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Evaluation of Total Phenolic Content and Antioxidant Activity in Ten Selected Mahaleb (*Prunus mahaleb* L.) Genotypes

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Abstract
Mahaleb (*Prunus mahaleb* L.) is an important rootstock for *P. avium* and *P. cerasus* cultivars. The present study has compared the phenolic contents and antioxidant activity of the methanolic extracts of the barks, leaves and fruits of ten selected mahaleb genotypes. The total phenolic content (5.11-131.77 mg GA g^{-1}) in barks and the total flavonoid (54.06-180.6 mg QE g^{-1}) and proanthocyanidin (8.89-25.33 mg CA g^{-1}) contents in fruits were greater than the other parts of the plants. The maximum contents of total phenol and total proanthocyanidin were in the stem bark and fruit of the genotype '249' (131.77 mg GA g^{-1}, 25.33 mg CA g^{-1}, respectively), while the maximum contents of flavonoid, and anthocyanin were in the fruits of genotype 271 (180.6 mg QE g^{-1} and 260.81 mg CY g^{-1}, respectively). Antioxidant activity of the samples was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and reducing power assay (RPA). The antioxidant activity was the highest with the genotype '249', which showed 80.9% and 89.3% in DPPH and RPA assays, respectively. This study showed that total phenolic, flavonoid, proanthocyanidin, and anthocyanin contents were affected by mahaleb genotypes. This information may be of assistance in the production of mahaleb genotypes with maximum levels of desired phenolic.

Keywords: Anthocyanin, DPPH, flavonoid, phenol, proanthocyanidin, rootstock.

Abbreviations: ***P. mahaleb*** ,*Prunus mahaleb* L. ;**DPPH** ,1,1-diphenyl-2-picrylhydrazyl ; **BHT**, butylatedhydroxytoluene; **BHA**, butylated hydroxyl anisole; **TBHQ**, tert-butyl hydroquinone; **TPC**, total phenolic content; **TFC**, total flavonoid content; **TPrAC** ,total proanthocyanidin content; **TAC**, total anthocyanin content; **CY**, cyanidin-3-glucoside; **CA**, catechin; **CE**, quercetin; **GA**, gallic acid; **RPA**, reducing power assay.

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Introduction

The genus *Prunus* has several plant species that produce a great deal of raw materials for the horticulture, ornamental, food, and pharmaceutical industries (Özcelik *et al.*, 2012). Crops of this genus, are mainly used as fresh fruits, processed sweets, jams, juices, candies, and natural sweeteners in pharmaceutical products (Özcelik *et al.*, 2012).

One of the best cherry rootstocks for Iranian nurseries and orchards is mahaleb. Mahaleb is tolerant to lime-induced iron chlorosis and zinc deficiency, and can grow in light, calcareous soils and arid climates in Iran (Moghadam and Khalighi, 2007). It is a shrub or tree belonging to the *Rosaseae* family under the scientific name of *Prunus mahaleb* L., which is native to the Mediterranean, Southeast Europe, West Asia, and Central Europe (Abedian *et al.*, 2012). This plant has white and fragrant flowers growing to 1-2 m (Ieri *et al.*, 2012).

In Turkey, the slightly bitter fruits and seeds of the mahaleb trees have been used as a tonic for the heart and a traditional medicine for diabetes and gastrointestinal problems (Halilova and Ercisli, 2010). The oil of the kernels is exploited in the preparation of lacquers and varnishes, and contains little amounts of cyanogenic glycosides and coumarin derivatives (Aydin and Konak, 2002). The seed kernels have also been used to treat pediatric diarrhea in Sudan, and as sedative and vasodilator in Arab countries (Heim *et al.*, 2002; Hinneburg *et al.*, 2006).

The presence of phenolic compounds, including cyaniding 3-glycosides, O-coumaric acids, and quercetin derivatives in mahaleb has been previously reported (Ieri *et al.*, 2012). Total phenolic content and antioxidant activity of natural extracts are of particular interest to food industry which is looking for plant extracts with significant medicinal properties to be used as food preservatives (Skotti *et al.*, 2014). Lipid oxidation is one of the main causes of chemical deterioration in food. The food oxidation process produces a rancid flavor and decreases the sensory and nutritional quality of the products, making them unacceptable to consumers. (Olmedo *et al.*, 2014). The use of lipid-soluble antioxidants is a useful method to preserve the nutritional quality, reduce oxidation and rancidity, and extend the shelf-life of food products (Maisuthisakul *et al.*, 2007).

