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Polyphenol Contents and Antioxidant Activities of Extracts from Flowers of Two *Crataegus azarolus* L. Varieties

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Abstract: Phenolic contents of the ethyl acetate extracts prepared from floral buds and opened flowers harvested on *Crataegus azarolus* trees native in two localities were performed. The antioxidant activity was measured by DPPH$^{\bullet}$ (2,2-diphenyl-picrylhydrazyl), ABTS$^{\bullet+}$ (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radicals scavenging using spectrophotometric method. The *C. azarolus* var. *aronia* (Willd.) Batt., producing yellow fruits, was richer in total phenols (1638.7±89.9 mg acid gallic/100 g dry weight) according to *C. azarolus* var. *eu-azarolus* Maire (1415.5±23.8 mg acid gallic/100 g dry weight), producing red ones. Ethyl acetate extract from opened flowers has less content in total phenols, proanthocyanidins and flavonoids compared to this from floral buds. Floral buds from the two *C. azarolus* varieties occurring in Siliana-Djebel Serdj showed the highest radical scavenging activities (2431.8±32.7 and 2267.7±22.7 µmol Trolox/100 g dry weight). Hawthorn from Tunisia contains eight antioxidants of phenolic type (chlorogenic acid, hyperoside, rutin, spiraeoside, isoquercitrin, quercetin, (-)-epicatechin and the dimer procyanidin B2). These compounds identified specially in floral bud extracts presented a strong radical-scavenging activity.

Key words: *Crataegus azarolus* L., phenolic contents, organic extract, DPPH free radical scavenger

INTRODUCTION

Excessive production of reactive oxygen species (superoxide anion; O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), by the organism is thought to be involved in a number of pathological phenomenons including anemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's and Alzheimer's diseases, ageing process, atherosclerosis and cancer diseases (Bermúdez-Soto *et al*., 2007; Boudet, 2007; Kao *et al*., 2007; Mandel *et al*., 2007; Seifried *et al*., 2007; Stangl *et al*., 2007). Free radicals can be scavenged through chemoprevention utilizing natural antioxidant compounds present in medicinal plants (Sabu and Kuttan, 2002).

Among plant compounds, a growing number of reports deal with the free radical scavenging and antioxidant properties of polyphenolics (Skerget *et al*., 2005; Katalinic *et al*., 2006; Zhou and Yu, 2006; Ruberto *et al*., 2007). Different classes of these active phenolics are found in hawthorn species (Bahorun *et al*., 1994, 1996, 2004; Zhang *et al*., 2001; Cai *et al*., 2004; Chang *et al*., 2006; Cui *et al*., 2006; Svedstrom *et al*., 2006; Urbonaviciute *et al*., 2006) and were used in therapy as a cardiosedative drugs. In fact, the pharmacological properties of hawthorn is growing in therapeutics, since derived pharmaceuticals and extracts are known and recognized to improve the coronary blood flow and cardiac contraction in moderate heart failure not requiring a major cardiotonic therapy, as well as in the ageing heart

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(Zapie Jun, 2001; Degenring *et al*., 2003; Schröder *et al*., 2003; Veveris *et al*., 2004; Long *et al*., 2006). In France, *Crataegus* is also generally prescribed as a sedative to treat nervosity and sleep disorders (Bruneton, 1993; Hanus *et al*., 2004). In China, hawthorn has also been used in herbal medicines (Cui *et al*., 2006). Studies indicate that Chinese hawthorn extracts have beneficial effects such as antioxidant (Zhang *et al*., 2001; Chu *et al*., 2003), anti-inflammatory (Kao *et al*., 2005) and also hypolipidemic effects (Zhang *et al*., 2002). Hawthorn extract exhibit other effects such as anticarcinogen (Kao *et al*., 2007) and antimicrobial (Güven *et al*., 2006; Orhan *et al*., 2007) ones.

The genus *Crataegus*, known as Zaarour in Tunisia, is represented by two species in the flora of Tunisia; *C. oxyacanthus* ssp. *monogyna* (Jacq.) Rouy and Camus and *Crataegus azarolus* L. (Pottier-Alapetite, 1979). *C. oxyacanthus* ssp. *monogyna* has been often studied and it's the major hawthorn species utilized in European Pharmacopoeia (Bahorun *et al*., 1994). Up to-date, no analytical study has been performed on the Tunisian *Crataegus* species mainly *Crataegus azarolus*, which is represented by two varieties: *Crataegus azarolus* var. *aronia* (Willd.) Batt. and *C. azarolus* var. *eu-azarolus* Maire, they differ by the color of fruit: yellow fruits for the former and red ones for the later.

