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# Antioxidative Constituents from the Twigs of *Vitex rotundifolia*

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**Abstract** − In the course of screening for antioxidant compounds by measuring the radical scavenging effect on DPPH (1,1-diphenyl- 2-picrylhydrazyl), a total extract of the twigs of *Vitex rotundifolia* (Verbenaceae) was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of three iridoid compounds, 10-*O*-vanilloylaucubin (1), 10-*O*-p-hydroxybenzoylaucubin (2) and aucubin (3), two C-glycoside flavones, vitexin (4) and orientin (5), and a quinic acid derivative, 3,4-di-*O*-caffeoylquinic acid (6). Their structures were elucidated by spectroscopic studies. Among them, compounds 5 and 6 showed the significant antioxidative effects on DPPH free radical scavenging test. In riboflavin-NBT-light and xanthine-NBT-xanthine oxidase systems, compounds 5 and 6 exhibited the formation of the blue formazan in a dose-dependent manner. Compounds 5 and 6 showed better superoxide quenching activities than vitamin C.

**Keywords:** *Vitex rotundifolia*, Verbenaceae, Iridoid, Phenolic compounds, DPPH, Superoxide quenching activity

## INTRODUCTION

*Vitex rotundifolia* L. fil. (Verbenaceae) is widely distributed in Asia including Korea, Japan and China, and its fruit has been used in folk medicine for cold, headache, migraine, sore eyes, night blindness and neuralgia (Kimura *et al*., 1996). Earlier investigation on the chemical constituents of *V. rotundifolia* dealt with sesquiterpenes, diterpenes, flavones, lignans, phenylpropanoids and iridoids in fruit of this plant (Ono *et al*., 1997, 1999, 2001, 2002; You *et al*., 1998; Okuyama *et al*., 1998; Ko *et al*., 2000; Yoshioka *et al*., 2004). But phytochemical and pharmacological studies of the twigs of this plant has not been performed yet.

In the course of searching for antioxidants from plants by measuring the radical scavenging effect on DPPH (1,1-diphenyl-2-picrylhydrazyl), a total extract of the twigs of *V. rotundifolia* was found to show potent antioxidant activity. Subsequent activity-guided fractionation of the methanolic extract led to the isolation of six compounds, 10-*O*-vanilloylaucubin (1), 10-*O*-p-hydroxybenzoylaucubin (2) and aucubin (3), two C-glycoside flavones, vitexin (4) and orientin (5), and a quinic acid derivative, 3,4-di-*O*-caffeoylquinic acid (6) from the active ethyl acetate fraction. Among them, compounds 5 and 6 showed the significant antioxidative effects on DPPH free radical scavenging test, and superoxide quenching activity tests. This paper deals with the isolation and structural characterization of these compounds and their scavenging activity of the stable DPPH free radical and superoxide quenching activity.

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## MATERIALS AND METHODS

### General experimental procedures

NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. Sephadex LH-20 was used for column chromatography (Pharmacia, 25-100 μm). Prep-HPLC was carried out on a Jaigel GS310 column (Japan). TLC was carried out on Merck precoated silica gel $F_{254}$ plates and silica gel for column chromatography was Kiesel gel 60 (230-400 mesh, Merck). Spots were detected under UV and by spraying with 10% $H_2SO_4$ in ethanol followed by heating at 100-120°C for 3 min. All other chemicals and solvents were of analytical grade and used without further purification. Ascorbic acid and BHA (butylated hydroxyanisole) were obtained from Sigma Chemical Co.

**Plant materials**

The twigs of *V. rotundifolia* were collected in October 2006 at Taeahn, Chungnam, Korea, and identified by Jong Pil Lim, College of Pharmacy, Woosuk University. A voucher specimen was deposited in the herbarium of the College of Pharmacy, Woosuk University (WSU-06-022).