Several synthetic antioxidants, such as butylatedhydroxytoluene (BHT), butylated hydroxyl anisole (BHA) and tert-butyl hydro-quinone (TBHQ) are widely used as food additives. However, the safety of these synthetic antioxidants has been argued owing to the presumed toxic properties. For this reason, there is currently a growing interest in searching antioxidants from plant sources. The purpose of this study is to determine the antioxidant capacity and total phenolic, flavonoid, anthocyanin, and proanthocyanidin contents of different parts (leaves, fruits, and barks) of ten selected mahaleb genotypes from Iran.

Materials and Methods

Plant materials

In the present study, various parts of ten genotypes of mahaleb ('186', '265', '271', '249', 101', '166', '108', '188', '99', '266'), including the barks, leaves, and fruits were collected from the farm of Khorasan Agricultural Natural Resources located in the Khorasan Razavi province, Mashhad, Iran, in spring 2013. Some main characteristics of the ten analyzed genotypes are described in Table 1. The samples were air-dried in the shade at room temperature.

Preparation of extracts

Dried leaves and barks were ground to powder using a mortar and pestle. Thirty grams of each sample were extracted with 300 ml of 95% methanol for 48 hours at room temperature. The extracts were filtered and dried with rotary evaporator, and then freeze-dried. For the fruits, all the

Table 1. Some characteristics of the mahaleb genotypes tested in this study

Genotypes	Growth habit	Suckering	Flowering period (Day)	Fruit ripening day in June	Fruit skin color
99	Erect	High	6	11	Black red
101	Erect	High	12	22	Light red
108	Erect	Medium	15	24	Very dark red
166	Erect	Low	18	32	Dark red
186	Erect	Low	8	14	Black red
188	Erect	Low	17	37	Black red
249	Erect	Medium	13	29	Black red
265	Erect	Medium	9	18	Very dark red
266	Erect	Medium	17	32	Very dark red
271	Erect	Low	8	18	Very dark red

extraction steps were the same as above except for adding of methanol to fresh fruits. Plant extracts were kept in refrigerator prior to the assays.

Determination of total phenolic content (TPC)

Samples were measured for TPCs colorimetrically using the Folin-Ciocalteu method (Heim *et al.*, 2002) with slight modifications as detailed in the following. A 100 µL portion of extract was mixed with 0.5 mL Folin-Ciocalteu reagent (diluted 10 times with distilled water). 7 mL of distilled water was added to the solution and it was allowed to stand at room temperature for 5 minutes. Then, 1.5 mL sodium bicarbonate (60 mg mL^{-1}) solution was added to the mixture and left at room temperature in dark place for 2 hours. Absorbance was read at 725 nm against blank using UV-visible spectrophotometer (Cecil, UK.). A calibration curve was constructed using a standard solution of gallic acid (0.2-1 mg mL^{-1}). Results were expressed as mg gallic acid g^{-1} extract (mg GA g^{-1} DE). All experiments were performed in triplicate.

Determination of total flavonoid content (TFC)

The TFC was determined using the method suggested by Huang *et al*. (Huang *et al.*, 2004) with minor modifications. Five mL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract (0.4 mg mL^{-1}). Absorption of the resulting solution was read at 367 nm using UV-visible spectrophotometer (Cecil, UK.) against a blank sample containing 5 mL extract solution with 5 mL methanol without AlCl3. The TFC was determined using a standard curve with quercetin as the standard. TFC was expressed as mg of quercetin equivalents (QE) g^{-1} dry extract (mg QE g^{-1} DE). All experiments were performed in triplicate.

Determination of total proanthocyanidin content (TPrAC)

The TPrAC was determined using the method suggested by Price *et al*. (Price *et al.*, 1978). A 0.5 mL of supernatant was mixed with 1.5 mL 4% vanilin methanolic solution and 0.75 mL dense HCl. Then, it was incubated at room temperature for 15 minutes. Absorbance was read at 500 nm against blank using UV-Visible spectrophotometer (Cecil. UK.). TPrAC was expressed as mg of catechin equivalents (CA) g^{-1} of dry extract (mg CA g^{-1} DE). All experiments were performed in triplicate.

