RESEARCH ARTICLE

Phenolic compounds and antioxidant activities of grape canes extracts from vineyards

Yanlun Ju[1]*, Ang Zhang[2]*, Yulin Fang[1,3], Min Liu[1], Xianfang Zhao[1], Hua Wang[1,3], and Zhenwen Zhang[1,3]

[1] *Northwest A&F University, College of Enology. Yangling, Shaanxi 712100, China.* [2] *Entry-Exit Inspection and Quarantine Bureau, QingHuangDao, HeBei 066004, China.* [3] *Shaanxi Engineering Research Center for Viti-Viniculture, Yangling, Shaanxi 712100, China.*

* The first two authors contributed equally to this work.

Abstract

Grape canes are the main agro-wastes from vineyards. This work studied the antioxidant activities of the defatted methanolic extracts (ME) of canes from 11 genotypes: 5 *Vitis vinifera* widely known cultivars and 6 Chinese wild varieties from three species (*V. amurensis*, *V. davidii*, and *V. pentagona*) and the antioxidant activities of the ME's chloroform fractions (CF), ethyl acetate fractions (EAF) and water fractions (WF). Among ME and its three fractions, EAF's total phenolic contents (TPC) and total flavonoid contents (TFC) were the highest, at 586 mg/g of gallic acid equivalent and 320 mg/g of quercetin equivalent, respectively. The antioxidant power of the fractions/extracts was in the order EAF > ME > WF > CF, based on the DPPH radical-scavenging power and ferric-reducing antioxidant activity, while the order was EAF > CF > WF >ME based on the *β*-carotene-linoleic acid bleaching activity. Methanolic extracts demonstrated the strongest Fe^{2+}-chelating activity. The antioxidant activities of the extracts/fractions generally correlated with the TPC and TFC in all assays, except with the Fe^{2+}-chelating test. Grape canes from *V. davidii* had the highest TPC, TFC and antioxidant activities compared with those from other grape species. Catechin, epicatechin and *trans*-resveratrol were the predominant phenolic components of fractions/extracts. In light of these valuable bioactivities, grape canes from annual pruning practice considered as waste material have good commercial potential for utilization as a promising natural antioxidant in the food, pharmaceutical and cosmetic industries, given its low cost and availability in large amounts.

Additional key words: vineyard; flavonoids; antiradical effect; agro-wastes.

Introduction

Antioxidants are defined as 'substances' that, in small quantities, are able to protect biological systems from the potentially harmful effects of excessive oxidations. Antioxidants are also widely used as dietary supplements to delay or inhibit food deterioration, such as endogenous or exogenous, enzymatic or non-enzymatic, and natural or synthetic sources (Nardini *et al.*, 2005). Natural phenolics possess strong antioxidant properties that enable them to donate hydrogen, scavenge free radicals, break radical chain reactions, chelate metal ions, and quench singlet oxygen *in vitro* and *in vivo* (Rice-Evan *et al.*, 1996).

Previous studies have revealed that one-third of all cancer cases and one-half of cardiovascular diseases

can be attributed to irrational diets (Willet, 1994). In particular, phenolic antioxidants are often claimed to protect against cardiovascular diseases and certain tumors. Concurrently, synthetic antioxidants are forbidden in food because they are linked to carcinogenic activity in animals (Gharavi *et al.,* 2007). Recently, great emphases have been placed on the importance of searching for and exploiting more safety antioxidants to replace synthetic products and investigate their use as dietary supplements, functional food ingredients, pharmaceuticals and cosmetic products.

The main solid agro-wastes derived from the grape and wine industries are grape pomace or marc (peels and seeds), grape leaves, grape canes and grape stems. Some researchers have found that these wastes could be sources of phenolic antioxidants, such as anthocyanins, flavonoids and phenolic acids (Dani *et al.,* 2010). Grapevines are pruned annually, and these wastes are usually burned or used as fuels (Garg & Gupta, 2009), thus offering no direct economic benefits. Only a few utilization options are available for grape canes. For example, recent studies suggested that grape canes can be used as raw materials for the production of activated carbon, compost and biosurfactants (Nabais *et al.,* 2010). Sierra *et al.* (2008) reported that the extraction yields of *trans*-resveratrol and *trans*-viniferin from *Vitis vinifera* cv. 'Pinot Noir' grape canes were 3.45 ± 0.04 and 1.30 ± 0.07 mg/g dw, respectively, and Zhang *et al.* (2007) determined that 'Pinot Noir' grape canes had high individual phenolic levels. Although the occurrence of bioactive compounds in grape canes, such as phenolic acids and *trans*-resveratrol, has been reported by several authors, there is currently a considerable lack of information regarding the antioxidant capacity of grape canes compared with other grape-derived wastes, such as pomace. China is playing an increasingly important role in the grape and wine industry. More than two million tons of vine shoot waste is produced in China. It will be helpful for the sustainable development of related industries if we use these low-cost residues in a suitable manner.

In recent years, researchers have made great efforts to investigate the antioxidant capacities of grape pomace, seed and stems but have neglected grape canes. As part of our on-going work on the potential utilization of grape cane wastes, the antioxidant properties of crude methanolic extracts from 5 *Vitis vinifera* widely known cultivars and 6 Chinese wild varieties from three species (*V. amurensis*, *V. davidii,* and *V. pentagona*) were assessed using different methods *in vitro*, such as free radical-scavenging and reducing power capabilities. The result of this work should promote a better understanding and exploration of potential phenolic antioxidants from pruning grape canes.

Material and methods

Plant material

We used the following genotypes: (i) 5 grapevine lines (*V. vinifera*), 'Hongmeigui', 'Cabernet Sauvignon', 'Chardonnay', 'Pinot Noir' and 'Victoria Blanc', collected from Yangling, Shaanxi Province; (ii) 6 Chinese wild varieties, including three wine grapes, 'Shuanghong', 'Beibinghong' and 'Shuangyou', of *V. amurensis* from Tonghua, Jilin Province; one variety, 'Maoputao', of *V. pentagona* from Lantian, Shaanxi Province; and two wine grapes, 'Junzi' and 'Baiyu', of *V. davidii* from Chongyi, Jiangxi Province. Ideal grape canes with moderate vigor (0.8-1.0 cm diameter) were collected during the 2008 pruning period (Fig. 1). First, all sampled vine shoot samples were frozen in liquid nitrogen. Then, samples were lyophilized under -50°C in an lyophilizer (VirTis Genesis 25 XL, Gardiner, NY, USA) and ground using a domestic electrical grinder (final particle size <0.5 mm). Powdered grape canes were packed in air-tight bags and stored at -20 °C before use.

Chemicals

Reagents as 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), tertiary butylhydroquinone (TBHQ), ferrozine, β-carotene, Folin−Ciocalteu's phenol regent, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), linoleic acid, and all the phenolic compounds (purity > 97%) were purchased from Sigma-Aldrich (Shanghai, China). Methanol and acetonitrile were obtained from Kermel Chemical Reagent Co. Ltd. (Tianjin, China) and were of HPLC

Figure 1. Vine shoots of four grape species. From left to right, Cabernet Sauvignon, Maoputao, Junzi and Beibinghong.

grade. Water was double distilled and purified through a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were obtained from Xi'an Chemical Reagent Co. Ltd. (Xi'an, China) and were of analytical grade.

