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Comparative Analysis of Tunisian Wild *Crataegus azarolus* (Yellow Azarole) and *Crataegus monogyna* (Red Azarole) Leaf, Fruit, and Traditionally Derived Syrup: Phenolic Profiles and Antioxidant and Antimicrobial Activities of the Aqueous-Acetone Extracts

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ABSTRACT: Quantitative and qualitative analyses of the yellow and red azarole phenolic extracts prepared from leaf, fruit peel/pulp, and syrup were comparatively investigated. The yellow azarole was found significantly richer in polyphenols than the red-fruit species. Hyperoside was the main phenolic in both yellow and red azarole leaves and only in yellow fruits, whereas procyanidin B2 was the major compound in red fruits. Yellow azarole leaf and fruit peel extracts exhibited the strongest antioxidant activities using DPPH (≈168 and 79 μmol TEAC/g fw, respectively) and FRAP (≈378 and 161 μmol Fe^{2+}/g fw, respectively) assays. The highest antibacterial activities were recorded for the yellow azarole leaf and fruit peel extracts, especially against *Staphylococcus aureus* and *Streptococcus faecalis*. The low phenolic content of the syrups contrasted with their significant antioxidant and antimicrobial potentials, which were correlated to their hydroxymethylfurfural (HMF) (furan derivative amounts) content.

KEYWORDS: *antimicrobial, antioxidant, C. azarolus, C. monogyna, LC-MS, polyphenols, RP-HPLC-DAD*

INTRODUCTION

Throughout history, man has used various natural materials to cure various diseases. During the past decades, bioactive compounds commonly found in fruits, vegetables, and herbs have been shown to exhibit health benefits, with anticarcinogenic, antimutagenic, and atherosclerosis inhibitory activities.[1,2] Many studies highlighted the implication of the excessive production of reactive oxygen species (superoxide anion, O_2^-; hydrogen peroxide, H_2O_2; and hypochlorous acid, HOCl) by the organism in the development of several acute and chronic pathological phenomena, including inflammation, neurodegeneration, and Parkinson's and Alzheimer's diseases.[3] Interestingly, many phytochemicals can act as antioxidants to cure or prevent these disorders.[4] Scientific papers continually emphasize the free radical scavenging potential and antioxidant properties of polyphenols as bioactive compounds.[5] These substances, contributing to color and sensory properties, such as bitterness and astringency, in fruits, vegetables, seeds, and flowers,[6] are an integral part of the human diet and are supposed to have health-promoting effects.[7]

Azarole species (*Crataegus* spp.) have recently attracted increasing attention in the field of food, nutraceuticals, and medicine because of their widely reported health benefits, for example, the risk reduction of cardiovascular diseases.[8] These species are widespread in the moderate zones of the northern hemisphere, including the Mediterranean region.[9] Different classes of active phenolics were identified in azarole species[10−12] and were involved in therapeutical virtues.[8,13] In vivo experiments showed that *Crataegus* fruit extract inhibited the oxidation of human low-density lipoprotein (LDL).[11] Considerable effects of this extract on the cardiovascular system have been also reported, including treatment of congestive heart failure and significant reduction of mortality after ischemia perfusion in animals.[14,15] Besides medicinal purposes, azarole fruits are consumed as foodstuffs and can be consumed fresh, dried, or canned fruit.[9,16,17] The Chinese, at the end of the month of September, harvest the azarole and transform it into juice, wine, jam, marmalade, and various sweet foods.[18,19]

In the Tunisian flora, the genus *Crataegus*, locally known as "Zaarour", is represented by two species: the red-fruit *Crataegus monogyna* and the yellow-fruit *Crataegus azarolus*. Traditionally, the azarole syrup prepared from the fruit is considered as a seasonal food byproduct widely consumed all the year because it has low free water content and is, thus, appropriate for conservation for many months and even years. The local traditional process of azarole syrup preparation consists of mixing the entire fruit with water (ratio 1:1 w/v) and boiling for about 90 min. Following filtration through a cloth, the juice is collected and, in the same way, the remaining press cake is

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then re-extracted three times with water. Sucrose is added (1:1 w/v) to the combined collected filtered juice before boiling down until reaching ≈70−75 °Brix. Herein, the objective of the study is to identify and quantify the total phenol content, phenolic compounds profile (LC-ESI-MS) and related antiradical (DPPH), and the capacity to reduce Fe(III) at neutral pH (FRAP) as well as antimicrobial activities of Tunisian wild *C. monogyna* and *C. azarolus* fruit peel/pulp and leaves and local syrup byproduct.

MATERIAL AND METHODS

Chemicals. 2,2-Diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), Folin−Ciocalteu reagent, phosphoric acid, and all solvents used, of the highest available purity, were obtained from Sigma-Aldrich, Fluka (Milan, Italy). Gallic acid, sodium carbonate, $FeSO_4$, and $FeCl_3$ were supplied by Carlo Erba (Milan, Italy). All of the chemicals used in this study were of analytical grade. Ultrapure water (18 mΩ) was obtained with an Elix/Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy).

Biological Material. Fruits and leaves were harvested and collected in October−November (2011) from native trees of *C. azarolus* and *C. monogyna* in northwestern Tunisia (Jendouba and Bou-Salem, 36° 36′ 35″ N, 8° 58′ 17″ E). Species were identified at the National Agricultural Research Institute of Tunisia (INRAT). Fruit samples were rapidly hand peeled, and then separated pulp and peel parts were cut into thin slices prior to freezing at −20 °C. The syrup and sampled leaves were conserved at −20 °C until used.

Polyphenolic Extraction. The extraction was carried out using the method developed by Fattouch et al.[20] with minor modifications. Each 1 g of fruit, leaves, and syrup was mixed with 10 mL of cold (−20 °C) 70% aqueous-acetone. The homogenate was sonicated for 10 min and then centrifuged at 8000*g* for 15 min at room temperature. Supernatants were concentrated using a rotary evaporator (40 °C) under vacuum to a final volume of 3 mL. To prevent oxidation of the polyphenols, extraction was achieved rapidly, and the final extract solutions were stored at −20 °C until analyzed.

