Comparison and Characterization of the Antioxidant Potential of 3 Wild Grapes–*Vitis thunbergii*, *V. flexuosa*, and *V. kelungeusis*

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Abstract: This study considers a laboratory examination of the antioxidant performance of methanolic extracts from the leaves and stems of 3 common wild grapes (*Vitis thunbergii*, *V. flexuosa*, and *V. kelungeusis*) by various *in vitro* methods. It also seeks to identify the specific antioxidant constituent. Results revealed that, of these specimens, stem extracts of *V. thunbergii* exhibited good 1,1-diphenyl-2-picrylhydrazyl radical-scavenging and superoxide radical-scavenging performance and ferrous ion-chelating ability, as well as the highest total phenolic content (179.5 mg of GAE/g). The principal antioxidant, (+)-lyoniresinol-2a-*O*-*β*-D-glucopyranoside, was isolated from the stem extracts of *V. thunbergii* and identified. Removal of this compound from the extracts caused an approximate 2- to 5-fold decrease in antioxidant performance. This showed that (+)-lyoniresinol-2a-*O*-*β*-D-glucopyranoside is the primary antioxidant in wild grapes. Results also indicated that the antioxidant performance of (+)-lyoniresinol-2a-*O*-*β*-D-glucopyranoside was stronger than its lignan aglycone, (+)-lyoniresinol.

Keywords: aglycone, antioxidant activity, HPLC, phytochemicals, plant extract

Practical Application: Of the 3 common wild grapes–*Vitis thunbergii*, *V. flexuosa*, and *V. kelungeusis*, the extracts or phytochemicals, derived from the *V. thunbergii* stems have excellent antioxidant properties, so they have great potential as a basis for natural health products that seek to prevent diseases caused by the overproduction of radicals.

Introduction

Molecular and cellular damage due to reactive oxygen species (ROS) and reactive nitrogen species (RNS) is widely believed to be the major cause of chronic degenerative diseases, such as aging, neural disorders, diabetes, atherosclerosis, inflammatory injuries, cancer, and cardiovascular disease (Halliwell 1997). In light of this information, it can be seen that potential scavengers of ROS and RNS have the potential to prevent for free radical-mediated diseases (Ames and others 1995). In the past few years, the antioxidant properties of dietary or medicinal plants have been extensively studied (Halvorsen and others 2002).

Vitis thunbergii, *V. flexuosa*, and *V. kelungeusis* are 3 wild grapes, common to Taiwan. They are traditionally used as medicinal plants. In Taiwan, aqueous extracts of wild grape stems are used for the treatment of diarrhea, fracture, injury, jaundice, and hepatitis. It is well known that plants of the genus *Vitis* commonly contain oligomers of resveratrol (Huang and others 2005). Recent studies have also indicated that resveratrols have cardioprotective and anti-inflammatory properties, which appear to be partly responsible for their antioxidant properties (Olas and Wachowicz 2002; Liu and others 2003). However, to the best of our knowledge, there is no prior report on the antioxidant performance of different species and parts of wild grapes.

Mitchell and others (1998) demonstrated that the assessments of antioxidant performance can be affected by many factors in the system to be analyzed, such as pH value, varieties of solvents, free radicals, and metal ions. In other words, there is no universal method by which antioxidant performance can be measured accurately, because the process may involve multiple reaction characters and mechanisms.

In this study, a number of *in vitro* assays were performed, to evaluate the antioxidant performance of methanolic extracts from the leaves and stems of 3 species of wild grapes, common to Taiwan. The lignans of *V. thunbergii*, published in the literature, generally belonged to isolariciresinol derivatives (He and others 2004). For this study, the major bioactive constituent, (+)-lyoniresinol-2a-*O*-*β*-D-glucopyranoside, was isolated and identified in *V. thunbergii* stems. This is the first time that lyoniresinol-type lignan glycoside has been identified as the principal bioactive phytochemical in this species. We studied not only the antioxidant activities of (+)-lyoniresinol-2a-*O*-*β*-D-glucopyranoside, but also compared this phytochemicals with its aglycone, (+)-lyoniresinol.

Materials and Methods

Chemicals

1,1-Dipheny1–2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), potassium dihydrogen phosphate (KH_2PO_4), 3-(-2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), gallic acid, and (+)-catechin were all purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

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The other chemicals and solvents used in this experiment were of analytical grade.

Extraction of samples from different species and parts of wild grapes

Fresh leaves and stems of *V. thunbergii, V. flexuosa* and *V. kelungeusis* were sampled from a 15 m×15 m plot, at the agricultural experimental station of Natl. Chung Hsing Univ., in Taichung County. The species were identified by Prof. Tzer-Kuan Hu (Dept. of Agronomy, Natl. Chung Hsing Univ.). The samples were cleaned with tap water and dried at room temperature (25 °C), for a week. Dried leaves and stems of *V. thunbergii* (VTL: 2.3 kg and VTS: 2.4 kg), *V. flexuosa* (VFL: 1.4 kg and VFS: 3.2 kg) and *V. kelungeusis* (VKL: 5.3 kg and VKS: 4.4 kg) were then extracted, with 95% methanol (20 L), by soaking each one for a week at room temperature 2 times. The extracts were decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The resulting crude extract of *V. thunbergii* stems (128.8 g) was fractionated successively with ethyl acetate (EtOAc), *n*-butanol (BuOH) and water to yield soluble fractions of EtOAc (27.6 g), BuOH (23.7 g) and H_2O (72.5 g).

