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Antioxidative Chemical Constituents from the Stems of *Cleyera japonica* Thunberg

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Abstract: Chemical investigation to identify the antioxidative constituents from the stems of a tree *Cleyera japonica* Thunb. resulted in the isolation of seven compounds: catechin (1), catechin 3-O- α -L-rhamnopyranoside (2), epicatechin (3), taxifolin (4), taxifolin 3-O- α -L-arabinopyranoside (5), taxifolin 3-O- α -L-rhamnopyranoside (6) and proanthocyanidin A-1 (7). These isolates were studied for their scavenging activities against DPPH radical, hydroxyl radical and superoxide anion radicals using spectrophotometry and/or electron spin resonance. All isolates 1-7 exhibited more potent DPPH radical inhibition activities than the positive control, ascorbic acid. In the hydroxyl radical scavenging test, compound 7 (SC₅₀ 301.6 μ M) showed potent activity higher than ascorbic acid (SC₅₀ 859.7 μ M). All of the compounds 1-7 exhibited comparable activities to ascorbic acid for superoxide anion radical scavenging. These results demonstrated that *C. japonica* stem extracts could be potentially used as antioxidative agents in food or cosmetic applications.

Key words: *Cleyera japonica*, isolation, DPPH radical, hydroxyl radical, superoxide anion radical

INTRODUCTION

Reactive Oxygen Species (ROS) include oxygen-centered free radicals such as superoxide (O₂^{•-}), hydroxyl radical (HO[•]), alkoxy radical (RO[•]) and peroxy radical (ROO[•]) as well as nonradical species such as singlet oxygen (¹O₂) and hydrogen peroxide (H₂O₂). These radical and nonradical ROS are formed in living organisms during the normal metabolic processes. ROS, especially free radicals are chemically very reactive and can attack molecules in cells or tissues. For example, ROS can react with lipids in cell membranes, proteins in tissues or enzymes and bases in DNA (Denisov and Afanas'ev, 2005). This oxidative damage is believed to be a primary factor not only in various diseases but also in the normal process of aging (Valko *et al.*, 2007). Humans have evolved with antioxidant systems to protect against free radicals, which include enzymatic defense such as superoxide dismutase, catalase and glutathione peroxidase. Through these enzymes, superoxide and hydrogen peroxides are metabolized and therefore production of detrimental hydroxyl radical is prevented. Even though this endogenous defense system is provided, under some physiopathological situations such as air pollutants, UV radiation and inflammation, ROS is produced in excess. In order to diminish the cumulative effects of oxidative damage, exogenous antioxidants are

needed. Vitamins (A, C and E) and flavonoids from plant sources are antioxidants in diet (Pietta, 2000). Antioxidants are applied in the food industry as well as in the cosmetic industry as the functional ingredient to prevent oxidative damages (Elzaawely and Tawata, 2012; Gajula *et al.*, 2009; Ham *et al.*, 2010). Since application of antioxidants become broad in various areas, it is necessary to develop different type of novel antioxidative agents. Especially, natural antioxidants from plant sources are more favorable in the industry due to their environmentally friendly properties (Adisa *et al.*, 2011; Chanda *et al.*, 2011; Hajimahmoodi *et al.*, 2008; Oboh and Ademosun, 2006).

We are continuously conducting phytochemical studies on plants growing in Jeju, the largest island located at the southernmost part in Korea (Kim *et al.*, 2010a, b; Kim *et al.*, 2011; Ko *et al.*, 2011). In the course of our investigation for the biologically active natural products, we observed antioxidative activities in the ethanol extract prepared from the stems of *Cleyera japonica*, which led us to identify the active constituents.

C. japonica Thunb. (Theaceae family) is an evergreen tree of height up to 10 m distributed over Korea, Japan and Taiwan. This tree has usually been used as lumber for household furniture in Korea. The acetone extract from the leaves of *C. japonica* var. *morii* collected in Taiwan

showed a strong free radical scavenging activities (Hou *et al.*, 2003). However, no chemical constituents have been presented responsible for the activities. We herein described the antioxidative constituents from the ethanol extract of *C. japonica* stems and their scavenging activities against DPPH, hydroxyl and superoxide anion radicals.

