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Research Article

Phenolic Contents and Antioxidant Potential of *Crataegus* Fruits Grown in Tunisia as Determined by DPPH, FRAP, and β-Carotene/Linoleic Acid Assay

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Crataegus fruit is one of most important fruits in Tunisian flora. Some fruits of this genus are edible. This study was undertaken in order to examine the benefits of these fruits in human health and their composition of antioxidants including total polyphenol, flavonoids, proanthocyanidins content, and total anthocyanins. The antioxidative properties of the ultrasonic methanolic extract were assessed by different in vitro methods such as the FRAP, DPPH, and β-carotene/linoleic acid assay. We concluded that peel fraction of red fruits possessed relatively high antioxidant activity and might be a rich source of natural antioxidants in comparison with the pulp and seed fruit extract. The results also showed that hawthorn yellow fruit presents lower amounts of phenolic content, absence of anthocyanins, and less antioxidant capacity. Most of peel and seed fractions were stronger than the pulp fractions in antioxidant activity based on their DPPH IC_{50}, FRAP values, and results of β-carotene/linoleic acid. The total phenolic compounds contents were also highly correlated with the DPPH method and the FRAP assay.

1. Introduction

Dietary phenolic compounds have received much attention during the recent years due to their antioxidant and other biological properties imparting possible benefits to human health [1, 2]. Crude extracts of fruits, herbs, and vegetables are rich sources of polyphenols. These compounds include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavones, flavanols, flavanones, isoflavones, anthocyanins, and proanthocyanidins), vitamins, and carotenoids. These bioactive molecules can delay or inhibit the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidative chain reactions [3].

Antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals [4]. In order to receive a reliable picture of antioxidants content in *Crataegus monogyna* and *Crataegus azarolus* fruits extract, total polyphenols, total flavonoids, proantnocyanidins content, and total anthocyanins content were determined quantitatively using spectrophotometer methods. It was also shown that the measure of antioxidant capacity in natural products by only one assay is often not reliable; therefore, in this investigation, we used three complementary assays such as DPPH radical scavenging assay, ferric-reducing/antioxidant power (FRAP) and β-carotene linoleic acid assay to check the antioxidant activity of these fruits.

2. Materials and Methods

2.1. Plant Material. Two series of *Crataegus azarolus* and *Crataegus monogyna* fruits (2 kg) were collected from Jendouba, north of Tunisia, in September 2011. Fruits were immediately transported to the Department of Chemistry of the Faculty of Sciences in Bizerte. Each fruit was separated into three parts such as peel, pulp, and seed. They were lyophilized and kept in desiccators, and the fresh fruit was stored at −4°C until analysis.

2.2. Chemical Reagents. The chemical reagent Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Linoleic acid, BHT, free radical DPPH, and FRAP reagent were from Sigma Co. (St. Louis, MO, USA). Folin-Ciocalteu's reagent and all standard antioxidants were from Fluka Chemie (Buchs, Switzerland). Tween 40 is from Merck. All other chemical reagents used for extract were obtained from Sigma Co. (St. Louis, MO).

2.3. Ultrasound-Assisted Procedures Extraction. The peel, pulp, and seed of red and yellow fruit of *Crataegus* were processed separately; approximately 2 g of lyophilized fruit parts was extracted 3 times by 15 mL of methanol/acidified water HCl 1.5 N (80/20 v/v) during 30 min in an ultrasonic bath (FALC Instruments, Italy) [5]. The extracts were then washed with hexane to remove chlorophyll and other low molecular weight compounds. The extracts were centrifuged, the solvent was evaporated under reduced pressure, and the residue was dissolved in ultrapure water and lyophilized. The crude extract was kept to quantify the total antioxidant contents.

3. Determination of Total Antioxidant Compounds

3.1. Total Phenolic Content. Total phenolic content was quantified using the modified Folin-Ciocalteu's method [6]. Folin-Ciocalteu's reagent/water (750 μL, 1 : 14) mixture was added to a 50 μL sample and the reaction was stopped exactly 3 min after adding 200 μL of 20% Na_2CO_3. The solution was homogenized, vortexed and heated at 100°C for 2 min, and kept in the dark room for 30 min for incubation. Absorbance was read at 760 nm using a UV-Vis spectrophotometer T60U. All assays were carried out at least in duplicate and MeOH was used as blank (50 μL instead of the extract). Methanolic dilutions of gallic acid were used as standard; results were expressed as gallic acid equivalent per gram of lyophilized sample.

