Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 63 (2003) 795–801
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Antioxidative polyphenols from walnuts (*Juglans regia* L.)

Toshiyuki Fukuda, Hideyuki Ito, Takashi Yoshida*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan

Received 30 January 2003; received in revised form 18 March 2003

Abstract

Three hydrolyzable tannins, glansrins A–C, together with adenosine, adenine, and 13 known tannins were isolated from the *n*-BuOH extract of walnuts (the seeds of *Juglans regia* L.). Glansrins A–C were characterized as ellagitannins with a tergalloyl group, or related polyphenolic acyl group, based on spectral and chemical evidence. The 14 walnut polyphenols had superoxide dismutase (SOD)-like activity with EC_{50} 21.4-190 μM and a remarkable radical scavenging effect against 1,1-diphenyl-2-picrylhydrazyl (DPPH) (EC_{50} 0.34–4.72 μM).

Keywords: Walnuts; *Juglans regia*; Juglandaceae; Glansrins A–C; Ellagitannin; Antioxidant; SOD-like effect; Radical scavenger

1. Introduction

Walnuts, the seeds of *Juglans regia* L. (Juglandaceae), are a highly nutritious food. They are also used as a traditional remedy for treating cough, stomach ache (Perry, 1980), and cancer in Asia and Europe (Duke, 1989). Walnut is rich in an oil composed of unsaturated fatty acids, such as linoleic and oleic acid, which are susceptible to oxidation. Although the content of α-tocopherol, an antioxidant, in walnut is lower than in other nuts, such as almonds, hazelnuts, peanuts, etc. (Kagawa, 2001), walnut is readily preserved. This implies that the nut contains antioxidants inhibiting lipid auto-oxidation. Recently, a walnut extract containing ellagic acid, gallic acid, and flavonoids was reported to inhibit the oxidation of human plasma and low density lipoproteins (LDL) *in vitro* (Anderson et al., 2001). Although the presence of ellagic acid suggests the occurrence of its bound forms, ellagitannins, there are no reports on the tannin constituents of walnut. In our continuing study of the antioxidative tannins and related polyphenols in foods and nuts, we isolated 16 polyphenolic constituents, including three new hydrolyzable tannins, along with adenosine and adenine from commercial walnuts. This paper describes the full details of the isolation and structure elucidation of the new tannins, named glansrins A (**1**), B (**2**), and C (**3**). We also report on their antioxidant effects in experimental models of lipid peroxidation.

2. Results and discussion

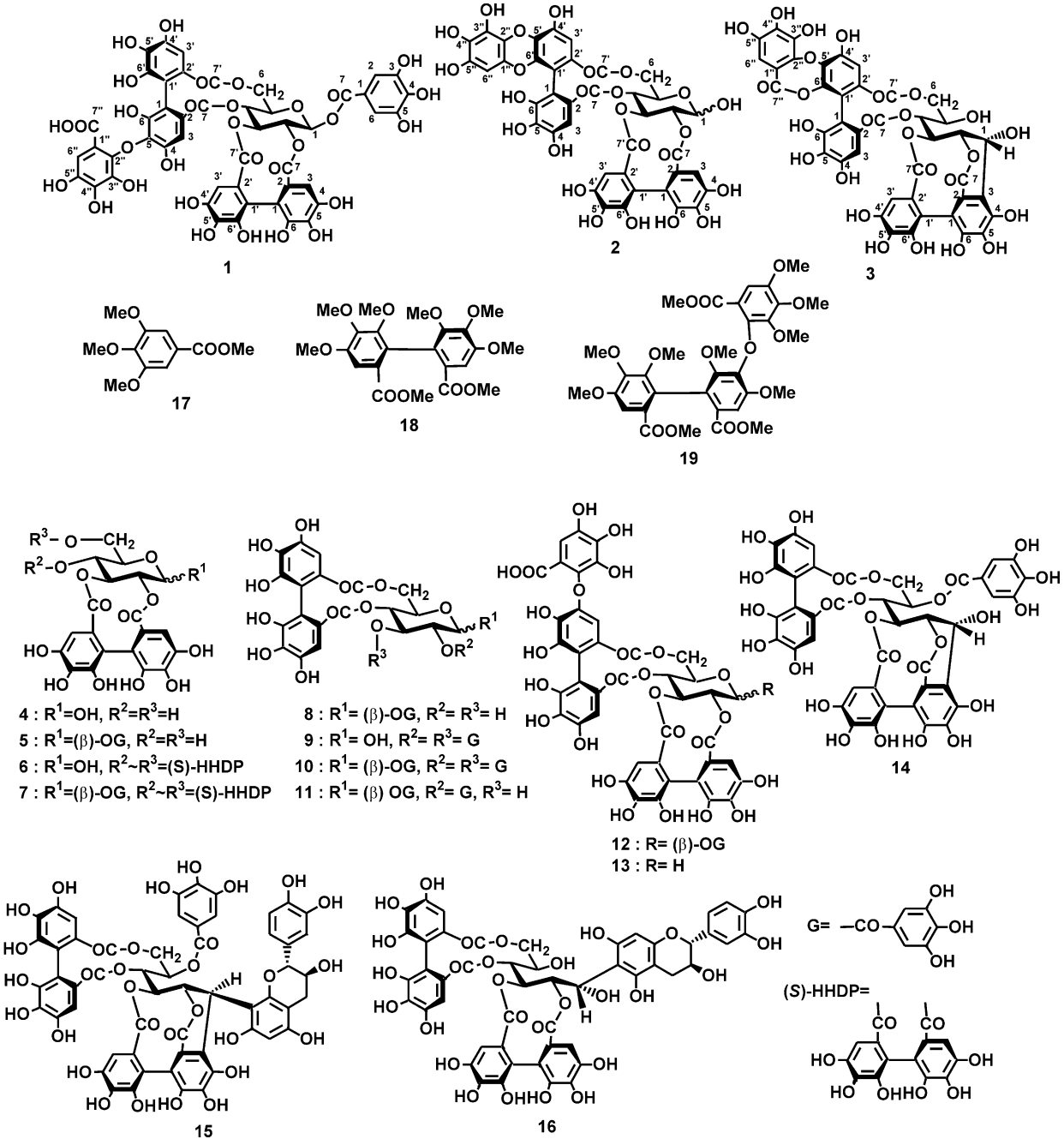
2.1. Characterization of the new tannins

