Bulgarian Chemical Communications, Volume 48, Special C (pp. 9–13) 2016

Total phenolic and flavonoid contents and antioxidant activity of extracts from *Vitis vinifera* L.

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Received June 26, 2016, Revised September 10, 2016

Plants naturally are a rich source of secondary metabolites and novel therapeutic compounds. These compounds are well known for their various beneficial effects on human health. In this study, variation in total phenolic and flavonoid contents and antioxidant activities (DPPH radical-scavenging,reducing power, superoxide radical scavenging, hydrogen peroxide scavenging, total antioxidant activity) of water, acetone and methanol extracts from the *Vitis vinifera* L. leaves collected from north Turkey was studied. Results indicated that it wassimilar total phenolic and flavonoid contents of methanol extract. Total phenolic content varied from 48.67 ± 1.15 to 70.87 ± 1.15 (mg GAE/g dry wt), and total flavonoid content ranged from 45.20 ± 0.86 to 72.90 ± 0.40(mg CE/g dry matters). Furthermore, results indicated that the extracts have good antioxidant activities. It was concluded that *V. vinifera* might be a potential source of antioxidants.

Key words: Flavonoids, Antioxidant activity, *Vitis vinifera*, Scavenging activity, Phenolic compound.

INTRODUCTION

Free radicals are naturally present in living systems; however, high amounts of free radicals can oxidise biomolecules, leading to tissue damage, cell death or degenerative processes, including aspects of ageing, cancer, cardiovascular diseases, arteriosclerosis, neural disorders, skin irritations and inflammation [1]. Natural antioxidants exist in the leaves, seeds, roots and fruits of most plants. These are the most effective free radical scavengers in living organisms [2].

Vitis vinifera L. (common grape) belong to Vitaceae family,which fruits have been used as a food and for wine or beverage production. In Ayurvedic (Indian) system, grapes leaves are used as a folk remedy for the treatment of diarrhea and vomiting. The grape leaves havebeen used to stop bleeding, to treat inflammatory disorder, pain, hepatitis, free radical related diseases [3]. The leaves are composed of wide range of polyphenols including anthocyanins, flavonoids and also organic acid [4]. Previous reports showed that leaves, fruits and juiceof *V. vinifera* have the hepatoprotective effect on acetaminophen induced hepatic DNA damage, apoptosis and necrotic cell death [5]. In this view, the present study was carried out to evaluate the antioxidant activity of *V. vinifera* leaves.

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EXPERIMENTAL

Plant Material

Fresh grape (*V. vinifera* L.) leaves were collected (during February) from Tekirdag, (Tekirdag, Turkey). Plant materials were washed with distilled water and dried at room temperature. For preparation of water extract (WE), 25 g sample was put into a fine powder in a mill and was mixed with 500 mL boiling water by magnetic stirrer for 15 min. The extract was then filtered and evaporated to dryness under reduced pressure and controlled temperature (40-50°C) in a rotary evaporator. For the preparation acetone (AE) and methanol (ME) extracts, 25 g sample was put into a fine powder in a mill and was mixed with 500 mL solvent. The residue was re-extracted until extraction solvents became colorless. The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected, then solvent was removed by a rotary evaporator (Buchi R-200, Switzerland) at 40°C to obtain dry extract. All the extracts were kept at -20°C and were dissolved in water or solvent before use.

Determination of total phenolic compounds

Total phenolics in *V. vinifera* extracts were determined according to Folin–Ciocalteu method [6] as described previously [7]. Briefly, 1 mL of the *V. vinifera* extracts (25–125μg/mL) was transferred into test tubes and their volumes made up to 4.6 mL with distilled water. After addition of 0.1 mL Folin–Ciocalteau reagent (previously diluted 3-fold with distilled water) and 0.3 mL 2% Na_2CO_3

solution, tubes were vortexed and then allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The total phenolic compounds in the *V. vinifera* extracts were expressed as gallic acid equivalents (GAE) (mg g^{-1}).