MATERIALS AND METHODS

Reagent and equipment: All solvents used in this experiment were of analytical grade. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a JEOL (JNM-ECX 400) instrument with chemical shift data reported in ppm relative to the solvent used. JES-FA200 (JEOL) Electron Spin Resonance (ESR) spectrometer was used for the radical scavenge tests. Merck silica gel (0.063-0.2 mm) was used for normal phase column chromatography. Silica gel 60 F_{254} coated on aluminum plates by Merck were used for Thin Layer Chromatography (TLC). Gel Filtration Chromatography (GFC) was performed using Sephadex LH-20 (25–100 μm) from Fluka. DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Aldrich. Xanthine, xanthine oxidase and DMPO (5,5-dimethyl-1-pyrroline N-oxide) were purchased from Sigma.

Plant material: The stems of *C. japonica* Thunb. were collected from the Halla Botanical Garden, Jeju Island in Korea. A voucher specimen (No. 124) was prepared and deposited at the laboratory of Natural Product Chemistry, Department of Chemistry, Jeju National University.

Extraction and isolation: The dried stems of *C. japonica* (1.1 kg) were extracted three times with 70% ethanol by stirring with a magnetic stirrer at room temperature each time for 24 h. The combined solutions were filtered and the filtrate was concentrated in a vacuum rotary evaporator at a maximum temperature of 40°C to afford a gummy extract (116.6 g). A portion of the extract (14.2 g) was suspended in water (1 L) and partitioned into n-hexane, ethyl acetate (EtOAc) and n-butanol soluble fractions. The EtOAc-soluble fraction (3.9 g) was subjected to Vacuum Liquid Chromatography (VLC) over silica gel with elution (each 300 mL) of n-hexane-EtOAc (0–100%) and EtOAc-methanol (0–50%) gradients to afford 18 fractions (frs. 1–18). Fraction 6 (138.7 mg) was further purified by silica gel Column Chromatography (CC) with chloroform-methanol (5:1) to provide compounds 1 (55.9 mg) and 4 (5.9 mg). Fraction 7 (144.5 mg) was purified by CC over Sephadex LH-20 with chloroform-methanol (2.5:1) to give the compounds 1 (2.3 mg), 3 (2.6 mg), 4 (3.0 mg) and 7 (11.5 mg). Fraction 8 (306.3 mg) was

subjected to Sephadex LH-20 CC with chloroform-methanol (3:1) to give compounds 5 (16.3 mg) and 6 (39.2 mg). Fraction 9 (520.9 mg) was also purified by Sephadex LH-20 CC with chloroform-methanol (3:1) to provide compound 5 (264.5 mg). Fraction 10 (953.1 mg) was subjected to silica gel CC with chloroform-EtOAc-methanol (2:2:1) to yield five fractions (frs. 10-1 to 10-5). Subfraction 10-2 (356.0 mg) was further purified over Sephadex LH-20 CC with chloroform-methanol (3:1) to afford compounds 2 (11.9 mg) and 5 (191.9 mg). Fraction 11 (313.3 mg) was purified by Sephadex LH-20 CC with chloroform-methanol (3:1) to give compounds 2 (9.6 mg) and 5 (80.5 mg). Fraction 12 was also purified by Sephadex LH-20 CC with chloroform-methanol (4:1) to give compound 5 (9.4 mg).

Catechin (1): $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 6.83 (1H, d, $J = 1.8$ Hz, H-2'), 6.76 (1H, d, $J = 8.2$ Hz, H-5'), 6.71 (1H, dd, $J = 1.8, 8.2$ Hz, H-6'), 5.92 (1H, d, $J = 2.3$ Hz, H-6), 5.85 (1H, d, $J = 2.3$ Hz, H-8), 4.56 (1H, d, $J = 7.6$ Hz, H-2), 3.97 (1H, m, H-3), 2.85 (1H, dd, $J = 5.3, 16.0$ Hz, H-4), 2.50 (1H, dd, $J = 8.2, 16.0$ Hz, H-4); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD) δ 158.0 (C-9), 157.8 (C-5), 157.1 (C-7), 146.5 (C-4'), 146.4 (C-3'), 132.3 (C-1'), 120.2 (C-6'), 116.2 (C-5'), 115.4 (C-2'), 100.9 (C-10), 96.4 (C-6), 95.6 (C-8), 83.0 (C-2), 69.0 (C-3), 28.7 (C-4).