Total Polyphenols. The total phenol content (TPC) was measured through spectrophotometric determination with the Folin−Ciocalteu method as reported by Tuberoso et al.[21] The absorbance was read at 725 nm on a 10 mm quartz cuvette using a Varian Cary 50 scan spectrophotometer (Varian, Leinì, TO, Italy) against a blank. The TPC results, expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight (fw), were obtained using a calibration curve of a freshly prepared gallic acid standard solution (10−200 mg/L). All of the measurements were taken in triplicate, and means and standard deviation values were calculated.

Antiradical Activity (DPPH Test). This assay is based on the ability of the antioxidant to scavenge the radical cation DPPH. The method described by Tuberoso et al.[21] was used starting from 50 μL of water-diluted extract. The sample was mixed with 2 mL of DPPH 0.04 mM in methanol. Antiradical activity was measured as the relative decrease in absorbance at 517 nm as the reaction between DPPH and antioxidant. Readings were carried out with a Cary 50 spectrophotometer (Varian, Milan, Italy) using a 10 mm plastic cuvette. A calibration curve in the range 0.02−0.8 mM was used for Trolox, and data were expressed as Trolox equivalent antioxidant capacity (TEAC, μmol/g fw).

Total Antioxidant Activity (FRAP Test). The ferric reducing antioxidant assay (FRAP) is based on the reduction, at low pH, of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to the ferrous complex followed by a spectrophotometric analysis.[21] The reagent was prepared by mixing 10 mM TPTZ with 20 mM ferric chloride in acetate buffer (pH 3.6). Twenty microliters of diluted extracts (with ultrapure water) was dissolved in 2 mL of FRAP reagent. The quantitative analysis was done using the external standard method (ferrous sulfate, 0.1−2 mmol), correlating the absorbance (λ = 593 nm) with the concentration. The results were expressed as micromoles of Fe^{2+} per gram of fw.

Antimicrobial Activity. Tests were performed on six bacterial reference strains: *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (CIP 106510), *Streptococcus faecalis* (ATCC 7830), *Pseudomonas aerugenosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and *Salmonella typhimurium* (NRRLB 4420). The antimicrobial tests were carried out using the well and disk diffusion methods as previously described by Fattouch et al.[20] The bacterial suspension in potato count broth (PCB), adjusted to 0.5 McFarland turbidity and evaluated using a serial 10-fold dilution method, was spread-plated on count agar medium (PCA) to give a population of 10^8 colony-forming units (cfu) per plate. The concentration of the suspension was standardized by adjusting the optical density to 0.1 at 600 nm wavelength using a Shimadzu UV−vis spectrophotometer (Columbia, MD, USA). Wells of 6 mm diameter were made on the inoculated agar surface and loaded with 100 μL of azarole extracts or standard antibiotics (ampicillin and oxytetracyclin). The inoculated plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone around the well using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates), and the average was considered. Inhibited microorganisms were then tested using the broth microdilution method[22] to determine the minimum inhibitory and bactericidal concentrations (MIC and MBC, respectively). A 100 μL amount of the diluted working extract and 100 μL of the bacterial suspensions were added into the microwells. The plates were incubated aerobically at 37 °C for 24 h. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria. Bacterial growth was revealed by the presence of turbidity and a pellet on the well bottom. To confirm MIC and establish MBC, 25 μL of broth was homogenically removed from each well and inoculated on new PCA plates. After overnight incubation at 37 °C, the number of surviving organisms was determined; MBC was determined when 99.9% of bacteria were dead.

RP-HPLC-DAD Analysis. Extracts were detected and quantified using an HPLC-DAD method as described in Tuberoso et al.[23] A Varian system ProStar HPLC fitted with a Thermo Separation diode array detector Spectro System UV 6000 lp (Thermo Separation, San Jose, CA, USA) set at 280, 350, and 520 nm was employed. Separation was obtained with a Gemini C18 column (150 × 4.60 mm, 3 μm, Phenomenex, Casalecchio di Reno, BO, Italy) using 0.22 M phosphoric acid (solvent A) and acetonitrile (solvent B) as mobile phase at a constant flow rate of 1 mL/min. The gradient (v/v) was generated by keeping 80% of solvent A for 20 min, then decreasing to 60% in 20 min and to 10% in 10 min, and remaining at this concentration for 5 min. Before each injection, the system was stabilized for 10 min with the initial A/B ratio (80:20, v/v). Chromatograms and spectra were elaborated with a ChromQuest V. 2.51 data system (ThermoQuest, Rodano, Milan, Italy). Stock solutions of standard polyphenols were prepared in methanol, and the working solutions were prepared in ultrapure water. Calibration curves were built with the method of external standard, correlating the area of the peaks with the concentration. Chlorogenic acid, procyanidin B2, delphinidin 3-*O*-glucoside, epicatechin, vitexin, rutin, hyperoside, and isoquercitrin standard solutions were prepared in methanol and working standard solutions, in ultrapure water. The identification of each phenolic compound was based on a combination of retention time and spectral matching. Quantification of individual phenolics was achieved from the areas of their peaks by comparison with calibration curves obtained with reference compound solutions of phenolics. The polyphenol concentrations in the analyzed fruits are expressed in milligrams per 100 g fw.

LC-Electrospray Ionization (ESI)-MS. The LC-ESI-MS system consisted of an Agilent LC 1100 series (Agilent Technologies, Inc., Santa Clara, CA, USA) controlled by Chemstation software. The HPLC instrument was coupled to an Esquire 3000p (Bruker Daltonics, Germany) mass spectrometer equipped with an ESI source and ion trap mass analyzer. The ESI was operated in the positive mode with ESI source probe at 250 °C, CDL at 250 °C, block at 240 °C,

flow gas (N_2) at 4.5 L/min, probe voltage at 4.5 kV, fragmentor voltage at 20 V, and a nominal mass range up to m/z 800.

Statistical Analyses. GraphPad InStat software (GraphPad software, San Diego, CA, USA) was used to calculate the means and standard deviations of three independent experiments involving triplicate analyses for each sample/condition. Evaluation of statistical significance of differences was performed by one-way analysis of variance (one-way ANOVA). Differences of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Determination of Phenolic Compounds. The TPCs of the aqueous-acetone extracts prepared from the two *Crataegus* species were measured according to the Folin−Ciocalteu colorimetric method, and data are shown in Table 1. TPCs ranged from 119 to 4118 mg/100 g fw, with higher amounts found with the yellow fruit *C. azarolus* extracts in comparison to their corresponding amounts obtained from the red fruit *C. monogyna*. Whereas fruit pulp extracts contained the lowest polyphenolic amounts, the highest TPCs were recorded for leaf extracts of both *C. azarolus* and *C. monogyna*. For the same fruit, the polyphenolic contents of peel extracts were found 6.4−6.6 times greater than those of pulps.

