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Phytochemical characterization and antioxidant activities of the fruit extracts of several *Crataegus* taxa

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ABSTRACT

This study was designed to comparatively evaluate the phytochemical composition and antioxidant potentials of fruit extracts from five *Crataegus* taxa (*C. monogyna*, *C. orientalis*, *C. pontica*, *C. rhipidophylla* and *C. turcicus*) growing wild in the flora of Turkey. Antioxidant activities of the samples were investigated using DPPH radical scavenging, FRAP, CUPRAC and total antioxidant capacity tests. Total phenol, phenolic acid, flavonoid, and proanthocyanidin contents were measured spectrophotometrically. Additionally, the presence of some phenolic compounds in the extracts was investigated by LC–MS/MS. Furthermore, the extracts used for the antioxidant activity studies were standardized for chlorogenic acid and hyperoside by HPTLC densitometry. Among the tested extracts, *C. turcicus* contained the highest phenolic (398.48 ± 0.98 GAE/g extract) and total flavonoid contents (23.87 ± 2.74 QE/g extract) content while *C. monogyna* contained by far the highest total proanthocyanidin (175.65 ± 10.59 EGCG-E/g extract), hyperoside (0.42%) and chlorogenic acid (0.90%) contents. *Crataegus monogyna* exhibited the highest antioxidant activity in FRAP, CUPRAC, and TOAC tests. Good correlation was found between the phenolic content and antioxidant parameters. This is the first report related with comparison of these five species in respect to phenolic profile and antioxidant activity.

1. Introduction

Crataegus species (Maloidea subfamily in Rosaceae) are widely distributed in Asia, Europe and America. The genus *Crataegus* is represented around 200 species including the hybridized species. (Kumar et al., 2012; Edwards et al., 2012). It is represented by 21 species in Turkey and ongoing studies shows that there are more taxa and all taxa are known as "alıç" (Dönmez, 2004). *Crataegus* (hawthorn) species are widely used as a remedy for hypertension, angina, arrhythmia, congestive heart failure, in different traditional systems of medicine. Moreover, in Traditional Chinese Medicine, hawthorn fruits are used to improve circulation, remove blood stasis, to treat indigestion, diarrhea, abdominal pain, hyperlipidemia and hypertension. In Europe, fruits, leaves and flowers of hawthorn species are used in the treatment of heart problems due to their antispasmodic, cardiotonic, hypotensive and antiatherosclerotic activities (Edwards et al., 2012). Furthermore, the fruits are traditionally used against intestinal problems and as a diuretic agent in Turkey (Baytop, 1999). In addition to their traditional utilization, some *Crataegus* species are included in several monographs, especially due to their positive inotropic activity and roles in the activation of heart muscle cells (German Commission E Monographys, 1998). For instance, the leaves together with flowers are approved in decreasing cardiac output in German Commision E Monographs. Hawthorn extract significantly augments the maximal workload and exercise tolerance whilst alleviating pressure-heart rate product (Edwards et al., 2012; Guo et al., 2008).

Current studies supporting the traditional usage of hawthorn species revealed that different parts of *Crataegus* species have radical scavenging, anti-lipoperoxidant antiviral, anti-inflammatory, antimicrobial, gastroprotective, antihyperlipidemic, hypoglyceamic, hepatoprotective and immunostimulant activities (Kumar et al., 2012; Meriçli and Melikoglu, 2002; Zheng et al., 2018). The bioactivities of hawthorn fruits, leaves and flowers were reported to be associated with their phenolic composition, particularly proanthociyanidins and flavonoid glycosides (Edwards et al., 2012).

Oxidative stress leads to the formation of free radicals which are extremely hazardous for the living organisms, causing detrimental diseases including cardiovascular diseases, cancer and diabetes (Valko et al., 2007). On the contrary, antioxidants are useful tools to prevent the unwanted effects of free radicals and lead to a reduction of the risk of some acute and chronic diseases related to the redox state of the human body. Due to the limited sources for antioxidants along with their importance, studies on medicinal plants and their usage as

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antioxidants gained importance in food, cosmetics and pharmaceutical industry. In addition, the antioxidant properties of plant extracts have been attributed to their phenolic contents. Many investigations have been performed since the early 1870s on the beneficial effects of phenolic compounds as natural antioxidants with well over 150,000 research papers related to antioxidants (Ndhala et al., 2010).

In the current study, LC–MS/MS is also used for the assignment of the phenolic compounds in addition to several conventional *in vitro* methods (total phenolic content, total phenolic acid content, total flavonoid content, total proanthocyanidin content). LC–MS/MS is the method choice with proven sensitivity and selectivity in plant metabolomics analyses. LC–MS/MS can be very extensive and thousands of metabolites can be scanned from complex plant extracts by the use of high resolution instruments (Ganzera and Sturm, 2018), or predefined metabolites can be analyzed in MRM scan mode, highly selective analytical strategy (Lu et al., 2008), used commonly in quantification of small molecules. Herein, we have adapted MRM for the identification of phenolic compounds in a targeted fashion. In the prospect of quantification of hyperoside and chlorogenic acid, HPTLC technique is preferred due to its being an analytical tool used in the plant metabolomics studies as well as its advantages of generating visual (compound specific colored) data, low running cost, high versatility, high throughput, flexibility, accuracy and reproducibility (Reich and Schibli, 2007).

Referring the importance and uses of hawthorn, as well as its industrial importance, this study was planned to evaluate phenolic profile, antioxidant activity and phytochemical composition of five *Crataegus* taxa, one of which is endemic (*C. turcicus*) from Turkish flora by using several *in vitro* methods and by a newly developed LC–MS/MS technique and HPTLC method.

2. Materials and methods

2.1. Chemicals

All chemicals, enzymes and references used in the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The quality of all chemicals was of analytical grade. Standards used in optimization of LC–MS/MS parameters were also purchased from Sigma except isoorientin, verbascoside, leucoseptoside A and martynoside which were previously isolated with a purity grade of >95% by our study group (Kırmızıbekmez et al., 2004; Bardakci et al., 2015; Kırmızıbekmez et al., 2018).

