Research Article **Open Access**

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Phenolic composition and antioxidant potential of different organs of Kazakh *Crataegus almaatensis* Pojark: A comparison with the European *Crataegus oxyacantha* L. flowers

https://doi.org/10.1515/chem-2018-0048
received December 21, 2017; accepted March 1, 2018.

Abstract: The aim of this study was to investigate polyphenolic composition of different parts (leaves, flowers and fruits) of *Crataegus almaatensis* Pojark, an endemic plant of Kazakhstan, and compare it to a well known European *Crataegus oxyacantha* L. flowers. A Qual-Quant analysis was performed based on HR-MS measurements on 22 secondary metabolites: flavonoids and phenolic acids. Another goal was to evaluate the antioxidant potency of hawthorn extracts which was expressed in the total phenolic content and DPPH scavenging potency tests. Leaf extracts from *C. almaatensis* were found to be the most rich in metabolites and the most active in antiradical tests (IC_{50} value of 48 μg/ml and TPC of 218 mg/g). The weakest potential was determined for the fruit extract of this species. According to the performed principal component analysis (PCA), the fruit extracts were not correlated with other organs of the plant, and the metabolites responsible for the extracts' differentiation were cyanidin 3-glucoside and quercetin 3-galactoside. Based on a high correlation factor, the flowers of the Kazakh species was found to be as rich in polyphenols as the European hawthorn. The results of this study indicate that *C. almaatensis* is a promising source of natural antioxidants.

Keywords: hawthorn; LC-MS; antioxidant tests; Kazakhstan; flavonoids.

1 Introduction

Hawthorn is one of the oldest widely used herbal plant popularly prescribed in central Europe and known in Asian countries, with a wide spread usage in China. It can be spread in the form of trees or shrubs, and encompasses around 280 species all over the world. This plant is distributed in temperate areas of Europe, North America, North Africa, India, China and Western Asia. Different species of hawthorn are characteristic to specific regions such as *Crataegus pinnatifida* Bunge (Chinese hawthorn), *C. pubescens* Steud. (Mexican hawthorn), *C. cuneate* Rehder ex C.K.Schneid (Japanese hawthorn), *C. laevigata* (Poir.) DC and C. *monogyna* Jacq. (Europe), *C. oxyacantha* L. and *C. aronia* var. *aronia* (L.) Bosc. Ex Dc (Middle East), *C. phaenopyrum* Borkh. (American hawtrorn) and *C. ambigua* A.K.Becker (Russian hawthorn). *C. monogyna*. and *C. laevigata* are described in the European and United states Pharmacopoeia, while as *C. pinnatifida*, *C. pinnatifida* are official species in the Chinese Pharmacopoeia [1-5]. It is always of particular interest to study the composition of endemic plants, as they may contain higher amount of chemicals. Often their phytochemical properties and

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medicinal usage are well documented by the traditional medicinal system of their own region [6].

In the case of *Crataegus*, a broad range of biological activities important for both folk and official medical practices has been described, including its anti-oxidant, anti-inflammatory, vasodilator, positive inotropic, and cholesterol synthesis inhibiting properties [7]. The above-mentioned activities are strongly affected by the presence of antioxidant molecules in this plant's extracts, which have an ability to scavenge free radicals, produced as a result of biochemical and physiological reactions in the human body. Free radicals, if produced in excess or in a haphazard way, can affect the human body and lead to various chronic diseases, such as cardiac diseases, diabetes, or cancer. Epidemiological studies have demonstrated that natural products with free radical scavenging activity can attenuate the hazard effects of free radicals and show anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, antiviral activities, among others [8]. This can explain the positive effect of hawthorn on conditions such as hypertension, arrhythmia or atherosclerosis [7]. Oligomeric procyanidins, triterpenes, flavonoids, polysaccharides, and catecholamines were identified in hawthorn extracts and are responsible for its pharmacological potential [6, 9].

Either fresh or dried fruits, flowers and leaves of *Crataegus* species are used for the preparation of teas or as a source of extracts for the production of various dosage forms of over-the-counter medicines or dietary supplements [10].

The Republic of Kazakhstan has several wild growing hawthorn species. One of which is spread in the Ile Alatau region (mountains) of Kazakhstan named *Crataegus almaatensis* Pojark. There are only a few scientific papers on the chemistry of cultivated *Crataegus almaatensis* fruits, which encouraged the authors to study the composition of this particular species. Scarce data suggest that *Crataegus almaatensis* fruits may contain carotenoids, sugars and organic acids [11,12], however there is no information on its phenolic composition.

In a continuous effort to understand the chemistry and pharmacology of endemic medicinal Kazakh plants [13,14] a comparison between the phenolic composition of the European and the Kazakh hawthorn species, and the establishment of their antioxidant activity were the aims of this study.

2 Materials and Methods

2.1 Plant Material

The extracts investigated in the study were obtained from fruits, leaves and flowers of *Crataegus almaatensis* collected at the foothills of Ile Alatau Mountains, in Medeo valley, Almaty region, Kazakhstan in September 2015 (fruits) and May 2016 (flower and leaves) and authenticated by Institute of Botany and Phytointroduction, Almaty, Kazakhstan, by the head of the High Plant Flora Laboratory, candidate of biological sciences Dr. G. Kudabayeva and confirmed by general director doctor of biological sciences G. Sitpayeva (reference letter 01-04/456 from 10.11.2015).

Dried and ground flower of *Crataegus oxyacantha* produced by Herbapol Lublin was purchased from a local pharmacy in September 2016 and were introduced into the comparative study. All studied plant samples were given voucher specimen numbers (WKK1601005 – for the fruits, WKK1601006 – for the leaves, WKK1601007 – for the flowers of *C. almaatensis,* and WKK1601008 – for the flowers of *C. oxyacantha*) and the samples are stored in the Chair and Department of Pharmacognosy with Medicinal Plants Unit at Medical University of Lublin, Poland.