Determination of total anthocyanin content (TAC)

TAC was measured using a spectrophotometric differential pH method (Rapisarda *et al.*, 2000, Scalzo *et al.*, 2008). Two samples of 1g were treated with 10 mL of buffer solution, pH: 1.0 (125 ml of 0.2 M KCl and 375 ml of 0.2 M HCl) and 10 mL of buffer solution, pH: 4.5

(400 mL of 1M sodiumacetate, 240 mL of 1M HCl and 360 ml of water), respectively. The mixture was homogenized and centrifuged twice at 4℃ at 5000 g for 15 minutes. The supernatant was collected and its absorbance was read at 510 nm. TAC was determined by the following equation:

$$C\ (mg\ CY/g\ DE) = (A_{pH:\ 1.0} - A_{pH4.5}) \times 484.82 \times 1000/24825 \times DF$$

where the term in parentheses is the difference of absorbance at 510 nm between pH 1.0 and 4.5 solution; 484.82 is the molecular mass of cyanidin-3-glucoside; 24825 is its molar absorptivity (ε) at 510 nm in the pH: 1.0 solution; and DF is the dilution factor.

Results were expressed as milligrams of cyanidin-3-glucoside (CY) equivalents per g of dry extract (mg CY g^{-1} DE). All experiments were performed in triplicate.

Measurement of antioxidant activity

DPPH radical scavenging activity assay. The free radical scavenging activity of extracts was evaluated by DPPH method (Brand-Williams *et al.*, 1995). In brief, 0.1 mM solution of DPPH in methanol was prepared and 3 mL of this solution was added to 1 mL of each extracts with different concentrations (100-300 µg mL^{-1}). The mixture was shaken vigorously for a few seconds and incubated at room temperature for 60 minutes. Then the absorbance was measured at 517 nm using a visible spectrophotometer. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity which is calculated using the following equation:

$$\text{Percent (\%) inhibition of DPPH activity} = ([(A_0 - A_1 / (A_0)] \times 100\%$$

where A_0 is the absorbance value of the blank sample or control reaction and A_1 is the absorbance value of the test sample.

A curve of percent inhibition or percent scavenging effect against samples concentrations was plotted and the concentration of the sample required for 50% inhibition was determined. The assay was carried out in triplicate. BHT, Vitamin C, and Vitamin E were used as standard antioxidants. All experiments were performed in triplicate.

Reducing Power Assay (RPA). The RPA of the extracts was determined using assay Yen and Duh (Yen and Duh, 1994). 2.5 mL (concentration of 100-300 µg/mL) of extracts were mixed with 2.5 mL of potassium phosphate buffer (0.2M, pH 6.6) and 2.5 mL of potassium ferricyanide (1 g 100 mL^{-1}). The mixture was incubated at 50℃ for 20 minutes. Trichloroacetic acid (10%) was added to the mixture to stop the reaction. Equal volume of distilled water was added followed by 0.5 mL ferricchloride (0.1 g 100mL^{-1}) ($FeCl_3$). The procedure was carried out in triplicate and was allowed to stand for 30 minutes before measuring the absorbance at 700 nm. BHT, Vitamin C, and Vitamin E were used as standard antioxidants. The percentage of antioxidant activity in reducing power assay of the samples was calculated according to the formula:

$$\text{Antioxidant activity (\%)} = ([(A_1 - A_0/(A_0)] \times 100$$

where A_0, Absorbance of the blank; A_1, absorbance of sample.

All experiments were performed in triplicate.

Statistical analysis

All data were analyzed using MSTAT C version 2.10 software. Analysis of variance (ANOVA) and Duncan's multiple range method (for post-hoc comparisons) were used for the comparisons. Differences were considered significant at $P<0.05$.