2.2. Plant samples

The fruits of *C. monogyna* ssp. *monogyna* (*C. monogyna* Jacq.), *Crataegus orientalis* Pall. Ex. M. Bieb. subsp. *orientalis* (*C. orientalis* Pallas ex Bieb.), *Crataegus azarolus* var. *pontica* C. Koch. (*C. pontica* C. Koch.), *C. rhipidophylla* Gand., and an endemic species *C. turcicus* Dönmez were gathered from town of Ardanuç (Artvin/Turkey) on October 19, 2015 at the same altitude. The authentication process of the plant samples was performed by one of us (YK). Voucher specimens were stored at Artvin Coruh University Herbarium as ARTH 13584, ARTH 13585, ARTH 13583, ARTH 13586 and ARTH 13587, respectively.

2.3. Preparation of plant extracts

The fruits (50 g each) were washed to remove contaminants and their seeds were separated. The remaining pulps were blended and macerated in EtOH (250 mL) at room temperature for one month. After filtration the extracts were evaporated to dryness under reduced pressure. The residue was dispersed in water and lyophilized. The extracts were protected from any possible oxidation by being stored in air-tight, aluminum foil covered amber colored flasks. The extract yields of *C. monogyna*, *C. orientalis*, *C. pontica*, *C. rhipidophylla* and *C. turcicus* were calculated as 6.6%, 9.0%, 8.5%, 12% and 8.7%, respectively.

2.4. Quantitative assessment of phenolic profile

2.4.1. Total phenolic content

The assessments of total phenolic content of the samples were performed according to the method Singleton and Rossi (1965). Diluted samples were inserted into the mixture of Na_2CO_3 (20%) and Folin Ciocalteu reagent (diluted with H_2O). At the end of the incubation period at 45 °C, the absorbance of the mixtures was measured at 765 nm. The results were expressed as gallic acid equivalents (GAE) per g dried extract (DE).

2.4.2. Total flavonoid content

Total flavonoid content of the samples were calculated according to the method described by Celep et al. (2012). Sample dilutions were combined with the mixture of $AlCl_3$ and sodium acetate, and left for incubation at room temperature for 30 min. The absorbance was read at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE) per g DE.

2.4.3. Total phenolic acid content

The total phenolic acid content of the samples was measured spectrophotometrically at 490 nm (Mihailovic et al., 2016). This method relies on the formation of a complex due to the interaction of sodium nitrite–sodium molybdate with phenolic acids. The results were estimated as caffeic acid equivalents (CAE) in per g DE.

2.4.4. Determination of total proanthocyanidin content

The total proanthocyanidin content of each extract was estimated using the method described by Ariffin et al. (2011). According to the method, 2.5 mL of vanillin (1%) in MeOH and 2.5 mL of 9 M HCl in MeOH were added to 1 mL of each extract. After incubation at 30 °C for 20 min, the absorbance was measured at 500 nm. Results were expressed as mg epigallocatechingallate equivalents (EGCG-E) per g DE.

2.5. LC/ESI-MS-MS analysis of phenolic compounds

Phenolic compounds in *Crateagus* fruit extracts were assigned and detected by Agilent 1260 infinity series HPLC system consisting of a vacuum degasser, a binary pump, an autosampler and directly connected to Agilent 6420 triple quadrupole mass spectrometer with electrospray ion source (ESI). Agilent Mass Hunter software program was used for data analysis and acquisition. 5–10 μg/mL individual solutions of phenolic compounds are prepared by dissolving powder of pure standard in methanol. Mixture solution of phenolic compounds with concentration of 5–10 μg/mL for each compound was prepared separately. Both individual solutions and mixture solution were used for optimization of instrument parameters.

Solutions are injected directly to the ion source of the mass spectrometer without using column for optimization of ion source and tandem mass spectrometer parameters. The mass range was adjusted to 10–1200 m/z for mass scan in negative ion mode for the generation of singly charged ions of deprotonated phenolic compounds, $[M\text{-}H]^-$. Optimized ion source parameters were as follows: 250 °C (N_2 drying gas temperature), 11 l/min (drying gas flow), 40 psi (nebulizer) and 4000 V (capillary voltage).

Fragmentation of the precursor ions in product ion (MS/MS) and multiple reaction monitoring (MRM) scan modes have been achieved by collision-induced dissociation (CID) using N_2 as the collision gas. MS/MS scans of the observed deprotonated ions, $[M–H]^-$ were performed for observation of the most intense fragment ions and establishing the precursor and product ion pairs *i.e.* MRM transitions, for each compound. Then, collision energy (CE) and the fragmentor voltage (FV) of the MRM transitions were optimized.

The combination Inertsil ODS column (Zorbax Eclipse 4.6 × 150 mm i.d., 3.5 μm particle size) from Agilent with gradient flow using mobile phase A:MeOH:H_2O:formic acid (FA) (10:89:1, *v*/*v*/*v*) and mobile

phase B:MeOH:H_2O:FA (89:10:1, *v*/*v*/*v*) were used for separation of phenolic compounds (Agalar et al., 2018). Flow rate was adjusted to 0.4 mL/min. The gradient started with 0% B and then changed to 10% B within 5 min and then increased to 100% B in 45 min and kept there for 5 min. All the compounds eluted within 38 min and the total run was lasted 50 min, including column washing. The column is flushed with 10 mL MeOH and conditioned with the initial composition of the mobile phase for at least 5 min after each sequence. Column temperature was not controlled, and room temperature was kept constant. Injection volume was optimized as 10 μL, since higher volumes caused peak distortions significantly.

