Arch Pharm Res Vol 26, No 7, 535-539, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Antioxidant Constituents from the Stem of *Sorghum bicolor*

Yong Soo Kwon and Chang Min Kim

College of Pharmacy, Kangwon National University, 200-701, Korea

(Received January 27, 2003)

The EtOAc soluble fraction from the stem of *Sorghum bicolor* showed a strong free radical scavenging activity. Five major compounds were isolated from this fraction. They were identified by spectral data as methyl ferulate (**1**), methyl *p*-hydroxycinnamate (**2**), *p*-hydroxybenzaldehyde (**3**), tricin (**4**), and quercetin 3,4'-dimethyl ether (**5**). Among these compounds, **1** exhibited a strong, free radical scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) with an IC_{50} value of 0.7 μM. We further studied the effects of these isolated compounds on the lipid peroxidation in rat liver microsomes induced by non-enzymatic method. All five compounds showed anti-lipid peroxidation activity (IC_{50} values of 0.5, 0.4, 0.3 and 0.3 μM, respectively).

Key words: *Sorghum bicolor*, Phenolics, Antioxidant, Free radical scavenging activity, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, Lipid peroxidation

INTRODUCTION

Sorghum represents one of the most important crops in the world. It is used as a human food resource and folk medicine in Asia and Africa (Kim *et al.*, 1997). The stem of *Sorghum propinquum* has been used as a digestive and antidiarrheal agent (Nanjing, 1999). Meanwhile, some researchers have reported the chemical constituents of Sorghum (Rimando *et al.*, 1998; Selmar *et al.*, 1996; Avato *et al.*, 1990; Gujer *et al.*, 1986; Haskins and Gorz, 1983 Woodhead *et al.*, 1982).

In the course of a screening to evaluate antioxidant constituents from medicinal plants, we found that EtOAc extract from the stem of *Sorghum bicolor* showed a strong, free radical scavenging activity. We used chromatography to isolate the effective antioxidants from this active fraction. Repeated column chromatography of the EtOAc soluble fraction led to the isolation of five compounds. This paper deals with structure elucidation of these compounds and their antioxidant activities using the DPPH free radical scavenging method and lipid peroxidation assay in rat liver microsomes induced by non-enzymatic methods.

Correspondence to: Chang Min Kim, College of Pharmacy, Kangwon National University, 200-701, Korea
Tel: 82-33-250-6912, Fax: 82-33-255-9041
E-mail kcmin@kangwon.ac.kr

MATERIALS AND METHODS

Instruments and reagents

Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. NMR spectra (^{1}H-NMR and ^{13}C-NMR spectra taken at 200 and 50 MHz, respectively) were recorded in deuterated solvents using TMS as the internal standard. The EI (70 eV) and FAB MS spectra were measured using an Autospec Micromass, UV spectra using a Hitachi U-2000, and IR spectra in a KBr disk using a Bio-Rad FTS-7. TLC work was carried out using plates coated with silica gel 60 F_{254} (Merck Co.). All solvents were routinely distilled prior to use. Silica gel column chromatography was performed on Merck silica gel 60 (70-230 mesh and 230-400 mesh). Other reagents were commercial grade without purification.

Plant materials

The stem of *Sorghum bicolor* was collected at Yanggu, Kangwon Province, Korea in October 2001, and identified by Chang Min Kim, Kangwon National University. A voucher specimen of the plant was deposited at the herbarium of the College of Pharmacy (KNUP-H-44), Kangwon National University.

Extraction and isolation

The air-dried stems (2.3 kg) were ground and extracted with hot MeOH three times, for 4 hours each time. The resultant extracts were combined and concentrated under

reduced pressure to produce 120 g of the residue. This MeOH extract was suspended in 10 volumes of water and then partitioned successively with equal volumes of *n*-hexane, EtOAc and *n*-BuOH, leaving a residual water soluble fraction. Each fraction was evaporated *in vacuo* to yield the residues of *n*-hexane fraction(fr.) (14 g), EtOAc fr., (26.7 g), and *n*-BuOH fr., (8 g).

The EtOAc soluble fraction (26.7 g) was column chromatographed on a silica gel (250 g, 70-230 mesh, Ψ15×50 cm) using stepwise gradient elution with the solvents benzene-acetone (9:1 → 1:1 v/v), in order to divide the fraction into five sub-fractions (Fr.1-Fr.5).