Table 1. Total Phenols and Free Radical Scavenging and Antioxidant Activities of Yellow and Red Azarole Extracts[a]

	total phenols[b] (mg GAE/100 g fw)	TEAC[c] (μmol/g fw)	FRAP[d] (μmol Fe^{2+}/g fw)
	Yellow Azarole		
A1	2023.21 ± 47.05	79.76 ± 2.95	161.45 ± 3.83
A2	305.44 ± 2.91	15.58 ± 0.90	33.75 ± 3.14
A3	4006.27 ± 112.17	168.18 ± 0.56	378.07 ± 6.70
A4	189.18 ± 70.22	5.27 ± 1.00	18.07 ± 2.62
	Red Azarole		
B1	1461.35 ± 175.40	74.12 ± 1.40	149.67 ± 5.11
B2	227.56 ± 29.27	10.81 ± 1.60	29.23 ± 4.02
B3	2683. 85 ± 106.19	166.50 ± 2.44	365.32 ± 20.25
B4	263.41 ± 21.05	5.52 ± 0.22	37.30 ± 9.72

[a]Fruit peel (A1, B1), pulp (A2, B2), leaves (A3, B3), and syrups (A4, B4). Data are expressed as mean ± SD of three measurements ($P < 0.05$). [b]Total phenolics using Folin−Ciocalteu. [c]Free radical scavenging activity using DPPH method. [d]Ferric reducing-antioxidant assay.

Obtained data roughly divide our materials into three classes: (1) high phenolic content, represented by leaves; (2) medium content, represented by fruit peel; and (3) low content, represented by fruit pulp. This result confirms that the external parts of the plant material are richer in polyphenolic compounds than the internal parts.[15,17] Azarole syrup TPC was estimated to 189.18 ± 70.2 and 263.41 ± 21.05 mg/100 g fruit syrup of *C. azarolus* and *C. monogyna*, respectively. It is known that, using the Folin−Ciocalteu colorimetric method, the levels of total phenols in the extracts are not absolute measurements of the real amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. Nevertheless, this estimative method allowed us to obtain preliminary quantitative data prior to chromatographic analysis, which could be helpful in choosing the appropriate extract dilution to be injected in HPLC.

HPLC-DAD and HPLC-MS Analyses. The fractionation of the different extracts by RP-HPLC coupled with diode array detection (DAD) followed by ESI-MS analysis allowed the identification of the main phenolic compounds (Table 2) by comparison with authentic standards. Chromatograms at 280 nm are shown in Figure 1. Hydroxycinnamic acid, flavonoids, and anthocyanins were quantified and are shown in Table 2. Ten of the obtained peaks matched the reference compounds used in this work and were attributed to a chlorogenic acid derivative, chlorogenic acid, procyanidin B2, epicatechin, vitexin, rutin, hyperoside (quercetin 3-*O*-galactoside), isoquercitrine (quercetin 3-*O*-glucoside), delphinidin 3-*O*-glucoside, and hydroxymethylfurfural (HMF). Mass chromatograms of the protonated ions ($[M - H]^+$) at m/z ratios was found similar to those obtained with authentic standards. In addition to compound identification, HPLC-DAD allowed their quantitative analyses. The total phenolic contents, calculated from the sum of the determined individual compounds concentrations, were significantly ($p < 0.05$) distinct among the different extracts.

Some of the obtained RP-HPLC data obtained herein (Figure 1) were similar to the typical chromatograms obtained in a previous preliminary work carried out on fruits and leaves of the yellow-fruit azarole. [22] With regard to the red-fruit species, the results obtained for the fruit polyphenols in the present work are comparable to previous results of Urbonavicuite et al.[24] Table 2 presents that the highest contents are found in the leaf extracts of *C. azarolus* and *C. monogyna*. Chromatographic quantitative analysis showed the same trends as found with Folin−Ciocalteu methods. When the corresponding parts were compared, the yellow-fruit species was richer in phenolics than the red-fruit one. Only *C. monogyna* fruit pulp and peel parts were characterized by the presence of the anthocyanin compound represented by delphinidin 3-*O*-glucoside (Table 2). HMF was the main compound found in the studied syrups of *C. azarolus* and *C. monogyna* reaching, respectively, 46.29 ± 0.42 and 30.44 ± 1.53 mg/100 g.

The purity of the observed peaks, as determined using DAD, generally reached 100%. It is expected to find similar phenolic compounds in the corresponding extracts from both azarole species because they belong to the same botanical family. Comparison among the different parts of the plant showed that the total phenolic content decreased from leaves to peel, then pulp and syrup. The richness of the azarole leaves in polyphenolic compounds was also reported by Ding et al.[25] Among flavonols detected in leaves, hyperoside (quercetin 3-*O*-galactoside) was the major compound, representing 34.42 and 30.51% of total phenolics in yellow- and red-fruit species, respectively, followed by procyanidin B2 (24.9 and 24.88%, respectively). Hydroxycinnamic acids were represented by the chlorogenic acid with low contents recorded in azarole leaves (11.89 and 3.65% for *C. azarolus* and *C. monogyna*, respectively). The high phenolic content of azarole leaves was expected because these compounds may have an important role of protection of fruits and cells (membranes, chlorophylls, and other fragile organelles) against damages caused by UV radiation. Traditionally, *Crataegus* leaves are used as a poultice for boils, sores, and ulcers due to the bioactivities of phenolic compounds present in the leaves.[25]

In the peel and pulp of yellow azarole fruit, hyperoside was the major compound, representing 48.34 and 27.86% of total phenolics, respectively, whereas in red azarole fruit peel and pulp, procyanidin B2 was the predominant compound, representing 27.6 and 33.18% of total phenols, respectively.