2.6. HPTLC quantification of hyperoside and chlorogenic acid

Hyperoside and chlorogenic acid contents of plant samples were measured by using the previously published method Cretu et al. (2013). The standard solutions of hyperoside and chlorogenic acid (31.25 μg/mL) were prepared in MeOH and 100 mg of total extract and each fraction were dissolved in 10 mL MeOH. Each extract were filtered through a 0.45 μm syringe filter. 5 μL of *C. monogyna* and *C. pontica*, 8 μL of other extracts and 2–8 μL of standard hyperoside solution were applied in triplicate. In addition 5 μL of *C. monogyna*, 10 μL of other extracts and 2–8 μL of standard chlorogenic acid solution were applied in triplicate. The sample and standard solutions were spotted in the form of bands with 8 mm length on silica gel glass HPTLC plates $60F_{254}$ with Camag Automatic TLC Sampler IV. A constant application rate was applied and the spaces between the tracks were 10 mm. The mobile phase was ethyl acetate (EtOAc):acetic acid (AA):FA:H_2O 10:1.1:1.1:2.3 (*v*/*v*/*v*/*v*). Developments were carried out in Camag Automatic Developing Chamber (ADC-2). Chamber was saturated for 20 min and the plate was preconditioned for 5 min before the development. The humidity is controlled by ADC-2 using MgCl2 (33% RH) for 10 min. Plate was sprayed with Natural product Reagent A (2-aminoethyldiphenylborinate reagent) reagent using Camag Derivatizer and then heated for 5 min by using Camag plate heater at 100 °C after development of 70 mm. Densitometric screening was performed by using Camag TLC Scanner IV and VisionCATS software in fluorescence mode after derivatization. The slit dimension was kept at 5 × 0.2 mm, micro and the scanning speed was set at 20 mm/s. Standard contents were afforded by comparing AUCs with the calibration curve of standards. The coefficient of variation (CV%) is under 1.00 and the correlation coefficient (R) of the calibration curve was above 0.998. The presence of standards in extracts was assured by comparison of both retention factors (R*f*) and overlaying UV spectra of each extract and standards.

2.7. Estimation of antioxidant activity based on free radical-scavenging activity

2.7.1. DPPH radical-scavenging activity

DPPH radical-scavenging activity test was conducted according to the method defined earlier by Celep et al. (2013). Freshly diluted samples were separately mixed with 100 μM methanolic DPPH solution. The mixture was kept at room temperature in the dark, and the absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as reference compound.

2.8. Estimation of antioxidant activity based on metal-related activity

2.8.1. Cupric reducing antioxidant capacity (CUPRAC)

Total determination of CUPRAC activity of the samples was performed according to the modified method of Apak et al. (2004). Same volumes of neocuproine, ammonium acetate buffer and $CuSO_4$ were separately mixed. After the addition of samples, the mixture was incubated for 1 h, and the absorbance was measured at 450 nm. The results were expressed as mg ascorbic acid equivalent (AAE) per g DE.

2.8.2. Ferric reducing antioxidant power (FRAP)

The spectrophotometric method applied was described previously by Benzie and Strain (1996). Properly diluted samples were mixed with FRAP reagent and the volume was adjusted to 0.3 mL. After incubating 30 min, the absorbance was read at 593 nm. BHT was used as reference compound. The results were expressed as mM $FeSO_4$ per g DE.

2.8.3. Determination of total antioxidant capacity by phosphomolybdenum method

The method described by Prieto et al. (1999) was employed for the determination of total antioxidant capacity. Sample solutions were added to the reaction mixture composed of ammonium molybdate, sulfuric acid and sodium phosphate monobasic. Following the incubation at 95 °C for 1.5 h, the absorbance was read 695 nm. Total antioxidant capacity was expressed as mg AAE per g DE.

2.9. Statistics

All tests and analyses were performed in triplicate. The mean ± standard deviation was measured according to the results in each test. These results were statistically compared with ANOVA test. The multiple comparisons were achieved by Tukey–Kramer *post hoc* test. Statistically significant difference was defined as $p < .05$.

3. Results and discussion

3.1. Quantitative evaluation of phenolic profile of Crataegus samples

Total phenolic, total phenolic acid, flavonoid and proanthocyanidin contents of five *Crataegus* taxa were shown in Table 1. *Crataegus monogyna* obviously showed the highest, while *C. orientalis* showed the lowest total proanthocyanidin content compared to other four species. On the other hand, *C. pontica* exhibited highest total phenolic acid content among the others. In terms of total phenolic and flavonoid contents, results were quite similar with each other.

In the total extract and EtOAc fraction prepared from *C. monogyna* dried fruits prepared by Froehlicher et al. (2009), total phenolic content was found to be 1282.3 ± 76.1 and 360.3 ± 8.7, respectively. Besides, total flavonoid content was found as 103.0 ± 3.4 in EtOAc fraction and not detected in total extract, similarly total procyanidin content was

Table 1
Spectrophotometric determination of phenolic profile of *Crataegus* spp.

Analysis	*C. monogyna*	*C. orientalis*	*C. pontica*	*C. rhipidophylla*	*C. turcicus*
Total phenolic content[A]	391.97 ± 2.30^{a}	380.93 ± 3.78^{b}	361.39 ± 3.78^{c}	360.81 ± 2.30^{c}	398.48 ± 0.98^{a}
Total flavonoid content[B]	17.60 ± 1.41^{abc}	20.05 ± 2.23^{b}	18.78 ± 3.09^{abcd}	13.69 ± 0.51^{c}	23.87 ± 2.74^{d}
Total phenolic acid content[C]	187.33 ± 7.07^{a}	114.56 ± 5.09^{c}	207.33 ± 2.36^{d}	199.00 ± 9.43^{ad}	169.00 ± 4.71^{b}
Total proanthocyanidin content[D]	175.65 ± 10.59^{a}	31.73 ± 1.36^{b}	16.24 ± 1.56^{c}	54.67 ± 4.72^{d}	59.76 ± 4.81^{d}

a–e Different letters in the same row indicate significance ($p < .05$).
[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg quercetin equivalents (QE) in 1 g sample.
[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg caffeic acid equivalents (CAE) in 1 g sample.
[D] Total proanthocyanidin content was expressed as mg epigallocathecin gallate equivalents (EGCG-E) per g dried extract ± SD.

