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Characterization of Four Famous Red Grapevine Cultivars

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Abstract

The grape berries due to containing organic acids, sugars, aromatic compounds, phenolic compounds (including anthocyanins, flavanols, flavonols, stilbenes (resveratrol)), tannins, and oil in the pulp, skin, and seed have numerous health benefits for human health. In this study, we investigated genetic and phytochemical characteristics of four famous grapevine cultivars (Shiraz, Sirch, Panje Arous, and Yaghouti) at the maturity stage in 10-20º Brix on a cultivar basis. This research was performed at university of Hormozgan in 2017. The results indicated that Sirch cultivar had the highest total anthocyanin content (2733 mg kg^{-1} FW), total phenolic content (1666 mg kg^{-1} FW) and total carotenoid in the skin. High correlation (R^2 = 0.951) was observed between cultivars skin's total anthocyanin and total carotenoid contents. The highest quercetin content (1593 mg kg^{-1} FW) among the studied cultivars was obtained in Panje Arous cultivar (a pink grape) and Sirch cultivar had the highest delphinidin specific anthocyanin content (65.03 mg kg^{-1} FW). Among the studied cultivars, Shiraz had the highest total soluble sugar (%19.90) and amount of vinegar (950 ml Kg $^{-1}$ grapes). Analysis of GC-MS results of vinegar, indicated that the highest rate of ethanol (%98.442) was found in Panje Arous cultivar. DNA sequencing and alignment analysis of *F3H*, *UFGT*, *DFR*, and *MybA*1 gene sequences showed that there was high homology (>%99) among the studied cultivars, therefore it can be concluded that they are derived from a common ancestor.

Keywords: Grape anthocyanin, Phenolic content, Antioxidant activity, HPLC, GC mass, DNA sequencing.

Introduction

Grapes are one of the most cultivated crops with the world production of 74.5 million tons in 2017, and 1.9 million tons in Iran alone. (http://www.fao.org/faostat/en/#data/QC). Approximately, 86.6% of fresh grapes are processed to produce raisin, grape wine, juice, vinegar, sweet spreads, grape butter, jelly and seed oil (Maier et al., 2009). Grapes are a complex medley of carbohydrates, vitamins, edible fiber, acids, minerals (such as potassium and calcium), phytochemicals, proteins and flavor components. The most important grapes phytochemicals are polyphenols including anthocyanins, flavanols, flavonols, stilbenes (resveratrol) and phenolic acids (Dopico-Garcia et al., 2008; Novaka et al., 2008; Spacil et al., 2008) that are responsible for many biological activities like antioxidant, cardioprotective, anticancer, anti-inflammation, anti-aging and

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antimicrobial properties, which promote human health (Shrikhande, 2000; Wada et al., 2007). Phenolic components of grape fruit are partly responsible of their antioxidant properties. Grapes are good source of phenolic antioxidant containing 115-361 mg kg^{-1} total phenolics (Pastrana-Bonilla et al., 2003). Anthocyanins are interested compounds for biologists because of their contribution in formation of almost all of the orange, pink, red, blue and purple colors of colored grapes and possess many important biological roles (Koes et al., 2005). Anthocyanins are synthesized via the flavonoid pathway and together with their glucosided components; catechin and gallic acid are mainly existed in grape seed and skin (Chacona et al., 2009).

It is mainly accepted that an increase in grape color is concomitant with an increase in aroma intensity, improvement in phenol structure and increase in vinegar quality. Red, pink, and dark color of grape berries are because of the presence of anthocyanin in grape skins. The basic upstream genes that leading to the biosynthesis of colored anthocyanidins in grapes are *CHS*, *chalcone synthase*; *CHI*, *chalcone isomerase*; *F*3*H*, *flavanone* 3β-*hydroxylase*; *F*3'*H*, *flavonoid* 3'-*hydroxylase*; *F*3'5'*H*, *flavonoid* 3',5'-*hydroxylase*; *DFR*, *dihydroflavonol* 4-*reductase*; *ANS*, *anthocyanidin synthase*, *UFGT*, *UDP-glucose:flavonoid-O-glycosyltransferase*, *MybA* and *Myeloblastosis A* (Jaakola, 2013). *DFR* is a key enzyme involved in anthocyanin biosynthesis and proanthocyanidin synthesis, which catalyzes the reduction of dihydroflavonols to leucoanthocyanidins. *F*3*H* encoded for Naringenin, 2-oxoglutarate 3-dioxygenase and catalyzes the 3-beta-hydroxylation of 2S-flavanones to 2R, 3R-dihydroflavonols that are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins. Glycosylation is an important modification for increasing the hydrophilicity and stability of anthocyanins, because anthocyanidins are inherently unstable under the physiological conditions. In grape, anthocyanidins can only be O-glycosylated at the C3 position with the addition of glucoses by the activity of *UFGT* (Ford et al., 1998). *MybA*, proto-oncogene, is from transcription family with an essential role in regulation of genes involving in anthocyanin pathway (Jeong et al., 2006; Xie et al., 2006; Xie et al., 2015).

Molecular technology is considered as a reliable alternative tool for the identification and clustering of plant species. Due to the important roles of anthocyanin in the biological activities and industry, cloning and sequencing of interfered genes in anthocyanin biosynthesis pathway are necessary and important.