Crushed walnuts were extracted with aq. EtOH at room temperature, and the concentrated filtrate was fractionated into *n*-hexane-, EtOAc-, and *n*-BuOH-soluble portions. The *n*-BuOH extract was separated by successive CC over Dia-ion HP-20, Toyopearl HW-40, and prep HPLC to give **1**–**3** along with 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucopyranose (**4**) (Seikel and Hillis, 1970), isostrictinin (**5**) (Okuda et al., 1982a), pedunculagin (**6**), casuarictin (**7**), strictinin (**8**) (Okuda et al., 1983), tellimagrandin I (**9**), tellimagrandin II (**10**) (Wilkins and Bohm, 1976), 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucopyranose (**11**) (Koppaka, 1977), rugosin C (**12**) (Hatano et al., 1990), praecoxin A (**13**) (Hatano et al., 1991), casuarinin (**14**) (Okuda et al., 1983), stenophyllanin A (**15**) (Nonaka et al., 1985), stachyuranin B (**16**) (Han et al., 1995), adenosine, and adenine.

Glansrin A (**1**) was obtained as an off-white amorphous powder. The negative FABMS of **1** showed an $[M–H]^-$ ion peak at *m*/*z* 1103, which corresponds to the

* Corresponding author.
E-mail address: yoshida@pharm.okayama-u.ac.jp (T. Yoshida).

0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00333-9



molecular formula $C_{48}H_{32}O_{31}$. The ^1H NMR spectrum of **1** was characteristic of an ellagitannin, as revealed by its close similarity to that of rugosin C (**12**) (Hatano et al., 1990), which has the same molecular formula. The spectrum exhibited a two-proton singlet (δ 7.20) and five one-proton singlets (δ 6.93, 6.62, 6.57, 6.48, 6.38) in the aromatic region. The presence of a β-D-glucopyranose residue taking the $^4C_1$ conformation was evident from the coupling patterns of the aliphatic proton signals, which were assigned by ^1H-^1H COSY. Methylation of **1** followed by methanolysis in MeONa–MeOH gave methyl tri-*O*-methylgallate (**17**), dimethyl hexamethoxydiphenate (**18**), and trimethyl octa-*O*-methyltergallate (**19**) (Lin et al., 1990). The aromatic proton signals of **1** were accounted for by the presence of one galloyl, one hexahydroxydiphenoyl (HHDP), and one tergalloyl group in the molecule. These constituent units were consistent with the ^{13}C NMR spectrum assigned by ^1H-^{13}C COSY (Table 1). The position of each acyl group on the glucose core was determined from the HMBC data for **1**, which showed connectivity between each aromatic proton and glucose proton via a carbonyl carbon signal. The signal at δ 6.62 was assigned to the tergalloyl (Ter) H-3 by a correlation with the carbon

Table 1
One-bond and long-range ^{1}H-^{13}C correlation data for glansrin A (**1**)