2.2 Reagents

Ethanol and methanol (reagent grade purity) used for the preparation of extracts, DMSO, and Folin-Ciocalteu reagents were obtained from Avantor Performance Materials (Center Valley, PA, USA). The LC-MS analysis was performed using acetonitrile, water and formic acid (spectroscopic grade), which were purchased from J. T. Baker (Center Valley, PA, USA). Ammonium formate and all reference compounds (including quercetine used as a reference in antioxidant studies) of purity >95% were purchased from Sigma Aldrich (St. Louis, LA, USA).

2.3 Extracts preparation

Two gram portions of dried and powdered plant material (both *Crataegus almaatensis*. flower, fruit, leaves, and *Crataegus oxyacantha* flowers) were each suspended in 10 mL of either 96% ethanol or 50% ethanol. The prepared solutions were sonicated at 30ºC for 30 min and then filtered through a nylon syringe filter (0.45 µm pore size diameter, Cronus) into HPLC vials and test tubes. The solutions were dried under vacuum at 30ºC (in Eppendorf

Concentrator Plus) until dryness, weighted and used in the LC-MS quantification experiments and antioxidant assays.

2.4 LC-MS determination of phenolic content of the studied extracts

Phenolic compounds were identified and quantified using HPLC-ESI-Q-TOF-MS and HRMS/MS method. The experiments were performed using an Agilent Technologies LC system (1260) (Santa Clara, CA, USA) with a binary pump (G1312C), an autosampler (G1329B), a column oven (G1316A), a degasser (G1322A) and a PDA detector (G1315D), coupled with an ESI-Q-TOF-MS detector (G6530B). The system operated both in positive and negative modes and enabled qualitative and quantitative determination of the extracts' constituents. An Agilent MassHunter software was employed for system operation and spectral data analysis.

The samples were filtered through a nylon syringe filter (0.45 µm pore size diameter, Cronus) prior to the LC-MS analyses. Chromatographic separation was carried out using an Agilent Technologies (Santa Clara, CA, USA) Zorbax Stable Bond RP-18 column (150 mm x 2.1 mm, dp = 3.5 µm), with a temperature set up at 20 °C. The injected volume was 10 µL. The following gradient of solvents was employed using two solvents – A 0.1% of formic acid solution - and B 95% acetonitrile 5% formic acid (0.1%): 0 min – 1% of B in A, 70 min – 55% of B in A, 77 min – 95% of B in A, 83 min – 95% of B in A. The run time was set at 90 min and the flow rate was 0.2 mL/min. The length of the method was influenced by the high quantity of metabolites present in each sample, especially those of high polarity.

The MS detector conditions were set up to optimize the fragmentor and source parameters. The gas temperature was 350°C, sheath gas temperature 400°C, gas and sheath gas flows: 12 L/min, nebulizer gas pressure: 35 psig, fragmentation, capillary, nozzle and skimmer voltages of: 130 V, 4000 V, 1000 V, and 65 V, respectively.

After optimization, the MS/MS spectra were recorded for two of the most abundant signals at a given time, which were subsequently excluded for the next 0.3 min to enable fragmentation of less intensive spectra. To determine the extracts' constituent structures, both mild and strong fragmentation results were provided by recording the product spectra using two collision energies (CID): 15 V and 25 V. The characteristic mass fragments were identified and compared to the ones reported in the scientific literature (see Table S2 in the Supplementary Material).

The system was tuned and calibrated with the application of an external calibration mixture produced by Agilent Technologies (Santa Clara, CA, USA).

For the quantitative analysis of major metabolites, reference compounds (rutin for flavonoid glucosides, gallic acid for benzoic acid derivatives, caffeic acid for cinnamic acid derivatives, catechin and epicatechin gallate for catechins and anthocyanins) at the concentration of 0.1, 0.075, 0.005, 0.0025 and 0.001 mg/mL, each, were injected in the same chromatographic conditions. For each standard compound, a calibration curve was obtained and subsequently used for the quantification of metabolites present in the prepared extracts. The peak areas of reference compounds covered the peak areas of the tested solutions.

Flavonoids, anthocyanins and catechins present in the prepared extracts were determined by positive mode of system operation, while phenolic acids and simple organic acids were identified in negative ionization mode (see Table 1).

2.5 Determination of Antioxidant Activity

All extracts from *Crataegus almaatensis* (flower, fruits, leaves) and *Crataegus oxyacantha* (flowers) were assayed for their ability to scavenge free radicals using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay modified by Lee and colleagues [15,16]. A solution of 0.2 mg/mL DPPH in ethanol was prepared right before the analyses, 5 mg of each extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and kept as a stock solution. The obtained 5 mg/mL stock solutions were used to prepare several dilutions of extracts corresponding to: 25, 50, 100, 250, 500, 1000, 1500 and 2000 µg/mL of extract in DMSO. Later 0.1 mL of the test solution and obtained dilutions were transferred to a test tube and 1.9 mL of DPPH radical solution was added. The reaction mixtures were left for 30 min at 37° in a dark place. A blank containing 1.9 mL of DPPH and 0.1 mL DMSO with no addition of extract was prepared. The absorbance of all samples was measured at 515 nm on a UV spectrometer (Genesys 10S VIS, Thermo Scientific, Waltham, MA, USA). The obtained absorbance values were plotted against the concentration for each sample. Antiradical DPPH activity was expressed as IC_{50} in mg/mL representing the sample's concentration needed to scavenge 50% of DPPH free radicals and was referred to the IC_{50} value of an active reference compound.

Table 1: LC-MS determination of major constituents from *Crataegus almaatensis* extracts. (*DBE – double bond equivalent, Rt – retention time, Delta – difference between experimental and calculated masses*).