The increasing human population, periodic food shortages, current and expected effects of climate change have all led to raised awareness regarding global and national food security. The growing demand for the development of new cultivars that can be adapted to marginal environments, stimulate the search for genetic materials. The natural genetic resources can help to form a basis of food and livelihood security.

Four famous red grapevine cultivars of Iran are 'Yaghouti', 'Sirch', 'Panje Arous', and 'Shiraz'. They have high nutrition value and price than any other cultivars in south and south-eastern of Iran. The objective of this research was to analyze the biochemical, genetic and pigment diversities among four famous grapevines from four important distribution centers. Furthermore, cloning, sequencing and recording of anthocyanin biosynthesis genes from studied grapevines can help to have proper breeding programs for improvement of nutrition value of red superior grapes cultivars.

Materials and methods

Plant Material

Berries from four famous red grapevines (*Vitis vinifera*) including: 'Shiraz', 'Yaghouti', 'Sirch' and 'Panje Arous' were harvested in maturity stage, based on 10-

20º Brix depended on the cultivar (Table 1). All regional information includes: latitude, longitude, altitude and harvesting date are presented in Table 1. For biological investigation, 10 bunches per each cultivar were randomly selected and for DNA extraction, 5 young leaves were collected from Sepidan garden ('Panje Arouse' and 'Shiraz Red'), Fars; Sirch garden ('Sirch Red'), Kerman; Roudan garden ('Yaghouti'), Hormozgan; Iran, in 2017. All samples were frozen immediately in liquid nitrogen and stored at -80 °C.

Table 1. Geographic distribution, harvesting date and continental information of four famous Iranian red grapevines.

Cultivars	Province	Local	Latitude	Longitude	Altitude	Harvesting date
Panje Arouse	Fars	Sepidan	E51.98	N30.15	2240	Sept. 2016-2017
Shiraz Red	Fars	Sepidan	E51.98	N30.15	2240	Sept. 2016-2017
Sirch Red	Kerman	Sirch	E57.34	N30.12	1700	Sept. 2016-2017
Yaghouti	Hormozgan	Roudan	E57.12	N27.25	200	May 2016-2017

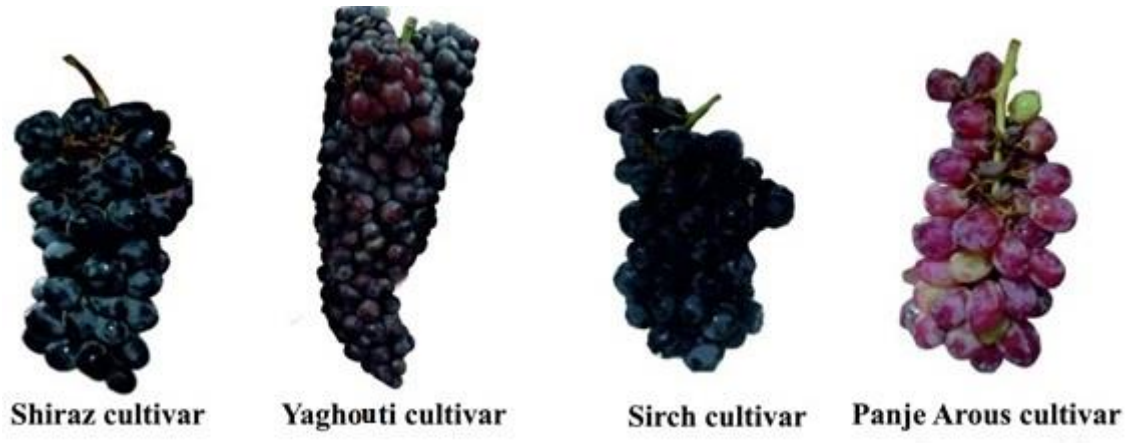

Fig. 1. Four famous red grapevines namely 'Shiraz', 'Yaghouti', 'Sirch', and 'Panje Arous' used in the present research.

Chemicals and reagents

Sodium bicarbonate (Na_2CO_3) was purchased from Panreac (Madrid, Spain). 2,2-diphenyl-1- picryl hydrazyl (DPPH), disodium ethylene diamine tetra acetatic acid (EDTA), Tris base, HCl, chloroform, isoamyl alcohol and isopropanol were from Sigma (Aldrich Chemie GmbH, Steinheim, Germany). Gallic acid, folin-ciocalteu phenol reagent, glucose, anthron sugar reagent, poly vinyl pyrrolidone (PVP), sulfuric acid, aceton, ethanol and methanol were purchased from Merck Co. (Darmstadt, Germany). Quercetin and delphinidin, UHPLC grade, formic acid, formaldehyde, and acetonitrile were from Roth Company, Germany. All other chemicals and reagents used in the present study had analytical grade.

Determination of total phenolic content

The content of total polyphenols was measured based on a colorimetric method using the folin-ciocalteau assay based on the Biglari et al. (2008). Twenty milligram of pulverized berries skin was dissolved in 1 mL of methanol. The solvent was incubated for 19 h in dark room at the 25 °C. The solvent was centrifuged at 14000 × g for 15 min at 4 °C. The supernatant was frozen at -20 °C and used for measuring of total phenolic content, quercetin and antioxidant activity. For measurement of total phenolic content, 100 µL of supernatant was taken and mixed with 750 µL folin-ciocalteu reagent. The mixture was mixed shortly and kept in room at the 25 °C for 5 min and then, 750 µL of %6 sodium bicarbonate was added to the

solvent and kept in dark room for 90 min. As blank, 750 µL phenol reagent and 750 µL of %6 sodium bicarbonate were mixed. Absorbance values were measured after 30 min of reaction at 725 nm using a UV-VIS spectrophotometer read set (CECIL, CE2501, 2000 series model). Gallic acid was used as a standard compound and the total phenolic content were expressed as mg g^{-1} gallic acid equivalent using the standard.