	δ_C	Correlated proton (δ_H)	
		Proton coupled *via* one bond	Proton coupled *via* two or three bonds
Glucose (glc)			
C-1	92.3	6.24	5.22 (glc H-2)
C-2	76.0	5.22	5.48 (glc H-3)
C-3	77.0	5.48	5.24 (glc H-4)
C-4	69.1	5.24	5.48 (glc H-3)
C-5	73.4	4.54	5.24 (glc H-4)
			3.92 (glc H-6)
C-6	63.3	5.37	
		3.92	
Galloyl (gall)			
C-1	119.8		
C-2, C-6	110.2	7.20	7.20 (gall)
C-3, C-5	146.3		7.20 (gall)
C-4	140.0		7.20 (gall)
C-7	165.1		6.24 (glc H-1)
Hexahydroxydiphenoyl (HHDP)			
C-1	115.1		6.48 (HHDP H-3)
C-2	126.0[a]		
C-3	107.3	6.48	
C-4	145.2		6.48 (HHDP H-3)
C-5	136.6		6.48 (HHDP H-3)
C-6	144.49[b]		
C-7	168.8		6.48 (HHDP H-3)
			5.22 (glc H-2)
C-1′	114.2		6.38 (HHDP H-3′)
C-2′	126.3[a]		
C-3′	107.1	6.38	
C-4′	145.2		6.38 (HHDP H-3′)
C-5′	136.1		6.38 (HHDP H-3′)
C-6′	144.53[b]		
C-7′	169.4		6.38 (HHDP H-3′)
			5.48 (glc H-3)
Tergalloyl (ter)			
C-1	116.9		6.62 (ter H-3)
C-2	131.9		
C-3	107.7	6.62	
C-4	150.0		6.62 (ter H-3)
C-S	137.2		6.62 (ter H-3)
C-6	150.0		
C-7	168.11		6.62 (ter H-3)
			5.24 (glc H-4)
C-1′	117.5		6.57 (ter H-3′)
C-2′	125.0		
C-3′	107.9	6.57	
C-4′	145.1		6.57 (ter H-3′)
C-5′	137.4		6.57 (ter H-3′)
C-6′	144.9[b]		
C-7′	168.15		6.57 (ter H-3′)
			3.92 (glc H-6)
C-1″	1142		
C-2″	1390		
C-3″	139.6		
C-4″	139.4		6.93 (ter H-6″)
C-5″	142.4		6.93 (ter H-6″)
C-6″	108.3	6.93	
C-7″	172.2		6.93 (ter H-6″)

[a] Values are interchangeable.
[b] Values are interchangeable.

signal at δ 150.0 (2C), which is characteristic of C-4 and C-6 of the tergalloyl group (Tanaka et al., 1996). This tergalloyl H-3 signal showed connectivity with the glucose H-4 at δ 5.24 via correlations with a common ester carbonyl carbon at δ 168.11 (Ter C-7). One of the other ester carbonyl carbon signals of the tergalloyl group [δ 168.15 (Ter C-7′)] was correlated with glucose H-6 (δ 3.92) and Ter H-3′ (δ 6.57) via three-bond couplings, establishing the position of the tergalloyl group at the O-4/O-6 of the glucose. Similar three-bond correlations [δ 7.20 (galloyl H-2, H-6)-δ 165.1 (galloyl C-7)-δ 6.24 (glucose H-1); δ 6.48 (HHDP H-3) δ 168.8 (HHDP C-7)-δ 5.22 (glucose H-2); δ 6.38 (HHDP H-3′)-δ 169.4 (HHDP C-7′)-δ 5.48 (glucose H-3)] were observed (Table 1). From these results, the structure of glansrin A was determined to be that represented by formula **1**. The atropisomerism at the chiral HHDP and tergalloyl groups in **1** was determined to be all *S*-series by the strong positive Cotton effects at 238 nm in the CD spectrum (Okuda et al., 1982b).

Glansrin B (**2**) was obtained as a light-brown amorphous powder. It exists as an anomeric mixture (α:β = 1:1), as indicated by duplication of each signal in its ^{1}H NMR spectrum. The spectrum was very similar to those of pedunculagin (**6**) and praecoxin A (**13**), especially to the signals of the sugar moiety, which were almost superimposable, indicating that the glucose hydroxyl groups in **2** are all acylated, except for the anomeric position. The number of aromatic proton signals was the same as for **13**, although the chemical shifts (five pairs at 6.73/6.71, 6.62/6.61, 6.60/6.55, 6.37/6.36, and 5.95/5.94) of **2** differed from those of **13**. The negative FABMS of **2** showed an $[M–H]^-$ ion peak at *m*/*z* 905, corresponding to the molecular formula $C_{40}H_{26}O_{25}$, which is 46 mass units (CH_2O_2) smaller than that of **13**. The ^{13}C NMR spectrum of **2** showed four ester carbonyl carbon signals, and lacked one free carboxyl carbon observed in **13**. This implies the presence of one syzygyl and one HHDP group in the molecule, which was supported by both the ^{1}H-^{13}C COSY and HMBC spectra. The syzygyl group has a dibenzo-1,4-dioxin structure, which is thought to be a metabolite derived from a tergalloyl group, and was first found in syzyginin B isolated from the leaves of *Syzygium aromaticum* (Tanaka et al., 1996). The location of the syzygyl group at O-4/O-6 of the glucose core was established by the HMBC correlations: 6.60/ 6.55 (Syz H-3)-δ 167.9/168.1 (Syz C-7)-δ 69.7/70.0 (glucose H-4) and δ 6.73/6.71 (Syz H-3′)-δ 167.6/167.7 (Syz C-7′)-δ 3.83/3.90 (glucose H-6) (Fig. 1). All of the biphenyl (HHDP and syzygyl) moieties were determined to have chirality in the *S*-configuration, due to the strong positive Cotton effect at 238 nm in the CD spectrum of **2**. Based on these findings, the structure of glansrin B was characterized as 2,3-*O*-(*S*)-hexahydroxydiphenoyl-4,6-*O*-(*S*)-syzygyl-β-D-glucopyranose (**2**).