3.2. Total Flavonoid Content. Total flavonoid content was measured using the modified colorimetric method of Zhishen et al. [7]. In sealed tubes, 1.5 mL of a 2% methanol solution of $AlCl_3 \cdot 6H_2O$ was added to 0.5 mL of sample and then kept in dark for 10 min. Absorbance was read at 430 nm, methanolic $AlCl_3$ was used as blank, and each measure was made in triplicate. A series of methanolic dilutions of rutin were prepared and assayed; flavonoid amounts in extract were expressed in mg rutin equivalent flavonoid/100 g dry matter [8].

3.3. Proanthocyanidin Content. In sealed tubes, 0.5 mL sample was added to a solution of 0.5 mL MeOH, 6 mL of n-BuOH/concentrated HCl (95 : 5 v/v), and 0.2 mL of a 2% $NH_4Fe\,(SO_4)_2 \cdot 12H_2O$ solution in 2 M HCl. Absorbance was read at 550 nm before and after heating for 40 min at 95°C (each measure in triplicate, blank n-BuOH/HCl mixture). A series of dilutions of cyanidin chloride in n-BuOH/HCl were assayed; proanthocyanidin amounts in extracts were expressed in mg cyanidin/100 g dry matter and were calculated from the following equation [9]:

$$\mathrm{PC} = \left[\frac{(A_{550}\mathrm{sample} - A_{550}\mathrm{control})}{(\varepsilon * L)}\right] * \mathrm{MW} * \mathrm{DF} * 1000, \quad (1)$$

where PC is proanthocyanidin contents expressed in mg cyanidin/100 g dry matter, A_{550}sample is the absorbance of the sample at 550 nm, A_{550}control is the absorbance of the control at 550 nm, ε = 17,360 $L^{-1}\,M^{-1}\,cm^{-1}$ molar absorptivity coefficiency of cyanidin, L is the cell path length (1 cm), MW = molecular weight of cyaniding (287 g mol^{-1}), DF is the dilution factor (g L^{-1}), and 1000 is the factor for conversion from g to mg.

3.4. Total Monomeric Anthocyanins (TMA). Total anthocyanins were quantified using the pH differential method described by Giusti and Wrolstad [10]. This method was based on reversible structural transformations of anthocyanin pigments in different pH solutions using a UV-Vis spectrophotometer (model T60U, PG Instruments). 960 μL of pH 1 (25 mL of 1.49% KCl + 67 mL of 1.7% HCl, pH corrected with HCl) and pH 4.5 (1.64% AcONa, pH corrected with AcOH) buffer solutions were each added to 40 μL of extract. Absorbance was read at 700 and 510 nm against water as blank. Each measure was made in triplicate. The results were expressed in mg cyanidin-3 glucoside/100 g dry matter:

$$\mathrm{TACY} = \left[\frac{\Delta A \times \mathrm{MW} \times \mathrm{DF} \times 1000}{\varepsilon}\right] \times 0.1,$$
$$\Delta A = \left[A_{512\,\mathrm{nm}} - A_{700\,\mathrm{nm}}\right]_{\mathrm{pH\ 1.0}} - \left[A_{512\,\mathrm{nm}} - A_{700\,\mathrm{nm}}\right]_{\mathrm{pH\ 4.5}}, \quad (2)$$

where TACY is the total anthocyanins expressed as mg cyanidin-3-glucoside/100 g DF fruit, MW is the molecular weight of cyaniding-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor, ε is the molar absorbance coefficient of cyaniding-3-glucoside (26,900 $M^{-1}\,cm^{-1}$), and 0.1 is the conversion factor per 1000 g to 100 g basis.

4. Determination of Antioxidant Activity

4.1. DPPH Radical Scavenging Assay. Radical scavenging activity of *Crataegus* fruits extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically. The method first introduced by Blois [11], developed by Brand-Williams et al. [12], and criticized by Molyneux [13] was employed. The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. Briefly, 100 μL of methanol hawthorn fruits extract was added to 1.9 mL of 410–3 mM of DPPH in methanol up to completing 2 mL. The free radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm every 2 min until the reaction reached its state. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve. The inhibition activity I(%) was calculated as follows:

$$I\,(\%) = 100 \times \frac{(A_0 - A_1)}{A_0}, \tag{3}$$

where A_0 is the absorbance of the control sample and A_1 is the absorbance of the test compound.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

