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# Anti-oxidative Activities of Phenolic Compounds from barks of *Pinus densiflora* Siebold et Zuccarini

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**Abstract** – Phytochemical examination of the barks of *Pinus densiflora* Siebold et Zuccarini has led to the isolation of one phenylpropanoid, one lignan, one flavonoid, one flavan 3-ol and two procyanidins : 4-*O*-β-D-glucopyranosyl- *p*-coumaric acid (**1**), 2,3-dihydro-2-(4-methoxy)-7-hydroxy-3-hydroxymethyl-5-(3-hydroxy propyl)-benzofuran 3-*O*-α-D-glucopyranoside (**2**), taxifolin 3'-*O*-β-D-glucopyranoside (**3**), (+)-catechin (**4**), procyanidin B1 (**5**) and epicatechin-(4β-8)-catechin-(4α-8)-catechin (**6**). Among them, Compound **4**, **5** and **6** showed potent anti-oxidative activities and these anti-oxidative activities were significantly different compared with ascorbic acid as positive control.

**Keywords** – *Pinus densiflora*, antioxidative activity, lignan, flavonoid, procyanidin, DPPH

## Introduction

The barks of *Pinus densiflora* Siebold et Zuccarini have been used for Korean traditional medicine as the remedies for bleeding, rheumatitis, bruise and scald, etc. From the barks of *Pinus densiflora* several phenoic compounds, icariside E4 (Miyase *et al*., 1989), (+)-isolariciresinol 9'-*O*-*β*-D-xylopyranoside, and (+) isolariciresinol (Hans *et al*., 1992), quercetin 3-*O*-*β*-D-glucopyranoside (Lee and Bae, 2001), 5,7,8,4'-tetrahydroxy-3-methoxy-6-methylflavone 8-*O*-*β*-D-glucopyranoside (Jung *et al*., 2001), and shikimic acid (Ishimaru *et al*., 2002), dihydroquercetin-3'-*O*-*β*-galactoside (Kim *et al*., 1991), (+)-catechin, catechin-(4*α*-6)-catechin, epicatechin-(4*β*-6)-catechin and catechin-(4*α*-6)-catechin-(4*β*-6)-catechin (Song and Oh, 1996) were isolated.

Pycnogenol which is polyphenols containing extract from the barks of *Pinus maritima* was known to have anti-inflammatory activities including inhibitory effect of NO production (Fabio *et al*., 1998), inhibition of inflammatory mediator from macrophage (Erben *et al,* 2000), inhibition of interlukin-1, proinflammatory cytokine (Cho *et al*., 2000) and anti-oxidative effect (Packer *et al*., 1999). As parts of our continuing search for new anti-oxidative and anti-inflammatory agents from natural sources, we isolated one phenylpropanoid, one lignan, one flavonoid, one flavan 3-ol and two procyanidins from the barks of *Pinus densiflora* and evaluated their anti-oxidative activities.

## Experimental

**General Experimental Procedures** – The NMR spectra were recorded on Varian Gemini 2000, 300 MHz (USA) and Bruker AMX-500, 500 MHz (Germany) spectrometer, using internal standard pulse program, with chemical shifts reported in ppm downfield from TMS. Column chromatography was carried out on Sephadex LH-20 (25 - 100 μm, Pharmacia, Sweden), MCI-gel CHP20P (75~150 μm, Mitsubishi, Japan) and YMC-gel ODS-A (230/70, 400/230, 500/400 mesh, YMC, Japan). TLC was performed on aluminum plates pre-coated with Kieselgel 60F254 (Merck). All other chemicals and solvents were of analytical grade.

**Plant Material** – The barks were prepared from the *Pinus densiflora* collected from Huk-seuk Dong, Seoul, South Korea, on September, 2008). A voucher specimen (PD2008) was deposited at the herbarium of the College of Pharmacy, Chung-Ang University.