Standard curve equation: Y= 0.0089X-0.0051 $R^2 = 0.999$

where Y is absorbance at 725 nm and X is total phenolic content in the extracts. All determinations were carried out in triplicate. The results were expressed as mg gallic acid equivalent per kg of fresh weight.

Determination of quercetin content by UHPLC method

Acidified methanol (% 0.1 HCl in methanol) extract was prepared based on a method described by He et al. (2010). Dry extract (100 mg) was refluxed in 20 ml HPLC grade acidified methanol (%12.5) and incubated for 1 h in 75 °C water bath. After cooling, filtration and dilution to known volumes with methanol, the solution was passed through 0.45 µm syringe filter and 30 µl of the sample injected into a Brisa C18 column. The elution mode of mobile phase composed of degassed mixture of a mobil phase A: water + %0.1 formic acid and B: acetonitrile + %0.1 formic acid. Quercetin (100 ppm) was used as the standard. A rapid, specific, reversed phase, HPLC-UV method with a flow rate of 0.4 mL min^{-1}, a column temperature of 25 °C, and ultraviolet (UV) detection at 370 nm was developed. Chromatography was performed using a reverse-phase HPLC (knauer, Germany) with a column (Brisa LC2 C18 5 µm 25 x 0.46 cm) (Teknokroma, Barcelona, Spain). The results were expressed as mg quercetin equivalent per kg of fresh weight. All determinations were carried out in triplicate.

Free radical-scavenging ability (DPPH)

The antioxidant activity or antiradical power (ARP) of the skin extract was determined by quantification of radical-scavenging ability using the stable 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) based on a modified method described by Ebrahimzadeh et al. (2008).

In a dark place, different concentrations of each extracts were added, at an equal volume, to methanolic solution of DPPH (100 µM). After 60 min standing at room temperature, the absorbance was recorded at 517 nm. The affinity of test material to quench DPPH radical (% Inh DPPH) was calculated using the following formula:

% Inh DPPH = {(A control-A sample)/A control} × 100

where A control was calculated as a sum of initial absorbance DPPH plus sample (100 µl) in methanol; A sample absorbance DPPH after 60 min. Sample concentration providing %50 inhibition (IC50) was calculated from the graph plotting inhibition percentage against extract concentration. IC50 values denote the concentration of sample that is required to scavenge %50 of DPPH free radicals. Data were expressed by mg GA L^{-1} of grape extract needed to reduce DPPH radical by %50 and µL extract (needed to reduce) 2000 μg^{-1} of DPPH. Methanol was used as blank.

Extraction and determination of anthocyanins

The grape pulp and skin anthocyanins were extracted Based on the method described by Boss et al. (1996). 10 to 20 frozen berries were peeled. The peel tissue was ground in liquid nitrogen using a mortar and pestle. A 0.5 g subsample of the tissue was then added to 1 mL of methanol, and the anthocyanins were extracted for 1 h at -20 °C. The grape tissue was pelleted by

centrifugation at 10,300 ×g for 15 min at 4 °C, and the supernatant was used as extract. For determination of total anthocyanin (TA), in a dark place, 10 µL of Anthocyanin extraction was diluted to 2 ml in methanol and %1 (v/v) HCL. The total anthocyanin contents were recorded at 520 nm and monomeric anthocyanin content was calculated using the molar absorption coefficient by the following formula:

$$\text{Total anthocyanins content}\left(\frac{\text{mg}}{\text{Kg}}\text{FW}\right) = \frac{A \times DF \times \text{MW} \times \text{volume of extract} \times 1000}{\varepsilon \times l \times \text{weight of plant material}}$$

where A = absorbance at 520 nm; MW (molecular weight) = 529 g mol^{-1} for malvidin-3-glucoside; DF = dilution factor (200); l = path length in cm; Ɛ = 26455 molar absorbance of malvidin-3-glucosid, in L * mol^{-1} * cm^{-1}; and 10^3 = factor for conversion from g to mg.

For the skin's delphinidin content determination, HPLC analysis was performed according to He et al. (2010) method, with some modification in total elution time. The above obtained extract was filtered through 0.4 µm nitrocellulose filter (CA). The filtrate (30 µL) was directly injected for each analytical measurement. The elution mode of mobile phase composed of degassed mixture of a mobil phase A: %2 formic acid and B: acetonitrile + %2 formic acid. A rapid, specific, reversed phase, HPLC-UV method with a flow rate of 1 mL min^{-1}, a column temperature of 30 °C, and ultraviolet (UV) detection at 525 nm was developed. The gradient profile began from %6 to %10 B for 4 min, from %10 to %25 B for 8 min, isocratic %25 B for 1 min, from %25 to %40 for 7 min, from %40 to %60 for l5 min, from %60 to %100 for 5 min, then returned to initial conditions at 40 min Chromatography was performed using a reverse-phase HPLC (knauer, Germany) with a column (Brisa LC2 C18 5 µm 25 x 0.46 cm) (Teknokroma, Barcelona, Spain). The results were expressed as mg delphinidin per kg of fresh weight. All determinations were carried out in triplicate.

