Natural Product Sciences
7(1) : 1~4 (2001)

(+)-Catechin, an Antioxidant Principle from the Leaves of *Pinus densiflora* that Acts on 1,1-Diphenyl-2-picrylhydrazyl Radical

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Abstract – The antioxidant activity of *Pinus densiflora* was determined by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The methanol extract of *P. densiflora* showed strong antioxidant activity, and thus fractionated with several solvents. The antioxidant activity potential of the individual fraction was in the order of ethyl acetate>n-butanol>water> dichloromethane> *n*-hexane fraction. The ethyl acetate soluble fraction exhibiting strong antioxidant activity was further purified by repeated silica gel column chromatography. Antioxidant (+)-catechin was isolated as one of the active principles from the ethyl acetate fraction, together with the inactive components, dihydrokaempferol and 1-*O*-benzoylglucose. The radical scavenging effect of (+)-catechin on DPPH radical exceeded to that of L-ascorbic acid which is a well known antioxidant.
Key words – *Pinus densiflora* Sieb. et Zucc., Pinaceae, (+)-catechin, antioxidant activity, DPPH radical

Introduction

Red pine (*Pinus densiflora* Sieb. et Zucc., Pinaceae) grows naturally or is planted in mountain regions of Korea, Japan and China. The leaves of red pine have long been used as a nourishing tonic drug in Korean folk medicines. The pine leaves are frequently used to brew a tea in Korea. We have previously reported that the ethanolic extract of the leaves of *P. densiflora* possess anti-aging effects on reactive oxygen radicals in animal experiments [Choi *et al.*, 1997, 1998a, 1998b, 1999a, 1999b]. The extensive human consumption in the form of tea in Korea and its exceptional anti-aging effects in our animal experiments prompted us to examine its further effect on antioxidant activity and to isolate its active principles. In this paper, we now report isolation and structure elucidation of the components from the methanolic extract of *P. densiflora* and describes their antioxidant effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Experimental

Instruments – Melting points are uncorrected. The UV spectrum was taken with a Shimadzu 202 UV spectrophotometer in MeOH solution and the IR spectrum on a JASCO IR-2 spectrometer in KBr disc. EIMS was taken on a Hewlett-Packard 5985B GC/MS spectrometer operating at 70 eV. ^{1}H- and ^{13}C-NMR spectra were taken with a Jeol JNM-ECP 400 spectrometer. The chemical shifts were referenced to residual solvent peaks (2.5 ppm in ^{1}H-NMR, 39.5 ppm in ^{13}C-NMR), and were recorded in δ values. Multiplicities of ^{1}H- and ^{13}C-NMR signals are indicated as s (singlet), d (doublet), and t (triplet). Column chromatography was done with silica gel (Merck, 70-230 mesh). TLC was carried out on precoated Merck Kieselgel 60 F_{254} plate (0.25 mm) and spots were detected under UV light using 50% H_2SO_4 reagent.

Plant material – The leaves of *P. densiflora* was purchased from a commercial supplier, in 1999 and authenticated by Prof. J. S. Choi of the Faculty of Food Science and Biotechnology, Pukyong National University. A voucher specimen has been deposited in the laboratory of Prof. J. S. Choi.

Extraction, fractionation and isolation – The powdered leaves (8.5 kg) of *P. densiflora* was refluxed with MeOH for three hr. (9L×3). The total filtrate was concentrated to dryness *in vaccuo* at 40°C to render the MeOH extract (2.1 kg), and this extract was suspended in distilled H_2O and partitioned with

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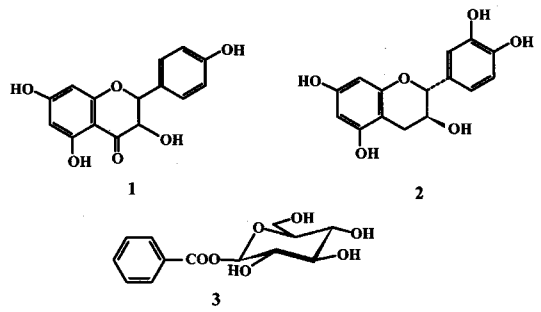

