



Extracts from *Vitis vinifera* Wastes exhibit Antioxidant activity

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ABSTRACT

The wide spectrum of side effects exhibited by synthetic compounds has attracted many researchers on replacing synthetic beneficial compounds with natural counterparts. Antioxidants are biochemical compounds with ability to capture free radicals and reduce their harmful effects to biological molecules. The use of plant wastes as natural sources of antioxidants could not only provide a nature based source, but it also is an environmental friendly task. *Vitis vinifera* (common grape) is one of the species from *Vitis* family native to various parts of the world including Iran. The potential of wastes from the common varieties of grape, as a source of natural antioxidants, was studied in this research. The leaves, stems, branches and barks of home grown grape plants were collected from homes and gardens in Rasht region. The methanol extract was examined for antioxidant activity by DPPH radical scavenging activity test, ferric-reducing antioxidant power, total phenol and flavonoid content. According to the results, the DPPH radical scavenging activity was 302.78 ± 5.21 mg/g dry weight, antioxidant activity by FRAP test was 0.224 ± 0.031 mmol/g DW. Total phenol content was 14.56 ± 0.24 mg/g dry weight of gallic acid equivalent. Total flavonoid content was 9.98 ± 0.31 mg/g DW and total anthocyanin was 0.705 ± 0.014 mg/g DW. Based on the results, we concluded that the use of almost priceless residual as a source of antioxidant has beneficial effect not only for management of wastes it could also find possible applications in food and pharmaceutical industries.

Key words: Waste management, environment, Grape, *Vitis vinifera*, secondary metabolites.

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INTRODUCTION

Plant derived wastes including stems, leaves, barks, dead flowers as well as low quality fruits and leftovers from fruit production industries are natural sources of secondary metabolites. If used in a proper way, they could be valuable sources of low price natural antioxidants. This could also provide a logical strategy to make best of them and control environmental pollution from their deterioration. Depending on the plant type, the part to be utilized and the way they are extracted the wastes may contain inorganic and organic secondary metabolites. For example, the content of iron can help to relieve anemia and have the power to counteract calcium loss and leading to bone strengthening. Considering the high volume of wastes produced in autumn from various plants and agricultural processes, the aim of this study was to investigate and compare the antioxidant activity of extracts from residual leaves, stems and barks of grape plant after its fruiting stage.

Vitis vinifera (common grape vine) is a member of *Vitis* that is grown in east and north parts of Iran. The plant could grow to about 32 m in length and its barks are flaky. The fruit is considered as a berry and best known as grape with a wide range of color and size. In cultivated plants, the fruit is quite large, up to 3 cm long, with red, green and purple (black) in color. Traditionally, the grape is used as a fresh fruit, processed to make wine or juice, or dried to produce raisins.

Reactive oxygen species (ROS) are free radicals produced by various natural metabolic pathways in the human body. They are also the result of exogenous chemical reactions which eventually enter the body [1]. They can be beneficial as signal transducers and growth regulators, if produced or used with normal doses [2]. However, large amounts of ROS can be dangerous to the system as they are capable of oxidizing bio-molecules including nucleic acids, proteins, lipids and DNA. The high amounts of ROS can initiate different degenerative diseases such as liver cirrhosis, various cancers, neurological disorders, atherosclerosis and arthritis [3]. The presence of natural antioxidants in cells such catalase, glutathione

peroxidase and superoxide dismutase can reduce the effect of ROS. Their harm can also be overcome by using exogenous antioxidants including α -tocopherol, β -carotene, ascorbic acid, glutathione and uric acid [4, 5].

Consumption of natural antioxidants such as fresh fruits and vegetables has protective effects against various diseases mostly related to the presence of several components, such as vitamins, flavonoids, anthocyanins and other phenolic compounds [6, 7].

Studies on natural antioxidants is mostly focused on activity of fruits and vegetables [8, 9]. However, broad information on the antioxidant activity of their wastes has attracted researcher during the last few decades. Many agricultural activities and food processing industries produce a large body of wastes including leaves, barks, peels and residuals of food factories. These types of natural wastes are not used or recycled as they have not yet found a beneficial commercial application [10]. However, it has been reported that peel and seed fractions of some fruits have higher antioxidant activity than the pulp fractions [11]. For example, a higher antioxidant activity has been found for pomegranate peel than its pulp [12]. Grape seed is higher than its pulp in antioxidant capacity and is a rich source of proanthocyanidin, which is very effective in scavenging various ROS [13].

MATERIALS AND METHODS

All types of wastes from various grape plants were collected, with permission of owners, from houses and gardens in Rasht. After separation of the contaminants, they were washed and dried at 37°C and kept in sealed plastic bags until experiments.

Buffers and solutions were freshly prepared in our laboratory. General chemical reagents including ethanol, acetone, sodium carbonate, acetic and hydrochloric acids were supplied by their local representatives. Folin-Ciocalteu's phenol reagent, tripyridyltriazine (TPTZ), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ferric and aluminum chlorides were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA).