Determination of total phenolic contents

Total phenolic contents were determined using the Folin–Ciocalteu method (Kujala and others 2000), using gallic acid as the standard. The test samples (5 mg) were dissolved in 5 mL of methanol/water (50:50, v/v). The extract solution (500 μL) was mixed with 500 μL of 50% Folin–Ciocalteu reagent. The mixture was allowed to rest, for 5 min, after which 1.0 mL of 20% Na_2CO_3 was added. After 10 min of incubation at room temperature, the mixture was centrifuged, for 8 min (12000 *g*), and the absorbance of the supernatant was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE), in milligrams per gram of sample. Each test was replicated 3 times.

1,1-Diphenyl-2-picrylhydrazyl assay (DPPH assay)

The DPPH radical-scavenging properties of the test extracts were examined using the method reported by Chang and others (2001). 10 μL of extract in methanol, with extract concentrations of 1, 5, 10, 50 and 100 μg/mL, respectively, was mixed with 200 μL of 0.1 mM DPPH-ethanol solution and 90 μL of 50 mM Tris-HCl buffer (pH 7.4). Methanol (10 μL), instead of sample solution, was used as the control for this experiment. After 30 min of incubation at room temperature, the reduction in DPPH radicals was measured by noting the absorbance (517 nm). (+)-Catechin was used as the positive control. Each test was replicated 3 times. The inhibition ratio was calculated using the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

Superoxide radical-scavenging assay (NBT assay)

Measurement of superoxide radical-scavenging activity was carried out, using the same method as Kirby and Schmidt (1997). Firstly, 20 μL of 15 mM Na_2EDTA in buffer (50 mM KH_2PO_4/KOH, pH 7.4), 50 μL of 0.6 mM NBT in buffer, 30 μL of 3 mM hypoxanthine in 50 mM KOH, 5 μL of test sample in methanol, and 145 μL of buffer were mixed in 96-well microplates (Falcon, Franklin Lakes, N.J., U.S.A.). The reaction was initiated by adding 50 μL of xanthine oxidase solution in buffer (1 unit in 10 mL buffer) to the mixture. The reaction mixture was incubated at room temperature and absorbance at 570 nm was determined every 1 min, for 9 min using a plate reader (Labsystems multiskan, Helsinki, Finland). The control was 5 μL of methanol, instead of the sample solution. (+)-Catechin was used as the positive control. Each test was replicated 3 times. The inhibition ratio was calculated using the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

Ferrous ion-chelating ability assay

The ferrous ion-chelating potential of the test samples was evaluated using the same method as Dinis and others (1994). In brief, 200 μL of the test samples in methanol (final concentrations of 125, 250, 500, 1000, and 2000 μg/mL, respectively) and 740 μL methanol were added to 20 μL of 2 mM $FeCl_2$. The reaction was initiated by adding 40 μL of 5 mM ferrozine. The mixture was shaken vigorously and then allowed to rest at room temperature for 10 min. Absorbance of the solution was then measured as 562 nm. Ethylenediaminetetraacetia acid (EDTA) was used as the positive control. Each test was replicated 3 times. The percent of inhibition of Fe^{2+}-ferrozine complex formation was calculated using the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

Reducing power assay

This assay was accomplished using the method reported by Oyaizu (1986), using (+)-catechin as the standard. In brief, 1 mL of reaction mixture, containing 500 μL of the test sample in 500 μL of phosphate buffer (0.2 M, pH 6.6), was incubated with 500 μL of potassium ferricyanide (1%, w/v) at 50 °C for 20 min. The reaction was terminated by addition of trichloroacetic acid (10%, w/v) and then the mixture was centrifuged at 12000 *g* for 10 min. The supernatant solution (500 μL) was mixed with distilled water (500 μL) and 100 μL of ferric chloride (0.1%, w/v) and then the optical density (OD) was measured at 700 nm. The reducing power ability is expressed as (+)-catechin equivalents (CEs) in milligrams per gram of sample, or in millimolars per molar pure phytochemical. Each test was replicated 3 times.