Catechin 3-O- α -L-rhamnopyranoside (2): $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 6.84 (1H, d, $J = 1.8$ Hz, H-2'), 6.77 (1H, d, $J = 8.0$ Hz, H-5'), 6.72 (1H, dd, $J = 1.8, 8.0$ Hz, H-6'), 5.94 (1H, d, $J = 2.3$ Hz, H-6), 5.86 (1H, d, $J = 2.3$ Hz, H-8), 4.62 (1H, d, $J = 8.0$ Hz, H-2), 4.29 (1H, d, $J = 1.4$ Hz, H-1''), 3.93 (1H, m, H-3), 3.68 (1H, m, H-5''), 3.57 (1H, dd, $J = 3.2, 9.6$ Hz, H-3''), 3.51 (1H, dd, $J = 1.8, 3.2$ Hz, H-2''), 3.31 (overlapped with CD_3OD , H-4''), 2.88 (1H, dd, $J = 5.5, 16.0$ Hz, H-4), 2.64 (1H, dd, $J = 8.5, 16.0$ Hz, H-4), 1.25 (3H, d, $J = 6.2$, H-6''); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD) δ 158.1 (C-9), 157.7 (C-5), 157.0 (C-7), 146.5 (C-4'), 146.4 (C-3'), 132.1 (C-1'), 120.0 (C-6'), 116.2 (C-5'), 115.2 (C-2'), 102.3 (C-1''), 100.8 (C-10), 96.5 (C-6), 95.6 (C-8), 81.3 (C-2), 76.1 (C-3), 74.1 (C-4''), 72.4 (C-3''), 72.1 (C-2''), 70.5 (C-5''), 28.1 (C-4), 18.1 (C-6'').

Epicatechin (3): $^1\text{H-NMR}$ (400 MHz, CD_3OD) δ 6.97 (1H, d, $J = 2.1$ Hz, H-2'), 6.80 (1H, dd, $J = 2.1, 8.0$ Hz, H-6'), 6.76 (1H, d, $J = 8.0$ Hz, H-5'), 5.94 (1H, d, $J = 2.3$ Hz, H-6), 5.91 (1H, d, $J = 2.3$ Hz, H-8), 4.82 (1H, s, H-2), 4.18 (1H, m, H-3), 2.86 (1H, dd, $J = 4.6, 16.7$ Hz, H-4), 2.73 (1H, dd, $J = 2.8, 16.7$ Hz, H-4); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD) δ 158.2 (C-5), 157.8 (C-7), 157.5 (C-9), 146.1 (C-3'), 145.9 (C-4'), 132.4 (C-1'), 119.5 (C-6'), 116.0 (C-5'), 115.5 (C-2'), 100.2 (C-10), 96.5 (C-6), 96.0 (C-8), 80.0 (C-2), 67.6 (C-3), 29.4 (C-4).

Taxifolin (4): ¹H-NMR (400 MHz, CD₃OD) δ 6.96 (1H, d, J = 1.8 Hz, H-2'), 6.85 (1H, dd, J = 1.8, 8.0 Hz, H-6'), 6.80 (1H, d, J = 8.0 Hz, H-5'), 5.92 (1H, d, J = 2.1 Hz, H-6), 5.88 (1H, d, J = 2.1 Hz, H-8), 4.91 (1H, d, J = 11.5 Hz, H-2), 4.50 (1H, d, J = 11.5 Hz, H-3); ¹³C-NMR (100 MHz, CD₃OD) δ 198.6 (C-4), 168.9 (C-5), 165.5 (C-7), 164.7 (C-9), 147.3 (C-3'), 146.5 (C-4'), 130.0 (C-1'), 121.0 (C-6'), 116.2 (C-5'), 116.0 (C-2'), 102.0 (C-10), 97.4 (C-6), 96.4 (C-8), 85.3 (C-2), 73.8 (C-3).

