Natural Product Sciences
10(5) : 244-247 (2004)

Inhibitory Effects of Methanol Extract, Phenolic Acids and Flavonoids from the Leaves of *Eucalyptus darylmpleana* against 1,1-Diphenyl-2-picrylhydrazyl Radical

Jong Cheol Park[1]*, Ju Gwon Park[1], Jae Seoun Hur[2], Myeong Rak Choi[3], Eun Jeong Yoo[3], Sung Hwan Kim[4], Jin Chang Son[4], and Moon Sung Kim[5]

[1]*Department of Oriental Medicine Resources,*
[2]*Department of Environmental Education, Sunchon National University, Suncheon, Jeonnam 540-742, Republic of Korea*
[3]*Department of Biotechnology, Yosu National University, Yosu 550-749 Republic of Korea*
[4]*Kyongbuk Institute of Health and Environment, Daegu 702-702, Republic of Korea*
[5]*Dong-A Pharm. Co., Yongin 449-900, Republic of Korea*

Abstract – The inhibitory effects of the leaves of *Eucalyptus darylmpleana* (Myrtaceae) on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was examined. The scavenging effect of the ethyl acetate fraction of *Eucalyptus darylmpleana* leaves on DPPH radical was stronger than the other fractions, and further purified by silica gel and Sephadex LH-20 column chromatography. 3, 4-Dihydroxybenzoic acid, gallic acid, quercetin, quercetin 3-O-α-$_L$-rhamnoside, quercetin 3-O-β-$_D$-glucoside and quercetin 3-O-rutinoside were isolated and elucidated by spectroscopic data. Among these components, gallic acid and quercetin 3-O-α-$_L$-rhamnoside exhibited potent scavenging activities on DPPH radical with IC_{50} values of 6.02 and 5.54 μM, respectively.
Keywords – *Eucalyptus darylmpleana*, myrtaceae, DPPH, radical scavenging effect, gallic acid, quercetin 3-O-α-$_L$-rhamnoside

Introduction

Eucalyptus trees (Myrtaceae) are among the most important hardwood forestry crops world-wide and pro vide a major source of pulp wood for high quality paper production, leaf material for distillation of essential oils, timber for construction and fuel (Turnbull, 1991). In addition, the trees have been known to produce several natural substances having antagonistic activities against several pathogenic microorganisms (Mireku and Wilkes, 1988). Chemical components such as torquatone (Ghisalberti *et al.*, 1995), phenolic compounds (Conde *et al.*, 1995), tannins (Seikel and Hillis, 1970; Yoshida *et al.*, 1992; Cadahia *et al.*, 1996), terpenoid (Santos *et al.*, 1997) and polyphenolic compounds (Hillis and Yazaki, 1973), and antimicrobial (Muller-Riebau *et al.*, 1995; Hur *et al.*, 2000) and antioxidative activities (Lee *et al.*, 1998; Yun *et al.*, 2000) reported from *Eucalyptus* species. In this paper, we report the isolation of phenolic acids and flavonoids from the methanolic extract of *Eucalyptus darylmpleana* leaves and describe their inhibitory effects on DPPH radical.

*Author for correspondence
Fax: +82-61-750-3608; E-mail: flavonoid@empal.com

Material and Methods

Plant material and apparatus – Five-years-old trees of *Eucalyptus darylmpleana* were used for the in vestigation. Young leaves of the trees were collected in the experiment plots of Research Institute of Industrial Science and Technology located at Gwangyang, Jeonnam in August, 2001. The voucher specimen (No. NM0800) is deposited in department of oriental medicine resources, Sunchon National University and identified by Prof. J. S. Hur of Sunchon National University. The NMR spectra were recorded with a Brucher AMX-400 spectrometer containing TMS as an internal standard and chemical shifts were given as δ (ppm).

Extraction, fractionation and isolation – The dried and powdered leaves (5 kg) of *Eucalyptus darylmpleana* was refluxed with MeOH. This extract has been par titioned with organic solvents of the different polarities to afford dichloromethane (30 g), ethyl acetate (20 g), *n*-butanol (40 g) and aqueous (60 g) fractions, respectively.