Isolation and identification of phytochemical

The phytochemical from the BuOH fraction of the *V. thunbergii* stems was separated and purified with semipreparative high-performance liquid chromatography (HPLC), using a pump equipped with a photodiode array (PDA) detector (200 to 650 nm) and a 250 mm × 10.0 mm i.d., 4-μm Synergi Polar-RP column (Phenomenex, Torrance, Calif., U.S.A.). The mobile phase was solvent **A**, 100% methanol; and solvent **B**, ultrapure water. Elution conditions were 0 to 40 min of 30% to 70% **A** to **B** (linear gradient) at a flow rate of 4 mL/min. Nuclear magnetic resonance (NMR) spectra were recorded, using a Bruker Avance 500 NMR spectrometer (Rheinstetten, Germany), in CD_3OD. Chemical shifts, δ (ppm), were determined relative to the solvent CD_3OD (δ_H 3.31, δ_C 49.1), as an internal standard. MS spectra were measured using a Finnigan LCQ ion-trap mass spectrometer (San Jose, Calif., U.S.A.). Spray voltage was 4.0 kV, capillary temperature was 280 °C, and sheath gas flow rate was 5 arbitrary units. The major antioxidant, that is, compound **1**, was detected at retention time of 16.9 min in the HPLC chromatogram, as shown in Figure 1A, and was identified using fast atom bombardment mass spectrometer (FABMS) and NMR. All spectral data for compound **1** were consistent with those in the literature (Dad and others 1989).

Acid hydrolysis of (+)-lyoniresinol-2a-O-β-d-glucopyranoside

The acid hydrolysis procedures were based on the method reported by Wang and others (2002). Twenty milligrams of (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside was dissolved in 2 mL of 95% ethanol/2 N HCl (1:1, v/v) solution in a securely sealed reaction bottle and then heated to 60 °C for 60 min. After repeated cycles of liquid/liquid partition with water/EtOAc (1:1), compound **2** (Figure 1) was then sampled from the pool of EtOAc extracts. The structure of compound **2** was identified using electron ionization mass spectrometer (EIMS) and NMR. All spectral data were consistent with those in literature (Rahman and others 2007).

(+)-Lyoniresinol-2a-*O*-β-D-glucopyranoside (**1**): an amorphous white powder; mp: 178 − 180 °C; $[\alpha]^{20}_{D}$ +22.4° (MeOH; *c* 1.01); UV (MeOH), λ_{max}, nm (log ε): 276 (3.68); FABMS *m/z*: 605 $[M + Na]^+$, 582 $[M]^+$, 420 [aglycone]$^+$, calcd for $C_{28}H_{38}O_{13}$; ^{1}H NMR (500 MHz, CD_3OD): δ 6.57 (s, 1, H-5), 6.42 (s, 2, H-2′, 6′), 4.41 (d, 1, *J* = 6.2 Hz, H-1), 4.27 (d, 1, *J* = 7.8 Hz, H-1″), 3.88 (dd, 1, *J* = 9.9, 5.6 Hz, H_b-2a), 3.85 (s, 3H, 6-OCH_3), 3.82 (m, 1, H_a-6″), 3.74 (s, 6H, 3′,5′-OCH_3), 3.66 (m, 1, H_b-6″), 3.63 (dd, 1, *J* = 10.9, 4.0 Hz, H_a-3a), 3.54 (dd, 1, *J* = 10.9, 6.6 Hz, H_b-3a), 3.45 (dd, 1, *J* = 9.9, 4.0 Hz, H_a-2a), 3.33 (s, 3H, 8-OCH_3), 3.21 to 3.38 (m, 4H, H-2″, 3″, 4″, 5″), 2.71 (dd, 1, *J* = 15.2, 4.7 Hz, H_b-4), 2.61 (dd, 1, *J* = 15.2, 11.8 Hz, H_a-4), 2.07 (m, 1, H-2), 1.70 (m, 1, H-3); ^{13}C NMR (125 MHz, CD_3OD): δ 149.0 (C-3′, 5′), 148.6 (C-6), 147.6 (C-8), 139.3 (C-1′), 138.9 (C-7), 134.5 (C-4′), 130.2 (C-10), 126.4 (C-9), 107.8 (C-5), 106.9 (C-2′, 6′), 104.8 (C-1″), 78.2 (C-3″), 77.9 (C-5″), 75.2 (C-2″), 71.7 (C-4″), 71.4 (C-2a), 66.2 (C-3a), 62.8 (C-6″), 60.2 (8-OCH_3), 56.8 (3′,5′-OCH_3), 56.6 (6-OCH_3), 46.7 (C-2), 42.8 (C-1), 40.6 (C-3), 33.8 (C-4).