**Extraction and isolation**

The shade dried and powdered twigs of *V. rotundifolia* (600 g) was extracted three times with hot MeOH, and then filtered. The extracts were combined and evaporated in vacuo at 40°C. The resultant methanolic extract (58 g) was successively partitioned as methylene chloride, ethyl acetate, *n*-butanol and water soluble fractions. Each fraction was tested for the radical scavenging effect on DPPH (1,1-diphenyl-2-picrylhydrazyl). Among these fractions, the ethyl acetate soluble fraction (7 g) showed the most significant free radical scavenging effect on DPPH. This fraction was subjected to chromatography on a Sephadex LH-20 column (MeOH only), and give five fractions (E1-E5). Fraction E1 (3.5 g) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-$H_2O$, 40:10:1) as an eluent to give six subfractions (E11-E16). Subfraction E12 (260 mg) was further chromatographed on a silica gel Lobar A column ($CHCl_3$-MeOH-$H_2O$, 3:1:1) and purified by a GS310 column LH-20 (MeOH) to give compound 1 (41 mg). Subfraction E14 (1.3 g) was crystallize with MeOH to give compound 2 (19 mg). Subfraction E15 (93 mg) was further chromatographed on a GS310 column (MeOH) to give compound 3 (10 mg) and 4 (9 mg). Fraction E3 (1.3 g) was chromatographed on silica gel column chromatography ($CHCl_3$-MeOH-$H_2O$, 40:10:1) as an eluent to give five subfractions (E31-E35). Subfraction E32 (136 mg) was crystallize with MeOH to give compound 5 (10 mg). Subfraction E35 (70 mg) was purified on a GS310 column (MeOH) to give compounds 6 (20 mg).

**10-*O*-Vanilloylaucubin (1)**

White powder (MeOH), $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$: 7.61 (1H, dd, *J*=8.4, 1.6 Hz, H- 6"), 7.59 (1H, d, *J*=1.6 Hz, H-2"), 6.86 (1H, d, *J*=8.4 Hz, H-5"), 6.34 (1H, dd, *J*=6.0, 2.0 Hz, H-3), 5.83 (1H, s, H-7), 5.11 (1H, dd, *J*=6.0, 4.3 Hz, H-4), 5.08 (1H, d, *J*=15.5 Hz, Ha-10), 5.03 (1H, d, *J*=7.2 Hz, H-1), 4.92 (1H, d, *J*=15.5 Hz, Hb-10), 4.70 (1H, d, *J*=8.0 Hz, H-1'), 4.47 (1H, m, H-6), 3.90 (3H, s, $OCH_3$), 3.84 (1H, dd, *J*=11.6, 1.8 Hz, Ha-6'), 3.66 (1H, dd, *J*=11.6, 6.1 Hz, Hb-6'), 3.39 (1H, dd, *J*=8.8, 8.8 Hz, H-3'), 3.00 (1H, t-like, *J*=7.3 Hz, H-9), 2.71 (1H, m, H-5). $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$: Table I.

