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Original Research

Antioxidant capacity and phylogenetic analysis of twenty native grape cultivars in Siirt province, Turkey

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Abstract: The quality of grape cultivars not only depends on the grape cultivar but also is influenced by the molecular concepts and agro-climatic factors. For this purpose, four different grape cultivars were collected from five different locations in Siirt province (Turkey). Totally twenty different grape cultivars were investigated. In the present study, the antioxidant activity (total phenolic, flavonoid, proanthocyanidin content, DPPH and FRAP activity) in seeds were indicated and phylogenetic analysis (cpDNA;*trn*L-F region) of twenty native grape cultivars were investigated to construct their phylogenetic tree. According to reported data on antioxidant activity and content of phytochemicals, all cultivars exhibited different values from each other, but Rutik and Gadüv cultivars were found as significantly higher in comparison to others. According to bioinformatics analysis, twenty grape cultivars were distributed into six different major groups. Rutik and Sevkeye cultivars exhibit significant distinction from other grape cultivars. The phylogenetic analysis was also associated and supported with the results of obtained data from bioactivity. The bioactivity and phylogenetic analysis were firstly identified and quantified in these grape cultivars, however, with regard to obtained data from the current study, the grape cultivars grown in Siirt province were indicated significant and valuable results and as a result, these cultivars have to be evaluated before extinction.

Key words: Vine; cultivars; Biological activity; Genetic relationship; Siirt; Turkey.

Introduction

Grape is the world's largest fruit crop with an annual production of more than 67 million tons of berries (1). Turkey is the world's sixth-leading producer of grapes with over 1.500,000 acres (6,100km^2) planted under vine grape. Ampelographers estimate that Turkey is home to between 600–1200 indigenous varieties of *Vitis vinifera* L. However, less than 60 of these cultivars were grown commercially (2).

Grapes (*V. vinifera*) contain a considerable amount of different phenolic compoundsin leaves, skins, pulp and seeds which may show biologicalproperties of interest, related to their antioxidant properties (3). Grape seeds are a more effective source of anti-oxidative constituents than skins ofgrape/wine byproducts. Functional ingredients of grapeseed include several flavonoids with a phenolic nature such as monomeric flavanols, procyanidins, and phenolic acids (4). The antioxidant activity of grape seed phenolic compounds is closely associated with activity against various cancer types, cardiovascular diseases and several dermal disorders (5).

It has been reported that the total phenolic and flavonoid content in seeds and shells of 18 different grape varieties were different (6). Among them, ‘‘Cabernet Sauvignon” and “Muscadine” grapes have the most plentiful phenolic compounds and antioxidant properties in seed, whilethe Oriental *Vitis* species ‘‘Black Pearl” and ‘‘Sangye” were found tobe the richest in phenolic contents in the skin. Grapes are rich in phytochemicals with many proven health benefits. It has been reported that grape extracts exhibited antioxidant activities, including scavenging of free radicals, inhibition of lipid oxidation and reduction of hydroperoxide formation (7). Also, proanthocyanidins are a class of biologically activeflavonoids found throughout the plant kingdom and are one of the most potent antioxidants in nature. Typically concentrated in the barkof trees and in the outer shells of fruits and seeds, proanthocyanidins serve to protect plants against oxidative elements such as oxygen and sunshine (8).

Phenolic compounds were clearly known that they have an important chemical and biological role in plant defence against to pathogens (9). In a previous study, different alterations in biosynthetic pathways of flavonoids and accumulation of individual phenol compounds were found to increase due to infections (10,11). Moreover, the genes responsible for secondary metabolism were involved in up-regulation or down-regulated by infections according to their molecular structure (12).

Phylogenetic analyses with a broader sampling of taxa and markers are needed to further understand the relationships within Vitaceae and test the generic delimitation within the family. The phylogenetic relationships among grape species are of keen interest for the

conservation and use of this germplasm. The plastid genome is an effective tool for inter-specific phylogenetic and intra-specific phylogeographic studies of angiosperms (13).

In this study, we aimed to analyze the total antioxidant capacity and carrying out the phylogenetic relationship between twenty grape cultivars belongs to *V. vinifera*. These data may provide valuable information for the characterization and evaluation of different grape cultivars and also can increase the economic value of the grape production.

