, **2**, and **3,** showed good inhibitory activities (IC_{50}) of 175.4, 94.4, and 126.6 μg/mL respectively, whereas a reversible ADH inhibitor, 4-methylpyrazole, showed the IC_{50} of 326.6 μg/mL against alcohol dehydrogenase (ADH).

Key Words: Persimmon, *Diospyros kaki*, Gamnamoside, (-) Catechin, Alcohol dehydrogenase

Introduction

The persimmon, *Diospyros kaki* Thunb.(Ebenaceae), is a ubiquitously cultivated plant in many farms or home gardens in Korea. The dried fruits and leaves have been traditionally used as a folk medicine for hiccups, reduction of internal bleeding, blood clotting, and dispelling of pathogenic heat. Traditionally fermented persimmon vinegar has been used as a folk medicine in Korea for eliminating the hangover caused by the heavy drinking of alcohol.[1,2] The chemical constituents and their medicinal usages of the genus *Diospyros* have been well documented in a recent review.[3]

The alcohol hangover is characterized by unpleasant physical and mental symptoms such as headache, nausea, vomiting, thirst, dizziness, vertigo, and other decreased sensory abilities.[4,5] The most of ethanol consumed is metabolized to acetaldehyde by NAD-dependent alcohol dehydrogenase (ADH) and further to acetic acid by NAD-dependent aldehyde dehydrogenase (ALDH) in the liver (Figure 1). The intermediate acetaldehyde contributes to several hangover symptoms and a highly pharmaco-active substance that binds with proteins and other biologically important molecules, causing hypertension,[6,7] atherosclerosis,[8] and cancers.[9,10] Traditional plant medicines for hangover symptoms include the use of *Evodiae fructus* extracts,[11] the roots extracts of *Pueraria lobata*,[12] and a mixture of Korean medicinal herbs (*Panax ginseng, Liriope platyphylla*, and others).[13] Recently, phytophenols in whiskey were shown to depress alcohol metabolism through ADH inhibition.[14]



ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase;
NAD^+ = nicotinamide adenine dinucleotide; NADH = reduced form of NAD^+

Figure 1. The metabolism of ethanol by alcohol metabolizing enzymes (ADH and ALDH).

Persimmon leaves and vinegar have been utilized as a traditional hangover remedy. However, little research has been performed on their chemical ingredients which are active against alcohol dehydrogenase (ADH). Detailed chemical investigations of the plant would establish the efficacy of the remedy. This paper describes the isolation and characterization of ADH-inhibitory phenyl glycosides (**1** and **2**) along with (-) catechin (**3**) from persimmon leaves.

Materials and Methods

General experimental procedures. High resolution TOF mass spectra were measured on a Waters LCT Premier mass spectrometer coupled with a Waters AQUITY HPLC system and data acquisition was achieved using MassLynx software, version 4.0. Optical rotations were measured on a Perkin Elmer 341-LC polarimeter. NMR spectra were recorded on a Varian Mercury 400 spectrometer with standard pulse sequences operating at 400 MHz in ^{1}H NMR and 100 MHz in ^{13}C NMR. The chemical shifts given in ppm were referenced to solvent peaks (CD_3OD δ_H 3.31 and δ_C 49.15). Flash column chromatography was carried out on a C_{18} column (cosmosil 75 C_{18}-prep, 100 id × 180 mm). C_{18} MPLC was performed on a C_{18} column (cosmosil 40 C_{18}-prep, 40 id × 320) with a solvent delivery pump (Yamazen GR-200, Pump 540, 6 mL/min). Thin-layer chromatography (TLC) analysis was performed on a precoated silica gel plate (Kieselgel 60, F_{254}, 20 × 20 cm, 0.25 mm thick, Merck). Spots were detected *via* staining with a solution of *p*-anisaldehyde-sulfuric acid in methanol followed by heating. Reversed phase HPLC was performed on a Gilson 321 model system (UV 220 nm) with a C_{18} HPLC column (YMC hydrosphere C_{18}, S-5 μm, 20 id × 250 mm) using H_2O-MeCN eluent with a flow rate of 7 mL/min. Optical density for a 96-well microplate was measured on a microplate reader (Tecan Sunrise A-5080) at 340 nm. ADH (alcohol dehydrogenase from *Saccharomyces cerivisiae*, EC 1.1.1.1, > 90%

protein, 331 units/mg), β-NAD^+ (β-nicotinamide adenine dinucleotide, oxidized form), ethanol (anhydrous, 200 proof, 95.5%), 4-methylpyrazole (hydrochloride salt), and antabuse (disulfiram) were obtained from Sigma-Aldrich Chemical Co.