Table 2
Retention times, MRM transitions and optimized fragmentor voltage (FV) and collision energy (CE) of phenolic compounds.

No	Compound	Molecular Weight	Retention Times	MRM transitions	FV	CE
1	*p*-coumaric acid	164.16 g/mol	21.89 min	163 m/z → 119 m/z	90 V	10 V
2	Gallic acid	170.12 g/mol	7.99 min	169 m/z → 119 m/z	110 V	10 V
3	Caffeic acid	180.159 g/mol	17.88 min	179 m/z → 135 m/z	120 V	10 V
4	Ferulic acid	194.186 g/mol	22.04 min	193 m/z → 134 m/z	100 V	15 V
5	Apigenin	270.240 g/mol	33.82 min	269 m/z → 117 m/z	150 V	30 V
6	Naringenin	272.256 g/mol	30.82 min	271 m/z → 151 m/z	140 V	15 V
7	CAPE	284.311 g/mol	38.10 min	283 m/z → 179 m/z	160 V	10 V
8	Catechin	290.271 g/mol	13.96 min	289 m/z → 245 m/z	130 V	5 V
9	Epicatechin	290.271 g/mol	17.65 min	289 m/z → 245 m/z	150 V	10 V
10	Quercetin	302.238 g/mol	30.53 min	301 m/z → 151 m/z	150 V	15 V
11	Chlorogenic acid	354.311 g/mol	15.39 min	353 m/z → 191 m/z	100 V	10 V
12	Rosmarinic acid	360.318 g/mol	26.02 min	359 m/z → 161 m/z	100 V	10 V
13	Apigenin-7-*O*-glucoside	432.381 g/mol	26.04 min	431 m/z → 268 m/z	200 V	30 V
14	Vitexin	432.381 g/mol	22.56 min	431 m/z → 311 m/z	130 V	15 V
15	Quercitrin	448.38 g/mol	26.72 min	447 m/z → 301 m/z	160 V	15 V
16	Luteolin-7-*O*-glucoside	448.38 g/mol	23.91 min	447 m/z → 285 m/z	190 V	20 V
17	Isoorientin	448.380 g/mol	21.29 min	447 m/z → 327 m/z	170 V	15 V
18	Hyperoside	464.379 g/mol	24.61 min	463 m/z → 300 m/z	170 V	25 V
19	Rutin	610.521 g/mol	24.62 min	609 m/z → 151 m/z	210 V	25 V
20	Verbascoside	624.592 g/mol	24.26 min	623 m/z → 161 m/z	210 V	25 V
21	Leucoseptoside A	638.619 g/mol	24.22 min	637 m/z → 461 m/z	240 V	20 V
22	Martynoside	652.646 g/mol	27.13 min	651 m/z → 175 m/z	230 V	25 V

found to be 96.8 ± 4.8 and 9.0 ± 0.6 in total extract and in EtOAc fraction, respectively (results are given as mg related compound/100 g DE). Plitszka et al. (2016) prepared aqueous citric acid and 80% MeOH extract from *C. monogyna*, and calculated phenolic contents as 913.0 ± 41.8 and 602.7 ± 17.0 mg GAE/100 g DE, respectively. Moreover, Cosmulescu et al. (2017), calculated total phenolic content as 203.01 ± 9.56 mg GAE/100 g fresh weight, total flavonoid content was calculated as 31.37 ± 1.67 mg QE/100 g fresh weight in alcoholic *C. monogyna* fruit extract.

There are only a few studies on *C. orientalis* var. *orientalis*. Bor et al. (2012) reported the total phenolic content as 98.24 ± 2.4 mg GAE/1 g dried extract (DE) in EtOH extract and Çalışkan et al. (2012) expressed total phenolic content of *C. orientalis* fruits 51.2 mg GAE/g DE in 60% MeOH extract.

3.2. LC/ESI-MS–MS analysis of phenolic compounds and HPTLC quantification of hyperoside and chlorogenic acid

Compounds eluted at very close retention times were identified by their distinct MRM transitions. Isobaric compounds (compounds with identical m/z) have been distinguished by either difference in their retention times or product ions or both. Catechin and epicatechin eluted at significantly different retention times (13.96 and 17.65 min, respectively). Apigenin-7-*O*-glucoside and vitexin with m/z 431 were identified based on their retention time at 26.04 and 22.56 min, and MRM transitions of 431 m/z → 268 m/z and 431 m/z → 311 m/z, respectively. Luteolin-7-*O*-glucoside, isoorientin and quercitrin with m/z 447 eluted at 21, 22 and 26 min with MRM transitions of 447 m/z → 285 m/z and 447 m/z → 327 m/z and 447 m/z → 301 m/z, respectively. As a result,