**Extraction and Isolation** – The fresh of barks of *Pinus densiflora* (1.5 kg) were extracted three times with 80% aqueous acetone at room temperature. After removal of $Me_2CO$ in vacuo, the aqueous solution was filtered. The filtrate was concentrated and then applied to a column of Sephadex LH-20. Elution with $H_2O$ containing increasing proportion of MeOH afforded 5 fractions. Repeated

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column chromatography of fraction 2, MCI gel CHP 20P with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100) and low pressure liquid column chromatography yielded **2** (200 mg). Repeated column chromatography of fraction 3, low pressure liquid column chromatography with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100) and MCI gel with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100) yielded **1** (40 mg). Repeated column chromatography fraction 4, followed by MCI with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100) yielded **3** (1.5 g). Repeated column chromatography fraction 5, followed by MCI-gel with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100), and followed by low pressure liquid column chromatography with a gradient solvent system of $H_2O$ : MeOH (100 : 0 → 0 : 100), (90 : 10 → 50 : 50) yielded **4** (300 mg), **5** (150 mg), and **6** (100 mg).

**4-O-*β*-D-Glucopyranosyl- *p*-coumaric aicd (1)** – White amorphous powder; $[\alpha]_D^{20}$ : –59.1° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3400 (OH), 1452, 1550 (aromatic C = C), $^1$H-NMR (500 MHz, MeOH-$d_4$ + $D_2O$) : δ 3.31-3.91 (5H in total, m, glc-3,5,2,4,6), 4.96 (1H, d, *J* = 7.5Hz, g-1), 6.40 (1H, d, *J* = 15.9Hz, H-1'), 7.08 (2H, d, *J* = 8.7Hz, H-3, 5), 7.39 (1H, d, *J* = 15.9Hz, H-2'), 7.49 (2H, d, *J* = 8.7Hz, H-2, 6). $^{13}$C-NMR (125MHz, MeOH-$d_4$ + $D_2O$) : δ 176.0 (COOH), δ 159.7 (C-4), δ 141.2 (C-1'), δ 131.5 (C-1), δ 130.1 (C-2, 6), δ 124.5 (C-2'), δ 118.0 (C-3, 5), δ 102.0 (C-1"), δ 78.1 (C-5"), δ 77.8 (C-3"), δ 74.8 (C-2"), δ 71.3 (C-4"), δ 62.4 (C-6").

**2,3-Dihydro-2-(4-methoxy)-7-hydroxy-3-hydroxymethyl-5-(3-hydroxypropyl)benzofuran 3-*O*-*β*-L-glucopyranoside (2)** – White amorphous powder; $[\alpha]_D^{20}$ : –24.3° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3400 (OH), 2840, 2800 (C-C), 1500, 1450 (aromatic C = C), Negative FAB MS : *m/z* 508 $[M-H]^-$; $^1$H-NMR (500 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 1.73 (2H, m, H-8'), 2..51 (2H, t, *J* = 7.5, 8 Hz, H-7'), 3.43-3.85 (8H in total, m, glu-3,5,2,4,6, H-8, 9a, 9b), 3.81 (3H, s, $OCH_3$), 4.91 (1H, d, *J* = 7.3 Hz, glu-1), 5.55 (1H, d, *J* = 5.7 Hz, H-7), 6.60 (2H, d, *J* = 12.2, H-2', 6'), 6.93 (1H, dd, *J* = 8.4, 1.2 Hz, H-6), 7.08 (1H, d, *J* = 1.2Hz H-2), 7.12 (1H, d, *J* = 8.4 Hz H-5). $^{13}$C-NMR (125MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 150.0 (C-4), δ 147.0 (C-3), δ 145.9 (C-3'), δ 141.7 (C-2'), δ 137.9 (C-1), δ 136.4 (C-10), δ 129.4 (C-4'), δ 119.0 (C-6), δ 116.9 (C-5), δ 116.8 (C-1'), δ 116.3 (C-5'), δ 110.1 (C-2), δ 102.1 ($OCH_3$), δ 87.5 (C-7), δ 77.6 (C-4"), δ 77.2 (C-2"), δ 74.3 (C-1"), δ 70.8 (C-3"), δ 64.7 (C-9), δ 62.2 (C-5"), δ 61.7 (C-8'), δ 56.5 (C-9'), δ 55.3 (C-8), δ 35.5 (C-7'), δ 32.3 (C-6').