4.2. Ferric-Reducing/Antioxidant Power (FRAP). The procedure of FRAP assay was according to Benzie and Strain [14]. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in presence of antioxidants. Briefly, the FRAP reagent contained 2.5 mL of 10 mmol L^{-1} TPTZ (2,4,6-tripyridyl-*s*-triazine, Sigma) solution in 40 mmol L^{-1} HCl plus 2.5 mL of 20 mmol L^{-1} $FeCl_3$ and 25 mL of 0.3 mol L^{-1} acetate buffer, pH 3.6, and was prepared freshly and warmed at 37°C. Aliquots of 40 μL sample supernatant were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min in a microplate (PowerWave XS, BioTek). The 1 mmol L^{-1} $FeSO_4$ was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol L^{-1} $FeSO_4$. Adequate dilution was needed if the FRAP value measured was over the linear range of standard curve.

4.3. β-Carotene/Linoleic Acid Assay. The antioxidant activity of extracts was evaluated using β-carotene/linoleic acid system according to the modified literature procedure [15, 16]. A stock solution of β-carotene/linoleic acid (Sigma-Aldrich) was prepared as follows: first, 0.5 mg of β-carotene was dissolved in 1 mL of chloroform (HPLC grade); then 25 μL of linoleic acid and 200 mg of Tween 40 (Merck) were added. The chloroform was subsequently evaporated using a vacuum evaporator (Buchi, Flawil, Switzerland). Then, 100 mL of distilled water saturated with oxygen (30 min at 100 mL/min) was added with vigorous shaking. Aliquots (2.5 mL) of this reaction mixture were transferred to test tubes, and 350 μL portions of the extracts (2 g/L in ethanol) were added before incubating for 48 h at room temperature. The same procedure was repeated with BHT at the same concentration and a blank containing only 350 μL of ethanol. After the incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidant capacities of the samples were compared with those of BHT and of the blank.

5. Results and Discussion

5.1. Total Phenolic Contents. The level of phenolic compounds in methanolic aqua acidified extracts measured according to the Folin-Ciocalteu method of peel, pulp, and seed of *Crataegus* varieties is presented in Table 1. All parts of red and yellow *Crataegus* varieties were a significant source of polyphenols; however, the total amount varied significantly between 45.7 and 123.35 mg gallic acid/100 g DW in the red variety and from 36.3 to 71.24 mg gallic acid/100 g DW in yellow fruit. The red peel had the highest amount of total polyphenols (123.35 mg gallic acid/100 g DW) whereas seed of yellow fruit had the lowest amount. Referring to Table 1, in both varieties, total flavonoid contents in peel extract were more than in pulp, followed by seed. Comparing the varieties, it was found that all different parts of *Crataegus monogyna* had higher contents of flavonoids (198.53 mg eq. rutin/100 g DW) in peel followed by (160.35 mg eq. rutin/100 g DW) in pulp and (96.01 mg eq. rutin/100 g DW) in seed; however, peel, pulp, and seed of *Crataegus azarolus* had the lowest TF (155.40, 60.45, and 14.71 mg eq. rutin/100 g DW, resp.). Findings also established the main source of total phenolics and total flavonoids in *Crataegus monogyna* to be the peel, which were about 123.35 mg gallic acid/100 g DW and 198.53 mg eq. rutin/100 g DW. The results of the present study indicated consistently the lowest values of total anthocyanins contents in the both varieties when compared to the other bioactive compounds. The red fruit contained higher amounts (5.85 mg eq. cyanidin/100 g DW) in peel and (0.31 mg eq. cyanidin/100 g DW) in fruit; however, yellow fruits are poured in anthocyanins. Proanthocyanidins or condensed tannins are ubiquitous and present as the second most abundant natural phenolics. The proanthocyanidins have been suggested to contribute to the phenomenon called health promoting effects, such as antioxidant, anticarcinogenic, and anti-inflammatory effects [17, 18]. According to Table 1, flavon-3-ols are the most abundant compounds present in *Crataegus* fruits; they represent more than half of the other compounds. Total proanthocyanidins content is most abundant in the red peel (873.58 mg eq. cyanidin/100 g DW), and a lower level was found in the yellow fruits (97.06 mg eq. cyanidin/100 g DW). We show that total antioxidants contents are influenced by the species of *Crataegus* and the different parts of fruit in the same variety, both peels were the rich source of antioxidant compounds.

5.2. Antioxidant Activities. In the present study, the antioxidant activities of *Crataegus* determined by free radical scavenging activity (DPPH) assay method indicated a steady

Table 1: Phenolics contents in different crude part extracts of red and yellow *Crataegus*.

