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Radical Scavenging Activities of Some *Vitis vinifera* Varieties Grown in Malatya (Turkey) and Their Preventive Effects on the Formation Lipid Peroxidation in Unsaturated Fatty Acids Mediated

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This study examined the phytochemical characteristics of five samples of *Vitis vinifera* grown in the Malatya region of Turkey and investigated their impacts on human health by determining their antioxidant activities *in vitro*. The present research includes five native grape varieties (white: Tahanebi, Sam and Kureys; red: Köhnü; black: Banazi Karasi). Antioxidative activities of the grape extracts were determined with the following modifications. The free radical scavenging effect in extracts was assessed by the decolouration of a methanolic solution of DPPH. Lipid peroxidation level was found to be significantly high in the Fenton reagent (FR) containing group when compared to the control group ($p < 0.0001$). However, lipid peroxidation level decreased significantly in the groups to which grape extracts were added ($p < 0.0001$). According to DPPH results, all grape samples showed effective antioxidant activity in different concentrations and these activities were observed to change in parallel with the increase in flavonoid concentration. The results indicate that the grape fruit extracts decrease lipid peroxidation level in the Fenton reagent environment and have scavenging effect on the DPPH$^{\bullet}$ depending on grape varieties.

Key Words: Grape, α,α-Diphenyl-β-picrylhydrazyl (DPPH), Lipid peroxidation, Flavonoids, Antioxidant capacity.

INTRODUCTION

Lipid peroxidation (LPO), a marker of oxidative damage, is associated with a progressive loss in membrane fluidity, reduction in membrane potential, an increase in membrane permeability to ions leading to cellular damage[1,2]. Numerous studies have pointed out that there is a link between elevated levels of lipid peroxidation and a wide range of diseases[3].

Recently there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics which are secondary plant metabolites naturally present in fruit and vegetables. The antioxidant activity of dietary polyphenols is considered to be much greater than essential vitamins. It is thefore contributes significantly to the health benefits of fruit[4,5].

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Grape (*Vitis vinifera*) is a rich source of phenolic compounds widely grown across the world[6]. A large amount of different phenolic compounds presents in skin, pulp and seeds[7,8]. The antioxidant compounds present in grape have been identified as phenolic acids (benzoic and hydroxy cinnamic acid), stilbene derivaties (resveratrol), flavan-3-ols (catechin acid, epicatechin), flavonols (kaempherol, quercetin, myricetin) and anthocyanins[9,10]. It has been found that resveratrol and quercetin inhibit human platelet aggregation *in vitro* and exhibit potential anticancer properties, by inducing cell differentiation[11] and inhibiting protein-tyrosine kinase activity[12]. In addition, previous studies demonstrated the importance of *V. vinifera* in the prevention of acetaminophen induced hepatic DNA damage, apoptosis and necrotic cell death[13].

The objective of this study is to investigate the antioxidant activities and phytochemical characteristics of five *Vitis vinifera* varieties. These species are planted widely in the Malatya region (Turkey) because Turkey, is the 5th-largest producer grapes and becoming one of the important wine producers in the world. This study underlies the significance of the antioxidant effects of food in order to ensure good health and quality of life. Previous studies related to this subject generally dealt with the antioxidant capacities of vegetative resources, while their chemical contents need to be evaluated. Therefore, in this study we aimed to examine the phytochemical characteristics of grape samples grown in the Malatya region of Turkey and investigated their impacts on human health by determining their antioxidant activities *in vitro*.

EXPERIMENTAL

Oleic acid (18:1, n 9), linoleic acid (18:2, n 6), linolenic acid (18:3, n 3), Twin 20, Tris-base and hydrochloride, quercetin, myricetin, resveratrol, cathechin, naringin, naringenin, kaempferol and HPLC grade methanol, acetonitrile, *n*-hexane, isopropyl alcohol, $FeCl_2 \cdot 2H_2O$, H_2O_2, KH_2PO_4, butylated hydroxytoluene (BHT), *n*-butanol, α,α-diphenyl-β-picrylhydrazyl, (DPPH), dimethyl sulphoxide, 2-thiobarbituric acid (TBA) and ethyl alcohol were purchased from Sigma-Aldrich.