Determination of total flavonoid content

Total flavonoid content was determined by using a method described by Wang et al. [8] with minor modification using catechin as standard flavonoid compound. Briefly, 10 mLof the extract (1000 µg/mL) or (+)-catechin standard solution (0–50 µg/mL) was mixed with 1 mL of a 5% sodium nitrite solution. After 6 min, 1mL of a 10% aluminium chloride solution was added and the mixture was allowed to stand for a further 5 minbefore 10 mL of NaOH (5%) was added. The mixture was brought to 25 mL with distilled water and mixed well. The absorbance was measured immediately at 510 nmusing a spectrophotometer. Results were expressed as the average of triplicates. The total flavonoid content was calculated as catechin equivalents (CE) (mg g^{-1}).

Antiradical activity

The DPPH radical scavenging activity of the *V. vinifera* extracts was measured according to the procedure described by Burits, Asresand Bucar [9]. Briefly, 0.1 mM solution of DPPH• in ethanol was prepared and 1 mL of this solution was added to 3 mL of *V. vinifera* extracts at different concentrations (25-125 µg/mL). The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 0.5h. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against ethanol. All measurements were made in triplicate and averaged. The DPPH radical scavenging activity was calculated usingthe following equation:

$$\text{Scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

ABTS assay

The ABTS•+ method was based on the procedure described by Siddhuraju and Becker [10]. Briefly, 10mg of ABTS was diluted in 2.6mL of potassium persulfate solution (2.45mM) and final concentration of ABTS•+ was 7mM. The mixture was left to stand in dark at room temperature for 12–16 h before use. The ABTS•+ was diluted to the absorbance of 0.70 ± 0.02 and stocked for off line and on line assays. 1mL of diluted extract was added with 3mL of ABTS•+ solution and stand in dark at room temperature for 60 min. The absorbance was measured at 734 nm. ABTS scavenging activity is presented as an EC50 value.

Reducing power

Reducing power was determined according to the method proposed by Oyaizu [11] with minor modifications [12]. Stock solutions were diluted. A 500 µL sample solution was mixed with 1.25 mL of 0.2 M phosphate buffer (pH 6.6) and 1.25 mL of 1.0% (w/v) potassium ferricyanide. The resulting mixture was incubated at 50°C for 30 min. After the addition of 1.25 mL of 10% (w/v) trichloroacetic acid, the mixture was centrifuged at 2500× g for 10 min. A 2.5 mL supernatant was mixed with water (2.5 mL) and 0.5 mL of 0.1% (w/v) ferric chloride before the absorbance was determined at 700 nm. In this assay, the presence of reductants, such as antioxidant compounds in the sample, causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Standards were used for comparison.

Superoxide anion scavenging activity

Measurement of the superoxide anion scavenging activity of *V. vinifera* extracts was based on the method described by Guo and Wei et al.[13] with slight modifications Superoxide radicals are generated in PMS-NADH systems by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, superoxide radicals were generated in 3 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 µM) solution, 1 mL NADH (78 µM) solution and the sample solution. The reaction was initiated by adding 1 mL of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against a blank. A decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion radical generation for three parallel measurements was calculated using the following formula: Inhibition $(\%) = [(Ac - As)/Ac] \times 100$ In this formula, Ac is the absorbance of control and As is the absorbance in the presence of the extract or a standard.

Scavenging of hydrogen peroxide

The hydrogen peroxide scavenging ability of extracts was determined according to the method of Ruch et al. [14]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a

spectrophotometer. *V. vinifera* extracts (125 µg/mL) were added to 1 mL of the hydrogen peroxide (40 mM) solution.The absorbance of hydrogen peroxide at 230 nm was read after ten minutes against a blank solution of phosphate buffer not having hydrogen peroxide. The percentage of hydrogen peroxide scavengedby extracts and standard solutionswas calculated as follows: H_2O_2 scavenging activity $(\%) = [(Ac - As)/Ac] \times 100$, where A_C and A_S are the absorbance values of the control sample and the test sample, at particular times, respectively.