Table 2. RP-HPLC-DAD and LC-MS Analyses of Main Phenolics in the Yellow and Red Azarole Leaf, Fruit, and Syrup Aqueous-Acetone Extracts

					concentration (mg phenolic/100 g fw)							
					yellow azarole (*Crataegus azarolus*)				red azarole (*Crataegus monogyna*)			
peak	Rt[a] (min)	λ_{max}	$[M + H]^+$ m/z	identity	peel	pulp	leaf	syrup	peel	pulp	leaf	syrup
1	16.0	280	355	derivative of chlorogenic acid	7.36 ± 0.02	4.67 ± 0.02	8.43 ± 0.36	1.93 ± 0.10	6.72 ± 0.22	4.46 ± 0.40	7.55 ± 0.55	3.24 ± 0.05
A	19.3	520	465	delphinidin 3-*O*-glucoside	–[b]	–	–	–	61.62 ± 0.33	3.44 ± 0.11	–	–
2	19.4	280	355	chlorogenic acid	21.23 ± 0.58	12.64 ± 0.39	87.06 ± 0.53	3.50 ± 0.44	22.49 ± 0.25	4.50 ± 0.08	17.39 ± 0.19	2.00 ± 0.08
3	20.6	280	579	procyanidin B2	22.33 ± 0.05	12.61 ± 0.59	182.24 ± 1.20	–	89.12 ± 0.96	14.50 ± 0.06	118.52 ± 0.48	–
4	21.5	280	291	epicatechin	2.84 ± 0,12	1.13 ± 0.07	11.34 ± 0.28	–	4.71 ± 0.12	1.38 ± 0.05	11.22 ± 0.07	–
5	25.5	350	433	vitexin	29.40 ± 0.07	4.83 ± 0.34	46.51 ± 0.41	3.15 ± 0.12	11.91 ± 0.92	6.60 ± 0.41	41.16 ± 0.41	6.13 ± 0.07
6	25.7	350	611	rutin (quercetin-3-rutinoside)	22.95 ± 0.06	5.98 ± 0.21	85.91 ± 0.86	1.78 ± 0.05	16.31 ± 0.34	1.39 ± 0.09	32.89 ± 0.97	1.57 ± 0.40
7	26.8	350	465	hyperoside (quercetin-3-*O*-galactoside)	146.49 ± 0.44	18.19 ± 0.26	251.88 ± 1.26	8.06 ± 0.11	80.54 ± 0.44	5.18 ± 0.25	145.34 ± 2.21	6.26 ± 0.06
8	26.9	350	465	isoquercitrin (quercetin-3-*O*-glucoside)	50.42 ± 0.47	5.22 ± 0.33	58.24 ± 0.29	3.61 ± 0.25	29.43 ± 1.14	2.24 ± 0.05	102.21 ± 1.12	3.22 ± 0.17
total					303.02 ± 1.81	65.27 ± 2.21	731.60 ± 1.30	22.03 ± 0.17	322.83 ± 2.88	43.69 ± 0.16	476.30 ± 1.51	22.42 ± 0.22

[a]HPLC retention time. [b]–, not detected.

The red-fruit species (*C. monogyna*) is distinguished by the presence of anthocyanins, essentially delphinidin 3-*O*-glucoside, representing 19.08 and 7.87% of total phenols in peel and pulp, respectively. The phytochemical constituents of *C. monogyna* have been the subject of intensive investigations for a long time,[10,12,26] but few studies were focused on the *C. azarolus* polyphenolic composition and antioxidant activity.[22,27] To our knowledge, no scientific study has been directed to the comparison of both *Crataegus* species, *C. azarolus* and *C. monogyna*, which correspond to yellow- and red-fruit azarole, respectively. Statistical analysis revealed that the comparison showed that quantitative differences among different aqueous-acetone extracts obtained from azarole samples are more consistent than qualitative ones and that extracts obtained from *C. azarolus* (yellow fruit) contain higher amounts of phenolic compounds than those from *C. monogyna* (red fruit). With regard to the traditionally made syrups, the polyphenolic contents were found lowest among all of the analyzed extracts. Taking into account that in syrups the *Crataegus* extracts were mixed with sugar and boiled to 75 °Brix, we expected the development of the Maillard reaction, which is generally characterized by 5-hydroxymethylfurfural (5HMF). This latter compound is known to react as phenolic compounds in the Folin–Ciocalteu assay;[28] thus, the total phenolic content of syrup extracts as determined using this colorimetric method is supposed to be overestimated and does not reflect real polyphenolic composition. This was confimred by the chromatographic (RP-HPLC) data, where only a few phenolic compounds were observed in the chromatograms concomitant with the presence of a high level of 5HMF (Table 2).

Antiradical Activity. The assessment of antiradical activity of the different polyphenolic extracts from *C. azarolus* and *C. monogyna* leaf, fruit peel, and pulp as well as syrup extracts showed that all studied extracts were able to scavenge the DPPH radical (Table 1). Results present that leaf extracts exhibited higher effects. The lowest TEAC values were obtained for *C. azarolus* and *C. monogyna* syrups, respectively. Many pharmacological studies have also suggested that *Crataegus* extracts scavenge superoxide anion (O_2^{-}), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxides (H_2O_2) and were able to inhibit lipid peroxidation.[12,26] The strong antioxidant activity of *C. azarolus* and *C. monogyna* leaf and fruit peel extracts could be attributed to their high total polyphenolic content. A positive correlation between phenolic composition and antiradical activity was proved by regression values of about 0.9301. Generally, it has been found that polyphenols are some of the most effective antioxidative constituents in the plant kingdom[29] that might contribute to protection against oxidative harm.