The present research includes five native grape varieties (white: Tahanebi, Sam and Kureys; red: Köhnü; black: Banazi Karasi). Grapes were harvested at their technological maturity, from Yesilyurt, Konak and Arapgir vineyards, region Malatya (Turkey), 2008 vintage. The fruits were immediately washed and frozen to be further freeze-dried. Freeze-dried samples were maintained at -20 ºC prior to analysis. Only healthy looking fruits (without mechanical damage or bacterial infection) were selected for examination. The antioxidant activity and flavonoid analysis were determined in DMSO extracts.

Preparation of grape extracts: 50 g samples of fresh fruits were homogenized in 100 mL of 80 % methanol. Homogenates were centrifuged at 5,000 rpm for 5 min at 4 ºC. The supernatant was then concentrated by drying under reduced

25 pressures at 50 ºC using a rotary evaporator. Each extract was re-suspended in DMSO to give a stock solution and stored at -20 ºC until analysis.

Antioxidative activity testing in the unsaturated fatty acid environment: Preventing effect on the lipid peroxidation of the grapes extracts were determined by the method of Shimoi *et al.*[14] with the following modifications: 1 mM $FeCl_2$ ($FeCl_2 \cdot 2H_2O$) and 3 µM hydrogen peroxide (H_2O_2) solutions were prepared freshly for every treatment, using doubly deionized water. Extracts of grape fruits were also prepared freshly using DMSO. 3.97 mM oleic acid (18:1, n-9), 10.44 mM linoleic acid (18:2, n-6, LA) and 2.30 mM linolenic acid (18:3, n-3, LNA) were dissolved in the DMSO. Buffer solutions were prepared with 0.2 % Twin 20, 0.05 M Tris-HCl and 0.15 M KCl (pH = 7.4).

During *in vitro* experiment, the first group was used as a control, the second group was Fenton reagent group, ($FeCl_2$ + H_2O_2, Fenton R), the third group was Fenton R plus Banazi extract, the fourth group Fenton R plus Kohnu extract and fifth group Fenton R plus Sam extract, sixth group Fenton R plus Tahnabi extract an seventh group Fenton R plus Kureys. The first group was prepared and 0.4 mL fatty acid mixture (LNA: 3.26 µM/1 mL; 14.82 µM/1 mL LA and 4.99 µM/1 mL 18:1 oleic acid) was suspended in 5 mL buffer solution. The second group was a Fenton reagent group and 0.4 mL fatty acid mixture 40 µM $FeCl_2$ and 60 µM H_2O_2 were suspended in 5 mL buffer solution. The third to seventh groups were Fenton reagent and grape fruit extracts and 0.4 mL fatty acid mixture 40 µM $FeCl_2$ and 60 µM H_2O_2 and 2 mL fruit extracts were suspended in 5 mL buffer solution.

All of the mixtures were incubated at 37 °C for 24 h. After incubation, 100 µL of 4 % (w/v) butylated hydroxy toluene solution was added to prevent further oxidation. Then, 1 mL of each mixture was taken and 1 mL 0.6 % 2-thiobarbituric acid solution was added to the reaction mixture and incubated at 90 °C for 40 min. Samples were allowed to cool to room temperature and the pigment produced was extracted with 3 mL of *n*-butanol. Samples were then centrifuged at 6,000 rpm for 5 min and the concentration of the upper *n*-butanol layer was measured using a HPLC-fluorescence detector.

Quantitation of lipid peroxidation level *in vitro* environment: The products of peroxidation of fatty acids *in vitro* environment were determined by reading the fluorescence detector set at λ (excitation) = 515 nm and λ (emission) = 543 nm. Formation of the malonaldehyde *in vitro* enviroment expressed as thiobarbituric acid-reactive substances (TBARS) calculated from a calibration curve using 1,1,3,3-tetraethoxypropane as the standard. The MDA-TBA complex was analyzed using the HPLC equipment. The equipment consisted of a pump (LC-10 ADVP), a UV-visible detector (SPD-10AVP), a column oven (CTO-10ASVP), an autosampler (SIL-10ADVP) a degasser unit (DGU-14A) and a computer system with class VP software (Shimadzu, Kyoto Japan). Inertsil ODS 3 column (15 × 4.6 mm, 5 µm) was used as the HPLC column. The column was eluted isocratically at 20 ºC with a 5 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (85:15, v/v) at a rate of 1 mL/min[15].

