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In vitro Antioxidant and Free Radical Scavenging Activities of Red Grape Seed Extracts

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Abstract: This study was carried out to evaluate the antioxidant activity of grape seed extracts (GSEs) by using various *in vitro* antioxidant assays. Total phenolic compounds and flavonoids were determined in GSEs (water and ethanol). Antioxidant activity of GSEs was evaluated by various antioxidant assays, including total antioxidant capacity, reducing power, DPPH radical scavenging, nitric oxide scavenging, hydroxyl radical scavenging and metal ion chelating activities. The various antioxidant activities were compared to ascorbic acid as standard antioxidant. The results showed that GSEs contained a logical amount of phenolic compounds and flavonoids. Therefore, it was demonstrated that the extracts of the grape seeds are good scavengers of reactive oxygen species (ROS). The higher antioxidative activity of ethanol grape seed extract (EGSE) in comparison with water grape seed extract (WGSE) was associated with its content of total phenolics and flavonoids. The results suggested that these extracts are a potential source of natural antioxidants that may be used in pharmaceutical or food industry.

Key words: Antioxidant activity · Free radical scavenging · Red grape seed · Phenolics · Flavonoids · *In vitro*

INTRODUCTION

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer, atherosclerosis, gastric ulcer and other conditions [1, 2]. Many antioxidant compounds, naturally occurring from plant sources, have been identified as a free radical or active oxygen scavengers. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity [3]. In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers. The intake of antioxidants such as polyphenols has been effective in the prevention of these diseases [4]. Polyphenols belong to a heterogeneous class of compounds possessing a variety of effects towards antioxidant behaviour. In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the last few decades [5].

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. It has various biological functions, due to its rich polyphenol ingredients, most of which are contained in its seeds (60-70%) and skin (30%). However, large quantities of grape seed wastes are produced annually by the food processing industry-wine, juice etc [6].

Grape seed extract, concentrated from the seeds of grape (*Vitis vinifera* L.), has a high content of compounds known as oligomeric proanthocyanidins (OPCs), which are made up of proanthocyanidin monomers. Grape seed polyphenol (GSP) is typical condensed tannin. Its active constituents are the proanthocyanidins, which represent a variety of polymers of flavan-3-ol such as catechin and epicatechin [7]. GSP is an antioxidant that is more powerful than catechins in aqueous system *in vitro* [8]. GSP has various physiological effects *in vivo*, such as antioxidant effects, protection against X-ray and ultraviolet rays, chemoprevention, anti-cancer or anti-tumor effects and inhibitory effects against atherosclerosis and hypercholesterolemia [9]. Recently, GSP has been considered as a potential health-food ingredient because of these beneficial properties. No toxicity of GSP has been reported, although GSP interacts with some kind of proteins [10]. To date, there have been

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no systematic reports of the effects of GSP on lipid metabolism such as the composition of fecal steroids, which alters in some hepatobiliary or colorectal diseases [11].