(+)-Lyoniresinol (**2**): an amorphous white solid; mp: 170 to 171 °C; $[\alpha]^{20}_{D}$ +13.3° (MeOH; *c* 0.32); UV (MeOH), λ_{max}, nm (log ε): 278 (3.65); EIMS *m/z*: 420 $[M]^+$ (100), 402 (6), 389 (5), 371 (21), 248 (15), 217 (33), 210 (18), 205 (39), 183 (54), 167 (49), calcd for $C_{22}H_{28}O_8$; ^{1}H NMR (500 MHz, CD_3OD): δ 6.57 (s, 1, H-5), 6.37 (s, 2, H-2′, 6′), 4.30 (d, 1, *J* = 5.7 Hz, H-1), 3.84 (s, 3H, 6-OCH_3), 3.72 (s, 6H, 3′,5′-OCH_3), 3.59 (dd, 1, *J* = 10.9, 5.0 Hz, H_a-3a), 3.48 (m, 2, H_2-2a), 3.48 (m, 1, H_b-3a), 3.36 (s, 3H, 8-OCH_3), 2.69 (dd, 1, *J* = 14.9, 4.8 Hz, H_b-4), 2.56 (dd, 1, *J* = 14.9, 11.5 Hz, H_a-4), 1.96 (m, 1, H-2), 1.62 (m, 1, H-3); ^{13}C NMR (125 MHz, CD_3OD): δ 149.0 (C-3′, 5′), 148.6 (C-6), 147.7 (C-8), 139.3 (C-1′), 138.9 (C-7), 134.5 (C-4′), 130.2 (C-10), 126.2 (C-9), 107.8 (C-5), 106.8 (C-2′, 6′), 66.8 (C-3a), 64.2 (C-2a), 60.2 (8-OCH_3), 56.8 (3′,5′-OCH_3), 56.6 (6-OCH_3), 48.8 (C-2), 42.3 (C-1), 40.9 (C-3), 33.5 (C-4).

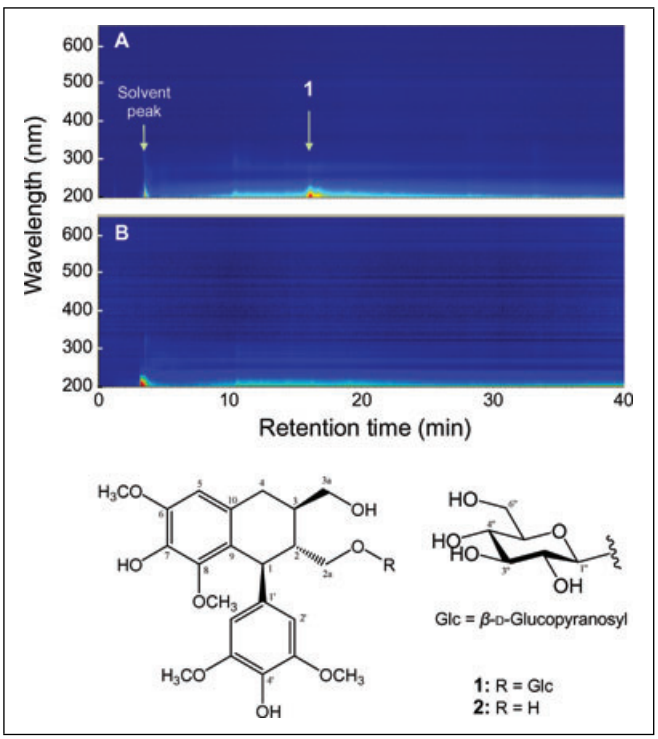

Figure 1–HPLC–PDA chromatograms of the BuOH fraction of *Vitis thunbergii* stem with (A) and without (B) compound 1. 1 = (+)-lyoniresinol-2a-*O*-β-d-glucopyranoside; 2 = (+)-lyoniresinol (prepared from the acid hydrolysis of 1).

Statistical analyses

All results are expressed as mean ± SD ($n = 3$). The significance of difference was calculated by Scheffe's test. $P < 0.05$ was considered to be statistically significant. Comparisons of total phenolic contents and various antioxidant properties were carried out using Pearson's correlation test.

Results and Discussion

Total phenolic contents of different species and parts of wild grapes

Phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects, including antioxidant properties (Da Silva and others 1991; Sato and others 1996). Many studies have revealed that phenolic content in plants can be correlated to their antioxidant properties (Yen and Hsieh 1998; Chang and others 2001; Wangensteen and others 2004).

In this study, Table 1 shows the total phenolic contents for VTL, VTS, VKL, VKS, VFL, and VFS, calculated as GAE, in milligrams per gram of sample. Total phenolic contents of the

Table 1–Antioxidant performance and total phenolic contents of the methanolic extracts from different species and parts of wild grapes.

Species	Plant parts	Extract yields (wt%)	IC_{50} (μg/mL) DPPH radical	IC_{50} (μg/mL) Superoxide radical	IC_{50} (μg/mL) Fe^{2+} chelating	Reducing power (mg CE/g)	Phenolic contents (mg GAE/g)
V. thunbergii	Leaf	5.4	14.6 ± 0.4^{C}	8.0 ± 0.2^{B}	>2000	257.7 ± 14.0^{A}	164.5 ± 1.4^{B}
	Stem	6.7	16.0 ± 0.3^{C}	3.6 ± 0.1^{B}	263.8 ± 13.6^{C}	184.1 ± 9.3^{C}	179.5 ± 4.9^{A}
V. kelungeusis	Leaf	1.6	16.5 ± 1.0^{C}	46.2 ± 14.8^{A}	>2000	244.3 ± 11.2^{AB}	167.3 ± 4.2^{AB}
	Stem	4.9	27.8 ± 0.3^{B}	60.3 ± 8.7^{A}	1888.6 ± 224.9^{A}	224.9 ± 9.7^{B}	175.2 ± 2.1^{AB}
V. flexuosa	Leaf	3.6	81.2 ± 1.5^{A}	>100	691.3 ± 141.0^{B}	81.0 ± 4.0^{E}	37.5 ± 1.9^{D}
	Stem	3.2	28.6 ± 1.0^{B}	4.0 ± 0.7^{B}	>2000	147.2 ± 3.1^{D}	118.8 ± 7.5^{C}

