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Antioxidative Constituents from Viticis trifoliae Fructus (Fruit of *Vitex rotundifolia* L.)

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Seven phenolic compounds, vanillic acid (1), *threo*-guaiacyl glycerol (2), *erythro*-guaiacyl glycerol (3), taxifolin (4), dihydrodehydrodiconiferyl alcohol (5), dihydrodehydrodiconiferyl alcohol-9-*O*-β-D-glucoside (6) and dihydrodehydrodiconiferyl alcohol-(4→8)-*erythro*-guaiacyl glycerol ether (7), were separated from the methanol extract of Viticis trifoliae Fructus (Fruit of *Vitex rotundifolia* L.) and their structures were identified on the basis of spectroscopic data. In addition, 1, 2, 4–7 and two previously isolated iridoid glucosides, agnuside (8) and VR-I (10-*O*-vanilloyl aucubin) (9) were tested for antioxidative activity using the ferric thiocyanate method. These compounds, except 8, exhibited stronger antioxidative activity than 3-*tert*-butyl-4-hydroxyanisole. Moreover, 1, 2 and 4–9 were investigated for the scavenging effect on 1,1-diphenyl-2-picrylhydrazyl. All tested compounds, except 8, showed a potent scavenging effect. Especially, the effect of 4 was almost twice that of α-tocopherol at a concentration of 0.02 mM.

Keywords: antioxidative constituent, radical scavenger, phenolic compound, ferric thiocyanate method, Viticis trifoliae Fructus, *Vitex rotundifolia* L.

Antioxidants function as protection from lipid peroxidation, which cause the rancidity of fats and oils in food. Furthermore, the peroxidation of unsaturated fatty acids in cell membranes causes various human diseases (Yagi, 1987; Yoshikawa *et al.*, 1994). Synthetic antioxidants such as 3-*tert*-butyl-4-hydroxyanisole (BHA) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and natural antioxidants such as α-tocopherol and ascorbic acid are currently used as food additives for the purpose of preventing the deterioration of foods. Although these synthetic antioxidants showed a stronger antioxidative effect than α-tocopherol and ascorbic acid, the use of synthetic ones is being reconsidered because of their toxic side effects (Ito *et al.*, 1986). Therefore, new antioxidants, especially from natural sources which would be safer for the human body, are required.

Vitex rotundifolia L. (Verbenaceae) is widely distributed in Asia, and its fruit (Viticis trifoliae Fructus) is used as folk medicine for headaches (Kimura & Kimura, 1981). Diterpenes, flavones, lignan, phenylpropanoides, hydroxybenzoic acid derivatives and iridoids have been previously reported from this fruit (Okuyama *et al.*, 1994, 1995; Kondo *et al.*, 1986; Kimura *et al.*, 1967).

In the course of our studies on natural antioxidants from edible plants and crude drugs (Ono *et al.*, 1995a, 1997a; Masuoka *et al.*, 1997), the methanol extract of this fruit showed a stronger antioxidative activity than BHA using the ferric thiocyanate method (Kikuzaki & Nakatani, 1993). We reported earlier the isolation and the structure elucidation of eight iridoids, agnuside (**8**), VR-I (10-*O*-vanilloyl aucubin) (**9**), eucommiol, 1-oxo-eucommiol, iridolactone, pedicularis-lactone, viteoid I and viteoid II from the methanol extract of Viticis trifoliae Fructus (Ono *et al.*, 1997b). The present paper describes the further separation and the structure elucidation of seven phenolic compounds, vanillic acid (**1**), *threo*-guaiacyl glycerol (**2**), *erythro*-guaiacyl glycerol (**3**) taxifolin (**4**), dihydrodehydrodiconiferyl alcohol (**5**), dihydrodehydrodiconiferyl alcohol-9-*O*-β-D-glucoside (**6**) and dihydrodehydrodiconiferyl alcohol-(4→8)-*erythro*-guaiacyl glycerol ether (**7**) from the methanol extract and the antioxidative activities of **1**, **2**, and **4**–**9**.

Materials and Methods

All instruments used in the present investigation were the same as cited in the preceding report (Ono *et al.*, 1995a, b). α-Tocopherol, BHA, L-cysteine monohydrochloride monohydrate and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Nacalai Tesque, Inc., Kyoto. Linoleic acid was purchased from Tokyo Kasei Kogyo Co., Tokyo. The separation procedure for fractions (frs.) 1, 2, 25, 35, 37, 39, 43, and agnuside (**8**) and VR-I (**9**) was also reported in the preceding paper (Ono *et al.*, 1997b).