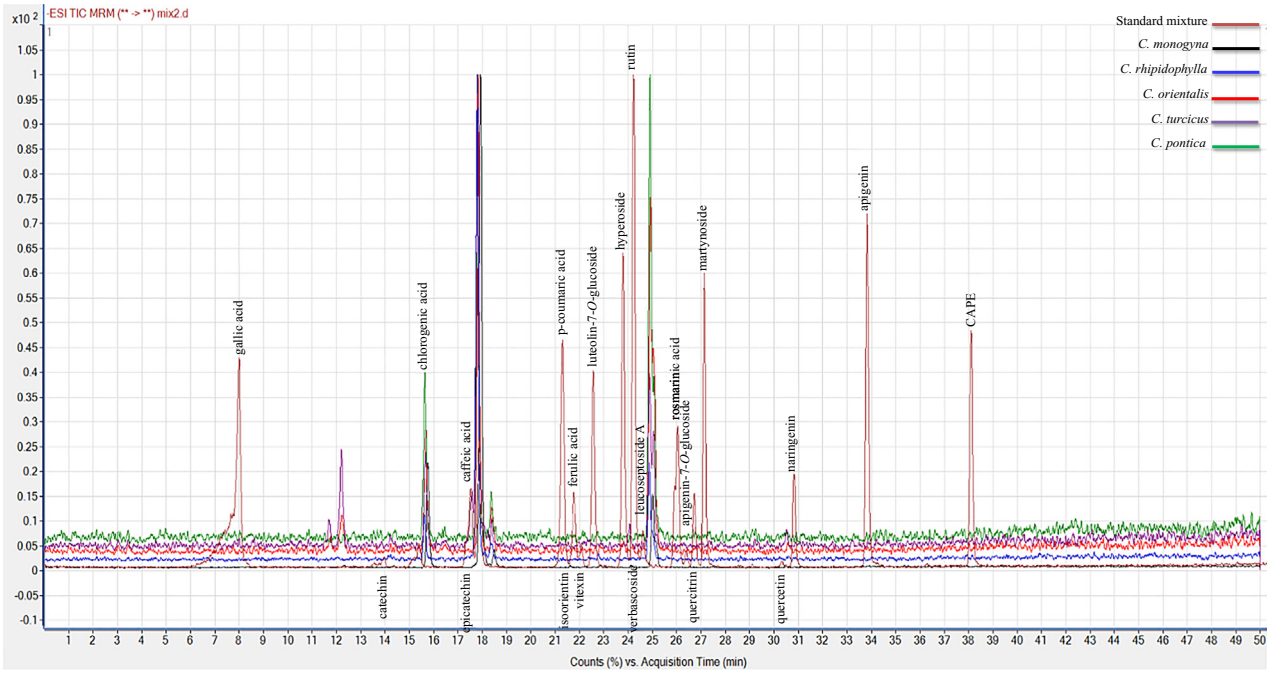

Fig. 1. Overlapped MRM profiles from LC–MS/MS analysis of standard compounds and *Crataegus* extracts.

Table 3
Phenolic profiling of *Crataegus* spp. (100 μg/mL) by ESI- LC–MS/MS.

No	Bileşik/Bitki*,**	*C. monogyna*	*C. orientalis*	*C. pontica*	*C. rhipidophylla*	*C. turcicus*
1	*p*-coumaric acid	–	–	–	–	–
2	Gallic acid	–	–	–	–	–
3	Caffeic acid	–	–	–	–	–
4	Ferulic acid	–	–	–	–	–
5	Apigenin	–	–	–	–	–
6	Naringenin	–	–	–	–	–
7	CAPE	–	–	–	–	–
8	Catechin	–	–	–	–	–
9	Epicatechin	+	+	+	+	+
10	Quercetin	Trace	Trace	Trace	Trace	Trace
11	Chlorogenic acid	+	+	+	+	+
12	Rosmarinic acid	–	–	–	–	–
13	Apigenin-7-*O*-glucoside	trace	–	–	–	–
14	Vitexin	+	Trace	–	Trace	Trace
15	Quercitrin	–	–	–	–	–
16	Luteolin-7-*O*-glucoside	+	–	–	–	+
18	Isoorientin	+	–	–	–	trace
19	Hyperoside	+	+	+	+	+
20	Rutin	–	–	–	–	–
21	Verbascoside	–	–	–	–	–
22	Leucoseptoside A	–	–	–	–	–
23	Martynoside	–	–	–	–	–

* Cut off point 10^3 (intensity, cps).
** Peaks under cut off point were evaluated as trace.

combination of retention time and MRM transitions characteristic to each compound facilitated their identification from plant material with confidence (Table 2 and Fig. 1). Although phenylethanoid glycosides were not previously detected in *Crataegus* sp., extensively found phenylethanoid glycosides in many medicinal plants, presence in *Crataegus* sp. were also investigated by LC–MS/MS. Results were consistent with the previous works in terms of phenylethanoid glycosides though they are not detected in *Crataegus* samples. Phytochemical characterization showed that extracts of five *Crataegus* species commonly contain epicatechin, quercetin, chlorogenic acid, and hyperoside. Similarly, whole extracts contain vitexin except for *C. pontica*. LC–MS/MS results of extracts are shown in Table 3 and Fig. 1.

Hyperoside and chlorogenic contents of each extract were quantified by using HPTLC. The R*f* values were found to be 0.46 and 0.51 for hyperoside and chlorogenic acid (Figs. 2–3) respectively. The authentication of standard compounds in plant samples were verified by comparison of R*f* values and overlapping their UV spectra (Figs. 2, 3, 4, 5). Quantification of standards were afforded by comparing AUCs with the calibration curve of standards.

HPTLC analysis revealed that *C. monogyna* contained by far the highest hyperoside (0.42%) and chlorogenic acid (0.90%) (*w*/*w*%) contents among the extracts. *C. turticus*, *C. rhipidophylla*, *C. orientalis* and *C. pontica* contain hyperoside in ascending order, respectively. Similarly, *C. rhipidophylla*, *C. pontica*, *C. turcicus* and *C. orientalis* contain chlorogenic acid in ascending order. The results of HPTLC study was given in Table 4.

Phenolic composition of *C. monogyna* has been investigated previously by several researchers (Froehlicher et al., 2009; Plitszka et al., 2016; Cosmulescu et al., 2017; Orhan et al., 2007; Prinz et al., 2007; Bernatoniene et al., 2008; Ruiz-Rodriguez et al., 2013). Orhan et al. (2007), quantified hyperoside in *C. monogyna* fruit samples from Turkey. Prinz et al. (2007) quantified vitexin, rutin and hyperoside in *C. monogyna* fruit extract from Austria. Bernatoniene et al. (2008) reported chlorogenic acid, hyperoside, rutin, quercetin, vitexin-2″-*O*-rhamnoside, epicatechin, catechin and procyanidin B_2 in both aqueous and ethanolic extracts of *C. monogyna* from Lithuania. Epicatechin and catechin were detected as the major antioxidant substances by Froehlicher et al. (2009) which are also detected in all five samples in present study. Froehlicher et al. (2009) quantified chlorogenic acid, hyperoside, caffeic acid and epicatechin in *C. monogyna* fruit extract from France. Ruiz-Rodriguez et al. (2013) detected gallic acid, chlorogenic acid, epicatechin, quercetin 3,4-diglucoside, quercetin 3,7,4-triglucoside and cyanidine 3-galactoside in samples from Spain. Also, Cosmulescu et al. (2017) recently quantified gallic acid, catechin hydrate, vanillic acid, chlorogenic acid, caffeic acid, syringic acid, epicatechin, coumaric acid, ferulic acid, sinapic acid, salicylic acid, rutin, elagic

Fig. 2. Densitometric chromatogram of hyperoside and *Crataegus* extracts (CM: *C. monogyna*/CO: *C. orientalis*/CP: *C.pontica*/CR: C. *rhipidophylla*/CT: *C. turcicus*).