The purpose of the present study was to evaluate phenol, flavonoid and procyanidin contents of each Ethyl Acetate (E.A.) extract prepared from reproductive organs harvested on native trees of two *Crataegus azarolus* varieties spreading out in two localities of the Tunisian territory. Estimation of the antioxidant activities of all extracts was performed.

MATERIALS AND METHODS

Chemicals: ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-picrylhydrazyl), Trolox C (6-hydroxy-2,5,7,8-tetramethychroma-2carboxylic acid), potassium sulphate (di-potassium peroxdisulfate), HPLC grade of (-)-epicatechin, procyanidin dimer B2, chlorogenic acid, hyperoside, rutin, spiraeoside and isoquercetrin were obtained from Sigma-Aldrich (Taufkirchen, Germany). HPLC grade of quercetin and cyanidin chloride were obtained from Extrasynthèse (Genay, France).

Plant material: Native trees of two *C. azarolus* varieties were spreading out at two localities; one in the centre of the country, on a mountainous region, in Siliana Village, called Serdj, the second locality was in the east coast near Hammam-Sousse Town: *C. azarolus* var. *aronia* with yellow fruits and *C. azarolus* var. *eu-azarolus* with red ones (Pottier-Alapetite, 1979).

Floral buds and opened flowers were collected in March 2004. All voucher specimens are deposited in the Higher Institute of Agronomy of Chott-Meriem, Botanic Laboratory Herbarium and were assigned for each one a corresponding number and code R401 and R404 for *C. azarolus* var. *aronia* and *C. azarolus* var. *eu-azarolus* respectively, together from Serdj; R412 and R414 for *C. azarolus* var. *aronia* and *C. azarolus* var. *eu-azarolus*, respectively, together from Hammam-Sousse (Table 1).

Extraction procedure: One hundred gram of fresh plant material was macerated in absolute methanol for 48 h at room temperature (thrice). The pooled methanol filtrates were concentrated using a vacuum rotary evaporator to eliminate the solvent. The methanolic extract was then dissolved in 500 mL of water and extracted subsequently using petroleum ether (150 mL×3), ethyl acetate (150 mL×3) and butanol (150 mL×3) in an order of increasing solvent polarity. The solvents were then evaporated to produce petroleum ether, ethyl acetate and butanol, respectively extracts. In this study, we give results related to the ethyl acetate extract.

Analysis of total phenols in ethyl acetate extracts: The concentration of total phenols in EA extracts was measured by UV spectrophotometry (Jenway 6300), based on a calorimetric oxidation/reduction reaction. The oxidizing agent used was Folin-Ciocalteu reagent (Merck) (Singleton and Rossi, 1965; AOAC, 1984). To 50 µL of diluted extract (1 mg/1 mL of methanol) was added, in screw-capped test tubes, 750 µL of distilled water/Folin-Ciocalteu solution (28/2; v/v). After 3 min, 200 µL of

Table 1: Abbreviations of studied organs, corresponding regions of harvest, localization, bioclimatic stage, latitude and longitude of each locality

Abbreviations	Reproductive organs	*Crataegus* species and varieties	Locality of harvest	Localization; bioclimatic stage; latitude-longitude
F.B.a.Sj.	Floral Buds	*Crataegus azarolus* var. *aronia*	Silana Serdj mountain	Centre of Tunisia; low arid;
F.B.e.Sj.	Floral Buds	*Crataegus azarolus* var. *eu-azarolus*	Silana Serdj mountain	36°4'-9°22'
O.F.a.Sj.	Opened flowers	*Crataegus azarolus* var. *aronia*	Silana Serdj mountain	
O.F.e.Sj.	Opened flowers	*Crataegus azarolus* var. *eu-azarolus*	Silana Serdj mountain	
F.B.a.H.S.	Floral Buds	*Crataegus azarolus* var. *aronia*	Hammam Sousse	East Coast of Tunisia; low subarid;
F.B.e.H.S.	Floral Buds	*Crataegus azarolus* var. *eu-azarolus*	Hammam Sousse	35°51'-10°35'
O.F.a.H.S.	Opened flowers	*Crataegus azarolus* var. *aronia*	Hammam Sousse	
O.F.e.H.S	Opened flowers	*Crataegus azarolus* var. *eu-azarolus*	Hammam Sousse	

sodium carbonate (Na_2CO_3) (200 g L^{-1}) was added and the test tubes were properly shacked before incubating in boiling water bath for 1 min. The tubes were then allowed to cool in the dark. The absorbance was measured at 765 nm and results were expressed in mg of gallic acid/100 g dry weight (DW) using appropriate standard curve. For a control sample, 50 µL of methanol was used.