Grape seeds, a rich source of phenolic compounds, possess a broad spectrum of antioxidative properties that protects various cells from free radicals and oxidative stress. Grape seed extract, best known for its anti-oxidant, has been shown to have significantly greater antioxidant activity than vitamins C, E or beta-carotene [12]. Grape seed extract (GSE) has a protective effect on oxidant-induced production and deposition of extracellular matrix components, which results in hepatic fibrosis. It also improves hepatic ischemia-reperfusion injury and reduces the size of the infarct in cardiac ischemia in the rat [13]. Several studies have indicated that extracts obtained from grape seed inhibit enzyme systems that are responsible for the production of free radicals and that they are antimutagenic and anticarcinogenic [14]. Antioxidative activity of grape seed extract has been confirmed by b-carotene linoleate and linoleic acid peroxidation methods as well as by DPPH and phosphomolybdenum complex methods [15]. Grape seed extract has been evaluated for its antioxidative effect on a few meat types and has been reported to improve the oxidative stability of cooked beef and turkey patties [16]. Grape seed extract added at 10 and 20 g/kg to turkey thigh meat decreased TBARS values nearly ten-fold as compared to the control [17]. Chis *et al.* [18] investigated the anti-hyperglycaemic and antioxidant effect of grape seed extract, a polyphenolic flavonoid, in normal and streptozotocin-induced diabetic Wistar rats. The results showed that oral administration of grape seed extract (100 mg/kg/day) reduced the levels of lipid peroxides and carbonylated proteins and improved the antioxidant activity in plasma and hepatic tissue in rats treated with grape seed natural extract as compared with the diabetic control rats. Chaimad *et al.* [19] determined the neuroprotective effect of grape seed extract (GSE) on oxidative stress and brain damage induced by high-fat diet in rats. Comparing to the untreated high-fat diet rats, feeding with high-fat diet containing GSE 0.5 and 1% to healthy male Wistar albino rats for 8 weeks resulted in a significant reduction in lipid peroxidation marker, malondialdehyde and nitric oxide in brain. Peng *et al.* [20] investigated the antioxidant activity change of breads added with grape seed extract (GSE). The results showed that bread with the addition of GSE had stronger antioxidant activity than that of blank bread and increasing the level of GSE addition further enhanced the antioxidant capacity of the bread.

The aim of this research was to evaluate the antioxidant activity of grape seed extracts using various *in vitro* antioxidant assays.

MATERIALS AND METHODS

Materials

Plant Material: Grape (*Vitis vinifera* L. cv. Red Roomy), as large clusters with red berries, was purchased from a local market at Giza, Egypt.

Chemicals: Ascorbic acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, 2-deoxyribose and ferrous sulphate were purchased from Sigma Chemical Co., USA. All other chemicals were of analytical reagent grade.

Methods

Preparation of Grape Seed Extracts (GSEs): Two different extracts were prepared from red grape seeds using the procedure described by Badavi *et al.* [21] with some modifications as follows:

Grape seeds were separated from the grapes manually, air dried (in shade, 25-30°C) for one week and milled to fine powder. The grape seed powder (0.2 g) was macerated in 20 ml of distilled water or ethanol 80% for 24 h at 5°C and was stirred three times. The mixture filtered with cheese cloth and the resulting filtrate was used as water grape seed extract (WGSE) or ethanol grape seed extract (EGSE). The total solids (%) in both extracts WGSE and EGSE were determined by the dry weights following drying at 100°C until constant mass was achieved. In all assays, concentrations of grape seed extracts (WGSE or EGSE) were calculated as grape seeds equivalent.

Determination of Total Phenolic Content (TPC): TPC was determined spectrophotometrically in grape seed extracts using Folin-Ciocalteau method as described by Gao *et al.* [22] as follows:

Grape seed extract (100 μl) was mixed with 0.2 ml of Folin-Ciocalteu reagent, 2.0 ml of H_2O and 1.0 ml of 15% Na_2CO_3 solution. The developing color was measured at 765 nm after 2 h at room temperature using Jenway 6300 spectrophotometer. The concentration was calculated from the standard curve prepared using serial concentrations of standard tannic acid solution.

Determination of Total Flavonoid Content (TFC): TFC was determined in grape seed extracts using the method described by Kumaran and Karunakaran [23] as follows:

One milliliter of each grape seed extract was mixed with 1.0 ml of aluminium trichloride in ethanol (20 mg/ml) and a drop of glacial acetic acid then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min using Jenway 6300 spectrophotometer. Blank was prepared using 1.0 ml of grape seed extract and a drop of glacial acetic acid then diluted to 25 ml with ethanol. The absorption of standard quercetin solution (0.5 mg/ml in ethanol) was measured under the same conditions. The amount of total flavonoids content in grape seed extracts in quercetin equivalents (QE) was calculated by the following equation:

$$X = (A \bullet m_0) / (A_0 \bullet m)$$

Where: X is the flavonoids content (mg/mg grape seed extract in QE), A is the absorption of grape seed extract solution, A_0 is the absorption of standard quercetin solution, m is the weight of grape seed (mg) in grape seed extract and m_0 is the weight of quercetin in the solution (mg).