No	*Ion (+/-)*	*Rt (min)*	*Molecular formula*	*m/z experimental*	*m/z calculated*	*Delta (ppm)*	*DBE*	*MS/MS fragments*	*Proposed compound*	*References*
1	(+)	24.194	$C_{15}H_{10}O_7$	303.0508	303.0499	-2.88	11	229, 165, 137	**Quercetin**	**18**
2	(+)	21.043	$C_7H_6O_4$	153.054	153.0546	4.08	5	135, 109	**Gentisic acid**	**39, 40**
3	(-)	26.161	$C_{21}H_{20}O_{11}$	447.0956	447.0922	-7.65	13	301, 205, 161	**Quercitrin**	**18**
4	(-)	25.687	$C_{11}H_{12}O_5$	223.06	223.0601	0.45	7	163	**Sinapinic acid**	**16, 18**
5	(-)	19.402	$C_{16}H_{18}O_9$	353.0895	353.0867	-3.1	9	191, 85	**Chlorogenic acid**	**16, 18, 39**
6	(+)	20.471	$C_{21}H_{21}O_{11}$	449.1083	449.1078	-0.36	12	195	**Cyanidin 3-glucoside**	**38**
7	(+)	20.117	$C_{15}H_{14}O_6$	291.0863	290.0785	12.07	9.5	177, 159, 215	**Catechin**	**37**
8	(+)	20.556	$C_{15}H_{14}O_7$	307.0785	307.0812	8.29	9	185, 289, 261	**Epigallocatechin**	**37**
9	(-)	21.585	$C_{27}H_{29}O_{16}$	609.1498	609.145	-7.87	14	461, 300, 151	**Rutin**	**18**
10	(-)	19.402	$C_7H_{12}O6$	191.0628	191.055	-14.13	3	127, 85	**Quinic acid**	**18, 41**
11	(-)	25.434	$C_{21}H_{20}O_{12}$	463.0879	463.0882	0.65	12	301, 127	**Quercetin 3-glucoside (isoquercetin)**	**38**
12	(-)	14.902	$C_7H_6O_4$	153.0261	153.0193	-0.44	5	x	**Protocatechuic acid**	**16, 18**
13	(-)	3.226	$C_9H_8O_3$	163.0468	163.0401	-2.64	6	x	**p-coumaric acid**	**16, 39**
14	(-)	19.57	$C_9H_8O_3$	163.0468	163.0401	-2.64	6	x	**m-Coumaric acid**	**16**
15	(-)	21.134	$C_9H_8O_3$	163.0468	163.0401	-2.64	6	x	**o-Coumaric acid**	**16, 39**
16	(-)	22.275	$C_{27}H_{30}O_{14}$	578.5176	577.1563	-2.28	13	451, 425, 413, 407, 289	**Vitexin 2“-O-rhamnoside**	**42**
17	(-)	24.177	$C_{27}H_{30}O_{14}$	578.519	577.1563	-5.22	13	413, 293, 173	**Vitexin 4“-O-rhamnoside**	**42**
18	(-)	22.628	$C_{21}H_{20}O_{10}$	432.378	431.0984	-7.47	12	311, 413	**Vitexin**	**39**
19	(-)	24.751	$C_{27}H_{30}O_{15}$	593.1522	593.1512	-1.69	13	x	**Vitexin 4“-O-glucoside**	**42**
20	(-)	24.926	$C_{23}H_{22}O_{13}$	505.0986	505.0988	0.32	13	423, 300	**Quercetin glucoside**	**39**
21	(-)	20.421	$C_{15}H_{18}O_9$	341.0873	341.0878	1.48	7	251, 179, 161	**Caffeic acid 3-glucoside**	**42**
22	(-)	19.496	$C_9H_8O_4$	179.0338	179.035	6.57	6	135	**Caffeic acid**	**16, 18**

2.6 Determination of Total Polyphenolic Content

Quantification of polyphenols in the obtained extracts was performed according to the previously published protocols [15,17,18] using the Folin–Ciocalteu assay with modifications. Since the antioxidant activity of the samples was confirmed by DPPH test, the total polyphenolic content was subsequently calculated.

Different concentrations of gallic acid solutions in (DMSO) were prepared: 25, 75, 100, 200 μg/mL. Half mililitre of each prepared solution was mixed with 2.5 mL of diluted Folin-Ciocalteu reagent (0.25 mL of the reagent with 2.25 mL of distilled water) and 2 mL of 7.5% sodium carbonate and left for 30 min in a dark container. After that time, the absorbance was measured at 765 nm on a UV spectrometer (Genesys 10S VIS, Thermo Scientific, Waltham, MA, USA). A blind probe (sample without gallic acid) was used as reference solution.

Next, the 2 mg/mL solution of gallic acid in DMSO was used as a stock solution for the preparation of calibration curve (25, 75, 100, 200, 250, 300, 350, 400, 800 and 1000 μg/mL), while all obtained extracts of hawthorns were stored at a concentration of 5 mg/mL and were further diluted to 500 and 1000 μg/mL prior to the test. The total polyphenolic content was calculated and expressed as gallic acid equivalents (GAE) according to the calibration curve for this phenolic acid, previously prepared, which provided the following equation $y = 1.3108\ln(x) - 4.1964$.

2.7 Statistical evaluation

Statistical analysis of data was made using the MSExcel 2013 and Statistica 12 program (StatSoft Inc., USA). The correlation between alcoholic and alcohol-water extracts (50%) from flowers, leaves and fruits of *C. almaatensis* and *C. oxyacantha* flower extracts was assessed. Also, the principal component analysis (PCA) was conducted. All analytical measurements were repeated six times for each sample and reference compounds. The obtained results were expressed as the mean values ± standard deviation (SD). The significance of the obtained results was determined at $P<0.05$ performing t-test for the applied methods.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

3.1 Qualitative and quantitative LC-MS analysis of the extracts

Polyphenolic compounds constitute a widespread group of secondary metabolites. As previously described many biological effects of various plant species depend on their secondary metabolites. In the case of *Crataegus* species, the phenolic ones play a very important role. This is why it is crucial to determine the phenolic content in different organs of *Crataegus almaatensis* and compare it to the European species *Crataegus oxyacantha* [8].