Fr. 1 was re-chromatographed on silica gel column (70 g, 70-230 mesh, Ψ2×50 cm) by elution with $CHCl_3$-MeOH (49:1) to give compounds **1** (34 mg), **2** (230 mg) and **3** (73 mg). Fr. 2 was re-chromatographed on Sephadex LH 20 column (70 g, Ψ2.5×50 cm) by elution with MeOH to give compounds **4** (25 mg) and **5** (60 mg).

Methyl ferulate (1)

mp: 63-64°C UV (nm): λ_{max} (MeOH) 235, 294 (sh), 324; UV (nm): λ_{max} (MeOH+NaOH) 255, 308, 376; IR ν_{max} (KBr) cm^{-1}: 3449 (OH), 1667 (C=O), 1055 (C-O); ^{1}H-NMR (Acetone-d_6) δ (ppm): 8.27 (1H, s, OH), 7.69 (1H, d, *J* = 16.2 Hz, H-7), 7.42 (1H, d, *J* = 1.8 Hz, H-2), 7.24 (1H, dd, *J* = 1.8 and 8.2 Hz, H-6), 6.97 (1H, d, *J* = 8.2 Hz, H-5), 6.49 (1H, d, *J* = 16.2 Hz, H-8)), 4.01(3H, s, $COOCH_3$), 3.82 (3H, s, OCH_3); ^{13}C-NMR (Acetone-d_6) δ (ppm): 167.13 (C-9), 149.25 (C-3), 147.92 (C-4), 144.94 (C-7), 126.60 (C-1), 123.07 (C-6), 115.26 (C-8), 114.71 (C-5), 110.51 (C-2), 55.48 (OCH_3), 50.70 ($COO\underline{C}H_3$); EI-MS, *m/z*: 208[M]$^+$.

Mmethyl *p*-hydroxycinnamate (2)

mp: 137°C; UV (nm): λ_{max} (MeOH) 225, 297 (sh), 311; UV (nm): λ_{max} (MeOH+NaOH) 231, 309, 356; IR ν_{max} (KBr) cm^{-1}: 3449 (OH), 1667 (C=O), 1055 (C-O); ^{1}H-NMR (Acetone-d_6) δ (ppm): 9.01 (1H, s, OH), 7.71 (1H, d, *J* = 16.2 Hz, H-7), 7.65 (2H, d, *J* = 8.4 Hz, H-2 and H-6), 6.99 (2H, d, *J* = 8.4 Hz, H-3 and H-5), 6.45 (1H, d, *J* = 16.2 Hz, H-8), 3.81(3H, s, $COOCH_3$); ^{13}C-NMR (Acetone-d_6) δ (ppm): 167.08 (C-9), 159.76 (C-4), 144.58 (C-7), 130.13 (C-2 and C-6), 126.16 (C-1), 115.85 (C-3 and C-5), 114.47 (C-8), 50.67 ($COO\underline{C}H_3$); EI-MS, *m/z*: 178[M]$^+$

***p*-Hydroxybenzaldehyde (3)**

mp: 115-116°C; UV (nm): λ_{max} (MeOH) 208, 221, 283; UV (nm): λ_{max} (MeOH+NaOH) 238, 330; IR ν_{max} (KBr) cm^{-1}: 3449 (OH), 1667 (C=O), 1055 (C-O); ^{1}H-NMR (Acetone-d_6) δ (ppm): 9.95 (1H, s, CHO), 9.55 (1H, s, OH), 7.90 (2H, d, *J* = 8.4 Hz, H-2 and H-6), 7.11 (2H, d, *J* = 8.4 Hz, H-3 and H-5); ^{13}C-NMR (Acetone-d_6) δ (ppm): 190.30 (CHO), 163.06 (C-4), 132.02 (C-2 and C-6), 129.62 (C-1), 115.85 (C-3 and C-5); EI-MS, m/z: 122 [M]$^+$.