Materials and Methods

Grape materials

Twenty grape cultivars belong to *V. vinifera* were used in this study. The name and location of grape cultivars were given in Table 1. All cultivars were identified by specialist according to the flora of Turkey's. The grapes were collected at harvest seasons times (June-August; 2013-2015). Grape seeds were manually separated from the pulp with the help of pens and forceps and dried in an oven at 30 ^{0}C until reached a constant weight. The grape seeds were powdered in methanol with mortar and pestle and stored at + 4 ˚C until the analysis. For each grape cultivars, the leaf tissue was protected in silica gel for phylogenetic analysis; the samples were homogenized with liquid nitrogen and stored at – 20˚C for evaluation of antioxidant capacity.

Preparation of grape cultivars

The procedure followed for extraction of antioxidants was according to Saura-Calixto*et al.* (2007) with modifications (14). The powdered seeds from twenty grape cultivars were weighted (1.0) g and mixed with methanol (80% v/v). The samples were homogenized with a homogenizer (Wiggin Hauser D-130, Germany) for 2 minutes and subjected to ultrasound (Bandelin-UW-2070, Germany) for five minutes. The extracts were shaken (JEIO Tech SI-600, Korea) on an orbital shaker for a midnight and evaporated (Biby RE-100B, Germany) to dryness. The concentration of all crude extracts was adjusted to the same value by addition of 80% methanol. All spectrophotometric data were acquired using Uvmini-1240 Spectrophotometer.

Total phenol contents

Total phenolic contents of grapes seeds were performed by the methods involving Folin–Ciocalteu reagent and gallic acid as standard (15). Extract solution (0.1 ml) containing 1000 mg extract was taken in a tube, and 1 ml Folin–Ciocalteu reagent was added and the flask was shaken thoroughly. After 3 min, 1 ml of a solution of 6% Na_2CO_3 was added and the mixture was allowed to stand for 1 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated to gallic acid solutions.

Total flavonoid content

The total flavonoid content was estimated using aluminium chloride colourimetric assay (16,17). The 0.5 ml of test samples solution in methanol was mixed with 2ml of distilled water and 150 µl of 5% $NaNO_2$. After 6 min, 150 µl of 10% $AlCl_3$ and 2mL of 1 M NaOH were added and left at room temperature for 15 min. The absorbance of the mixtures was measured at 510 nm. Quercetin was used as a standard to determine flavonoid contents of grape seed extracts.

DPPH assay

DPPH assay was performed in according to Villano*et al*., (2007) (18). Briefly, 1 ml of seed extract sample were added to 4 ml of 0.01 mM DPPH (dissolved in methanol), incubated for 15 min in dark conditions and measured at 517 nm.

DPPH activity (% incubation) = $(AC– A_1) / A_C \times 100$ (A_C: Control Absorbance, $A_{1:}$ Sample Absorbance)

FRAP assay

FRAP assay was performed in according to Benzie and Strain (1996)(19). Briefly, 100 µL of seed extract sample appropriately diluted were added to 3 ml of the FRAP reagent. FRAP solution; Sodium acetate solution (300 mM, PH 3.6), 10 mM TPTZ (2,4,6- Tris (2-pyridyl)-s- triazin) in solution of 40 mM HCl and 20 mM ferric chloride solution. Then, after incubation

Table 1. Grape cultivars collected from five different regions of Siirt Province in Turkey (2013-2015).

