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Research Article

Comparative evaluation of physicochemical properties of four main Iranian cultivars of *Olea europaea* (Mary, Roghani, Fishomi and Zard)

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Abstract

Oleuropein, Hydroxytyrosol and Tyrosol are three major phenolic compounds present in olive with remarkable pharmacological effects. In this study, leaves and fruits of four olive cultivars including 'Fishomi', 'Mary', 'Roghani' and 'Zard' growing in the north of Iran were collected for comparative evaluation of the concentration of Oleuropein, Hydroxytyrosol and Tyrosol as well as antioxidant properties. According to the results, the highest and lowest levels of Tyrosol were detected in 'Mary' and 'Zard' leaves, respectively. There were significant differences between levels of Hydroxytyrosol in 'Mary' leaves compared with other examined cultivars. 'Mary' fruits had the highest level of Oleuropein, Hydroxytyrosol and Tyrosol, while 'Fishomi' fruits had the lowest level of these compounds. The total phenol content of leaf extracts was significantly higher than those detected in fruit extracts. In the leaves and fruits of the examined cultivars, the highest and the lowest Phenylalanine ammonia-lyase (PAL) activity was found in 'Mary' and 'Fishomi' cultivars, respectively. PAL activity in the leaves of all examined cultivars was significantly higher than their fruits. Meanwhile, the results showed that the antioxidant activities of the leaves in all cultivars were higher than the fruits. These results suggest that olive leaves, as well as fruits, might also be used as rich sources of natural antioxidants.

Keywords: Hydroxytyrosol, Oleuropein, Olive, tyrosol, PAL activity

Introduction

Olive (*Olea europaea* L.) is one of the most important commercial fruits in Iran. Different olive genotypes are grown in some regions of Iran, such as Guilan province in the north of Iran. The cultivars of Zard, Roghani, Mari, and Fishomi are among the major cultivars of olive in Iran (Delkash-Roudsari *et al*., 2015). In recent years, due to higher olive oil demand, the cultivation of olive has been expanded in various regions of Iran. However, the cultivation of olive trees is restricted because of terrible environmental conditions and water shortage in most of the new olive farm areas. The limitations of water and hot summers have remarkably decreased the amount of olive fruit as well as oil quality (Gholami and Zahedi, 2019a). However, despite this water deficit condition, some of the olives cultivars perform well (Gholami and Zahedi, 2019b). Regarding diverse environmental conditions, it is very important to know about the physiological characteristics of different olive cultivars in different places under different conditions (Arji, 2015).

Olive has various useful pharmacological applications including important antioxidant properties. Some of the pharmacological benefits of olive are related to phenolic composition, especially to oleuropein (Ole) and flavonoids (Fls) content (Bonechi *et al*., 2019). Moreover, oleuropein, hydroxytyrosol (Htyr) and tyrosol (Tyr) are three of the main phenolic compounds present in the fruits, leaf and oil of the olive tree (*Olea europaea* L.) (Irakli *et al*., 2018). These phenolic compounds have outstanding pharmacological effects including anticancer, spasmolytic, immune-stimulant, hypotensive, hypoglycemic, antiviral (even against HIV), and enzyme modulator effects due to their antioxidative properties (Karkovic Markovic *et al*., 2019).

As a protective action, Ole may also directly neutralize radicals by providing hydroxyl groups (Gavahian *et al*., 2019). Both Ole and Htyr have been reported to be scavengers of superoxide anions and inhibitors of the respiratory burst of neutrophils and hypochlorus acid-derived radicals (Al-Azzawie and Alhamdani, 2006). Many molecules isolated from olive fruits and leaves are derived from Ole. Some of these are simple phenols that have several important functions; for example, they can act as antioxidants with high free-radical scavenging activity and as substrates for oxidation reactions (Toric *et al*., 2019). Many of the

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nutritional and organoleptic properties of olive oil depend on its content of phenols in general and of Ole and Htyr in particular. Nevertheless, the usage of these compounds has been limited, because of the limitation in the commercial availability of these products (Karkovic Markovic *et al*., 2019).