Plant material. The leaves of persimmon *Diospyros kaki* (gamnamoo in Korean) were collected at the mountainous area of Yeongdong, Chungbuk, Republic of Korea, in October, 2007. A voucher specimen (SM1380) has been deposited in the Natural Products Chemistry Laboratory, Kongju National University, Republic of Korea.

Extraction and isolation. The persimmon leaves (25 kg) were extracted by percolation with 90% aqueous MeOH (75 L) at room temperature for a week. The extract was concentrated under a reduced pressure at 30 °C to yield a deep green residue (1.64 kg). The residue was suspended in 90% aqueous methanol (2 L) and extracted with hexane (2 L) four times to yield a hexane layer as a green solid (1 kg) and the remaining aqueous layer as a deep brown syrup (640 g). The aqueous layer was concentrated and partitioned between water (2 L) and BuOH (3 × 1 L), yielding a butanol layer as a brownish solid (180 g) and an aqueous layer as a brownish syrup (460 g). The brownish syrup (200 g) from the aqueous layer was chromatographed on a C-18 flash column (cosmosil 75) with a stepwise gradient elution of a mixture of water and methanol to afford eight fractions. The 30% MeOH eluate (2 g out of 15 g sample) was further subjected to C-18 MPLC (cosmosil 40) with a gradient elution of 10% aqueous MeOH to 50% aqueous MeOH to yield five subfractions. Subfraction 3 (30% aqueous MeOH eluate, 300 mg) was further purified by preparative HPLC using 10 - 20% aqueous MeCN for 45 min to yield compound **1** as a brownish syrup (20 mg, R_t 25.2 min) and compound **2** as a yellowish syrup (12 mg, R_t 27 min). Subfraction 4 (40% aqueous MeOH eluate, 280 mg) was further purified by preparative HPLC using 12 - 22% aqueous MeCN for 60 min to yield compound **3** as a brownish syrup (27 mg, R_t 39.2 min).

Osmanthuside H [2-(4-hydroxyphenyl)ethanol β-D-apiofuranosyl(1→6)-β-D-glucopyranoside] (1): A brownish syrup. It was identified as osmanthuside H upon detailed comparison of spectral data (see Table 1 for ^{1}H and ^{13}C NMR) with literature values. $[\alpha]_D^{20}$: -61.6 (*c* 0.48, MeOH) (reported: -78.9, *c* 0.3, MeOH).[15]

Gamnamoside [4-(3-hydroxypropyl)-2-methoxyphenol β-D-apiofuranosyl(1→6)-β-D-glucopyranoside] (2): A yellowish syrup; $[\alpha]_D^{20}$ -67.8 (*c*: 0.27, MeOH); UV (MeOH): λ_{max} (log ε) 208 (3.71), 276 (3.05) nm; IR (neat): ν_{max} 3320, 2924, 1613, 1515, 1229, 1010 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR: Table 1; HRTOFMS (positive ESI mode): *m/z* 494.2225 $[M+NH_4]^+$ (calcd. for $M+NH_4$, 494.2238) and 515.1519 (calcd. for $[M+K]^+$, 515.1531); R_f 0.45 (70:27:3 CH_2Cl_2-MeOH-H_2O).

(-) Catechin (3): A brown powder. It was identified as (-) catechin by comparison of spectral data and specific rotation in the literature.[16]

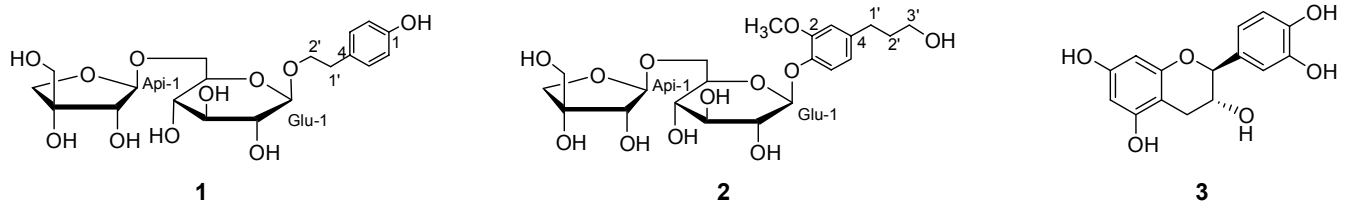
Identification of carbohydrates in compound 2: A solution of compound **2** (1 mg) in 5% HCl (1 mL) was heated at 90 °C for 2 h. After evaporation in vacuum, the mixture was analyzed by TLC with authentic carbohydrates. Carbohydrates were identified as glucose and apiose. TLC (70: 27: 3 CH_2Cl_2-MeOH-H_2O) R_f 0.13 (D-glucose) and 0.23 (D-apiose).