Separation of 1–7 Fraction 25 was subjected to high performance liquid chromatography (HPLC) (YMC-pack S-5 120A ODS, 20 mm i.d.×250 mm; solv., 10% CH_3OH; YMC Co., Ltd., Kyoto) to give **2** (15 mg) and fr. 51 (12 mg). HPLC [Kusano C.I.G. prepacked column Si-10, 22 mm i.d.×100 mm; solv., $CHCl_3$-CH_3OH-H_2O=8:2:0.2); Kusano Kagakukikai Co., Tokyo] of fr. 51 furnished **3** (6 mg). Fractions 37, 39 and fr. 43 were each subjected to HPLC under conditions similar (solv., 60% CH_3OH) to those for fr. 25 to

give **5** (87 mg) from fr. 37, fr. 52 (18 mg) and **7** (11 mg) from fr. 39, and fr. 53 (320 mg) and fr. 54 (45 mg) from fr. 43. Fraction 52 and fr. 54 were each subjected to HPLC under conditions similar (solv., $CHCl_3$-CH_3OH-H_2O=14:2:0.1) to those for fr. 51 to afford **1** (11 mg) from fr. 52, and **6** (15 mg) from fr. 54. Fraction 35 was successively chromatographed over MCI gel CHP 20P (eluting with 40% CH_3OH; 50% CH_3OH; 60% CH_3OH; 70% CH_3OH; Mitsubishi Chemical Industries, Ltd., Tokyo), Chromatorex ODS (eluting with 10% CH_3OH; 20% CH_3OH; 30% CH_3OH; 40% CH_3OH; 50% CH_3OH; 60% CH_3OH; 70% CH_3OH; Fuji Silysia Chemical, Ltd., Aichi) and silica-gel (silica gel 60, 230–400 mesh, eluting with $CHCl_3$-CH_3OH-H_2O=10:2:0.1; 8:2:0.2; 7:3:0.5; Merck, Darmstadt, Germany) to give **4** (19 mg).

Acetylation of 7 Solution of **7** (1 mg) in Ac_2O-pyridine (1:1, 1 ml) was allowed to stand at room temperature overnight. After removal of the reagent under a stream of N_2, the residue was partitioned between ether (1.0 ml) and H_2O (0.5 ml). The ether layer was concentrated to afford an acetate (**10**, 1 mg).

10: proton nuclear magnetic resonance (^{1}H-NMR) (in $CDCl_3$, 500 MHz) δ: 7.05 (1H, s, H-2),[1] *ca.* 6.98 (2H, H-5 and H-6),[2] 6.91 (1H, s, H-2″),[1] 6.86 (1H, d, J=8.5 Hz, H-6″),[2] 6.80 (1H, d, J=8.5 Hz, H-5″),[2] 6.65 (1H, s, H-2′),[3] 6.63 (1H, s, H-6′),[3] 6.06 (1H, d, J=5.5 Hz, H-7″), 5.44 (1H, d, J=6.7 Hz, H-7), 4.63 (1H, m, H-8″), *ca.* 4.43 (1H, Ha-9), *ca.* 4.42 (1H, Ha-9″), 4.29 (1H, dd, J=7.3, 11.6 Hz, Hb-9), *ca.* 4.23 (1H, Hb-9″), 4.09 (2H, t, J=6.5 Hz, H_2-9′), 3.89 (3H, s, OCH_3), 3.82 (3/2H, s, OCH_3), 3.81 (3/2H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.70 (1H, m, H-8), 2.64 (2H, t, J=7.5 Hz, H_2-7), 2.30 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 1.94 (2H, tt, J=6.5, 7.5 Hz, H_2-8′).[1–3]Assignments may be interchanged in each number.

Assay of antioxidative activity by the ferric thiocyanate method The antioxidative activity of the test sample was evaluated based on the ferric thiocyanate method as described in a preceding paper (Ono *et al.*, 1995a). A mixture of 2.51% linoleic acid EtOH solution (1.00 ml), 0.05 M phosphate buffer (pH 7.0, 2.00 ml) and H_2O (1.00 ml) was added to the 0.1% EtOH solution (1.00 ml) of each sample in a vial with a cap and placed in the dark at 40°C to accelerate the oxidation. At intervals during incubation, this assay solution (0.05 ml) was diluted with 75% EtOH (4.85 ml), which was followed by adding 30% ammonium thiocyanate (0.05 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.05 ml) to the reaction mixture, the absorbance of the developed red color was measured at 500 nm. The control sample was prepared from the mixture containing all ingredients except a test sample. α-Tocopherol and BHA were used as standard samples.