Additionally, phenylpropanoid compounds of olive have an important role to control cancer, acting as quenchers of singlet oxygen formation and free radical scavengers. One of the important gateway enzymes in the secondary metabolic pathway that induced the synthesis of phenylpropanoids is phenylalanine ammonia-lyase (PAL) (Sirin and Aslim, 2019). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the first reaction in the pathway of phenylpropanoid biosynthesis, in which the ammonia is eliminated from L-phenylalanine to yield trans-cinnamate (MacDonald and D'Cunha, 2007; Machado *et al*., 2013). In higher plants, trans-cinnamate is the precursor of a broad spectrum of phenylpropanoid derivatives such as flavonoids, stilbenes, coumarins, lignins, other wall-bound phenolics, soluble esters and amides, and some alkaloids (Kumar and Geol, 2019). Moreover, PAL regulates the synthesis and accumulation of these compounds in different tissues and plants. Hence, PAL is directly involved in the regulation of the circulatory system, lignin accumulation, and the response against pathogens or mechanical damage (MacDonald and D'Cunha, 2007). Formulations of antioxidant polyphenols derived from olive extract, with an effective amount of substantially purified Htyr or a substantially purified mixture of Htyr and Ole, are now being used as a therapeutic and/or an antioxidant for a variety of health purposes (Lopez-Huertas and del Rio, 2014). Ortega-Garcia *et al*. (2009a) have detected PAL activity and phenolic compounds in leaves and fruits of *Olea europaea* L. cv. 'Picual' during ripening. During the fruit ripening process, a significant increase in the concentration of total phenols and Ole was detected in the leaf. In contrast, the concentration of Ole, Htyr and Tyr significantly decreased in fruit during ripening (Ortega-Garcia *et al*. (2009a).

Since olive contain a variety of phenolic compounds that is important in terms of biological attributes and positive health effects, more efforts and studies are essential to evaluate the phenolic compounds of various cultivars of olive and their derived products in different areas. A affect variety of factors the phenolic compounds of olive cultivars impacting the quality of olive fruit and olive oil in different geographical regions. Some studies indicated that some olive cultivars, especially two main Iranian olive cultivars (Zard and Roghani) in different microclimates and different elevations had more fruits and better oil quality in the region with higher elevation (Ahmadipour and Arji, 2012). Therefore, it is necessary to conduct agricultural practices with different cultivation systems and evaluate the pharmacological compounds, because the reduction of these compounds has serious adverse effects on the quality, stability, and favorable health properties of the olive products (Malheiro *et al*., 2015). Due to the increasing usage of phytochemicals in pharmacy and food industries, and inadequate studies regarding the pharmacological compounds of Iranian olive cultivars, especially the cultivars grown in the north of Iran, this study was carried out to evaluate PAL activity and some valuable phenolics (Ole, Htyr and Tyr) in olive leaves and fruits of four cultivars (namely 'Mary','Roghani', 'Fishomi' and 'Zard') grown in the north of Iran, Guilan province, Rudbar.

Materials and methods

Plant materials: The olive leaves and fruits were collected from eight-year-old trees grown in Rudbar Olive Research Station, Guilan province, north of Iran. Table 1 demonstrated the climatic characteristics of Rudbar city. The Green ripe fruits and six-month-old leaves (the third leaf from the top of the branches) were collected in late September. 100 leaves and 100 fruits from the southern position of three different trees were collected for each cultivar and washed with distilled water. Leaves were dried for 162 h at 40 °C (Abaza *et al*., 2015). Olive fruits were homogenized with liquid nitrogen and then dried as described above. Extraction process was carried out for both leaf and fruit powders. All chemical reagents were of analytical grade and purchased from Sigma and Merck Companies.