The ethyl acetate fraction (20 g) was subjected to ch romatograph using silica gel with CH_2Cl_2-MeOH-H_2O (5:1:1, lower layer; 25:7:5, lower layer; 7:3:1, lower layer) and Sephadex LH-20 with MeOH-H_2O to give E-1~E-25 subfractions (volume of each tube: 40 mL, tubes 1-600). We isolated purely compound A (**1**, 110 mg) from subfraction E-3 (tubes 23-24) and compound B (**2**, 12 mg) from subfraction E-6 (tubes 78-87), compound C (**3**, 198 mg) from subfraction E-10 (tubes 146-179), com pound D (**4**, 196 mg) from subfraction E-15 (tubes 269-271), compound E (**5**, 12 mg) from subfractions E-17 (tubes 277-279) and compound F (**3**, 105 mg) from subfraction E-23 (tubes 511-518). And compound G (**1**, 7 mg) from subfraction B-10 (tubes 105-113), compound H (**3**, 35 mg) from subfraction B-14 (tubes 195-208) and compound I (**6**, 105 mg) from subfraction B-18 (tubes 342-358) were obtained by silica gel and Sephadex LH-20 column chromatography of *n*-BuOH soluble fraction (20 g) with the elution of CH_2Cl_2-MeOH-H_2O (7:3:1, lower layer; 65:35:10, lower layer) and MeOH-H_2O, respectively.

Compound 1 (quercetin) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.64 (1H, d, *J*=2.4 Hz, H-2'), 7.53 (1H, dd, *J*=2.4 & 8.4 Hz, H-6'), 6.85 (1H, d, *J*=8.4 Hz, H-5'), 6.38 (1H, d, *J*=2.0 Hz, H-8), 6.16 (1H, d, *J*=2.0 Hz, H-6); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 175.5 (C-4), 163.5 (C-7), 160.4 (C-5), 155.8 (C-9), 147.4 (C-4'), 146.5 (C-2), 144.8 (C-3'), 135.5 (C-3), 121.7 (C-1'), 119.8 (C-6'), 115.4 (C-5'), 114.6 (C-2'), 102.8 (C-10), 98.0 (C-6), 93.2 (C-8).

Compound 2 (3,4-dihydroxybenzoic acid) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.31 (1H, d, *J*=2.0 Hz, H-2), 7.26 (1H, dd, *J*=2.0 & 8.0 Hz, H-6), 6.77 (1H, d, *J*=8.0 Hz, H-5); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 167.1 (C-7), 149.8 (C-4), 144.7 (C-3), 121.8 (C-1), 121.5 (C-6), 116.4 (C-2), 115.0 (C-5).

Compound 3 (gallic acid) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 6.90 (2H, s, H-2 & 6); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 167.2 (C-7), 145.2 (C-3 & 5), 137.8 (C-4), 120.3 (C-1), 108.5 (C-2 & 6).

Compound 4 (quercetin 3-O-α-$_L$-rhamnoside) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.54 (1H, d, *J*=2.0 Hz, H-2'), 7.54 (2H, dd, *J*=2.0 & 8.4 Hz, H-6'), 6.81 (1H, d, *J*=8.4 Hz, H-5'), 6.38 (1H, d, *J*=1.9 Hz, H-8), 6.18 (1H, d, *J*=1.9 Hz, H-6), 5.24 (1H, *J*=1.4 Hz, anomeric H), 0.80 (3H, d, *J*=5.5 Hz, rha-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 178.2 (C-4), 164.8 (C-7), 161.8 (C-5), 157.8 (C-2), 157.0 (C-9), 149.0 (C-4'), 145.7 (C-3'), 134.8 (C-3), 121.7 (C-6'), 121.3 (C-1'), 116.3 (C-2'), 116.5 (C-5'), 104.7 (C-10), 102.4 (C-1''), 99.3 (C-6), 94.2 (C-8), 71.9 (C-4''), 71.3 (C-5''), 71.1 (C-3''), 70.8 (C-2''), 18.3 (C-6'').

Compound 5 (quercetin 3-O-β-$_D$-glucoside) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.26 (1H, d, *J*=2.0 Hz, H-2'), 7.26 (1H, dd, *J*=2.0 & 8.4 Hz, H-6'), 6.84 (1H, d, *J*=8.4 Hz, H-5'), 6.36 (1H, d, *J*=2.0 Hz, H-6), 6.18 (1H, d, *J*=2.0 Hz, H-8), 5.23 (1d, *J*=7.7 Hz, anomeric H); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 177.9 (C-4), 164.6 (C-7), 161.8 (C-5), 156.8 (C-2), 156.7 (C-9), 149.0 (C-4'), 145.3 (C-3'), 133.9 (C-3), 122.2 (C-6'), 121.8 (C-1'), 116.8 (C-5'), 115.8 (C-2'), 104.6 (C-10), 101.5 (C-1''), 99.3 (C-6), 94.2 (C-8), 78.3 (C-5''), 77.2 (C-3''), 74.8 (C-2''), 70.7 (C-4''), 61.7 (C-6'').