Alcohol dehydrogenase activity assay. ADH activity was measured according to the method in the literature with a modification.[17,18] The test solutions were prepared by dissolving the isolates in DMSO and were subsequently diluted with the sodium phosphate buffer solution (10 mM, pH 7.4) by a two-fold serial dilution method. All the following solutions were prepared in a sodium phosphate buffer solution (10 mM, pH 7.4). An ethanol solution (100 μL, 5%), β-NAD^+ (20 μL, 1.87 mM) and a serially diluted test solution (20 μL) were added to a 96-well plate followed by making up to a volume of 180 uL with a sodium phophate buffer solution. After 5 min of equilibration at 24 °C, the absorbance was measured for the sample blank at 340 nm using a microplate reader. After the addition of ADH solution (20 μL, 0.24 unit) the mixture was incubated at 24 °C. The absorbance was measured at 340 nm at intervals of 5 min for a period of 2 h to determine the amount of NADH produced. A control experiment was done in parallel to study the impact of the solvent DMSO itself (the final highest concentration of DMSO, 2% v/v). The inhibitory concentration of the sample (IC_{50}, μg/mL) against the enzyme was expressed as the concentration of the sample at which the enzyme activity was inhibited to 50% at 2 h. 4-Methylpyrazole and antabuse were used as positive controls. The experiments were performed in triplicate.

Results and Discussion

Compound **1** was obtained as a brownish syrup. The ^{1}H and ^{13}C NMR spectra of compound **1** were in a good agreement with those of osmanthuside H published in the literature (Table 1).[15] The specific rotation of compound **1** was found to be -61.6 in methanol, comparable to that of osmanthuside H (-78.9, MeOH).[15]

Compound **2** was obtained as a yellowish syrup with a specific rotation of -67.8. The mass spectrum (HRTOFMS) displayed an ammonium-cation added molecular ion $[C_{21}H_{32}O_{12} + NH_4]^+$ at *m/z* 494.2215 (calcd. for $C_{21}H_{32}O_{12} + NH_4$, 494.2238). The IR absorptions at 3320 and 1613 cm^{-1} were indicative of



1 2 3

Figure 2. Structures of osmanthuside H (**1**), gamnamoside (**2**), and catechin (**3**).

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2** in CD_3OD*

Position	Compound **1**		Compound **2**	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
1	-	156.9	-	146.2
2	6.68 (d, 8.4)	116.3	-	150.8
2-OMe	-	-	3.84 (3H) (s)	56.8
3	7.06 (d, 8.4)	131.1	6.85 (d, 1.6)	114.2
4	-	130.9	-	138.7
5	7.06 (d, 8.4)	131.1	6.76 (dd, 8.0, 1.6)	122.2
6	6.68 (d, 8.4)	116.3	7.06 (d, 8.0)	118.5
1′	2.83 (d, 8.4)	36.5	2.63 (2H) (t, 7.4)	32.8
2′	3.95 (m), 3.70 (m)	72.3	1.81 (2H) (tt, 7.4, 6.8)	35.6
3′	-	-	3.55 (2H) (t, 6.8)	62.3
Glu-1	4.26 (d, 8.0)	104.5	4.78 (d, 7.2)	103.3
Glu-2	3.16 (t, 8.0)	75.1	3.46 (dd, 8.4, 7.2)	75.06
Glu-3	3.32 (m)	78.1	3.43 (t, 8.4)	77.9
Glu-4	3.26 (t, 8.4)	71.8	3.34 (m)[a]	71.8
Glu-5	3.39 (m)	77.0	3.51 (m)	77.2
Glu-6-H*a*	3.98 (dd, 11.2, 6.0)	68.8	3.98 (dd, 11.2, 6.4)	68.8
H*b*	3.60 (dd, 11.2, 2.0)	-	3.61 (dd, 11.2, 2.0)	-
Api-1	5.00 (d, 2.8)	111.1	4.96 (d, 2.2)	111.1
Api-2	3.89 (d, 2.8)	78.1	3.88 (d, 2.2)	78.2
Api-3	-	80.7	-	80.6
Api-4-H*a*	3.96 (d, 9.6)	75.2	3.94 (d, 9.6)	75.12
H*b*	3.75 (d, 9.6)	-	3.73 (d, 9.6)	-
Api-5	3.64 (2H) (br s)	65.7	3.57 (2H) (br s)	65.7

*Assignments were made from the interpretation of ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and TOCSY data. [a]Overlapped with the solvent peaks.