Taxifolin 3-O-α-L-arabinopyranoside (5): ¹H-NMR (400 MHz, CD₃OD) δ 6.97 (1H, d, J = 2.1 Hz, H-2'), 6.85 (1H, dd, J = 2.1, 8.2 Hz, H-6'), 6.79 (1H, d, J = 8.2 Hz, H-5'), 5.92 (1H, d, J = 2.1 Hz, H-6), 5.90 (1H, d, J = 2.1 Hz, H-8), 5.13 (1H, d, J = 10.5 Hz, H-2), 4.80 (1H, d, J = 10.5 Hz, H-3), 3.92 (1H, dd, J = 7.6, 11.7 Hz, H-5''), 3.84 (1H, d, J = 3.9 Hz, H-1''), 3.80 (1H, m, H-4''), 3.59 (1H, dd, J = 3.9, 6.0 Hz, H-2''), 3.55 (1H, dd, J = 3.2, 6.0 Hz, H-3''), 3.38 (1H, dd, J = 3.7, 11.7 Hz, H-5''); ¹³C-NMR (100 MHz, CD₃OD) δ 196.1 (C-4), 169.6 (C-7), 165.6 (C-5), 164.4 (C-9), 147.6 (C-4'), 146.7 (C-3'), 129.1 (C-1'), 120.9 (C-6'), 116.4 (C-5'), 115.8 (C-2'), 102.4 (C-1''), 101.5 (C-10), 97.6 (C-6), 96.7 (C-8), 83.9 (C-2), 76.4 (C-3), 73.2 (C-2''), 71.2 (C-4''), 66.9 (C-3''), 63.5 (C-5'').

Taxifolin 3-O-α-L-rhamnopyranoside (6): ¹H-NMR (400 MHz, CD₃OD) δ 6.96 (1H, d, J = 1.8 Hz, H-2'), 6.84 (1H, dd, J = 1.8, 8.2 Hz, H-6'), 6.81 (1H, d, J = 8.2 Hz, H-5'), 5.92 (1H, d, J = 2.1 Hz, H-6), 5.90 (1H, d, J = 2.1 Hz, H-8), 5.07 (1H, d, J = 10.5 Hz, H-2), 4.57 (1H, d, J = 10.5 Hz, H-3), 4.25 (1H, m, H-5''), 4.05 (1H, d, J = 1.4 Hz, H-1''), 3.66 (1H, dd, J = 3.2, 9.6 Hz, H-4''), 3.54 (1H, dd, J = 1.6, 3.2 Hz, H-3''), 3.31 (overlapped with CD₂HOD, H-2''), 1.19 (3H, d, J = 6.2 Hz, H-6''); ¹³C-NMR (100 MHz, CD₃OD) δ 196.1 (C-4), 169.0 (C-7), 165.7 (C-5), 164.2 (C-9), 147.5 (C-4'), 146.7 (C-3'), 129.3 (C-1'), 120.6 (C-6'), 116.4 (C-5'), 115.6 (C-2'), 102.6 (C-10), 102.3 (C-1''), 97.6 (C-6), 96.5 (C-8), 84.1 (C-2), 78.7 (C-3), 73.9 (C-4''), 72.3 (C-3''), 71.9 (C-2''), 70.6 (C-5''), 18.0 (C-6'').

Proanthocyanidin A-1 (7): ¹H-NMR (400 MHz, CD₃OD) δ 7.13 (1H, d, J = 2.1 Hz, H-12), 7.02 (1H, dd, J = 2.1, 8.2 Hz, H-16), 6.92 (1H, d, J = 2.0 Hz, H-12'), 6.81 (3H, overlapped, H-15, 15', 16'), 6.09 (1H, s, H-6'), 6.07 (1H, d, J = 2.3 Hz, H-8), 5.96 (1H, d, J = 2.3 Hz, H-6), 4.73 (1H, d, J = 7.8 Hz, H-2'), 4.23 (1H, d, J = 3.4 Hz, H-4), 4.15 (1H, m, H-3'), 4.07 (1H, d, J = 3.2 Hz, H-4''), 2.94 (1H, dd, J = 5.5, 16.5 Hz, H-4'), 2.57 (1H, dd, J = 8.5, 16.5 Hz, H-4''); ¹³C-NMR (100 MHz, CD₃OD) δ 158.3 (C-7), 156.9 (C-5), 156.3 (C-5'), 154.4 (C-9), 152.4 (C-7'), 151.6 (C-9'), 146.9 (C-13), 146.9 (C-13'), 146.5 (C-14'), 145.8 (C-14), 132.4 (C-11), 130.7 (C-11'), 120.8 (C-16'), 120.0 (C-16), 116.5 (C-15), 115.9