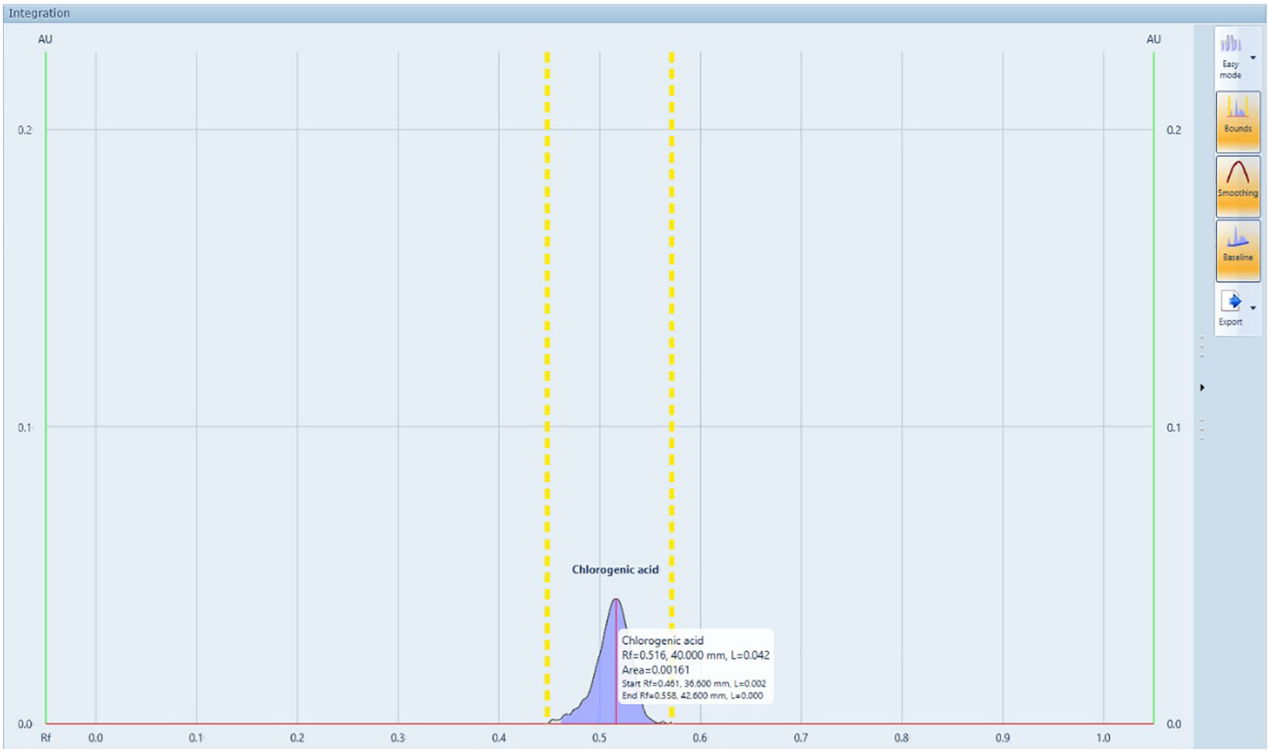

Fig. 3. HPTLC chromatogram of chlorogenic acid.