Extraction, identification and quantification of phenolic compounds: Phenolic extraction was carried out using 15 percent acetic acid in methanol added to 1 g of finely powdered tissue and then kept at 4 ˚C for 24 hours. These samples were centrifuged at 10,000 g for 15 minutes at 0 ˚C (Himac CR 15, HITACHI, Japan). The supernatant of samples was filtered through a 0.45 µm disposable syringe filter and used for Ole, Htyr and Tyr quantification by high-performance liquid chromatography (HPLC) coupled to a diode array detector with a wavelength set at 280 nm. The column was a 1.5 mm I.D. × 250 mm (Grand C18-UG 120-5 SE, MASIS, Inc., Aomori, Japan) with a 1.5 mm I.D. × 35 mm guard column (Bakhshi and Arakawa, 2006). Elution solvents were carried out at a flow rate of 1 mL/min, using as mobile phase (a) a mixture of water/acetic acid (97.5:2.5 v/v) and (b) methanol/acetonitrile (1:1 v/v) according to the method of Tasioula-Margari and Tsabolatidou (2015). To identify and calculate the amount of each component, the method of the external standard was used. In this method, the pure and standard components were injected into the device under completely identical and separate conditions, and a peak was obtained which had a retention time and a surface area. To identify any combination of the peak retention time, it was compared with its corresponding standard. Furthermore, to determine its quantity, the calibration curve was first drawn, and then, the line equation was obtained. Finally, the concentration of the component is calculated using

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Table 1- Climatic characteristics of Rudbar city

Geographical latitude (min:s)	Geographical longitude (min:s)	Altitude (m)	Mean annual Temp. (°C)	Annual rainfall (mm)
36:44	49:25	338	15.5	650.0

the area of the subquery surface obtained from the desired component and the calibration curve line equation. In the HPLC device used in this study, data recording, qualitative identification, and the rest of the quantitative calculations were carried out using Chemstation Software (Agilent Chemstation A.10.01) (Magwaza *et al*., 2016).

Total phenol content (TPC): The total phenolic content of the extract was determined using the Folin–Ciocalteu method according to Kaur and Kapoor (2002). Briefly, 200 µL of crude extract (1 mg/mL) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. Then, the mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Determination of antioxidant activity: DPPH radical scavenging activities (DSA) were evaluated as described by Lee *et al*. (2009b). The antioxidant potential of extracts was determined using the ferric reducing antioxidant power (FRAP) assay according to the method of Benzie and Strain (1999a).

Phenylalanine ammonia-lyase (PAL) measurements: PAL extraction and activity measurements were carried out according to a modified method of Rivero *et al*. (2001). Five grams of leaf and fruit were pulverized in a mortar with liquid nitrogen. The powder was then homogenized in a proportion of 1:5 w/v with extraction buffer composed of 0.1 $molL^{-1}$ borate buffer pH 8.9, 50 gL^{-1} polyvinylpolypyrrolidone, and 2 mmol L^{-1} β-mercaptoethanol. Extracts were centrifuged at 4°C for 30 min at 12000 g. The supernatant was collected and was centrifuged at 4°C for 5 min at 12000 g. Then the supernatant was used for further measurements. PAL activity was determined by measuring the amount of cinnamic acid formed in the assay medium, using spectrophotometry. The mixture containing 500 µl of samples and 1000 µl of 80 mM borate buffer at pH 8.9 with 30 mM phenylalanine were incubated for 1 h in a water bath at 37°C. Then 1.5 ml of 2 M HCl was added. An aliquot of 3 mL of ethylacetate was added to each sample and shaken in the vortex for 3 min to extract the cinnamic acid. For each sample, one aliquot of 1 mL of ethylacetate phase was collected in a new tube and evaporated in an extraction chamber. The residue resulting after evaporation was dissolved in 0.05 mol L^{-1} NaOH and the concentration of trans-cinnamic acid was measured spectro photometrically at A= 290 nm. One unit of PAL activity was defined as the amount of enzyme that catalyzed for the formation of 1 µmol of cinnamic acid min^{-1} in a cuvette of 1 cm under optimum assay conditions.

Statistical analysis: This experiment were conducted in a completely randomized design with three replications. The results for the three samples were expressed as mean values and standard error for all sampling. The differences were analyzed using analysis of variance followed by a Duncan Test with $\alpha = 0.05$. These analyses were carried out using SAS v. 9.1.3 software.