Results and Discussion

Total phenolic contents

The TPCs in samples were determined from equation of calibration curve ($y=0.004x + 0.0672$, $R^2= 0.9912$) and expressed as gallic acid equivalent. Results showed that the levels of phenolic compounds in different parts of the ten

selected genotypes of mahaleb were significantly (P<0.01) different from each other. The TPC in the tested genotypes decreased in the following order: bark>leaf>fruit. The highest TPC of bark, leaf, and fruit belonged to genotypes '249' (131.70 mg GA g^{-1} DE), '188' (23.13 mg GA g^{-1} DE), and '271' (1.99 mg GA g^{-1} DE). The lowest content of phenols of bark, leaf, and fruit was recorded for genotypes '101' (5.11 mg GA g^{-1} DE), '249' (7.25 mg GA g^{-1} DE), and '166' (0.66 mg GA g^{-1} DE). It has been reported that the TPC of methanolic extract of mahaleb seedcake is 71.9 ± 0.31 (Mariod *et al.*, 2010), that is lower than that of the bark of genotype '131'. Among the ten genotypes, stem bark of '249' was distinguishable by its high TPC. The difference in the mahaleb genotypes in terms of total phenolic may be due to genetic variations, as all genotypes were at the same age and grown under the same ecological conditions (Fig. 1).

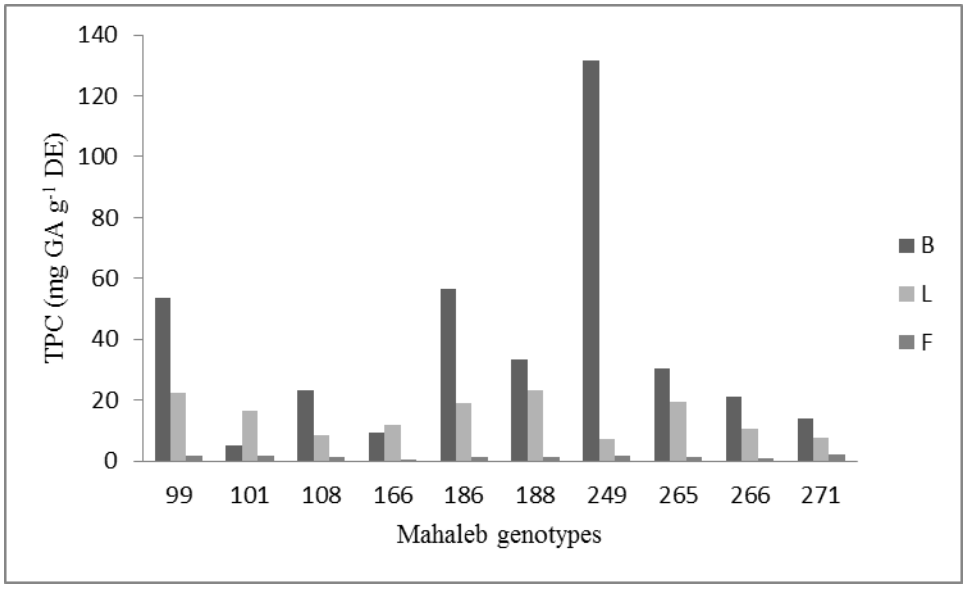

Fig. 1. Total phenolic content of methanolic extract from mahaleb genotypesexpressed in terms of gallic acid equivalent (mg of GA g^{-1} of dry extract)

Total flavonoid contents

Flavonoids are a very diverse group of polyphenolic compounds in plants and possess interesting pharmacological properties, such as anti-proliferative effects and antioxidant activity (Xi *et al.*, 2014). It has been reported that the flavonoids of red sorghums vary among genotypes (Dykes *et al.*, 2009). Our results revealed that the TFC of tested genotypes decreased in the following order: fruit>leaf> bark. The highest TFC of fruit, leaf, and bark belonged to '271' (180.6 mg QE g^{-1}), '99' (22.81 mg QE g^{-1}), and '249' (16.09 mg QE g^{-1}) mahaleb genotypes. The lowest content of flavonoids in bark and leaf samples was recorded for the genotype '101' (1.43 and 6.90 mg QE g^{-1}, respectively), while the genotype '166' had the lowest TFC in fruit (54.06 mg QE g^{-1}). The flavonoid content of mahaleb seed has been previously reported to be 28.5 ± 0.37 mg rutin g^{-1} DW (Oskoueian *et al.*, 2012). Among the tested genotypes, the higher TFC was found in fruits *compared with* leaves and barks. Overall, the results suggested the influence of genotype on the TFC of mahaleb (Fig. 2).