Ferric reducing antioxidant potential (FRAP) assay

The total antioxidant activity of the *V. Vinifera* extracts was determined according to the thiocyanate method described by Mitsuda et al. [15] For stock solutions, 10 mg of extracts was dissolved in 10 mL deionized water. The solution, which contains the same concentration of extracts or standard samples (75 µg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Fifty millilitres linoleic acid emulsion contained 175 µg Tween-20, 155 µL linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0).On the other hand, 5 mL control was composed of 2.5 mL linoleic acid emulsion and 2.5 mL, 0.04 M potassium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37 ºC in a glass flask. At regular intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 3.7 mL of solvent, followed by the addition of 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % hydrochloric acid. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer. This step was repeated every 10 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid oxidation. All data on total antioxidant activity are the average of triplicate experiments.

RESULTS

Recovery Percent, Total Phenolics, and Flavonoid Contents

It is well known that phenolics and flavonoids are the important antioxidant substances that are obtained from most natural plants.In the present study, the percent yield, total phenolics and flavonoid contents obtainedfrom extracts of *Vitis vinifera* are shown in Table 1

The extraction was carried out with three different solvents, including water, methanol, and acetone, to obtain extracts from dried plant material, which will be used in all assays. The extraction with methanol resulted in the highest amount of total extractable compounds. The extraction yields were found to be 118.8, 131.2, and 61.2 mg/g dried leaf for the water, methanol, and acetone extracts, respectively. These extraction yields indicated that the solvents used for extract preparation from *V. vinifera* leaves showed different capacities to extract the leaf compounds and probably different compositions of the extracts.

Among the different leave extracts, the methanol extract of *V. vinifera* showed the highest total phenolic content (70.87 ± 1.15 mg GAE/g extract) when compared to other extracts. Moreover, we determined the total flavonoid contents of the*V. Vinifera* extracts. Flavonoids are important secondary metabolites in plants with high antioxidant activity properties.In our study they wereestimated using the linear regression equation obtained from the standard catechin curve (absorbance = 0.013 [CE] x 0.003), r^2 = 0.9990 as catechin equivalents per 1 mg of extract (CE/mg extract). The methanol extract of *V. vinifera* showed the highest total flavonoid content (72.90 ± 0.40 mg CE/g extract). The total flavonoid contents exhibited the descending order among: methanol extract > water extract > acetone extract. In different studies, various amounts of flavonoids have been detected in vegetables. For example, 11.88 ± 1.46 µg epicatechin equivalent was detected in the water extracts of chard[16].

Antiradical activity

Antiradical activity of extracts was carried out by measuring the decolourisation of DPPH solution at 517 nm. The scavenging effects of extracts on DPPH increased with the increase in concentration. The highest inhibition ratio was 55.2% at 125 µg mL^{-1} for methanol extract. Ascorbic acid had an activity of 64.2% at 125 µg mL^{-1} and BHA had an activity of 61.7% (Fig. 1). *V. vinifera* extracts, on interacting with DPPH, might have transferred an electron to it, thus neutralizing its free radical nature as observed by Oyaizu [11].

ABTS assay

The antioxidant capacity of leaf extracts was determined by the ABTS method. The antioxidant ability of *V. vinifera* extracts to scavenge the blue-green colored ABTS+ radical cation was measured. ABTS+ scavenging activity increased with increasing concentration.

Table 1. Extraction yields and contents of total phenols, total flavonoids in *Vitis vinifera* extracts.

Extracts	Extraction yield[a] (mg g^{-1} dry wt)	Total phenols[b] (GAE) (mg g^{-1})	Total flavonoids [c] (CE) (mg g^{-1})
Water extract	118.8	60.33 ± 0.58	59.87 ± 0.23
Acetone extract	61.2	48.67 ± 1.15	45.20 ± 0.86
Methanol extract	131.2	70.87 ± 1.15	72.90 ± 0.40

Note: The data are expressed as mean ± SD (n = 3). [a]Expressed as milligram of extract per gram dry material. [b]Expressed as milligram of gallic acid per gram dry extract. [c]Expressed as milligram of catechin per gram dry extract.