Compound 6 (quercetin 3-O-rutinoside) – ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.53 (1H, d, *J*=2.0 Hz, H-2'), 7.52 (2H, dd, *J*=2.0 & 8.4 Hz, H-6'), 6.81 (1H, d, *J*=8.4 Hz, H-5'), 6.36 (1H, d, *J*=2.0 Hz, H-8), 6.17 (1H, d, *J*=2.0 Hz, H-6), 5.32 (1d, *J*=7.2 Hz, anomeric H), 4.36 (1H, s, anomeric H), 0.98 (3H, d, *J*=6.0 Hz, rha-CH_3); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 176.9 (C-4), 163.7 (C-7), 160.9 (C-5), 156.2 (C-2), 156.1 (C-9), 148.1 (C-4'), 144.4 (C-3'), 133.0 (C-3), 121.3 (C-1'), 120.9 (C-6'), 116.0 (C-5'), 115.0 (C-2'), 103.7 (C-10), 101.0 (C-1''), 100.1 (C-1'''), 98.5 (C-6), 93.4 (C-8), 76.3 (C-3''), 75.8 (C-5''), 74.0 (C-2''), 71.7 (C-4'''), 70.5 (C-4''), 70.3 (C-2'''), 69.9 (C-3'''), 68.2 (C-5'''), 66.9 (C-6''), 17.8 (C-6''').

DPPH radical scavenging effect – One hundred microliter of MeOH solution of varying sample co nce ntrations (control: 100 μL MeOH) was added to an EtOH solution of DPPH (60 μM) in microwell, according to the method of Hatano *et al.* (1989). After being mixed gently and left for 30 min at room temperature, the DPPH radical level of each well was determined using a Microplate Reader. The antioxidant activity of each samples was expressed in terms of the IC_{50} value (concentration in μM required to inhibit DPPH radical fo rmation by 50%) determined from the log dose-inhibition curve. L-Ascorbic acid was used as positive control.

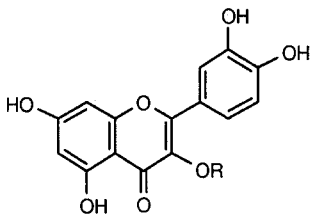
Result and Discussion

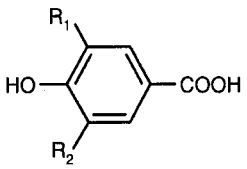
The methanol extract of the leaves of *Eucalyptus darylmpleana* was fractionated with dichloromethane, ethyl acetate and *n*-butanol successively. Column chro matography of ethyl acetate and *n*-butanol soluble fra ctions afforded six compounds.

The ^{1}H-NMR spectrum of compound 2 indicated the presence of aromatic signals of an ABX type at δ 6.77 (*J*=8.0 Hz), δ 7.26 (*J*=2.0 and 8.0 Hz) and δ 7.31 (*J*=2.0 Hz), respectively, assignable to H-5, H-6 and H-2. Its ^{13}C-NMR spectrum also showed the signals of two oxygen-bearing aromatic ring (δ 149.8, δ 144.7) and a ketone

group (δ 167.1). These data were expected that compound 2 is 3,4-dihydroxybenzoic acid. The identity with 3,4-dihydroxybenzoic acid was identified by comparison of ^{1}H- and ^{13}C-NMR spectra with those reported literature (Pouchert and Behnke, 1993).