Grape Cultivars	Region	Location
Karrot	Şirvan	38º 09′ 27.06 N 42º 00′ 16.74 E
ÇiçekeNator	Şirvan	37º 41′ 47.94 N 42º 16′ 14.58 E
Gadüv	Şirvan	37º 53′ 46.50 N 41º 54′ 20.88 E
Meyan	Şirvan	38º 09′ 12.54 N 42º 00′ 11.76 E
Reşealye	Eruh	37º 57′ 37.50 N 41º 59′ 41.88 E
Kıtılnefs	Eruh	38º 09′ 12.96 N 42º 00′ 11.46 E
Turture	Eruh	37º 58′ 23.94 N 42º 37′ 44.28 E
Besirane	Eruh	37º 42′ 07.08 N 42º 16′ 25.92 E
Rutik	Pervari	37º 57′ 38.04 N 41º 59′ 42.48 E
Spiyo	Pervari	38º 09′ 12.36 N 42º 00′ 11.70 E
Mevazer	Pervari	37º 44′ 29.78 N 42º 26′ 14.70 E
Gevri	Pervari	37º 58′ 23.04 N 42º 37′ 46.50 E
Emiri	Tillo	38º 09′ 26.04 N 42º 00′ 18.36 E
Heseni	Tillo	37º 41′ 39.78 N 42º 16′ 14.70 E
Şevkeye	Tillo	37º 58′ 23.88 N 42º 37′ 45.66 E
Aşkar	Tillo	37º 57′ 37.14 N 41º 59′ 41.94 E
Sinciri	Centrum	37º 58′ 17.22 N 42º 37′ 17.40 E
Binetati	Centrum	37º 58′ 26.10 N 41º 59′ 49.56 E
Tayfi	Centrum	37º 58′ 25.98 N 42º 37′ 44.10 E
Gozane	Centrum	37º 31′ 19.78 N 42º 56′ 14.70 E

at room temperature for 6 min in dark condition the absorbance at 593 nm was measured. Calibration was prepared with ferrous sulfate (100-1000 µg/ml). Results were identified as µM Fe^{+2} corresponding to each gram of dried weigh.

Total proanthocyanin content

Total proanthocyanidins (PAC) concentration was determined colourimetrically using the DMAC method (20). DMAC solution was prepared with cold methanol and 6 N HCl mixtures. A series of dilutions of standard catechin were prepared in 80% ethanol ranging from 1–100 µg/ml. Blank, standard and diluted samples were analyzed in triplicates and read absorbance at 640 nm. Concentrations of grape solutions were expressed as mg/100 g procyanidin catechin equivalent.

DNA isolation and PCR conditions

DNA was extracted by using CTAB method (21) with somemodification(22).Foramplificationthe*trn*L-Fregion of cpDNA, trnLf:ATTTGAACTGGTGACACGAG and trnLr: GGTTCAAGTCCCTCTATCCC primer pairs were used.

PCR reaction was carried out with a 50 µl total volume containing 0.7 µl units Taq polymerase, 5 µl Taq buffer, 1 µl dNTP (10 mM), 4 µl of template DNA 4 µl $MgCI_2$ (25 mM), 2 µl of each primer (50 pmol/µ) and complete mix with ddH_2O to get final volume. The specific primers used in that study was obtained from a previous study belong to Taberlet *et al.* (1991)(23).

Amplification was performed in programmable Thermal Cycler (Eppendorf-22331, Germany) as follows; initial denaturation at 95 °C for 7 min, followed by 35 cycles at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min, final extension at 72 °C for 10 min. The PCR products were separated by gel electrophoresis on agarose gel. Sequencing was achieved with the procurement of services (IonTek Company, Istanbul).

Phylogenetic analyses

After getting sequences for each grape cultivar, all sequences were aligned by using the CLUSTAL W algorithm (24). All sequences were combined with the sequences of these same regions in a text document. Additionally, for phylogenetic analysis, the *trn*L-F sequences were manually/visually checked by using the BioEditv7.2.5 software. Afterthe alignment analysis, trimmed of sequences was achieved. The phylogenetic trees were generated with Neighbor-joining (NJ), Maximum Parsimony (MP) by using MEGA 6.0.6 software.

Statistical analysis

Data are presented as the average of three independent biological replicates. Statistical analyses were made by using SPSS 15.0 program. Significant differences and correlation analysis were determined by Duncan's multiple range tests. $P<0.05$ were regarded as significant.

Results

The total phenolic content of twenty different grape cultivars measured by FCR method was given in Table 2. The obtained values were arranged from 8.79 (Şevkeye) to 70.86 mg/g (Rutik). There were significant differences between the total content of cultivars and locations. The phenolic content of grape cultivars from the location of Şirvan and the location of Pervari

Table 2 - Total phenolic, flavonoid, proantocyanine, DPPH and FRAP content in different grape seeds cultivars from five different regions of Siirt Province, Turkey.