Chromatographic conditions for flavonoid analysis: Chromatographic analysis was carried out using PREVAIL C_{18} reversed-phase column (15 × 4.6 mm) 5 μm diameter particles. The mobile phase was methanol/water/acetonitile (46/46/8, v/v/v) containing 1.0 % acetic acid[16]. This mobile phase was filtered through a 0.45 μm membrane filter (Millipore), then deaerated ultrasonically prior to use. Catechin (CA), naringin (NA), rutin (RU), resveratrol (RES), myricetin (MYR), morin (MOR), naringenin (NAR), quercetin (QU) and kaempferol (KA) were quantified by DAD following RPHPLC separation at 280 nm for catechin and naringin, 254 nm for rutin, myricetin, morin and quercetin, 306 nm for resveratrol and 265 nm for kaempferol. Flow rate and injection volume were 1.0 mL/min and l0 μL, respectively. The chromatographic peaks of the analyses were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using the external standard method. All chromatographic operations were carried out at a temperature of 25 °C.

Antioxidant assay by DPPH radical scavenging activity: The free radical scavenging effect in extracts was assessed by the decolouration of a methanolic solution of DPPH according to the method of Brand-Williams *et al.*[17]. A solution of 25 mg/L DPPH in methanol was prepared and 4.0 mL of this solution was mixed with 25, 50, 100, 250, 500 and 1000 μL of extract in DMSO. The reaction mixture was stored in darkness at room temperature for 0.5 h. The absorbance of the mixture was measured spectrophotometrically at 517 nm.

The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs control - Abs sample)]/ (Abs control)] × 100 where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract/standard.

Determination of sugars using liquid chromatography: Sugars in the combined extracts were determined using high-performance liquid chromatography (HPLC) with a refractive index detector (RID). The mobile phase was acetonitrile/water (75/25, v/v) and the elution was performed at a flow-rate of 1 mL/min. The temperature of the analytical column was kept at 40 ºC. The column used was a Supelcosil-NH_2, (25 × 4.6 mm, 5 μm, Sigma, USA). The analyses were performed in triplicate batches. Prior to the quantitative and qualitative determination of sugars in the sample, standard solutions were prepared of different sugars: sucrose, glucose and fructose. These standard solutions of different sugars were used to make calibration lines for each of the sugars, which were later used for assessing the concentrations corresponding to the different peaks in the chromatograms.

Statistical analysis: Statistical analysis was performed using SPSS software (Ver 15.0). The experimental results were reported as mean ± SEM Analysis of variance (ANOVA) and an LSD (Least Significant Difference) test were used to compare the experimental groups with the controls.

RESULTS AND DISCUSSION

Lipid peroxidation: The lipid peroxidation level was found to be significantly high in the group containing Fenton reagent (FR) ($p < 0.0001$) when compared to the control group. When the groups containing grape extracts and the control group were compared a significant increase was observed in Kureys group ($p < 0.001$, $p < 0.05$). No statistical difference was observed in Banazi and Kohnu groups when compared to the control group. On the other hand, the lipid peroxidation level in the groups containing Banazi, Kohnu and Kureys fruit extracts was found to decrease significantly and partial decrease were observed in Sam and Tahanebi compared to the group containing Fenton reagent. When the groups containing grape extracts were compared among each their, the lipid peroxidation level in the Banazi, Kohnu and Kureys groups was observed to be lower than the Sam and Tahanebi groups. No statistical difference was observed between the Banazi and Kohnu groups (Fig. 1).