Analysis of proanthocyanidins in ethyl acetate extracts: The proanthocyanidins were determined by UV spectrophotometry method based on acid hydrolysis and colour formation. The HCl/butan-1-ol assay was used to quantify the total proanthocyanidins (Porter *et al*., 1986).

One milligram of each EA extract was dissolved in 1 mL of methanol. 0.25 mL of this solution was added 3 mL of a 95% solution of n-butanol/HCl (95/5; v/v) in stoppered test tubes followed by addition of 0.1 mL of a solution of $NH_4 Fe (SO_4)_2 \cdot 12 H_2O$ in 2 M HCl (0.2%; w/v). The tubes were incubated for 40 min at 95°C. For a control sample, 0.25 mL of methanol was used. After incubation, the samples were cooled and analyzed by measuring absorbance at 540 nm. The results were expressed as mg of cyanidin chloride/100 g DW.

Analysis of total flavonoids in ethyl acetate extracts: The $AlCl_3$ method (Lamaison and Carnat, 1991) was adopted for the purpose of determining the total flavonoid content of the EA extracts. A quantity of E.A. extract (0.5 mg) was dissolved in 1 mL of methanol. 0.5 mL of this solution was added equal volumes of a solution of 2% $AlCl_3$. $6H_2O$ (2 g in 100 mL methanol). The mixture was thoroughly mixed and incubated for 10 min. After incubation, the samples were cooled and analyzed by measuring absorbance at 367.5 nm. The results were expressed in mg rutin equivalents/100 g DW.

High performance liquid chromatography analysis: HPLC analysis of E.A. extracts was carried out using a Hewlett Packard 1500 series (Waldbronn, Germany) liquid chromatography system equipped with a vacuum degasser, quaternary pump, auto-sampler, thermostated column compartment and diode array detector. After filtration on millipore filter paper (0.22 µm) (Whatman), 20 µL of each diluted ethyl acetate extract (1 mg dry weight/1 mL of absolute methanol) were injected on a Spherisorb ODS2 RP18 (5 µm) reversed phase, C18 column (4.6 mm i.d.×150 mm) (Sigma-Aldrich, Taufkirchen, Germany) eluted by an acidified acetonitrile-water gradient. Elution with a flow rate of 0.7 mL min at 25°C was as follow: 0-5 min, 0-7.5% B in A; 5-13 min, 20% B in A; 13-20 min, 80% B in A; 20-25 min, 100% B in A (solvent A: acetonitrile/water, 1/9 v/v, pH 2.5; solvent B: acetonitrile/water, 9/1 v/v, pH 2.5). Chlorogenic acid, (-)-epicatechin and procyanidin dimer B2 were identified and quantified by comparison with authentic standards at 280 nm. Hyperoside, rutin, spiraeoside, isoquercitrin and quercetin were identified and quantified by comparison with authentic standards at 360 nm.

DPPH radical scavenging activity: Measurement of antiradical activity was adapted from Soler-Rivas *et al*. (2000). The extracts were diluted (1 mg dry weight mL^{-1}) using absolute methanol. Twenty microliter of ethyl acetate extract was added to 980 µL of DPPH radical (90 µM in methanolic solution) in a test tube. Methanol was used in the place of antioxidant solution as a control. The solution was immediately mixed vigorously for 10 sec by a vortex mixer and transferred to the cuvette holder of the spectrophotometer against the blank, which did not contain the extract. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 515 nm. All experiments were performed in triplicate.