Extraction and determination of total skin carotenoid

To extraction carotenoid, Arnon method (1949) was used. For this purpose, in a dark place, 0.5 g of skin tissue was ground to a fine powder with mortar and pestle, and then 10 mL of %80 acetone was added. After centrifuge for 5 min, the supernatant was used as extract. The absorbance of the supernatant at 470 nm was determined by a spectrophotometer. Absorbance was read at 470 nm on a UV-VIS double beam spectrophotometer (model Spectro UV-Vis 336.001). The total carotenoids were calculated using the following formula (Kirk and Allen, 1965):

$$\textit{total carotenoids}\left(\frac{\text{mg}}{100\text{g}}\text{FW}\right) = \frac{\textit{Absorbance at}\,480 \times \textit{Volume of extract} \times 10 \times 100}{2500 \times \textit{Weight of plant material}}$$

where, 2500 = extinction coefficient

Data were expressed by mg carotenoid per 100 g fresh weight. Acetone %80 was used as blank.

Extraction and measurement of total soluble sugar

Total soluble sugar was extracted based on a modified method described by Omokolo et al. (1996). Pulp tissue (40 mg) was fine homogenized with 5 ml %80 ethanol. The tubes were incubated at 70 °C for 10 min in a water bath. The alcoholic extracts were centrifuged for 15 min at 1000 × g. Then, the clear solution was transferred to a sterile container and stored at -20 °C.

Total soluble sugar was determined based on a modified method described by McCready et al. (1950). The condensed extract (25 µL) was diluted with 175 µL distilled water to total of 200 µL. The extract samples (200 µL diluted) were mixed with 3 mL Anthron reagent and the tubes were incubated at 93°C for 21 min in a water bath. The absorbance of each

sample was measured after cooling by spectrophotometer (model Spectro UV-Vis 336.001) at 620 nm. The calibration curve was determined with glucose standards. The same procedure was applied to all standard glucose solutions and Anthron reagent (0, 20, 40, 60, 80, 100 mg L^{-1}) and standard curve was obtained. The glucose-free test tube was used as blank. The results were expressed as g glucose per 100g berries pulp of fresh weight.

Standard curve equation: $Y = 0.0151X + 0.0023$ $R^2 = 0.998$

where Y is absorbance at 620 nm and X is total soluble sugar content in the extracts.

GC-MASS analysis for ethanol content of vinegar

Vinegar was obtained by a traditional method. The fresh grapes were washed and berries separated from the peduncle, cap stem and rachis. The berries were placed in a clean plastic container. The water (150 ml) was added per 1 kg of berries. Then, the lid of the container was closed. The container was placed at 25 °C. After 4-5 days, the vinegar contains fruit fly in the container, which plays an important role in the fermentation of the materials and the production of vinegar. During incubation, the ingredients are mixed with a clean mixer, (3-4 times). After about 1 month, the mold was formed on the surface of the liquid. This step was lasted between 40-80 days depending on the cultivars. The grapes pomace was separated and the juice transferred in a clean glass container to become completely sour and turn into vinegar. It took 10-20 days for the liquid to become quite sour and turn into vinegar. Each vinegar sample filtered and centrifuged at 4000 × g for 20 min. supernatant used for the analysis. The GC-MS system was including the Aglient 5975 C mass tracker with a source of ionization electron ionization (EL), and an Aglient 7890 gas chromatography column HP-5MS (with a length of 30 m, an inner diameter of 0.25 mm, a film thickness of 0.25 µl). The temperature was set at 280 °C (the inlet temperature), 150 °C (the temperature of the ionization source of the mass detector), 230 °C (the temperature of the analyzer (coadrople)), and 280 °C (the temperature of the interface between MS and GC).

DNA extraction

Genomic DNA was extracted from young leaves by using the method described by Piccolo et al. (2012). The frozen leaves were taken out of the freezer and quickly ground to a fine powder with mortar and pestle (frozen rapidly at -80 °C). The powders (100 mg) were immediately transferred to a 1.5 mL microfuge tube containing 700 µL of preheated (60-65 °C) CTAB buffer. The tubes were shaken and incubated at 65 °C for 60 min in a water bath, mixing by inversion 3-4 times during incubation. Samples were centrifuged at 10000 rpm for 10 min at 4 °C and the aqueous phase was gently transferred into a new tube. Equal volume of chlorophorm: isoamyl alcohol (24:1) was added and mixed by inversion. Samples were centrifuged at 10000 rpm for 10 min at 4 °C and the aqueous phase was transferred into a new tube. Equal volume of isopropanol (-20 °C) was added to separate the DNA; samples were mixed and incubated at -20°C for 30 min. Precipitated DNA was centrifuged at 14000 rpm for 10 min at 4 °C and supernatant was discarded. DNA pellets were washed with 500 µL of %70 ethanol (-20 °C) and centrifuged at 14000 rpm for 5 min at 4 °C. The resulting DNA pellets were air-dried at room temperature and dissolved in 200 µL of TE buffer. RNase A was added to each sample (1/100 µL DNA sample) and was incubated at 37 °C for 1 h. Samples were stored at -20 °C. The quality of the DNA was estimated on an agarose gel (%1). The concentration of DNA was estimated with a spectrophotometer "Bio Photometer" (Eppendorf, Germany). DNA was diluted up to 100 ng μL^{-1} for use in the PCR.