Assay of scavenging effect on DPPH We applied the method of Uchiyama *et al.* (1968) with slight modification. An EtOH solution (1.00 ml) of each test sample was added to a mixture of 0.1 M acetic acid buffer (pH 5.5, 1.00 ml) and 0.5 mM DPPH EtOH solution (0.50 ml) in a test tube. The solution was shaken and allowed to stand at room temperature for 30 min, and then the absorbance of the resulting solution was measured at 517 nm. The control sample was prepared from the mixture containing all ingredients except a test sample. The scavenging effect on DPPH was expressed as follows: ΔO.D.=O.D. of control−O.D. of sample. α-Tocopherol and L-cysteine were used as standard samples.

Results and Discussion

Antioxidative activities of methanol extract, fr. 1 and fr. 2 The methanol extract of Viticis trifoliae Fructus showed a stronger antioxidative activity than BHA using linoleic acid as the substrate by the ferric thiocyanate method (Fig. 1). This extract was partitioned between hexane and methanol. The methanol soluble fr. (fr. 2) showed a stronger antioxidative activity than hexane soluble fr. (fr. 1) (Fig. 2). Fraction 2 was fractionated by repeated column chromatographies (MCI gel CHP 20P, Chromatorex ODS, silica gel), and HPLC on octadecyl silica (ODS) and silica gel to give seven compounds (**1**–**7**).

Structure elucidation of 1–7 Compounds **1**–**6** were identified as vanillic acid (Nishibe *et al.*, 1981), *threo*-guaiacyl glycerol (Deyama *et al.*, 1986), *erythro*-guaiacyl glycerol (Deyama *et al.*, 1986), taxifolin (Nonaka *et al.*, 1987; Harborne & Mabry, 1982), dihydrodehydrodiconiferyl alcohol (Agrawal & Thakur, 1985; Rimando *et al.*, 1994; Fukuyama *et al.*, 1996) and dihydrodehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (Abe & Yamauchi, 1986), respectively, on the basis of spectral data and physical data (Fig. 3).

Compound **7** was obtained as a white powder, $[\alpha]_D^{25}$−2.4° (c=0.9, methanol). The negative ion fast atom bombardment mass spectrum (negative FAB-MS) and positive FAB-MS of **7** exhibited an $[M-H]^-$ ion peak at m/z 555 and an $[M+Na]^+$ ion peak at m/z 579, respectively, indicating its molecular weight to be 556, A high-resolution positive FAB-MS [m/z: 579.2200 $[M+Na]^+$ (Calcd. for $C_{30}H_{36}O_{10}Na$: 579.2206] indicated the molecular formula of **7** to be $C_{30}H_{36}O_{10}$. The ^{1}H-NMR spectrum (in methanol-d_4) of **7** indicated 1 mol of guaiacyl glycerol signals together with similar signals of **5**. However, the signals of H-7 and two

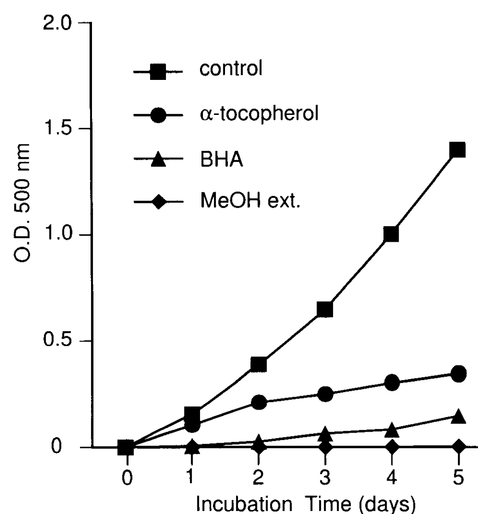

Fig. 1. Antioxidative activity of the methanol extract from Viticis trifoliae Fructus. Each compound was added at a final concentration of 0.02% based on the total volume of solution.