**Table I.** $^{13}$C-NMR spectral data of compounds 1-5

| C | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 97.8 | 98.0 | 97.7 | — | — |
| 2 | — | — | — | 167.8 | 166.2 |
| 3 | 141.7 | 141.7 | 141.6 | 104.1 | 103.9 |
| 4 | 105.5 | 105.5 | 105.7 | 184.2 | 184.0 |
| 5 | 46.2 | 46.3 | 46.2 | 162.3 | 162.0 |
| 6 | 82.8 | 82.8 | 82.8 | 95.6 | 95.2 |
| 7 | 132.5 | 132.4 | 130.2 | 164.6 | 164.9 |
| 8 | 142.9 | 142.9 | 148.0 | 110.0 | 109.1 |
| 9 | 48.5 | 48.5 | 47.9 | 158.8 | 158.7 |
| 10 | 63.8 | 63.6 | 62.6 | 105.6 | 105.2 |
| 1' | 100.2 | 100.2 | 99.9 | 122.5 | 123.5 |
| 2' | 74.9 | 74.9 | 74.9 | 133.0 | 114.1 |
| 3' | 78.0 | 77.9 | 77.9 | 116.5 | 147.0 |
| 4' | 71.5 | 71.4 | 71.5 | 164.4 | 151.0 |
| 5' | 78.2 | 78.2 | 78.2 | 116.5 | 116.8 |
| 6' | 62.7 | 62.7 | 61.4 | 133.0 | 120.3 |
| 1" | 122.4 | 122.1 | | 75.0 | 75.3 |
| 2" | 113.6 | 132.9 | | 71.5 | 71.8 |
| 3" | 153.1 | 116.2 | | 81.1 | 80.1 |
| 4" | 148.8 | 163.6 | | 71.1 | 72.6 |
| 5" | 116.0 | 116.2 | | 82.9 | 82.6 |
| 6" | 125.2 | 132.9 | | 62.8 | 62.9 |
| 7" | 167.8 | 167.8 | | | |
| $OCH_3$ | 56.5 | | | | |

Recorded at 100 MHz for $^{13}$C-NMR in $CD_3OD$.

**10-*O*-p-Hydroxybenzoylaucubin (2)**

White powder (MeOH), $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$: 7.82 (2H, d, *J*=8.8 Hz, H- 2", 6"), 6.74 (2H, d, *J*=8.8 Hz, H-3", 5"), 6.24 (1H, dd, *J*=6.0, 2.0 Hz, H-3), 5.73 (1H, s, H-7), 5.03 (1H, dd, *J*=6.0, 4.3 Hz, H-4), 4.99 (1H, d, *J*=15.6 Hz, Ha-10), 4.89 (1H, d, *J*=7.6 Hz, H-1), 4.80 (1H, d, *J*=15.6 Hz, Hb-10), 4.60 (1H, d, *J*=8.0 Hz, H-1'), 4.38 (1H, m, H-6), 3.76 (1H, dd, *J*=11.6, 1.8 Hz, Ha-6'), 3.57 (1H, dd, *J*=11.6, 6.1 Hz, Hb-6'), 3.26 (1H, dd, *J*=8.8, 8.8 Hz, H-3'), 2.89 (1H, t-like, *J*=7.4 Hz, H-9), 2.61 (1H, m, H-5). $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$: Table I.

**Aucubin (3)**

White powder (MeOH), $^1$H-NMR (400 MHz, $CD_3OD$) $\delta$: 6.26 (1H, dd, *J*=5.6, 2.0 Hz, H-3), 5.72 (1H, s, H-7), 5.04 (1H, dd, *J*=5.6, 4.3 Hz, H-4), 4.90 (1H, d, *J*=7.6 Hz, H-1), 4.62 (1H, d, *J*=8.0 Hz, H-1'), 4.39 (1H, m, H-6), 4.29 (1H, d, *J*=15.2 Hz, Ha-10), 4.12 (1H, d, *J*=15.2 Hz, Hb-10), 3.82 (1H, dd, *J*=11.6, 1.8 Hz, Ha-6'), 3.65 (1H, dd, *J*=11.6, 6.1 Hz, Hb-6'), 2.84 (1H, t-like, *J*=7.6 Hz, H-9), 2.61 (1H, m, H-5). $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$: Table I.

**Vitexin (4)**

Yellow powder (MeOH), $^{1}$H-NMR (400 MHz,) δ: 7.93 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.83 (1H, s, H-3), 6.81 (2H, d, *J*=8.8 Hz, H-3', 5'), 6.41 (1H, s, H-6), 4.67 (1H, d, *J*=9.1 Hz, H-1"). $^{13}$C-NMR (100 MHz, $CD_3OD$) δ: Table I.