Isolation of flavonoid 3 hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT), and MybA1 genes

The gene-specific Primers were developed based on the conserved sequence of the *F*3*H*, *DFR*, and *UFGT* genes in *Vitis* species plants using the FPCR and DNAMAN package software (Microsoft visual studio 6.0, visual Basic 6.0 SP6 Company). *Myb*A1 gene primers were selected from a prior study by Kobayashi et al. (2004) (Table 2). The primers were synthesized by Germans Metabion Company. PCR reactions were performed in a Thermal Cycler (from BIO-RAD) with a total volume of 40 µL containing 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, , 2.5 µM of each primer, 40 ng template DNA and 1.5 U of *Taq* DNA polymerase. PCR condition was set at 94 °C for 1 min, following with 35 cycles of 94 °C for 30 s, 59 °C (*F*3*H*, *UFGT*, and *DFR* genes) and 61°C (*Myb*A1 gene) for 45 s, 72 °C for 1 min, which followed by a final extension of 7 min at 72 °C. Samples were run in %1 agarose gel in 0.5 × Tris-borate-EDTA (TBE, pH 8.3) buffer. Gels were run at voltage 100 for 35 min and visualized with ultraviolet light after SYBR Green staining as described by Sambrook and Russell (2001). The extracted genes were purified and sequenced with 3730xl DNA analyzer by Bioneer Company (Korea).

Table 2. The primers pair sequences of grapevine anthocyanin pathway genes.

Gene name	Primer name	Sequences (5'→3')	Annealing temp. (°C)
F3H	Forward primer	GCCGTCGTGATGA GATTTGC	59.0
	Reverse primer	TTCAGCCTTTCGCCGGTTAT	59.1
DFR	Forward primer	CGGTTTCATCGGTTCATGGC	59.0
	Reverse primer	TTCGTTTCCAGTGATCGGGG	59.1
UFGT	Forward primer	GCCCCAGGAGGATATTGAGC	59.0
	Reverse primer	CCTCAGCTAGGGCTACAAGC	58.9
*Myb*A1	Forward primer1c	GGACGTTAAAAAATGGTTGCACGTG	61.3
	Reverse primer1c	GAACCTCCTTTTTGAAGTGGTGACT	60.4

Comparison of homologues sequences for queries and drawing phylogenetic trees

Homology of anthocyanin pathway genes sequence (*F*3*H*, *UFGT*, *DFR*, and *Myb*A1 genes) of above mentioned famous red grapes cultivars were compared with themselves and database sequences in NCBI by nucleotide blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The results for seeding were used for calculating the triangular matrix of similarity and drawing a phylogenetic tree by MEGA, software version 7 (Kumar et al., 2015). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura and Nei (1993) method.

Statistical Analysis

All determinations were carried out in two years with three repetitions. The phytochemical recorded data were subjected to analysis of variance (ANOVA) with SAS software (SAS Institute, version 9.13 Cary, NC). Statistical significance was determined at the $p \leq 0.05$. Duncan's Multiple- Range Test was used to compare the means.

Results

There was no significant difference between the years because the sampling was performed in a unique maturity stage.

Anthocyanin content

Total anthocyanin content in skin and pulp of the studied cultivars were expressed in terms of mg malvidin 3-glucoside per kg fresh weight. The skin total anthocyanin in all four cultivars showed significant differences. The cultivars Sirch, Shiraz, Yaghouti and Panje Arous, were

respectively categorized based on the total amount of anthocyanin. Sirch cultivar with 2733 mg malvidin 3-glucoside per kg fresh weight, had the highest total monomeric anthocyanin and Panje Arous cultivar with 542.5 mg malvidin 3-glucoside per kg fresh weight indicated the lowest amount of total monomeric anthocyanin (Table 3). In pulp extracts, the anthocyanins were present in the Yaghooti (406 mg MglcE kg^{-1} FW) and Shiraz (21.37 mg MglcE kg^{-1} FW) cultivars; but, in Sirch and Panje Arous cultivars, no anthocyanin was determined.

Delphinidin-specific anthocyanin

The highest amount of individual anthocyanin delphinidin was found in Sirch cultivar (65.03 mg kg^{-1} FW). Shiraz, Panje Arous and Yaghouti cultivars did not show any significant differences; the lowest amount of delphinidin was obtained in Panje Arous with 23.5 mg kg^{-1} of fresh weight (Table 3).

Quercetin-specific flavonoids content

The highest quercetin content was found in Panje Arous cultivar with 1593 mg kg^{-1} of fresh weight. Yaghouti and Sirch cultivars did not show any statistically significant difference in quercetin content. The lowest quercetin content was obtained in Shiraz cultivar with 270 mg kg^{-1} of fresh weight (Table 3).

