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Antioxidant Activities and Several Bioactive Substances of Different Extracts of *Vitis vinifera* L.

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ABSTRACT: This study has aimed to determine the amount of flavonoids, anthocyanins, total phenolic compounds, ascorbic acid and antioxidant activity of the grape in the polar and nonpolar sub-fractions of methanolic extracts with two extraction methods, maceration and ultrasonic. The phenolic compounds content and antioxidant activity of selected grape (*Vitis vinifera* L. "Red Seedless") from Maragheh region, East Azerbaijan (Iran) were investigated with a view to their exploitation as a potential source of natural antioxidants. Antioxidant activities of the samples were determined by three testing systems namely DPPH, β-carotene/linoleic acid and reducing power assays. In the DPPH system, the highest radical scavenging activity was observed by the polar sub-fraction of the methanolic extract in frozen fruits (IC_{50} = 26.34 ± 6.5 µg/ml). In order to evaluate the efficiency of different methods for the extraction of the main polyphenols in grape, two methods were tested and compared. The results showed that the maceration method is better method as compared to the ultrasonic method. These advantages are visible on extracting of the flavonoids, anthocyanin, total phenolic, ascorbic acid contents and antioxidant capacity. Our findings demonstrate that the methanolic extracts of frozen and dried fruits of *Vitis vinifera* may be suggested as a strong potential source of natural antioxidant.

Keywords: Anthocyanin, Ascorbic Acid, Flavonoids, Grape, Total Phenols.

Introduction

In the past few years, there has been increasing interest in determination of suitable dietary sources of phenolic antioxidants. Iran is one of the most appropriate countries for producing grapes and raisins due to its climate and weather conditions. In terms of grape varieties, Iran is ranked as the first country in the world. According to the Food and Agriculture Organization of the United Nation report in 2014, 80.3% of raisin export in the world has been conducted by Turkey, the United States, Chili, Iran and Uzbekistan; and share of Iran in raisin export was 13.79 % on the same year (Mekouar, 2020). Polyphenols have been associated with the bioactive potential of grapes due to their antioxidant, anti-inflammatory, anticarcinogenic and antibacterial activities (Bagchi *et al.*, 2000; Gris *et al.*, 2011). Phenolics in grapes and red wines

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have been reported to inhibit oxidation of human low-density lipoproteins (LDL*) in vitro*. Phenolic compounds extracted from 12 different varieties of grapes showed antioxidant activity towards LDL oxidation *in vitro* (Tselepis *et al.*, 2005). An important field of research today is the control of 'redox' status by consuming foods with high antioxidant properties. Natural antioxidants present in the diet increase the resistance to oxidative stress and they may have a substantial impact on human health (Dimitrios, 2006).

Several methods have been developed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds, which contribute towards the total capacity of a plant in scavenging free radicals. The methods are typically based on inhibition of the accumulation of oxidized products, since the generation of free radical species is inhibited by the addition of antioxidants and this gives rise to a reduction of the end point by scavenging free radicals. The reliable method to determine RSC involves the measurement of the disappearance of free radicals, such as the 2, 2-azino-bis 3-ethylbenzenthiazoline-6-sulphonic acid radical (ABTS+), the 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^o$) or other colored radicals, with a spectrophotometer (Choi *et al.*, 2002; Gholivand *et al.*, 2014).

This research work intends to study the total phenolics, total flavonoids, anthocyanins, ascorbic acid contents, and antioxidant activity of *Vitis vinifera* frozen and dried fruits in the polar and non-polar sub-fractions with two methods of extraction, maceration and ultrasonic.

Materials and Methods

- *Chemicals and Plant material*

Linoleic acid, 2, 6 -di- tert-butyl- 4-methylphenol (butylated hydroxytoluene, BHT), 2, 2-diphenyl-1- picrylhydrazyl (DPPH, 95%), gallic acid, oxalic acid, ascorbic acid (AA), catechin, PVPP (polyvinyl polypyrrolidone), cyanidin -3-glucoside and β-carotene, were procured from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, and HPLC grade chloroform, standard Folin–Ciocalteu's phenol reagent, anhydrous sodium sulfate, ferric chloride, sodium carbonate, potassium ferricyanide, phosphate buffer solution (PBS), and Tween 40 were obtained from Merck (Darmstadt, Germany). The fruits of *Vitis vinifera* "Red Seedless" were gathered in the summer of 2018 from Maragheh region, East Azerbaijan, Iran. Fruits were stored both frozen and dried.