Because hyperoside was present at high amounts in the leaf and fruit peel/pulp extracts (Table 2), the recorded antioxidant properties of these extracts could be attributed to the presence of this flavonol. Hyperoside has many bioactivities such as scavenging reactive oxygen species (ROS), preventing free radical induced oxidation, increasing superoxide dismutase activity, and protecting neural PCE12 cells against apoptosis and *tert*-butyl hydroperoxide induced cytotoxicity.[30]

Antioxidant Activity. FRAP values are shown in Table 1. The highest activity was recorded for leaves, followed by peel and then pulp extracts; syrup extracts exhibited the lowest potentials. Whatever the azarole species, extracts of fruit peel presented more potential than those of fruit pulps. Syrup

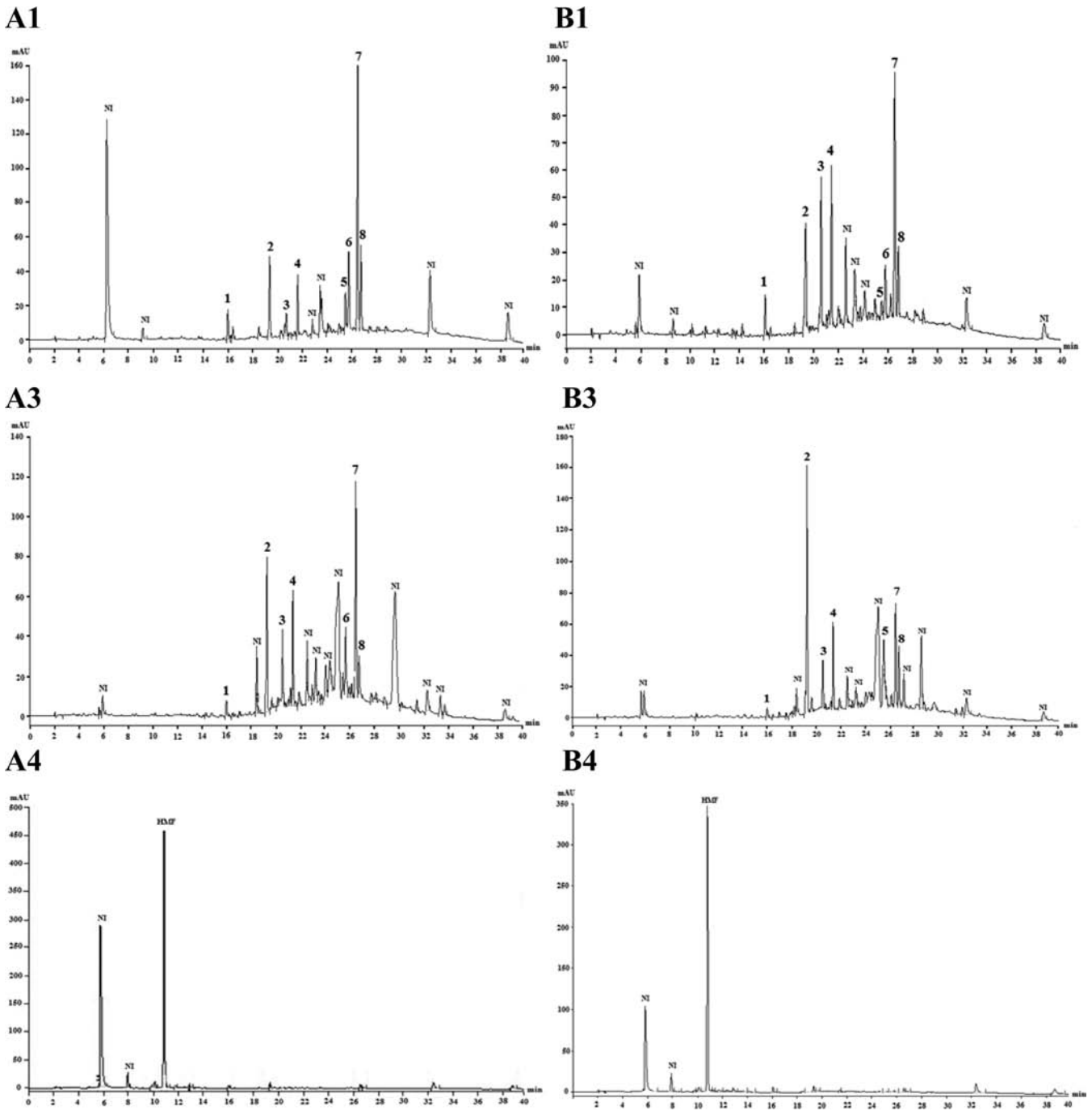

Figure 1. Typical chromatograms of yellow azarole peel (A1), pulp (A2) fruit, leaf (A3), and syrup (A4) and red azarole peel (B1), pulp (B2) fruit, leaf (B3), and syrup (B4) aqueous-acetone extracts recorded at 280 nm. NI, not identified. For peak identification, see Table 2.

extracts showed the lowest potentials (18.07−37.30 μmol Fe^{2+}/g fw) in the FRAP assay.

The FRAP assay used is one of the most rapid and useful tests for routine analysis, because a lot of samples can be analyzed within a short time. In our study, a strong correlation was found between FRAP values and phenolic contents (R^2 = 0.9276), suggesting that phenolic compounds are the major contributors to this activity. Obtained results are in agreement with independent studies of *Crataegus*, which revealed their richness in proanthocyanidins and flavonoids.[12] The latter compounds have been shown to reduce oxidative stress and protect human LDL from oxidation.[11] Chatterjee et al.[13] already proposed that the *Crataegus* antioxidant content could account for the observed cytoprotective and cardioprotective properties.

The high level of procyanidin B2 in the studied leaf and fruit peel/pulp extracts (Table 2) is related to the antioxidant properties of these extracts. Sakano et al.[31] studied the mechanism of preventive action of procyanidin B2 against oxidative DNA damage in human cultured cells and proved that this flavonol exerts antioxidant properties by interacting with H_2O_2 and metal ions (Fe(II)). Other minor phenolic compounds detected in the herein analyzed extracts should not be neglected, because the synergy of the different chemicals with each other should be taken into consideration for the biological activity.[20]

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Antimicrobial Tests. The consistent and reproducible results obtained using the standard antimicrobial well and disk diffusion techniques showed that Tunisian *C. azarolus* and *C. monogyna* extracts exhibited interesting antimicrobial potentials in comparison to ampicillin and oxytetracyclin used as positive controls (Table 3). *Staphylococcus aureus* and *Streptococcus faecalis* were the most sensitive microorganisms to the extracts examined in this study. No effect was observed against *Salmonella typhimurium*, except the weak inhibition observed in the well diffusion assay using leaves and fruit peel extracts at higher concentrations. The strongest antibacterial activity of the polyphenolic extracts was recorded against Gram-positive *S. aureus* and *S. faecalis* bacteria, whereas the lowest activity was against the Gram-negative *Salmonella* strain.