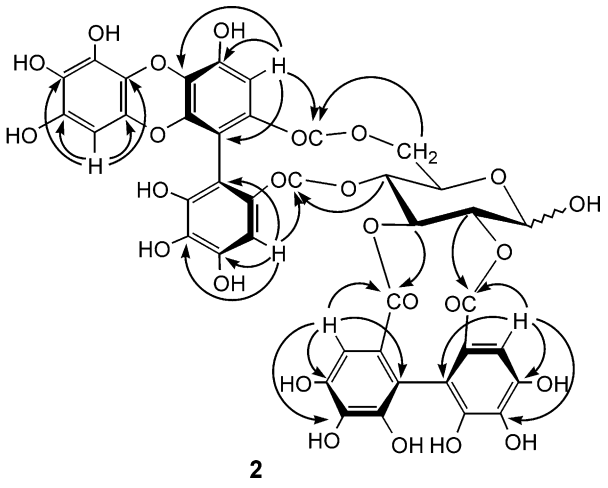


Fig. 1. Selected HMBC correlations observed in **2**.

Glansrin C (**3**) was obtained as a light-brown amorphous powder. The negative FABMS of **3** showed an $[M–H]^-$ ion peak at *m*/*z* 933, which corresponds to the molecular formula $C_{41}H_{26}O_{26}$. The coupling patterns of the sugar proton signals in the ^1H NMR spectrum were characteristic of a *C*-glucosidic ellagitannin, such as casuarinin (**14**). However, the signal assignable to H-5 was shifted to an upper field by 1.14 ppm, as compared to the corresponding signal of **14**, suggesting that the C-5 hydroxyl group of glucose is not acylated. Other differences in the spectra of **3** and **14** were the absence of a galloyl signal and the presence of an extra one-proton singlet (total four at δ 6.93, 6.89, 6.76, 6.43) in the former. The ^{13}C NMR spectrum of **3** indicated the presence of a tergalloyl group or a related acyl group with three aromatic protons besides an HHDP group. The presence of the acyl group related to tergalloyl group was confirmed by methylation of **3** followed by methanolysis yielding **19**. The HHDP group was shown to participate in a *C*-glucosidic linkage by the HMBC correlations between H-3′ (δ 6.43) and glucose H-3 (δ 5.49), via a common ester carbonyl carbon at δ 170.3 (HHDP C-7′), and between the anomeric proton (δ 5.65) and the HHDP C-2∼C-4 signals. Based on these data, and taking the molecular formula and HMBC data (Table 2) into consideration, glansrin C was assumed to have structure **3**, with a lactonized tergalloyl group at O-4/O-6. The direction of the lactone ring was determined by comparing the ^{13}C NMR signals of **3** with those of **1**. The carbon signals due to the C-7″ (δ 163.3) carboxyl group and C-6′ (δ 145.3) of **3** were observed in the upper field, while the C-1′ (δ 120.3), C-3′ (δ 111.5), and C-5′ (δ 142.4) signals were shifted to the lower field, as compared with those of **1** [C-7″ (δ 172.2), C-6 (δ 150.0), C-1 (δ 116.9), C-3 (δ 107.7), C-5 (δ 137.2)]. These shifts were consistent with the data reported for the depsidic tergalloyl moiety (Lin et al., 1990), establishing the lactone structure shown in formula **3**. The *S*-configuration of the biphenyl (HHDP and tergalloyl) moieties was evidenced by the CD spectrum of **3**, as found for **1** and **2**.

Table 2
One-bond and long-range ^1H-^{13}C correlation data for glansrin C (**3**)

	δ_C	Correlated proton (δ_H)	
		Proton coupled *via* one bond	Proton coupled *via* two or three bonds
Glucose (glc)			
C-1	67.7	5.65	
C-2	77.0	4.68	5.65 (glc H-1)
C-3	70.8	5.49	5.65 (glc H-1)
			4.17 (glc H-5)
C-4	77.2	5.12	4.68 (glc H-2)
			3.92 (glc H-6)
C-5	68.3	4.17	3.92 (glc H-6)
C-6	68.9	4.62	
		3.92	
Hexahydroxydiphenoyl (HHDP)			
C-1	116.1		
C-2	120.5		5.65 (glc H-1)
C-3	116.8		5.65 (glc H-1)
C-4	143.68[a]		5.65 (glc H-1)
C-5	138.6		
C-6	143.9[b]		
C-7	164.9		4.68 (glc H-2)
C-1′	116.5		6.43 (HHDP H-3′)
C-2′	127.7[c]		
C-3′	105.2	6.43	
C-4′	145.8		6.43 (HHDP H-3′)
C-5′	135.0		6.43 (HHDP H-3′)
C-6′	145.27[b]		
C-7′	170.3		6.43 (HHDP H-3′)
			5.49 (glc H-3)
Tergalloyl (ter)			
C-1	114.2		6.76 (ter H-3)
C-2	124.7[c]		
C-3	107.5	6.76	
C-4	145.7		6.76 (ter H-3)
C-5	136.3		6.76 (ter H-3)
C-6	145.33[b]		
C-7	168.6		6.76 (ter H-3)
			5.12 (glc H-4)
C-1′	120.3		6.89 (ter H-3′)
C-2′	133.4		
C-3′	111.5	6.89	
C-4′	148.4		6.89 (ter H-3′)
C-5′	142.4		6.89 (ter H-3′)
C-6′	146.3[b]		
C-7′	168.3		6.89 (ter H-3′)
			3.92 (glc H-6)
C-1″	112.7		6.93 (ter H-6″)
C-2″	140.5		6.93 (ter H-6″)
C-3″	136.6		
C-4″	143.74[a]		6.93 (ter H-6″)
C-5″	144.1		6.93 (ter H-6″)
C-6″	109.8	6.93	
C-7″	163.3		6.93 (ter H-6″)

[a] Values are interchangeable.
[b] Values are interchangeable.
[c] Values are interchangeable.