Extraction

The procedure used to prepare the extracts/fractions is presented in Fig. 2. Pulverized vine canes of each cultivar (100 g, dw) were extracted with 1000 mL of 1 mol/L HCl/methanol/water (1:80:19, v/v/v) and centrifuged using a high-speed refrigerated centrifuge for 20 min at 12000 rpm and 4 °C. The resulting supernatant was collected and extracted thrice. The combined extracts were evaporated (Büchi RE-111, Switzerland)

Figure 2. Scheme of extraction and fractionation of phenolics from vine shoots.

under 35 °C to remove methanol and were then defatted with petroleum ether (3:1, v/v). The remaining aqueous extract was lyophilized to obtain methanolic extracts (ME), which were re-suspended in distilled water (1:10, w/v) and then successively partitioned with an equal volume of chloroform and ethyl acetate (3:1, v/v) to afford chloroform fractions (CF) and ethyl acetate fractions (EAF), respectively. The remaining extract was freeze-dried and considered to be water fractions (WF). The ME and its three fractions were dissolved in methanol (5 mg/mL) and stored at -20 °C for further analysis. The yields of extracts/fractions were calculated and expressed as percent of the dry plant sample (%, w/w).

Determination of total phenolics and flavonoids

The determination of total phenolic contents (TPC) of ME and its fractions was performed according to Folin-Ciocalteu colorimetric method (Singleton *et al.,* 1999). The determination of total flavonoids contents (TFC) was performed according to the aluminum chloride colorimetric method (Chang *et al.,* 2002).

DPPH radical-scavenging activity

The free radical-scavenging activity of each extract/fraction was measured according to the procedure of Brand-Williams *et al.* (1995). The results were expressed as the IC50 values (μg/mL).

Fe^{2+} reducing antioxidant power

The ability to reduce Fe^{2+} was measured according to the protocol of Benzie & Strain (1996). The fresh working solution was prepared by mixing 300 mM acetate buffer (pH 3.6) with 10 mM 2,4,6-tri(2-pyridyl)-s-triazine solution (dissolved in 40 mM HCl) and 20 mM FeCl$_3$ solution (10:1:1, v/v/v). The solution was stored at 37 °C before use with 100 μL distilled water, followed by 2.85 mL of working solution. The absorbance value was measured at 593 nm after a 30-min incubation at 37 °C. The results were calculated using regression equations and expressed as mM Trolox equivalents/g extracts or fractions (mM TE/g).

Chelating of metal ions

This test was performed according to the protocol of Višnja *et al.* (2010). Briefly, the adequately diluted
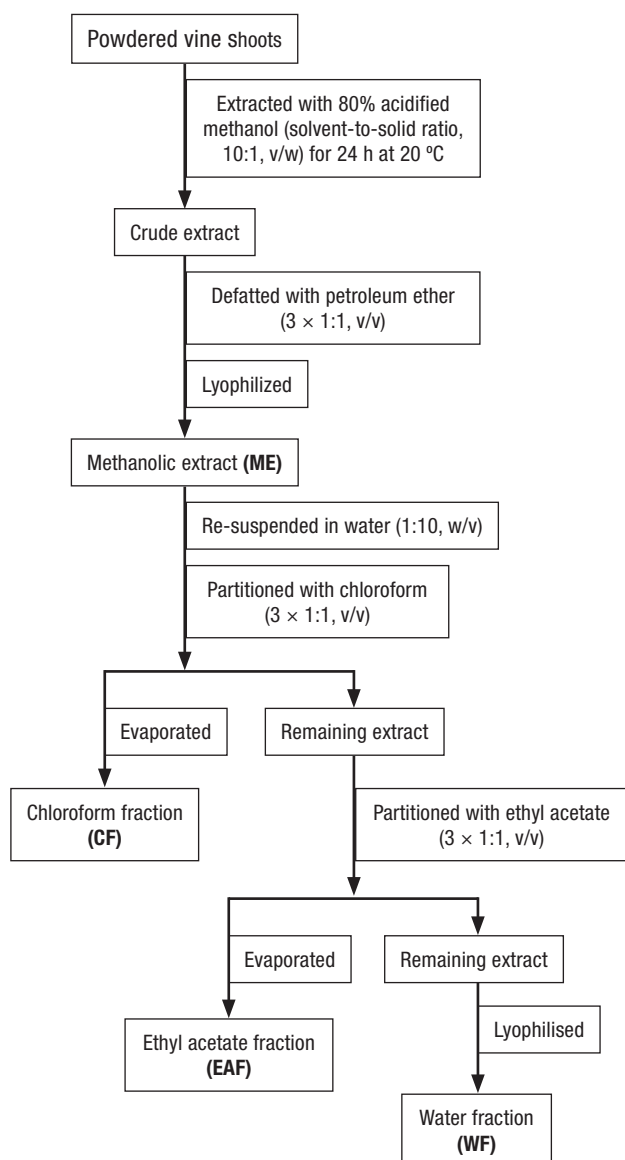
grapevine extract (1 mL) was mixed with methanol (3.7 mL), 2 mM $FeCl_2$ (0.1 mL) and 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and left standing at room temperature in the dark for 10 min. The absorbance of the resulting solution was measured spectrophotometrically at 562 nm. A low absorbance of the resulting solution indicated a strong Fe^{2+}-chelating ability. The ability to chelate ferrous ion and prevent the formation of ferrous ion-ferrozine complex was calculated using the following equation:

$$\text{Chelating effect (\%)} = [1-(A_{sample}/A_{control})]\times100,$$

where $A_{control}$ was the absorbance of a mixture of methanol (4.7 mL), 2 mM $FeCl_2$ (0.1 mL) and 5 mM ferrozine (0.2 mL). All analyses were performed in triplicate and averaged. Sample concentration providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentage against extract concentration.

β-carotene-linoleic acid bleaching assay

The assay was performed in a modified β-carotene/linoleic acid emulsion system. Briefly, an aliquot (10 mL) of 0.2 mg/mL β-carotene chloroform solution was thoroughly mixed with 200 mg of linoleic acid and 2000 mg Tween 20. The mixture was then evaporated at 40 °C to remove chloroform, and the residue was diluted with 1000 mL distilled water to form an emulsion after a vigorous agitation. Each sample (1 mL) diluted in 5 mL of the emulsion was tested at a final concentration of 200 mg/L. The reaction mixture with samples displaced by the same volume of methanol was the control, and for the positive control, *tert*-butylhydroquinone (TBHQ) was used. After 3 h in a water bath (50 °C), the zero time absorbance was immediately recorded at 470 nm and successively at 180 min against a blank consisting of an emulsion without β-carotene. The antioxidant activity (AA) was calculated using the following equation: AA (%) = $[(As_0 - As_{180}) / (Ac_0 - Ac_{180})] \times 100$. As_0 and Ac_0 represent the absorbance values for the samples and control, respectively, which were measured at the initial incubation time (t = 0 min), whereas As_{180} and Ac_{180} are the absorbance values for samples and control, respectively, which were measured at the end of the incubation time (t = 180 min).