		Source of extract		
	Variety	Peel	Pulp	Seed
Total phenolics[a]	*C. monogyna*	123.35 ± 0.02	122.26 ± 0.16	45.72 ± 0.04
	C. azarolus	71.24 ± 0.01	60.89 ± 0.04	36.03 ± 0.02
Total flavonoids[b]	*C. monogyna*	198.53 ± 0.11	160.35 ± 0.1	96.01 ± 0.01
	C. azarolus	155.40 ± 0.23	60.45 ± 0.06	14.71 ± 0.02
Proanthocyanidins[c]	*C. monogyna*	873.58 ± 0.33	507.31 ± 0.32	399.68 ± 0.29
	C. azarolus	352.91 ± 0.29	291.16 ± 0.19	97.06 ± 0.21
Total anthocyanins[d]	*C. monogyna*	5.58 ± 0.05	0.31 ± 0.03	0
	C. azarolus	0	0	0

[a]Total phenol: Folin-Ciocalteu, in mg eq. gallic acid/100 g DW.
[b]Flavonoids: $AlCl_3$ method, in mg eq. rutin/100 g DW.
[c]Anthocyanins: direct colorimetry in mg eq. cyanidin-3-O-glucoside/100 g DW.
[d]Procyanidins: butanol-HCl methods, in mg eq. cyanidins/100 g DW.

Table 2: Free radical scavenging capacity, FRAP assay, and standard antioxidants of *Crataegus* fruit extract.

		DPPH IC_{50} (μg/mL)	FRAP (mM Trolox)	FRAP (mM AA)
C. monogyna	Peel	750	8.88	9.12
	Pulp	720	5.44	5.68
	Seed	540	5.71	5.95
C. azarolus	Peel	780	6.89	7.13
	Pulp	560	4.40	4.64
	Seed	240	6.15	6.39
Control	BHT	820	—	—

increase in the scavenging activity of free radicals in all extracts and standard range between 240 and 800 μg/mL (Table 2). It was observed that the ability of test materials (pure antioxidants and fruits extracts) to scavenge DPPH was assessed on the bases of their IC_{50} values, defined above as the concentration of test material to decrease the absorbance at 515 nm (or concentration) of DPPH solution to half of its initial value. These IC_{50} values of *Crataegus* fruit extract are given in Table 2. It can be seen that seed from yellow variety shows higher IC_{50} value (780 μg/mL) than red peel (750 μg/mL). This result can be attributed to the higher phenolic content of the peel and pulp. The higher DPPH radical scavenging activity is associated with a lower IC_{50}. FRAP values of peel, pulp, and seed fractions of *Crataegus* fruits are also summarized in Table 2. The reducing ability of different parts of red *Crataegus* fruits extracts is expressed, respectively, in mM Trolox equivalent and mM ascorbic acid equivalent: they ranged from 5.44–8.88 mM Trolox/100 g DW to 5.68–9.12 mM ascorbic acid/100 g DW. These values are more important than the reducing ability of yellow fruits; however, they ranged from 4.64 to 7.13 mM Trolox/100 g DW and, 4.4 to 6.89 mM A A/100 g DW. The decreasing order efficiency in FRAP system is as follows: peel > pulp > seed in both varieties of *Crataegus* fruits extract. These results agreed with the DPPH values. The basis of β-carotene/linoleic acid assay is discoloration of β-carotene in reaction with linoleic acid free radical. That radical is formed at elevated temperature upon removal of hydrogen atom located between two double bonds of linoleic acid [16].

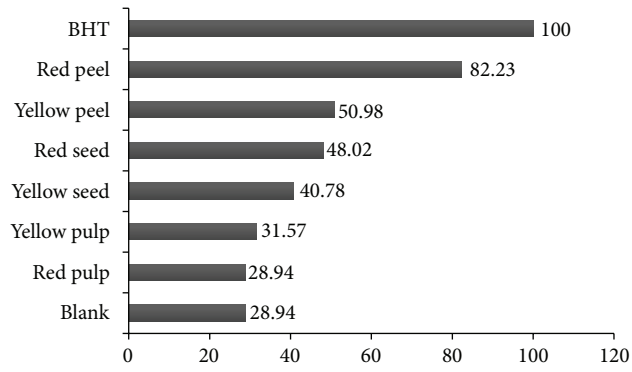

Figure 1: Relative antioxidant activity of *Crataegus* extracts and positive control (BHT) in β-carotene/linoleic acid assay.