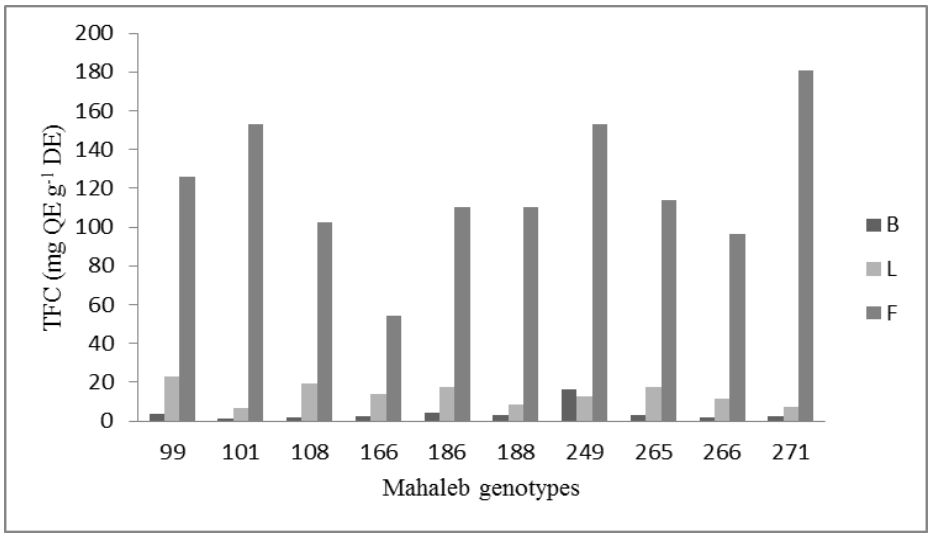

Fig. 2. Total flavonoid content of methanolic extract from mahaleb genotypes expressed in terms of quercetin equivalent (mg of QE g^{-1} of dry extract)

Total proanthocyanidin contents

Proanthocyanidins are the most abundant polyphenols in human diet (Hort *et al.*, 2012). They are oligomers or polymers of polyhydroxy falvan-3-ol units, such as (+)-catechin and (−)-epicatechin, that are linked through an interflavan carbon bond. Several reports have demonstrated pharmacological effects of proanthocyanidins, such as antiinflammatory, antiallergic, antitumor, free radical scavenging, and antioxidant activities (Li *et al.*, 2012). The contents of proanthocyanidin found in mahaleb are shown in Fig. 3. The TPrAC decreased in the following order: fruit>bark>leaf. Higher proanthocyanidin contents were observed in fruit and bark samples of the genotype '249' (25.33 mg CA g^{-1} and 23.62 mg CA g^{-1}, respectively) and in the leaf sample of the genotype '266' (20.75 mg CA g^{-1}). Many studies have shown that the proanthocyanidin level is genetically controlled, and environmental conditions may modify those levels (Scioneaux *et al.*, 2011). Our results showed that TPrAC is affected by mahaleb genotype.

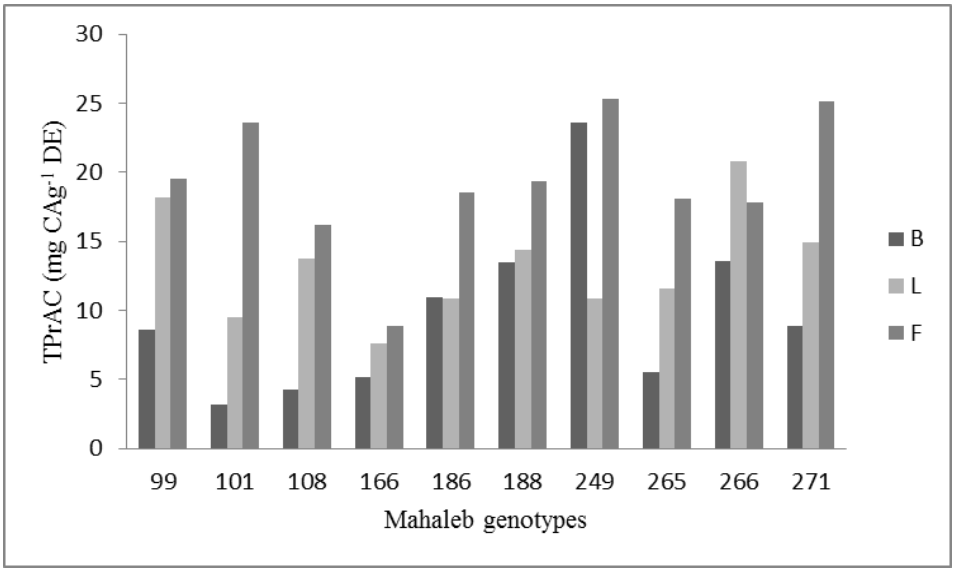

Fig. 3. Total proanthocyanidin content of methanolic extract from mahaleb genotypes expressed in terms of catechin equivalent (mg of CA g^{-1} of dry extract)