Fig. 1. Structures of compounds **1-3.**

n-Hexane (450 g), CH_2Cl_2 (150 g), EtOAc (200 g), *n*-BuOH (450 g) and H_2O (750 g) in sequence. The EtOAc fraction showed strong scavenging activity against DPPH radicals; Thus, the EtOAc (100g) fraction was chromatographed on a Si gel column using EtOAc-MeOH (gradient) as solvent to yield 8 subfractions. The fraction 5 (12 g) was further chromatographed on a Si gel column eluting with CH_2Cl_2-MeOH (5:1) to give dihydrokaempferol (**1**, 24 mg) and D-catechin (**2**, 3.2 g). The fraction 6 (2.5 g) was chromatographed on a Si gel column eluting with CH_2Cl_2-MeOH (10:1) to give 1-*O*-benzoylglucoside (**3**, 50 mg). The chemical structures of these compounds are shown in Fig. 1.

Compound 1 – Colorless needles (MeOH), mp 225~6°C, $[\alpha]_D^{24}$ = +18.0°(c 0.12, MeOH). $FeCl_3$; positive. UV λ_{max}^{MeOH} (log ε) nm 292 (4.65), 329 (sh., 4.03), ^{1}H-NMR (DMSO-d_6 + D_2O, 400 MHz) δ : 4.55 (1H, d, J=11.4 Hz, H-3), 5.02 (1H, d, J=11.4 Hz, H-2), 5.85 (1H, d, J=2.0 Hz, H-6), 5.91 (1H, d, J=2.0 Hz, H-8), 6.78 (2H, d, J=8.5 Hz, H-3' & 4'), 7.31 (2H, d, J=8.5 Hz, H-2' & 6'), 11.5 (1H, s, OH). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ : 83.4 (C-2), 72.0 (C-3), 198.1 (C-4), 163.5 (C-5), 96.5 (C-6), 167.3 (C-7), 95.6 (C-8), 163.1 (C-9), 100.9 (C-10), 128.1 (C-1'), 130.0 (C-2'), 115.4 (C-3'), 158.0 (C-4'), 115.4 (C-5'), 130.0 (C-6').

Compound 2 – Colorless needles (Aqueous-MeOH), mp 174~7°C, $[\alpha]_D^{24}$ = + 30°(c 0.12, MeOH). $FeCl_3$; positive. UV λ_{max}^{MeOH} (log ε) nm 281 (3.51). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ : 2.51 (2H, ddd, J=16.1, 8.1, 5.4 Hz, H-4), 5.69 (1H, d, J=2.2 Hz, H-6), 5.89 (1H, d, J=2.2 Hz, H-8), 6.60 (1H, dd, J=8.1, 2.0 Hz, H-6'), 6.69 (1H, d, J=8.1 Hz, H-5'), 6.73 (1H, d, J=2.0 Hz, H-2'). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ : 80.7 (C-2), 66.0 (C-3), 27.5 (C-4), 155.9 (C-5), 93.8 (C-6), 156.1 (C-7), 94.9 (C-8), 155.2 (C-9), 99.0 (C-10), 130.5 (C-1'), 114.3 (C-2'), 144.5 (C-3'), 144.5 (C-4'), 115.0 (C-5'), 118.4 (C-6').

Compound 3 – Amorphous powder, IR bands (KBr) 3,427, 3,312, 1,716, 1,600, 1,583, 1,078 cm^{-1}, HREIMS (70 eV) *m/z* $[M]^+$ absent, 122.0374 (calculated for $C_7H_6O_2$ 122.0368 ($[M-C_6H_{10}O_5]^+$, 88), ^{1}H-NMR (DMSO-d_6, 400 MHz) δ : 5.73 (1H, d, J=7.87 Hz, H-1'), 7.49 (2H, dd, J=7.5 & 1.5 Hz, H-3 & 5), 7.62 (1H, t, J=7.5 Hz, H-4), 8.09 (2H, d, J=7.5 Hz, H-2 & 6), ^{13}C-NMR (DMSO-d_6, 100 MHz) δ : 130.76 (C-1), 130.89 (C-2), 129.59 (C-3), 134.71 (C-4), 166.73 (COO), 96.25 (C-1'), 78.86 (C-3'), 78.03 (C-5'), 74.01 (C-2'), 71.02 (C-4'), 62.28 (C-6').