Tricin (4)

mp: 291-292°C; UV (nm): λ_{max} (MeOH) 247 (sh), 269 342; UV (nm): λ_{max} (MeOH+NaOH) 264, 275 (sh), 417; UV (nm): λ_{max} (MeOH+NaOAc) 275, 321 (sh), 362; UV (nm): λ_{max} (MeOH+NaOAc+H_3BO_3) 271, 342; IR ν_{max} (KBr) cm^{-1}: 3449 (OH), 1667 (C=O), 1055 (C-O); ^{1}H-NMR (DMSO-d_6) δ (ppm): 12.96 (1H, s, 5-OH), 7.32 (2H, s, H-6'and H-2'), 6.97 (1H, s, H-3), 6.55 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6) and 3.87 (6H, s, $2OCH_3$); ^{13}C-NMR (DMSO-d_6) δ (ppm): 181.90 (C-4), 164.22 (C-2), 163.76 (C-7), 161.51 (C-5), 157.43 (C-9), 148.29 (C-3'and C-5'), 139.95 (C-4'), 120.49 (C-1'), 104.48 (C-3), 103.81 (C-2'and C-6'), 103.67 (C-10), 98.89 (C-6), 94.25 (C-8), 56.41 (OCH_32); Positive FAB-MS, *m/z*: 331 [M+H]$^+$.

Quercein-3,4'-dimethyl ether (5)

mp: 235-236°C; UV (nm): λ_{max} (MeOH) 221, 272, 326; UV (nm): λ_{max} (MeOH+NaOH) 222, 278, 299 (sh); UV (nm): λ_{max} (MeOH+NaOAc) 230, 277, 306 (sh); UV (nm): λ_{max} (MeOH+NaOAc+H_3BO_3) 231, 273, 325; UV (nm): λ_{max} (MeOH+$AlCl_3$) 239, 264, 312, 361, 379; IR ν_{max} (KBr) cm^{-1}: 3450 (OH), 1685 (C=O), 1055 (C-O); ^{1}H-NMR (DMSO-d_6) δ (ppm): 12.96 (1H, s, 5-OH), 7.12 (1H, d, *J* = 1.8 Hz, H-2'), 7.05 (1H, d, *J* = 1.8 and 8.0 Hz, H-6'), 6.79 (1H, d, *J* = 8.0 Hz, H-5'), 6.65 (1H, d, *J* = 2.0 Hz, H-6), 6.30 (1H, d, *J* = 2.0 Hz, H-8), 3.95 and 3.82 (each 3H, s, $2OCH_3$); ^{13}C-NMR (DMSO-d_6) δ (ppm): 181.98 (C-4), 164.47 (C-7), 161.52 (C-5), 157.51 (C-9), 153.01 (C-4' and C-2), 147.02 (C-3'), 140.03 (C-3), 125.41 (C-1'), 120.51 (C-6'), 115.89 (C-2'), 114.79 (C-5'), 104.45 (C-10), 99.03 (C-6), 94.40 (C-8), 60.53 (OCH_3), 55.62 (OCH_3); EI-MS, m/z: 330[M]$^+$.

Sample preparation

The tested samples were dissolved in MeOH-DMSO (1:1) to various concentrations (2.5-120 μg/mL).

Preparation of rat liver microsomal suspension

Male Sprague-Dawley rat liver was removed and washed with ice-cold 0.9% saline. The liver was homogenized in 9 volumes of ice-cold 1.15% KCl. To prepare the liver microsomal fraction, the liver was rapidly homogenized in ice-cold 0.25 M sucrose and centrifuged at 9,000 g at 4°C for 20 min. The supernatant was centrifuged at 105,000 g at 4°C for 60 min. The microsomal pellets were washed 3 times with ice-cold 0.15 M KCl, and then stored at -20°C until the experiments (Nguyen *et al.*, 1998). The protein content of tissue homogenates was measured using a Bio-Rad protein assay kit (Maxwell *et al.*, 1999; Na *et al.*, 2001).

DPPH radical scavenging activity

The scavenging activity was corresponded to the intensity quenching DPPH radical (Yoshida *et al*, 1989). Four

milliliters of MeOH solution of test compounds at various concentrations (2.5-120 μg/mL) was added to a solution of DPPH (1.5×10^{-4} M) in MeOH (1 mL), and the reaction mixture (total volume, 5 mL) was shaken vigorously. After storage at room temperature for 30 min in air, the remaining DPPH was determined by colorimetry at 520 nm, and the radical scavenging activity of each sample was expressed by the ratio of the reduction in DPPH absorption (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (control). The mean values were obtained by triplicate experiments.