Total anthocyanin contents

Anthocyanins are naturally occurring phenolic compounds that give color to the plants, vegetables, and fruits. They protect plants against a variety of oxidants through a number of mechanisms (Kong *et al.*, 2003). Anthocyanins possess known pharmacological activities and are used by humans for therapeutic purposes. TACs of the ten mahaleb fruits are shown in Figure 4. Of the genotypes studied, '271' had the highest TAC (260.81 mg CY g^{-1}) followed by '249' (235.99 mg CY g^{-1}) and '101' (228.60 mg CY g^{-1}). The differences in TACs between these three genotypes and other genotypes were statistically significant ($P<0.05$) (Fig. 4). Reports on black bean have confirmed that genotype affects TAC (Marles *et al.*, 2010). The '166' genotype had the lowest concentration of total anthocyanins (118.64 mg CY g^{-1}). It has been reported that the contents of total anthocyanins of two *Prunus caroliniana* cultivars ranges from 9.3 to 174.3 mg CY 100 g^{-1} of fresh weight (Alasalvar *et al.*, 2005), and also TAC of eight *Prunus caroliniana* genotypes changes from 164.4 to 206.4 mg/100 g fresh weight (Halilova and Ercisli, 2010). In the present study, TACs of the ten mahaleb genotypes ranged from 67.52 to 260.81 mg CY g^{-1} DE. TAC has been estimated to be 6.9-15.1 mg of cyaniding 3-rutinose equivalents of 100 g FW in red grape genotypes, and 34-515 mg of cyaniding 3-glucoside equivalents of 100 g FW in genotypes of blue berries (Moyer *et al.*, 2002). Our results indicated that mahaleb genotypes can serve as a rich source of anthocyanins.

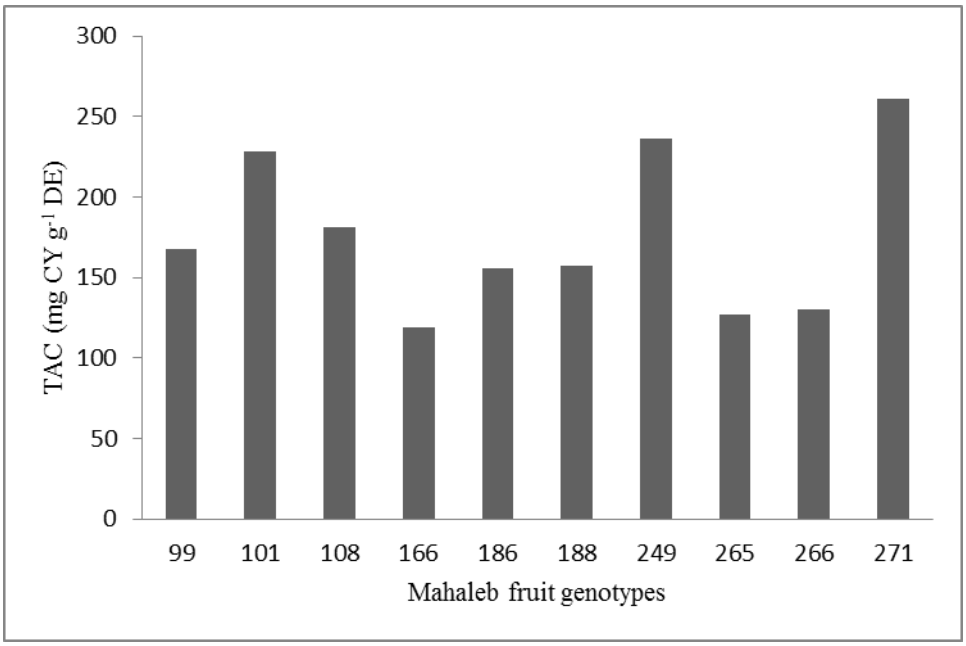

Fig. 4. Total anthocyanin content of methanolic extract from mahaleb genotypes expressed in terms of cyaniding 3-glycoside equivalent (mg of CY g^{-1} of dry extract)