To determine the MICs for all of the sensitive bacterial strains observed in the diffusion assay, the microdilution method was used, and results are shown in Table 4. The MIC values varied from 810 to 88.08 μg phenolics per milliliter of medium culture. The extracts showed bacteriostatic and/or bactericide activities depending on the starting plant material and the targeted bacterial strain (Table 4). The lowest MICs were recorded with *C. azarolus* leaf extracts tested against *S. typhimurium* and *E. coli*, whereas the highest MICs were recorded with *C. monogyna* syrup extracts tested against *S. aureus* and *S. faecalis*. *C. monogyna* leaf extracts were found bactericidal against all of the strains, whereas fruit pulp and syrup extracts did not exhibit bactericide effects against any tested bacteria (Table 4). Extracts prepared from *C. azarolus* leaf and fruit peel as well as *C. monogyna* fruit peel presented bactericidal effects against three bacterial strains at the highest concentrations reachable in the test. MBCs against *P. aeruginosa* were found about 607.5, 336.66, and 358.7 μg/mL using the extracts obtained from *C. azarolus* leaf and fruit peel as well as *C. monogyna* fruit peel, respectively, whereas, using these latter extracts, MBCs against *S. typhimurium* and *E. coli* were, respectivel,y 810, 505, and 538.05 μg/mL. With *S. aureus*, *S. epidermidis*, and *S. faecalis*, no bactericidal effects were observed even at the highest concentrations of the extracts reachable in the test.

The obtained results suggest that aqueous-acetonic extracts of azarole were more efficient in inhibiting Gram-positive than Gram-negative bacteria. The greater resistance of Gram-negative bacteria to plant extracts was previously documented.[32] These results are also in agreement with the report of Taguri et al.,[33] who tested polyphenolic extracts of 10 different plants against a range of microbial cells and found that Gram-positive were more sensitive than Gram-negative bacteria. The dissimilar bacterial sensitivity to polyphenolic compounds is likely to be the result of the differential cell wall structure between Gram-negative and Gram-postive. The Gram-negative outer membrane is thought to interact with phenolic substances.[34] Results showed also that leaf and fruit peel extracts exhibited more antibacterial potential than the fruit pulp and syrup extracts, which reflects differences in biochemical qualitative and quantitative contents. These data are confirmed by the work of Rodriguez et al.,[35] who explained that the antibacterial activities were due to the high level of bioactive molecules in plants.

This study contributes to the knowledge of the beneficial properties of the phenolic extracts of azarole in Tunisia. The analytical characterization and evaluation of the exhibited biological activities of both azarole yellow and red fruits as well as their leaves could support the exploitation of these natural

Table 3. Antibacterial Activity of Phenolic Extracts of Yellow and Red Azarole Using Disk and Well Diffusion Assays

	diameter[a] of the inhibition zone (mm)											
	Staphylococcus aureus (ATCC 6538)		*Staphylococcus epidermidis* (CIP 106510)		*Streptococcus faecalis* (ATCC 7830)		*Pseudomonas aeruginosa* (ATCC 9027)		*Salmonella typhimurium* (NRRLB 4420)		*Escherichia coli* (ATCC 8739)	
	disk	well	disk	well	disk	well	disk	well	disk	well	disk	well
fruit peel[b]	12.7 ± 0.3	14.1 ± 0.8	9.3 ± 0.5	12.5 ± 0.6	10.8 ± 0.4	12.3 ± 1.0	–[c]	8.2 ± 0.5	–	7.3 ± 0.1	–	9.6 ± 0.2
fruit peel[d]	12.5 ± 0.6	13.4 ± 0.3	9.0 ± 0.3	10 ± 0.9	11.0 ± 0.3	12.0 ± 1.1	8.6 ± 0.1	8.0 ± 0.2	7.0 ± 0.7	7.0 ± 0.9	–	–
fruit pulp[b]	9.2 ± 0.7	10.6 ± 0.7	9.5 ± 0.6	10.9 ± 0.2	9.5 ± 0.2	10.8 ± 0.6	–	–	–	–	–	8.6 ± 0.9
fruit pulp[d]	9.08 ± 0.1	10.1 ± 0.9	9.2 ± 0.2	10.1 ± 0.1	9.11 ± 0.4	10.5 ± 0.9	–	–	–	–	9.2 ± 0.5	9.0 ± 0.2
leaf[b]	12.7 ± 0.9	14.3 ± 0.5	–	–	11.7 ± 0.4	12.4 ± 0.5	7.5 ± 0.7	8.5 ± 0.4	–	7.1 ± 0.2	–	10.2 ± 0.3
leaf[d]	11.4 ± 0.3	13.5 ± 0.8	–	–	10.6 ± 0.5	11.1 ± 0.3	7.1 ± 0.8	9.9 ± 0.3	–	6.9 ± 0.5	–	–
syrup[b]	9.1 ± 0.9	9.9 ± 0.1	8.11 ± 0.2	8.8 ± 0.8	9.3 ± 0.1	10.1 ± 0.6	–	–	–	–	–	7.2 ± 0.1
syrup[d]	10.0 ± 0.8	11.12 ± 0.4	9.03 ± 0.3	9.8 ± 0.7	10.3 ± 1.3	11 ± 1.4	–	–	7.6 ± 0.2	7.1 ± 0.1	7.3 ± 0.6	8.2 ± 1.0
Amp[e]	11.2 ± 0.5	15.5 ± 1.3	11.2 ± 0.6	13.5 ± 1.2	11.3 ± 0.5	16.2 ± 1.0	10.5 ± 0.5	15.4 ± 0.7	11.4 ± 0.3	13.9 ± 1.0	12.7 ± 1.3	16.5 ± 1.6
Tet[f]	13.5 ± 1.3	17.3 ± 1.1	12.5 ± 1.3	14.8 ± 0.6	12.7 ± 0.3	18.2 ± 0.9	12.8 ± 0.7	17.3 ± 1.5	12.1 ± 0.2	15.2 ± 0.7	14.0 ± 0.7	18.2 ± 0.9

[a]Including the diameter of the disk or the well (ø 6 mm). Values are means ± SD of three separate experiments done in triplicate. ; [b]Yellow azarole extract. [c]–, no antimicrobial activity, ø = 6 mm. [d]Red azarole extract. [e]AMP, ampicillin (50 μg/mL). [f]TET, oxytetracyclin (200 μg/mL).