Table 3
SOD-like activity and DPPH radical scavenging activity of walnut polyphenols

Compound	SOD-like activity EC_{50} (μM)	DPPH radical scavenging activity EC_{50} (μM)	Galloyl	Number of HHDP (+other)	Phenol-OH	M.W.
Glansrin A (**1**)	190	0.36	1	1 + 1[a]	17	1104.7
Glansrin B (**2**)	41.9	0.93	0	1 + 1[b]	13	906.6
Glausrin C (**3**)	21.4	0.57	0	1 + 1[c]	13	934.6
2,3-HHDP-D-glucopyranose (**4**)	166	4.35	0	1	6	482.3
Isostrictinin (**5**)	47.3	1.73	1	1	9	634.5
Pedunculagin (**6**)	63.7	4.72	0	2	12	784.5
Casuarictin (**7**)	77.9	0.40	1	2	15	936.6
Strictinin (**8**)	48.9	2.68	1	1	9	634.5
Tellimagrandin I (**9**)	53.4	0.79	2	1	12	786.6
Tellimagrandin II (**10**)	94.8	0.44	3	1	15	938.7
1,2-di-*O*-galloyl-4,6-HHDP-β-D-glucopyranose (**11**)	76.3	1.27	2	1	12	786.6
Rugosin C (**12**)	45.3	0.34	1	1 + 1[d]	17	1104.7
Casuarinin (**14**)	57.7	0.78	1	2	15	936.6
Stenophyllanin A (**15**)	35.6	0.41	1	2 + 1[e]	19	1208.9
Adenosine	> 1000	> 1000	–	–	–	267.2
Adenine	695	> 1000	–	–	–	135.1
Gallic acid	31.7	5.88	1	0	3	170.1
L-Ascorbic acid	34.6	6.25	–	–	–	176.1

[a] Tergalloyl group.
[b] Syzygyl group.
[c] Tergalloyl group (depsidone form).
[d] Valoneoyl group.
[e] Catechin.

2.2. Antioxidant effects of the walnut polyphenols

The antioxidant effects of the compounds obtained in this study were evaluated in two ways: using superoxide-dismutase (SOD)-like activity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. SOD-like activity was estimated by suppression of the superoxide anion radical (O_2^-) generated from the xanthine-xanthine oxidase (XOD) system in the presence of test samples. The results are shown in Table 3. Glansrin C (**3**) and stenophyllanin A (**10**) exhibited a strong SOD-like effect comparable to L-ascorbic acid and gallic acid used as positive controls. The other polyphenols showed activity with an EC_{50} on the order of 10^{-5} M, with the exception of glansrin A (**1**) and 2,3-HHDP-D-glucopyranose (**4**). Conversely, all of these polyphenols had a more potent reducing ability (EC_{50} value 10^{-7}–10^{-6} M) toward the DPPH radical than L-ascorbic acid and gallic acid, while adenosine and adenine had no activity. Although the structure-activity relationships are unclear, ellagitannins with a galloyl group plus an HHDP group, or valoneoyl and its isomeric group, such as **1**–**3**, **7**, **9**–**12** and **14**–**15**, tend to exhibit a more potent radical scavenging effect against DPPH than those with only an HHDP group, like **4** and **6**. This indicates that ellagitannin polyphenols should act as effective antioxidants in walnut.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO DIP-370 digital polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-500 or BRUKER DRX-500 (500 MHz for ^1H and 126 MHz for ^{13}C) in $(CD_3)_2CO + D_2O$. The chemical shifts are given in δ (ppm) values relative to that of the solvent [$(CD_3)_2CO$ (δ_H 2.04; δ_C 29.8)] on a tetramethylsilane scale. FAB-MS was performed on a JEOL JMS-SX102A using 3-nitrobenzyl alcohol as the matrix agent. CD spectra were measured on a JASCO J-720W spectrometer. Reversed-phase HPLC was performed on a Develosil RPAQUEOUS (C-30) (Nomura Chemical Co. Ltd., Japan) column (i.d. 4.6×250 mm), developed with H_2O–MeOH (3:7) (flow rate, 1 ml/min; detection, UV 280 nm) at 40 °C. The solvent was evaporated under reduced pressure at below 40 °C.

3.2. Materials

Walnuts were purchased from TABATA Inc. (Tokyo, Japan). DPPH, xanthine, L-ascorbic acid, and gallic acid of reagent grade were purchased from Wako Chem. Ind. (Tokyo, Japan). XOD (from

almond) was purchased from Sigma Chem. (St. Louis, MO, USA).