The ^{1}H-NMR spectrum of compound **3** showed one singlet at δ 6.90 (2H) attributable to galloyl protons. A comparison of the ^{13}C-NMR spectrum of compound **2** with literature data (Park *et al.*, 1993) showed it to be gallic acid. Compound **1** were identified as a well known compound, quercetin by comparison of reported NMR data (Markham *et al.*, 1978). The ^{1}H-NMR spectra of compounds **4** and **5** showed one anomeric proton signal at δ 5.24 (J=1.4 Hz) and 5.23 (J=7.7 Hz), respectively. Two anomeric proton signals at δ 4.36 (s) and 5.32 (J=7.2 Hz) in the ^{1}H-NMR spectrum of compound **6** indicated that 2 mole of sugars was linked, one of which was assumed to be rhamnose by the diagnostic methyl signal at δ 0.98. The ^{1}H-NMR spectra of compounds **4-6** showed two meta-coupled doublets ascribable to H-8 and H-6 of A-ring in the flavonoid skeleton, and a meta-coupled doublet, an ortho, meta-coupled doublet-doublet and an ortho-coupled doublet attributable to H-2', H-6' and H-5' of B-ring, respectively. These data indicated that com pounds **4-6** were quercetin glycosides. The sugar moiety was attached to 3-hydroxyl group of aglycone by the ^{13}C-NMR analysis, which showed glycosylation shift for the carbon signals of C-2, C-3 and C-4, by the comparison with those of the genin reported in the literature. The sugar moieties of compounds **4-6** were determined to be α-$_{L}$-rhamnopyranose, β-$_{D}$-glucopyranose and rutinose, respectively, by the J values of the anomeric proton signals and the ^{13}C-NMR data. From the above results,





	R		R_1	R_2
1	H	2	OH	H
4	α-$_{L}$-rhamnose	3	OH	OH
5	β-$_{D}$-glucose			
6	rutinose			

Fig. 1. Chemical sttuctures isolated from the leaves of *Eucalyptus darylmpleana.* 1) quercetin; 2) 3,4-dihydroxybenzoic acid; 3) gallic acid; 4) quercetin-3-O-α-$_{L}$-rhamnoside; 5) quercetin 3-O-β-$_{D}$-glucoside; 6) quercetin 3-O-rutinoside.

compounds **4-6** were characterized as quercetin 3-O-α-$_{L}$-rhamnoside, quercetin 3-O-β-$_{D}$-glucoside and qu ercetin 3-O-rutinoside, respectively (Fig. 1).

The DPPH system is a stable radical-generating pro cedure. Free radicals are reactive molecules which occur normally in aerobic organisms and mediate a number of important biological processes. Physiologically, the fo rmation and elimination of free radicals are balanced by the antioxidant system in the body. However, when a dis turbance results in enhanced generation of free radicals and decreased antioxidant capacity, excess radicals can overwhelm the defence system and cause cell and tissue damage. Certain free-radical scavengers might reduce such damage. The inhibitory effects of the leaves of *Eucalyptus darylmpleana* on DPPH radical was examined. The MeOH extract of *Eucalyptus darylmpleana* leaves was fractionated into dichloromethane, ethyl acetate and *n*-butanol soluble fractions. As shown Table 1, the radical scavenging effects of the ethyl acetate fraction was stronger than the others. The IC_{50} value of this fraction was 3.69 μg/mL. This results suggest that the MeOH extract and ethyl acetate fraction of *Eucalyptus dary lmpleana* contain the effective radical scavengers. Therefore the ethyl acetate fraction having strong effect on DPPH radical, this was chromatographed by silica gel and Sephadex LH-20 to afford five compounds. Among these components, quercetin 3-O-α-$_{L}$-rhamnoside, gallic

Table 1. The radical scavenging effect of the extract and fractions of *Eucalyptus darylmpleana* on DPPH radical

Sample	IC_{50} (μg/mL)[a]
MeOH extract	7.10 ± 0.43
CH_2Cl_2 fraction	12.76 ± 0.16
EtOAc fraction	3.69 ± 0.07
n-BuOH fraction	8.00 ± 0.10

[a]Concentration giving a 50% decrease of DPPH radical.

Table 2. The radical scavenging effect of compounds isolated from the *Eucalyptus darylmpleana* on DPPH radical

Sample	IC_{50} (μM)[a]
compound 1	6.56 ± 0.05
compound 2	9.91 ± 0.38
compound 3	6.02 ± 0.51
compound 4	5.54 ± 0.05
compound 5	10.19 ± 0.21
compound 6	8.37 ± 0.13
L-ascorbic acid	8.04 ± 0.32

[a]Concentration giving a 50% decrease of DPPH radical.

acid, quercetin and quercetin 3-O-rutinoside exhibited high scavenging activities on DPPH radical with IC_{50} values of 5.54 μM, 6.02 μM, 6.56 μM and 8.37 μM, respectively (Table 2). Quercetin isolated from the stem bark of *Eucalyptus globulus* has been reported to have lipid peroxidation inhibitory activity (Lee *et al.*, 1998; Yun *et al.*, 2000). The radical scavenging effects of four components were comparable to that of L-ascorbic acid.