Total carotenoid content

Based on the total carotenoid content in the skin extract, the four studied grapevine cultivars classified into two groups: one group exhibiting clearly higher carotenoid content (berries with a darker color: Shiraz and Sirch cultivars), and the other group exhibiting low level of carotenoid (Yaghouti and Panje Arous cultivars). Sirch and Shiraz cultivars did not show statistically significant difference; Sirch cultivar with carotenoid content of 5.832 mg 100 g^{-1} of fresh weight, showed the highest amount of carotenoid and there was no significant difference between Panje Arous and Yaghouti cultivars; The Panje Arous cultivar showed the lowest carotenoid content in comparison with the other studied cultivars (1.533 mg 100 g^{-1} FW). (Table 3).

Total phenolic content

The highest total phenolic content was obtained in Sirch cultivar with 1666 mg GAE kg^{-1} of fresh weight, followed by Shiraz cultivar with 1460 mg GAE kg^{-1} of fresh weight. The lowest total phenol content of skin was obtained in Yaghouti cultivar with 1041 mg GAE kg^{-1} of fresh weight (Table 3).

TSS

Analysis of the results of TSS showed that Shiraz, Panje Arous, Sirch and Yaghouti cultivars ranked in a descending order for the TSS%. Accordingly, the highest and the lowest TSS contents were detected in Shiraz cultivar with %19.90 and 'Yaghouti' with %10.70, respectively (Table 3).

Table 3. Total phenolics (TPC), total anthocyanin (TA), cartonoids (CAR), individual anthocyanin delphinidin and flavonoid quercetin contents in the grape berry skin extracts and total sugar solution (TSS) in pulp extracts of four famous red grapevines *Vitis vinifera* L. cultivars.

Grape cultivars	TPC mg GAE kg^{-1} FW	TA mg MglcE L^{-1}	Del mg Kg^{-1} FW	Que mg Kg^{-1} FW	CAR mg $100g^{-1}$ FW	TSS Pulp %
Yaghouti	1041^{c}	697.8^{c}	26^{b}	405^{b}	2.253^{b}	10.70^{c}
Sirch	1666^{a}	2733^{a}	65.03^{a}	414^{b}	5.832^{a}	14.54^{bc}
Panje Arous	1083^{c}	542.5^{d}	23.5^{b}	1593^{a}	1.533^{b}	16.34^{b}
Shiraz	1460^{b}	1997^{b}	25.46^{b}	270^{c}	5.728^{a}	19.90^{a}

The mean comparison was performed according to Duncan's Multiple-Range Test (SSR test) at a ($p \leq 0.5$). Similar letters within columns are not significantly different.

Table 4. Antioxidant properties for grape skin extracts of four famous red grapevines *Vitis vinifera* cultivars determined as DPPH radical-scavenging capacity.

Grape cultivars	DPPH radical-scavenging activity IC50 (mg GA L^{-1})*	DPPH radical-scavenging activity (µl extract 2000µg^{-1}of DPPH)
Yaghouti	130.391[b]	12.52[b]
Sirch	148.588[a]	8.879[c]
Panje Arous	149.617[a]	13.82[a]
Shiraz	129.794[b]	9.039[c]

* IC50 – sample concentration in mg GAE L-1 of grape skin extracts providing %50 inhibition, The mean comparison was performed according to Duncan's Multiple-Range Test (SSR test) at a ($p \leq 0.5$). Similar letters within columns are not significantly different.

Antioxidant properties

Antioxidant properties of the skin extracts of these cultivars were analyzed by measuring the stability of 2, 2-diphenyl-2-picril hydrazyl hydrate (DPPH) against plant extracts. The antioxidant activity based on two units of the extract content required to reduce 2000 mg of DPPH and the amount of gallic acid present in the extract to reduce the %50 amount of free radicals was calculated. Accordingly, the lower amount of extracts used in present study the greater the antioxidant properties. Sirch, Shiraz, Yaghouti and Panje Arous cultivars were ranked from the highest to the lowest antioxidant amount according to the IC50, respectively. In the amount of extract needed to evaluate the antioxidant properties of the skin, the highest amount of used extract was found in 'Panje Arous' (13.82 µL) and the lowest value was found in Sirch cultivar (8.879 µL) (Table 4).

Vinegar

Shiraz red grape had the highest amount of vinegar (950 ml Kg^{-1} grapes). It was found that the vinegar color was directly correlated with the grape color (Fig. 1, 2).

Fig 2. Vinegar from Shiraz, Yaghooti, Sirch, Panje Arous grapevine cultivars from left to right, respectively.

The results of gas chromatography showed that 'Sirch' vinegar has the most measured components. During gas chromatography, 27, 8, 5, 4 compounds were identified in Sirch, Shiraz, Yaghooti and Panje Arous cultivars, respectively. Ethanol was the common and the first peak in all cultivars. It was obtained with the highest rate in Panje Arous cultivar with a retention time of 1.491 min and a relative percentage of 98.442. Yaghouti cultivar was placed in the second order, in terms of ethanol, with a retention time of 1.496 minutes and %53.533. The ethanol retention time was identical for Shiraz and Sirch cultivars (1.502 min). The ethanol contents were %14.238 and %8.120 in Sirch and Shiraz cultivars, respectively. The highest known component in Sirch cultivar, was isobutyl alcohol, with a retention time of 2.361 min and value of %29.677. In Shiraz cultivar, acetic acid was obtained with a retention time of 2.647 min and the highest value of %75.101. In the Yaghooti cultivar, after ethanol, 2-Butanone 3-Hydroxy and acetic acid were identified with a retention time of 3,614 and 2.506 min and value of %29.206 and %6.980, respectively. In Panje Arous cultivar, the other identified compounds were ethyl acetate with a retention time of 2.139 and value of %0.684, 1-butanol 3-methyl with a retention time of 4.251 min and value of %0.448, and 1-propanol-2-methyl, with a retention time of 0.426 min.