HPLC analysis of individual phenolics

The chromatographic analyses were performed using a liquid chromatograph system (Shimadzu LC-2010AHT, Kyoto, Japan) equipped with a quaternary pump, a photodiode array detector (DAD) and a UV detector. The DAD detector was applied to scan phenolic compounds of interest to ascertain their maximal absorbance wavelengths. The variable UV detector was used for quantitative purposes with the external standard method. All standards were dissolved in methanol at a stock concentration of 1 mg/mL. A calibration standard mixture was prepared by appropriate dilutions with methanol from the stock solution. Known amounts of extracts/fractions were dissolved in methanol. All solutions were stored in the dark at -40 °C and filtered through 0.22-µm membranes before injection.

The chromatographic conditions used were similar to those of the method published by Zhang *et al.* (2007). Briefly, a gradient solvent system was employed, where solvent A was water-acetic acid (97:3, v/v) and solvent B was acetonitrile. The elution profile had the following proportions (v/v) of solvent B: 0.00-5.00 min, 0-8.5%; 5.00-16.50 min, 8.5-2.0%; 16.50-35.00 min, 2.0-18%; 35.00-50.00 min, 18-20%; 50.00-65.00 min, 20-30%; 65.00-70.00 min, 30-0%. The wavelength-switching program was employed. The column was held at 30 °C and was flushed at a flow rate of 0.8 mL/min. The UV detector was used for quantitative purposes with the external standard. The injection volume for all solutions was 10 µL, and the procedure was performed in triplicate. The linearity of the method was established by automatic injections of the standard mixture solutions at six calibration levels in three replicates from low to high concentrations. All data were processed using the Shimadzu Workstation CLASS-VP 6.12 software.

Statistical analysis

All determinations were performed in triplicate, and the results were expressed as the means and standard deviations (SD). The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to compare the difference between means by Duncan's *t*-tests, and $p < 0.05$ was considered significant. Correlations between antioxidant capacities and phenolic contents were computed as Pearson's correlation coefficients (*r*).

Results and discussion

Extraction yields, TPC and TFC of extracts/fractions

Different extraction mediums have significant effects on extraction efficiency, which is an important factor for the recuperation of bioactive compounds. The most widely used solvents for extracting phenolics from solid

grape wastes are methanol, acetone, ethanol and their water mixtures. Previous studies have reported that methanol provides relatively higher amounts of extractable compounds compared with other solvents (Zielinski & Kozlowska, 2000). The addition of acids to extraction solutions could enhance the extraction efficiency due to denaturing cellular membranes and facilitating the solubilization of phenolics (Zhang *et al.,* 2001). Therefore, in this study, an acidified methanol solution was employed as a crude extraction medium,

and methanolic ME were selected as a basis for further partitioning and purifying by petroleum ether, CF, EAF and WF. Petroleum ether was used to remove non-polar concomitant compounds, such as lipids and chlorophyll, in the crude extracts.

The extraction yield, TPC, and TFC of the extracts/fractions from different vine canes are presented in Tables 1 and 2. The results clearly show that ME had the highest extraction yield, ranging from 15.0% for cv. 'Junzi' to 25.9% for 'Cabernet Sauvignon', where-

Table 1. Extraction yields of vine shoot extracts/fractions.

Cultivars	Extraction yield (%, w/w)[1]			
	ME	CF	EAF	WF
Cabernet Sauvignon	25.9 (1.5)[b]	3.1 (0.3)[a]	2.0 (0.4)[a]	16.7 (2.2)[b]
Pinot Noir	21.1 (0.4)[a]	3.4 (0.2)[a]	2.0 (0.2)[a]	11.9 (1.1)[a]
Chardonnay	24.6 (1.7)[b]	3.2 (0.4)[a]	1.6 (0.1)[a]	15.6 (1.0)[b]
Junzi	15.0 (0.8)[c]	2.8 (0.2)[b]	0.95 (0.1)[b]	10.9 (0.9)[c]
Average	21.7 (1.4)	3.1 (0.3)	1.6 (0.3)	13.8 (1.9)

[1] ME, methanolic extract; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction. Values are the mean of three replicates (± standard deviation). Values with different letters within each column denote significant differences at $p < 0.05$.

Table 2. Total phenolic contents (TPC) and total flavonoids contents (TFC) of extracts/fractions.

Cultivars[1]	TPC (mg GAE/g)				TFC (mg QE/g)			
	ME[2]	CF	EAF	WF	ME	CF	EAF	WF
Shuangyou[VA]	120a (3.11)	78.9a (3.91)	590a (10.04)	75.2a (3.99)	80a (2.01)	7.8a (1.03)	213a (12.01)	40.2a (1.03)
Shuanghong[VA]	129a (5.32)	79.1a (2.62)	581ab (12.07)	73.8a (2.24)	60.2b (1.01)	7.1a (2.13)	199b (14.01)	20.7b (2.02)
Beibinghong[VA]	141b (1.51)	75.7ab (3.21)	595a (13.08)	80.1b (2.54)	60.7b (2.00)	8.3ab (1.05)	221c (10.01)	38.2c (2.00)
Maoputao[VP]	97c (2.20)	85.3c (2.58)	491c (15.17)	76.4c (4.24)	70.4c (3.00)	11.2c (4.07)	287d (10.01)	20.8b (1.04)
Junzi[VD]	225d (6.95)	80.5d (3.75)	833d (10.04)	97.3d (3.73)	147d (2.01)	13.2d (2.01)	589e (12.02)	67.5e (2.02)
Baiyu[VD]	170e (3.94)	65.8e (2.65)	701e (9.26)	72ae (2.99)	110e (3.00)	9.43e (1.07)	474d (10.01)	72.3f (3.00)
Cabernet Sauvignon[VV]	100.5f (2.43)	67.9ef (3.12)	623f (13.02)	69.8ef (2.50)	100.8f (4.02)	10.4f (1.02)	330f (8.01)	37.8g (1.03)
Hongmeigui[VV]	68.5g (4.49)	66e (5.45)	521g (10.19)	60.9g (1.75)	61.7bg (5.01)	9.7e (1.03)	312g (6.01)	35.6h (2.04)
Pinot Noir[VV]	70.8gh (1.14)	68.5f (3.02)	512gh (10.11)	58.6gh (1.42)	70.3h (2.00)	9.6e (1.22)	320g (7.01)	36.8hi (2.12)
Chardonnay[VV]	62.5i (2.56)	58.5g (2.91)	498gh (13.21)	57.5h (2.47)	44j (1.01)	8.7bg (1.15)	275h (2.02)	29.7j (1.04)
Victoria Blanc[VV]	52.5j (1.46)	60.7gh (3.22)	428i (10.08)	47.9i (4.03)	33.1k (2.01)	7.9a (1.02)	262i (3.01)	11.9k (0.92)

[1] VA, *V. amurensis*; VP, *V. pentagona*; VD, *V. davidii*; VV, *V. vinifera*. [2] ME, methanolic extract; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction. Values are the mean of three replicates (± standard deviation). Values with different letters within each column denote significant differences at $p < 0.05$.

as EAF exhibited the lowest yield, varying from 0.95% (Junzi) to 2.03% (Cabernet Sauvignon). The high extraction yield of ME could be attributed to the fact that aqueous methanol was effective at extracting polyphenols linked to polar fibrous matrices (Hussein *et al.,* 1990). Conversely, grape canes are a lignocellulosic material, and the main constituents (lignin, cellulose and hemicelluloses) can be hydrolyzed during exposure to low pH values (Spigno *et al.,* 2004). Similar extraction yield orders were observed in other plant materials, such as grape pomace and bamboo shoots (Campos *et al.,* 2008; Park & Jhon, 2010).