(C-12'), 115.8 (C-12), 115.8 (C-15'), 106.9 (C-10), 104.2 (C-8'), 103.3 (C-10'), 98.3 (C-6), 96.7 (C-8), 96.7 (C-6'), 84.7 (C-2'), 68.3 (C-3'), 68.0 (C-3), 29.4 (C-4), 29.2 (C-4').

DPPH radical scavenging activity: Sample solutions (20 μL) of different concentrations (100, 50, 25, 12.5, 6.25 and 3.125 μg mL⁻¹ in DMSO) were added to a 0.2 mM DPPH ethanol solution (180 μL) and allowed to react at room temperature. The absorbance values were measured after 10 min at 515 nm with a UV/Vis spectrophotometer. The DPPH radical scavenging activities of samples were calculated according to the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100$$

where, Abs_{sample} is the absorbance of the experimental sample, Abs_{blank} is the absorbance of the blank, Abs_{control} is the absorbance of the control. Ascorbic acid (vitamin C) was used as a positive control. Each treatment was replicated thrice.

DPPH radical scavenging activity with ESR: Test solution was prepared by mixing 10 μL of sample and 90 μL of DPPH (0.2 mM) in a methanol solution. After mixing vigorously for 10 sec, the solution was then transferred into a 100 μL Teflon capillary tube fitted into the cavity of the ESR spectrometer. The ESR spectrum was recorded 2 min after test solution preparation. ESR spectrometer parameters were set as follows: magnetic field of 337 mT, power of 1.00 mW, frequency of 9.4375 GHz, modulation amplitude of 0.8 mT, gain of 500, scan time of 0.5 min, scan width of 10 mT and time constant of 0.03 sec at room temperature (Kim *et al.*, 2010c). Ascorbic acid was used as a positive control and each treatment was replicated thrice.

Hydroxyl radical scavenging activity: Hydroxyl radicals generated by the Fenton reaction (H₂O₂ plus FeSO₄) were reacted with a radical spin trap, DMPO. The resulting DMPO-OH radicals were detected by using an ESR spectrometer. Briefly, ESR signaling was recorded after 2.5 min of test solution preparation by mixing of sample (10 μL) with 0.3 M DMPO (30 μL), 10 mM FeSO₄ (30 μL) and 10 mM H₂O₂ (30 μL). ESR spectrometer parameters were set as follows: magnetic field of 337 mT, power of 1.00 mW, frequency of 9.4354 GHz, modulation amplitude of 0.2 mT, gain of 200, scan time of 0.5 min, scan width of 10 mT and time constant of 0.03 sec at room temperature (Kim *et al.*, 2010c). Ascorbic acid was used as a positive control and each treatment was replicated thrice.

Superoxide anion radical scavenging activity: Superoxide anion radicals produced by a xanthine/xanthine oxidase system were reacted with the spin trap agent, DMPO. The generated DMPO-OOH radicals were detected using ESR spectrometry. Briefly, ESR signaling was recorded 5 min after of test solution preparation by mixing of sample (10 μL) with 1.5 M DMPO (30 μL), 5 mM xanthine (30 μL) and 0.25 U mL^{-1} xanthine oxidase (30 μL). The parameters of the ESR spectrometer were: magnetic field of 337 mT, power of 5.00 mW, frequency of 9.4374 GHz, modulation amplitude of 0.2 mT, gain of 700, scan time of 0.5 min, scan width of 10 mT, time constant of 0.03 sec and a temperature of 25°C (Kim *et al.*, 2010c). The ascorbic acid was used as a positive control and each treatment was replicated thrice.