Free radical scavenging activity (DPPH)

The DPPH method has been recommended as an easy and accurate technique to measure the antioxidant activity of fruit and vegetable juices or extracts. The results of DPPH assay are highly reproducible and comparable to the free radical scavenging methods such as ABTS (Wanyo *et al.*, 2014). The extracts and reference antioxidants at the 100 µg ml^{-1} concentration inhibited DPPH significantly ($P<0.05$). The percentage inhibition values

of vitamin C, BHT, and vitamin E were 92.4%, 92.5%, and 95.3%, respectively. Methanolic extracts showed lower activity compared with the reference antioxidant. The radical scavenging activity of mahaleb genotypes are shown in Figure 5. A statistically significant difference was found among the tested genotypes. The DPPH values for investigated extracts varied in a wide range, between 21.9% and 80.9%. The highest antioxidant activity (80.9%) was found in stem bark of the genotype '249', followed by stem bark of '186' (79.5%) and stem bark of '99' (78.02%). A previous study reported lower antioxidant activity of the methanolic extract of mahaleb seed (100 µg ml^{-1}) with the inhibition percentage value of 44.3% (Oskoueian *et al.*, 2012). In the present study, stem bark samples contained the highest TPC that may explain the high antioxidant capacity of the stem bark of mahaleb. Our results are in agreement with previous studies (Wan *et al.*, 2011; Parejo *et al.*, 2002) which reported a correlation between free radical scavenging activity and TPC.

Reducing power assay (RPA)

The reducing powers of various concentrations of extracts and standards (vitamin E, vitamin C, and BHT) are presented in Figures 5 and 6. The reducing powers of positive controls (vitamin C, vitamin E, and BHT), at the concentration of 100 µg ml^{-1}, were 91.6%, 90.9% and 91.6%, respectively, while the values for the methanolic extracts were lower. The extract of the stem bark of the genotype '249' exhibited a higher reducing power (89.30%) that can be attributed to its high phenolic content (131.77 mg g^{-1}). When the genotype '249' was compared with the above-mentioned positive controls, it showed considerable reducing capacity. These results were in accordance with those of the DPPH method, where the stem bark of genotype '249' had the highest antioxidant activity (80.9%).

