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Scientific Research

Investigation the antioxidant activity and total phenol and flavonoid contents of wild grapevines (*Vitis vinifera* subsp. sylvestris) fruit and leaf extracts

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ABSTRACT

Vitis vinifera subsp. Sylvestris, belonging to Vitis family and one of indigenous plants of Iran, was collected from Miankalleh. In this research, different plant extracts (methanol, water, chloroform, ethyl acetate) were obtained from leaves and fruits of wild grapes. The yield of extracts was determined. Also, total phenol and total flavonoid content of the extracts were analyzed and the antioxidant activity of the extracts were assessed using DPPH radical (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) methods. Our investigation showed that the highest extraction yields was related to aqueous extract with values of 14.35% for leaves and 12.71% for plant fruits. The total phenolic content of the fruit was higher than the leaf. Also, the methanolic extracts had the higher total phenolic content than other extracts of leaf (2.9 ± 0.25 mg/g) and fruit (12.3 ± 0.1 mg/g). The total flavonoid content of the methanolic extract of the fruit was obtained: 9.7 ± 0.03 mgGAE/g dried plant, which showed the highest amount. Investigation of the antioxidant activity using DPPH assay revealed that the aqueous extract of leaf with IC50 of 26.74 ± 0.12 µg/mL had the best antioxidant activity. The methanolic extract of the leaf was more potent than other extracts in FRAP assay. In conclusion, the phytochemical analysis of leaf and fruit of wild grapes showed that the methanolic extract had the best antioxidant potential.

1- Introduction

The genus Vitis (grape), belonging to the family Virtaceae, consists of 70 species worldwide, characterized by woody, climbing, and twisting trees [1]. This genus has opposite and palmate leaves. The grape species (*Vitis vinifera*) is native to Central Europe, the Mediterranean region, and southwestern Asia, and is now cultivated worldwide. This plant is also found growing wild in various parts of the world [2]. Over time, it has adapted to different climatic and environmental conditions and is now cultivated in various regions around the globe. In traditional medicine, grapes have been used to treat ailments such as rheumatoid arthritis and rheumatism. Additionally, due to its antioxidant constituents like ellagic acid, grapes have been known to reduce symptoms of arthritis and exhibit good anti-cancer properties [3]. Wild grapes grow in regions with fertile soil, including various geographical areas in countries such as Iraq, Turkey, Afghanistan, Syria, Lebanon, and Iran [4]. Wild grapes (*Vitis vinifera* subsp. Sylvestris) are a subspecies of grape plants that naturally grow in the forests of northern Iran and the moist regions at the foothills of the Zagros Mountains. These plants have three-lobed leaves with a hairy underside [5]. Plants are valuable sources of natural secondary metabolites. These compounds possess numerous properties such as antimicrobial [6], antioxidant [7], anticancer [8], antidiabetic [9], and more. Flavonoids and phenolic compounds are a group of secondary metabolites that are associated with many plant properties. For instance, if a plant has high levels of phenolic and flavonoid content, the likelihood of having anticancer [10,11] and antimicrobial [12] properties increase. Moreover, the high content of these compounds is related to the level of oxidative stress inhibition [13]. The antioxidant activity of a plant can also have a direct relationship with its biological effectiveness [14]. Since limited studies on the phytochemistry of this valuable plant have been published so far, the study of the wild grape plant from the protected area of Miankalle in Mazandaran province was collected in this pioneering study. Various extracts from the leaves and fruits of the plant were obtained by maceration method in four solvents (methanol, ethyl acetate, chloroform, and water). Subsequently, the phytochemical properties of the extracts, including total phenolic content, total flavonoid content, and antioxidant activity using DPPH and FRAP methods, were evaluated.

2- Experimental

Materials and apparatuses

In this study, all materials and solvents with a purity of over 99% were purchased from Merck Millipore. A double beam spectrophotometer (SPEKOL 2000 Analytik Jena) was used for UV-VIS spectrum recording.

Plant Collection

The leaves and fruits of wild grape plants were collected from the Miandoroud region (Behshahr, Mazandaran, Iran) and registered in the biology department of University of Mazandaran under the herbarium number 5524. The leaves and fruits of this plant were powdered after drying in dark condition.