**Orientin (5)**

Yellow powder (MeOH), $^{1}$H-NMR (400 MHz, $CD_3OD$) δ: 7.33 (1H, dd, *J*=8.8, 2.0 Hz, H-6'), 7.32 (1H, d, *J*=2.0 Hz, H-2'), 6.87 (1H, d, *J*=8.8 Hz, H-5'), 6.50 (1H, s, H-3), 6.44 (1H, s, H-6), 4.19 (1H, d, *J*=7.2 Hz, H-1"). $^{13}$C-NMR (100 MHz, $CD_3OD$) δ: Table I.

**3,4-Di-*O*-caffeoylquinic acid (6)**

Yellow powder (MeOH), $^{1}$H-NMR (400 MHz, $CD_3OD$) δ: 7.57 (1H, d, *J*=15.6 Hz, H-3"), 7.51 (1H, d, *J*=15.6 Hz, H-3'), 7.05 (1H, d, *J*=2.0 Hz, H-5"), 7.01 (1H, d, *J*=2.0 Hz, H-5'), 6.92 (1H, dd, *J*=8.0, 2.0 Hz, H-9"), 6.87 (1H, dd, *J*=8.0, 2.0 Hz, H-9'), 6.76 (1H, d, *J*=8.0 Hz, H-8"), 6.75 (1H, d, *J*=8.0 Hz, H-8'), 6.29 (1H, d, *J*=15.6 Hz, H-2"), 6.24 (1H, d, *J*=15.6 Hz, H-2'), 5.62 (1H, m, H-3), 5.18 (1H, m, H-4), 4.15 (2H, m, H-5). $^{13}$C-NMR (100 MHz, $CD_3OD$) δ: 181.8 (COO), 168.5 (C-1"), 168.4 (C-1'), 149.6 (C-7'), 149.5 (C-7"), 147.4 (C-3'), 147.2 (C-3"), 146.8 (C-6'), 146.7 (C-6"), 127.8 (C-4'), 127.7 (C-4"), 123.2 (C-9'), 123.1 (C-9"), 116.5 (C-8'), 116.4 (C-8"), 115.3 (C-5'), 115.2 (C-5"), 115.1 (C-2'), 114.9 (C-2"), 75.6 (C-1), 74.6 (C-4), 70.0 (C-5), 67.6 (C-3), 38.09 (C-2) and 38.05 (C-6).

**DPPH radical scavenging effect**

Ethanol solutions of test samples at various concentrations (0.1-100 μg/ml) were added to a solution of DPPH in methanol (0.2 mM) in 96 well plates. After storing these mixtures for 30 minutes at room temperature, the remaining amounts of DPPH were determined by colorimetry at 520 nm on a microplate reader (Yoshida *et al*., 1989). And the radical scavenging activity of each compound was expressed by the ratio of the lowering of the DPPH solution in the absence of compounds. The mean values were obtained from triplicate experiments.

**Riboflavin and superoxide quenching activity (Choi *et al*., 2001)**

Superoxide quenching activities of test samples were measured photochemically, using an assay system consisting of methionine, riboflavin, and nitrobluetetrazolium (NBT) (Ginnopolitis and Ries, 1977). The reaction mixture was composed of 0.13 μM riboflavin, 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, PBS buffer (pH 7.4), and various concentrations of test samples. The sample was randomly placed in a light storage box and replaced randomly every 5 min for 15 min. The temperature within the light storage box was 20 ± 1°C during the light illumination. The light intensity at the sample level was 5,500 lux. During the light illumination, NBT was reduced to blue formazane formation was measured by the absorbance at 560 nm. The inhibition of blue formazane formation was taken as superoxide quenching activity.