ABTS radical scavenging activity: ABTS radical scavenging activity was measured using a modified Re *et al*. (1999) method. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration). The reaction mixture was allowed to stand in the dark at room temperature for 12-16 h prior to use. ABTS solution was diluted with methanol to an absorbance of 0.70 (±0.02) at 734 nm. To a diluted ABTS solution (980 µL) was added 20 µL of the extract solution (1 mg dry weight/mL). The solution was immediately mixed vigorously for 10 sec by a vortex mixer and transferred to a cuvette. The absorbance was monitored at 734 nm after 6 min.

Radical-scavenging expression: For the two test ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$), Trolox, the water-soluble a tocopherol (Vitamin E) analog, served as standard. A concentration-response curves, for $ABTS^{\bullet+}$ (734 nm) and for $DPPH^{\bullet}$ (515 nm), as a function of different Trolox concentrations were prepared. The decrease in absorption of tested samples was used for calculating the TEAC (Trolox equivalent antioxidant capacity for the two radicals).

Statistical analysis: Simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as test samples. Linear regression analysis was performed,

quoting the correlation coefficient r_{xy} between antioxidant activities and concentration in phenolic classes. All results are expressed as Mean±SD of three parallel measurements. The results were processed using Microsoft Excel 2003 and the data were subjected to one way analysis of variance (ANOVA) and the significance of differences between sample means were calculated by Duncan multiple range test using SPSS for Windows (Standard Version 12.0 SPSS Inc., Chicago, IL.), p-value ≤0.05 were regarded as significant.

RESULTS

Polyphenolic contents of *Crataegus* ethyl acetate extracts prepared from floral buds and opened flowers: The total phenol, flavonoid and proanthocyanidin contents of the two varieties of *Crataegus azarolus* reproductive organ ethyl acetate extracts are shown in Table 2. The total phenol contents ranged from 931.31 to 1638.7 mg/100 g DW. The highest level was measured in floral buds (F.B.) ethyl acetate extract from *Crataegus azarolus* var. *aronia* developed in Serdj locality. Comparable and significant amounts were obtained in F.B.a.H.S. (1033.3±7.9 mg/100 g DW), F.B.e.H.S. (1023.7±32.1 mg/100 g DW), O.F.a.H.S. (1014.2±11.8 mg/100 g DW), O.F.e.H.S. (991.3±0.8 mg/100 g DW), O.F.e.Sj. (945.6±33.8 mg/100 g DW) and O.F.a.Sj. (931.1±26.6 mg/100 g DW).

Opened flowers from the two varieties from Serdj locality contained the lowest amounts. The total flavonoid contents of the eight E.A. extracts ranged from 317.8±19.2 to 753.6±54.0 mg/100 g DW. F.B.e.Sj. contained the highest total flavonoid contents. Comparable and lowest amounts were obtained in O.F.a.Sj. (317.8±19.2 mg/100 g DW), O.F.e.Sj. (346.4±29.8 mg/100 g DW), F.B.e.H.S. (358.9±0.4 mg/100 g DW) and O.F.e.HS (345.6±0.1 mg/100 g DW). Similar moderate levels were obtained in F.B.a.H.S. (472.7±1.4 mg/100 g DW) and O.F.a.H.S. (535.8±1.8 mg/100 g DW). Values obtained for the total proanthocyanidin contents of the eight ethyl acetate extracts varied from 520.9±0.6 to 925.3±8.1 mg/100 g DW. F.B.e.Sj. had also the highest level of total proanthocyanidin while the lowest was measured in O.F.e.H.S. Comparable levels were measured in ethyl acetate extracts from O.F.a.Sj. (673.5±10.5 mg/100 g DW), O.F.e.Sj. (640.1±19.5 mg/100 g DW) and O.F.a.H.S. (659.9±10.3 mg/100 g DW). Table 3 shows the two flavan-3-ol ((-)-epicatechin and the procyanidin B2 dimer), the chlorogenic acid and the five flavonoids (hyperoside, rutin, spiraeoside, isoquercitrin and quercetin) contents. Here (-)-epicatechin and the dimeric procyanidin B2, which were the catechins characterized in the eight ethyl acetate extracts of *Crataegus azarolus* were detected at the upper concentrations of 198.7±0.8 mg/100 g DW in F.B.a.Sj. and 55.2±0.2 mg/100 g DW in F.B.a.Sj. The summation of the amounts of (-)-epicatechin and procyanidin B2 dimer gave an indication of the catechin richness of E.A extracts from the two varieties of *Crataegus azarolus* in the following order F.B.a.Sj.>F.B.a.H.S. >F.B.e.Sj.>O.F.a.Sj.>F.B.e.H.S. >O.F.e.Sj.>O.F.a.H.S.>O.F.e.H.S. Chlorogenic acid was the main phenolic acid in ethyl acetate extract found at the upper concentration of 244.1±0.6 mg/100 g DW in F.B.a.Sj. The lowest level is 16.6±0.1 mg/100 g DW (O.F.e H.S.). The concentrations of hyperoside, rutin, spiraeoside, quercetin and isoquercitrin ranged from 24.7±0.0 to 412.2±0.1 mg/100g DW; 9.7±0.1 to 198.3±0.6 mg/100 g DW; 65.0±0.2 to 245.9±0.6 mg/100 g DW; 1.8±0.0 to 72.5±0.3 mg/100 g DW and 6.2±0.0 to 101.9±0.2 mg/100 g DW, respectively (Table 3). F.B.a.Sj. present the highest amount of the different individual polyphenols except for hyperoside.