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Table 4. Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of Yellow and Red Azarole Extracts

	Staphylococcus aureus (ATCC 6538)	*Staphylococcus epidermidis* (CIP 106510)	*Streptococcus faecalis* (ATCC 7830)	*Pseudomonas aeruginosa* (ATCC 9027)	*Salmonella typhimurium* (NRRLB 4420)	*Escherichia coli* (ATCC 8739)
			MIC (μg/mL)			
fruit peel[a]	125.25 ± 0.75	252.5 ± 1.50	125.25 ± 0.75	336.66 ± 2.01	505.00 ± 3.01	505.00 ± 3.01
fruit peel[b]	134.51 ± 1.20	269.02 ± 2.40	134.51 ± 1.20	358.70 ± 3.20	538.05 ± 4.80	538.05 ± 4.80
fruit pulp[a]	108.50 ± 3.68	108.50 ± 3.68	108.50 ± 3.68	>[c]	>	>
fruit pulp[b]	72.81 ± 0.26	72.81 ± 0.26	72.81 ± 0.26	>	>	>
leaf[a]	243.90 ± 0.43	303.75 ± 0.54	243.90 ± 0.43	607.50 ± 1.08	810.00 ± 1.44	810.00 ± 1.44
leaf[b]	198.45 ± 0.62	396.91 ± 1.25	198.45 ± 0.62	529.22 ± 1.66	793.83 ± 2.50	793.83 ± 2.50
syrup[a]	113.80 ± 1.50	>	113.80 ± 1.50	>	>	>
syrup[b]	88.08 ± 3.11	>	88.08 ± 3.11	>	>	>
			MBC (μg/mL)			
fruit peel[a]	252.50 ± 1.50	336.66 ± 2.01	252.50 ± 1.50	336.66 ± 2.01	505.00 ± 3.01	505.00 ± 3.01
fruit peel[b]	269.02 ± 2.40	358.70 ± 3.20	269.02 ± 2.40	358.70 ± 3.20	538.05 ± 4.80	538.05 ± 4.80
fruit pulp[a]	>	>	>	>	>	>
fruit pulp[b]	>	>	>	>	>	>
leaf[a]	303.75 ± 0.54	607.50 ± 1.08	303.75 ± 0.54	607.50 ± 1.08	810.00 ± 1.44	810.00 ± 1.44
leaf[b]	198.45 ± 0.62	396.91 ± 1.25	198.45 ± 0.62	529.22 ± 1.66	793.83 ± 2.50	793.83 ± 2.50
syrup[a]	>	>	>	>	>	>
syrup[b]	>	>	>	>	>	>

[a]Yellow azarole extract. [b]Red azarole extract. [c]>, no inhibition with the highest concentration in the test conditions.

sources of polyphenols in food and medicinal applications. Besides, traditional syrup preparation seems to allow heavy losses in bioactive compounds that could be preserved using soft procedures, a track that should be worthy of deep investigation. Synergetic/antagonistic effects between different phenolic compounds should not be neglected in future works to deepen our knowledge about the behavior of the whole chemical composition of the extracts, known as "totum", so as we can accurately elucidate the phenomena behind the observed bioactivities.

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Notes

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REFERENCES

(1) Bermudez-Soto, M. J.; Larrosa, M.; Garcia-Cantalejo, J. M.; Espin, J. C.; Tomas-Barberan, F. A.; Garcia-Conesa, M. T. Up-regulation of tumor suppressor carcinoembryonic antigen-related cell adhesion molecule 1 in human colon cancer Caco-2 cells following repetitive exposure to dietary levels of a polyphenol-rich chokeberry juice. *J. Nutr. Biochem.* **2007**, *18*, 259−271.

(2) Geleijnse, J. M.; Launer, L. J.; Hofman, A.; Pols, H. A. P.; Witteman, J. C. M. Tea flavonoids may protect against atherosclerosis: the Rotterdam Study. *Arch. Intern. Med.* **1999**, *159*, 2170−2174.

(3) Mandel, S.; Amit, T.; Bar-Am, O.; Youdim, M. B. H. Iron dysregulation in Alzheimer's disease: multimodal brain permeable iron chelating drugs, possessing neuroprotective-neurorescue and amyloid precursor protein-processing regulatory activities as therapeutic agent. *Prog. Neurobiol.* **2007**, *82*, 348−360.

(4) Seifried, H. E.; Anderson, D. E.; Fisher, E. I.; Milner, J. A. A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.* **2007**, *18*, 567−579.

(5) Skerget, M.; Kotnik, P.; Hadolin, M.; Hras, A. R.; Simonic, M.; Knez, Z. Phenols, procyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem.* **2005**, *89*, 191−198.

(6) Macheix, J. J.; Fleuriet, A.; Billot, J. *Fruits Phenolics*; CRC Press: Boca Raton, FL, 1990; pp 24−31, 295−342.

(7) Halliwell, B. Antioxidants in human health and disease. *Annu. Rev. Nutr.* **1996**, *16*, 33−50.

(8) Chang, W.; Dao, J.; Shao, Z. Hawthorn: potential roles in cardiovascular disease. *Am. J. Chin. Med.* **2005**, *33*, 1−10.

(9) Bignami, C.; Poalocci, A.; Scossa, A.; Bertazza, G. Preliminary evaluation of nutritional and medicinal components of *Crataegus azarolus* fruits. *J. Food Eng.* **2000**, *5*, 241−247.

(10) Cui, T.; Nakamura, K.; Tian, S.; Kayahara, H.; Tian, Y. L. Polyphenolic contents and physiological activities of chinese hawthorn extracts. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 2948−2956.

(11) Zhang, Z.; Min Zhu, Q.; Huang, Y.; Hoz, W. K. K.; Chen, Z. Y. Characterization of antioxidants presents in hawthorn fruits. *J. Nutr. Biochem.* **2001**, *12*, 144−152.

(12) Bahorun, T.; Aumjaud, E.; Ramphul, H.; Rycha, M.; Luximon-Ramma, A.; Trotin, F.; Aruoma, O. I. Phenolic constituents and antioxidant capacities of *Crataegus monogyna* (hawthorn) callus extracts. *Nahrung* **2003**, *47*, 191−198.