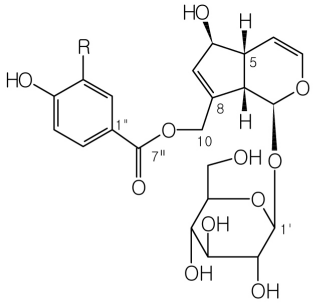
**Xanthine and superoxide scavenging assay**

Superoxide radicals were generated by xanthine/xanthine oxidase and measured by previously reported method (Thuong *et al*., 2007). In briefly, test samples were mixed with 20 mM phosphate buffer (PH7.8) containing 0.48 mM NBT and 1.6 mM xanthine. After 5 min, xanthine oxidase (0.05 U/ml) 100 μl was added. The absorbance of reaction mixture was read at 570 nm after 30 min incubation at 37°C. Superoxide radical scavenging activity was expressed by the degree of NBT reduction of a test group in comparison to that of control.

## RESULTS AND DISCUSSION

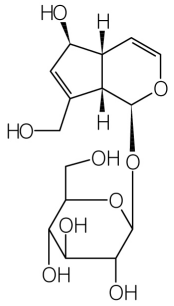
In the course of our screening for antioxidative components from natural plants, the ethyl acetate soluble fraction of methanolic extract of the twigs of *V. rotundifolia* was found to show scavenging activity on DPPH radical (Fig. 2). Subsequent activity-guided fractionation of the ethyl acetate soluble fraction led to the isolation of three iridoid compounds, two C-glycoside flavones, and a quinic acid derivative (Fig. 1).

Compounds 1-3 have similar patterns in their NMR spectra. Compound 1 was obtained as an amorphous powder from MeOH. The $^{1}$H-NMR spectrum of 1 showed two double doublet peaks at δ 6.34 (1H, dd, *J*=6.0, 2.0 Hz) and 5.11 (1H, dd, *J*=6.0, 4.3 Hz), which were assigned to protons at C-3 and C-4, a singlet peak at δ 5.83 (C-7), and a methoxy signal at δ 3.90 (3H, s). And also, the $^{1}$H-NMR spectrum of 1 showed the typical pattern of a coupling group of 1,3,4-trisubstituted benzene ring at δ 7.61 (1H, dd, *J*=8.4, 1.6 Hz, H-6"), 7.59 (1H, d, *J*=1.6 Hz, H-2") and 6.86 (1H, d, *J*=8.4 Hz, H-5"). In the $^{13}$C-NMR spectrum of 1, 22 carbon signals were observed, which included a carbonyl group at δ 167.8, ten aromatic or olefinic carbons at δ 141.7, 105.5, 132.5, 142.9, 122.4, 113.6, 153.1, 148.8, 116.0 and 125.2, six sugar carbons at δ 100.2, 74.9, 78.0, 71.5, 78.2 and 62.7, and a methoy group at δ56.3. From these results, compound 1 was deduced to be an iridoid glycoside bearing a 1,3,4-trisubstituted aromatic ring. The structure of 1 was determined to be 10-*O*-vanilloylaucubin