Different superscript capital letters within a column indicate significant difference at $P < 0.05$.

methanolic extracts decrease in the order of VTS (179.5 mg/g), VKS (175.2 mg/g), VKL (167.3 mg/g), VTL (164.5 mg/g), VFS (118.8 mg/g), and VFL (37.5 mg/g). This result suggests that the antioxidant activity of *Vitis* species extracts may correlate to their phenolic contents, and it is proposed here that the abundant phenolic compounds are present in VTS.

Antioxidant performances of different species and parts of wild grapes

Table 1 shows the yields of methanolic extracts obtained from different species and parts of wild grapes to be variable, for example, VTL (5.4%) provides over 3 times more than VKL (1.6%) and VTS (6.7%) provides over 2 times more than VFS (3.2%). To determine the antioxidant properties of these methanolic extracts from wild grapes, DPPH, NBT, reducing power, and ferrous ion-chelating ability assays were performed. As shown in Table 1, the IC_{50} values (the concentration required to inhibit radical formation by 50%) for DPPH radical in VTL, VTS, VKL, VKS, VFL, and VFS were 14.6, 16.0, 16.5, 27.8, 81.2, and 28.6 μg/mL, respectively. This result shows that both leaves and stems of *V. thunbergii* significantly inhibit formation of the DPPH radical. Similarly, methanolic extracts of the leaves showed the same order (VTL > VKL > VFL) for the superoxide radical-scavenging activity, with IC_{50} values of 8.0, 46.2, and >100 μg/mL, respectively. It can also be seen that the order of efficiency for superoxide radical scavenging, for the stems, was VTS (IC_{50} = 3.6 μg/mL) ≥ VFS (IC_{50} = 4.0 μg/mL) > VKS (IC_{50} = 60.3 μg/mL). With regard to ferrous ion-chelating ability, VTS showed the best inhibitory performance, with an IC_{50} value of 263.8 μg/mL. However, VTL (257.7 mg CE/g) exhibited the highest reducing power, while VFL (81.0 mg CE/g) exhibited the lowest. These results indicate that, with the exception of reducing power, the antioxidant performance of the methanolic extracts from the *Vitis* species is effectively greatest for VTS.

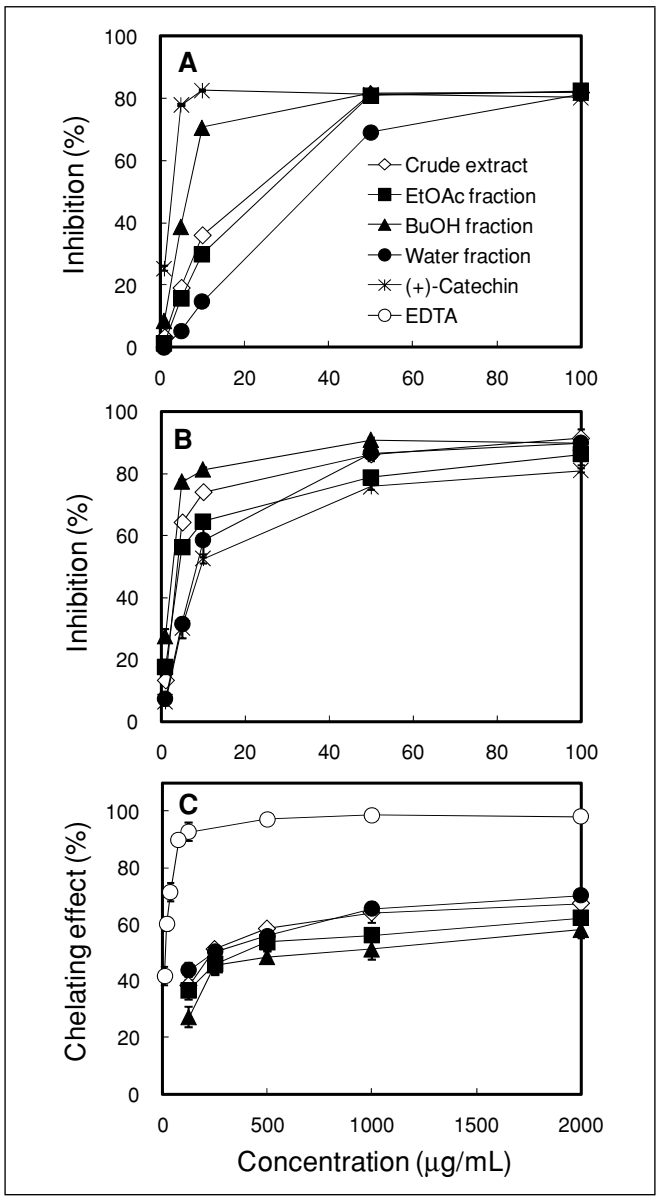

Figure 2–Antioxidant performance of methanolic extract from the stem of *V. thunbergii* and its derived soluble fractions. (A) DPPH radical-scavenging performance, (B) superoxide radical-scavenging performance, (C) ferrous ion-chelating ability. Results are expressed as mean ± SD (n = 3).