Determination of Antioxidant Activities of Grape Seed Extracts

Determination of Total Antioxidant Capacity: Total antioxidant capacity of grape seed extracts was assayed by the phosphomolybdenum method as described by Kumaran and Karunakaran [23] as follows:

Known volumes (0.1-0.3 ml) of each grape seed extract were added to test tube then completed to a constant volume (0.3 ml) with DW. 3.0 ml of reagent solution (0.6 *M* sulfuric acid, 28.0 m*M* sodium phosphate and 4.0 m*M* ammonium molybdate) were added to each tube and mixed well then incubated at 95°C for 90 min. Blank was prepared by the same procedure without grape seed extract. Ascorbic acid solution (0.03%, w/v) was used as positive control. After cooling to room, the absorbance of the solution was measured at 695 nm using Jenway 6300 spectrophotometer against blank. Increased absorbance of the reaction mixture indicated increased total antioxidant capacity.

Determination of Reducing Power: The reducing power of grape seed extracts was determined by the method of Mathew and Abraham [24] as follows:

In clean test tubes, a serial of known volumes (0.2-1.0 ml) of each grape seed extract were added. The solutions were completed to 1.0 ml with DW. 2.5 ml of phosphate buffer solution (0.2 *M*, pH 6.6) and 2.5 ml of potassium ferricyanide solution (1%, w/v) were added to each tube then mixed well. The mixtures were incubated at 50°C for 20 min. After incubation, 2.5 ml of trichloroacetic acid solution (10%, w/v) were added to each mixture then centrifuged at 5,000 rpm for 10 min. A known volume (2.5 ml) of each clear solution obtained after centrifugation (supernatant) was taken in another clean test tube then 2.5 ml of DW and 0.5 ml of ferric chloride solution (0.1%, w/v) were added and mixed well. The absorbance was measured at 700 nm using Jenway 6300 spectrophotometer. Blank was prepared by the same procedure without grape seed extract. Ascorbic acid solution (0.03%, w/v) was used as positive control. Increased absorbance of the reaction mixture indicated increased reducing power.

Assay of DPPH Radical Scavenging Activity: The antioxidant activity of grape seed extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method described by Lee *et al.* [25] as follows:

Known volumes (50-150 μl) of grape seed extract were individually added to test tubes then completed to a known volume (1.0 ml) by DW. 1.0 ml of DPPH solution (0.2 m*M* in ethanol) was added to each tube then mixed well and incubated at room temperature for 30 min. Control was prepared by the same procedure without grape seed extract. Ascorbic acid solution (0.03%, w/v) was used as a positive control. The absorbance (A) of the solution was measured at 517 nm using Jenway 6300 spectrophotometer. Inhibition of DPPH free radical in percent (I%) was calculated from the following equation:

$$I\% = [(A_c - A_s)/A_c] \times 100$$

Assay of Nitric Oxide Scavenging Activity: The scavenging activity of nitric oxide by grape seed extracts was determined by the method described by Kumaran and Karunakaran [23] as follows:

In clean test tubes, 0.5 ml of sodium nitroprusside solution (10 m*M* in 0.1 *M* phosphate buffer saline, pH 7.4) was mixed with different volumes of grape seed extract (0.1-0.5 ml) then DW was added to each tube to complete the solution to a known volume (1.0 ml). The test tubes were incubated at room temperature for 150 min then 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride)

was added to each tube and mixed well. The absorbance (A) was measured at 546 nm using Jenway 6300 spectrophotometer. Control was prepared by the same procedure without pollen extract. Ascorbic acid solution (0.03%, w/v) was used as positive control. Scavenging activity of nitric oxide was calculated from the following equation:

$$\text{Scavenging activity (\%)} = [(A_c - A_s)/A_c] \times 100$$

Assay of Hydroxyl Radical Scavenging Activity: The scavenging activity of hydroxyl radical by grape seed extracts was assayed by deoxyribose method as described by Nagai *et al*. [26] as follows:

In a clean test tubes, 0.45 ml of sodium phosphate buffer solution (0.2 *M*, pH 7.0), 0.15 ml of 2-deoxyribose solution (10 m*M*), 0.15 ml of $FeSO_4$-EDTA solution (10 m*M* $FeSO_4$, 10 m*M* EDTA), 0.15 ml of H_2O_2 solution (10 m*M*) and grape seed extract (50-100 µl) were added. The solutions were completed to a final volume (1.5 ml) with DW then incubation at 37°C for 4 h. After incubation, the reaction was stopped by adding 0.75 ml of trichloroacetic acid solution (2.8%, w/v) and 0.75 ml of thiobarbituric acid solution (1% in 50 m*M* NaOH solution) then the solutions were boiled for 10 min and cooled in water. The absorbance (A) of the solution was measured at 520 nm using Jenway 6300 spectrophotometer. Control was prepared by the same procedure without pollen extract. Ascorbic acid solution (0.03%, w/v) was used as positive control. Inhibition of deoxyribose degradation in percent (I%) was calculated using the following equation:

$$I\% = [(A_c - A_s)/A_c] \times 100$$

Assay of Fe^{2+} Chelating Activity: The ability of grape seed extracts to chelate ferrous (Fe^{2+}) ion was determined using a modified method of Minotti and Aust [27] as described by Oboh *et al*. [28].

In a clean test tube, 150 µl of freshly prepared ferrous sulphate solution (500 µ*M*) were added to a reaction mixture consisted of 168 µl of Tris–HCl buffer solution (0.1 *M*, pH 7.4) and grape seed extract (10-25 µl). The solution was completed by saline solution (0.9% NaCl, w/v) to a known volume (561 µl). The reaction mixture was incubated for 5 min at room temperature before the addition 13 µl of 1, 10-phenanthroline solution (0.25%, w/v). The absorbance (A) was measured at 510 nm using Jenway 6300 spectrophotometer. Control was prepared by the same procedure without pollen extract. Ascorbic acid solution (0.03%, w/v) was used as positive control. The Fe^{2+} chelating activity (%) was calculated from the following equation:

$$Fe^{2+} \text{ chelating activity (\%)} = [(A_c - A_s)/A_c] \times 100$$

Statistical Analysis: The results were analysed by an analysis of variance ($P<0.05$) and the means separated by Duncan's multiple range test. The results were processed by CoStat computer program (1986).

RESULTS AND DISSCUSION

Grape Seed Extracts a Source of Antioxidants: The systematic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Flavonoids, as one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics [23]. So that total phenolic compounds and flavonoids were chemically determined in both grape seed extracts (water and ethanol). The antioxidant activity of grape seed extracts was evaluated using various antioxidant assays, including total antioxidant capacity, reducing power, DPPH radical scavenging, nitric oxide scavenging, hydroxyl radical scavenging and metal ion chelating activities. The various antioxidant activities of grape seed extracts were compared to standard antioxidant (ascorbic acid).