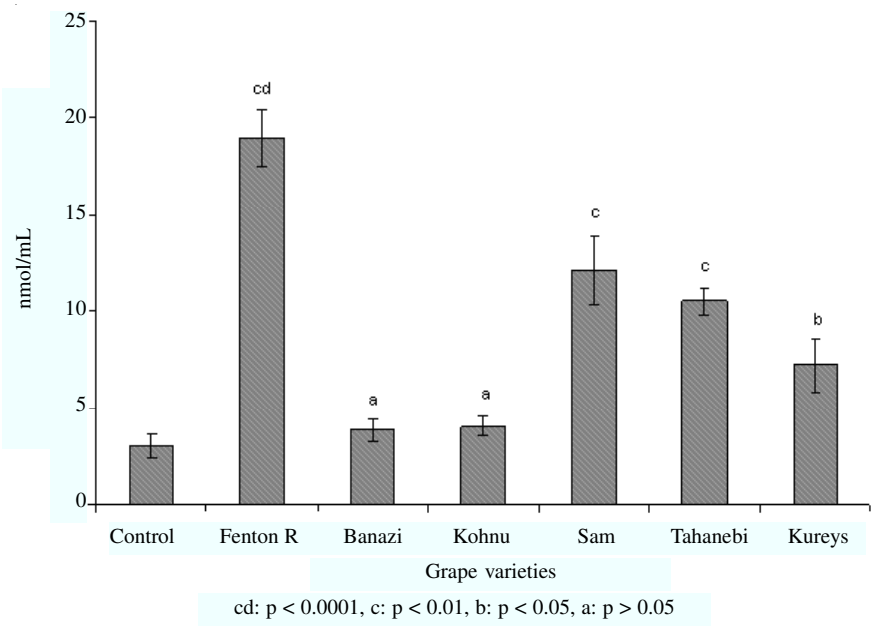

Fig. 1. Levels of MDA-TBARS in the environments of Fenton reagent and Fenton reagent with grape fruit extracts (Fenton R: Fenton reagent)

In recent years, there has been a growing interest in the use of grape extracts as a dietary antioxidant supplements[18,19]. Grapes are rich in polyphenols, of which about 8 % or less are present in pulp, 46-69 % in the seed and 12-50 % in the skin[20]. Phenolic compounds are known as high level antioxidants because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals[21]. They have prooxidant properties *in vitro*

particularly in the presence of transition metal ions such as iron and copper[22,23]. This may be related to the ability of flavonoids to undergo autoxidation catalyzed by transition metals to produce free radicals such as hydroxyl radicals *via* Fenton chemistry[23]. It is presumed that despite its higher levels of flavonoid, grape varieties showed a low performance in decreasing lipid peroxidation level because of the above-mentioned reasons.

Sugar analysis: Sugar analysis showed that fructose and glucose were present in all grape samples. When grape samples are compared in terms of fructose and glucose content, it is observed that the level of Banazi group is significant high in comparison with other groups and show almost no significant difference statically among other groups ($p < 0.0001$), ($p > 0.05$) (Fig. 2).

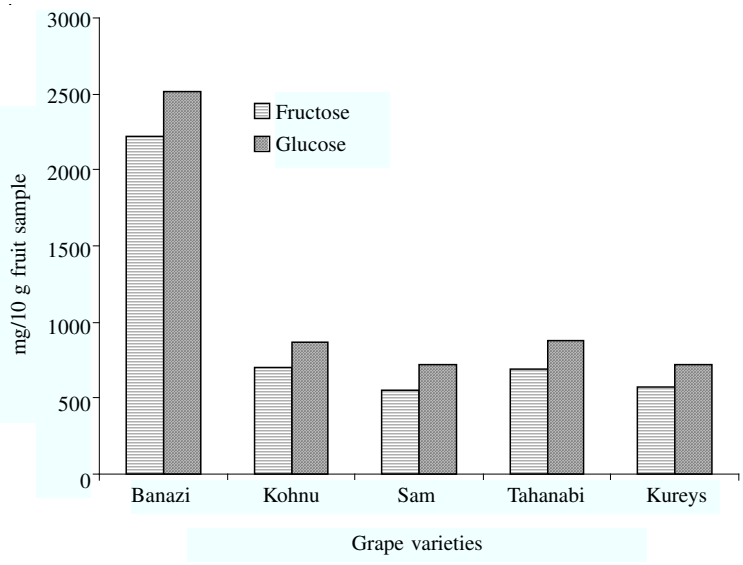

Fig. 2. Fruit sugar amount (mg/10 g)

Varandas *et al.*[24] examined the sugar contents (glucose and fructose) on grape skin were analyzed for five Portuguese *Vitis vinifera* grape varieties and compared in grape juice and whole grapes. They reported that grape skin has shown higher levels of glucose than fructose and their contents (total sugar) were higher in the whole grape than in the juice and much higher than in the grape skin itself. Predominantly located in the pulp, glucose and fructose represent about 99 % of the sugar contents at the end of grape maturation. Sugar determination on grape skins (fructose and glucose) has already been reported in studies related to berry ripening by Takayanagi and Yokotsuka[25].