Antioxidant activities of VTS extract and its derived soluble fractions

Based on the bioactivity-guided isolation principle, stem extracts of *V. thunbergii* have potential for use as a natural antioxidant, so the antioxidant performance of VTS extracts was further assessed. As shown in Figure 2A, the DPPH radical-scavenging performance of the VTS methanolic extract and its derived soluble fractions, including the soluble fractions of EtOAc, BuOH, and water, is dose-dependent. The BuOH fraction showed the

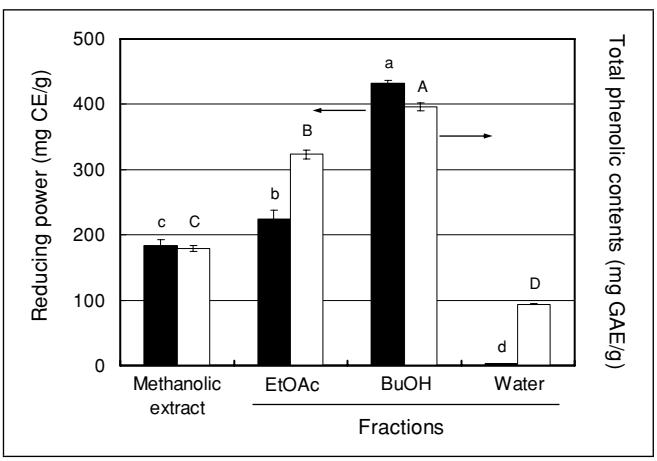

Figure 3–Reducing power (black bar) and total phenolic contents (white bar) of the methanolic extract from the stem of *V. thunbergii* and its derived soluble fractions. Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences between groups (P < 0.05).

Table 2– Antioxidant performance of the BuOH fraction of *V. thunbergii* stem with and without the major compound 1.

Extracts	IC_{50} (μg/mL) DPPH radical	Superoxide radical	Reducing power (mg CE/g)
BuOH fraction[a]	7.3 ± 0.4[B]	6.4 ± 0.6[B]	379.3 ± 11.8[A]
BuOH fraction without compound 1[b]	25.7 ± 0.6[A]	34.6 ± 2.1[A]	199.2 ± 5.9[B]

Different superscript capital letters within a column indicate significant difference at P < 0.05.
[a]The extract was collected and concentrated from all eluents of HPLC during the retention time of 0 to 40 min in Figure 1A.
[b]The extract was collected and concentrated from all eluents, except for compound 1, during the retention time of 0 to 40 min in Figure 1A.

strongest performance. With the exception of the water fraction, all extracts showed good inhibitory performance with respect to the DPPH radical. The IC_{50} values of the crude extract, EtOAc fraction, BuOH fraction, and water fraction were 16.0, 17.7, 6.9, and 36.2 μg/mL, respectively.

Similarly, the BuOH fraction exhibited the highest superoxide radical-scavenging performance (Figure 2B). The IC_{50} values of (+)-catechin, crude extract, EtOAc fraction, BuOH fraction, and water fraction were 4.2, 3.6, 4.4, 2.3, and 8.2 μg/mL, respectively. The chelating effect of the test samples on ferrous ions is shown in Figure 2C. As can be seen, the chelating ability of the test samples increases, as concentration increases from 125 to 2000 μg/mL. The crude extract and water fraction exhibited excellent chelating ability. The IC_{50} values for crude extract, EtOAc, BuOH, and water fractions were 263.8, 362.4, 742.5, and 248.9 μg/mL, respectively.

It has been reported that chelating agents are effective as secondary antioxidants, because they reduce redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon 1990). According to data in Figure 2C, the BuOH fraction is not a good secondary antioxidant, because of its poor capacity for metal ion binding, but it is an excellent primary antioxidant (or free radical scavenger). Figure 3 shows that the reducing power of the BuOH fraction (431.9 mg CE/g) is higher than that of the crude extract (184.1 mg CE/g), EtOAc fraction (224.8 mg CE/g), and water fraction (22.4 mg CE/g). Again, the BuOH fraction exhibited the greatest reducing power.