- *Maceration*

400 g of frozen and dried fruits put in the methanol at 45°C for frozen and 50°C for dried fruits during 45 and 60 hours respectively. The extracts were filtered and concentrated under vacuum at 50°C by using a rotary evaporator. These extracts were suspended in water and extracted with chloroform (3 ×500 ml). The extracts were stored in darkness at 4°C until used within a maximum period of one week. The extractions separated with water (polar) and chloroform (non-polar).

- *Ultrasonic Extraction*

Frozen and dried fruits of *Vitis vinifera* was weighed exactly (400 g) and mixed with 1000 mL of the solvent in a reagent bottle. The bottle was then closed and placed in the ultrasonic bath with temperature maintained at 45°C for frozen during 15 min and 50°C for dried fruits in 20min using ultrasonicator (42 kHz, heat power 250 W, KQ-500DE, MTH, China). The mixture was then filtered through Whatman filter paper No. 4 and the filtrate was evaporated under reduced pressure at

50°C. These extracts were suspended in water and extracted with chloroform (3 ×500 ml). The extracts were stored in darkness at 4°C until used within a maximum period of one week. The extractions separated with water (polar) and chloroform (non-polar).

- ***Determination of antioxidant properties***

1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity assay

The free radical-scavenging activities of extract were measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Sharififar *et al*. with some modifications (Sharififar *et al.*, 2007). 3 ml of various concentrations of the extract (500, 1000, 1500, 2000 and 2500 µg/mL) were added to 1 ml of a 0.5 mM methanol solution of DPPH. The mixture was strongly shaken and left to stand at room temperature for 60 min in the dark. The absorbance was measured at 517 nm against a blank. Inhibition of free radical, DPPH, in percent (I %) was calculated according to formula:

$$I\% = ((A_b - A_s)/A_b) \times 100$$

Where A_b is the absorbance of the control reaction (containing all reagents except the test compound), and A_s is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against sample concentration. Tests were carried out in triplicate. Ascorbic acid (AA) was used as positive control.

β –Carotene/linoleic acid assay

The antioxidant activity was evaluated according to the method described by Gholivand *et al*. with some modifications (Gholivand *et al.*, 2014). Briefly, 1.5 ml of β-carotene solution (1 mg/ml in chloroform), 3 ml of linoleic acid solution (10 mg/ml in chloroform), and 1.0 ml of Tween 40 solution (300 mg/ml in chloroform) were pipetted into a 250 ml flask. The chloroform was removed by rotary vacuum evaporator, and 150 ml deionized water was added to the residue and the mixture was shaken to form an emulsion. 350 µl of test sample in methanol (2 mg/ml) was mixed with 2.5 ml of this reagent, and the emulsion system was incubated for up to 24 h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT as positive control, and a blank containing only 350 µl of methanol. After this incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidative capacities of the extract were compared with those of BHT and blank.

Reducing power assay

The reducing power of extract was determined according to the method of Jahanban-Sfahlan *et al*. with some modifications (Jahanban-Sfahlan *et al.*, 2009). Different concentrations of methanolic extract (polar and nonpolar) of fruits in methanol (1.0 ml) were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Ascorbic acid was used as positive control.

- ***Determination of total phenolic contents***

Total phenolic contents of the extracts

were determined using the Folin-Ciocalteu reagent according to the method of Gholivand and Piryaei using gallic acid as standard, with some modifications (Gholivand & Piryaei, 2014). The extract solution (0.1 ml) containing 1000 µg of the extract was mixed with 46 ml of distilled water in a volumetric flask and 1 ml Folin-Ciocalteu reagent was added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and 3 ml aqueous solution of 2% Na_2CO_3 was added. At the end of incubation of 2 h at room temperature, absorbance of each mixture was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. Total phenol contents were expressed as µg gallic acid equivalents per mg of the extract. All tests were carried out in triplicate, and gallic acid equivalent values were reported as X ± SD of triplicates.