Determination of scavenging activities of grape varieties: According to the results of the $DPPH^{\bullet}$ free radical scavengers, effective antioxidant activity of different concentrations of grape samples is appeared and the higher the concentration of

these activities increase or decrease according to the grape samples has been observed. The Banazi group was found to have more significant radical scavenging characteristics than the other groups in 25 µL concentration ($p < 0.0001$) (Fig. 3). That the group of Banazi rather than other examples having significant radical scavenging effect is clear. It is determined that this group has gradually decreasing antioxidant capacity as of 250 µL depending on the concentration of this group and showing almost no difference for 500 and 1000 µL.

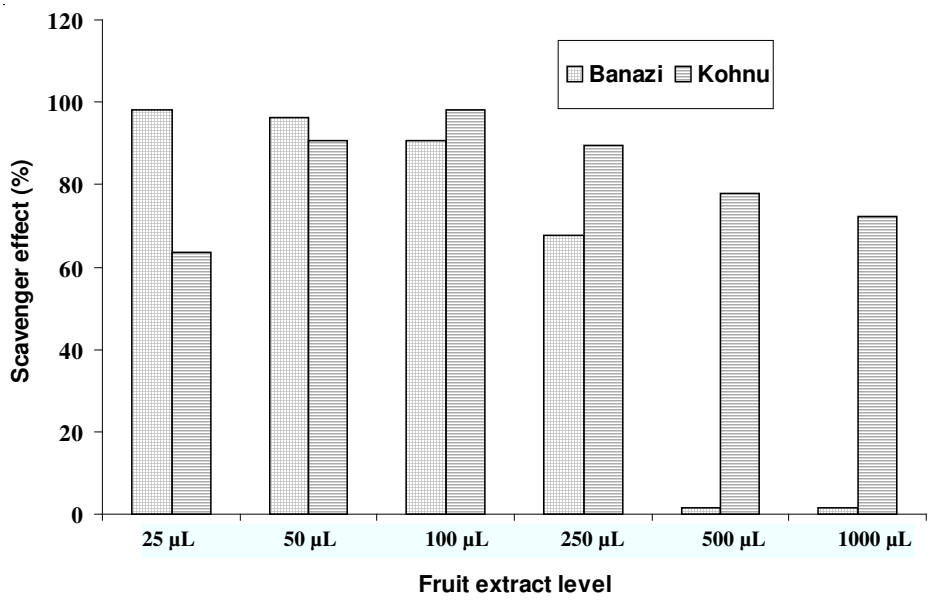

Fig. 3. DPPH$^{\bullet}$ scavenger effect of Banazi and Kohnu extracts (%)

When 50 and 100 µL concentrations are compared among themselves it is observed that the Banazi and Kohnu groups have the highest radical showed cleaning activity ($p < 0.0001$) (Fig. 3). There is no difference statically among groups including other samples but the Sam, Tahanebi and Kureys samples have significant increasing amounts of flavanoids as of 250 µL depending on increasing concentrations (Fig. 4).

It is established that antiradical activity is dependent on the structure of the free radical-scavenging compounds, the substituents present on the ring of the flavonoids and the degree of polymerization. The structural criteria for the potent free radical-scavengers are that these should possess either (I) a 3-hydroxy group on an unsaturated C ring or (ii) a 2,3-double bond with the 3-OH group and 4-one in the C ring or (iii) an *ortho*-OH substitution pattern in the B ring where the OH groups are not glycosylated[26]. Moreover, it is known that the polyhydroxylated phenolic compounds have a higher polarity than those of the other phenols[27]. The polarity of the flavonoids depends primarily on the nature of the radicals on rings and in particular on the number of OH groups.