Many studies have reported a direct correlation between antioxidant performance and reducing power, for various plant extracts (Duh and others 1999; Kumaran and Joel karunakaran 2006). In this study, a similar correlation was also observed. In addition, the total phenolic contents of the BuOH fraction also showed the highest value (396.2 mg GAE/g), followed by EtOAc fraction (323.1 mg GAE/g), crude extract (179.5 mg GAE/g), and water fraction (93.7 mg GAE/g) (Figure 3). Zhao and others (2006) reported that the total phenolic contents in 3 barley varieties ranged from 1.03 to 1.87 mg GAE/g. Additionally, the result reported by Luximon-Ramma and others (2006) showed that the crude extract of black tea leaves exhibited high total phenolic contents of 39 to 94 mg GAE/g. A comparison of our results with these earlier studies indicates that VTS extracts are richer in phenolic compounds than those of daily phyto-beverages.

Correlations between total phenolic contents and antioxidant activities

Many studies have revealed that the phenolic contents of plants are related to their antioxidant performance, and that the antioxidant performance of phenolic compounds is probably related to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Chang and others 2001).

According to the previous studies, antioxidant performance seems to increase in proportion to phenolic contents. In this study, we noted a linear relationship between total phenolic contents and antioxidant performance, verified using Pearson's correlation test. Our results show that strong correlations exist between total phenolic contents and DPPH, NBT, ferrous ion-chelating ability, and reducing power, with R^2 values of 0.86, 0.81, 0.85, and 0.87 (all P-values < 0.01), respectively. Similarly, there is a strong correlation between total phenolic contents and antioxidant performance, for common grape berries (*V. vinifera* L.) (Kedage and others 2007). This study shows that phenolic compounds in VTS play an important role in its performance as an antioxidant.

Identification of the major antioxidant in *V. thunbergii* stems

In previous antioxidant assays, we found that the BuOH soluble fraction of VTS exhibited powerful antioxidant properties and that it has potential as a natural antioxidant, so the BuOH soluble fraction was further separated, using semipreparative HPLC. As shown in Figure 1A, the principal phytochemical, (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside (**1**), was isolated from the BuOH soluble fraction and identified. In order to evaluate the effect of this compound on antioxidant performance, the remaining extract (that is, remove compound **1** from the BuOH fraction) was prepared using HPLC. Its PAD profile is shown in Figure 1B. Comparing the data for the BuOH fraction, in Table 2, it can be clearly seen that the remaining extract exhibits an approximate 2- to 5-fold decrease in antioxidant performance. This proves that lyoniresinol-type lignan glycoside, (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside, is an important antioxidant in VTS. Only a few papers have discussed the subjects of lignan derivatives in grapes and the lignans of *V. thunbergii*, published in the literature, belong to isolariciresinol derivatives (He and others 2004). However, the results of this study demonstrate that the antioxidant properties of VTS depend on the presence of lyoniresinol-type lignan glycoside.

It has also been reported that many phytochemicals containing glycoside may be less effective, as antioxidants, than their aglycone forms, under *in vitro* assay conditions (Kiato and others 1993; Wang and others 2002). However, Table 3 demonstrates that the antioxidant performance of (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside is stronger than that of (+)-lyoniresinol (an aglycone of (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside). The IC_{50} values of (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside and (+)-lyoniresinol were 14.6 μM and 22.0 μM in DPPH assay, and 22.4 μM and >100 μM in NBT assay. The reducing powers of both compounds were 909.6 mM CE/M and 560.7 mM CE/M, respectively. These results show that lignan glycoside also has excellent antioxidant activities. Similar results have also been reported by Sadhu and others (2007). Our results provide promising baseline information for the potential use of crude VTS extract and the isolated compound, as antioxidative supplements.

Table 3–Antioxidant performance and content of the principal phytochemical from the BuOH fraction of *V. thunbergii* stem.

Phytochemicals	Content (mg/g of crude extract)	IC_{50} (μM)		Reducing power (mM CE/M)[a]
		DPPH radical	Superoxide radical	
(+)-Lyoniresinol-2a-*O*-β-D-glucopyranoside	2.5 ± 0.2	14.6 ± 0.9^{B}	22.4 ± 0.7^{B}	909.6 ± 17.9^{A}
(+)-Lyoniresinol[b]	–	22.0 ± 0.1^{A}	>100	560.7 ± 4.5^{B}
(+)-Catechin (positive control)	–	11.3 ± 0.5^{C}	27.4 ± 1.4^{A}	–

Different superscript capital letters within a column indicate significant difference at $P < 0.05$.
[a](+)-Catechin equivalent in millimolars per molar sample.
[b]The compound was prepared from the acid hydrolysis of (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside.

Conclusions

It is well-known that free radicals are one of the causes of several diseases. This study demonstrated that the *Vitis* species, especially *V. thunbergii* stems, perform excellently as antioxidants. The specific antioxidant, (+)-lyoniresinol-2a-*O*-β-D-glucopyranoside, which was isolated and identified in the *V. thunbergii* stems, exhibited stronger antioxidant performance than its lignan aglycone form, (+)-lyoniresinol. Our results show that these extracts, or the phytochemical derived from the *V. thunbergii* stems, could be used to prevent diseases caused by the overproduction of radicals and might also be suitable for the treatment of degenerative diseases. Future studies should focus on *in vivo* pharmacological research.