**Taxifolin 3'-*O*-*β*-D-glucopyranoside (3)** – Brown amorphous powder; $[\alpha]_D^{20}$ : +22.1° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3400 (OH), 2840, 2800 (C-C), 1550, 1450 (aromatic C = C), Negative FAB MS : *m/z* 467 $[M-H]^-$; $^1$H-NMR (300 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 3.31-3.92 (5H in total, m, glc-3,5,2,4,6), 4.67 (1H, d, *J* = 11.4Hz, H-3), 4.88 (1H, d, *J* = 7.5Hz, glc-1), 5.07 (1H, d, *J* = 11.4Hz, H-2), 5.96 (1H, d, *J* = 2.1Hz, H-6), 5.99 (1H, d, *J* = 2.1Hz, H-8), 6.93 (1H, d, *J* = 8.1Hz, H-5'), 7.13 (1H, dd, *J* = 8.1, 2.1Hz, H-6'), 7.45 (1H, d, *J* = 2.1Hz, H-2'). $^{13}$C-NMR (75 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 198.4 (C-4), δ 168.4 (C-7), δ 165.0 (C-5), δ 164.2 (C-9), δ 149.2 (C-4'), δ 146.1 (C-3'), δ 129.7 (C-1'), δ 124.8 (C-6'), δ 118.9 (C-2'), δ 116.8 (C-5'), δ 104.0 (C-1"), δ 101.5 (C-10), δ 97.2 (C-6), δ 96.2 (C-8), δ 84.3 (C-2), δ 78.0 (C-5"), δ 77.4 (C-3"), δ 74.6 (C-2"), δ 72.9 (C-3), δ 71.1 (C-4"), δ 62.4 (C-6").

**(+)-Catechin (4)** – Brown amorphous powder; $[\alpha]_D^{20}$ : +14.7° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3329 (OH), 1628 (C = C), 1550, 1452 (aromatic C = C), Negative FAB MS : *m/z* 289 $[M-H]^-$; $^1$H-NMR (300 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 2.51 (1H, dd, *J* = 16.2, 8.7 Hz, H-4ax), 2.92 (1H, dd, *J* = 16.2, 5.4 Hz, H-4eq), 4.00 (1H, m, H-3), 4.56 (1H, m, *J* = 8.1Hz, H-2), 5.88 (1H, d, *J* = 2.4 Hz, H-6), 6.04 (1H, d, *J* = 2.4 Hz, H-8), 6.75 (1H, dd, *J* = 8.1, 1.8 Hz, H-6'), 6.79 (1H, d, *J* = 8.1, H-5'), 6.92 (1H, d, *J* = 1.8 Hz, H-2'). $^{13}$C-NMR (75MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 157.9 (C-7), δ 157.4 (C-9), δ 157.0 (C-5), δ 145.9 (C-3'), δ 145.8 (C-4'), δ 132.1 (C-1'), δ 120.1 (C-6'), δ 115.8 (C-2'), δ 115.4 (C-5'), δ 100.6 (C-10), δ 96.2 (C-6), δ 95.3 (C-8), δ 82.7 (C-2), δ 68.3 (C-3), δ 28.8 (C-4).

**Procyanidin B-1 (5)** – Brown amorphous powder; $[\alpha]_D^{20}$ : –12.4° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3467 (OH), 1620, 1550 (aromatic C = C), Negative FAB MS : *m/z* 579 $[M-H]^-$; $^1$H-NMR (300 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 2.60 (1H, dd, H-4t ax), 2.80 (1H, dd, H-4t eq), 3.98 (1H, m, H-3t), 4.12 (1H, s, H-3u), 4.66 (1H, s, H-4u), 4.80 (1H, s, H-2t), 5.10 (1H, s, H-2u), 5.99-6.04 (3H, in total, H-8u, 6u, 6t), 6.71-7.01 (6H, in total, H-2', 5', 6' ut). $^{13}$C-NMR (75 MHz, $Me_2CO$-$d_6$ + $D_2O$) : 27.5 (C-4t), 36.7 (C-4u), 67.7 (C-3t), 72.5 (C-3u),6.9 (C-2u), 81.9 (C-2t), 95.5 (C-8u), 96.1 (C-6u), 96.9 (C-6t), 101.0 (C-10u,t), 107.7 (C-8t), 114.8-115.9 (C-2',5'u,t), 119.3-119.4 (C-6'u,t), 132.1-132.4 (C-1'u,t), 145.2-145.6 (C-3',4'u,t), 155.4-157.7 (C-9,7,5 u,t)