- Determination of total flavonoids

A modified protocol of that described by Kim *et al*. was employed (Kim *et al.*, 2003). A 0.1 ml aliquot of methanolic extract, appropriately diluted, was mixed with 0.4 ml distilled water in a 1.5 ml micro-centrifuge tube 0.03 ml of 5% $NaNO_2$ was added and the mixture allowed to react for 5 min. Following this, 0.03 ml of 10% $AlCl_3$ was added and the mixture stood for a further 5 min. Finally, the reaction mixture was treated with 0.2 ml of 1 M Na_2CO_3 and 0.24 ml distilled water, and the absorbance at 510 nm was obtained against a blank prepared similarly, by replacing extract with distilled water. Total flavonoid content was calculated from a calibration curve using catechin as standard, and expressed as mg catechin equivalents (CTE) per 100 g (Dourtoglou *et al.*, 2006).

- Determination of ascorbic acid content

Ascorbic acid contents of the extracts was determined using ascorbic acid as standard with some modifications. The samples (1 g) and 4 ml oxalic acid (1%) were mixed, homogenized for 1 min, and filtered. PVPP (polyvinylpolypyrrolidone) (100 g) was added to 2.5 ml of the filtered sample, to remove phenols, and 2–3 drops of H_2SO_4 (25%) were added, to reduce the pH to below 1. Absorbance of the mixture was determined at 254 nm. The results were expressed as µg ascorbic acid (AA) 100 mg^{-1} sample weight (sw) (Pantelidis *et al.*, 2007).

- Determination of anthocyanin

Total anthocyanin content was measured with the pH differential absorbance method, as described by Gholivand and Piryaei (2014). Briefly, absorbance of the extracts was measured at 510 and 700 nm in buffers at pH of 1.0 (hydrochloric acid–potassium chloride, 0.2 M) and 4.5 (acetate acid– sodium acetate, 1 M). Calculate the absorbance of the anthocyanin content by using a molar extinction coefficient of 29,600 (cyanidin-3- glucoside) as follows:

$$A = [(A_{510}-A_{700})\ pH_{1.0}-(A_{510}-A_{700})\ pH_{4.5}]$$

The results were expressed as µg cyanidin-3-glucoside equivalents per 100 mg sw (Gholivand & Piryaei, 2014).

Results and Discussion

Fruits and vegetables contain phytochemicals with antioxidant activity. These antioxidants have multifunction. Their activity and mode of action in a particular test system may depend on the oxidation conditions, which may in turn affect both the kinetics of oxidation and the composition of the system. Therefore, a multi-dimensional assay protocol would

be an advantage by reducing these limitations (Saoudi *et al.*, 2020). The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical-scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form 1, 1-diphenyl-2-picryl hydrazine (non-radical) with the loss of this violet color (Molyneux, 2004). DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Lower IC_{50} value reflects better DPPH radical-scavenging activity.

In this study, the effect of two extraction methods ultrasound and maceration for the efficient extraction of antioxidative compounds from *Vitis vinifera* in the case of frozen and dried fruits was investigated. Methanol was chosen as the solvent because alcohols are most widely used in antioxidant extraction work. During extraction, it was seen that maximum extraction yield was achieved with maceration extraction method.

The polar subfraction in frozen fruits of methanol extract provided the highest radical-scavenging activity with the lowest IC_{50} value of 26.34 ± 6.5 µg/ml for maceration method. In addition, DPPH scavenging abilities of the methanolic extracts were lower than that of synthetic antioxidant BHT. Ascorbic acid and BHT were used as standards. In Figures 1 and 2 present the comparison between polar and non-polar subfraction in frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with maceration and ultrasonic extraction methods.