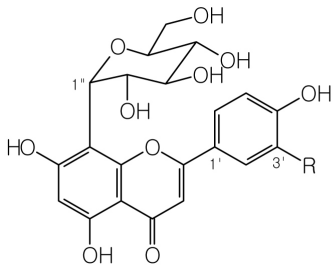


**1** R = $OCH_3$

**2** R = H

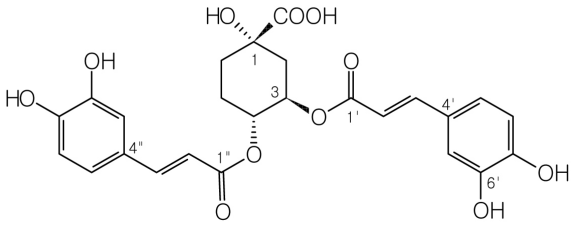


**3**



**4** R = H

**5** R = OH



**6**

**Fig. 1.** Structures of compounds 1-6 isolated from *Vitex rotundifolia*.

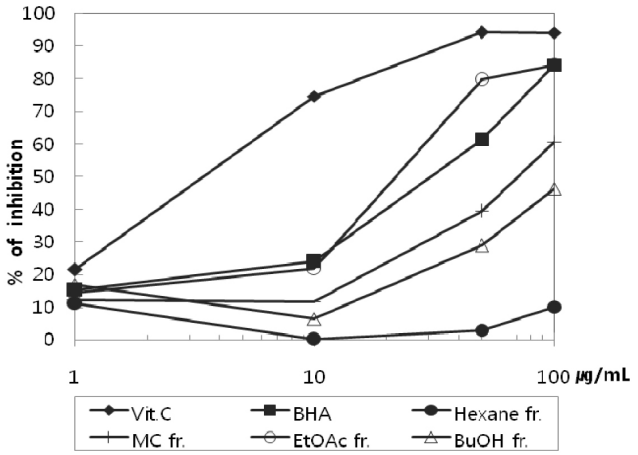



**Fig. 2.** Radical scavenging effects on DPPH radical of the fractions from the twigs of *V. rotundifolia*.

(VR-1) on the basis of the above evidences, together with a comparison of the above data with those published in the literature (Ono *et al*., 1997).

The NMR spectrum of 2 was very similar to that of 1. The main differences were chemical shifts of aromatic ring and the absence of methoxy group. The $^1$H-NMR spectrum of 2 showed two ortho-coupled doublets each of two protons with a J value of 8.8 Hz at δ 7.82 (2H, d, H-2", 6") and 6.74 (2H, d, H-3", 5"), indicating the presence of a 1,4-disubstituted aromatic ring. From these results, compound 2 was deduced to be an iridoid glycoside bearing a 1,4-disubstituted aromatic ring. The structure of 2 was determined to be 10-*O*-p-hydroybenzoylaucubin (agnuside) on the basis of the above evidences, together with a comparison of the above data with those published in the literature (Ono *et al*., 1997). The NMR spectrum of 3 was similar to that of 2 except for the absence of aromatic group. According to this result, compound 3 was deduced to be a simple iridoid glycoside. The structure of 3 was determined to be aucubin on the basis of the above evidence, together with a comparison of the above result with those published in the literature (El-Naggar and Beal, 1980).

Compounds 4 and 5 have similar patterns in their NMR spectra. Compounds 4 and 5 were obtained as an amorphous powder from MeOH. The $^1$H-NMR spectrum of 4 showed two ortho-coupled doublets each of two protons with a *J* value of 8.8 Hz at δ 7.93 (2H, d, H-2', 6') and 6.81 (2H, d, H-3', 5'), indicating the presence of a 1,4-disubstituted aromatic ring, and two singlet protons at δ 6.83 and 6.41 (each 1H, s) were observed in olefinic area. In the $^{13}$C-NMR spectrum of 4, twenty one carbon signals were observed, which included a carbonyl carbon (δ 184.2, C-4), and six oxygenated aliphatic carbons (δ 82.9, 81.1, 75.0, 71.5, 71.1, and 62.8). From these results, compound 4 was deduced to be a flavonoid glycoside bearing a 1,4-disubstituted aromatic B-ring. The structure of 4 was determined to be vitexin on the basis of the above evidences, together with a comparison of the above data with those published in the literature (Cheng *et al*., 2007). The NMR spectrum of 5 was very similar to that of 4. The main differences were chemical shifts of aromatic B-ring. The $^1$H-NMR spectrum of 5 showed the typical pattern of a coupling group of 1,3,4-trisubstituted benzene ring at δ 7.33 (1H, dd, *J*=8.4, 1.6 Hz, H-6'), 7.32 (1H, d, *J*=1.6 Hz, H-2'). In the $^{13}$C-NMR spectrum, twenty one carbon signals were

observed, which included a carbonyl group at δ 184.0, and six oxygenated aliphatic carbons (δ 82.6, 80.1, 75.3, 72.6, 71.8, and 62.9). From these results, compound 5 was deduced to be a flavonoid glycoside bearing a 1,3,4-trisubstituted aromatic B-ring. The structure of 5 was determined to be orientin on the basis of the above evidences, together with a comparison of the above data with those published in the literature (Hwang *et al*., 1994).