Plant extraction

For extracting from the dried parts of the wild grape plant's fruits and leaves, maceration method was used. Initially, 2 g of powdered dried plant part was poured into a 250 mL Erlenmeyer flask and 75 ml of the solvent was added until the surface of the powdered plant was completely covered with solvent. The flask was sealed and placed on a shaker for 24 hours Then the extract was poured in another flask and new solvent was added. This process was repeated three times, with 75 mL of solvent added in each repetition. The obtained extract was filtered through Whatman filter paper No. 1. To prevent energy loss, high temperatures, and compound degradation, solvent separation from the extract was done using a rotary evaporator. The amount and frequency of extraction were optimized. Ultimately, four aqueous, ethyl acetate, methanol, and chloroform extracts were obtained from fruit and leaf samples.

Determination of extraction yield and concentration of each extract

To determine the extraction yield, the solvent in 2 g of each of the four extracts was evaporated, and the weight of the extracts was measured. The extracts were yellowish-brown in color and oily in texture. The weight of the extracts was measured using a precise balance with an accuracy of 0.1 mg to calculate the extraction efficiency in each case.

Determination of total phenolic content

Measurement of total phenolic content in wild grape plants was conducted using the Folin-Ciocalteu method[15]. Specifically, 50-250 µg of the desired extracts were pipetted into test tubes and brought to a volume of 1600 µL with distilled water. Subsequently, 100 µL of Folin-Ciocalteu reagent was added and thoroughly mixed with a vortex. After 5 min, 300 µL of 7% sodium carbonate was added. Following a 2-hour incubation period in darkness at room temperature, the absorbance of the extract was measured using a spectrophotometer at a wavelength of 760 nm compared to the control sample. The control sample contained all reaction components except for the plant extract.

To obtain the calibration curve, gallic acid was used as a standard. Gallic acid was prepared at concentrations of 2.5, 5, 10, 15, and 20 µg/µL, and 100 µL of Folin-Ciocalteu reagent and 300 µL of 7% sodium carbonate were added to each concentration following the above method. After a 2-hour incubation period in darkness at room temperature, their absorbance at a wavelength of 760 nm was read, and the absorption curve versus gallic acid concentration was plotted. These experiments were repeated three times for each concentration. Finally, the total phenol content was expressed as mg of gallic acid per g of dry weight of the plant.

Determination of total flavonoid content

The amount of total flavonoid contents was measured using the aluminum chloride colorimetric method[16]. Specifically, 50-400 µg/µL of extracts were placed in a test tube and diluted to a volume of 1500 µL with distilled water. Then, 75 µL of $NaNO_2$ (5% w/w) were added, after 5 min followed by the addition of 150 µL of aluminum chloride (10% w/v), and left for 6 min. Subsequently, 500 µL of 1 M sodium hydroxide and 275 µL of water were added. After thorough mixing, the absorption against the blank was read at a wavelength of 510 nm. Quercetin was used as the standard. The amount of flavonoid content was reported based on the mg of quercetin per g of dry powder. A standard curve was plotted based on concentrations of 50, 100, 250, 300, and 400 mg/mL of quercetin. This

experiment was repeated three times for each concentration.

Determination of DPPH radical scavenging activity

The antioxidant activity of the samples was evaluated by measuring the DPPH radical scavenging capacity [17]. Concentrations ranging from 75 to 1200 µg/µL of various extracts were placed in test tubes. Ascorbic acid (5 mM) was used as a standard. Methanol (50%) was added to various volumes of the extracts and adjusted to total volume of 3 mL. Then, a 1 mM DPPH solution was added to a volume of 1 mL. The mixture was vigorously vortexed and incubated in darkness for 30 min. Subsequently, the absorbance of the mixture was measured using a UV-visible spectrophotometer at a wavelength of 517 nm. A blank was reaction without the plant extract sample. The IC_{50} value (the concentration of each extract required to scavenge 50% of the radicals) was determined for the extracts. This experiment was repeated three times for each plant part. In this method, the antioxidant capacity based on DPPH radical scavenging (RSD%) was calculated using the following formula.

$$\%\text{RSD} = \frac{\text{Blank absorbstion} - \text{Sample absorbtion}}{\text{blank absorbstion}} \times ١٠٠$$

Determination of reducing power (FRAP)

In this method, Fe (III) is reduced to Fe (II). Antioxidants capable of reducing Fe^{3+} to Fe^{2+} convert the colorless TPTZ-Fe^{3+} complex to the blue TPTZ-Fe^{2+} complex [18]. For this purpose, the concentration of 250 µg/mL of various plant extracts from fruit and leaves was adjusted to a final volume of 2 mL. The FRAP solution containing 10 mM TPTZ (in 40 mM HCl), 20 mM iron chloride, and 300 mM acetate buffer at pH 3.6 was added to the extract. The sample was then placed at a temperature of 37°C for 10 min, and the color intensity obtained at a wavelength of 593 nm was read against the control.