The consequence is the loss of conjugation and, accordingly, a decrease in absorbance at 470 nm. Antioxidants present in solution can prevent the degradation of β-carotene by reacting with the linoleate free radical or any other radical formed in the solution.

The reduction in absorbance of β-carotene-linoleate emulsion in presence of the extracts is shown in Figure 1. Relative antioxidant activity of *Crataegus* extracts increased with the species and the parts of fruits. In the β-carotene/linoleic acid model system, we could conclude that results were consistent with the data obtained from DPPH test and FRAP assay. Peel extract of red fruit showed markedly relative

Table 3: Linear correlation of Trolox equivalent antioxidant capacity (TEAC) versus the total phenolic content of *crataegus* fruit extract.

Variety	Phenolic contents	Correlation coefficients	
		$TEAC_{DPPH}$	$TEAC_{FRAP}$
C. monogyna	TP	$y = 2.5328x + 424.06$ $R^2 = 0.9856$	$y = 0.0245x + 4.2986$ $R^2 = 0.3249$
	TF	$y = 2.1267x + 347.53$ $R^2 = 0.9412$	$y = 0.0305x + 2.054$ $R^2 = 0.6819$
	PC	$y = 0.3507x + 461.84$ $R^2 = 0.5885$	$y = 0.0076x + 2.1564$ $R^2 = 0.9783$
C. azarolus	TP	$y = 14.884x - 307.64$ $R^2 = 0.983$	$y = 0.0707x + 1.8527$ $R^2 = 1$
	TF	$y = 3.6082x + 249.36$ $R^2 = 0.9094$	$y = 0.0162x + 4.5682$ $R^2 = 0.8267$
	PC	$y = 1.9996x + 32.668$ $R^2 = 0.9666$	$y = 0.0096x + 3.4515$ $R^2 = 0.9964$

TP: Total polyphenols.
TF: Total flavonoids.
PC: Proanthocyanidin content.

antioxidant activity (82.23%), as did the peel extract of yellow fruit (50.98%). These results implied that the potential antioxidant capabilities in *Crataegus monogyna* were attributed to the phenolic compounds in this species compared with *Crataegus azarolus* species. Pulp of yellow fruit showed the weakest activity potential in this test system (28.24%) (Table 3).

5.3. Relationships amongst Different Antioxidants. Free radical scavenging of phenolic compounds is an important property underlying their various biological and pharmacological activities. Recently, Awika et al. [19] found positive correlations between the determinations of phenolic antioxidant using the oxygen radical absorbance capacitys (ORAC), ABTS, and DPPH assays. Results revealed that polyphenols from *Crataegus* fruit extract had high antioxidant activities (Table 1). The total phenolic compounds contents were also highly correlated with the antioxidant activities with the DPPH method and the FRAP assay. Data of the correlations (R^2) summarized in Table 2 with total polyphenols content in red fruits were 0.98, 0.94, and 0.58, respectively. DPPH was also highly correlated with TP, TF, and PC of red fruit; the data show 0.98, 0.90, and 0.96, respectively. The correlation between Trolox equivalent antioxidant capacity (TEAC) (Y) and total phenolic contents (X) of *Crataegus* monogyna had a coefficient (R^2) varied from 0.32 to 0.97 while the correlation coefficient of *Crataegus* azarolus are 1, 0.99 and 0.82 (Table 1). This result suggests that higher percentage of the antioxidant capacity of Tunisia *Crataegus* accessions results from the contribution of phenolic compounds. Also, it can be concluded that antioxidant activity of plant extracts is not limited to phenolics content but also comes from the presence of other antioxidant secondary metabolites, such as flavonoids, proanthocyanidins, and anthocyanins. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

They may also have a metal chelating potential [20]. Besides flavoring purposes, spices and herbs have also been used for their medical or antiseptic properties because of their richness in bioactive molecules and consequently their benefits for human health [21].

6. Conclusion

Comparison of phenolic contents and antioxidant activities of methanol extracts of *Crataegus azarolus* and *Crataegus monogyna* fruits cultivated in Tunisia shows the presence of total phenols, proanthocyanidins, and flavonoids with some difference. Anthocyanins are present only in red fruit. This richness in antioxidants contributes to the antioxidative effect. A linear correlation of Trolox equivalent antioxidant capacity (TEAC) versus the total phenolic content of *Crataegus* was established. The richest composition in antioxidant compounds and the higher antioxidant capacity activity of Crataegus can improve the use of these fruits in various fields such as agroalimentary and pharmaceutical industry.

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