The $^{1}$H-NMR spectrum of 6 showed the typical pattern of two coupling groups of 1,3,4-trisubstituted benzene rings at δ 7.05 (1H, d, *J*=2.0 Hz, H-5"), 7.01 (1H, d, *J*=2.0 Hz, H-5'), 6.92 (1H, dd, *J*=8.0, 2.0 Hz, H-9"), 6.87 (1H, dd, *J*=8.0, 2.0 Hz, H-9'), 6.76 (1H, d, *J*=8.0 Hz, H-8") and 6.75 (1H, d, *J*=8.0 Hz, H-8'), and two coupling groups of trans coupled olefinic protons at δ 7.57 (1H, d, *J*=15.6 Hz, H-3"), 7.51 (1H, d, *J*=15.6 Hz, H-3'), 6.29 (1H, d, *J*=15.6 Hz, H-2") and 6.24 (1H, d, *J*=15.6 Hz, H-2'). In the $^{13}$C-NMR spectrum of 6, twenty five carbon signals were observed, which included three carbonyl groups at δ 181.8 (COO), 168.5 (C-1") and 168.4 (C-1'), and six aliphatic carbons at δ 75.6 (C-1), 74.6 (C-4), 70.0 (C-5), 67.6 (C-3), 38.09 (C-2) and 38.05 (C-6). From these results, compound 6 was deduced to be a dihydoxyquinic acid bearing two caffeoyl moieties. The structure of 6 was determined to be 3,4-di-*O*-caffeoylquinic acid on the basis of the above evidences, together with a comparison of the above data with those published in the literature (Timmermann *et al*., 1983; Kim *et al*., 1999).

There has been interest in finding various pharmacological active constituents from the fruit of *V. rotundifolia*. Polymethoxyflavonids have been studied the inhibitory effects on proliferation (You *et al*., 1998; Ko *et al*., 2000). It was reported that phenylnaphthalene compounds isolated from this plant showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (Kawazoe *et al*., 2001).

The radical scavenging effects of six compounds obtained from *V. rotundifolia* were shown in Fig. 3. The positive control vitamin C showed the DPPH radical scavenging effect with the $IC_{50}$ value of 6.1 μg/ml. Compounds 5 and 6 exhibited scavenging activities dose-dependently on DPPH with $IC_{50}$ values of 10.8 and 9.9 μg/ml, respectively.