Preparation of waste extracts

Various parts of the plant were mixed and ground into a fine powder and incubated in 75% methanol for two days, at room temperature. The mixture was filtered using whatman paper and the resulting filtrate concentrated by rotary evaporator at 40°C. The extract was then stored in a desiccator at 4°C until use.

Determination of total phenol content

The colorimetric Folin-Ciocalteu method was used to measure the content of phenolic compounds. In brief, 100 μ L of extracts were added to 100 μ L of Folin-Ciocalteu reagent (%50) and mixed thoroughly. A mixture of 2ml of sodium carbonate (%2) and 2.8ml deionized water was then added and well mixed. The resulting solution was incubated at room temperature for 30 minute and its absorbance was read at 720 nm on a UV-Vis spectrophotometer. Total phenol content was calculated using a standard curve of aqueous solutions of gallic acid and expressed as mg gallic acid equivalents (GAE)/g DW [14].

Total flavonoids content

Flavonoid content of the samples was determined using a modified method introduced by Chang *et al.* (2002). In practice, 500 μ L from each extracted sample was mixed with 1.5ml methanol (%85), 100 μ L of %10 aluminum chloride methanolic solution, 100 μ L of 1 M potassium acetate solution and 2.8 ml distilled water. The resulting solution was incubated for 40 minutes at room temperature and its absorbance was measured at 415 nm. Total flavonoid content of mixed grape wastes was calculated using a standard curve of quercetin and expressed as mg quercetin equivalents (QEs)/g DW.

Total anthocyanin content

To measure total anthocyanin content of extracts, 0.02 grams of each sample was mixed with 4 ml 1% methanolic solution and stored at 4°C for 24. The mixture was then centrifuged at 13000 rpm for 10 min and the 530 and 675 nm absorbance of supernatant were read on UV-Vis spectrophotometer. The anthocyanin content for each extract was calculated from the following equation [15].

$$\text{Anthocyanin content (mg/g DW)} = A_{530} - (0.25 \times A_{675})$$

DPPH Radical scavenging power

A slightly modified method described in [16] was used to evaluate the antioxidant capacity of extracts on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. In practice, aliquots of extracts were mixed with 2ml of DPPH methanolic solution (0.004%). A control tube was also prepared containing 2ml DPPH and 2ml methanol. Both mixtures were mixed thoroughly and kept in the dark for 30 min. The absorbance at 517nm was measured against a blank of methanol without DPPH. The inhibition rate (%) of the DPPH radical was then calculated from the following equation.

$$\text{Inhibition rate of DPPH (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

A plot of extract concentrations versus inhibition rate (%) of the DPPH radical was used to evaluate IC₅₀. The results were expressed as antioxidant activity equivalent ascorbic acid as standard (AEAC).

$$\text{AEAC (mg AA/G DW)} = \text{IC}_{50 \text{ ascorbate}} / \text{IC}_{50 \text{ sample}} \times 1000$$

Measurement of Ferric reducing antioxidant power (FRAP)

For this purpose, a procedure based on the ability of extracts to reduce ferric ions was used [13]. The test was started by preparing the FRAP reagent in sodium acetate buffer (300 mmol/L, pH 3.6), tripyridyltriazine (TPTZ) (10 mmol/L in 40 mmol/L HCl) and FeCl₃ solution (20 mmol/L) 10:1:1 (v:v:v) proportion. A 0.05 ml volume of mixed waste extracts prepared in distilled water was added to 1.5 ml of FRAP reagent. The mixture was stored at room temperature for 30 minutes and its absorbance measured at 593nm. The control solution was a mixture of 0.05ml distilled water and 1.5ml FRAP reagent. The antioxidant capacity was determined from the calibration curve in mmol FeSO₄ equivalents per one gram of sample's dry weight ($\mu\text{mol FeSO}_4/\text{g DW}$).

Statistical analysis

To statistically analyze the result, SAS for Windows version 9.1 was used. All data were reported as mean \pm standard deviation (SD) of three replicates. Differences between means were determined by One-Way ANOVA using Tukey's test. Statistical significance was defined at a level of $P \leq 0.05$.