To draw a standard curve for the FRAP method, ferrous sulfate ($FeSO_4.7H_2O$) with concentrations of 125, 250, 500, and 1000 µM was used, and the antioxidant activity of the extracts was expressed based on the µMol of Fe^{2+}. Ascorbic acid was used as a positive control. This experiment was repeated three times for each concentration.

3- Discussion and Conclusion

Extraction Efficiency

Extraction from wild grape leaves and fruit was carried out using aqueous, methanol, chloroform, and ethyl acetate solvents. Methanol and water, with high polarity, are among the most suitable solvents for plant extraction and are commonly used, especially methanol, in most plant studies. As shown in Table 1, the highest extraction efficiency was related to the aqueous extract with values of 14.35% and 12.71% in the leaves and fruit, respectively.

Table 1: Yield of extraction for leaf and fruit of wild grapes in different solvents

Solvent	Plant organ	Yield (w/w %)
Water	leaf	14.35
	fruit	12.71

Methanol	leaf	6.44
	fruit	4.63
Chloroform	leaf	2.23
	fruit	1.68
Ethyl acetate	leaf	1.98
	fruit	1.34

Determination of Phenolic Content in the Extracts

The total phenolic content was determined using the standard Gallic acid. The total phenolic content was reported as mg of Gallic acid equivalents per g of dry weight of the plant. The results of the analysis are presented in Table 2.

Table 2: Total phenolic content of leaf and fruit (mgGAE/g) [a]

Solvent	Total phenolic content of leaf	Total phenolic content of fruit
Water	±0.02 2.7	0.06 ± 4.7
Methanol	0.25 ± 2.9	0.10 ± 12.3
Chloroform	0.03 ± 0.9	0.06 ± 1.7
Ethyl acetate	0.03 ± 0.7	0.07 ± 1.0

a : mg of Gallic acid equivalent /g of dried plant

Based on the data which represented in Table 2, the methanolic extract of the fruit contains the highest total phenol content (12.3 ± 0.10 mgGAE/g dried plant), while the ethyl acetate extract of the leaf has the lowest content (0.7 ± 0.03 mgGAE/g dried plant). Generally, wild grape fruits have higher total phenolic content compared to plant leaves. Methanol and water are the solvents with the highest phenolic compounds. The study indicates that polar solvents are more effective in extracting phenolic compounds from various plant parts. The phenolic content in wild grape fruit and leaves is notably lower than that of two grape varieties, Ghazal and Ozum, as reported by Rezazad and co-workers in 2021. The study utilized 70% methanol and ultrasonic waves for extraction. The extraction method significantly influences the extraction of phenolic and flavonoid compounds, potentially explaining discrepancies between different studies [19]. Another study by Rabiei and co-workers in 2021 assessed phenolic, flavonoid, and antioxidant activity in 15 grape varieties from Zanjan province. Shahani grapes exhibited the highest phenolic content at 1.82 mg gallic acid /g of extract, while most cultivars had phenolic content below 1 mg gallic acid /g of extract. Comparing these findings with wild grape subspecies, it is evident that wild grape possesses higher phenolic and flavonoid content [20] .

Determining the Flavonoid Content of Extracts

,Flavonoids include flavones ,flavanones, flavonols and flavanonols which form the largest group of secondary metabolites in plants. Based on the absorption values related to different concentrations of the extracts and their comparison with the standard quercetin solution, the results of the

evaluation of the flavonoid content of the extracts are shown in Table3.