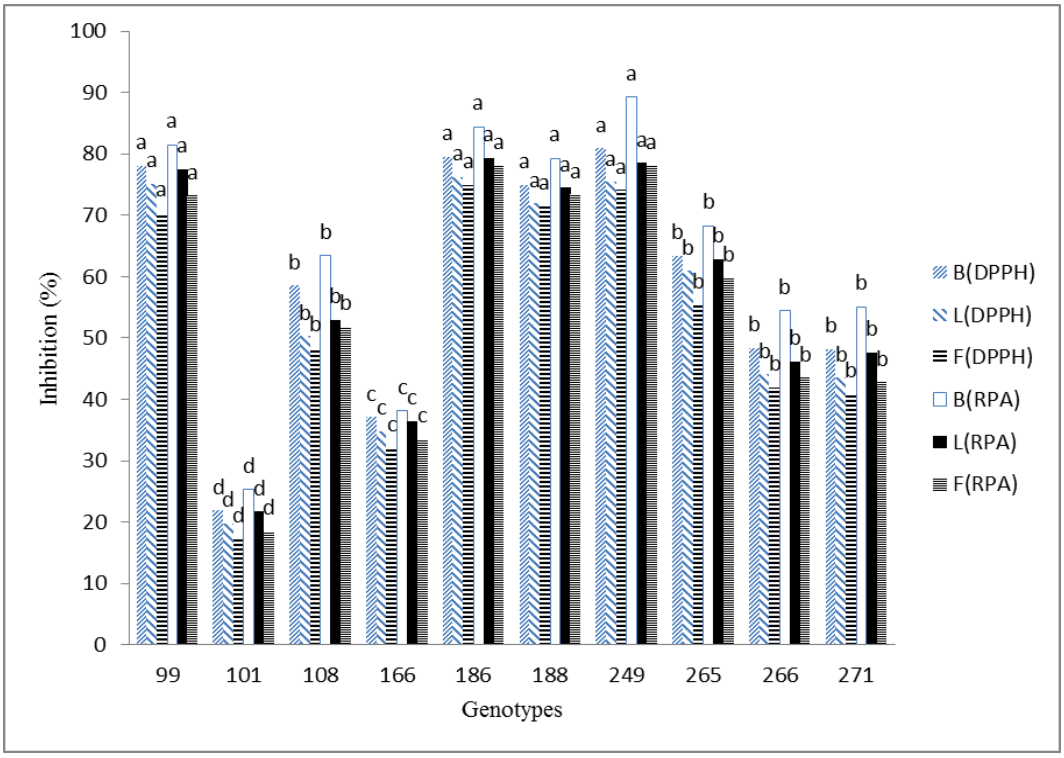

Fig. 5. Total antioxidant activity of bark (B), leaf (L) and fruit (F) in ten mahaleb genotypes measured by DPPH and reducing power assay (RPA)

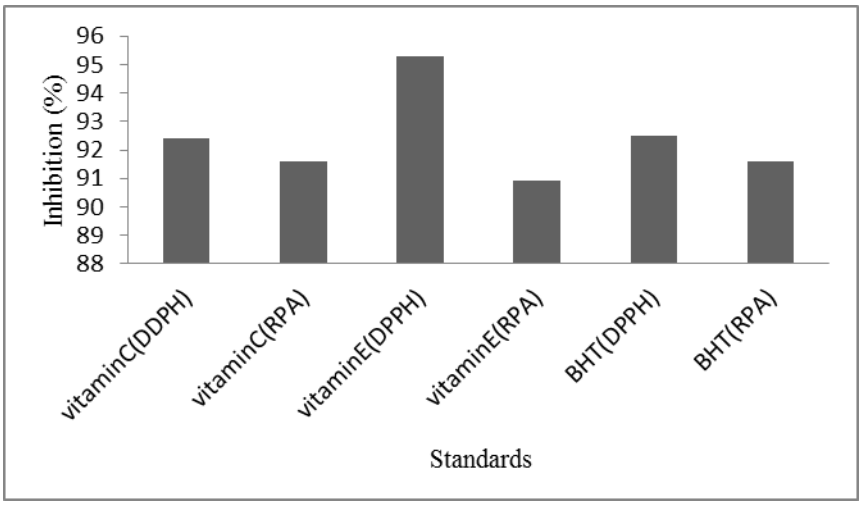

Fig. 6. Antioxidant activity of standard antioxidants measured by DPPH and reducing power assay (RPA)

Our results suggest that, among the 10 tested genotypes, '249' was the richest in terms of total phenols, and it was the most powerful antioxidant. Structurally, phenols comprise an aromatic ring bearing one or more hydroxyl substituents. The antioxidant activity of phenolic is due to their capacity to scavenge free radicals, donate hydrogen atoms or electrons, and chelate metal cations (Medini *et al.*, 2014). The TPC, TFC, and TPrAC of the stem bark of genotype '249' were also higher compared with other tested genotypes. The phenol and flavonoid contents depended on the part of the plant. Our study revealed that the stem bark contains the highest level of phenols, while the fruit had the highest level of flavonoids.

Overall, the present results indicated that the antioxidant activity of mahaleb extracts could be mainly attributed to the reducing and electron donating capacity of the extract rather than a direct free radical scavenging effect. In a complex system such as food, different mechanisms may contribute to oxidative reactions. Therefore, it is important to characterize the extracts by a variety of antioxidant assays (at least two) to obtain reliable results (Ismail *et al.*, 2010; Oskoueian *et al.*, 2011; Armin *et al.*, 2011).The results obtained in our study showed that the highest antioxidant activity was observed in the parts that have the highest phenolic content. Although some studies have shown a close relationship between the TPC and antioxidant capacity of plants (Farasat *et al.*, 2014; Djeridane *et al.*, 2006), others have reported opposite results with poor or no correlation (Wong *et al.*, 2006; Sengul *et al.*, 2009). Therefore, major classes of phytochemical(s) that determine the antioxidant capacity of plant extracts are still a matter of controversy that require elucidation in future studies.

In conclusion, the findings of the present study showed that total phenolic, flavonoid, proanthocyanidin, and anthocyanin contents of mahaleb are affected by genotypes, and provided the first evidence on the contents of these compounds in different plant parts. This information may be of assistance in the production of mahaleb genotypes with maximum levels of desired phenolic and implies the importance of mahaleb as a natural source of bioactive compounds that could be applicable in food, pharmaceutical, and cosmetic industries.

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