The applied LC-MS method enabled the qualitative and quantitative analysis of the studied samples. According to the scientific literature, flavonoids and proanthocyanidins are the main constituents of *Crataegus* species [19]. In our study 22 compounds (12 flavonoids and 10 phenolic acids) were identified in either 96% or 50% ethanol extracts of *Crataegus almaatensis* flower, fruit and leaves and in *Crataegus oxyacantha* flowers (Table 1). The identification was performed based on the scientific literature, spectra of some reference compounds, accurate mass measurements and MS/MS spectra of the determined compounds. The application of HRMS-MS analysis succeeded in high accuracy mass measurements, with an error of less than 15 ppm. Clear MS/MS spectra were recorded for the major compounds present in the extracts at the given collision energies (see Supplementary Material).

The quantitative analysis was based on the calibration curves equations obtained for reference compounds: rutin, gallic acid, caffeic acid, catechin and epicatechin gallate at the following concentrations: 0.1, 0.075, 0.005, 0.0025 and 0.001 mg/mL. The values of R-squared for all calculated calibration curves were higher than 0.997 and the equations were as listed below: y= 8073128x+210308 for rutin, y=38044768x+119640 for gallic acid, y=17244017x-683396 for caffeic acid, y=4120762x-89363 for catechin and y=524315083x+247706 for epicatechin gallate.

The comparative results of the quantitative studies are collected in the table 2.

Ethanol at 50% was found to be a better solvent to extract hawthorn's metabolites. Among the selected compounds for the studies, only two - vitexin and gentisic acid were present in similar quantities in both extracts. Because of this fact, the quantitative analysis of extracts is only discussed for 50% ethanol extracts.

The most predominant components of the studied extracts were mono- and di- glycosylated derivatives of flavonols and flavones. The major flavonol present in all parts of *Crataegus almaatensis* was hyperoside, which is in accordance with former studies on other *Crataegus* species [20]. According to the literature, hyperoside is known to be the main component of *Crataegus* flowers [21]. Its quantity in *C. almaatensis* flowers calculated as 3.34 mg/g DW was almost two times higher than in the European species (1.58 mg/g DW) [22], flowers of *Crataegus microphylla* C. Koch (0.25 mg/g) [23] and was not detected in *Crataegus pinnatifida* by Liu et al [24]. Leaves of *Crataegus almaatensis* contained 2.19 mg/g DW of this metabolite, which is comparable with 2.51mg/g fresh weight in *Crataegus azarolus* L. species, but higher than *Crataegus monogyna* 1.45mg/g fresh weight [25] and *Crataegus microphylla* 0.38 mg/g content [23]. Hyperoside content in *Crataegus pinnatifida* collected in May at the same time as *Crataegus almaatensis* is much lower being 0.01 mg/g DW [26] and this compound was not present in *Crataegus pinnatifida* [24,27].

Regarding the content of flavonol glycosides, fruits were found to contain smaller quantities of these compounds in comparison to other parts of the plant. The amount of hyperoside found in the fruits of *Crataegus almaatensis* (0.70 mg/g DW) corresponded to the one obtained for the *Crataegus aronia var aronia* 0.61 mg/g fruit extract [28], but was higher than those from the three Chinese hawthorn varieties (0.25-0.50 mg/g DW) [29-31].

The second major compound - rutin, was also present in all parts of the plant material. The content of rutin (0.66 mg/g DW) in the flowers of *Crataegus almaatensis* was slightly higher than the one found in *Crataegus oxyacantha* samples (0.53 mg/g DW) and stays within the average value of rutin content (0.097-1.186 mg/g) determined for *Crataegus azarolus var aronia*. On the other hand, *Crataegus azorolus*

Table 2: Percentage content of determined component in the studied 96% and 50% ethanol extracts.