Results and discussion

Total phenol contents: The levels of TPC in each cultivar are presented in Figure 1. 'Zard' and 'Fishomi' leaves had the highest TPC as 13.3 and 12.16 $mg{\cdot}g^{-1}$ dry weights (DW), respectively which were significantly higher than 'Mary' and 'Roghani' leaves as well as the fruits of all examined cultivars. However, the concentrations of TPC showed no significant difference in the examined fruits. Reports showed that TPC of 'Zard' leaf was significantly higher than TPC in 'Cobrancosta' (Ferreira *et al*., 2007). This difference may be related to cultivars, environmental conditions and sampling stage differences. The higher amount of TPC might cause stronger radical scavenging effect in 'Zard' and 'Fishomi' leaves. It has been reported that phenolic compounds can perform antioxidant activity by several potential pathways. The main pathway is likely through free radical scavenging which the phenolic molecules can cleave the free radical chain reaction (Jabalbarezi Hukerdi *et al*., 2018). However, the total phenolic content (TPC), DPPH•, and ferric reducing antioxidant power (FRAP) differed significantly among genotypes (Orak *et al*., 2019).

DSA: The DSA (DPPH radical scavenging activities) values of extracts were examined (Figure 1). The DPPH was linked with the neutralization of free radicals generated in both assays systems by the compounds present in the extracts with antioxidant capacity (Martinez-Patino *et al*., 2019). According to the current results, the antioxidant activity was different in all examined cultivars. This study showed that Iranian olive cultivars could be considered as strong radical scavengers and as good sources of natural antioxidants for medicinal and commercial uses. The highest DSA were measured in 'Zard' and 'Fishomi' leaves that were higher than the leaves and fruits of all examined cultivars. The lowest DSA was found in 'Roghani' fruit. According to Figure 1, results showed that all samples had DSA higher than 50%. These results was in good agreement with the findings of Briante *et al*. (2003). Olive leaves are rich in phenols, such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, which have exhibited antioxidant and antimicrobial properties (Caturla *et al*., 2005;

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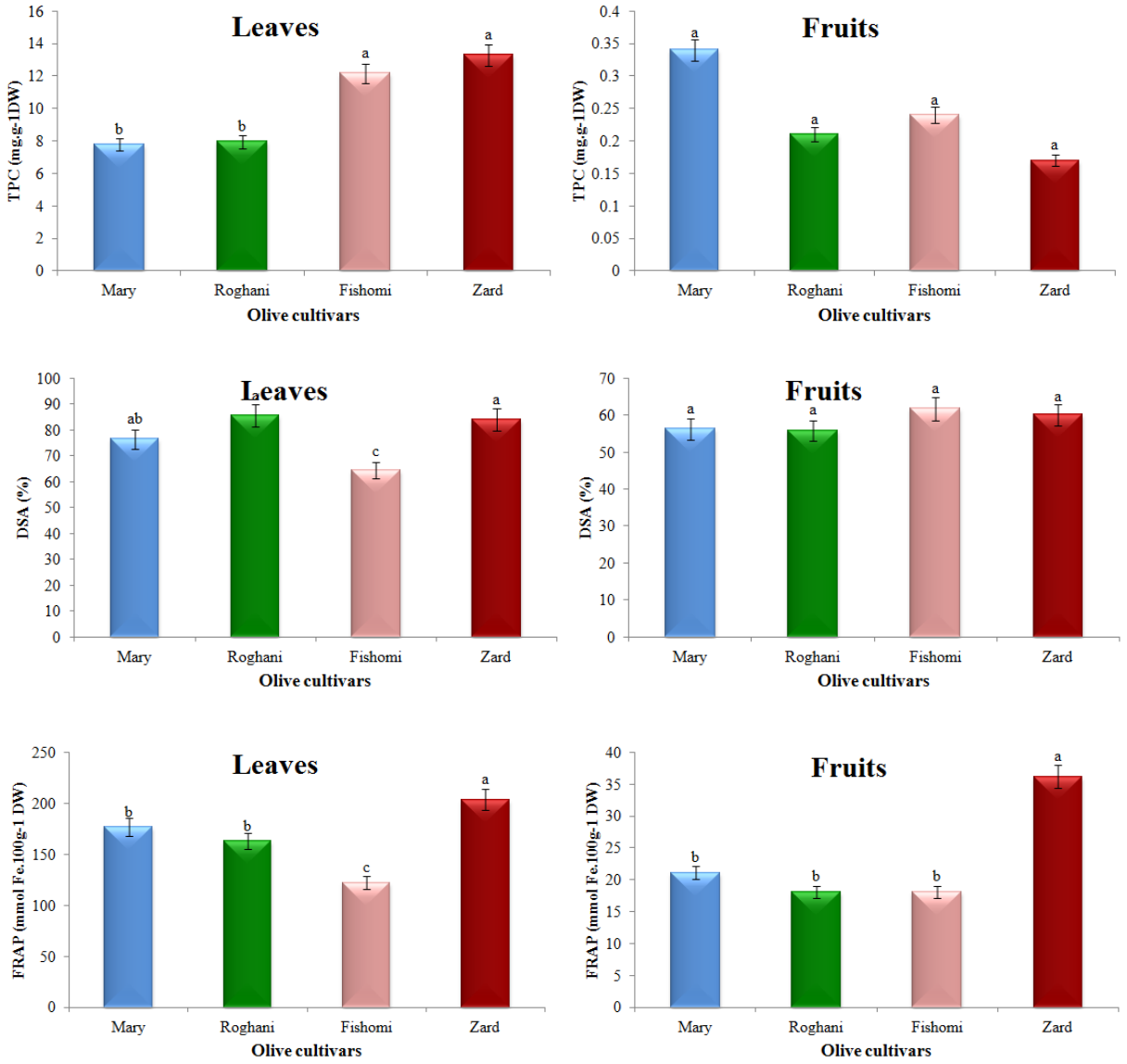