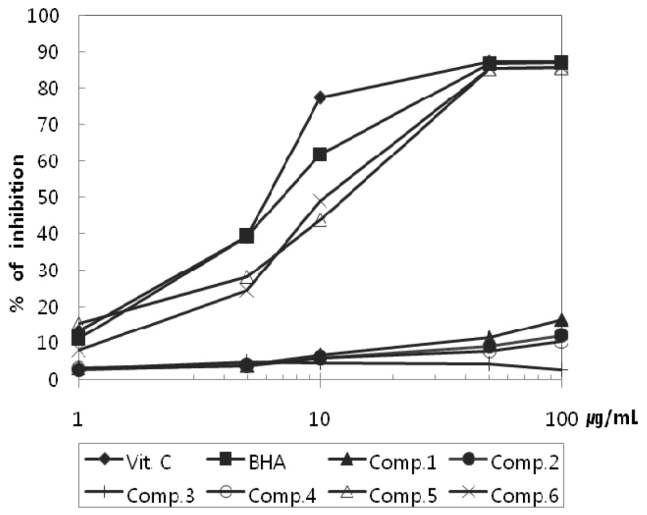



**Fig. 3.** Radical scavenging effects on DPPH radical of the isolated compounds from the twigs of *V. rotundifolia*.

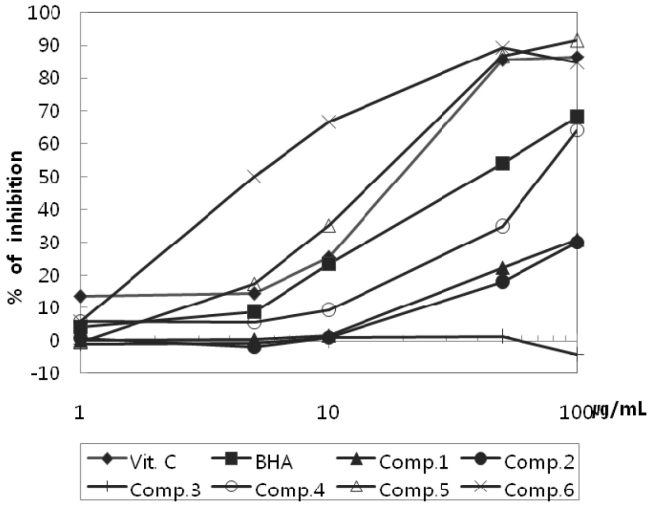



**Fig. 4.** Riboflavin originated superoxide quenching activities of the isolated compounds from the twigs of *V. rotundifolia*.

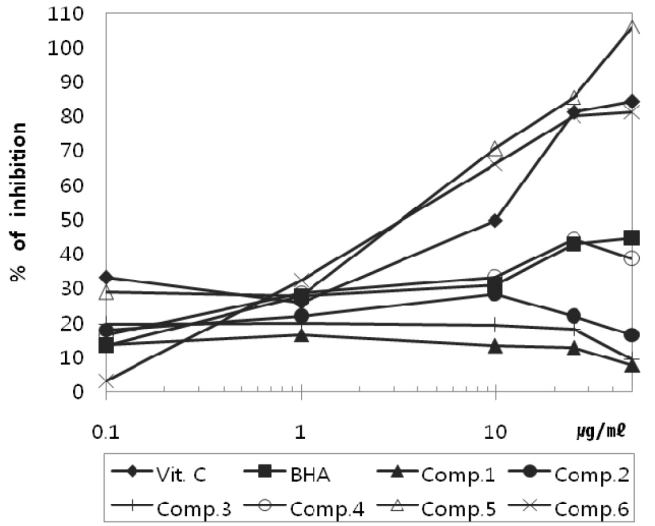



**Fig. 5.** Xanthine originated superoxide scavenging effects of the isolated compounds from the twigs of *V. rotundifolia*.

However, compounds 1-4 showed no activities in comparison with reference antioxidants such as ascorbic acid and BHA. Fig. 4 and 5 show the superoxide quenching activities of the isolated compounds 1-6, as measured by the riboflavin-NBT-light and xanthine-NBT-xanthine oxidase systems. Compounds 5 and 6 were found to be potent scavengers of superoxide radical generated in two systems. In riboflavin-NBT-light system, compounds 5 and 6 exhibited the formation of the blue formazan in a dose-dependent manner with $IC_{50}$ values of 10.3 and 5.1 μg/ml, respectively (vitamin C, positive control, $IC_{50}$ value, 39.7 μg/ml) (Fig. 4). In xanthine-NBT-xanthine oxidase system, compounds 5 and 6 also exhibited the formation of the blue formazan in a dose-dependent manner with $IC_{50}$ values of 3.2 and 3.0 μg/ml, respectively (vitamin C, positive control, $IC_{50}$ value, 9.9 μg/ml) (Fig. 5). Superoxide quenching activities of compounds 5 and 6 more pronounced than vitamin C, used as a positive control.

Free radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as by-products of metabolic processes (Devi *et al*., 2008). Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Kumaran and Karunakaran, 2006). The results from free radical scavenging systems revealed that the ethyl acetate soluble fraction of the twigs of *V. rotundifolia*, and compounds 5 and 6 had significant antioxidant activities. Therefore compounds 5 and 6 may be useful for the treatment of various oxidative damage.

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