Table 3: Total flavonoid content of wild grapes leaf and fruit extracts (mgQE/g) [a]

extract	Total flavonoid content of leaves	Total flavonoid content of fruit
Water	4.9 ± 0.40	3.3 ± 0.02
Methanol	6.8 ± 0.40	9.70 ± 0.03
Chloroform	1.7 ± 0.03	1.0 ± 0.03
Ethyl acetate	0.60.± 0.01	0.7 ± 0.02

a : mg of quercetin equivalent /g dried plant

According to Table 3, the highest total flavonoid content (in terms of mg of quercetin per g of dry weight of the plant) is related to the methanolic extract of the fruit (9.7 ± 0.03 mg/g) and the lowest amount is related to the ethyl acetate extract of the leaves (0.60 ± 0.01 mg/g). Generally, based on the results obtained, it can be concluded that the methanolic extract is a more powerful source of antioxidants compared to other extracts. A study on the phenolic content, flavonoids, and antioxidant activity of the methanolic extract of leaves, skins, and grape juice of the red grape variety grown in the Urmia region revealed that the highest amount of flavonoid content belonged to the leaves of this grape variety with a level of 3.8 mg catechin /g plant. Furthermore, the highest amount of phenolic content was reported in the leaves with a level of 8.9 mg/g gallic acid. The leaves of this grape variety have less flavonoid content compared to the leaves of wild grapes studied, which could be due to the specific variety under investigation and the season and time of sample collection. Additionally, it was revealed that the leaves of this grape variety have higher phenolic and flavonoid content than grape pomace and juice [21].

Investigation of the antioxidant activity of extracts by the DPPH method

In this study, ascorbic acid has been used as standard. The results of the antioxidant activity of the extracts are presented in Table 4 and Figures 1 and 2.

Table 4: Antioxidant activity of wild grapes fruit and leaf extracts using DPPH assay (µg/ml)

sample	IC_{50} of standard	IC_{50} of leaves	IC_{50} of fruits
Ascorbic acid	16.62 ± 0. 1		
Aqueous extract	-	26.740.± 0.12	59.67 ± 0.25
Methanolic extract	-	54.65 ± 0.16	34.36 ± 0.23
Chloroform extract	-	81.20 ± 0.33	78.39 ± 0.14
Ethyl acetate extract	-	55.5 ± 2.30	75.10 ± 3.40