Figure 1- Concentrations of TPC, DPPH antioxidant activities (DSA %) and FRAP (mmol Fe.g^{-1} DW) in leaves and fruits of *Olea europaea* cv. 'Mary', 'Roghani', 'Fishomi' and 'Zard' (Values were compared by Duncan test. Results are expressed as mean ± SE of three values. In each row values followed by different letters are significantly different (P < 0.05)).

Romani *et al*., 2017). It has been reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds (Skerget *et al*., 2005). As Kiritsakis *et al*. (2010) suggested it seemed that the differences in the antioxidant activities may be attributable to other unidentified compounds or synergistic interactions among components. However, Yu *et al*. (2002) claimed that there has been no correlation between the TPC and the radical scavenging capacity in the wheat extract. According to this correlation, higher TPC may be indicated as a lower EC50 value of DPPH• scavenging activity (Orak *et al*., 2012).

FRAP: The FRAP (ferric reducing antioxidant power) values of extracts were detected (Figure 1); the highest amount of FRAP found in 'Zard' leaves had significantly higher than leaves and fruits of other examined cultivars. Figure 1 demonstrated the levels of FRAP. These results suggested that olive leaf had great potential as a functional food ingredient, particularly as a source of phenolic compounds. The FRAP experiment measures the antioxidant activity for the reduction of Fe^{3+} (ferric iron) to Fe^{+2} (ferrous iron) (Martinez-Patino *et al*., 2019). Moreover, the FRAP test has already been applied to measure the antioxidant properties of several dietary components such as orange juice, wines, and teas (Benzie and Szeto, 1999b). The highest reducing power among examined olive fruits was in 'Zard' (36.2 mmol Fe.100 g^{-1} DW). The difference between the inhibition percentage of reducing the power of DSA and FRAP may be related to the diversity of phenolic components. Also, decreased FRAP antioxidant activities of the fruits may be due to polymerized compounds exhibiting hindrance effects that prevent