(13) Chatterjee, S. S.; Koch, E.; Jaggy, H.; Krzeminski, T. In vitro and in vivo studies on the cardioprotective action of oligomeric procyanidins in a *Crataegus* extract of leaves and blooms. *Arzneim.-Forsch.* **1997**, *47*, 821−825.

(14) Degenring, F. H.; Suter, A.; Weber, M.; Saller, R. Randomised double blind placebo controlled clinical trial of a standardised extract

of fresh *Crataegus berries* (Crataegisan) in the treatment of patients with congestive heart failure NYHA II. *Phytomedicine* **2003**, *10*, 363−369.
(15) Zhang, D. L.; Zhang, Y. T.; Yin, J. J.; Zhao, B. L. Oral administration of *Crataegus* flavonoids protects against ischemia/reperfusion brain damage in gerbils. *Neurochemistry* **2004**, *90*, 211−219.
(16) Ercisli, S. A short review of the fruit germplasm resources of Turkey. *Genet. Resour. Corp Ev.* **2004**, *51*, 419−435.
(17) Koyuncu, T.; Pinar, Y.; Lule, F. Convective drying characteristics of azarole red (*Crataegus monogyna* Jacq.) and yellow (*Crataegus aronia* Bosc.) fruits. *J. Food Eng.* **2006**, *78*, 1471−1475.
(18) Espiard, E. *Introduction à la Transformation Industrielle des Fruits*; Tec et doc: Paris, France, 2002; 360 pp.
(19) Cui, T.; Li, J. Z.; Kayahara, H.; Ma, L.; Wu, L. X.; Nakamura, K. Quantification of the polyphenols and triterpene acids in Chinese hawthorn fruit by high-performance liquid chromatography. *J. Agric. Food Chem.* **2006**, *54*, 4574−4581.
(20) Fattouch, S.; Caboni, P.; Coroneo, V.; Tuberoso, C. I. G.; Angioni, A.; Dessi, S.; Marzouki, N.; Cabras, P. Antimicrobial activity of Tunisian quince (*Cydonia oblonga* Miller) pulp and peel polyphenolic extracts. *J. Agric. Food Chem.* **2007**, *55*, 963−969.
(21) Tuberoso, C. I. G.; Bifulco, E.; Jerković, I.; Caboni, P.; Cabras, P.; Floris, I. Methyl syringate: a chemical marker of asphodel (*Asphodelus microcarpus* Salzm. et Viv.) monofloral honey. *J. Agric. Food Chem.* **2009**, *57*, 3895−3900.
(22) Belkhir, M.; Rebai, O.; Dhaoudi, K.; Sioud, B.; Fattouch, S.; Amri, M. Antioxidant and antimicrobial activities of Tunisian azarole (*Crataegus azarolus* L.) leaves and fruit pulp/peel polyphenolic extracts. *Int. J. Food Prop.* **2013**, *16*, 1380−1393.
(23) Tuberoso, C. I. G.; Bifulco, E.; Caboni, P.; Cottiglia, F.; Cabras, P.; Floris, I. Floral markers of strawberry-tree (*Arbutus unedo* L.) honey. *J. Agric. Food Chem.* **2010**, *58*, 384−389.
(24) Urbonavicuite, A.; Jakstas, V.; Kornysova, O.; Janulis, V.; Maruska, A. Capillary electrophoretic analysis of flavonoids in single-styled hawthorn (*Crataegus monogyna* Jacq.) ethanolic extracts. *J. Chromatogr.* **2006**, *1112*, 339−344.
(25) Ding, X. P.; Wang, X. T.; Chen, L. L.; Qi, J.; Xu, T.; Yu, B. Y. Quality and antioxidant activity detection of Crataegus leaves using on-line high-performance liquid chromatography with diode array detector coupled to chemiluminescence detection. *Food Chem.* **2009**, *3*, 929−933.
(26) Bahorun, T.; Gressier, B.; Trotin, F.; Brunet, C.; Dine, T.; Luyckx, M.; Vasseur, J.; Cazin, M.; Cazin, J. C.; Pinkas, M.; Battez-Lebegue, S. Oxygen species scavanging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparations. *Arzneim.-Forsch.* **1996**, *46*, 1086−1089.
(27) Ljubuncic, P.; Portnaya, I.; Cogan, U.; Azaizeh, H.; Bomzon, A. Antioxidant activity of *Crataegus aronia* aqueous extract used in traditional Arab medicine in Israel. *J. Ethnopharmacol.* **2005**, *101*, 153−161.
(28) Jerković, I.; Kasum, A.; Marijanovic, Z.; Tuberoso, C. I. G. Contribution to the characterisation of honey-based Sardinian product abbamele: Volatile aroma composition, honey marker compounds and antioxidant activity. *Food Chem.* **2011**, *124*, 401−410.
(29) Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total polyphenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113−4117.
(30) Liu, Z.; Tao, X.; Zhang, C.; Lu, Y.; Wei, D. Protective effects of hyperoside (quercetin 3-*O*-galactoside) to PC12 cells against cytotoxicity induced by hydrogen peroxide and tert-butyl hydroperoxide. *Biomed. Pharmacother.* **2005**, *59*, 481−490.
(31) Sakano, K.; Mizutani, M.; Murata, M.; Oikawa, S.; Hiraku, Y.; Kawanishi, S. Procyanidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. *Free Radical Biol. Med.* **2005**, *39*, 1041−1049.
(32) Kudi, A. C.; Umoh, J. U.; Eduvie, L. O.; Gefu, J. Screening of some Nigerian medicinal plants for antibacterial activity. *J. Ethnopharmacol.* **1999**, *67*, 225−228.
(33) Taguri, T.; Tanaka, T.; Kouno, I. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol. Pharm. Bull.* **2004**, *27*, 1965−1969.
(34) Tortora, G. J.; Funke, B. R.; Case, C. L. *Microbiology: An Introduction*; Benjamin Cummings: San Francisco, CA, 2001; p 88.
(35) Rodriguez Vaquero, M. J.; Alberto, M. R.; Manca de Nadra, M. C. Antibacterial effect of phenolic compounds from different wines. *Food Control* **2007**, *18*, 93−101.