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References

Ames BN, Gold LS, Willett WC. 1995. The causes and prevention of cancer. Proc Natl Acad Sci 92:5258–65.

Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. 2001. Antioxidant activity of extracts from *Acacia confusa* bark and heartwood. J Agric Food Chem 49:3420–4.

Da Silva JMR, Darmon N, Fernandez Y, Mitjavila S. 1991. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J Agric Food Chem 39:1549–52.

Dad G, Corbani A, Manitto P, Speranza G, Lunazzi L. 1989. Lignan glycosides from the heartwood of European oak *Quercus petraea*. J Nat Prod 52:1327–30.

Dinis TCP, Madeira VMC, Almeida, LM. 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 315:161–9.

Duh PD, Du PC, Yen GC. 1999. Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. Food Chem Toxicol 37:1055–61.

Gordon MH. 1990. The mechanism of antioxidant action *in vitro*. In: Hudson BJF, editor. Food Antioxidants. London: Elsevier. p 1–18.

Halliwell B. 1997. Antioxidants and human disease: a general introduction. Nutr Rev 55:S44–S52.

Halvorsen BL, Holte K, Myhrstad MCW, Barikmo I, Hvattum E, Remberg SF, Wold AB, Haffner K, Baugerød H, Andersen LF, Moskaug Ø, Jacobs DR Jr, Blomhoff R. 2002. A systematic screening of total antioxidants in dietary plants 1. J Nutr 132:461–71.

He YH, Dou DQ, Terashima K, Takaya Y, Niwa M. 2004. Two lignan glycosides from *Vitis thunbergii*. Heterocycles 63:871–7.

Huang YL, Tsai WJ, Shen CC, Chen CC. 2005. Resveratrol derivatives from the roots of *Vitis thunbergii*. J Nat Prod 68:217–20.

Kedage VV, Tilak JC, Dixit GB, Devasagayam TP, Mhatre M. 2007. A study of antioxidant properties of some varieties of grapes (*Vitis vinifera* L.). Crit Rev Food Sci Nutr 47:175–85.

Kiato S, Ariga T, Matsudo T, Sekine H. 1993. The syntheses of catechin-glucosides by transglycosylation with *Leuconostoc mesenteroides* sucrose phosphorylase. Biosci Biotechnol Biochem 57:2010–5.

Kirby AJ, Schmidt RJ. 1997. The antioxidant activity of Chinese herbs for eczema and of placebo herbs–I. J Ethnopharmacol 56:103–8.

Kujala TS, Loponen JM, Klika KD, Pihlaja K. 2000. Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. J Agric Food Chem 48:5338–42.

Kumaran A, Joel karunakaran R. 2006. Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. Food Chem 97:109–14.

Liu JC, Chen JJ, Chan P, Cheng CF, Cheng TH. 2003. Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. Hypertension 42:1198–205.

Luximon-Ramma A, Neergheen VS, Bahorun T, Crozier A, Zbarsky V, Datla KP, Dexter DT, Aruoma OI. 2006. Assessment of the polyphenolic composition of the organic extracts of Mauritian black teas: a potential contributor to their antioxidant functions. Biofactors 27:79–91.

Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG. 1998. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 360:142–8.

Olas B, Wachowicz B. 2002. Resveratrol and vitamin C as antioxidants in blood platelets. Thromb Res 106:143–8.

Oyaizu M. 1986. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr 44:307–15.

Rahman MD, Katayama T, Suzuki T, Nakagawa T. 2007. Stereochemistry and biosynthesis of (+)-lyoniresinol, a syringyl tetrahydronaphthalene lignan in *Lyonia ovalifolia* var. *elliptica* I: isolation and stereochemistry of syringyl lignans and predicted precursors to (+)-lyoniresinol from wood. J Wood Sci 53:161–7.

Sadhu SK, Khatun A, Phattanawasin P, Ohtsuki T, Ishibashi M. 2007. Lignan glycosides and flavonoids from *Saraca asoca* with antioxidant activity. J Nat Med 61:480–2.

Sato M, Ramarathnam N, Suzuki Y, Ohkubo T, Takeuchi M, Ochi H. 1996. Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. J Agric Food Chem 44:37–41.

Wang SY, Kuo YH, Chang HN, Kang PL, Tsay HS, Lin KF, Yang NS, Shyur LF. 2002. Profiling and characterization antioxidant activities in *Anoectochilus formosanus* Hayata. J Agric Food Chem 50:1859–65.

Wangensteen H, Samuelsen AB, Malterud KE. 2004. Antioxidant activity in extracts from coriander. Food Chem 88:293–7.

Yen GC, Hsieh CL. 1998. Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models *in vitro*. J Agric Food Chem 46:3952–7.

Zhao H, Dong J, Lu J, Chen J, Li Y, Shan L, Lin Y, Fan W, Gu G. 2006. Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). J Agric Food Chem 54:7277–86.