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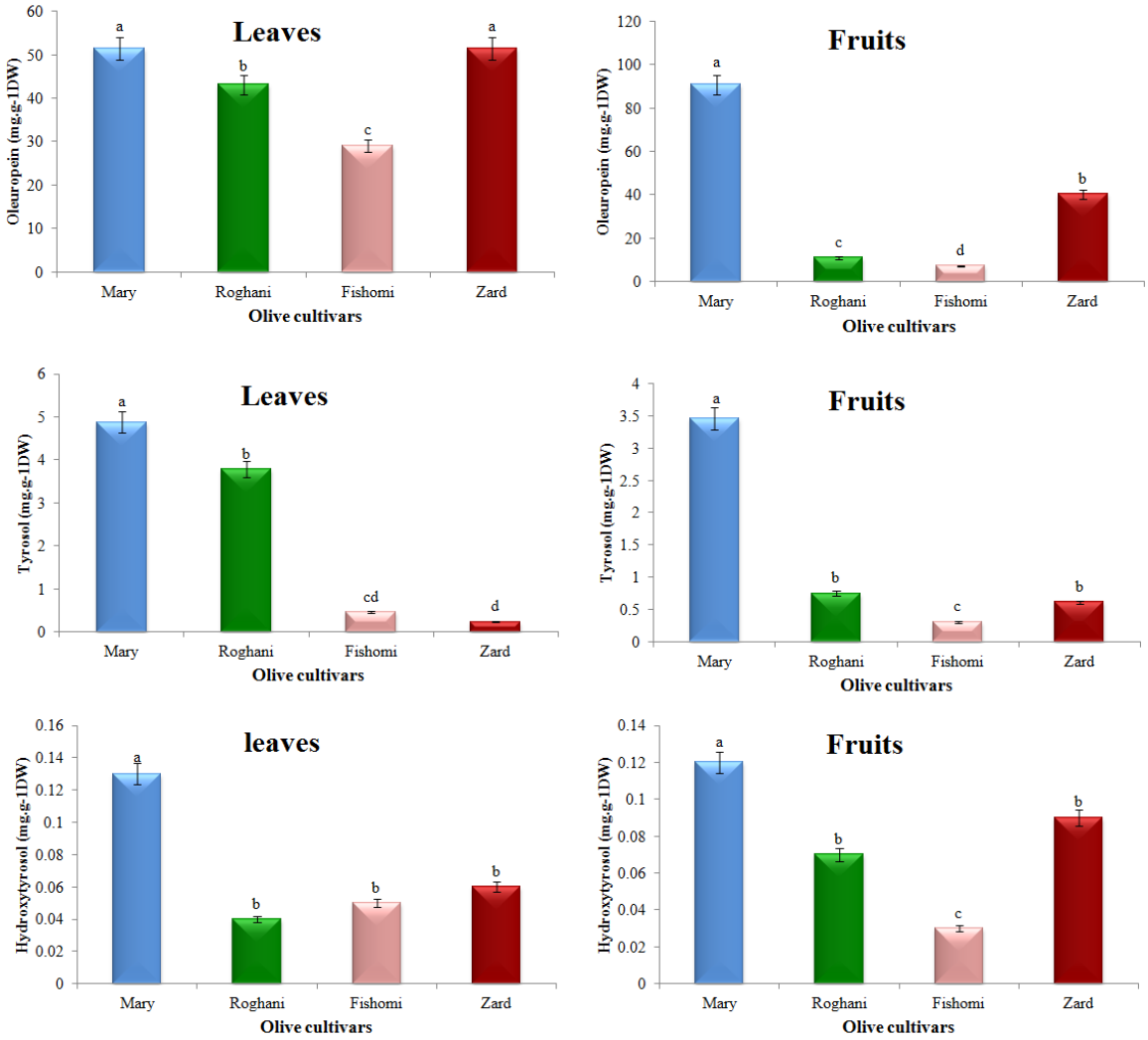

Figure 2- oleuropein, hydroxytyrosol and tyrosol (mg. g^{-1}DW) of extracts of leaves and fruits of *Olea europaea* cv. 'Mary', 'Roghani', 'Fishomi' and 'Zard' (Values were compared by Duncan test. Results are expressed as mean ± SE of three values. In each row values followed by different letters are significantly different (P < 0.05)).

close contact between the frap reagent and OH groups (Hu *et al*., 2016). Regarding DSA and FRAP test, in addition to the fruits, olive leaf extracts revealed high antioxidant properties because of the valuable phenolic compounds especially Ole (Xie *et al*., 2015).