**Epicatechin (4*β*-8)-catechin (4*α*-8)-catechin (6)** – Brown amorphous powder; $[\alpha]_D^{20}$ : –97.4° (*c* = 0.01, MeOH), IR $\nu^{KBr}{}_{Max}$ $cm^{-1}$ : 3467 (OH), 1620, 1550 (aromatic C = C), Negative FAB MS : *m/z* 867 $[M-H]^-$; $^1$H-NMR (300 MHz, $Me_2CO$-$d_6$ + $D_2O$) : δ 2.60 (1H, dd, H-4t ax), 2.90 (1H, dd, H-4t eq), 4.04-4.06 (3H, in total,

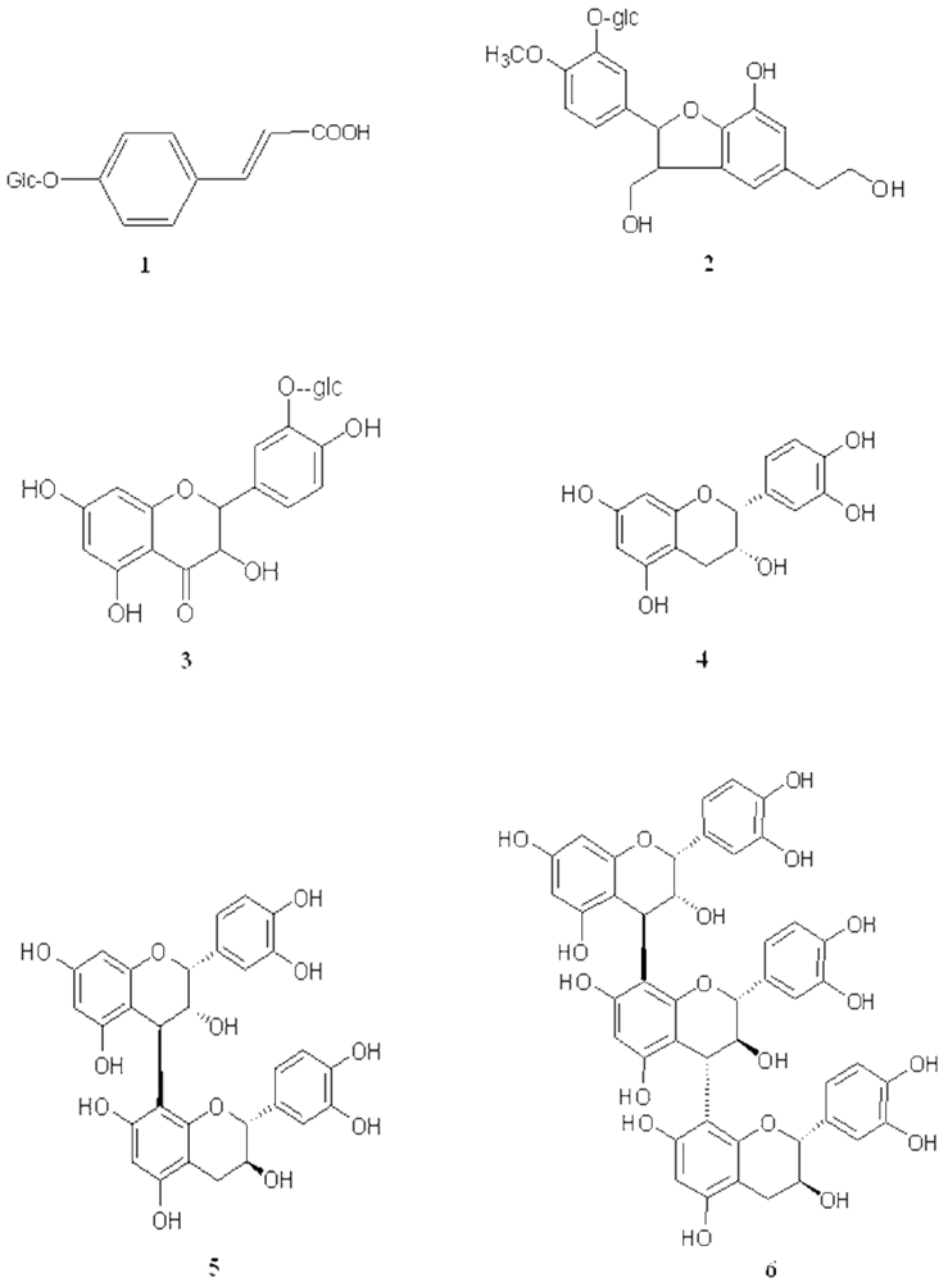


**Fig. 1.** Structures of compound **1-6**.

H-3u, 3m, 3t), 4.48-4.72 (5H, in total, H-4u, 4m, 2u, 2m, 2t), 5.95-6.20 (4H, in total, H-8u, 6u, 6m, 6t), 6.68-7.08 (9H, in total, H-2', 5', 6' u,m,t). $^{13}$C-NMR (75 MHz, $Me_2CO$-$d_6$ + $D_2O$) : 29.1 (C-4t), 36.6 (C-4u), 38.1 (C-4m), 68.2 (C-3t), 72.9 (C-3 u,t), 76.9 (C-2u), 82.9 (C-2t), 84.1 (C-2m), 95.8 (C-6 u,m,t, 8u), 100.6 (C-10 u,m,t), 107.7-108.3 (C-8 m,t), 115.1-115.9 (C-2',5'u,m,t), 119.1-120.6 (C-6'u,m,t), 131.7-132.2 (C-1'u,m,t), 145.7-145.9 (C-3',4'u,m,t), 155.2-157.4 (C-9,7,5 u,m,t)

**Biological Assay**

**DPPH radical scavenging activity** – The antioxidant activities of the six compounds were determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrzyl (DPPH) free radical by a previously described method with a slight modification (Hatano *et al*., 1989). 20 μl of the compound in EtOH (99.5% ethanol) was added to 180 μl of a DPPH solution (7.887 mg DPPH in 200 mL EtOH). After mixing gently and standing at for 30 min, the optical density was measured

**Table 1.** Anti-oxidative Activities of Compound **1 - 6**

| Compounds | $IC_{50}$ (μg/ml) |
|---|---|
| **1** | None |
| **2** | 35.79 |
| **3** | 22.97 |
| **4** | 3.91 |
| **5** | 2.98 |
| **6** | 3.44 |
| Ascorbic acid | 5.28 |

at 515 nm using an ELISA reader (BIO-RAD, Japan). The free radical scavenging activity was expressed as follow:

DPPH scavenging activity (%) = {(Ac – As)/(Ac –Ab)} × 100