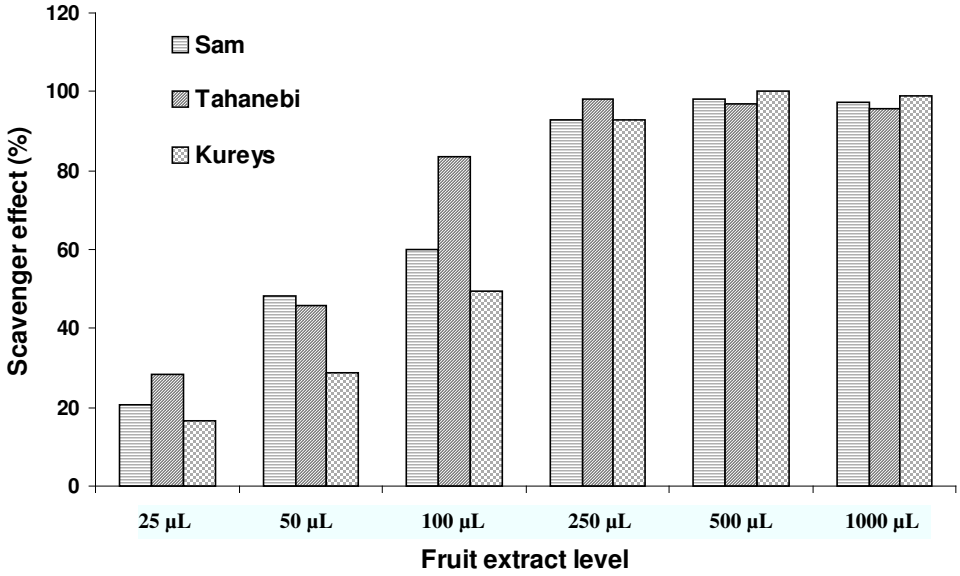

Fig. 4. DPPH• scavenger effect of Sam, Tahanebi and Kurays extracts (%)

Flavonoid and resveratrol analysis: According to the results of flavonoid analysis, rutin and catechin flavonoids were high in all grape samples whereas other flavonoids were present at lower levels (Table-1).

TABLE-1
CONTENT OF FLAVONOIDS AND RESVERATROL IN GRAPE FRUIT EXTRACTS (µg/1 g)

Flavonoids	Banazi	Kohnu	Sam	Tahanebi	Kureys
Cathechin	1980.00±2.88	2367.00±4.58[cd]	100.66±1.76	Trace	390.66±3.48
Rutin	769.33±2.33[d]	489.00±2.08	429.33±2.33	594.35±2.33	587.00±5.56
Resveratrol	2.16±0.16	1.16±0.16	2.00±0	3.16±0.16[b]	2.83±0.16
Naringenin	Trace	Trace	Trace	Trace	Trace
Quercetin	Trace	Trace	Trace	Trace	Trace
Total	2751.49[cd]	22857.16[cd]	531.99	597.51	980.49

cd: $p < 0.0001$ d: $p < 0.001$ c: $p < 0.01$ b: $p < 0.05$ a: $p > 0.05$.

Comparison of catechin content was found that the Banazi and Kohnu groups contained a significant amount and that there was no significant difference between the other groups ($p < 0.0001$, $p > 0.05$). Comparison of rutin amount showed that the Banazi group had the highest amount and partially statistically difference was observed among other groups ($p < 0.001$, $p < 0.05$).

In addition, analysis showed the presence of resveratrol in all grape groups. Although no significant differences were observed between all groups in term of resveratrol levels, the Tahanebi group was found to contain slightly more resveratrol when compared to the other groups ($p < 0.05$).

When total phenolic compound levels were compared in accordance with the results, it was found that the Banazi and Kohnu groups contained significantly high levels of phenolic compounds ($p < 0.0001$) and a partial difference was observed between Sam, Tahanebi and Kureys groups ($p < 0.05$). Present results almost parallel with previous results suggesting that the phenolic compound of grape is comprised of anthocyanins, resveratrol, catechin and epicatechin[20,28].

In conclusion, we have determined that the grape varieties grown in the Malatya region contain a substantial amount of phenolic compounds and sugar, depending on the location where the plant was grown. Phenolic compounds are closely related to the prevention of lipid peroxidation of grapes and reduced risk of chronic diseases. There may be some differences in the amounts and the diversity of these phytochemical characteristics contained in the fruit. This is not due to a single factor, but may be affected by sunlight, soil type, climatic differences and the location where the plant grows and differences between fruit species[29]. The amount of vitamins, minerals and sugar may vary from region to region because of the elements contained in the soil where the fruit grows[30]. Not only environmental factors, but also genetic factors, may mean that vitamin and mineral levels are different among the same grape species and also in the same species in different cultural forms.

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