DPPH radical scavenging effect – Evaluation on the DPPH radical scavenging effect was carried out according to the method first employed by M. S. Blois (Blois, 1958). Four milliliters of MeOH solution of varying sample concentrations was added to 1.0 ml DPPH methanol solution (1.5×10^{-4} M). After standing at room temperature for 30 min., the absorbance of this solution was determined at 520 nm using a spectrophotometer and the remaining DPPH was calculated. The results were calculated by taking the mean of all triplicate values.

Results and Discussion

Structures of isolated compounds – Column chromatography on silica gel of the ethyl acetate soluble fraction of the methanolic extract furnished compounds **1-3** in the order of increasing polarity, respectively.

Compound **1**, mp 225~6°C, $[\alpha]_D^{24}$ = +18.0°, showed hydroxyl (3500~3000 cm^{-1}) and α, β-unsaturated C = O (1600 cm^{-1}) absorptions in its IR spectrum, and UV absorptions at 292 and 329 nm (sh) which suggested the presence of a flavanonol skeleton [Mabry *et al.*, 1970]. It was clear from the ^{13}C-NMR spectrum [signals at 72.0 ppm (C-3), 83.4 (C-2) and 198.1 ppm (C-4)] that **1** was a flavanonol [Agrawal, 1989]. The ^{1}H-NMR spectral data also justified the assignment of a flavanonol skeleton for **1**, which was substantiated by two one proton doublet (each, *J*=11.4 Hz) signals at δ4.55 and 5.02 ascribable to H-3 and H-4. In the aromatic region of the ^{1}H-NMR spectrum of **1**, two signals at δ5.85 (1H, d, *J*=2 Hz) and 5.91 (1H, d, *J*=2 Hz) was assigned to H-6 and H-8 on the A-ring of a flavanonol skeleton, and two doublet signals of an A_2B_2 system at δ6.78 (2H, *J*=8.6 Hz, H-3',5') and 7.31 (2H, *J*=8.6 Hz, H-2',6') revealed the presence of a 4'-hydroxylated ring B. The C=O signal at δ198.1

in the ^{13}C-NMR spectrum as well as appearence of the chelated OH group in a lower field in the ^{1}H-NMR spectrum supported the presence of a hydroxyl group at C-5. The MS spectrum showed a molecular ion at *m/z* 288 (21.2%) and other fragment peaks at *m/z* 136 (RDA fragment with B ring, 23.2), 153 (RDA fragment with A ring H, 100) and 107 (136-CHO, 43.2). These spectral data were in agreement with those for the structure of dihydrokaempferol [Choi *et al.*, 1990]. It was further identified by direct comparisons with an authentic sample (mmp, co-TLC and ^{1}H-& ^{13}C-NMR).

Compound 2, mp 174~8°, [α] +30°, showed positive $FeCl_3$ test (green) and hydroxyl (3300 cm^{-1}), aromatic ring (1630 and 1525 cm^{-1}) absorptions in its IR spectrum, and a single absorption peak characteristic of a catechin at 281.5 nm in its UV spectrum. The MS spectrum showed a molecular ion at *m/z* 290 (21.1%) and other fragment peaks at *m/z* 152 (RDA fragment with B ring, 43.4), 139 (RDA fragment with A ring H, 100), 123 (152-CHO, 69.7) and 109 (123-CO, 8.0). The ^{1}H-NMR spectrum showed the two signals at 5.89 (1H, d, *J*=2 Hz) and 5.69 (1H, d, *J*=2 Hz) ascribable to H-8 and H-6 on the A-ring of a flavan skeleton and the signals at 6.72 (1H, d, *J*=2 Hz), 6.69 (1H, d, *J*=8 Hz) and 6.60 (1H, dd, *J*=8 and 2 Hz) assignable to the protons of a 1,3,4-trisubstituted benzene ring. The signals at 4.48 (1H, d, *J*=7 Hz, H-2), 3.88~3.75 (1H, m, H-3), 2.51 (2H, ABX type, *J*=16.0, 8.0 and 5.5 Hz, H_2-4) suggested the presence of a catechin moiety. These spectral data were in agreement with those for the structure of (+)-catechin. It was further identified by direct comparisons with an authentic sample (mmp, co-TLC and ^{1}H-& ^{13}C-NMR).