	C. almaatensis												*C. oxyacantha* flowers			
Proposed compounds	flower EtOH 96%	percent in dry weight	flower EtOH 50%	percent in dry weight	leaves EtOH 96%	percent in dry weight	leaves EtOh 50%	percent in dry weight	fruit EtOH 96%	percent in dry weight	fruit EtOH 50%	percent in dry weight	EtOH 50%	percent in dry weight	EtOH 96%	percent in dry weight
Quercetin	0.1234	0.0139	0.572	0.0514	0.2024	0.0202	1.094	0.0898	0.392	0.0477	0.4907	0.0539	0.4533	0.0373	0.1565	0.014611
SD	0.009	0.001014	0.0093	0.001461	0.0043	0.00043	0.0336	0.002758	0.0251	0.003054	0.0122	0.001342	0.0085	0.000704	0.0047	0.000439
Gentisic acid	0.095	0.0107	0.086	0.0077	ND	ND	0.0422	0.0035	tr	ND	ND	ND	0.0676	0.0056	0.071	0.006629
SD	0.0042	0.000473	0.0071	0.001115	ND	ND	0.0018	0.000148	ND	ND	ND	ND	0.0064	0.00053	0.0024	0.000224
Quercitrin	0.26	0.0292	0.5117	0.046	0.1603	0.016	0.83	0.0681	0.0451	0.0055	0.0385	0.0042	0.782	0.0648	0.31	0.028942
SD	0.0081	0.000912	0.0106	0.001665	0.0089	0.00089	0.0326	0.002676	0.0043	0.000523	0.0037	0.000407	0.0056	0.000464	0.0103	0.000962
Sinapinic acid	ND	ND	0.057	0.0051	0.0389	0.0039	ND	ND	0.1208	0.0147	0.363	0.0399	0.0018	0.0002	0.0245	0.002288
SD	ND	ND	0.0033	0.000518	0.0024	0.00024	ND	ND	0.0065	0.000791	0.0055	0.000605	0.0006	4.97E-05	0.0017	0.000159
Chlorogenic acid	0.749	0.0842	1.496	0.1345	0.6087	0.0609	0.479	0.0393	0.279	0.0339	0.326	0.0358	1.367	0.1132	0.862	0.080478
SD	0.0131	0.001476	0.0825	0.01296	0.0108	0.00108	0.0113	0.000928	0.0137	0.001667	0.0087	0.000957	0.0825	0.006834	0.0077	0.000719
Cyanidin 3-glucoside	0.0052	0.0006	ND	ND	ND	ND	0.0026	0.0002	0.721	0.0877	0.5357	0.0589	0.0804	0.0067	0.0217	0.002026
SD	0.0001	1.13E-05	ND	ND	ND	ND	0.00011	9.03E-06	0.0052	0.000633	0.0209	0.002299	0.0016	0.000133	0.0014	0.000131
Catechin	ND	ND	ND	ND	0.0036	0.0004	ND	ND	ND	ND	ND	ND	0.0162	0.0013	0.0295	0.002753
SD	ND	ND	ND	ND	0.0001	0.00001	ND	ND	ND	ND	ND	ND	0.00063	5.22E-05	0.00062	5.79E-05
Epigallocatechin	ND	ND	ND	ND	0.0009	0.0001	0.0006	ND	ND	ND	ND	ND	0.0008	0.0001	0.0022	0.000205
SD	ND	ND	ND	ND	0.00007	0.000007	0.00001	ND	ND	ND	ND	ND	0.00001	8.28E-07	0.0001	9.34E-06
Rutin	0.563	0.0633	0.73	0.0656	0.372	0.0372	0.427	0.0351	0.103	0.0125	0.368	0.0405	0.6351	0.0526	0.4083	0.03812
SD	0.0107	0.001205	0.0302	0.004744	0.0111	0.00111	0.0103	0.000845	0.0037	0.00045	0.0173	0.001903	0.0036	0.000298	0.0055	0.000513
Quinic acid	0.0113	0.0013	0.0218	0.002	0.0912	0.0091	0.281	0.0231	0.0217	0.0026	0.035	0.0038	0.0328	0.0027	0.015	0.0014
SD	0.0009	0.000101	0.0004	6.28E-05	0.0007	0.00007	0.0109	0.000895	0.0021	0.000256	0.0018	0.000198	0.0017	0.000141	0.0011	0.000103
Quercetin 3-galactoside (hyperoside)	1.805	0.2022	3.712	0.3336	1.6328	0.1633	2.671	0.2192	0.1709	0.0207	0.6428	0.0704	1.91	0.1582	1.08	0.100831
SD	0.0537	0.006049	0.0612	0.009614	0.0884	0.00884	0.0772	0.006337	0.0055	0.000669	0.0255	0.002805	0.0625	0.005178	0.0392	0.00366

Continued **Table 2:** Percentage content of determined component in the studied 96% and 50% ethanol extracts.

	C. almaatensis												*C. oxyacantha* flowers			
Protocatechuic acid	ND	ND	ND	ND	ND	ND	0.0126	0.001	ND	ND	0.035	0.0038	0.0715	0.0059	0.0677	0.006321
SD	ND	ND	ND	ND	ND	ND	0.00055	4.51E-05	ND	ND	0.0014	0.000154	0.003	0.000249	0.0022	0.000205
p-Coumaric acid	0.044	0.0049	0.0359	0.0032	ND	ND	ND	ND	0.022	0.0027	ND	ND	0.046	0.0038	0.027	0.002521
SD	0.0011	0.000124	0.0015	0.000236	ND	ND	ND	ND	0.0013	0.000158	ND	ND	0.0007	5.8E-05	0.0009	8.4E-05
m-Coumaric acid	0.0399	0.0045	ND	ND	ND	ND	0.0139	0.0011	0.0229	0.0028	ND	ND	ND	ND	ND	ND
SD	0.0007	7.88E-05	ND	ND	ND	ND	0.00094	7.72E-05	0.0007	8.52E-05	ND	ND	ND	ND	ND	ND
o-Coumaric acid	0.031	0.0035	0.027	0.0024	ND	ND	0.01	0.0008	0.018	0.0022	ND	ND	0.026	0.0022	0.027	0.002521
SD	0.0018	0.000203	0.0008	0.000126	ND	ND	0.0008	6.57E-05	0.0011	0.000134	ND	ND	0.0008	6.63E-05	0.0008	7.47E-05
Vitexin 2“-O-rhamnoside	0.2101	0.0236	0.345	0.031	0.13	0.013	0.251	0.0206	ND	ND	ND	ND	0.184	0.0152	0.009	0.00084
SD	0.0054	0.000608	0.0062	0.000974	0.0051	0.00051	0.0091	0.000747	ND	ND	ND	ND	0.0121	0.001002	0.0001	9.34E-06
Vitexin 4“-O-rhamnoside	0.128	0.0144	0.722	0.0649	0.6491	0.0649	1.054	0.0865	0.074	0.009	0.097	0.0107	1.102	0.0913	0.681	0.063579
SD	0.0062	0.000698	0.0068	0.001068	0.0073	0.00073	0.0709	0.005819	0.0062	0.000754	0.0022	0.000242	0.0633	0.005244	0.0136	0.00127
Vitexin	0.024	0.0027	0.0104	0.0009	0.0302	0.003	0.0125	0.001	0.017	0.0021	ND	ND	0.014	0.0012	0.0247	0.002306
SD	0.0049	0.000552	0.0001	1.57E-05	0.0017	0.00017	0.0011	9.03E-05	0.0008	9.73E-05	ND	ND	0.0006	4.97E-05	0.0017	0.000159
Vitexin 4“-O-glucoside	0.0014	0.0002	0.0086	0.0008	ND	ND	ND	ND	ND	ND	ND	ND	0.074	0.0061	ND	ND
SD	0.0001	1.13E-05	0.00004	6.28E-06	ND	ND	ND	ND	ND	ND	ND	ND	0.0013	0.000108	ND	ND
Quercetin glucoside	tr	tr	tr	tr	ND	ND	ND	ND	ND	ND	ND	ND	0.0051	0.0004	tr	ND
SD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0004	3.31E-05	ND	ND
Caffeic acid 3-glucoside	0.0011	0.0001	0.0046	0.0004	ND	ND	ND	ND	ND	ND	ND	ND	0.0054	0.0004	0.011	0.001025
SD	0.00007	7.88E-06	0.0001	1.57E-05	ND	ND	ND	ND	ND	ND	ND	ND	0.0004	3.31E-05	0.0006	5.6E-05
Caffeic acid	0.0076	0.0009	tr	tr	ND	ND	ND	ND	ND	ND	ND	ND	0.0126	0.001	0.0096	0.000896
SD	0.0002	2.25E-05	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0008	6.63E-05	0.0002	1.87E-05