Table 2: Total phenol, flavonoid and proanthocyanidin levels of ethyl acetate extracts prepared from floral buds (FB) and opened flowers (OF) harvested on *Crataegus azarolus* var. *aronia* (a) and *Crataegus azarolus* var. *eu-azarolus* (e) trees from Serdj (Sj.) and Hammam Sousse (HS) localities

Reproductive organ, variety and locality	Total phenols (mg acid gallic/100 g DW)	Total flavonoids (mg rutin/100 g DW)	Total proanthocyanidins (mg chloride cyanidin/100 g DW)
F.B.a.Sj.	1638.7±89.9^{b}	640.3±42.4^{c}	922.1±10.9^{d}
F.B.e.Sj.	1415.5±23.8^{b}	753.6±54.0^{d}	925.3±8.1^{d}
O.F.a.Sj.	931.1±26.6^{a}	317.8±19.2^{a}	673.5±10.5^{b}
O.F.e.Sj.	45.6±33.8^{a}	346.4±29.8^{a}	640.1±19.5^{b}
F.B.a.H.S.	1033.3±7.9^{a}	472.7±1.4^{b}	897.9±6.8^{d}
F.B.e.H.S.	1023.7±32.1^{a}	358.9±0.4^{a}	850.2±15.1^{c}
O.F.a.H.S.	1014.2±11.8^{a}	535.8±1.8^{b}	659.9±10.3^{b}
O.F.e.H.S.	991.3±0.8^{a}	345.6±0.1^{a}	520.9±0.6^{a}

DW: Dry weight; Data are expressed as Mean±SD of three measurements. In the same column, contents with same superscript letter(s) are not significantly different at p>0.05

Table 3: Levels of individual phenols from eight ethyl acetate extracts (mg/100 g DW) prepared from floral buds (FB) and opened flowers (OF) harvested on *Crataegus azarolus var. aronia* (a) and *C. azarolus* var. *eu-azarolus* (e) trees from Serdj (Sj.) and Hammam Sousse (HS) localities

Individual phenol	F.B.a.Sj.	F.B.e.Sj.	O.F.a. Sj.	O.F.e.Sj.	F.B.a.H.S.	F.B.e.H.S.	O.F.a.H.S.	O.F.e.H.S.
Chlorogenic acid	244.1±0.6^{g}	162.8±0.5^{e}	89.0±0.7^{d}	29.6±0.4^{c}	210.0±0.9^{f}	24.6±0.5^{b}	17.8±0.1^{a}	16.6±0.1^{a}
Hyperoside	410.7±0.6^{f}	412.2±0.1^{f}	176.5±0.3^{c}	112.4±0.1^{b}	340.4±0.3^{e}	318.9±0.4^{d}	24.7±0.0^{a}	35.0±8.6^{a}
Rutin	198.3±0.6^{h}	174.3±0.1^{g}	118.6±1.1^{f}	61.5±0.3^{c}	85.6±0.8^{d}	92.5±0.5^{e}	9.7±0.1^{a}	16.1±0.4^{b}
Spiraeoside	245.9±0.6^{g}	190.9±0.3^{e}	167.6±0.7^{d}	135.3±0.3^{c}	206.8±0.3^{f}	190.2±0.5^{e}	68.0±0.2^{b}	65.0±0.2^{a}
(–) -Epicatechin	198.7±0.8^{f}	113.6±0.1^{c}	125.0±0.9^{d}	88.1±2.4^{b}	135.6±0.2^{e}	89.7±0.4^{b}	5.6±0.1^{a}	7.7±0.1^{a}
Quercetin	72.5±0.3^{h}	41.2±0.1^{g}	24.8±0.5^{e}	18.1±0.1^{d}	27.2±0.3^{f}	4.7±0.2^{c}	3.2±0.0^{b}	1.8±0.0^{a}
Isoquercitrin	101.9±0.2^{h}	57.4±0.1^{f}	37.6±0.8^{e}	9.0±0.2^{c}	59.2±0.4^{g}	25.4±0.2^{d}	8.0±0.1^{p}	6.2±0.0^{a}
Procyanidin B2 dimer	55.2±0.2^{g}	49.7±0.1^{f}	12.7±0.8^{b}	17.0±0.2^{c}	39.0±0.2^{e}	29.8±0.2^{d}	2.9±0.0^{a}	3.3±0.0^{a}