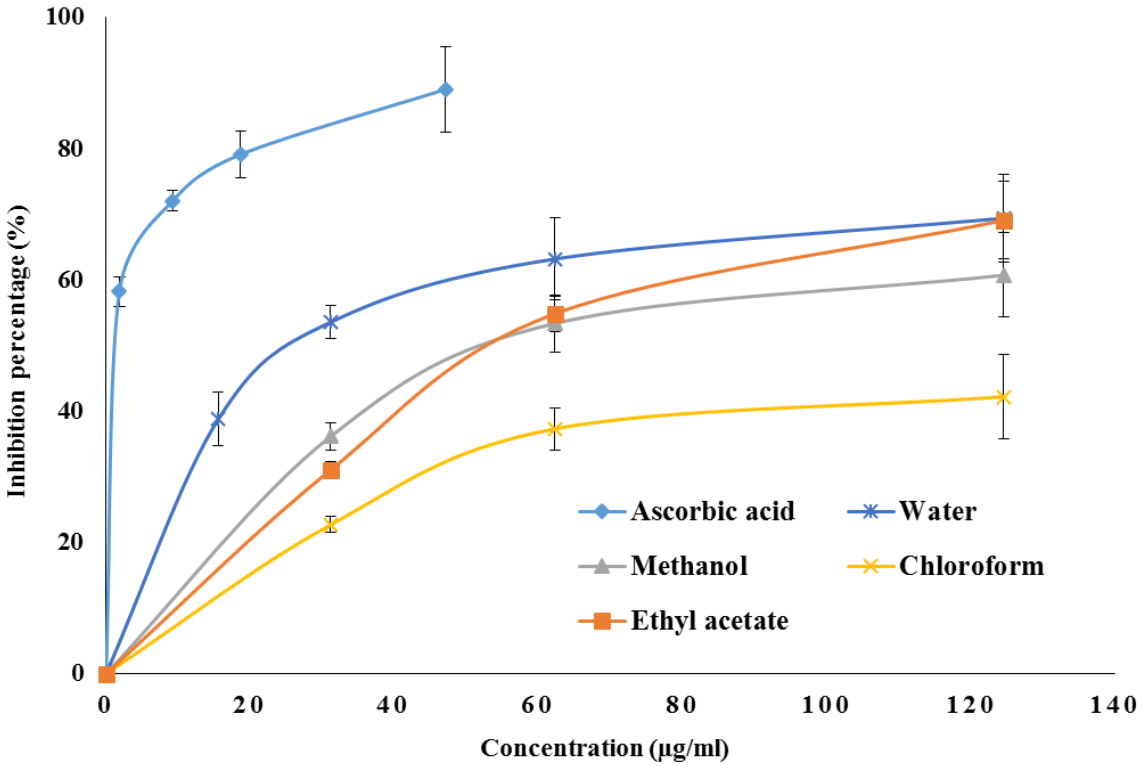

Fig. 1: DPPH assay results for wild grape leaf extracts

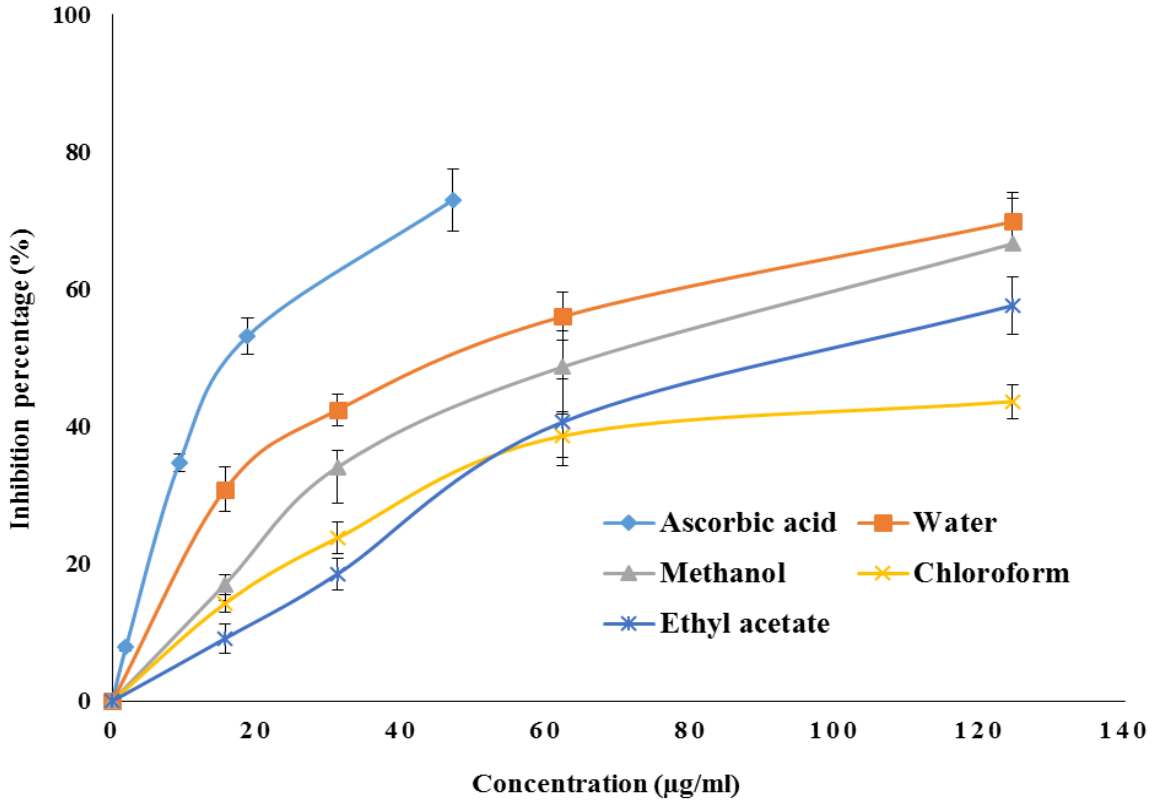

Fig. 2: DPPH assay results for wild grape fruit extracts

As shown in Figure 2, the graph curves upwards for values above 50%, indicating that only concentrations with inhibitory activity up to this point are reported as IC50. For leaf extracts, the order of effectiveness is water < methanol < ethyl acetate < chloroform, while for fruit extracts, the order is methanol < water < ethyl acetate < chloroform in demonstrating anti-radical activity compared to ascorbic acid standards. Among the extracts, the water extract of leaves showed a high radical scavenging activity with a value of 26.74 µg/ml, significantly higher than the ascorbic acid standard of 16.60 µg/ml. In 2013, Croatian researchers investigated the antioxidant properties of red grape leaves. The results indicated a radical scavenging activity of 142 µg/ml for the ethanol extract compared to the ascorbic acid standard. The results showed that the ethanol extract of this species exhibits high anti-radical activity, attributed to the presence of flavonoid compounds in this extract [22].