The TPC and TFC of extracts/fractions varied from 47.9 to 833 mg gallic acid equivalent (GAE)/g and 7.10 to 589 mg quercetin equivalent (QE)/g, respectively. Among all extracts/fractions, both the highest TPC (accounting for 37.7% of total extractable phenolics) and the highest TFC (41.3% of total extractable flavonoids) were detected in the EAF, whereas the lowest contents were found in WF and CF, respectively. These results suggest that medium polar phenolic compounds might be a major component of vine shoot phenolics and that ethyl acetate is suitable to extract phenolic compounds from grape canes. Ethyl acetate fractions with the highest polyphenolic content were also reported for other plant materials (Campos *et al.,* 2008; Conde *et al.,* 2008). The mean TPC and TFC of all extracts/fractions were in the following orders: EAF > ME > CF ≥ WF and EAF > ME > WF > CF, respectively. The results also indicate that the phenolic level in each fraction was not directly related to the corresponding extraction yield (Table 1). For example, methanolic extracts and their fractions from the varieties of *V. davidii,* with the lowest extraction yields, had the highest TPC and TFC values. Similar findings were also found in buckwheat extracts by Sun *et al.* (2005), who reported that the extraction of buckwheat also had the highest TPC and TFC values.

Among all cultivars, 'Junzi' had the highest TPC in its ME, EAF and WF (225, 833 and 97.3 mg GAE/g, respectively), whereas 'Victoria Blanc' exhibited the lowest CF, EAF and WF contents (52.5, 428 and 47.9 mg GAE/g, respectively) followed by 'Chardonnay', with values of 58.5, 465 and 57.5 mg/g, respectively, in the corresponding fractions. The trend of the TFC level was similar to those observed for the TPC of the cultivars analyzed, where 'Junzi' had the highest TFC values of 147, 13.2, 589 and 67.5 mg/g QE in its ME, CF, EAF and WF, respectively, followed by 'Baiyu', with corresponding values of 110, 9.43, 474 and 53.3 mg QE/g, respectively. In contrast, 'Victoria Blanc' had the lowest TFC in ME (33.1 QE/g) and WF (11.9 QE/g), and 'Shuanghong' showed the lowest values in CF (7.10 QE/g) and EAF (199 QE/g). It is worth noting that 'Junzi' of *V. davidii* is the varieties with vine shoot extracts that are rich in phenolic compounds. Further statistical analysis in Table 3 indicate good correlations between TPC and TFC for ME (r = 0.92, $p < 0.05$) and WF (r = 0.84, $p < 0.05$). Thus, it can be presumed that flavonoids are a major group of phenolics present in vine shoot extracts.

Comparisons of the mean values of TPC and TFC in all extracts/fractions among 4 grape species are depicted in Fig. 3. As expected, considerable variability in TPC or TFC values was observed within the corresponding fractions. For example, significant differences ($p < 0.05$) in TPC levels were observed among *V. amurensis*, *V. pentagona* and *V. davidii* for ME, EAF and WF (Fig. 3A). TFC levels in EAF of *V. amurensis*, *V. vinifera* and *V. davidii* exhibited significant differences ($p < 0.05$) (Fig. 3B). Zhang *et al.* (2011a,b) found that phenolic compounds of grapevine exhibit variation among numerous factors, such as cultivar, environmental conditions and management practice. However, in our case, the considerable differences may depend on the grape species and the distinct habitats in which the vine shoot samples were collected.

Table 3. Pearson's correlation coefficients of antioxidant activity and phenolic contents.

Variables[1]	ME		CF		EAF		WF	
	TPC	TFC	TPC	TFC	TPC	TFC	TPC	TFC
ME (TPC)	1	0.92**						
CF (TPC)			1	0.50[ns]				
EAF (TPC)					1	0.58[ns]		
WF (TPC)							1	0.84**
DPPH	0.92**	0.93**	0.98**	0.51[ns]	0.89**	0.74**	0.78**	0.88**
FRAP	0.79**	0.76**	0.82**	0.60*	0.98**	0.67*	0.82**	0.80**
Fe²⁺-chelating	0.09[ns]	0.29[ns]	0.42[ns]	0.52[ns]	0.42[ns]	−0.05[ns]	0.92**	0.81**
β-CLAB	0.89**	0.80**	0.91**	0.61*	0.88**	0.67*	0.87**	0.85**

[1] ME, methanolic extract; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction; TPC, total phenolic content; TFC, total flavonoid content; DPPH and Fe²⁺-chelating assay (1/IC50 values); FRAP assay (Trolox equivalents values); β-CLAB, β-carotene/linoleic acid bleaching assay. *,**: Correlation is significant at the 0.05 level and 0.01 level (2-tailed), respectively. [ns]: non-significant.
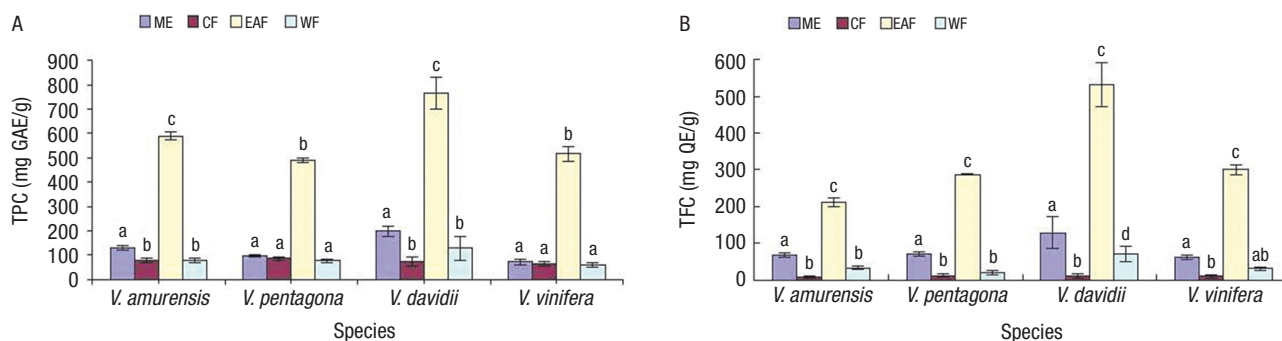
Figure 3. Comparison of average phenolics contents among four grape species: (A) Total phenolic content (TPC), (B) Total flavonoid content (TFC). Different lowercases on the histogram of the corresponding fraction imply significant differences ($p < 0.05$).