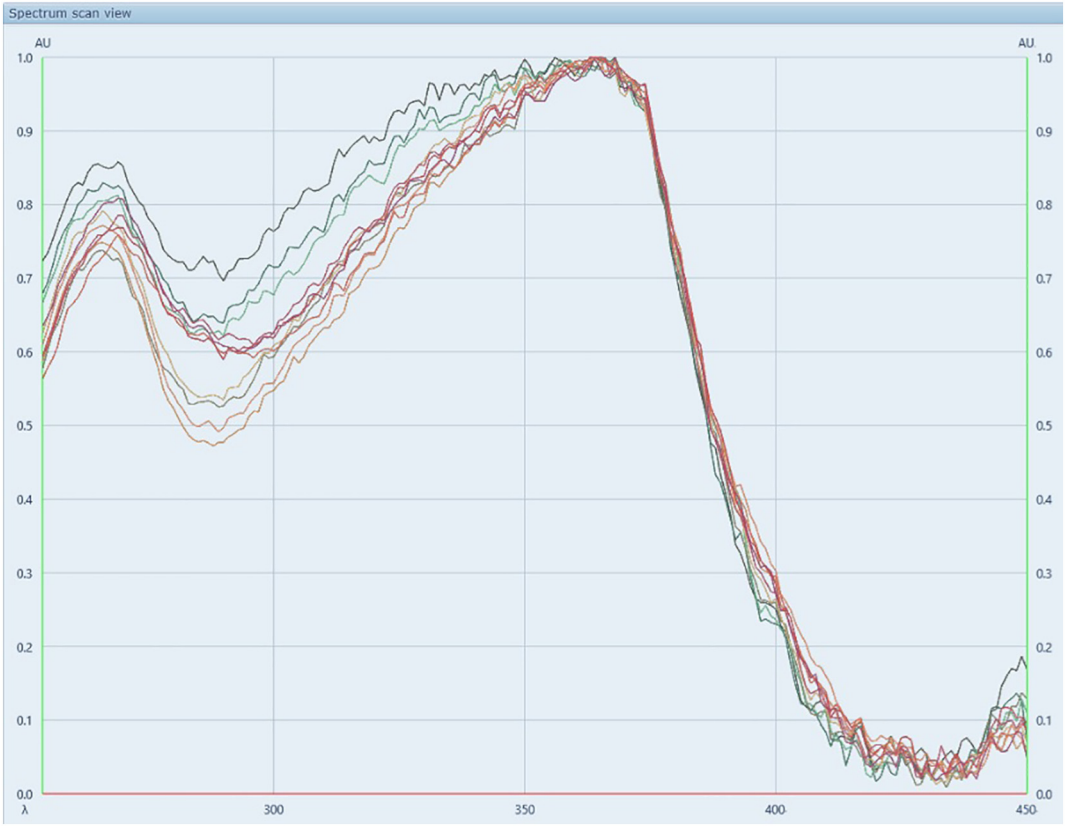

Fig. 4. Overlapped UV spectra of hyperoside and *Crataegus* extracts.

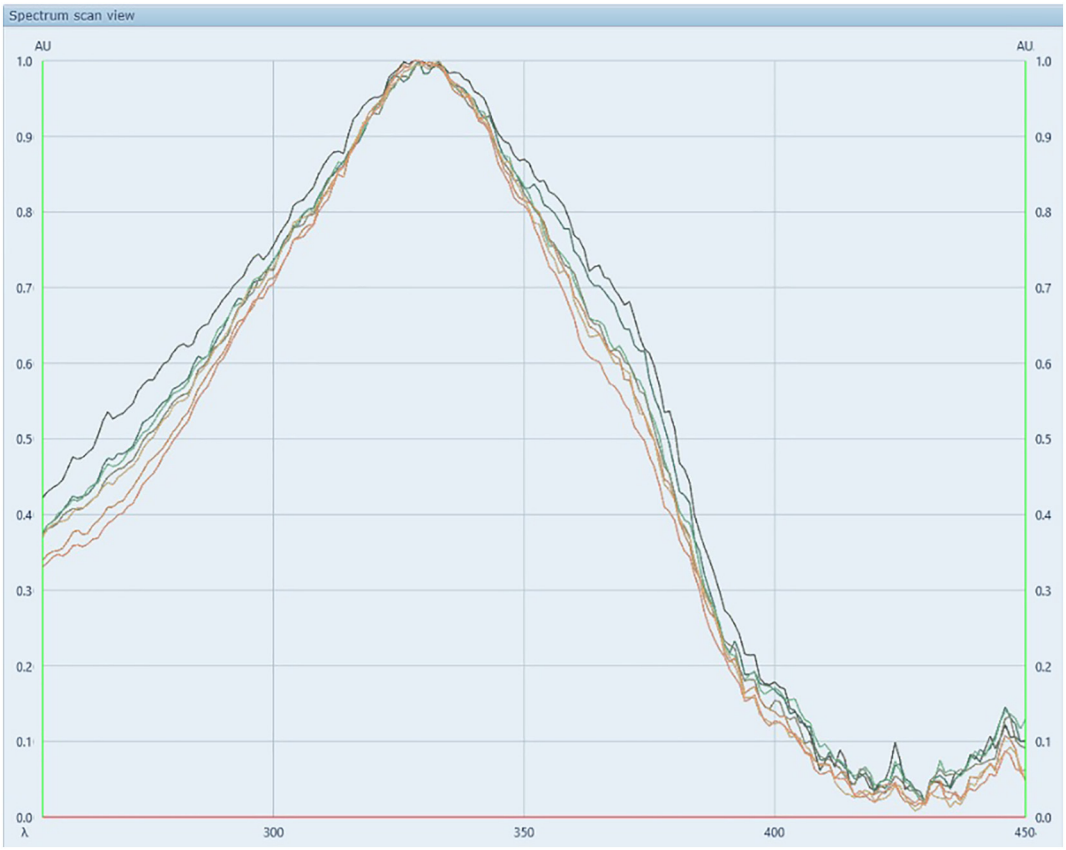

Fig. 5. Overlapped UV spectra of chlorogenic acid and *Crataegus* extracts.

acid, myricetin, trans-cinnamic acid and quercetin in samples from Romania by using HPLC-PDA. Similarly with the previously published data, we have detected epicatechin, quercetin, vitexin, isoorientin and hyperoside in *C. monogyna* samples from Turkey.

LC–MS/MS indicated that *C. monogyna* and *C. turcicus* have similar composition. While luteolin-7-*O*-glucoside and isoorientin have been observed only in *C. monogyna* and *C. turcicus*. Epicatechin, quercetin, chlorogenic acid, vitexin (except for *C. pontica*) and hyperoside were detected in all species. The results of phytochemical characterization were consistent with the antioxidant activities of the species that *C. monogyna* and *C. turcicus* demonstrated the highest activities. With respect to DPPH radical scavenging, it is obvious that hyperoside and chlorogenic acid shows its scavenging effect along with other phenolic compounds that the highest DPPH scavenging is seen with the extract containing moderate amount of those standard compounds. *C. monogyna* with the highest hyperoside and chlorogenic acid contents showed the greatest FRAP and CUPRAC activities. Total antioxidant capacities of whole extracts were consistent with their hyperoside and chlorogenic acid contents. Eventually, except that DPPH scavenging, results of remaining antioxidant assays were correlated with the hyperoside and chlorogenic acid contents of the berries.

3.3. Estimation of antioxidant capacity

For a more precise comparison, all the fruits were collected in from the same geographical region on the same day. By this way, any differences that might be caused by these factors were avoided. In order to evaluate antioxidant spectrum of *Crataegus* species, their effect on DPPH radical and metals were investigated. The results are summarized in Table 5. *Crataegus monogyna* showed the highest FRAP and CUPRAC activity as well as the highest antioxidant capacity among five *Crataegus* taxa. EC_{50} values were calculated for comparison of DPPH radical sweeping capacities and calculations showed that *C. monogyna* and *C. turcicus* have similar and highest scavenging activities.