Phenolic compounds: The content of individual standard phenolic acids viz. Ole, Htyr and Tyr in the examined olive cultivars (Mary','Roghani', 'Fishomi' and 'Zard') grown in the north of Iran were quantified by HPLC and presented in Figure 2 and 3. The retention time of the standard acids at 280 nm ranged from 10.75 to 25.5 min in the following order Ole>Tyr>Htyr. This suggested that Hydroxytyrosol derivatives were the first compounds to be oxidized; therefore, it provided oxidative stability to the oil. α-Tocopherol was oxidized after a significant decrease in hydroxytyrosol derivatives content. Tyrosol derivatives are the antioxidants that decrease with the lowest rate; causing the creation of the oil with less antioxidant activity (Nissiotis and Tasioula-Margari, 2002). Ole was most abundant in 'Mary' fruit extract and significantly higher than other examined cultivars. The lowest level of Ole is found in 'Fishomi' fruit. There were no considerable differences among levels of Htyr in both studied fruits and leaves. The highest and lowest levels of Tyr were detected in 'Mary' and 'Zard' leaves, respectively. According to Figure 2, the highest and lowest levels of Tyr were detected in 'Mary' and 'Zard' leaves, respectively. Oleuropein, the main phenolic compound of olive fruit, has important antioxidant properties that are responsible for some of the nutritional assets of fruits and the defense mechanism of leaves (Ortega-Garcia *et al*., 2008). Oleuropein was probably decomposed into hydroxytyrosol and oleanolic acid under the action of the light, acid, base, high temperature (Yuan *et al*., 2015). The amounts of Tyr recorded in the present study were higher than the Spanish as well as Italian cultivars studied by Romani (1999). The Concentration of Ole determined in the olive leaf extract of three Greek cultivars was significantly lower than the amounts observed in all cultivars investigated in this study. During fruit

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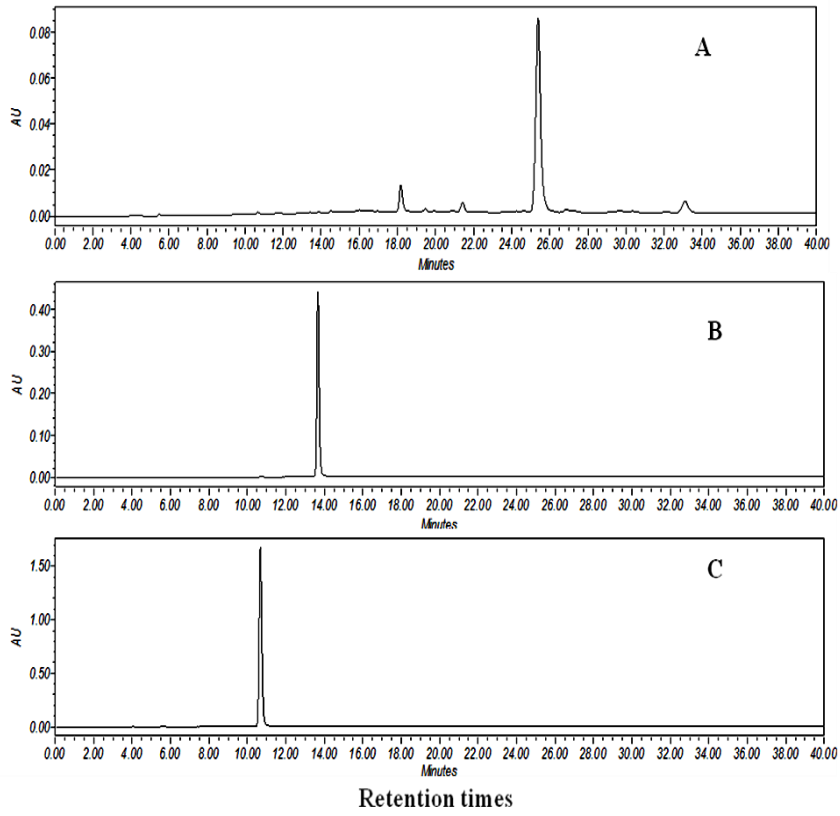

Figure 3- HPLC chromatogram of standard phenolic acids mixture of concentration at 280 nm. (A) Oleuropein, (B) Tyrosol and (C) Hydroxytyrosol.