Analysis of antioxidant activity of extracts using the FRAP method

Figure 3 and Figure 4 respectively show the calibration curve and the reducing power diagram of different leaf and fruit extracts of wild grape plant. The methanolic extract in the leaves and the ethyl acetate extract in the fruit have higher reducing power compared to other extracts. For the plant's leaves, the order of extracts in terms of reducing power is methanolic < aqueous < ethyl acetate < chloroform, and for the fruit of the plant, the order is ethyl acetate < methanolic < aqueous < chloroform compared to the standard ascorbic acid. In 2013, Croatian researchers investigated the antioxidant properties of red grape leaves. The results of these studies indicate that this compound contains high levels of phenolic and flavonoid compounds. Furthermore, a study on the ethanol extract of red grape leaves shows high levels of reducing power compared to the standard [22]. According to research conducted on other species of grape genus, the high antioxidant properties of this species can also be attributed to the presence of phenolic and flavonoid compounds in it [23].

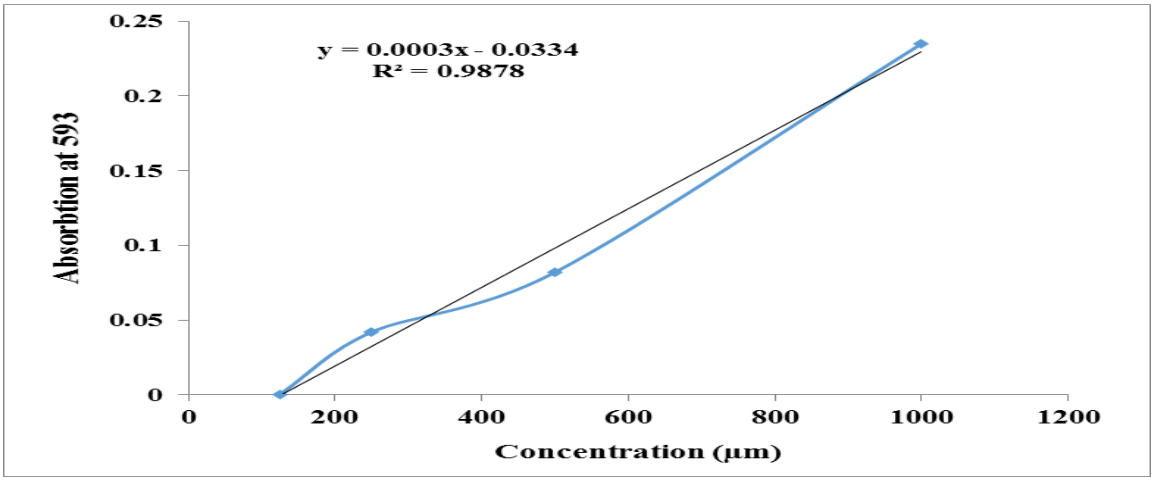

Fig.3: Calibration curve for FRAP assay

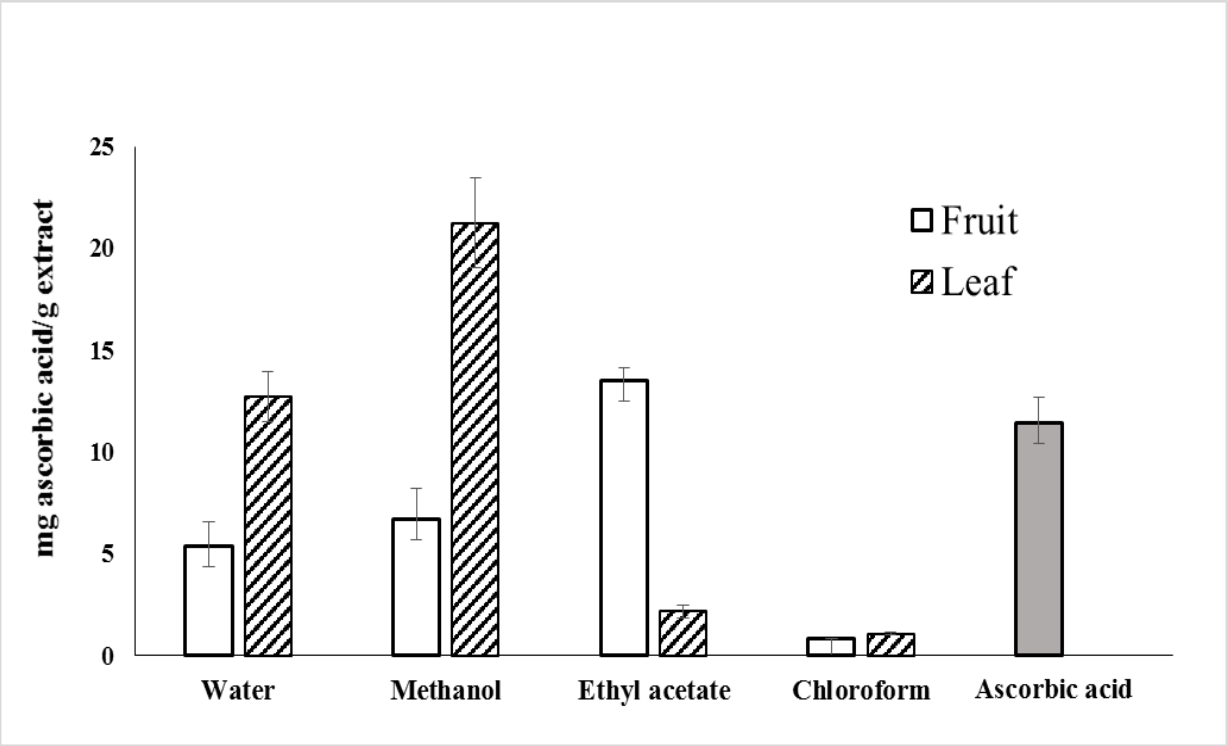

Fig. 4: FRAP assay results for wild grape fruit and leaf extracts