DW: Dry Weight; Data are expressed as Means±SD of three measurements. In the same line, contents with same superscript letter(s) are not significantly different at p>0.05

Antioxidant capacities of *Crataegus* ethyl acetate extracts from floral buds and opened flowers: The $TEAC_{DPPH}$ values ranged from 317.6±12.2 to 1681.4±58.6 µmol/100 g DW and $TEAC_{ABTS}$ values from 966.2±10.4 to 2431.8±32.7 µ mol/100 g DW. The F.B.a.Sj. have the highest $TEAC_{DPPH}$ and $TEAC_{ABTS}$ values while, O.F. from *C. azarolus* var. *azarolus* developed in Sj. show weak free radical scavenging potentialities (Table 4). Both $TEAC_{DPPH}$ and $TEAC_{ABTS}$ assays show similar trend in antioxidant potentials (r = 0.99) (Table 5). There was a strong correlation between antioxidant activities and total phenol contents ($TEAC_{DPPH}$: r = 0.91; $TEAC_{ABTS}$: r = 0.89) and with total flavonoids contents ($TEAC_{DPPH}$: r = 0.88; $TEAC_{ABTS}$: r = 0.88).

Total proanthocyanidin contents influenced the antioxidant potency of the *Crataegus* ethyl acetate extract ($TEAC_{DPPH}$: r = 0.84; $TEAC_{ABTS}$: r = 0.83). Regression correlation coefficients also show important contribution in the antioxidant activity of the contents of each phenolic compound identified of the E.A. extract (i.e., for procyanidin B2 dimer, $TEAC_{DPPH}$: r = 0.85 and $TEAC_{ABTS}$: r = 0.86), for isoquercitin, $TEAC_{DPPH}$: r = 0.78; $TEAC_{ABTS}$: r = 0.99). Within the antioxidant capacities (according to $TEAC_{ABTS}$) of ethyl acetate extracts, we can classify the reproductive organs in the following order F.B.a.Sj.> F.B.e.Sj.> F.B.a.H.S.> F.B.e.H.S.> O.F.a.H.S.> O.F.e.Sj.> O.F.e.H.S. > O.F.a.Sj.

Table 4: Antioxidant activities, as assessed by DPPH· and ABTS·+ radicals, of *Crataegus* ethyl acetate extracts prepared from floral buds (FB) and opened flowers (OF) harvested on *Crataegus azarolus* var. *aronia* (a) and *C. azarolus* var. *eu-azarolus* (e) trees from Serdj (Sj.) and Hammam Sousse (HS) localities

	Antioxidant activity (µmol Trolox/100 g dry weight)	
Organ, variety and locality	$TEAC_{DPPH}$	$TEAC_{ABTS}$
F.B.a.Sj.	1681.4±58.6^{f}	2431.8±32.7^{g}
F.B.e.Sj.	1401.5±9.6^{e}	2267.7±22.7^{f}
O.F.a.Sj.	317.6±12.2^{a}	966.2±10.4^{a}
O.F.e.Sj.	605.5±19.4^{b}	1449.1±19.8^{c}
F.B.a.H.S.	967.6±7.4^{d}	1699.7±26.8^{e}
F.B.e.H.S.	893.7±11.4^{c}	640.7±48.3^{de}
O.F.a.H.S.	893.4±29.4^{c}	1608.5±70.8^{d}
O.F.e.H.S.	359.8±25.0^{a}	1055.8±61.3^{b}