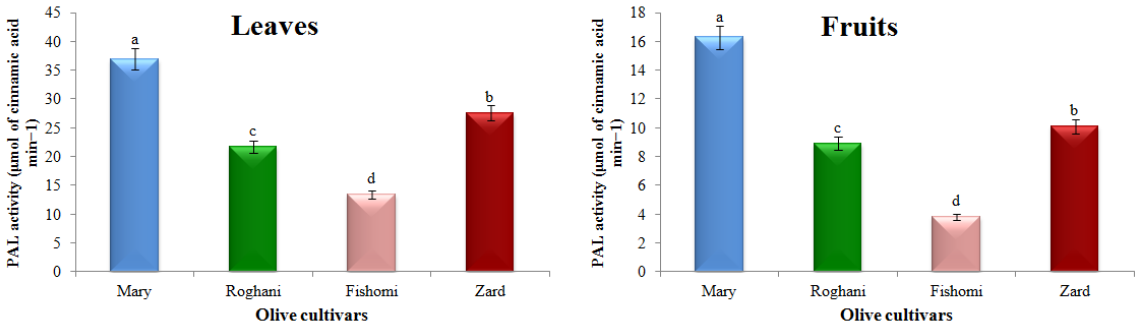

Figure 4- PAL activity (µmol cinnamic acid mg protein^{-1} min^{-1}) in leaves and fruits of *Olea europaea* cv. 'Mary', 'Roghani', 'Fishomi' and 'Zard' (Values were compared by Duncan test. Results are expressed as mean ± SE of three values. In each row values followed by different letters are significantly different (P < 0.05)).

maturation, oleuropein concentration might be decreased in fruits and increased in leaves (Ortega-Garcia *et al*., 2008). On the other hand, the concentration of Ole and Tyr in Mary fruit was higher than those detected in 'picual' fruit in Jaén, Spain (Ortega-Garcia *et al*., 2009b). There were important differences for all the compounds among the studied cultivars from Iran and other cultivars in other parts of the world. In all cases, the differences found can be related to the differences in diversity of cultivars, environmental factors, and the degree of ripeness of the olives as well as different extraction methods.

PAL activity: PAL activity was assayed in leaves and fruits of olive tree cv. 'Mary', 'Roghani', 'Fishomi' and 'Zard'. The results are shown in Figure 4. In leaf and fruit, the highest and the lowest PAL activity was found in Mary's and Fishomi's cultivars, respectively.

PAL activity in leaves of all examined cultivars was significantly higher than their fruits. The activity of PAL in the leaf and fruit of examined cultivars indicated that it must be involved in the biosynthesis of phenylpropanoids in both sections, in which the concentration of phenols was very high. High PAL activities found in olive leaf caused an increase in biosynthesis and accumulation of phenolics and other compounds derived from phenylpropanoids, which act as antibiotics and powerful phytoalexins (Ortega-Gracia and Peragon, 2009b). In this same sense, an increase in

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the concentration of total phenolic compounds and Ole has been observed in leaf rather than fruit, demonstrating that, maybe, a relationship between PAL activity and total phenol and Ole biosynthesis can exist in the olive cultivars (Tovar *et al*., 2002). PAL generally happens at low levels in normal tissues, though its activity greatly increases upon infection and stress. However, PAL activity is an important physical indicator of a plant's ability to resist damage. Additionally, some studies indicated that PAL can catalyze the synthesis of anthocyanidin which is an important component of the color of fruits and leaves (Wang *et al*., 2015).

Conclusions

It could be concluded that an increase in phenolic compounds reflects an important improvement in the quality of olive fruits and their derivative products, due to the beneficial effects of phenolic compounds on the health and the stability of olive oil against oxidation (Rajabiesterabadi *et al*., 2020; Manna *et al*., 2002). The high phenolic compounds of the olive leaves of some cultivars such as 'Zard' and 'Roghani' can be used for the enrichment of commercial oil. Regarding DSA and FRAP test, in addition to the fruits, olive leaves extracts revealed high antioxidant properties because of the valuable phenolic compounds, especially Ole. The high levels of PAL and phenols in an olive leaf could be related to some of the defense processes or with the biosynthesis of phenolic compounds that accumulated in the olive tree. The current results showed that among the four examined Iranian cultivars of *Olea europaea*, the highest and the lowest antioxidants activity was found in the leaf and fruit of Mary's and Fishomi's cultivars, respectively.

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Conflict of interest

The authors declare that no conflict of interest exists.

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