*ND-not detected, tr – traced, SD - standard deviation

var azarolus (0.161-0.615mg/g DW) contained smaller quantities of this flavonoid diglycoside [22]. Rutin in the leaves of the Kazakh species was calculated as 0.35 mg/g DW and this quantity is comparable with that previously published for the *Crataegus monogyna* 0.329 mg/g in fresh leaves [25], but significantly higher than those found for *C. pinnatifida* leaves (0.09 mg/g in the samples) [24,27]. In the fruits of *Crataegus almaatensis* rutin was present at the concentration 0.40 mg/g DW, a bit higher than the *Crataegus azarolus* and *Crataegus monogyna* sum of the peel and pulp rutin content (0.29 and 0.18 mg/g fresh weight respectively) [25], however, Chinese hawthorn fruits were proven to contain very low concentration of this flavonoid – namely, 0.007 mg/g in the samples and 0.026 mg/g DW [24,27,29].

Rhamnoside glucosides of quercitrin and vitexin (4''-*O*-rhamnoside) were abundantly present in the flowers. It is worth mentioning that *Crataegus almaatensis* flowers and fruits contained less quercitrin (0.46 mg/g and 0.042 mg/g DW, respectively) than *Crataegus oxyacantha* (0.65 mg/g DW), but its leaves contain similar quantity (0.68 mg/g) of this metabolite when compared to the reference extract of hawthorn. The same pattern was recorded for vitexin 4''-*O*-rhamnoside. According to Melikoglu and co-workers, *Crataegus microphylla* leaf extracts contained 0.01 mg/g of vitexin 4''-*O*-rhamnoside, which was not present in its flowers [23]. This amount is far lower than the one obtained in this study. Vitexin 4''-*O*-rhamnoside was calculated to be one of the major components of the *Crataegus almaatensis* extract and its quantity accounts for 0.87mg/g DW in the leaf extracts and 0.65 mg/g DW in the flowers). There is not much research work on the quantification of quercitrin and vitexin 4''-*O*-rhamnoside in other *Crataegus* species. Also, Vitexin 2''-*O*-rhamnoside was found to be abundantly present in the Kazakh hawthorn leaves and flowers in comparison to its quantity in *Crataegus oxyacantha*. According to our study, fruits did not contain this glycoside, which stays in accordance with scientific literature. Orhan and co-workers state that this flavonoid glycoside was not present in the fruits of *Crataegus aronia var. aronia*, *C. monogyna* or *C. pseudoheterophylla* Pojark. [28]. However, its presence was identified in *C. pinnatifida* fruits [24,27].

Also, *Crataegus almaatensis* flowers are a richer source of the flavonol quercetin (0.51 mg/g DW) in comparison to *C. oxyacantha* (0.37 mg/g DW), *C. azarolus var. aronia* (0.032-0.248 mg/g DW), *C. azorolus var. azarolus* (0.02-0.18 mg/g DW) [22], and *C. microphylla* (0.06 mg/g) [23]. Quercetin in the leaves of *Crataegus almaatensis* (0.90 mg/g DW) is also higher than in the leaves of hawthorn species collected in west Azerbaijan and Iran (0.12 mg/g in the dried extracts) [32] and *C. laevigata* (0.24 mg/g methanol extracts) determined by Mojka and co-authors [33]. Its presence is reduced in fruits (0.54 mg/g DW). The latter concentration is high in comparison with *Crataegus monogyna* fruits (0.046 mg/g) [32] and the fruits of Chinese species (0.009 mg/g DW) [29].

Among simple phenolics, hydrocinnamic acid derivatives were mostly present in the studied samples. Chlorogenic acid, the most important one, has been found in all investigated hawthorn species so far [20]. Its quantity in *Crataegus almaatensis* and *C. oxyacantha* flowers were on a similar level, ranging around 1.13-1.35 mg/g DW. This amount is much higher than the results obtained for *Crataegus azarolus var aronia* (0.178-0.890 mg/g DW), and *C. azorolus var azarolus* (0.166-0.296 mg/g DW) [22]. However, Belkhir and co-workers determined its presence in the leaves of *Crataegus azorolous* to be 0.87 mg/g fresh weight, which was higher than what is here described for *C. almaatensis* content (0.39 mg/g DW) and also higher than in *C. monogyna* (0.17 mg/g fresh weight) [25]. This phenolic acid was also found in the fruits (0.36 mg/g DW), at a lower concentration from previously reported Chinese samples [30]. Interestingly, the fruits themselves contained antocyanin – cyanidin -3-glucoside at 0.59 mg/g DW, compound that was not found in other parts of the plant.

The performed quantitative studies of the extracts composition showed that *Crataegus almaatensis* is a rich source of polyphenols – both phenolic acids and flavonoids and contains larger quantities of the majority of the studied compounds in comparison to the previously characterized hawthorn species. Both flowers and leaves contained a multitude of components at a higher concentration, which is shown in the Figure S1 of the Supplementary Material. Also, the similarities between the obtained extracts are presented in the dendrogram - figure S3 of the Supplementary File. Based on the obtained results it can be concluded, that the content of active compounds in the fruits of the Kazakh hawthorn was average, as other species were found to contain higher quantities of polyphenols in their fruit extracts.