Correlation among the traits

There was a high correlation among the

measured traits. A significant correlation was found between the antioxidant and total anthocyanin contents. It is observed that the more total phenolic content and total anthocyanin content, the lower the amount of extract needed to reduce 2000 µg of DPPH. There was a positive correlation between the total carotenoid, the total anthocyanin and the antioxidant properties. There were significant positive correlations between the total anthocyanin and total phenol ($R^2 = 0.911$) and between the total anthocyanin content and total carotenoid ($R^2 = 0.951$).

Isolation of partial F3H, UFGT, DFR, and MybA1 genes and their homology analysis

The amplified PCR fragments of *F3H*, *UFGT*, *DFR*, and *MybA*1 genes from four famous grape cultivars were obtained by using forward and reverse specific primers. The amplified PCR fragments were sequenced in both directions by specific reverse and forward primers, using the Automatic DNA Sequencer 3730Xl (Macrogene, Korea). The sequencing data were edited using Chromas software Version 1.41. They were submitted to GenBank (KY235308.1 accession number - *dihydroflavonol reductase* gene from Yaghooti cultivar 1787 bp, KY089083.1 accession number - *dihydroflavonol reductase* gene from Panje Arous cultivar 1771 bp, KY050779.1 accession number - *dihydroflavonol reductase* gene from Sirch cultivar 1811 bp, KY129904.1 accession number - *dihydroflavonol reductase* gene from Shiraz cultivar 1770 bp, KY346963.1 accession number - *MybA*1 gene from Sirch cultivar 958 bp, KY406229.1 accession number - *MybA*1 gene from Shiraz cultivar 950 bp, KY406228.1 accession number - *MybA*1 gene from Yaghouti cultivar 985 bp, KY406230.1 accession number - *MybA*1 gene from Panje Arous cultivar 810 bp, MF774609 accession number - *flavonoid* 3-*O*-*glucosyltransferase* gene from Panje Arous cultivar 664 bp, KY293689.1 accession number - *flavonoid* 3-*O*-*glucosyltransferase* gene from Yaghouti cultivar 669 bp, KY305473.1 accession number - *flavonoid* 3-*O*-*glucosyltransferase* gene from Shiraz cultivar 676 bp, KY305474.1 accession number - *flavonoid* 3-*O*-*glucosyltransferase* gene from Sirch cultivar 676 bp, KX984343.1 accession number- *flavanone* 3-*hydroxylase* gene from 'Yaghouti' 801 bp, KX984342.1 accession number- *flavanone* 3-*hydroxylase* gene from 'Sirch' 808 bp, KX984344.1 accession number- *flavanone* 3-*hydroxylase* gene from Panje Arous cultivar 797 bp, KY006128.1 accession number- *flavanone* 3-*hydroxylase* gene from Shiraz cultivar 810 bp). Phylogeny tree analysis based on distance matrix of *F3H* gene sequences from the four famous red grape cultivars and Pinot Noir cultivar indicated *F3H* genes sequences of three cultivars: 'Yaghouti', 'Shiraz', and 'Panje Arous' had highest homology (%100) and these three cultivars placed in the cluster 1. Sirch cultivar with 0.00063 distance from the other famous red grapevine cultivars placed in the cluster 2 and Pinot Noir cultivar with 0.16626 distance with others placed in the cluster 3 (Fig. 3A). Cluster analysis of *DFR* gene sequences from the four famous red grapevines and *Vitis Belulla* grape revealed that Sirch and Yaghouti cultivars with 0.0005 distance placed in the cluster 1. Panje Arous and Shiraz cultivars with 0.007 distance from the cluster 1 placed in cluster 2 and *Vitis Belulla* grape placed in the cluster 3 with 0.3385 distance based on their *DFR* gene sequences (Fig. 3B). Phylogeny tree of *UFGT* gene sequences from studied cultivars and PN40024 grape cultivar indicated that Sirch and Panje Arous cultivars had highest homology (100 %) and placed in the first cluster. The second cluster was related to PN40024 cultivar with 0.002267 distance and the third cluster was belonged to Yaghouti and

Shiraz cultivars with 0.0052 distance based on *UFGT* gene sequences (Fig. 3C). Cluster analysis of *MybA*1 gene sequences from the four famous red grapevine cultivars and Koshu cultivar showed that cultivars of Sirch, Shiraz, Yaghouti, and Koshu with the highest homology (%100) placed in the first cluster and Panje Arous cultivar with 0.008668 distance placed in the second cluster (Fig. 3D).