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چکیده

زیر گونه انگور وحشی (*Vitis vinifera* subsp. Sylvestris) متعلق به جنس Vitis و از گیاهان بومی ایران، از منطقه میانکاله جمع‌آوری شد. در این پژوهش،عصاره‌های متانولی،آبی،کلروفرمی و اتیل استاتی به روش خیساندن از میوه و برگ گیاه انگور وحشی تهیه شد. پس از آن بازده عصاره گیری تعیین گردید. همچنین محتوای فنولی کل و فلاونوئیدی کل میوه و برگ مورد ارزیابی قرار گرفت و به منظور تعیین فعالیت آنتی‌اکسیدانی عصاره‌ها از دو روش (۲و۲–دی فنیل–۱–پیکریلی هیدرازیل) DPPH و روش (قدرت احیاکنندگی یون آهن) FRAP استفاده گردید. بررسی‌ها نشان داد که بیشترین بازده عصاره‌گیری مربوط به عصاره آبی با مقادیر٪۱٤/۳۵برای برگ و ٪۱۲/۷۱ برای میوه گیاه بوده است. از نظر محتوای فنولی، میوه گیاه دارای محتوای بالاتری نسبت به برگ است. همچنین می‌توان گفت که عصاره متانولی دارای بالاترین مقادیر محتوای فنولی در برگ (mg/g ۰/۲۵ ± ۲/۹) و میوه گیاه (mg/g ۰/۱ ± ۱۲/۳) است. در مورد محتوای فلاونوئیدی بیشترین میزان مربوط به عصاره متانولی میوه (mg/g ۰/۰۳± ۹/۷) بوده است. بررسی فعالیت آنتی اکسیدانی به روش‌های DPPH نشان داد که عصاره آبی برگ با $IC_{۵۰}$ برابر با µg/ml ۰/۱۲ ± ۲٦/۷٤ دارای بهترین نتیجه در مقایسه استاندارد می‌باشد. عصاره متانولی برگ نیز دارای بیشترین قدرت احیای آهن در روش سنجش فعالیت آنتی‌اکسیدانی به روش FRAP بود. به طور کلی ارزیابی فیتوشیمیایی برگ و میوه گیاه انگور وحشی نشان داد که عصاره متانولی بهترین عملکرد را در بین چهار عصاره مورد بررسی داشته است.