DW: Dry weight; Data are reported as Mean±SD of three measurements; In the same column, contents with same superscript letter(s) are not significantly different at p>0.05

Table 5: Correlation coefficients (r) between $TEAC_{DPPH}$/$TEAC_{ABTS}$ and phenolic contents of the *Crataegus azarolus* ethyl acetate extracts and their individual phenolic compounds

	Correlation coefficients (r)	
Polyphenols	$TEAC_{DPPH}$	$TEAC_{ABTS}$
Total phenols	0.91	0.89
Total flavonoids	0.88	0.88
Total proanthocyanidins	0.84	0.83
Chlorogenic acid	0.72	0.69
Hyperoside	0.77	0.76
Rutin	0.70	0.70
Spiraeoside	0.65	0.63
Quercetin	0.76	0.74
(–) -Epicatechin	0.58	0.56
Isoquercitrin	0.78	0.99
Procyanidin B2 dimer	0.85	0.86
DPPH/ABTS	0.99	

DISCUSSION

The medicinal use of *Crataegus* has a long tradition with written records dating back to ancient Roman times (Veveris *et al*., 2004). The constituents of *Crataegus* have been the subject of intensive investigations for a long time (Shahat *et al*., 1995; Bahorun *et al*., 1996; Cui *et al*., 2006; Soko Ł-Łetowska *et al*., 2007) but intention has not been directed at *Crataegus azarolus*. In fact, a few studies were interested for the polyphenolic composition of *Crataegus azarolus* and its antioxidant activity (Ker'y *et al*., 1987; Twaij *et al*., 1987; Ljubuncic *et al*., 2005). Present results show that ethyl acetate extracts obtained from *Crataegus azarolus* var. *aronia* (with yellow fruits) are richer in polyphenols than those from *Crataegus azarolus* var. *eu-azarolus* (with red fruits). Whatever was the variety and the region of harvest, the floral buds are richer in polyphenols than opened flowers,

this is in accordance with results reported by Bahorun *et al*. (1994) on *Crataegus monogyna*. Nevertheless, *Crataegus azarolus* ethyl acetate extract present lower contents in total phenols, total flavonoids and total proanthocyanidins in regard to the same extract prepared from *Crataegus monogyna* (Bahorun *et al*., 1994). This difference could be attributed to genotype (different species), locality and/or to extraction protocol.

HPLC analysis of ethyl acetate extracts of *Crataegus azarolus*, demonstrate the presence of chlorogenic acid as the main phenolic acid. It's in accordance with studies of Bahorun *et al*. (1994) and of Urbonaviciute *et al*. (2006) realized on *Crataegus monogyna* and with those done by Zhang *et al*. (2001), Cai *et al*. (2004, 2006) on *Crataegus pinnatifida.* Ethyl acetate *Crataegus azarolus* extract present lower amount in this phenol acid compared with other species, such as *C. monogyna* which present 322 mg/100 g DW chlorogenic acid in floral buds and 69 mg/100 g DW in opened flowers (Bahorun *et al*., 1994). Contrast results in amount of this phenolic acid in *C. pinnatifida* fruits were identified. In fact, Zhang *et al*. (2001) demonstrate the presence of 64.9 mg/100 g DW, when Cui *et al*. (2006) suggest the presence of 1210 mg/100 g DW.

Hyperoside is the main flavonoid detected in ethyl acetate reproductive organ extracts of *Crataegus azarolus* for the two varieties. This is in accordance with of all studies in hawthorn species (Bahorun *et al*., 1994; Zhang *et al*., 2001; Cai *et al*., 2004; Cui *et al*., 2006; Urbonaviciute *et al*., 2006). According to Bahorun *et al*. (1994), ethyl acetate extract of *Crataegus monogyna* floral buds present 547 mg/100 g DW hyperoside. This amount is higher than this found in this study (maximum of 410.7±0.6 mg/100 g DW). On the other hand, ethyl acetate extract of *Crataegus pinnatifida* fruits contains 24.6 mg/100 g DW (Zhang *et al*., 2001) and 280 mg/100 g DW hyperoside (Cui *et al*., 2006). Rutin and spiraeoside were detected with higher amounts than in other species of *Crataegus* such as *C. monogyna* and *C. pinnatifida*. In fact, Zhang *et al*. (2001), demonstrate the presence of 2.6 mg/100 g DW rutin in ethyl acetate fruit extract.