Evaluation of antioxidant properties of extracts/fractions

Mechanisms of antioxidant action include several aspects, such as inhibiting reactive oxidants generation, scavenging or destroying free radicals to break chain reactions, and binding the transition metal ions (Dinis *et al.,* 1994; Brand-Williams *et al.,* 1995; Jayaprakasha *et al.,* 2001). The specificity and sensitivity of a single method does not typically accurately reflect the complete examination of all antioxidants in complex matrices, such as botanical extracts (Frankel & Meyer*,* 2000). In this study, a combination of several different test systems, including DPPH radical-scavenging activity, ferric reducing antioxidant power (FRAP), iron-chelating capacity and *β*-carotene-linoleic acid bleaching assays, were used to provide a reliable antioxidant assessment of grape canes extracts/fractions. The phenolic content of plant extracts is associated with their antioxidant properties. Thus, the relationship between the antioxidant activity and the TPC and TFC was also investigated.

DPPH radical-scavenging activity

The ability of different extracts/fractions from grape canes to quench free radicals was measured using a DPPH radical-scavenging activity assay. This method depends on the reduction of the purple DPPH radical by accepting an electron or hydrogen radical from antioxidants to form the corresponding yellow-colored α, α-diphenyl-β-hydrazine (a stable diamagnetic molecule) in a methanolic solution (Frankel & Meyer*,* 2000). To obtain the concentration of each sample, a 50% decrease in initial DPPH radicals, referred to as an IC50 value, was required. Lower IC50 values indicate higher DPPH radical-scavenging power. The degree of discoloration indicated the scavenging potential of the extract/fraction in terms of its hydrogen donating ability. Based on the calculated IC50 values of samples and positive controls, the order of the antiradical activity was as follows: gallic acid > TBHQ > Trolox > EAF > ME > WF > CF with IC50 values of 0.85, 1.90, 3.86, 5.24, 43.4, 92.2 and 120 µg/mL, respectively (Table 4). Among the extracts/fractions of all cultivars, the EAF exhibited the highest activities with IC50 values from 2.03 µg/mL for 'Junzi' to 5.50 µg/mL for 'Victoria Blanc', whereas the CF with IC50 values from 90.4 µg/mL for 'Cabernet Sauvignon' to 159 µg/mL for 'Victoria Blanc' exhibited the lowest radical-scavenging activities. Most of the chloroform fractions (CF) exhibited weak activity, with IC50 values greater than 100 µg/mL. However, it should be emphasized that the EAF from 'Shuangyou', 'Beibinghong', 'Junzi', 'Baiyu', and 'Pinot Noir' exhibited significantly increased scavenging activities compared with Trolox ($p < 0.05$). In addition, the EAF of 'Junzi' possessed a comparable activity with TBHQ. This finding suggests that ethyl acetate fractions of grape canes may be useful in the future. For correlation analysis, the IC50 values were transformed into their reciprocal values (1/IC50). With reference to Table 3, the correlation coefficient between the antiradical activity monitored by DPPH assay and the TPC and TFC of all of the extracts/fractions of the eleven grape canes were satisfactory ($r > 0.74$, $p < 0.01$), with the single exception of the slight correlation between the TFC and the antiradical activity of the CF ($r < 0.55$). The results indicate that phenolic compounds in grape canes extracts/fractions are major constituents that can scavenge the DPPH radical due to the presence of the hydroxyl groups in their electron donating ability. These results are consistent with those of many research groups, who also reported such relationships between the phenolic content and free-radical scavenging activity (Sun & Ho*,* 2005; Conde *et al.,* 2008; Park & Jhon, 2010).

*Ferric reducing antioxidant power (FRAP)
and iron-chelating capacity*

The reducing power of different extracts/fractions from grape canes was evaluated using the FRAP assay. In this assay, the antioxidants present in the test solution can reduce Fe^{3+} to Fe^{2+} by donating an electron in the presence of TPTZ, thereby forming the intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. The antioxidant ability of extracts/fractions was expressed using the Trolox equivalent (TE) value. Higher TE values indicate higher antioxidant activity. As shown in Table 4, the strongest antioxidant activity was noted for EAF, with a mean TE value of 5.12 ± 2.70 mM TE/g, compared with other extracts/fractions. No significant differences ($p < 0.05$) in the mean TE values were observed among the ME, CF and WF (0.75 ± 0.40, 0.40 ± 0.07 and 0.50 ± 0.21 mM TE/g, respectively). There were 6.0-, 2.0-, 7.0- and 5.3-fold differences in TE values between the high-

Table 4. Antioxidant capacities analyzed by DPPH, FRAP and metal ions chelating assays. Values are the means of three assessments and SD (in parenthesis). Values labeled the same lowercases within each column are not significantly different according to Duncan's new multiple range test ($p < 0.05$).

Cultivars[1]	DPPH assay IC$_{50}$ values (µg/mL)				Fe^{2+}-chelating assay IC$_{50}$ values (mg/mL)				FRAP values (mM TE/g)			
	ME[2]	CF	EAF	WF	ME	CF	EAF	WF	ME	CF	EAF	WF
Shuangyou[VA]	36.41e (1.01)	114.43ef (2.91)	3.37d (0.04)	80.03c (2.99)	0.17f (0.01)	2.31ef (0.02)	0.28d (0.01)	0.54c (0.03)	0.63d (0.01)	0.45g (0.01)	5.10f (0.03)	0.52e (0.02)
Shuanghong[VA]	49.82i (0.92)	111.22e (2.62)	3.55de (0.07)	92.34d (2.74)	0.12c (0.01)	1.60c (0.03)	0.29de (0.01)	0.48c (0.02)	0.66e (0.02)	0.44fg (0.01)	5.12f (0.11)	0.40c (0.02)
Beibinghong[VA]	43.11fg (1.51)	111.21e (4.21)	3.25d (0.08)	98.11e (3.54)	0.14d (0.00)	1.64c (0.04)	0.28d (0.01)	0.51c (0.00)	1.07h (0.03)	0.44fg (0.02)	6.05g (0.04)	0.67g (0.02)
Maoputao[VP]	49.14i (1.20)	133.12g (3.58)	5.32hi (0.17)	100.16ef (3.24)	0.16ef (0.00)	2.03d (0.07)	0.37f (0.01)	0.70de (0.04)	0.36c (0.01)	0.39d (0.02)	2.64c (0.01)	0.48d (0.01)
Junzi[VD]	22.05c (0.95)	97.08c (2.75)	2.03b (0.04)	76.84bc (2.73)	0.34h (0.01)	1.33b (0.01)	0.26c (0.02)	0.38b (0.02)	1.55j (0.03)	0.53h (0.01)	10.92j (0.14)	0.90h (0.03)
Baiyu[VD]	30.71d (0.94)	118.53f (2.65)	2.74c (0.26)	73.11b (2.69)	0.37i (0.00)	2.24e (0.07)	0.24c (0.01)	0.48c (0.00)	0.87g (0.01)	0.42e (0.02)	6.64h (0.31)	0.70g (0.01)
Cabernet Sauvignon[VV]	44.00gh (1.43)	90.43b (4.12)	4.33f (0.02)	95.70de (3.50)	0.12c (0.02)	2.78h (0.09)	0.31e (0.01)	0.78ef (0.03)	1.27i (0.02)	0.39d (0.01)	3.78d (0.03)	0.59f (0.03)
Hongmeigui[VV]	52.34j (1.49)	131.72g (4.45)	4.67fg (0.19)	97.92e (3.75)	0.15de (0.01)	2.05d (0.04)	0.29de (0.01)	0.68d (0.04)	0.72f (0.01)	0.37c (0.01)	4.29e (0.04)	0.39c (0.00)
Pinot Noir[VV]	45.56h (1.14)	104.83d (4.02)	3.26d (0.11)	91.06d (3.42)	0.04b (0.00)	2.54g (0.22)	0.10b (0.01)	0.70de (0.12)	0.62d (0.01)	0.42ef (0.02)	7.85i (0.11)	0.47d (0.02)
Chardonnay[VV]	60.92k (1.56)	153.36h (3.91)	5.04gh (0.21)	104.52f (3.47)	0.28g (0.00)	3.09i (0.15)	0.37f (0.02)	0.80f (0.04)	0.29b (0.00)	0.29b (0.01)	2.37b (0.03)	0.24b (0.01)
Victoria Blanc[VV]	42.01f (1.46)	159.27i (3.22)	5.50i (0.08)	104.33f (3.03)	0.34h (0.01)	2.45fg (0.02)	0.40f (0.01)	1.07g (0.01)	0.26a (0.00)	0.26a (0.00)	1.55a (0.08)	0.17a (0.00)
Gallic acid	0.85a (0.03)	0.85a (0.03)	0.85a (0.03)	0.85a (0.03)								
Trolox	3.86b (0.04)	3.86a (0.04)	3.86e (0.04)	3.86a (0.04)								
TBHQ	1.90a (0.07)	1.90a (0.07)	1.90b (0.07)	1.90a (0.07)								
EDTA					0.02a (0.00)	0.02a (0.00)	0.02a (0.00)	0.02a (0.00)				
Average [3]	43.41B (10.71)	120.47D (21.94)	5.24A (1.41)	92.19C (10.95)	0.20A (0.11)	2.19C (0.53)	0.29A (0.08)	0.65B (0.20)	0.75A (0.40)	0.40A (0.07)	5.12B (2.70)	0.50A (0.21)