Statistical analysis: Means \pm SEM of the data were calculated; statistical analysis of the results was performed by Student's t-test for independent samples. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

In the course of preliminary screenings, the ethanol extract of *C. japonica* stems was found to have significant radical scavenging activities. These antioxidative activities were measured against DPPH and hydroxyl radicals by using Electron Spin Resonance (ESR) spectrometry. ESR spectroscopy, whose signal intensities depend on the concentrations of free radicals, is a sensitive and specific method for the detection of radical species in chemical and biological systems. Direct measurement of radical species with ESR in the reaction mixture has advantages over traditional spectrophotometric method leading to high reliability and precision. This technique has been successfully applied for systematic studies on the evaluation of antioxidant capacity of natural extracts (Jiang *et al.*, 2010). Upon the DPPH radical inhibition activities, the ethanol extract exhibited SC_{50} 13.6 $\mu\text{g mL}^{-1}$ which was comparable to ascorbic acid (SC_{50} 4.6 $\mu\text{g mL}^{-1}$; positive control). In addition, the extract was comparable (SC_{50} 442.5 $\mu\text{g mL}^{-1}$) to ascorbic acid (SC_{50} 153.6 $\mu\text{g mL}^{-1}$) in the hydroxyl radical inhibition tests.

As the ethanol extract exhibited considerable free radical inhibition properties, a phytochemical study was conducted in order to identify the active constituents. The extract was partitioned successively into n-hexane, ethyl acetate (EtOAc), n-butanol and water soluble fractions. The EtOAc fraction was chosen and subjected to repeated column chromatography over silica gel and Sephadex LH-20. Compounds 1-7 (Fig. 1) were isolated

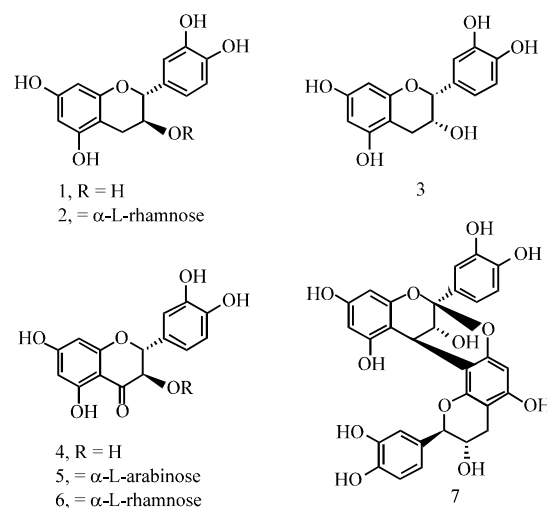


Fig. 1: Structures of isolated compounds 1-7 from *C. japonica*

Table 1: DPPH radical scavenging activities for compounds 1-7 determined by UV-VIS spectrophotometry

Compounds	SC_{50} (μM)	Compounds	SC_{50} (μM)
1	32.7	5	32.8
2	28.4	6	26.2
3	31.7	7	15.1
4	30.6	Ascorbic acid	44.9

SC_{50} : Concentration (μM) at which radical scavenging activity is 50%

from these purification procedures. These isolates were identified as catechin (1) (Martin *et al.*, 2000), catechin 3-O- α -L-rhamnopyranoside (2) (Bonefelda *et al.*, 1986; Ishimarua *et al.*, 1987), epicatechin (3) (Martin *et al.*, 2000), taxifolin (4) (Han *et al.*, 2007), taxifolin 3-O- α -L-arabinopyranoside (5) (Chosson *et al.*, 1998), taxifolin 3-O- α -L-rhamnopyranoside (6) (Lucas-Filbh *et al.*, 2001) and proanthocyanidin A-1 (7) (Lou *et al.*, 1999). The chemical structures of these compounds were elucidated based on the spectroscopic data, including 1D and 2D NMR spectra, as well as comparison to those in the literature. As far as we know, all of the compounds 1-7 were isolated for the first time from the plant *C. japonica*.