The antioxidant activities of the plant extract were also evaluated by the spectrophotometric β-carotene bleaching test. In a β-carotene/ linoleic acid model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (Kulisic *et al.*, 2004). The relative antioxidative activities (RAAs) of the extracts were calculated from the equation, RAA = A sample/A BHT, where A BHT is the absorbance of the control (BHT) and A sample is the absorbance of the extract. The calculated RAAs of the extract with maceration and ultrasonic methods are given in Figures 3 and 4. In both assays, the polar subfraction of methanolic extract in frozen fruits of *Vitis vinifera* showed better antioxidative capacity than the others subfraction (RAAs for maceration: 98.14% and for ultrasonic: 75.30%).

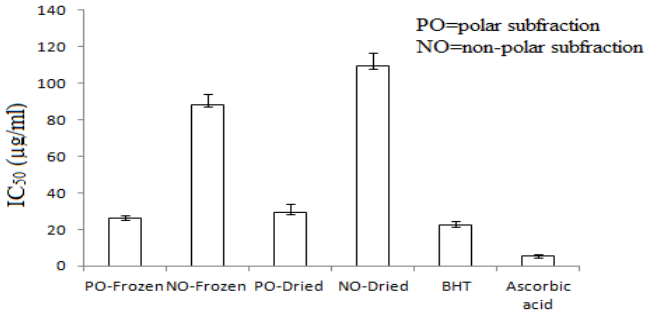

Fig. 1. Comparison between polar and non-polar subfraction in the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with maceration method.

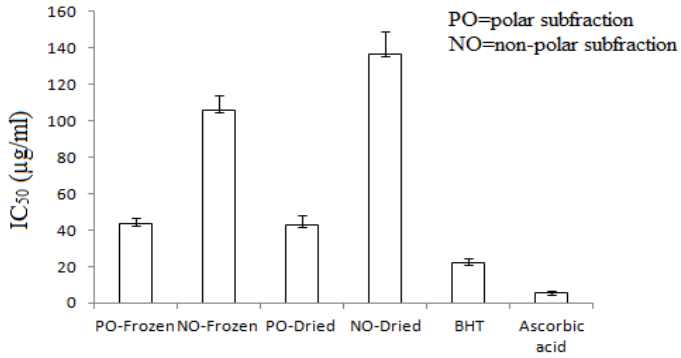

Fig. 2. Comparison between polar and non-polar subfraction in the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with ultrasonic method.

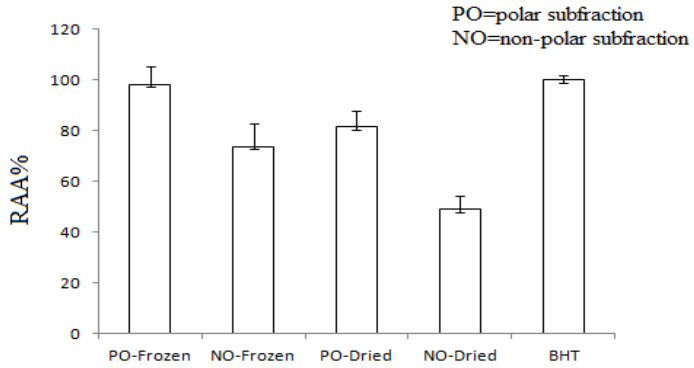

Fig. 3. Relative antioxidative activities (RAAs) of the methanolic extracts of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with maceration method.

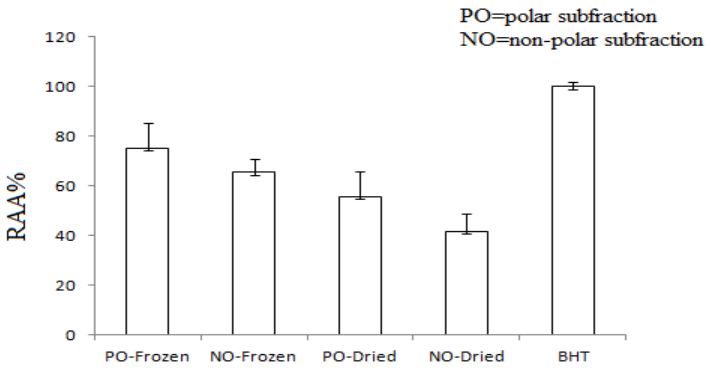

Fig. 4. Relative antioxidative activities (RAAs) of the methanolic extracts of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with ultrasonic method.