the hydroxyl and aromatic groups. The presence of the aromatic group was supported by the UV absorptions at 208 and 276 nm. Detailed examination of the ^{1}H and ^{13}C NMR spectral data (Table 1) revealed that the sugar moieties present in compound **2** were similar to those of compound **1**. Upon acid hydrolysis, compound **2** afforded two carbohydrate moieties, which were identified as glucose and apiose upon TLC comparison with authentic samples. In the COSY spectra, a 1, 2, 4-trisubstituted aromatic ring was deduced from the correlations between signals at δ 6.85 (1H, d, *J* = 1.6 Hz, H-3), δ 6.76 (1H, dd, *J* = 8, 1.6 Hz, H-5), and δ 7.06 (1H, d, *J* = 8 Hz, H-6).The protons at δ 3.55 (H-3′) were coupled with the protons at δ 1.81 (H-2′) which in turn was coupled with the proton at δ 2.63 (H-1′), indicating the presence of hydroxypropyl moiety. The HMBC experiment established the attachment of a sharp singlet of a methoxy group at δ 3.84 (3H, s) to the aromatic ring and the hydroxypropyl group to the carbon C-4 at δ 138.7. Important HMBC correlations were observed between sugar moiety and aglycone: glu-H-1 at δ 4.78 to C-1 at δ 146.2; api-H-1 at δ 4.96 to glu-C-6 at δ 68.8; and glu-H-6 at δ 3.98 and δ 3.61 to api-C-1 at δ 111.1. These connectivities were also confirmed from the NOEs between H-6 at δ 7.06 and glu-H-1, and glu-H-6 and api-H-1 (Figure 3). The β-configuration of the glucose unit was deduced from the coupling constant (7.2 Hz) of the anomeric proton at δ 4.78. The β- configuration of the apiose unit was procured from the chemical shift (δ 111.1) of the anomeric carbon and the coupling constant (2.2 Hz) of the anomeric proton (*quasiaxial*) at δ 4.96.[15] The coupling constant of the anomeric protons with α configuration in apiofuranosides appears in 4.3 - 4.9 Hz compared to that of β-configuration.[19] From an observed specific rotation (-67.8, MeOH) of compound **2** the stereochemical nature of the sugar moiety was deduced as identical with the known osmanthuside H (-78.9, MeOH), implying the stereochemistry of both sugar units were in D form.[15,20] Thus, the structure of compound **2** was determined to be 4-(3-hydroxypropyl)-2-methoxyphenol β-D-apiofuranosyl(1→6)-β-D-glucopyranoside, designated gamnamoside from the Korean name of the plant.

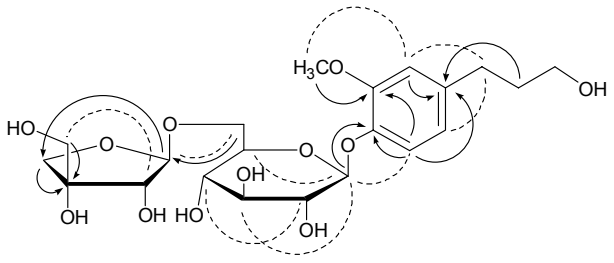


Figure 3. HMBC (solid arrows, H to C) and NOE (dashed curves) correlations of gamnamoside (**2**).

Yeast alcohol dehydrogenase (ADH) used in the present study is a metallodehydrogenase containing stoichiometric amounts of zinc having a high specificity towards to ethanol among alcohols making it appropriate for *in vitro* ADH assay.[21-23] The inhibitory activities (IC_{50}) of isolates **1, 2,** and **3** against ADH were found to be 175.4, 94.4, and 126.6 μg/mL, respectively, while a well-known reversible ADH inhibitor, 4-methylpyrazole, showed the IC_{50} of 326.6 μg/mL. (Table 2). An alcohol aversive drug, antabuse (disulfiram) known as an excellent inhibitor of acetaldehyde dehydrogenase (ALDH),[24]

Table 2. The inhibitory activity of compounds **1**, **2**, and **3** against alcohol dehydrogenase

Compound	IC_{50} (μg/mL)*
1	175.4 ± 3.5
2	94.4 ± 4.5
3	126.6 ± 8.7
antabuse	73.5 ± 0.5
4-methylpyrazole	326.6 ± 7.9

*The experiments were performed in triplicate, and the average values were expressed with the standard errors of the mean. The concentrations of ADH, NAD, and EtOH used were 1.2 units/mL, 1.87 mM, and 2.5% v/v, respectively, in the sodium phosphate buffer (10 mM, pH 7.4).

also inhibited ADH at 73.5 μg/mL. (-) Epigallocatechin and (-) epigallocatechin gallate were reported to inhibit the ethanol metabolism in ICR mice.[25] However, there are no reports on the effect of (-) catechin on ethanol metabolism. Isolates **1** and **2** did not show cytotoxicity below 100 μg/mL against cancer cell lines (small lung carcinoma A549),[26,27] DPPH-radical scavenging activity,[28] or antimicrobial activities (*Staphylococcus aureus* and *Candida albicans*).[27,29] The results presented suggest that the isolates could perform as effective ADH inhibitors to alleviate the formation of acetaldehyde during the oxidation process of ethanol metabolism and hence reduce the possibility of hangover symptoms.

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