3.2 Statistical analysis

The difference in the composition of the fruits in comparison to the other studied extracts was well visualized in the statistical tests. Within *Crataegus almaatensis* there is a very high correlation between the components of ethanol extracts from flowers and leaves, namely: 0.8528 to 0.9819 (Table 3) regardless of the solvent used. Also, a high

Table 3: The correlation values obtained for all tested extracts.

Variable	*C. almaatensis*					
	FL_96%	FL_50%	LE_96%	LE_50%	FR_96%	FR_50%
FL_96%	1.0000	0.9819*	0.9418*	0.8528*	0.1853	0.6320*
FL_50%	0.9819*	1.0000	0.9783*	0.9168*	0.2212	0.6530*
LE_96%	0.9418*	0.9783*	1.0000	0.9264*	0.1963	0.6248*
LE_50%	0.8528*	0.9168*	0.9264*	1.0000	0.2308	0.6268*
FR_96%	0.1853	0.2212	0.1963	0.2308	1.0000	0.7841*
FR_50%	0.6320*	0.6530*	0.6248*	0.6268*	0.7841*	1.0000

*statistical significance at $p < 0.05$

correlation with the remaining extracts was found for the 50% ethanol fruit extract (0.6248-0.7841). Particularly noteworthy is the ethanolic (96%) fruit extract. This extract only correlated with 50% alcohol-water extract from the same part of the plant, whereas with other samples tested it showed only a weak correlation at the values of 0.1853 to 0.2308. This correlation was outside the assumed level of significance $p < 0.05$. The above may indicate that the extract contained a different chemical composition.

The evaluation of the correlation matrix carried out for alcoholic and alcohol-water extracts from flowers, leaves and fruits showed similar conclusions – a much lower correlation of ethanol fruit extract with the remaining extracts. Figure 1A proves these conclusions and shows the first two main components of PC1 and PC2, representing, respectively, 73.74% and 21.46%, which gives a total of 95.20% of the variance of the primary variables. In addition, the first two components resemble the original variables to a very good degree, as evidenced by the length of the vectors reaching almost the edge of the circle, which supports the conclusions on the difference of fruit extracts from the remaining parts of the plant.

In the next stage of the statistical analysis, the quantity of the single components of the extracts was evaluated. In order to assess which compounds differentiated the composition of all extracts, classification tests and the principal component analysis (PCA) were performed (figure 1B). As can be seen from figure 1B, the metabolites that discriminate between the extracts from different parts of *C. almaatensis* were cyanidin 3-glucoside and quercetin 3-galactoside. Their content in the tested extracts was 0.0-0.0877 um/ g and 0.0207-0.3336 um/ g, respectively.

Comparative quantitative analysis of flower extracts of both studied species - *Crataegus almaatensis* (Ca) and *C. oxyacantha* (Co) - revealed a very high correlation (0.8681-0.9201) between alcoholic and water-alcoholic extracts (Table 4). This conclusion confirms similar potential of both species.

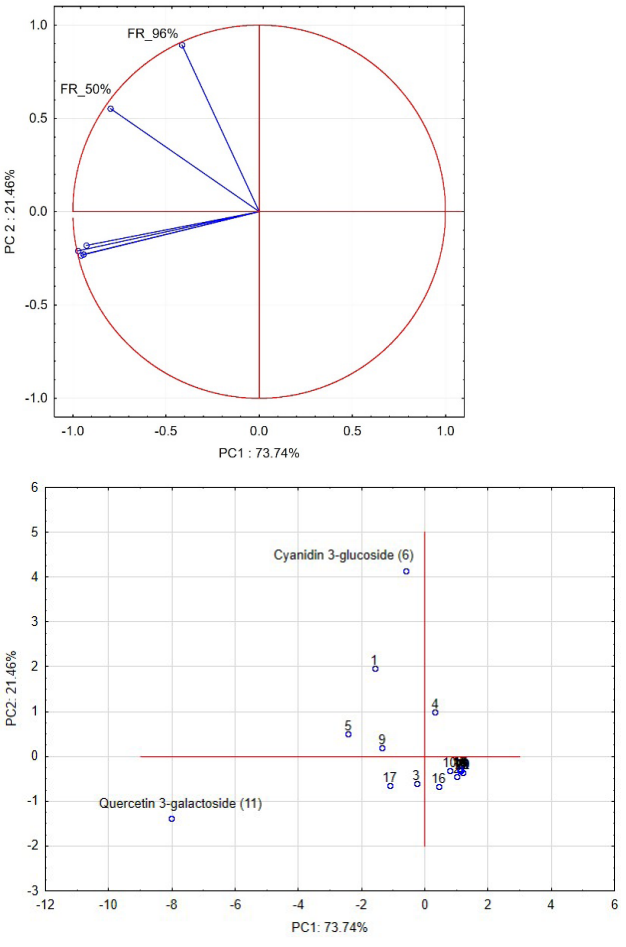

Figure 1: (A) Factor loads of PC1 vs PC2; (B) The determination of components that discriminate the differences between the extracts based on PCA analysis.

3.3 Radical scavenging tests

LC-MS analysis of *Crataegus almaatensis* extracts from different organs clearly showed a wide variety of phenolic compounds present in high concentrations in the studied samples. Since antioxidant potential is essential for the establishment of health benefits in food products [34,35], the authors found it important to determine the antioxidant capacity of phytochemical constituents present in *Crataegus almaatensis*. For this purpose two assays were applied (DPPH radical test and Folin-Ciocalteu assay) to determine the actual scavenging power of *Crataegus almaatensis* extracts, in comparison to the commonly available *C oxyacantha* (Table 5). In case of DPPH radical, IC_{50} values were calculated and used as a tool for comparing the antioxidant strength. For the Folin-Ciocalteu assay the GAE value was used for that purpose.