Previously, antioxidant activity of *C. monogyna* from different regions have been examined by various groups (Froehlicher et al., 2009; Plitszka et al., 2016; Cosmulescu et al., 2017; Bernatoniene et al., 2008; Ruiz-Rodriguez et al., 2013). It has been found that aqueous and alcoholic extracts were different in terms of phenolic composition and antioxidant activity. Antioxidant activities of alcoholic extracts were relatively higher due to the presence of flavonoids (Bernatoniene et al., 2008).

Froehlicher et al. (2009), calculated the DPPH radical scavenging activity of total extract and EtOAc fraction of *C. monogyna* as 6.3 ± 1.1 and 1.4 ± 0.1 mM Trolox/100 g DE, respectively. Similarly, Serteser et al. (2008) measured the DPPH radical scavengings of *C. orientalis* and *C. monogyna* with the EC_{50} values of 1.57 and 1.55 respectively. Moreover, DPPH inhibition of *C. orientalis* was also quantified by Bor et al. (2012) and Çalışkan et al. (2012) with the values of 51.37% ± 4.47 (1 mg/mL) and 21.4%, in EtOH and in 60% MeOH extracts, respectively. Cosmulescu et al. (2017), quantified total antoxidant capacity of 70% MeOH *C. monogyna* fruits as 0.32 ± 0.01 mmoL Trolox/100 g as well.

Table 4
Quantification data for hyperoside and chlorogenic acid from *Crataegus* extracts.

Extract	Hyperoside (w/w%)	CV%*	Chlorogenic acid (w/w%)	CV%
C. monogyna	0.420	0.03	0.900	1.63
C. orientalis	0.180	1.46	0.156	1.70
C. pontica	0.192	1.17	0.139	2.20
C. rhipidophylla	0.124	0.37	0.128	0.16
C. turcicus	0.121	1.57	0.149	0.37

* CV: Coefficient of variation.

Table 5
In vitro antioxidant activities of *Crataegus* spp.

Analysis	*C. monogyna*	*C. orientalis*	*C. pontica*	*C. rhipidophylla*	*C. turcicus*
DPPH scavenging activity[A]	1209.04 ± 4.61^{ade}	1567.39 ± 21.31^{b}	2413.13 ± 27.55^{c}	1237.47 ± 16.90^{d}	1179.60 ± 7.68^{e}
FRAP[B]	2.83 ± 0.04^{a}	0.73 ± 0.01^{b}	0.50 ± 0.00^{c}	1.05 ± 0.09^{d}	0.86 ± 0.16^{bd}
CUPRAC[C]	560.08 ± 0.10^{a}	190.37 ± 8.99^{b}	133.80 ± 4.94^{c}	226.72 ± 5.96^{d}	254.72 ± 3.43^{e}
Total antioxidant capacity[C]	61.89 ± 1.48^{a}	39.52 ± 0.37^{b}	31.63 ± 0.37^{c}	37.94 ± 1.11^{b}	44.00 ± 0.74^{d}

P.S. 1) EC_{50} value of the reference compound "BHT" in DPPH scavenging activity is found to be 350 ± 10 μg/mL. 2) FRAP activity of the reference compound "BHT" is found to be 4.24 ± 0.48 mM FeSO4 eq. in 1 g sample.
[a–e] Different letters in the same row indicate significance ($p < .05$).
[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and DPPH activity was expressed as EC50 in μg/mL equivalents.
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as Mm FeSO4 equivalents in 1 g sample.
[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.

4. Conclusion

The utilization of plants as food and medicinal remedies since ancient times is partially due to the biological efficacy of secondary metabolites that exert antioxidant activities such as phenolic compounds, vitamins, and carotenoids. Many investigations have been performed since the early 1870s on the beneficial effects of phenolic compounds as natural antioxidants and over 150.000 research papers related to antioxidants were published (Ndhala et al., 2010). The best expressed property of phenolics is the ability to trap free radicals. They also have the optimal chemical properties as an antioxidant because they act both as hydrogen and electron donors and have the ability of chelating metal ions (Leopoldini et al., 2011).

According to the Council of Europe, hawthorn berries should not contain less than 1.0% procyanidins, while the leaf and flower material must contain a minimum of 1.5% flavonoids (The Council of Europe, 2004). American Herbal Pharmacopoeia permits the utilization of hybrids of *Crataegus* sp. considering the requirements given in Council of Europe as well (The Council of Europe, 2004; American Herbal Pharmacopoeia and Therapeutic Compendium, 1999a, 1999b). Although the berries of *Crataegus* sp. were ivestigated in this work, proanthocyanidin contents of whole samples and flavonoid contents of whole samples except *C. rhipidophylla* met the requirements of *Cratagus* sp., utilized for therapeutic purposes.

As mentioned earlier, there are numerous works related with *Crataegus* spp., and majority of these studies were about the transgenomic investigation of hybid *Crataegus* species (Dönmez, 2004; Dönmez, 2014; Yilmaz et al., 2010) as well as the antioxidant evaluation of this genus, primarily *C. monogyna* (Cosmulescu et al., 2017; Bor et al., 2012; Ruiz-Rodriguez et al., 2013). Even though, there are several studies including either chemistry or biological activity of few *Crataegus* species, none of the published literatures explains the antioxidant activities and the phytochemical characterization of *C. pontica*, *C. rhipidophylla* and *C. turcicus*. This work constitutes the first report on comparison of five *Crataegus* spp. fruits in terms of their biological activities and phytochemical characterization. We have also utilized LC–MS/MS MRM scan mode for selective identification of phenolic compounds simultaneously with the intent of extending the instrumental set up presented in this report towards quantification of the most prominent phenolic compounds from extracts in *Crataegus* species in future. And quantified the two major components of whole extracts, hyperoside and chlorogenic acid by HPTLC.

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