Grape Cultivars	Region	Total Phenolic (mg/g)	Total Flavonoid (mg/g)	Total Proanth (mg/100 g)	DPPH (inhibition%)	FRAP (µmol Fe^{+2}/g)
Karrot	Şirvan	20.68± 2.13	20.37± 1.16	28.02±3.2	93.39±0.1	27.80±2.1
Çiçekenator	Şirvan	43.27±6.12	22.40± 5.98	113.44±5.4	93.73±0.3	50.1±3.54
Gadüv	Şirvan	62.93±3.75	28.07± 4.39	126.04±2.9	94.07±0.4	67.61±2.4
Meyan	Şirvan	23.96±4.42	16.06± 2.93	45.32±6.12	93.73±0.3	39.4±1.18
Reşealye	Eruh	19.65±2.63	16.15± 1.67	17.97±1.19	93.62±0.2	33.44±1.19
Kıtılnefs	Eruh	13.27±1.14	10.52± 3.56	21.48±1.1	85.87±0.5	26.49±1.0
Turture	Eruh	15.1±3.75	10.44± 4.44	36.58±2.14	91.57±0.3	31.09±2.1
Besirane	Eruh	51.72±6.9	21.33± 3.02	118.63±5.2	93.28±0.6	48.97±0.95
Rutik	Pervari	70.86±1.13	32.89± 5.91	120.9±4.92	95.73±0.7	69.01±1.16
Spiyo	Pervari	32.58±4.56	22.25± 3.21	90.62±3.65	93.50±0.5	41.16±2.67
Mevazer	Pervari	27.58±3.16	20.89± 4.01	83.29±7.9	93.73±0.6	42.38±1.18
Gevri	Pervari	35.13±2.43	18.42± 1.12	74.49±4.76	93.96±0.6	48.20±2.79
Emiri	Tillo	22.24±1.11	12.94± 3.74	75.79±3.48	93.73±0.8	38.77±1.28
Heseni	Tillo	14.65±2.22	9.88± 3.51	50.51±2.19	89.06±0.2	32.45±1.43
Şevkeye	Tillo	8.79±4.32	4.06± 4.48	2.73±0.98	83.87±0.4	28.88±2.34
Aşkar	Tillo	10.68±2.56	6.18± 1.19	9.62±2.12	85.87±0.9	33.44±1.98
Sinciri	Centrum	25.86±5.12	18.56± 3.85	92.19±4.47	93.84±1.1	49.69±3.14
Binetati	Centrum	25.68±5.67	13.65± 2.69	107.2±6.23	93.50±0.98	40.66±3.76
Tayfi	Centrum	14.31±1.19	9.51± 1.1	12.12±1.12	92.48±1.25	32.09±1.63
Gozane	Centrum	22.93±6.23	15.74± 3.65	12.20±3.2	91.57±1.15	31.68±4.44

were higher than the other locations in generally. The amounts of total flavonoids content were given in Table 2. The values of flavonoids were showed almost similar contents with each other and ranged between 4.06 (Şevkeye) to 32.89mg/g (Rutik).

The total amounts of proanthocyanin content of seed extracts were reported in Table 2. According to obtained results, seed extracts of Gaduv (126.04 mg/100 g) and Rutik (120.9 mg/100 g) cultivars were higher than the others. The proanthocyanin results of other cultivars were arranged between 2.73 to 113.44 mg/100 g. The results indicated that different proanthocyanin content was determined and independent on location.

The antioxidant activity of grape seeds determined as DPPH radical scavenging ability ranged from 83.87 to 95.73 % (Table 2). The highest antioxidant activity was determined for Rutik cultivar which was also richest in phenolic and flavonoids. The lowest DPPH value for seed extract of grape samples was found for Şevkeye cultivar. The antioxidant activity of grape seed extracts determined as FRAP ranged from 26.49 (Kıtılnefs) to 69.01 (Rutik) µmol/g Fe^{+2}. Also, the high positive correlation was observed between FRAP activity and flavonoid content.

The DNA extracted from the grape cultivars was found to be appropriate for PCR amplified of the trnL-F region. Constructed phylogenetic trees by using the MP (Maximum Parsimony) and NJ (Neighbor-joining) methods were indicated in Fig. 1. The *trn*L-F region of cp DNA of grapevine cultivars has been known important to detect the genetic relationships (25). In this study, also a *trn*L-F region of cpDNA was found to be more valuable in term of phylogenetic analysis of grape varieties. According to dendrogramof MP method, twenty grape varieties were distributed into six major groups (Group A to F). Group A that involved only Rutik cultivar from Pervari region and also Group F contained only Sevkeye variety from Tillo region.