The EC50 values ofthe extracts were between 12.32±0.16 and 45.87±0.47µg/mL. It was found that the ABTS+ scavengingactivity of water extract of chard increased with increasing concentration, reaching 18.56 ±1.77% at 400 µgmL^{-1} [16].

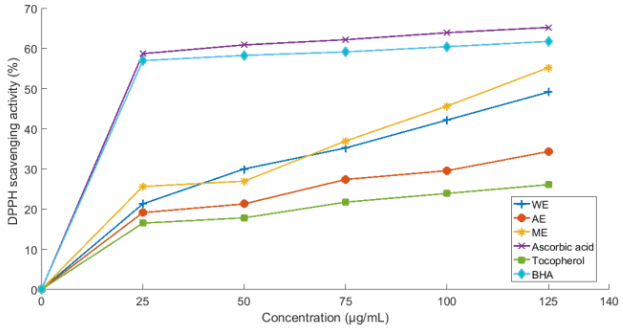

Fig. 1 DPPH radical scavenging activity of the *V. vinifera* extracts. BHA, ascorbic acid and α-tocopherol were used as reference antioxidants.

Reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [17]. Fig. 2 shows, the extent of the reduction, in terms of absorbance values at 700 nm. The reducing power of *V. vinifera* extracts was not concentration dependent and was found to be below those of ascorbic acid (1.617), BHA (1.042) and BHT (1.004) at 250 µg mL^{-1}. The extracts showed lower reducing power than the standards. Reducing power of extracts and standards decreased in order of ascorbic acid > BHA > BHT > methanol extract > water extract > acetone extract. Previous studies have correlated the reducing capacity of phytocompounds to their electron-donating ability [13]. Hence it can be started that the effective electron (hydrogen) donating ability of the *V. vinifera* extracts contributed to the observed overall antioxidant property.

Superoxide anion scavenging activity

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals. It is considered to play an important role in the peroxidation of lipids [18].Figure 3 shows the superoxide radical scavenging activity of *V. vinifera* extracts (50 and 125 µg mL^{-1}) in comparison with the same doses of standard antioxidants BHA, BHT and ascorbic acid. Ascorbic acid had stronger superoxide radical scavenging activity than BHA and BHT. The inhibition of superoxide radical formation by *V. vinifera* extracts and standard antioxidants decreased in the following order: Ascorbic acid (37.3 %), BHA (9 %), BHT (11.1%), water extract (18.2 %), acetone extract (28.9 %) and methanol extract (9.2 %) in presence of 125 µg mL^{-1} test sample.

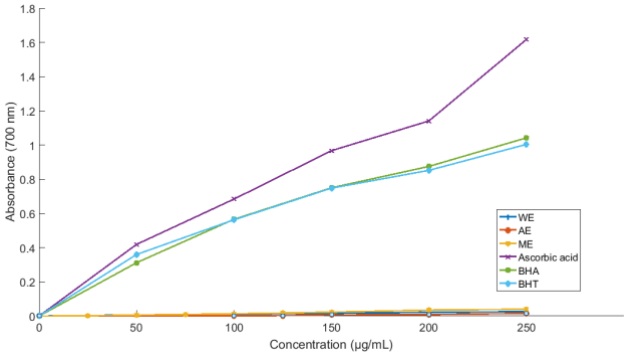

Fig. 2 Reducing power of the extracts from *V. vinifera*. BHA, BHT and ascorbic acid were used as reference antioxidants.