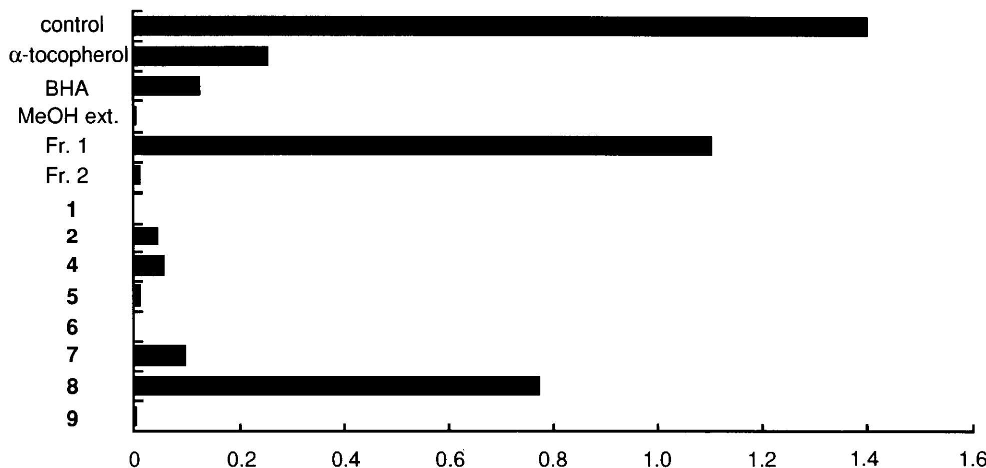

Fig. 2. Antioxidative activity of fr. 1, fr. 2, **1**, **2**, **4**-**9** for the 5th day of the lipid peroxidation. The final concentration of the sample was 0.02%.

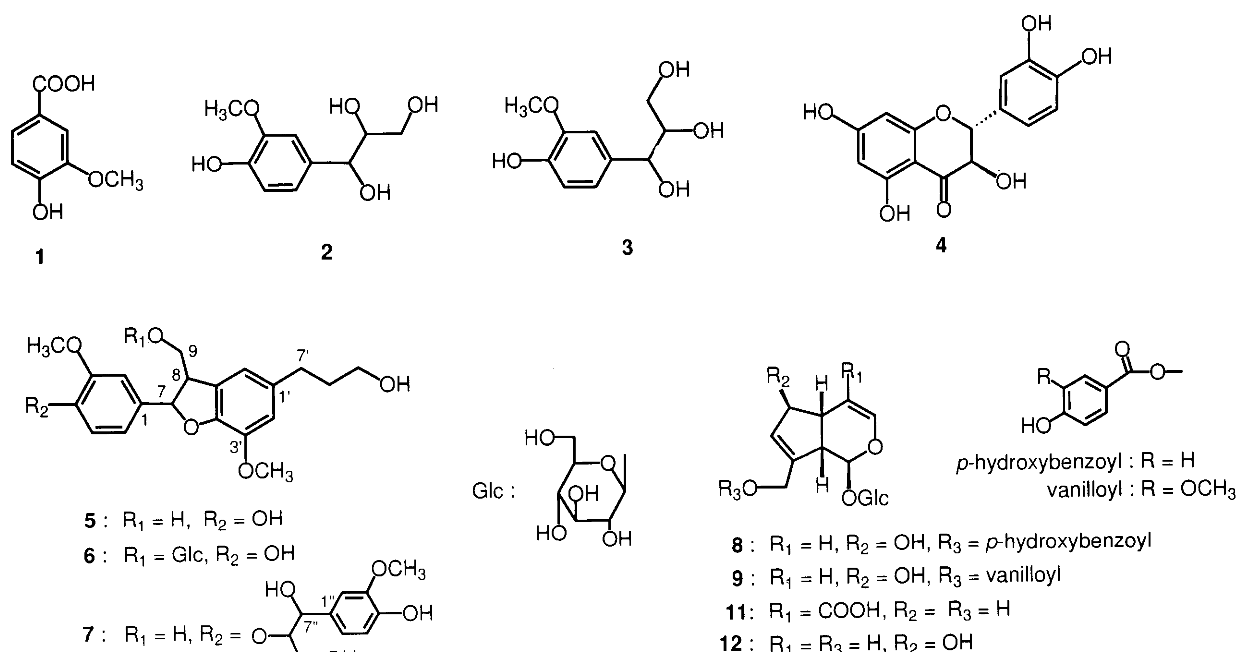

Fig. 3. Structures of **1**-**9**, **11** and **12**.