Phenolic and Flavonoid Contents: The results showed that grape seed extracts contain a logical amount of phenolic compounds and flavonoids (Table 1). Ethanol grape seed extract contains high amount of phenolic compounds and flavonoids (66.60 and 11.56 mg/g grape seeds, respectively) in comparison with water grape seed

Table 1: Total solids, phenolic and flavonoid contents of grape seed extracts

Grape seed extract	Total solids (%)	Phenolics (mg/g grape seeds)	Flavonoids (mg/g grape seeds)
Water extract	51.66^{b}±0.87	31.20^{b}±0.42	6.85^{b}±0.85
Ethanol extract	55.83^{a}±0.60	66.60^{a}±1.96	11.56^{a}±1.44
L.S.D	2.96	5.58	4.64

-Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

Table 2: Total antioxidant capacity of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid

Treatment	Conc. (ppm)	Total antioxidant capacity ($O.D_{695\ nm}$)
WGSE	303	$0.504^{g}\pm0.001$
	606	$0.856^{e}\pm0.002$
	909	$1.274^{d}\pm0.002$
EGSE	303	$0.754^{f}\pm0.006$
	606	$1.309^{c}\pm0.001$
	909	$1.866^{a}\pm0.006$
Ascorbic acid	27.3	$1.708^{b}\pm0.008$
LSD 0.05	-	0.015

-Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

Table 3: Total reduction capability of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid

Treatments	Conc. (ppm)	Total reduction capability ($O.D_{700\ nm}$)
WGSE	235.2	$0.024^{g}\pm0.003$
	705.8	$0.590^{e}\pm0.002$
	1176.4	$1.233^{c}\pm0.003$
EGSE	235.2	$0.306^{f}\pm0.002$
	705.8	$0.808^{d}\pm0.002$
	1176.4	$1.929^{a}\pm0.001$
Ascorbic acid	35.3	$1.796^{b}\pm0.002$
LSD 0.05	-	0.0072

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

extract (31.20 and 6.85 mg/g grape seeds, respectively). Total solids of each extract were WGSE 51.66% and EGSE 55.83%. The phenolic contents in grape seeds are different from one variety or cultivar to another [29]. The results are supported by many authors [30-35] who found that the phenolics content of grape seeds may range from 5% to 8% by weight. The most abundant phenolics isolated from grape seeds are catechins (catechin, epicatechin and procyanidins) and their polymers. Kallithraka *et al.* [36] investigated the effect of different solvents on the extraction of some phenolic compounds from grape seeds. The results showed that methanol was the best solvent for the extraction of (+)-catechin, (-)-epicatechin and epigallocatechin, whereas 70% acetone yielded the largest amounts of procyanidins and 75% ethanol yielded the largest amount of gallic acid. The greatest amount of total phenols was extracted by 70% acetone. Savova *et al.* [37] studied the polyphenols recovery from the seeds of red-grape (*Vitis vinifera* L.) in different ethanol-water mixtures at different temperatures and times of extraction. Total polyphenols extracted varied sensibly over the range of 0.7-24 mg/g depending on operating conditions.

Total Antioxidant Capacity: The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. In the present data (Table 2), total antioxidant capacity of grape seed extracts and ascorbic acid was demonstrated. The study revealed that the antioxidant activity of the grape seed extracts increased with increasing concentration of the grape seed extract. As demonstrated previously, ethanol grape seed extract seemed to be having a higher capacity than water grape seed extract. The antioxidant activities of grape seed extracts and standard were in the following order: EGSE at 909 ppm > ascorbic acid > EGSE at 606 ppm > WGSE at 909 ppm > WGSE at 606 ppm > EGSE at 303 ppm > WGSE at 303 ppm.

Reducing Power: Data in Table 3 showed that the reductive capability of grape seed extracts compared to ascorbic acid. For the measurements of the reductive ability, it has been investigated from the Fe^{3+}–Fe^{2+} transformation in the presence of tested materials. Like the antioxidant activity, the reducing power of grape seed extracts increases with increasing concentration. Earlier studies, Tanaka *et al.* [38] and Duh [39] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [40]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The present data on the reducing power of the tested extracts suggest that it is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity of antioxidants has been attributed by various mechanisms, among which some of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [41].