Inhibitory lipid peroxidation in rat liver microsomes

The antioxidant activity was determined by quantification of thiobarbituric acid reactive substance (TBA-RS) using a slightly modified method of Buege and Aust (Nguyen *et al.*, 1998). The reaction mixture consisted of 0.5 tissue homogenate, 0.9 mL phosphate buffer (50 mM, pH 7.4), 0.5 mL of one of the chemical system generating free radicals (0.01 mM $FeSO_4$ + 0.1 mM ascorbic acid (Fe-VC) or 0.01 mM $FeSO_4$-H_2O_2 (the Fenton reagent)), and 0.1 mL of vehicle or a solution of test samples. The reaction mixtures were incubated by adding 1 mL of 10% (w/v) trichloroacetic acid to the mixture. After centrifugation at 8,000 g for 10 min, the supernatants were incubated with 1 mL of 0.8% (w/v) TBA at 100°C for 15 min. After cooling, TBA-RS generated was spectrophotometrically determined at 532 nm.

RESULTS AND DISCUSSION

The EtOAc extract of the stem of *S. bicolor* showed a strong free radical scavenging activity. Thus, detailed laboratory investigation was performed on this active extract. Bioassay- guided fractionation led to the isolation of compounds **1**, **2**, **3**, **4**, and **5**.

A phenylpropanoid skeleton for **1** was presumed from the UV and IR spectra. Bathochromic shift resulting from the addition of NaOH indicated the presence of a phenolic hydroxyl group in the phenylpropanoid skeleton (Dey and Harborne, 1989). The ^{1}H- and ^{13}C-NMR spectral data of **1** were very similar to those of ferulic acid except for a methoxy presence at δ4.01 and δ50.70. From a comparison of our data with that reported in the literature (Wandji *et al.*, 1990; Joshi *et al.*, 1986), compound **1** was identified as methyl ferulate.

The structures of **2**, **3**, and **4** were identified as methyl *p*-hydroxycinnamate (Dey and Harborne, 1989; Pouchert and Behnke, 1993), *p*-hydroxybenzaldehyde (Pouchert and Behnke, 1993), and tricin (Agrawal, 1989; Ulubelen *et al.*, 1984), respectively, by analysis of their NMR data and comparison of their physical and spectral data with those of literature values.

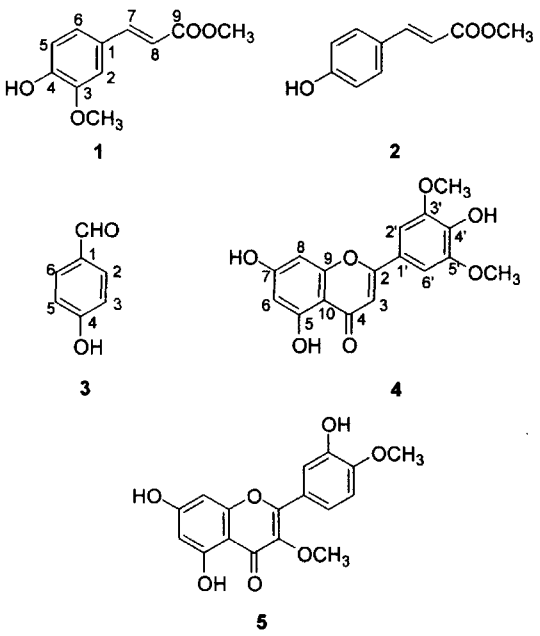

Fig. 1. Structures of compounds **1-5**

Compound **5** was shown to be a quercetin derivative from UV spectrum and the bathochromic shift resulting from the addition of NaOAc and $AlCl_3$ indicated the presence of free hydroxyl groups at 5 and 7 positions (Mabry *et al.*, 1970). The ^{1}H-NMR spectrum of **5** exhibited signals due to the H-6 and H-8 position of the flavone ring at δ6.65 (1H, d, J = 2.0 Hz) and δ6.30 (1H, d, J = 2.0 Hz), respectively, H-2' position at δ7.12 (1H, d, J = 1.8 Hz), the H-5' position at δ7.05 (1H, d, J = 8.0 Hz), the H-6' position at δ7.05 (1H, dd, J = 1.8 and 8.0 Hz) and the two methoxyl groups at δ3.95 and δ3.82. From a comparison of these results with the values previously reported in the literature (Agrawal, 1989; Alberto Marco *et al.*, 1986), compound **5** was identified as quercetin 3,4'-dimethylether. Among these compounds, the antioxidant activities of *p*-hydroxybenzaldehyde (**3**) and tricin (**4**) were previously reported using antilipid peroxidation assay (Ha *et al.*, 2000) and DPPH free radical scavenging method (Kwon *et al.*, 2002), respectively.