Acknowledgement

One of the authors (J.G.P.) wishes to thank Sunchon National University for providing financial support.

Reference

Cadahia, E., Conde, E., Garcia-Vallejo, M.C., and Fermandez de Simon, B., Gel permeation chromatographic study of the molecular weight distribution of tannins in the wood, bark and leaves of *Eucalyptus* spp. *Chromatographia* **42**, 95-100 (1996).

Conde, E., Cadahia, E., and Garcia-Vallejo, M.C., HPLC analysis of flavonoids and phenolic acids and aldehyde in *Eucalyptus* spp. *Chromatographia* **41**, 657-660 (1995).

Ghisalberti, E.L., Skelton, B.W., and White, A.H., Structure study of torquatone, an acylphloroglucinol derivative from *Eucalyptus* species. *Aust. J. Chem.* **48**, 1771-1774 (1995).

Hatano, T., Edamatsu, R., Hiramatsu, M, Mori, A., Fujita, Y., Yasuhara, T., Yoshida, T., and Okuda, T., Effects of the interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols and superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. *Chem. Pharm. Bull.* **37**, 2016-2021 (1989).

Hillis, W.E. and Yazaki, Y., Wood polyphenols of *Eucalyptus polyanthemos*. *Phytochemistry* **12**, 2969-2977 (1973).

Hur, J.S., Ahn, S.Y., Koh, Y.J., and Lee, C.I., Antimicrobial properties of cold-tolerant Eucalyptus species against phytopathogenic fungi and food-borne bacterial pathogens. *Plant Patho. J.* **16**, 286-289 (2000).

Lee, I.K., Yun, B.S., Kim, J.P., Chung, S.H., Shim, G.S., and Yoo, I.D., Antioxidative compounds isolated from the stem bark of *Eucalyptus globulus*. *Kor. J. Pharmacogn.* **29**, 163-168 (1998).

Markham, K.R., Ternai, B., Stanley, R., Geiger, H., and Mabry T.J., Carbon-13 nmr studies of flavonoids-III, Naturally occurring flavonoid glycosides and their acylated derivatives. *Tetrahedron* **34**, 1389-1397 (1978).

Mireku, E. and Wilkes, J., Production of phenols in the sapwood of *Eucalyptus maculata* after wounding and infection. *Eur. J. For. Pathol.* **18**, 121-127 (1988).

Muller-Riebau, F., Berger, B., and Yegen, O., Chemical composition and fungitoxic prosperties to phytopathogenic fungi of essential oils of selectied aromatic plants growing wild in Turkey. *J. Agric. Food Chem.* **43**, 2262-2266 (1995).

Park, J.C., Young, H.S., Yu, Y.B., and Lee, S.H., Studies on the Chemical Components and Biological Activities of Edible Plants in Korea(1) : Phenolic compounds from the leaves of *Cedrela sinensis* A. Juss. *Yakhak Hoeji* **37**, 306-310 (1993).

Pouchert, C.J. and Behnke, J., The Aldrich Library of ^{13}C and ^{1}H-NMR Spectra. Vol. 2, Aldrich, 1993, p.1116.

Santos, G.G., Alves, J.C.N., Rodilla, J.M.L., Duarte, A.P., Lithgow, A.M., and Urones, J.G., Terpenoids and other constituents of *Eucalyptus globulus*. *Phytochemistry* **44**, 1309-1312 (1997).

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

Turnbull, J.W., Future use of Eucalyptus: opportunities and problem. In: IUFRO symposium on Intensive Forestry: the role of eucalytus. Vol. I., Durban, South Africa, 1991, pp. 2-29.

Yoshida, T., Maruyama, T., Nitta, A., and Okuda, T., Eucalbanine A, B and C, monomeric and dimeric hydrolyzable tannins from *Eucalyptus alba*. *Chem. Pharm. Bull.* **40**, 1750-1754 (1992).

Yun, B.S., Lee, I.K., Kim, J.P., Chung, S.H., Shim, G.S., and You, I.D., Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globus*. *Arch. Pharm. Res.* **23**, 147-150 (2000)

(Accepted October 3, 2004)