DPPH Radical Scavenging Activity: DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Data indicated that the grape seed extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine (Table 4). The scavenging effect

Table 4: Scavenging activity of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid against DPPH radical

Treatments	Conc. (ppm)	Scavenging activity (%)
WGSE	250	$68.32^{g}\pm0.06$
	500	$74.87^{f}\pm0.08$
	750	$83.57^{c}\pm0.04$
EGSE	250	$79.08^{e}\pm0.08$
	500	$88.58^{b}\pm0.06$
	750	$94.75^{a}\pm0.12$
Ascorbic acid	23	$81.96^{d}\pm0.32$
LSD 0.05	-	0.42

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

Table 5: Nitric oxide scavenging activity of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid

Treatments	Conc. (ppm)	Scavenging activity (%)
WGSE	666.6	$26.52^{e}\pm0.94$
	2000	$69.88^{c}\pm1.64$
	3333.3	$83.14^{b}\pm3.06$
EGSE	666.6	$30.82^{e}\pm1.99$
	2000	$74.90^{c}\pm3.42$
	3333.3	$91.75^{a}\pm0.35$
Ascorbic acid	100	$57.34^{d}\pm0.94$
LSD 0.05	-	6.24

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

of each grape seed extract at high concentration (750 ppm) and ascorbic acid standard solution with the DPPH radical was in the following order: EGSE (94.75%) > WGSE (83.57%) > ascorbic acid (81.96%). Data also revealed that the scavenging activity of both grape seed extracts was increased with increasing the concentration of each extract. From the obtained results, it could be concluded that the highest concentration of EGSE (750 ppm) almost possesses scavenging activity higher than 23 ppm of ascorbic acid and the higher concentration of other one (750 ppm of WGSE) possesses scavenging activity equal to 23 ppm of ascorbic acid. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins and aromatic amines (*p*-phenylene diamine, *p*-aminophenol, etc.), reduce and decolourise DPPH by their hydrogen donating ability [23]. Phenolic compounds of the grape seed extracts are probably involved in their antiradical activity.

Nitric Oxide Scavenging Activity: Nitric oxide (reactive nitrogen species), formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions [42]. Incubation of sodium nitroprusside resulted in linear time-dependent nitrite production. Data as shown in Table 5 revealed that grape seed extracts have the nitric oxide scavenging activity. In general, the ability of EGSE on nitric oxide scavenging activity was higher than that of WGSE. There was a positive correlation between the concentration of grape seed extract and scavenging activity against nitric oxide. As shown in Table 5, higher concentration of EGSE (3333.3 ppm) almost possesses nitric oxide scavenging activity equal to higher concentration of WGSE and more than that of 100 ppm ascorbic acid. Accordingly, grape seed extracts may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health.

Table 6: Hydroxyl radical scavenging activity of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid

Treatments	Conc. (ppm)	Scavenging activity (%)
WGSE	166.6	$5.82^{g}\pm0.21$
	250	$15.04^{f}\pm1.00$
	333.3	$17.73^{e}\pm0.19$
EGSE	166.6	$70.57^{c}\pm0.24$
	250	$73.18^{b}\pm0.31$
	333.3	$74.95^{a}\pm0.50$
Ascorbic acid	10	$24.82^{d}\pm0.18$
LSD 0.05	-	1.42

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

Hydroxyl Radical Scavenging Activity: Free radicals and other reactive species are constantly generated *in vivo* both by accidents of chemistry and for specific metabolic purposes. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its radical derivates (superoxide anion and hydroxyl radicals), peroxides and transition metals. Reactive species are thought to play an important role in aging and in the pathogenesis of numerous degenerative or chronic diseases, such as cancer, cardiovascular diseases, diabetes and atherosclerosis [43]. The scavenging abilities of ascorbic acid and grape seed extracts on hydroxyl radical inhibition were shown in Table 6. Hydroxyl radical scavenging activity was seen with both grape seed extract, whilst EGSE was more effective than