Methyl ferulate (**1**) exhibited a potent, free radical scavenging effect with an IC_{50} value of 0.7 μM, whereas *p*-hydroxybenzaldehyde (**3**), tricin (**4**) and quercetin 3,4'-dimethylether (**5**) showed weak DPPH radical scavenging effects with IC_{50} values of 3.3, 2.5 and 3.4 μM, respectively. The reason for the weak antioxidant activities of **4** and **5** was considered to be the lack of a catechol moiety at the B-ring of the flavonoid skeleton (Rafat Husain *et al.*, 1987; Torel *et al.*, 1986). Methyl *p*-hydroxycinnamate (**2**) was inactive in the antioxidant assay system using DPPH radical scavenging activity as shown in Table I.

Table I. DPPH radical scavenging activities of the isolated compounds

Compounds	IC_{50}[a] (μM)
Methyl ferulate (**1**)	0.7
Methyl *p*-hydroxycinnamate (**2**)	>26.9
p-Hydroxybenzaldehyde (**3**)	3.3
Tricin (**4**)	2.5
Quercetin 3,4'-dimethyl ether (**5**)	3.4
BHT*	0.6

*Used as a positive control.
[a]Concentration giving a 50% decrease of DPPH radical. The values are the means of triplicate experiments.

Table II. Effects of the isolated compounds on free radical-induced lipid peroxidation in the liver homogenates

Compounds	IC_{50}[a] (μM)
Methyl ferulate (**1**)	0.5
Methyl *p*-hydroxycinnamate (**2**)	0.4
p-Hydroxybenzaldehyde (**3**)	0.5
Tricin (**4**)	0.3
Quercetin 3,4'-dimethyl ether (**5**)	0.3
BHT*	0.2

[a]Concentration giving a 50% inhibition of lipid peroxidation. The values are the means of triplicate experiments.
*Used as a positive control.

We further studied the effects of these compounds on the lipid peroxidation in rat liver microsomes induced by non-enzymatic method. Among the tested compounds, methyl ferulate (**1**), methyl *p*-hydroxycinnamate (**2**) and *p*-hydroxybenzaldehyde (**3**) exhibited moderate inhibiton, while tricin (**4**) and quercetin 3, 4'-dimethyl ester (**5**) exhibited potent inhibition, on ascorbic acid/Fe^{2+} induced lipid peroxidation in rat liver microsomes with IC_{50} values of 0.5, 0.4, 0.5, 0.3 and 0.3 μM, respectively (Table II).

In conclusion, methyl ferulate (**1**) exhibited significant, free radical scavenging activity, and tricin (**4**) and quercetin 3, 4'-dimethyl ester (**5**) exhibited anti-lipid peroxidation activity in the present study.

REFERENCES

Agrawal, P. K., Carbon-13 NMR of Flavonoids. Elsevier (1989).

Alberto Marco, J. O. B., Sanz, F. J., and Sanchez-Parareda, J., 3-Methoxyflavones and coumarins from *Artemisia incnescens*. *Phytochemistry*, 25, 2357-2360 (1986).

Avato, P., Bianchi, G., and Murelli, C., Aliphatic and cyclic lipid components of sorghum plant organs. *Phytochemistry*, 29, 1073-1078 (1990).

Dey, P. M. and Harborne, B J., Methods in plant biochemistry, Vol. 1. Plant phenolics, Academic press, San Diego, pp. 75-112 (1989).

Gujer, R., Magnolato, D., and Self R., Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry*, 25, 1431-1436 (1986).