The antioxidant properties for compounds (1-7) were examined in radical inhibition assays. DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activities can be assayed by a spectrophotometer and it forms a stable radical species with a strong absorption at 515 nm bearing purple color; its degradation by test sample can be monitored by disappearance of absorption. Using the DPPH radical inhibition assay, all isolates 1-7 showed more potent activities than the positive control ascorbic acid (SC_{50} 44.9 μM) (Table 1). Proanthocyanidin A-1 (7) displayed the most potent activity. The disappearance of DPPH

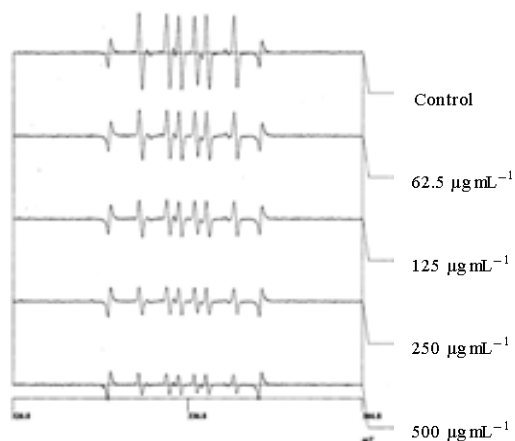


Fig. 2: DPPH and hydroxyl radical ESR spectra for the compound 7

Table 2: DPPH radical scavenging activities for compounds 1-7 determined by ESR spectrometry

Compounds	SC ₅₀ (µM)	Compounds	SC ₅₀ (µM)
1	17.2	5	30.2
2	22.9	6	19.1
3	22.7	7	9.4
4	20.7	Ascorbic acid	23.3

SC₅₀: Concentration (µM) at which radical scavenging activity is 50%

radical species can also be monitored by an ESR spectrum. As shown in Table 2, this assay also showed that compound 7 was the most potent inhibitor with a SC₅₀ of 9.4 µM, indicating better activity than ascorbic acid (SC₅₀ 23.3 µM). The ESR spectrum for compound 7 is shown in Fig. 2.

As hydroxyl radical is the most reactive chemical species among ROS, this species produces the most deleterious effect on cells in living organisms (Denisov and Afanas'ev, 2005). In the scavenging activity assays, hydroxyl radical was generated by the reaction of hydrogen peroxide and Fe²⁺ ion known as the Fenton reaction. The hydroxyl radical inhibition was verified using ESR by monitoring the DMPO-OH radical peak. In this experiment, the reactive hydroxyl radical was trapped by a nitrogen N-oxide, DMPO to yield a relatively stable radical DMPO-OH, which can be detected by ESR. Using this hydroxyl radical inhibition assay, compound 7 exhibited more potent activity (SC₅₀ 301.6 µM) than ascorbic acid (SC₅₀ 859.7 µM) (Table 3). Figure 2 indicated that hydroxyl radical was scavenged by compound 7 in a dose-dependent manner. The other compounds appeared to have relatively lower activities.

Superoxide anion is a ROS generated during the respiratory metabolic process in mitochondria. Even though this radical anion is not as reactive as hydroxyl

Table 3: Hydroxyl radical scavenging activities for compounds 1-7 determined by ESR spectrometry

Compounds	SC ₅₀ (µM)	Compounds	SC ₅₀ (µM)
1	2118.2	5	>2500.0
2	>2500	6	>2500.0
3	2157.8	7	301.6
4	1300.7	Ascorbic acid	859.7

SC₅₀: Concentration (µM) at which radical scavenging activity is 50%

Table 4: Superoxide anion radical scavenging activities for compounds 1-7 determined by ESR spectrophotometry

Compounds (100 µg mL ⁻¹)	Scavenging activity (%)
1	45.8
2	40.6
3	46.0
4	46.4
5	39.7
6	37.4
7	46.4
Ascorbic acid	72.0

radicals, it can initiate a cascade of ROS. Therefore, inhibition of the superoxide reduces ROS production. In the scavenging activity assay, superoxide was generated by the xanthine and xanthine oxidase system. At the concentration of 100 µg mL⁻¹, compounds 1-7 showed comparable scavenging activities to ascorbic acid (Table 4).

In conclusion, phytochemical investigation of the stems of *C. japonica* led to the isolation of seven compounds. We demonstrated that compounds 1-7 possessed relatively potent inhibition activities against DPPH, hydroxyl and superoxide anion radicals. Natural antioxidants may be responsible for the protective effects against the risk of many physiological and pathological processes. Based on these results, it is suggested that the extract of *C. japonica* stems containing potent antioxidative constituents could have potential in many industrial applications.

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