Different studies have indicated that the electron donation capacity reflects the reducing power of bioactive compounds in associated with antioxidant activity. Antioxidants can be explained as reducers, and inactivation of oxidants by reducers can be described as redox reaction in which one reaction species is reduced at the expense of the oxidation of the other. Fe^{3+} reduction is often used as an indicator

of electron donating activity, which is an important mechanism of phenolic antioxidant action (Yıldırım *et al.*, 2001). In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe^{3+}–Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then being monitored by measuring the formation of Perl's Prussian blue (Fe_4 [-Fe (CN) $_6]_3$) at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figures 5 and 6 shows the reducing power of the methanolic extract of *Vitis vinifera* as a function of their concentrations with maceration and ultrasonic methods. It was found that the reducing power of extract increased with the increase of their concentrations.

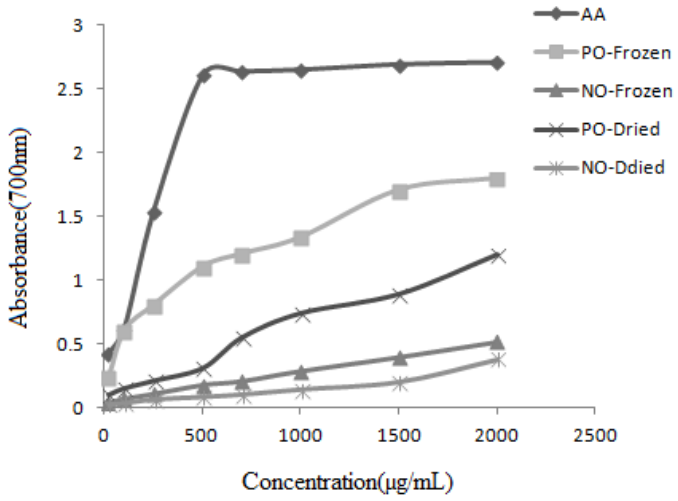

Fig. 5. Reducing power for the methanolic extract of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" as a function of their concentrations with maceration method.

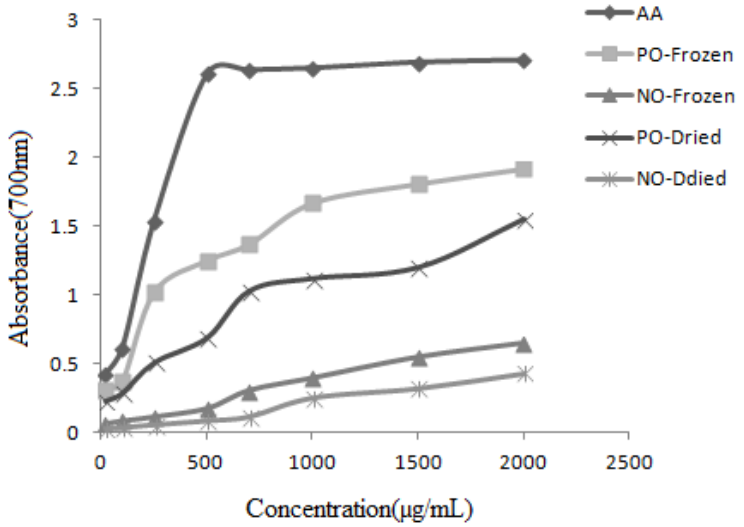

Fig. 6. Reducing power for the methanolic extract of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" as a function of their concentrations with ultrasonic method.