[1] VA, *V. amurensis*; VP, *V. pentagona*; VD, *V. davidii*; VV, *V. vinifera*. Gallic acid, Trolox, TBHQ and EDTA were used as positive controls. [2] ME, methanolic extract; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction. [3] Average row (n = 11): values followed by the same uppercases are not significantly different within each assay ($p < 0.05$).

est- and lowest-ranked ME, CF, EAF and WF, respectively. Interestingly, for the corresponding extract/fraction, the TE values of cv. 'Junzi' and 'Victoria Blanc' were the highest and lowest, respectively, of the different cultivars. Significant correlation was noted between the TE values and the TPC ($r > 0.79$, $p < 0.01$) and TFC ($r > 0.60$, $p < 0.05$ or 0.01) of ME and its three fractions (Table 3), indicating that the reducing power is highly related to the amounts of phenolic compounds present in grape canes extracts/fractions. It should be noted that the correlation coefficients of TFC were weaker than those of TPC, which might be explained by the fact that the presence of other non-flavonoid constituents contributed to the overall reducing power.

As mentioned above, chelating transition metals is an antioxidant mechanism. Among the various species of metal ions, the Fe^{2+} ion, which promotes the formation and propagation of many radical reactions, is the most powerful pro-oxidant (Dinis *et al.,* 1994). Therefore, the Fe^{2+}-chelating capacity of extracts/fractions was characterized and reported as IC50 values (Table 4). All extracts/fractions (ME, CF, EAF and WF) from different grape canes exhibited iron-chelating capacities (interfering with the formation of red-colored Fe^{2+}/ferrozine complex), with mean IC50 values of 0.20 ± 0.11, 2.19 ± 0.53, 0.29 ± 0.08 and 0.65 ± 0.20 mg/mL, respectively, which were less than the positive control EDTA (0.02 ± 0.00 mg/mL). No significant difference was observed ($p < 0.05$) between ME and EAF with respect to the mean IC50 values. The ME from grape canes exhibited the strongest chelating capacity among all extracts/fractions, with IC50 values ranging from 0.04 mg/mL for 'Pinot Noir' to 0.37 mg/mL for 'Baiyu', whereas CF yielded the weakest from 1.33 mg/mL for 'Junzi' to 3.09 mg/mL for 'Chardonnay'. The results revealed that some of these extracts/fractions, such as the ME and EAF from 'Pinot Noir', can be considered ideal iron chelators. Although the ME from grape canes exhibited the highest metal chelating activity, its reducing power and ability to scavenge DPPH radical were relatively low in this study (Table 4). These differences might be due to the intrinsic heterogeneity of extracts/fractions from grape canes because the chelating activities of compounds are related to their structure-function configuration (Rice-Evans *et al.,* 1996). Plant extracts or phytochemicals that exhibit high antioxidant activities both with and without iron chelating capacity were observed in previous studies (Rohman *et al.,* 2000). Diverse correlations were found between the iron-chelating property and the phenolic contents for all extracts/fractions, with cor-

relation coefficients (r) ranging from -0.05 to 0.92 (Table 3). These correlations suggest that the iron-chelating effects by grape canes extracts could not be explained exclusively by their phenolic contents. In fact, in the literature, contradictory data exist concerning the correlation between the metal chelating activity and phenolic content of plant extracts. Our results partially agree with those reported by Hinneburg *et al.* (2006).