WGSE. The scavenging activity was increased with increasing the concentration of grape seed extract. From the obtained results, it could be arranged these substances according to their hydroxyl radical scavenging activities in the following decreasing order: 333.3 ppm of EGSE (74.95%) > 250 ppm of EGSE (73.18%) > 166.6 ppm of EGSE (70.57%) > ascorbic acid (24.82%) > 333.3 ppm of WGSE (17.73%) > 250 ppm of WGSE (15.04%) > 166.6 ppm of WGSE (5.82%). As previously shown, EGSE was more effective than WGSE in hydroxyl radical scavenging activity. Generally, grape seed extracts demonstrated the antioxidant effects against peroxidation of biomolecules to scavenge the hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxyl radicals.

Metal Chelating Activity: The method of metal chelating activity is based on chelating of Fe^{2+} ions by the reagent 1, 10-phenanthroline, which is a quantitative formation of a complex with Fe^{2+} ions. The formation of a complex is probably disturbed by the other chelating reagents, which would result in the reduction of the formation of red-coloured complex. Measurement of the rate of reduction of the colour, therefore, allows estimation of the chelating activity of the coexisting chelator [44]. In this assay both extracts interfered with the formation of ferrous complex with the reagent 1,10-phenanthroline, suggesting that it has chelating activity and captures the ferrous ion before 1,10-phenanthroline. The percentages of metal scavenging capacity of tested grape seed extracts are shown in Table 7. The metal scavenging effect of the extracts follows the order: EGSE (435.5 ppm) > WGSE (435.5 ppm) > EGSE (261.3 ppm) > WGSE (261.3 ppm) > ascorbic acid (13.1 ppm) > WGSE (174.2 ppm) > EGSE (174.2 ppm). The data also showed that the metal chelating activity of both grape seed extracts increased with increasing the concentration of extract. It was reported that chelating agents, which form *ó*-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised form of the metal ion [40]. The obtained data reveal that extracts demonstrate an effective capacity for iron binding, suggesting that its action as antioxidant may be related to its iron-binding capacity.

In general, it was demonstrated that the extracts of the grape seeds are good scavengers of active oxygen species, including superoxide anion radical, hydroxyl radical and nitric oxide radical. The antioxidative activity of grape seed extracts is attributed to their contents of antioxidant agents, including phenolics, flavonoids, ascorbic acid and tocopherols [45-47]. The higher antioxidative activity of ethanol grape seed extract in comparison with water grape seed extract was associated with its content of total phenolics and flavonoids (Table 1). Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [48]. Guendez *et al.* [31] found that there is a significant correlation between DPPH scavenging activities of grape seed extracts and total phenolic content. Mielnik *et al.* [49] found that grape seed extract could be very effective in inhibiting lipid oxidation of cooked turkey meat during chill-storage. Spranger *et al.* [50] found that grape seed procyanidins presented higher antioxidant activities than other well-known antioxidants such as vitamin C, suggesting that grape seed procyanidins might be of interest to be used as alternative antioxidants. The results of antioxidant activity were supported by Rababah *et al.* [51], Li *et al.* [52], Choi and Lee [53], Chedea *et al.* [54] and Shan *et al.* [55]. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans [38].

Table 7: Metal chelating activity of water grape seed extract (WGSE), ethanol grape seed extract (EGSE) and ascorbic acid

Treatments	Conc. (ppm)	% Fe Chelation
WGSE	174.2	10.02^{e}±0.61
	261.3	42.65^{c}±0.20
	435.5	77.27^{a}±0.92
EGSE	174.2	6.40^{f}±0.41
	261.3	45.80^{b}±0.20
	435.5	78.90^{a}±0.59
Ascorbic acid	13.1	22.72^{d}±1.57
LSD 0.05	-	2.53

- Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$

Concluding Remarks: Finally, it could be concluded that red grape seed extracts are a potential source of natural antioxidants that may be used in pharmaceutical or food industry.

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