Polyphenolic compounds such as flavonoids, phenolic acids are considered the major contributors to the antioxidant activity of fruits and vegetables. The antioxidant activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and free radicals quenchers, as well as their metal chelating abilities (Vladimir-Knezevic *et al*., 2011). The amounts of total phenolics in the extract for the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" were determined spectrometrically according to the Folin–Ciocalteu procedure and calculated as gallic acid equivalents. Gallic acid is a water-soluble polyhydroxyphenolic compound that can be found in various natural plants. The standard curve equation was, y (absorbance) = 0.0003 ×gallic acid (µg) + 0.00534. The absorbance value was inserted in the above equation and the total amount of phenolic compound was calculated. The amounts of total phenols found in the plant methanolic extract are shown in Figures 7 and 8.

The results indicated that the polar subfraction in frozen fruits in methanolic extract with maceration method has higher total phenolic compounds than the other subfractions. In addition, according to these results, there is a relationship between total phenol contents and antioxidant activity. Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sun *et al.*, 2002).

The standard curve equation for determination flavonoids with catechin is y (absorbance) =0.004× catechin (µg) +0.0495. The amounts of total flavonoids for frozen and dried fruits of *Vitis vinifera* with two extraction methods were shown in table 1. Husain (1987) reported that flavonoids were OH scavengers. They also noted that the effectiveness of such compounds increases with increasing the number of hydroxyl groups attached to the aromatic B ring.

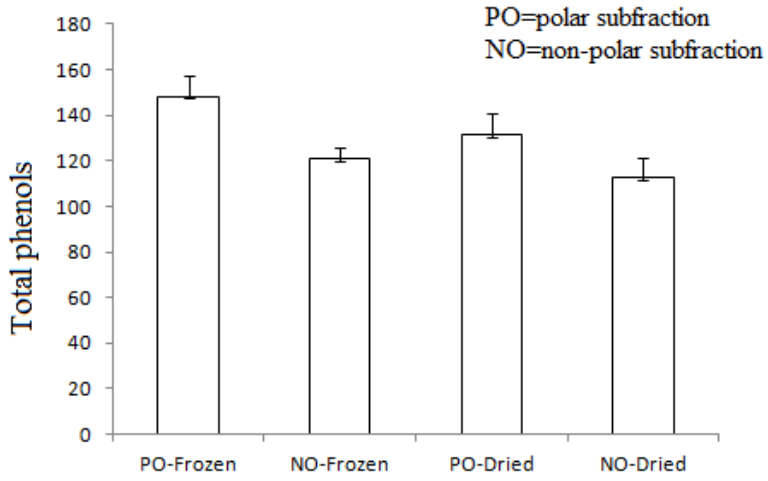

Fig. 7. The amounts of total phenols found in the methanolic extracts of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with maceration method.

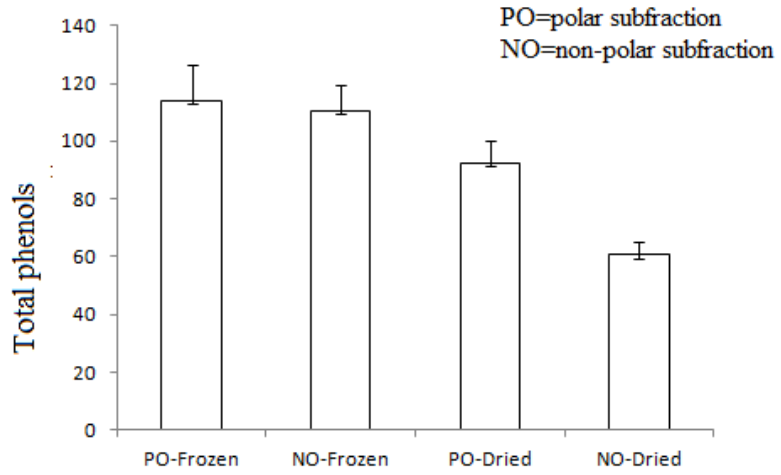

Fig. 8. The amounts of total phenols found in the methanolic extracts of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with ultrasonic method.