β-carotene-linoleic acid bleaching assays

To stimulate the oxidation of real biological systems, such as fluid foods containing oil, an aqueous *β*-carotene-linoleic acid emulsion system incubated at elevated temperatures was used to assess the anti-lipid peroxidation activities of extracts/fractions from grape canes. The mechanism of this method is a free radical-mediated phenomenon that results from the peroxyl radicals formed from the oxidation of linoleic acid. These free radicals subsequently attack the highly unsaturated *β*-carotene molecules. Subsequently, *β*-carotene undergoes a rapid fading of orange color, which can be minimized under the action of antioxidants (Jayaprakasha *et al.,* 2001). In this study, TBHQ, a commercial antioxidant that is widely used in many countries (Christian & Liliane, 2006), was chosen as the positive control. The discoloration rate of *β*-carotene depends on the antioxidant capacity of different extract/fraction. As shown in Fig. 4, all extracts/fractions were capable of inhibiting the bleaching of *β*-carotene by scavenging the linoleate-derived free radicals at a concentration of 200 mg/L, which is the maximum permissible level of synthetic antioxidants used in edible oils (Sun & Ho, 2005). The mean antioxidant activity (AA, %) values were 21.8 ± 7.71, 44.5 ± 4.87, 81.1 ± 7.92, 18.8 ± 6.61 and $97.9 \pm 1.89\%$ for ME, CF, EAF, WF and TBHQ, respectively. No significant difference was found in the average AA values between ME and WF ($p > 0.05$). Contrary to the results obtained from the three previous assays in this study, the CF exhibited significantly increased antioxidant activity compared with the ME and WF ($p < 0.05$). This finding could be explained by the 'polar paradox' theory (Frankel *et al.,* 1994). In relatively more polar media, such as oil-in-water emulsions, non-polar or less polar antioxidants are more effective because these compounds can be enriched at the interface between the oil and aqueous phase, thus protecting the oil phase from oxidation. In contrast, the polar antioxidants in the ME and WF were diluted in the bulk phase (aqueous) and thus exhibited relatively weak protecting
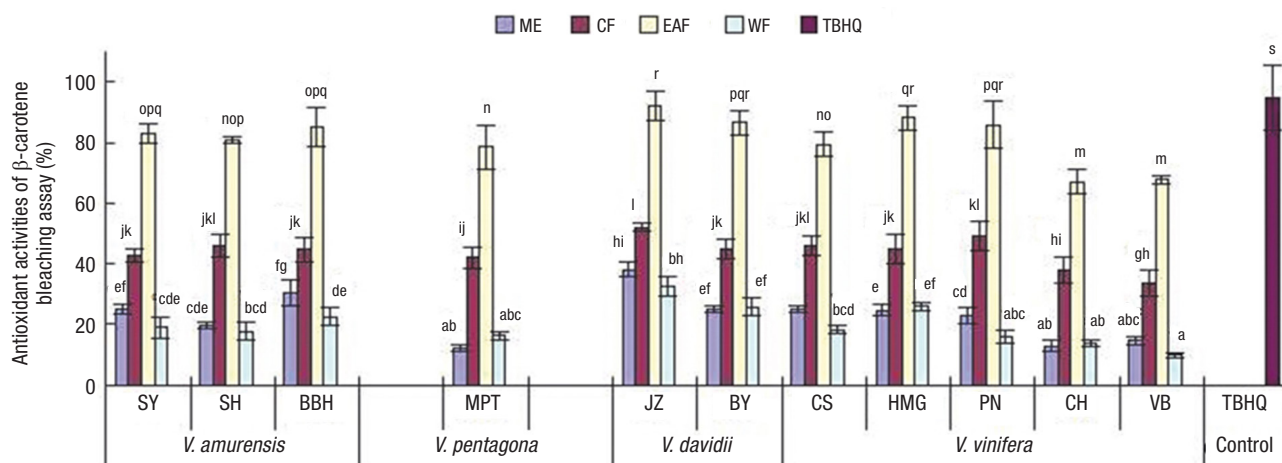
Figure 4. Antioxidant activities of all extracts/fractions (200 mg/L) in β-carotene-linoleic acid emulsion system. TBHQ (*tert*-butylhydroquinone) was used as positive control. SY, Shuangyou; SH, Shuanghong; BBH, Beibinghong; MPT, Maoputao; JZ, Junzi; BY, Baiyu; CS, Cabernet Sauvignon; HMG, Hongmeigui; PN, Pinot Noir; CH, Chardonnay; VB, Victoria Blanc. Different lowercases on the histograms imply significant differences at *p* < 0.05.

effects. It should be noted that the antioxidant capacity of EAF from 'Junzi', 'Baiyu', 'Hongmeigui' and 'Pinot Noir' was comparable to that of TBHQ, with AA values of 91.7, 86.5, 88.3 and 86.4%, respectively. The results suggested that the EAF and CF from grape canes have the potential to complement or replace synthetic antioxidants, and they could be considered safe and used at a concentration more than 200 mg/L in aqueous and oil-based foods. A significant correlation was found between the AA values and the TPC (r > 0.87, *p* < 0.01) and TFC (r > 0.61, *p* < 0.05 or 0.01) of the extracts/fractions (Table 3), indicating that the anti-lipid peroxidation activity is closely associated with the phenolic content. Although these correlations were similar to Ozsoy *et al.* (2008)'s findings, they were inconsistent with those of Sun & Ho (2005), who found that the methanol extract had the highest antioxidant activity coefficient but did not find a positive relationship between the total phenolic content and *β*-carotene bleaching assay for buckwheat extracts.

Identification and determination of phenolic constituents in extracts/fractions

Extracts/fractions from grape canes indicated different antioxidant capacities in different *in vitro* testing systems. To determine which constituent(s) is/are the most important active components in different extracts/fractions, HPLC-DAD-UV was used for both qualitative and quantitative analyses. Phenolic compounds were identified and confirmed by comparing their retention times and the spectral characteristics of their peaks with those of authentic standards and by spiking

the sample with standards and peak purity detection. The typical chromatographic profiles of phenolic composition of extracts/fractions are presented in Fig. 5. Of note, satisfactory separation with good resolution was achieved by the modified HPLC condition. Seven phenolic compounds, including gallic acid (GA), protocatechuic acid (PA), vanillic acid (VA), syringic acid (SYA), catechin (CAT), epicatechin (EC) and trans-resveratrol (RES), were identified and quantified in different extracts/fractions. These phenolic compounds, which occur widely in lignified organs (tissues) of grapevines, such as stems, shoots, canes and xylem, have already been studied (Luque-Rodríguez *et al.,* 2006; Zhang *et al.,* 2007). The quantitative data of all of the extracts/fractions from grape canes are summarized in Table 5. The results of total individual phenolic contents quantified by HPLC were similar to those obtained using the Folin-Ciocalteu method (Table 1). The composition profiles of ME and the other two fractions were more complex than those of the CF, which was devoid of EC in most cultivars analyzed and of GA in all cultivars. This finding is largely attributed to different extraction solvents with different extracting capacities (Park & Jhon, 2010). The highest contents of phenolic compounds were observed in the EAF, with mean values of 3.50 ± 0.67, 10.7 ± 3.65, 3.98 ± 0.92, 10.4 ± 3.02, 79.9 ± 33.75, 79.9 ± 29.02 and 127 ± 37.9 mg/g for GA, PA, VA, SYA, CAT, EC and RES, respectively. The results indicate that most of the phenolic compounds in ME go to the EAF via solvent-solvent partitioning. This finding may explain why EAF exhibited increased antioxidant activity compared with the other fractions (Table 1). Flavan-3-ols (CAT and EC) and stilbene (RES) were detected as the two main phenolic groups in extracts/fractions, accounting for
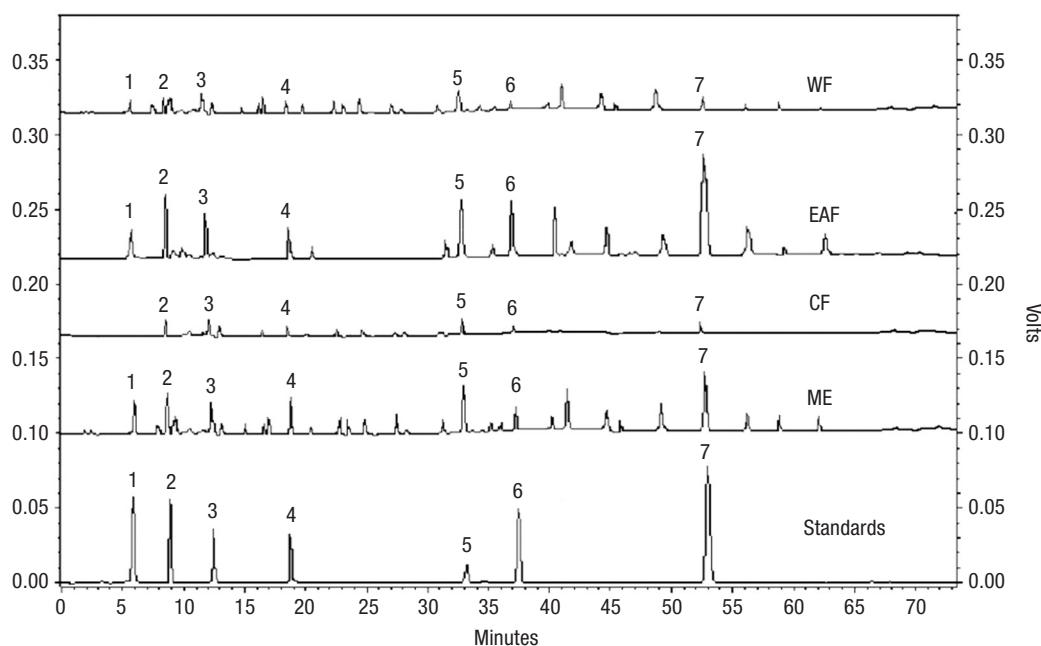
Figure 5. Typical HPLC chromatograms of phenolic standards and extracts/fractions (cv. Junzi). Peaks: 1-gallic acid; 2-protocatechuic acid; 3-catechin; 4-vanillic acid; 5-syringic acid; 6-epicatechin; 7- *trans*-resveratrol.