Discussion

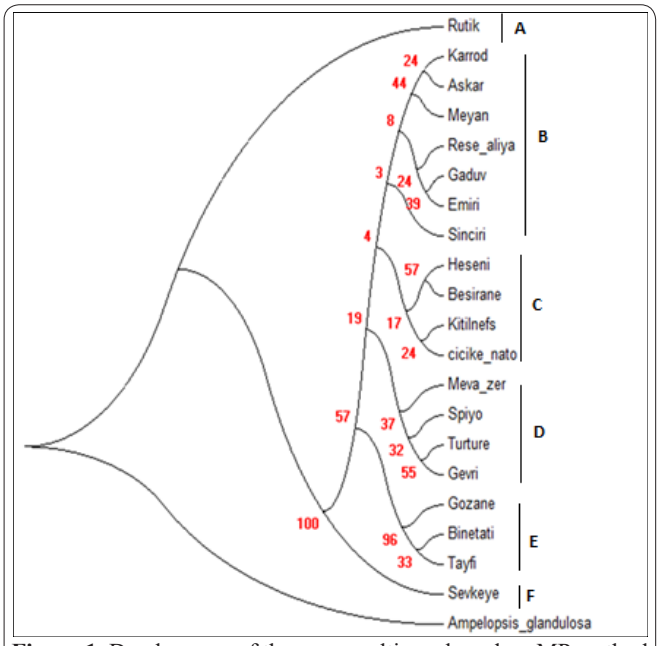

Figure 1. Dendrogram of the grape cultivars based on MP method with bootstrap value.

Comparison of obtained data from phenolic results with reference, the amount of total phenolic material of red and white grape seeds farming in Turkey is changing between 47.6 mg/g with130mg/g gallic acid equivalence. A phenolic compound in the grape seeds contains 60-70%of grape total polyphenols (26). Grape seeds contain more phenols than grape skin and its amount change according to region and climate (27)**.** In a previous study, it was reported that flavonoid content varied between 8.82 and 9.37 mg/g in some grape cultivars (28).

In a previous study, phenolic compounds and antioxidant activity of seed and skin extract from the pomace of Brazilian grape were analyzed. According to results, the phenolic and flavonoid contents of seeds were determined between 16.51 to 8963 mg/100 g and 11.18 to 6812 mg/100 g respectively. (29). The researchers are offer different suggestion for different values. The results were attributed to multiple factors like climate, the degree of ripeness, berry size and colour, grapevine variety, geographic conditions, infections etc. (30).

The chemical composition of grape extracts varies with cultivar, section and country. Therefore, this by-product is an important material for human health and it is used a natural antioxidant source. The resources show that proanthocyanidin in grape seeds are a 20 times stronger antioxidant than vitamin E and 50 times stronger than vitamin C (31).

The high positive correlation was observed between DPPH activity and total phenolic and flavonoid content. The correlation between DPPH and phenolic content was reported in several studies (29, 32, 33). A similar correlation was determined in a study of Rockenbach*et al.* (2011), who analyze the Brazilian grapes for antioxidant activity. In the same study the FRAP activity was arranged between 21.49 to 9262 µ mol/ Fe^{+2}/100g (29). Also, our results indicated similar and better results according to references.

In DNA analysis, Rutik and Sevkeye cultivars were exhibit significant distinctionthan the other grape cultivars. In this context, it can be argued the result of both antioxidant (Table 2) and phylogeny studies were interact with each other and showed that Rutik variety has a quite difference at DNA level and antioxidant capacity. The specific features of these varieties may be the result of its adaptation to the environmental and cultivation practices in their special region. In some previous studies, genetic relationships of various grape cultivars were found in multiple groups as consonant with our result (34, 35).

Grapes and their extracted phytochemicals played a significant role in human health. In our study, seed extracts of grape cultivars had high total phenolic, flavonoid content and antioxidant activity. In this study, it was tried to reveal the relationship between biological activities and genetic structures of culture forms. Biological activities and antioxidant capacities varied depending on climate and genetic structure. Finally, our results also confirm that grape seeds of Siirt (Turkey) have a big potential source for antioxidant activity and we expect to these cultivars will be evaluated in future.

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