Ha, J. H., Lee, D. U., Lee, J. T., Kim, J. S., Yong, C. S., Kim, J. A., Ha, J. S., and Huh, K., 4-Hydroxybenzaldehyde from *Gastrodia elata* B1. is active in the antioxidation and GABAergic neuromodulation of the rat brain. *J. Ethnopharmacology*, 73, 329-333 (2000).

Haskins, F. A. and Gorz, H. J., Is *p*-hydroxybenzaldehyde a major constituent of epicuticular wax from *Sorghum bicolor* seedlings?. *Phytochemistry*, 22, 611-612 (1983).

Joshi, C. K., Sharma, K. A., and Singh, P., A new ferulic ester from *Tecomella undulata*. *Planta Medica*, 52, 71-72 (1986).

Kim, C. M., Sin, M. K., Lee, K. S., and Ahn, D. K., The dictionary of Chinese medicines, Jungdam press, Seoul, p. 317 (1997).

Kwon, Y. S., Kim, E. Y., Kim, W. J., Kim, W. K., and Kim, C. M., Antioxidant constituents from *Setaria viridis*. *Arch. Pharm. Res.*, 25, 300-305 (2002).

Mabry, T. J., Markham, K. R., and Thomas, M. B., The systematic identification of flavonoids, Springer-Verlag, Berlin (1970).

Maxwell, A. G., Masato, Y., and Yoko, A., Free radical scavenging action of medicinal herbs from Ghana Thonningia sanguinea on experimentally-induced liver injuries. *General Pharmacol.*, 32, 661-667 (1999).

Na, M. K., An R. B., Lee, S. M., Hong, N. D., Yoo, J. K., Lee, C. B., Kim, J. P., and Bae, K, H., Screening of crude drugs for antioxidative activity. *Kor. J. Pharmacogn.*, 32, 108-115 (2001)

Nanjing University of TCM (edited), *Chinese Herb Encyclopedia (Zhonghua Bencao)*, vol. 8, Shanghai Science & Technology Press, Shanghai, pp. 424-425 (1999).

Nguyen, T. T. H., Matsumoto, K., Kasai, R., Yamasaki, K., and Watanabe, H., *In vitro* antioxidant activity of Vietnamese ginseng saponin and its components. *Biol. Pharm. Bull.*, 21, 978-981 (1998).

Pouchert, J. C. and Behnke J., The Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra vol. 2, Aldrich Chemical Company, Inc. (1993).

Rafat Husain, S., Cillard, J., and Cillard, P., Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry*, 26, 2489-2491 (1987).

Rimando, M. A., Dayan, E. F., Czarnota, A. M., Weston, A. L., and Duke, O. S., A new photosystem II electron transfer inhibitor from *Sorghum bicolor*. *J. Nat. Prod.*, 61, 927-930 (1998).

Selmar, D., Irandoost, Z., and Wray, V., Dhurrin-6'-glucoside, A cyanogenic diglucoside from *Sorghum bicolor*. *Phytochemistry*, 43, 569-572 (1996).

Torel, J., Cillard, J., and Cillard, P., Antioxidant activity of flavonoids and reactivity with peroxy radical. *Phytochemistry*,

25, 383-385 (1987).

Ulubelen, A., Mabry, T. J., Dellamonica, G., and Chopin, J., Flavonoids from *Massiflora palmeri*. *J. Nat. Prod.*, 47, 384-385 (1984).

Wandji, J., Nkengfack, E. A., Tanee Fomum, Z., Ubillas, R., Brian Killday, K., and Tempesta, S. M., A new prenylated isoflavonoid and long chain esters from two *Erythrina* species. *J. Nat. Prod.*, 53, 1425-1429 (1990).

Woodhead, S., Galeffi, C., and Marini Betolo, G. B., *p*-Hydroxybenzaldehyde as a major constituent of the epicuticular wax of seedling *Sorghum bicolor*. *Phytochemistry*, 21, 455-456 (1982).

Yoshida, T., Hatano, K., Okumura, T., Uehara, I., Komagoe, K., Fujita, Y., and Okuda, T., Studies on inhibition mechanism of autoxidation by tannins and flavonoids. V. Radical scavenging effects of tannins and related polyphenols on 1, 1-diphenyl-2-picrylhydrazyl radical. *Chem. Pharm. Bull.*, 37, 1919-1921 (1989).