Table 5. Identified phenolics (mg/g) in extracts/fractions.

Extracts [1]	Cultivars	Gallic acid	Protocatechuic acid	Vanillic acid	Syringic acid	Catechin	Epicatechin	Resveratrol
ME	Cabernet Sauvignon	0.41 (0.01)^c	0.93 (0.04)^b	0.42 (0.02)^c	1.22 (0.04)^c	6.32 (0.25)^b	6.15 (0.24)^b	13.59 (0.53)^a
	Pinot Noir	0.35 (0.03)^b	1.08 (0.04)^c	0.35 (0.01)^b	1.02 (0.04)^b	7.52 (0.30)^c	7.12 (0.28)^c	19.00 (0.75)^b
	Chardonnay	0.17 (0.01)^a	0.53 (0.02)^a	0.29 (0.01)^a	0.93 (0.04)^a	4.29 (0.17)^a	4.11 (0.16)^a	12.65 (0.50)^a
	Average (n = 3)	0.31 (0.11)	0.85 (0.25)	0.35 (0.06)	1.06 (0.13)	6.05 (1.43)	5.80 (1.35)	15.08 (3.01)
CF	Cabernet Sauvignon	ND	0.05 (0.00)*	ND	0.07 (0.01)^b	0.06 (0.01)*	0.05 (0.01)	0.81 (0.08)^c
	Pinot Noir	ND	0.07 (0.01)	0.04 (0.00)	0.09 (0.01)^c	0.22 (0.02)	ND	0.67 (0.07)^b
	Chardonnay	ND	ND	0.02 (0.00)*	0.04 (0.00)^a	ND	ND	0.52 (0.05)^a
	Average (n = 3)		0.06 (0.01)	0.03 (0.01)	0.06 (0.02)	0.14 (0.09)	0.05 (0.01)	0.67 (0.14)
EAF	Cabernet Sauvignon	3.66 (0.12)^b	8.14 (0.52)^b	3.76 (0.10)^b	14.54 (0.35)^c	83.33 (4.56)^c	81.54 (4.43)^c	126.6 (6.13)^a
	Pinot Noir	3.64 (0.12)^b	11.16 (0.47)^c	3.50 (0.05)^a	10.11 (0.19)^a	73.09 (3.09)^b	72.54 (3.27)^b	201.2 (10.7)^b
	Chardonnay	2.23 (0.05)^a	7.18 (0.27)^a	3.50 (0.01)^a	12.42 (0.21)^b	58.70 (3.21)^a	53.82 (1.31)^a	141.9 (9.96)^a
	Average (n = 3)	3.17 (0.71)	8.83 (1.84)	3.59 (0.14)	12.36 (1.93)	71.71 (11.2)	69.30 (12.6)	156.6 (35.1)
WF	Cabernet Sauvignon	0.05 (0.00)^c	0.11 (0.01)^b	0.04 (0.01)^b	0.08 (0.01)^b	0.77 (0.06)^b	0.69 (0.06)^b	1.48 (0.12)^a
	Pinot Noir	0.04 (0.00)^b	0.11 (0.01)^b	0.03 (0.00)^a	0.06 (0.01)^a	0.82 (0.09)^b	0.72 (0.08)^b	1.86 (0.21)^b
	Chardonnay	0.02 (0.00)^a	0.06 (0.00)^a	0.02 (0.00)^a	0.06 (0.00)^a	0.52 (0.02)^a	0.46 (0.02)^a	1.36 (0.06)^a
	Average (n = 3)	0.04 (0.01)	0.09 (0.03)	0.03 (0.01)	0.07 (0.01)	0.70 (0.15)	0.62 (0.13)	1.57 (0.26)

[1] ME, methanolic extract; CF, chloroform fraction; EAF, ethyl acetate fraction; WF, water fraction. Values are the mean of three replicates (± standard deviation). Values with different letters in each column of same extracts/fractions denote significant differences according to Duncan's new multiple range tests ($p < 0.05$); * indicate significant difference between two mean values at $p < 0.05$ according to Student's *t*-test; ND: not detected.

92, 83, 90 and 93% of total identified phenolic compounds in the ME, CF, EAF and WF, respectively. Regarding these predominant compounds in the corresponding extract/fraction of different grape canes, varieties from *V. davidii* exhibited significantly increased amounts of flavan-3-ols compared with *V. vinifera* and *V. amurensis* ($p < 0.05$), whereas *V. vinifera* and *V. amurensis* possessed significantly increased

trans-resveratrol levels compared with *V. davidii* ($p < 0.05$). Furthermore, the extracts/fractions with the highest levels of single compounds did not typically demonstrate the highest antioxidant activities. For example, the ME of cv. 'Pinot Noir' and EAF of 'Baiyu', with the highest amounts of RES and CAT, respectively, were not the strongest in most antioxidant assays. Thus, the antioxidant activity of extracts/fractions more

likely depends on the combination of several or more phenolic compounds. Flavan-3-ols and stilbenes may contribute significantly; however, other yet-unidentified phytochemicals and the possible interactions among them also should be considered in the overall antioxidant effects of extracts/fractions from grape canes.

This study is a first report on the antioxidant activities and phenolic composition of methanolic extract (ME) and its three fractions of vine shoots from *V. amurensis*, *V. davidii*, *V. pentagona* and *V. vinifera* from a waste utilization perspective. All extracts/fractions have potent antioxidant activity based on the DPPH radical-scavenging, FRAP, Fe^{2+}-chelating and β-carotene bleaching assays. The ME of grape canes demonstrated the highest iron-chelating activity, whereas ethyl acetate fractions, which have the highest TPC and TFC, exhibited the highest free-radical scavenging and reducing power activities, as well as the highest anti-lipid peroxidation activity. Among all grape genotypes analyzed, varieties from *V. davidii* possessed higher antioxidant activities than did those from the three other grape species. Antioxidant activities of extracts/fractions correlated positively with their total phenolic and flavonoids contents in all assays except the metal chelating power test. Qualitative and quantitative analyses of phenolic compounds by HPLC-DAD-UV indicated that catechin, epicatechin and *trans*-resveratrol were the main phenolic components of extracts/fractions. Further work in our laboratory is in progress to identify and characterize more inherent phytochemicals from different grape extracts and to evaluate their *in vivo* antioxidant potential.

Acknowledgements

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