Compound 3, showed showed the three signals at δ7.49 (2H, dd, J=7.5 & 1.5 Hz, H-3 & 5), 7.62 (1H, t, J=7.5 Hz, H-4) and 8.09 (2H, d, J=7.5 Hz, H-2 & 6) assignable to the protons of a 1,4-disubstituted benzene ring in the ^{1}H-NMR spectrum. The presence of an anomeric proton at δ5.73 (1H, d, J=7.87 Hz) in the ^{1}H NMR spectrum and the presence of six signals in the ^{13}C NMR spectrum at δ62.28 (C-6), 71.02 (C-4), 74.01 (C-2), 78.03 (C-5), 78.86 (C-3), and 96.23 (C-1) implied that **3** contained β-glucose moiety. The ^{13}C-NMR spectrum also showed a carbonyl carbone (δ166.73). Hydrolysis of **3** yielded benzoic acid and one mole of glucose. These results indicated that **3** is a benzoylglucoside. The configuration and conformation of D-glucose moiety was determined to be β-glucopyranose not only by the *J* value of the anomeric proton signal, but also by comparison of the ^{13}C-NMR data with those of corresponding methyl α-D- and β-D-glycosides. The glycosidic linkage site of β-D-glucopyranose was determined to be C-1 based on the HMBC and HMQC data analysis. In the HMBC spectrum, a correlation between the anomeric proton of the glucose moiety and benzoyl carbonyl carbon was detected. On the basis of these results, the structure of **3** was established as 1-*O*-benzoyl-β-D-glucopyranoside.

Radical scavenging effect of the methanol extract and their fractions of *P. densiflora* on DPPH radical – Active oxygen species such as superoxide radicals, hydrogen peroxide and hydrogen radicals has been recognized as the principle agent responsible for the deterioration of polyunsaturated fatty acids, or lipid containing foods when exposed to air (Slater *et al.*, 1987). The DPPH stable radical loses its characteristic purple color when supplied with electrons or hydrogen ions. The capacity of the tested substances to donate electrons can be estimated from the degree of their loss of color. The DPPH radical scavenging effect for the methanol extract and their fractions are shown in Table 1.

As can be seen in Table 1, a MeOH extract of *P. densiflora* showed a potent radical scavenging activity on DPPH radicals at a concentration of 32.54 μg/ml and was further fractionated into *n*-Hexane- CH_2Cl_2-, EtOAc-, *n*-BuOH-, and H_2O-soluble fractions. These fractions were also assayed for DPPH radical scavenging activity. Among them, the EtOAc-fraction showed a significant scavenging activity on DPPH radical at concentration of 13.24 μg/ml. Therefore, the EtOAc-soluble fraction was subjected to further chemical analysis and, after column chromatographic separation, the three compounds (**1, 2 & 3**) were isolated.

The radical scavenging effect of isolated components on DPPH radical – The radical scavenging

Table 1. The radical scavenging effect of the MeOH extract and its subsequent fractions of *Pinus densiflora* leaves on DPPH radical

Samples	SC_{50} (μg/ml)[a]
MeOH ext.	32.54
Hexane fr.	>480
CH_2Cl_2 fr.	45.44
EtOAc fr.	13.24
n-BuOH fr.	24.26
H_2O fr.	25.07
L-ascorbic acid	9.11

a : 50% scavenging concentration.