3.3. Extraction and isolation

Crushed walnuts (5 mm mesh-passed, 10 kg) were extracted in 70% aq. EtOH (3×15 l) for 24 h at room temp. and filtered. The filtrate was concentrated and extracted with hexane (1 l×3), EtOAc (1 l×3), and *n*-BuOH (1 l×3), successively. The *n*-BuOH extract (19.98 g) was passed through a porous polymer gel, Diaion HP-20 (Mitsubishi Chemical Co., Japan) column, and the adsorbed materials were eluted successively with H_2O, 10, 20, and 40% aq. EtOH, and EtOH. These eluates were concentrated to give residues (yield: 4.51, 2.08, 5.09, 7.50, and 0.50 g, respectively). Part (0.95 g) of the eluate, with 10% aq. EtOH, was chromatographed over Toyopearl HW-40 (i.d. 22×350 mm: Tosoh, Japan) with aq. MeOH (10→20→30→40→50% MeOH)→MeOH–H_2O–Me_2CO (7:1:2), to give adenosine (16.4 mg), adenine (14.7 mg), 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucopyranose (**4**) (19.4 mg), and pedunculagin (**6**) (253.9 mg). The 20% aq. EtOH eluate (4.60 g) was purified by CC on Toyopearl HW-40 (i.d. 22×350 mm) with aq. MeOH (20→30→40→50→60→70% MeOH)→MeOH–H_2O–Me_2CO (7:1:2), and prep HPLC [Develosil C30-UG-5, i.d. 20×250 mm (Nomura Chemical Co., LTD, Japan); 10 mM H_3PO_4–10 mM KH_2PO_4–CH_3CN (42.5:42.5:15) or 10 mM H_3PO_4–10 mM KH_2PO_4–EtOH–EtOAc (42.5:42.5:10:5)], to give isostrictinin (**5**) (94.1 mg), casuarictin (**7**) (163.5 mg), strictinin (**8**) (153.0 mg), tellimagrandin I (**9**) (585.8 mg), tellimagrandin II (**10**) (7.6 mg), 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucopyranose (**11**) (7.7 mg), rugosin C (**12**) (37.1 mg), praecoxin A (**13**) (4.6 mg), casuarinin (**14**) (176.9 mg), stenophyllanin A (**15**) (42.3 mg), stachyuranin B (**16**) (3.5 mg), glansrin A (**1**) (11.9 mg), glansrin B (**2**) (19.9 mg), and glansrin C (**3**) (7.0 mg).

Known compounds were identified by direct comparisons with authentic samples (normal and reversed phase-HPLC, NMR).

3.4. Glansrin A (*1*)

Off-white amorphous powder; $[\alpha]_D^{23}$ −11° (MeOH; *c* 0.3); negative FABMS *m/z*: 1103 $[M-H]^-$; (Found: C, 49.6; H, 3.8. $C_{48}H_{32}O_{31}\cdot 3H_2O$ requires:C, 49.7; H, 3.3%). CD (MeOH) [θ] (nm) $+2.3\times10^5$ (238), -6.9×10^4 (263), $+6.0\times10^3$ (282); ^{1}H NMR *δ*: 7.20 (2H, *s*, galloyl H-2, 6), 6.93 [1H, *s*, tergalloyl (Ter) H-6″], 6.62 (1H, *s*, Ter H-3), 6.57 (1H, *s*, Ter H-3′), 6.48 (1H, *s*, HHDP H-3), 6.38 (1H, *s*, HHDP H-3′), glucose protons *δ*: 6.24 (*d*, *J*=9 Hz, H-1), 5.22 (*t*, *J*=9 Hz, H-2), 5.48 (*dd*, *J*=9, 10 Hz, H-3), 5.24 (*t*, *J*=10 Hz, H-4), 4.54 (*dd*, *J*=6.5, 10 Hz, H-5), 5.37 (*dd*, *J*=6.5, 13 Hz, H-6), 3.92 (*d*, *J*=13 Hz, H-6); ^{13}C NMR, see Table 1.