Table 1. The amounts of total flavonoids in methanolic extract of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless"

Fruit type	Extraction	Flavonoids (mg CAE/100 g plant)
Frozen	Maceration	88.26± 6.9
	Ultrasonic	61.47±2.5
Dried	Maceration	78.53±1.6
	Ultrasonic	48.35±8.1

Significant differences in anthocyanin content were recorded, since these pigments are responsible for the red and blue color. The polar subfraction in frozen fruits contained the highest anthocyanin content expressed as cyanidin-3-glucoside. The results were shown in Tables 2 and 3. Anthocyanins are considered very good antioxidant agents, their high activity being attributed to their peculiar structure, namely the oxonium ion in the C ring. The antioxidant functions of anthocyanins have been ascribed to the aglycone moiety, and this was demonstrated for cyanidin and some of its glycosides, but the number of sugar residues at the three position), the oxidation state of the C ring, the hydroxylation and methylation pattern, as well as the acylation by phenolic acids are considered crucial factors for the expression of antioxidant effects (Kallitraka *et al*., 2009).

Significant differences in ascorbic acid content among the different subfraction were recorded Tables 2 and 3. The polar subfraction in frozen fruits had the highest content of ascorbic acid (4.89 mg/ 100 g sw). The ascorbic acid ranges from 4.4 to 57.2 mg (%) in organic and conventional grape juices (Dani *et al.* 2007). Several factors influence the ascorbic acid content, including preharvest factors, such as climatic conditions (sunlight exposure and weather) and farming practices, maturity at harvest, harvesting method, postharvest handling conditions (storage), species, cultivars and tissues as well as genotype. All these factors are responsible for the wide variation in ascorbic acid content of fruits and vegetables.

Table 2. The amounts of anthocyanin and ascorbic acid in methanolic extract of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with maceration method

Fruit type	Fraction	Ascorbic acid[a]	Anthocyanin[b]
Frozen	Polar-Subfraction	5.62±0.05	18.83±1.91
	Nonpolar-Subfraction	2.04±0.11	10.25±2.23
Dried	Polar-Subfraction	3.15±0.41	1.73±0.08
	Nonpolar-Subfraction	2.21±0.14	0.18±0.05

[a] Results are expressed as µg cyanidin-3-glucoside equivalents /100 mg fresh weight (fw).
[b] Results are expressed as µg ascorbic acid (AA)/100 mg fresh weight (fw).

Table 3. The amounts of anthocyanin and ascorbic acid in methanolic extract of the frozen and dried fruits of *Vitis vinifera* L. "Red Seedless" with ultrasonic method

Fruit type	Fraction	Ascorbic acid[a]	Anthocyanin[b]
Frozen	Polar-Subfraction	3.22±1.51	15.45±3.62
	Nonpolar-Subfraction	1.18±0.08	8.25±3.41
Dried	Polar-Subfraction	2.15±0.39	8.72±0.25
	Nonpolar-Subfraction	1.02±0.12	2.68±0.88

[a] Results are expressed as µg cyanidin-3-glucoside equivalents /100 mg fresh weight (fw).
[b]Results are expressed as µg ascorbic acid (AA)/100 mg fresh weight (fw).

Several works with extracts of various plants have reported a reduction in the oxidative stress due to the presence of high antioxidants amount such as polyphenols. Vijayakumar (2004), found these effects for black pepper and Gladine (2007) reported these effects for rosemary, grape, citrus, and calendula; whereas, Papandreou (2009) reported the same results for blue berries (*Vaccinium angustifoli um*).

Conclusion

According to the results of this study, the methanolic extracts of frozen and dried fruits of *Vitis vinifera* may be suggested as a strong potential source of natural antioxidant. The methanolic extract in different subfraction were found to be effective antioxidants in different *in vitro* assays including β-carotene bleaching, DPPH radical scavenging and reducing power which can be proposed as a natural additive in food and pharmaceutical industries. Maceration method is better than the ultrasonic. These advantages are visible on extracting of Phytochemicals and antioxidant capacity. The extension of this work to future vintages should refine these conclusions and further studies on individual phenolic compounds are needed to elucidate the different antioxidant mechanisms and possible synergism.

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