The dimer procyanidin B2 and (–)-epicatechin are the main catechins detected in *Crataegus azarolus* ethyl acetate extracts. It's in accordance with earlier studies on ethyl acetate extract of *Crataegus monogyna* (Bahorun *et al*., 1994; Urbonaviciute *et al*., 2006), *Crataegus pinnatifida* (Zhang *et al*., 2001; Cai *et al*., 2004; Cui *et al*., 2006), *Crataegus laevigata* (Svedstrom *et al*., 2006) and *Crataegus oxyacanthus* (Svedstrom *et al*., 2006; Sokoł-Łetowska *et al*., 2007). Amounts of (–)-epicatechin and procyanidin B2 dimer in ethylacetate extract of *C. monogyna* floral buds were 884 mg/100 g DW and 135 mg/100 g DW respectively, whereas opened flowers contains 157 mg/100 g DW and 44 mg/100 g DW (Bahorun *et al*., 1994). Ethyl acetate extract of *Crataegus pinnatifida* fruits contains 178.3 mg/100 g DW according to Zhang *et al*. (2001) but according to Cui *et al*. (2006) this extract contains 9860 mg/100 g DW of (–)-epicatechin and 5900 mg/100 g DW of procyanidin B2 dimer.

We have observed rapid and strong inhibition of both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals after the addition of ethyl acetate extracts from floral buds and opened flowers of *Crataegus azarolus* showing a high antioxidant activity of those extracts. Floral buds present the higher antioxidant activity. This is in accordance with Bahorun *et al*. (1994) study, where authors demonstrate that floral buds present the higher percent of inhibition of malondialdehyde formation compared to opened flowers. Ljubuncic *et al*. (2005) demonstrated that a decoction of leaf and green fruit of *Crataegus azarolus* var. *aronia* inhibit β-carotene and plasmatic oxidation, lipidic peroxidation and scavenged the radical $O_2^{\bullet-}$. Zhang *et al*. (2001) demonstrated the inhibition of Low Density Lipoprotein (LDL) peroxidation with ethyl acetate fraction of *Crataegus pinnatifida* fruits. Cui *et al*. (2006) demonstrated the oxygen radical scavenging capacity and enzyme inhibition of ethyl acetate fraction from *Crataegus pinnatifida* fruits. We demonstrate that ethyl acetate extract from *Crataegus azarolus* var. *aronia* floral buds (F.B.a.) present a high antioxidant capacity (1681.4±58.6 µmol Trolox/100 g DW) compared to tomato (from 5.4 to 20.9 µmol Trolox/g DW), to potato (from 2.3 to 9.9 mol Trolox/g DW) (Zhou and Yu, 2006) and to lettuce (1.14 µmol Trolox/g DW) extracts (Luximon-Ramma *et al*., 2005). According to those researchers, fresh tea leaves extract present an antioxidant activity equivalent to 1637 µmol Trolox/g DW. Ruberto *et al*. (2007) studies suggest similar antioxidant activity (1.58 mmol Trolox/g DW) for Nero d'Avola grape pomace extract.

Strong antioxidant capacity of *Crataegus azarolus* EA extracts has been related mainly to chlorogenic acid and hyperoside (Bahorun *et al*., 1994; Zhang *et al*., 2001), to (–)-epicatechin and procyanidin B2 dimer (Bahorun *et al*., 1994; Zhang *et al*., 2001; Cui *et al*., 2006) and to rutin, quercetin and isoquercitrin (Zhang *et al*., 2001). Ethyl acetate extract of Tunisian

Crataegus azarolus presents all this compounds and we demonstrate a strong correlation with all this compounds and the antioxidant capacity of this extract.

In conclusion, the present study supported the view that hawthorn contains antioxidants of phenolic type. The HPLC analysis led to identify eight antioxidants (chlorogenic acid, hyperoside, rutin, spiraeoside, isoquercitrin, quercetin, (-)-epicatechin and the dimer procyanidin B2). These compounds presented a strong radical-scavenging activity. Floral bud extracts could be used in clinical trials to study the modulation of risk factors of cardiovascular diseases, diabetes, cancer and neurodegenerative diseases.

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