3.5. Glansrin B (*2*)

Light-brown amorphous powder; $[\alpha]_D^{23}$ +90° (MeOH; *c* 0.1); negative FABMS *m/z*: 905 $[M-H]^-$; (Found: C, 50.9; H, 3.9. $C_{40}H_{26}O_{25}\cdot 2H_2O$ requires:C, 50.9; H, 3.2%). CD (MeOH) [θ] (nm) $+1.9\times10^5$ (238), -5.6×10^4 (266), $+1.7\times10^4$ (285); ^{1}H NMR *δ*: 6.73, 6.71 [1H in total, each *s*, syzygyl (Syz) H-3′], 6.60, 6.55 (1H in total, each *s*, Syz H-3), 6.614, 6.618 (1H in total, each *s*, HHDP H-3), 6.373, 6.368 (1H in total, each *s*, HHDP H-3′), 5.947, 5.941 (1H in total, each *s*, Syz H-6″), glucose protons *δ*: 5.46 (*d*, *J*=4 Hz, H-1), 5.08 (*dd*, *J*=4, 10 Hz, H-2), 5.49 (*t*, *J*=10 Hz, H-3), 5.12 (*t*, *J*=10 Hz, H-4), 4.62 (*ddd*, *J*=1.5, 6.5, 10 Hz, H-5), 5.19 (*dd*, *J*=6.5, 13 Hz, H-6), 3.83 (*dd*, *J*=1.5, 13 Hz, H-6)(α-anomer); 5.06 (*d*, *J*=8 Hz, H-1), 4.87 (*dd*, *J*=8, 9 Hz, H-2), 5.25 (*dd*, *J*=9, 10 Hz, H-3), 5.11 (*t*, *J*=10 Hz, H-4), 4.23 (*ddd*, *J*=1, 6.5, 10 Hz, H-5), 5.21 (*dd*, *J*=6.5, 13 Hz, H-6), 3.90 (*dd*, *J*=1, 13 Hz, H-6)(β-anomer); ^{13}C NMR *δ*: 64.1, 64.2 [glucose (Glc) C-6, α and β], 67.3 (Glc C-5, α), 69.7, 70.1 (Glc C-4, α and β), 72.3 (Glc C-5, β), 75.6 (Glc C-2, α), 75.7 (Glc C-3, α), 77.5 (Glc C-3, β), 78.3 (Glc C-2, β), 91.7 (Glc C-1, α), 95.2 (1C) (Syz C-6″), 95.5 (Glc C-1, β), 106.77, 106.83 (Syz C-3), 107.2, 107.30, 107.34, 107.7 (HHDP C-3 and C-3′), 110.4 (1C) (Syz C-3′), 113.9 (1C) (Syz C-1), 114.3, 114.4 (HHDP C-1′), 114.9, 115.0 (HHDP C-1), 115.7 (1C) (Syz C-1′), 124.7 (1C) (Syz C-4″), 125.37, 125.42, 126.5 (1C), 126.6, 126.7 (HHDP C-2, C-2′ and Syz C-2), 130.2, 130.3 (Syz C-2′), 130.5 (1C) (Syz C-2″), 133.03, 133.05 (Syz C-5′), 135.3 (1C) (Syz C-3″), 135.5 (1C) (Syz C-5″), 136.0, 136.1 (HHDP C-5′), 136.2 (1C) (Syz C-5), 136.4 (1C) (HHDP C-5), 142.1 (1C) (Syz C-6′), 142.2 (1C) (Syz C-1″), 144.3 (1C), 144,4 (1C) (HHDP C-6 and C-6′), 145.0 (1C) (HHDP C-4′), 145.13, 145.19 (HHDP C-4), 145.27, 145.32 (Syz H-4′), 145.5 (1C) (Syz C-4), 145.6 (1C) (Syz C-6), 167.6, 167.7 (Syz C-7′), 168.1 (1C) (Syz C-7), 168.9, 169.0 (HHDP C-7), 169.5, 169.6 (HHDP C-7′).

3.6. Glansrin C (*3*)

Light-brown amorphous powder; $[\alpha]_D^{23}$ +79° (MeOH; *c* 0.1); negative FABMS *m/z*: 933 $[M-H]^-$; (Found: C, 49.1; H, 3.8. $C_{41}H_{26}O_{26}\cdot 4H_2O$ requires:C, 48.9; H, 3.4%). CD (MeOH) [θ] (nm): $+1.6\times10^5$ (228), -3.4×10^4 (252), $+2.8\times10^4$ (287); ^{1}H NMR *δ*: 6.93 (1H, *s*, Ter H-6″), 6.89 (1H, *s*, Ter H-3′), 6.76 (1H, *s*, Ter H-3), 6.43 (1H, *s*, HHDP H-3′), glucose protons *δ*: 5.65 (*d*, *J*=5 Hz, H-1), 4.68 (*dd*, *J*=2, 5 Hz, H-2), 5.49 (*t*, *J*=2.5 Hz, H-3), 5.12 (*dd*, *J*=3, 8 Hz, H-4), 4.17 (*dd*, *J*=2, 8 Hz, H-5), 4.62 (*dd*, *J*=3, 12 Hz, H-6), 3.92 (*d*, *J*=12 Hz, H-6); ^{13}C NMR, see Table 2.

3.7. Methylation of 1 and 3 followed by methanolysis

A mixture of **1** (2 mg), K_2CO_3 (100 mg), and Me_2SO_4 (0.01 ml) in dry Me_2CO (2 ml) was stirred overnight at room temp., and then refluxed for 4 h. After removing the inorganic material by centrifugation, the supernatant was evaporated to dryness. The reaction mixture was directly methanolyzed with 1% NaOMe (0.1 ml) in MeOH (1 ml) at room temp. overnight. After acidification with a few drops of HOAc, the solvent was removed in vacuo. The residue was further treated with CH_2N_2-etherate (2 ml) for 2 h and the solvent was evaporated. The reversed-phase HPLC of the residue showed peaks identical with those of the authentic compounds, methyl tri-*O*-methylgallate (**17**) (t_R 6.1 min), dimethyl hexamethoxydiphenate (**18**) (t_R 6.6 min), and trimethyl octa-*O*-methyltergallate (**19**) (t_R 12.0 min).

Glansrin C (**3**) (1 mg) was similarly treated to yield **19** (HPLC). Small amounts of **17** and **18** were also detected on HPLC, which were produced by cleavage of the depside and ether linkages of the lactonized tergalloyl moiety in the reaction (Hatano et al., 1991).