When Ac was the absorbance of the control, As was the absorbance of the sample and Ab was the absorbance of the blank. The $IC_{50}$ values were defined as the concentration that could scavenge 50% DPPH free radical. L-ascorbic acid was used as positive control.

## Results and Discussion

The fresh barks of *Pinus densiflora* were extracted with 80% $Me_2CO$ and the extract was subjected to a combination of sephadex LH-20 for the fractionations. And repeated column chromatography on each fraction to afford 4-*β*-D-glucopyranosyloxyl-*p*-coumaric acid (**1**, Park *et al.,* 1996), 2,3-dihydro-2-(4-methoxy)-7-hydroxy-3-hydroxymethyl-5-(3-hydroxypropyl)-benzofuran 3-*O*-*β*-D-glucopyranoside (**2,** Lundgren *et al*., 1981), taxifolin 3'-*O*-*β*-D-glucopyranoside (**3**, Oleszek *et al*., 2002), (+)-catechin (**4**, Choi *et al*., 2001), procyanidin B-1 (**5**, Lee *et al*., 1992) and epicatechin (4*β*-8)-catechin (4*α*-8)-catechin (**6**, Hsu *et al*., 1985) and their structures were identified by the comparisons with the reported spectral and physical data in the literatures. The anti-oxidative activities were tested by DPPH radical scavenging method. Especially, compound **4** ($IC_{50}$ = 3.91 μg/ml) which is flavan-3ol and **5** ($IC_{50}$ = 2.98 μg/ml) and **6** ($IC_{50}$ = 3.44 μg/ml) which are procyanidin exhibited potent of free radical scavenging activity compared with positive control, ascorbic acid ($IC_{50}$ = 5.28 μg/ml). But compound **1** which is phenylpropanoid did not exhibit DPPH radical scavenging activity. Compound **2** ($IC_{50}$ = 35.79 μg/ml) and **3** ($IC_{50}$ = 22.97 μg/ml) which are lignan and flavonoid showed mild DPPH radical scavenging activities compared with **4**, **5** and **6** (**Table 1.**). These results showed that flavan 3-ol (**4**) and procyanidins (**5** and **6**) have potent free radical scavenging activities and the barks of *Pinus densiflora* is good source as anti-oxidative agent.

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