Table 2. The radical scavenging effect of compounds **1-3** isolated from EtOAc fraction on DPPH radical

Samples	SC_{50} (μM)[a]
Compound **1** (dihydrokaempferol)	> 80.0
Compound **2** ((+)-catechin)	>4.9
Compound **3** (1-*O*-benzoylglucoside)	>80.0
L-ascorbic acid	8.1

a : 50% scavenging concentration.

effect of isolated components from the ethyl acetate fraction of the methanol extract of *P. densiflora* was shown in Table 2. Among three isolated compounds, (+)-catechin exhibited higher scavenging activity on DPPH with IC_{50} of 4.9 μM. The antioxidant activity of catechin was two-fold more potent than that of L-ascorbic acid, which is well known antioxidant. However, compounds 2 and 3 were found to be inactive even at the higher concentration. These results suggest that the radical scavenging effect in the original methanol extract of *P. densiflora* was partially attributable to (+)-catechin.

(+)-Catechin was reported as an antioxidant from various plants. As (+)-catechin have an catechol moiety, the antioxidative potency of this compound may be attributable to this moiety. The antioxidative potency of phenolic acids are inter-related. These compounds react with the free radicals formed during autoxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992). The higher radical scavenging property of catechol phenolic acids is probably due to a superior stability of radical derived from catechol compared to that of phenoxyl radical (Ruiz-Larrea *et al.*, 1994). The present work indicate that (+)-catechin may be useful for the treatment of oxidative damage.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-98-MS-0001).

References

Agrawal, P. K., *Carbon-13 NMR of flavonoids*. Elsevier. Amsterdam. 1989.

Blois, M. S., Antioxidant determinations by the use of a stable free radical. *Nature* **26**, 1199-1200 (1958).

Choi, J. H., Kim, D. W., Kim, J. H., Kim, K. S. and Lee, J. S., Effect of pine needle extract (PNE) on physiological activity of SD rats I. Feeding effect of PNE on lipid and oxygen radical metabolisms in serum of SD rats. *Korean J. Life Sci.* **7**(4), 371-376 (1997).

Choi, J. H., Kim, J. H., Kim, D. W., Kim, K. S. and Lee, J. S., Effect of pine needle extract (PNE) on physiological activity of SD rats. II. Feeding effect of PNE on oxygen radicals and their scavenger enzymes in brain membranes of SD rats. *Korean J. Life Sci.* **8**(1), 91-96 (1998a).

Choi, J. H., Kim, J. H., Kim, D. W., Hwang, C. H. and Lee, J. S., Effect of pine needle extract (PNE) on physiological activity of SD rats. III. Feeding effect of PNE on fluidity and neurotransmitter-related enzymes in brain membranes of SD rats. *Korean J. Life Sci.* **8**(2), 167-172 (1998b).

Choi, J. H., Kim, D. I., Park, S. H., Kim, D. W., Lee, J. S. and Kim, S. H., Investigation of anti- aging effect and determination of chemical structures of pine needle extract (PNE) through the animal experiments . Effects of PNE on oxygen radicals and their scavenger enzymes in liver of SD rats. *Korean J. Life Sci.* **9**(4), 466-472 (1999a).

Choi, J. H., Kim, D. I., Park, S. H., Kim, D. W., Lee, J. S. and Kim, H. S., Investigation of anti- aging effect and determination of chemical structure of pine needle extract (PNE) through animal experiments II. Effects of PNE on membrane fluidity and oxidative stress in liver of SD rats. *Korean J. Life Sci.* **9**(4), 473-480 (1999b).

Choi, J. S., Woo, W. S., Young, H. S. and Park, J. H., Phytochemical study on *Prunus davidiana*. *Arch. Pharm. Res.* **13**(4), 374-378 (1990).

Cuvelier, M-E., Richard, H. and Berset, C., Comparison of the antioxidative activity of some acid-phenols: Structure-activity relationship. *Biosci. Biotech. Biochem.* **56**(2), 324-325 (1992).

Mabry, T. J., Markham, K. R. and Thomas, M. B., *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin, 1970.

Ruiz-Larrea, M. B., Leal, A. M., Liza, M., Lacort, M. and De Groot, H., Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. *Steroids* **59**, 383-388 (1994).

Slater, T. F., Cheeseman, K. H., Davies, M. J., Proudfoot, K. and Xin, W., *Proc. Nutr. Soc.* **46**, 1 (1987).

(Received November 24, 2000)