3.8. Radical scavenging effect on DPPH radical

EtOH solutions of each walnut polyphenol at various concentrations (0.5–10 μg/ml) were added to a solution of DPPH (30 μM) in EtOH (0.6 ml), and the reaction mixture (total volume, 1.2 ml) was shaken vigorously. After keeping at room temp. for 30 min, the remaining DPPH was determined by colorimetry at 520 nm, and the radical-scavenging activity of each compound was expressed using the ratio of the absorption of DPPH (%) relative to the control DPPH solution (100%) in the absence of sample. The mean values were obtained from triplicate experiments.

3.9. SOD-like activity by nitrite method

A mixture consisting of the sample solution (10–500 μg/ml) (0.1 ml), 0.5 mM xanthine solution (pH 8.2) (0.2 ml), buffer solution (65 mM KH_2PO_4, 35 mM $Na_2B_4O_7$, and 0.5 mM EDTA·2Na, pH 8.2) (0.2 ml), 10 mM hydroxylamine (0.1 ml), water (0.2 ml), and XOD (12.4 mU/ml in buffer) (0.2 ml) was incubated for 30 min at 37 °C and coloring reagent was added (30 μM *N*-naphthylethylenediamine, 3 mM sulfanilic acid, and 25% acetic acid) (2.0 ml). The final mixture was allowed to stand for 30 min at room temp., and the optical absorption was measured at 550 nm. A blank was measured in the same way with no enzyme. Water was used as a control. SOD-like activity (%) was calculated using the following equation, and was given as the mean value of three experiments.

$$\text{SOD-like activity (\%)} = \left\{1 - \frac{(\text{sample } A_{550}) - (\text{blank}_{\text{sample}} A_{550})}{(\text{control } A_{550}) - (\text{blank}_{\text{control}} A_{550})}\right\} \times 100$$

Acknowledgements

We would like to thank the SC-NMR Laboratory of Okayama University for conducting the NMR experiments.

References

Anderson, K.J., Teuber, S.S., Gobeille, A., Cremin, P., Waterhouse, A.L., Steinberg, F.M., 2001. Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. J. Nutr. 131, 2837–2842.

Duke, J.A., 1989. Handbook of Nuts. CRC Press, London.

Han, L., Hatano, T., Okuda, T., Yoshida, T., 1995. Tannins of *Stachyurus* species. III. Stachyuranins A, B and C, three new complex tannins from *Stachyurus praecox* leaves. Chem. Pharm. Bull. 43, 2109–2114.

Hatano, T., Ogawa, N., Yasuhara, T., Okuda, T., 1990. Tannins of Rosaceous plants. VIII. Hydrolyzable tannin monomers having a valoneoyl group from flower pet als of *Rosa rugosa* Thunb. Chem. Pharm. Bull. 38, 3308–3313.

Hatano, T., Yazaki, K., Okonogi, A., Okuda, T., 1991. Tannins of *Stachyurus* species. II. Praecoxins A, B, C and D, four new hydrolyzable tannins from *Stachyurus praecox* leaves. Chem. Pharm. Bull. 39, 1689–1693.

Kagawa, Y., 2001. Standard Tables of Food Composition in Japan. Kagawa Nutrition University Press, Tokyo.

Koppaka, V.R., 1977. Toxic principle of *Hippomane mancinella*. II. Structure of hipppomanin A. Lloydia 40, 169–171.

Lin, J.-H., Ishimatsu, M., Tanaka, T., Nonaka, G., Nishioka, I., 1990. Tannins and related compounds. XCVI. Structures of macaranins and macarinins, new hydrolyzable tannins possessing macaranoyl and tergalloyl ester groups, from the leaves of *Macaranga sinensis* (Baill.) Muell.-Arg. Chem. Pharm. Bull. 38, 1844–1851.

Nonaka, G., Nishimura, H., Nishioka, I., 1985. Tannins and related compounds. Part 26. Isolation and structures of stenophyllanins A, B, and C, novel tannins from *Quercus stenophylla*. J. Chem. Soc., Perkin Trans. 1, 163–172.

Okuda, T., Yoshida, T., Hatano, T., Yazaki, K., Ashida, M., 1982a. Ellagitannins of the Casuarinaceae, Stachyuraceae and Myrtaceae. Phytochemistry 21, 2871–2874.

Okuda, T., Yoshida, T., Hatano, T., Koga, T., Toh, N., Kuriyama, K., 1982b. Circular dichroism of hydrolyzable tannins, I: ellagitannins and gallotannins. Tetrahedron Lett. 23, 3937–3940.

Okuda, T., Yoshida, T., Kuwahara, M., Yazaki, K., 1983. Structures of pedunculagin, casuarictin, strictinin, casuarinin, casuariin and stachyurin. J. Chem. Soc., Perkin Trans. 1, 1765–1772.

Perry, L.M., 1980. Medicinal Plants of East and Southeast Asia. MIT Press, Cambridge.

Seikel, M.K., Hillis, W.E., 1970. Hydrolysable tannins of *Eucalyptus delegatensis* wood. Phytochemistry 9, 1115–1128.

Tanaka, T., Nonaka, G., Nishioka, I., Kouno, I., 1996. Syzyginins A and B, two ellagitannins from *Syzygium aromaticum*. Phytochemistry 43, 1345–1348.

Wilkins, C.K., Bohm, B.A., 1976. Ellagitannins from *Tellima grandiflora*. Phytochemistry 15, 211–214.