methoxyl groups appeared as two sets each. It was thought that the signals had appeared due to the occurrence of conformational isomerism. Therefore, the ^{1}H-NMR spectrum (in dimethylsulfoxide-d_6) of **7** was measured at 90°C. In this spectrum, the signals of H-7 and one methoxyl group were observed as one set, but that of one methoxyl group was still split by 1.2 Hz. From these data, **7** was considered to be a mixture of conformational isomers or diastereomers. In the NOE difference spectra, irradiation of the signals of methoxyl groups at δ 3.76, 3.78 and 3.85 gave enhancements of the signals at δ 6.94 (1H, d, $J=1.8$ Hz, H-2″), 6.99 (1H, d, $J=1.8$ Hz, H-2) and 6.72 (2H, s, H-2′ and H-6′), respectively. The ^{1}H-NMR spectrum of acetate of **7** (**10**) revealed the signals of four alcoholic acetyl groups (δ 2.09, 2.07, 2.03, 2.02) and one phenolic acetyl group (δ 2.30). Moreover, comparing the chemical shifts of the ^{1}H-NMR signals of **7** with **10**, the signals at H_2-9, H_2-9′, H_2-9″ and H-7″ in **10** were shifted downfield. These observations indicated that the guaiacyl glycerol group, which was supposed to be an *erythro* isomer from the coupling constant of the benzylic proton signal of the guaiacyl glycerol group in the ^{1}H-NMR spectrum of **10** (Deyama *et al.*, 1986), was located at OH-4 of **5**.

Accordingly, **7** was characterized as dihydrodehydrodiconiferyl alcohol-(4→8)-*erythro*-guaiacyl glycerol ether, which was considered to be leptolepisol A (Miki *et al.*, 1979) (Fig. 3).

Compounds **1**, **2** and **3** have been reported to be constituents of Viticis trifoliae Fructus (Okuyama *et al.*, 1994, 1995; Kondo, 1986), but, as far as we know, this is the first example of the separation of **4**, **5**, **6** and **7** from this fruit.

Table 1. Reduction of 1,1-diphenyl-2-picrylhydrazyl.

Test compound	Concentration ($\times 10^{-5}$ M)	ΔO.D.
1	2	0.404
1	4	0.614
1	8	0.852
2	2	0.480
2	4	0.716
2	8	0.962
4	1	0.554
4	2	0.982
4	4	1.294
5	2	0.332
5	4	0.512
5	8	0.770
6	2	0.320
6	4	0.472
6	8	0.672
7	2	0.440
7	4	0.674
7	8	0.954
8	4	0.024
8	8	0.084
8	16	0.108
9	2	0.224
9	4	0.324
9	8	0.482
α-tocopherol	1	0.342
α-tocopherol	2	0.564
α-tocopherol	4	1.016
cysteine	2	0.264
cysteine	4	0.442
cysteine	8	0.884

ΔO.D.=O.D. of control at 517 nm (1.444)−O.D. of sample. 1,1-diphenyl-2-picrylhydrazyl; 1×10^{-4} M.

Antioxidative activities of 1, 2, and 4–9 The antioxidative activities of **1**, **2**, and **4**–**9** were investigated in the same manner as that used for the methanol extract. All these compounds, except **8**, showed stronger antioxidative activities than BHA (Fig. 2).

Furthermore, the scavenging effects of **1**, **2** and **4**–**9** on the stable free radical DPPH were examined. These tested compounds, except **8**, exhibited potent scavenging effects (Table 1). Compound **4** showed the strongest activity among the tested compounds, almost twice that of α-tocopherol.

Accordingly, **1**, **2**, **4**, **5**, **6**, **7** and **9** were considered to be the antioxidative principles of the methanol extract of Viticis trifoliae Fructus. The antioxidative properties of these compounds seem to explain the radical scavenging effects due to the phenolic hydroxyl groups. In addition, it was supposed that the guaicyl (4-hydroxy-3-methoxyphenyl) group showed a stronger antioxidative activity than the 4-hydroxyphenyl group, because **8** exhibited no significant activity in spite of its structural similarity to **9** whose activity was stronger than that of BHA using the ferric thiocyanate method. Further, Toda *et al.* (1985) reported that geniposidic acid (**11**), an iridoid glucoside, showed a stronger antioxidative activity than α-tocopherol using their test method based on the air oxidation of linoleic acid, but aucubin (**12**), which is lacking in the vanilloyl group of **9**, showed no activity (Toda *et al.*, 1985). Thus, the antioxidative activity of **9** is derived from the vanilloyl group.

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