The comparison of different parts of *Crataegus almaatensis* revealed that the total phenolic content decreased from flower to leaves, and then to fruits. The amount of total phenolic compounds found in the studies show that flowers of *Crataegus almaatensis* were slightly more active than flowers of *C. oxyacantha*. We found that the richest part of the *Crataegus almaatensis* in polyphenols were extract of leaves with their concentration at 218±9mg/g, followed by *C. almaatensis* flowers extract with 180±7mg/g, which was almost 20 percent higher from *C. oxyacantha* flowers extract, which has 151±8mg/g of total phenolic content. Fruits of *Crataegus almaatensis* have the lowest value of total phenolics at 88-92 mg/g. Similar pattern was observed for the free radical scavenging activity. The most potent extract was the extract obtained from leaves (IC_{50} 48±2µg/ml), then the one obtained from flowers (IC_{50} 80±5 µg/ml) with the one obtained from fruits the weakest (IC_{50} = 230±19 µg/ml). *Crataegus almaatensis* flowers extract can be directly compared to a commonly available tea from *C. oxyacantha* flowers. The latter, commonly used material in Europe, exhibited a slightly lower antioxidant potential (IC50=100±9 µg/ml) when compared to the Kazakh species, however, according to the above described results of statistical analysis, the composition of flower extracts of both species are comparable and correlated. The radical scavenging results can confirm high antioxidant activity of the tested Kazakh species of hawthorn in relation to other known species. Also, it is worth mentioning that the leaf extract was found to be more active than the flower extract, which can shed new light on the application of hawthorn leaves in the pharmacotherapy. The tests were performed also on a solution of quercetine – a flavonoid known as a radical scavenger. Its antioxidant potential (IC50 of 24) confirms strong antioxidant properties of hawthorns' extracts – the most active one: 50% ethanol extract from the leaves of the Kazakh species was only two times weaker from pure quercetin.

Table 4: The comparison of flower extracts from both tested hawthorn species: *C. almaatensis* and *C. oxyacantha* expressed in correlation values.

Variable	Co_FL_50%	Co_FL_96%
Ca_FL_96%	0.8792	0.8681
Ca_FL_50%	0.9201	0.9037

Table 5: The results of antioxidant study and total phenolic content determination on the extracts prepared from *Crataegus almaatensis* and *Crataegus oxyacantha*. (*SD – standard deviation*).

Plant source	Plant organ	Solvent	DPPH : IC_{50} (µg/mL) ± SD	GAE (mg/g) ±SD
Crataegus almaatensis	flowers	EtOH 96%	80±2	160±4
		EtOH 50%	80±5	180±7
	fruits	EtOH 96%	215±11	92±3
		EtOH 50%	230±19	88±3
	leaves	EtOH 96%	160±5	110±5
		EtOH 50%	48±2	218±9
Crataegus oxyacantha	flowers	EtOH 96%	400±11	64±7
		EtOH 50%	100±9	151±8
Quercetin			24±3	-

Extracts obtained with ethanol 50% were more antioxidant in this model than the extracts obtained with ethanol 96%. This might be due to a higher concentration of polar phenolic glycosides, which were better extracted with higher percentage of water.

There are similar works on other species of hawthorn, Abu-Gharbieh and Shehad have determined total phenolic content using Folin-Ciocalteu reagent and DPPH radical scavenging activity for *Crataegus azarolous* var. *eu-azarolous* Maire leaves, the results for ethanol extract were 1.5mg CAE/g and IC_{50}129.2 µg/ml [36]. In comparative studies of Tunisian wild *Crataegus azarolus* and *C. monogyna* leaves the total phenols content were in the range of 4006.27 and 2683.85 mg CAE/100 fresh weight for two species respectively, while antioxidant activity determined by DPPH and ferric reducing-antioxidant assay were166.50-168.18 µmol/g fw and 365.32-378.07 µmol Fe^{2+}/g fw respectively [25]. Bahri-Sahloul and co-authors have found the total phenols of *Crataegus azarolous* var. *aronia*

(L.) Rouy & E.G.Camus and *C. azarolous* var. *eu-azarolus* flowers to be in range 45.6-1014.2 mg GAE/100 dw, while antioxidant activity by DPPH and ABTS+ radicals showed results in the range of $TEAC_{DPPH}$ 317-893 µmol Trolox/100 g DW and $TEAC_{ABTS+}$ 966-1608 µmol Trolox/100 g DW [22]. Studies determining total phenolic content of *Crataegus pentagyna* Waldst. & Kit. ex Willd on leaf and flower extract showed 206 GAE mg/g and 184 GAEmg/g extract respectively and scavenged ABTS (TEAC 0.64 and 0.65 µmol Trolox equivalent to1 mg/ml extract respectively) [37].

Our study shows the importance of *Crataegus almaatensis* in delivering active phenolics and being able to produce high quantities of active compounds similarly to the European officinal species. This was confirmed by a marked diversity of the extracts and also significant antioxidant potential of *Crataegus almaatensis*. Results obtained herein point out to the need for another pharmacopoeial monograph, which could find its place in the Pharmacopoeia of the Republic of Kazakhstan.

4 Conclusions

Our study shows the importance of *Crataegus almaatensis* in delivering active phenolics, similarly to the European officinal species. A multitude of secondary metabolites – flavonoids and phenolic acids were identified and quantified in the extracts of both species, which is certainly expressed by their high antioxidant capacity. The leaves of *Crataegus almaatensis* were found to deliver the highest amount of natural products among the tested parts of the plant, and 50% ethanol was selected as a better extractant in comparison with 96% ethanol. Statistical analysis performed on the quantitative data showed a significant difference of the fruit extracts, based on the content of two metabolites: cyanidin 3-glucoside and quercetin 3-galactoside. Leaf and flower extracts (the latter – of both species) were highly correlated. These findings underline a high value of *C. almaatensis* species, in relation to the European species: *Crataegus oxyacantha*.

Conflict of interest: The authors state no conflict of interests.

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