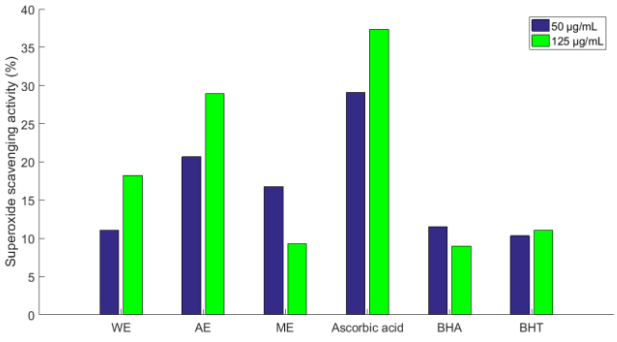

Fig. 3 Superoxide anion scavenging activity of the extracts from *V. vinifera*. BHA, BHT and ascorbic acid were used as reference antioxidants.

Hydrogen peroxide scavenging activity

The highest percentage H_2O_2 scavenging activity of 94.7% was obtained with BHT followed by α-tocopherol which had 91.4% scavenging activity. BHA, aceton extract, ascorbic acid and trolox had 66.95, 50.0, 47.97, 30.68%H_2O_2 scavenging activities respectively (Fig. 4).Hydrogen peroxide can accept protons (H^+) or electrons and by so doing be reduced to H_2O. In a H_2O_2 scavenging activity, the acetone extract act as hydrogen peroxide scavengers by donating hydrogen atoms to reduce the hydrogen peroxide to H_2O.

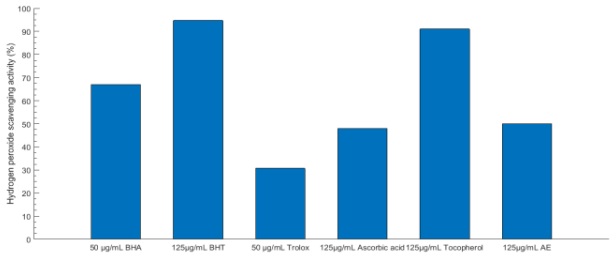

Fig. 4 H_2O_2 scavenging activity of the aceton extract from *V. vinifera*. BHA, BHT, ascorbic acid, trolox and α-tocopherol were used as reference antioxidants.

Total antioxidant activity

Total antioxidant activity of *V. vinifera* extracts was determined by the thiocyanate method. All of extracts exhibited effective antioxidant activity. The effects of same amounts of *V. vinifera* extracts of (75 µg/mL) on peroxidation of linoleic acid emulsion are shown in Fig. 5. The effects on lipid peroxidation of linoleic acid emulsion of extracts and standards decreased in that order: water extract > acetone extract > methanol extract > ascorbic acid > tocopherol > BHT > BHA. The total antioxidant capacity of plant extract may be attributed to their chemical composition and phenolic acid content.

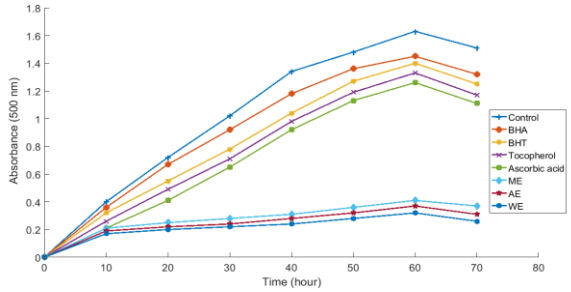

Fig. 5 Inhibitory effect of the extracts from *V. vinifera* on lipid peroxidation. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3).

CONCLUSION

The extracts of *V. vinifera* leaves exhibited different levels of antioxidant activity in all the models studied. The results from various free radical-scavenging systems revealed that the *V. vinifera* had significant antioxidant activity and free radical-scavenging activity. The free radical-scavenging property may be one of the mechanisms by which this drug is useful as a foodstuff as well as a traditional medicine. However, further investigation of individual compounds, their *in vivo* antioxidant activities and in different antioxidant mechanisms is warranted.

***Acknowledgements**: The authors are thankful to the Trakya University Research Fund, Edirne-Turkey (Project number: TUBAP-2012-37).*

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