RESULTS AND DISCUSSIONS

Radical scavenging activity of plant extracts is partly due to the presence of polyphenols and flavonoids. Table 1 has gathered the amount of total phenols, flavonoids and antocyanins. Based on our results, total phenol content was 14.56 ± 0.24 mg/g dry weight of gallic acid equivalent, total flavonoids content 9.98 ± 0.31 mg/g DW and total antocynins 0.705 ± 0.014 . The experiments were repeated triplicate and the results were calculated as the average obtained from three test. A number of research has reported that leftovers of fruit juicing factories contain considerable amounts of total phenol, antocyanins and flavonoid contents. In support of our results, it has been found that pomegranate peels contain considerable antioxidant activity mostly due to the content of antocyanins [11, 17]. The results obtained in our research are also in agreement with a previous research within our team [18]. Other research groups have reported the effect of ultrasonic treatment on antioxidant capacity of citrus species [19]. Many beneficial effects are reported for flavonoids found in fresh fruits and vegetables. It is suggested that they could protect human heart when taken as a supplement [6]. That research has emphasized that when flavonoids are taken as drug supplements they highly reduce the mortality rate from coronary heart diseases. Interestingly, the presence of flavonoids plays a major role in the traditional benefits known for honey. It has been found that considerable amounts of flavonoids exist in propolis [20] and honey [14]. In the present research, the total anthocyanin content of methanolic waste extracts from grape plant was also evaluated. Although only a few reports were found on the use of agricultural wastes as a source of antioxidants, our results could be compared to other wastes. For example, the antioxidant activity in seeds of some fruits has been reported to be mostly due to total phenols [10]. The medicinal benefits of fruits and their natural juices is a well-known fact and have been studied specifically. For example the hepatoprotective effect of orange juice on the injuries caused by carbon dioxide has been studied in detail [8].

The extraction conditions used to obtain antioxidant capacity by plant wastes as well as the environmental conditions are determinant factors on results [21]. In most cases, methanol is reported as the solvent of choice for extraction of phenolic compounds. This is reported to be due to its ability to inhibit the reaction of poly phenol oxidase (PPO) that causes oxidation of phenolic and its ease of evaporation as compared to water [22]. On the other hand, when compared to a non-polar solvent such as acetone, both methanol and ethanol offer better ability for extraction of phenolic compounds [23].

Our flavonoid results are in contrast to what was found by other researchers who reported that value was very low compared to their other antioxidant experiments [23]. However, total flavonoid content of some fruit wastes has been studied by another research team and their results are in agreement with our findings [20].

The DPPH assay is based on the ability of the extract to scavenge free radicals through donation of hydrogen. DPPH is a stable free radical that is reduced to diphenylpicryl hydrazine through a reaction with antioxidant compounds [16]. The initial reaction mixture has a deep violet color that is changed to light yellow after reduction of DPPH. As shown in Table 2, the extracts from grape plant leftovers had a considerable power to scavenge DPPH. The average of three tests for DPPH, showed an activity of 302.78 ± 5.21 mg/g dry weight. The antioxidant power to scavenge DPPH free radical has been used for determination of antioxidant capacity in a number of medicinal and aromatic plant extracts (Miliauskas *et al.* 2004). The method has also been used to investigate the effect of far infrared radiation on antioxidant activity of rice hulls (Lee *et al.* 2003). We found the method to be suitable for determining the antioxidant capacity in wastes extracts from leaves and various parts of grape plant. The DPPH method could also be modified to determine antioxidant capacity in human saliva [2, 20].

The results of FRAP test are also presented in Table 2 showing that antioxidant activity from the FRAP test was 0.224 ± 0.031 mmol/g DW. Similar to our experiments, the assay has been used to compare antioxidant power in peels, pulps and seeds of some fruits [16]. They have reported that fruit peels have stronger antioxidant power than seeds. The FRAP method has been slightly modified to measure the antioxidant power of celandine extracts [26]. They reported that the extract had a considerable power to reduce ferric ions, a result that is in agreement with our findings. We recommend the FRAP assay as a precise and straight forward method for measurement of antioxidant capacity in many natural sources including wastes from plants after harvesting.

Table 1. Total phenol, flavonoids and anthocyanins in extracts of selected fruits peels.

Tests	1 st	2 nd	3 rd	Average \pm SD
Total phenol (mg /g DW)	14.12	15.05	14.28	14.56 \pm 0.24
Total flavonoids (mg /g DW)	10.22	9.86	9.95	9.98 \pm 0.31
Total anthocyanins (mg/g DW)	0.775	0.689	0.710	0.705 \pm 0.014

Table 2. The results of DPPH and FRAP tests on methanolic extracts of selected fruit peels.

Tests	1 st	2 nd	3 rd	Average \pm SD
DPPH (mg AA.g ⁻¹ DW)	305.21	298.18	275.35	302.78 \pm 5.21
FRAP (mmol FeSO ₄ . g ⁻¹ DW)	0.212	0.229	0.214	0.224 \pm 0.03

CONCLUSIONS

In this research, antioxidants were measured in methanolic extracts obtained from mixed wastes of various grapes grown in Rasht. The results indicated total phenol content of about 15 mg/g DW, total flavonoids of around 10mg/g DW and total anthocyanin in the range of 0.7mg/g DW which are considerable values when compared to the main parts of fruits and vegetable. Besides, taking into account the very low price of grape wastes, their high volume and the benefit this action provides for waste management, the design of a pilot plant seems necessary.

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