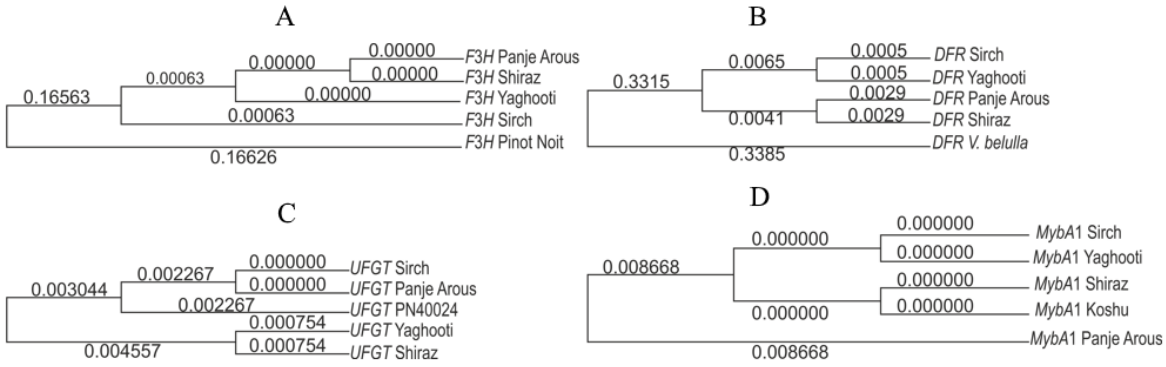

Fig. 3 Phylogeny tree base on distance matrix of A: *F3H*, B: *DFR*, C: *UFGT*, and D: *MybA*1 genes sequences from the four famous red grapevines *Vitis vinifera* cultivars and other grape cultivars.

Evolutionary rate of genes

In counting of the percentage of genes bases, *DFR* and *MybA*1 genes showed the highest percentage of adenin and thymin in all cultivars. Based on the Tamura and Nei gamma (G^+) model (1993), evolutionary rates of the genes were studied. Accordingly to the evolutionary ratio, genes are classified into five nucleotide groups. Sites that have an evolutionary rate of less than 1, will evolve at less than moderate speeds. The moderate speed relates to the sites which obtained grade 1 and the high evolutionary speed relates to the sites which take more than grade 1. In the 5th site, the evolutionary rate was found to be 4.83, 4.75, 1.10 and 1.10 for *MybA*1, *F3H*, *UFGT*, and *DFR* genes, respectively. For the sites: 1, 2, 3, and 4 evolutionary rates were between 0.90-1.10 for the entire studied four genes.

Discussion

Sirch, Panje Arous, Yaghouti, and Shiraz cultivars are the most famous native red grapevine cultivars, which widely cultivated in Iran. Many years ago Iranians recognized their properties, cultivated and domesticated them. They showed good properties for many industrial and pharmaceutical applications. In the present study, total anthocyanin content had a direct correlation with the color of the cultivars among studied cultivars. The grape cultivars with darker color (Sirch and Shiraz cultivars) showed higher levels of anthocyanin than those of pink (Panje Arous) and red (Yaghouti) berries. Panje Arous cultivar showed the lowest anthocyanin level. Castellarin and Gaspero (2007) showed that *GST* and *UFGT* genes are not expressed in white grapevines, and in pale red and pink varieties are expressed at least ten times less than dark and red cultivars. There was no anthocyanin in the pulp of Sirch and Panje Arous cultivars. However, Yaghouti cultivar can be described as a rare and seedless cultivar, which has significant anthocyanin in its pulp. Today, due to the high importance of the anthocyanin in various industries, scientists are trying to produce cultivars with having anthocyanin in both pulp and skin. This feature has been identified in rare wild species, and most of the cultivars produce anthocyanin just in their pulp. Sirch and Shiraz cultivars showed the highest total phenolic content. A high correlation was obtained between total anthocyanin and total phenol contents (R^2

= 0.911). Therefore, the studied cultivars (as rich sources of phenol, anthocyanins, and antioxidant) can be considered as natural sources of mentioned worthy compounds that can be used in various industries as a suitable alternative to industrial additive and chemical preservatives. The phenolic component content depends on the cultivar, and environmental conditions such as soil compositions, climatic conditions, region geography, kind of cultivation and pruning, and exposure to disease such as fungal contamination (Conde et al., 2016; Del-Castillo-Alonso et al., 2016; Sivilotti et al., 2016; Teixeira et al., 2013; Xia et al., 2010). In the studied cultivars, it was observed that dark skin cultivars showed more carotenoids than the other cultivars, which is consistent with the previous findings of Derradji-Benmenziane et al. (2014) based on the presence of carotenoids in the dark cultivars. Martinez de Toda et al. (2013) reported that in addition to genetics, kind of cultivation, and environmental conditions can also be considered as factors influencing these differences. 'Shiraz Red' grapevine showed the highest total sugar content among the studied grapevine cultivars. The results of vinegar preparation showed positive relation between sweetness and vinegar yield. Taste is one of the effective factors in the selection of each cultivar. The amount of sugar and sweetness are factors that influence the taste and quality of vinegar. Anthocyanins in the grapevine determine vinegar yield and color. Therefore, Sirch and Shiraz cultivars showed the highest vinegar yield and red color in vinegar preparation.

The homology analysis of the studied genes in the four famous red grapevines cultivars was revealed that they had more than %99 identities in all studied genes. It can be concluded that the studied genes evolution proceeds from common ancestors. Evolutionary analysis on the studied genes revealed that they were derived with a moderate speed.

In conclusion, it will be remembered that the studied cultivars are the most important grown red grapevine that developed over time through adaptation to their natural and cultural environments of agriculture. They are worthy germplasm sources that can be used in breeding program and organic agriculture. This study helps to select and produce hybrid cultivars with desirable features by breeding schedules and precipitate genetic promotion.

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