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Research Article

Phytochemical composition and antioxidant activity of *Malus baccata* (L.) Borkh. fruits

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Abstract

Crab apple (*Malus baccata* (L.) Borkh) has been cultivated throughout Europe as an ornamental plant, but the nutritional properties of its edible fruits were not fully revealed. The aim of the current study was to characterize the phytochemical composition of ripen crab apple fruits and to evaluate their nutritional and antioxidant potentials. The fruits were assayed for moisture, ash, protein, lipid, carbohydrate content, titratable acidity, pH, total phenolic compounds and natural pigments. Among the analyzed carbohydrates cellulose was found in the highest content (6% dw), followed by sugars (sucrose, glucose and fructose) and 1.8 % dw uronic acids. The total chlorophylls and carotenoid contents in their fruits were 6.51 and 4.80 μg/g fw, respectively. Total monomeric anthocyanins were not detected. The highest content of total phenolic compounds (2.67 mg GAE/g fw) was found in 95 % ethanol extract from fruits, while the total flavonoids were relatively low – 0.1 mg QE/g fw. DPPH assay (17.27 mM TE/g fw) and FRAP assay (14.34 mM TE/g fw) demonstrated *in vitro* antioxidant activities of crab apple. *Malus baccata* fruits were evaluated as a rich source of dietary fibers and phenolic compounds with significant antioxidant potential that could be used in human nutrition.

Keywords: crab apple, *Malus baccata*, carbohydrates, carotenoids, total phenols, antioxidant activity

Abbreviations: AIS – alcohol insoluble solids; DPPH – 2,2-diphenyl-1-picrylhydrazyl; dw – dry weight; FRAP – ferric-reducing ability of plasma; fw – fresh weight; HPLC – high-performance liquid chromatography; GAE – gallic acid equivalents; QE – quercetin equivalents; RID – refractive index detector; SD – standard deviation; TE – Trolox equivalent; TPTZ – tripyridyl-s-triazine

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Introduction

Wild edible fruits attract attention nowadays as potential sources of nutraceuticals with many health benefits. Their phytonutrients include many bioactive substances, such as organic acid, phenolic compounds and dietary fibers. However, many studies demonstrated the unrevealed and under evaluated potential of these fruits for human health and nutrition. One of these fruits is *Malus baccata* (L.) Borkh (*Rosaceae*). The genus *Malus* consists of about 30–35 species of deciduous shrubs or trees. *Malus baccata* (L.) Borkh commonly known as Crab apple, berry apple, wild apple, Siberian crab apple, Manchurian crab apple and Chinese crab apple. The fruit is known by the name of sheed palek, palanu and palek locally in India (Kumari and Dhaliwal 2017). This plant is widely distributed throughout the world, as its natural populations occur in Asia (mainly Russia - eastern Siberia, Primorsky Krai; Mongolia, Northern China, India, especially in Himachal Pradesh) and cultivated varieties grown in Europe and China (Hallmann et al. 2011; Rudikovskaya et al. 2014; Kumari and Dhaliwal 2017; Dadwal et al. 2018). *Malus baccata* was widely grown as a popular ornamental tree in the gardens and parks of Europe, North America and South America (Yoshizawa et al. 2004; Aladedunye and Matthäus 2014; Kumari and Dhaliwal 2017). The fruit is yellowish green when mature. It ripens in September-October. Fruits are usually of small size, cold-field edible fruit, sub-acidic to sweet in taste with some astringent taste. The plant grows in river valleys, on islands, and on the steppes and shrub steppes (Rudikovskaya et al. 2014). The species are widely used as an apple rootstock because of its high cold tolerance (Li et al. 2017). It is an exceptionally frost-resistant representative of the genus, as the trees can withstand temperatures under–50°C (Rudikovskaya et al. 2014). Wild crab apple fruits are a rich source of phenolic compounds, including anthocyanins (Sharma and Nath 2016). Moreover, these fruits were also described to contain α-sitosterol, campesterol and ursolic acid and their D-glucosides (Mulabagal et al. 2007). Major phenolic compounds found in extracts from its fruits were chlorogenic acid, quercetin-3-Gal/Glu, quercetin-3-Xyl/Ara, phloretin-2-xyloside,quercetin-3-rhamnoside, epicatechins and phloridzin (Tsao et al. 2003; Wang et al. 2013; Rudikovskaya et al. 2015). The vitamin C content in cultivated varieties fruit grown in Russia can reach from 5 to 50 mg% (Rudikovskaya et al. 2014) and 17 mg/100 g for Indian representatives (Kumari and Dhaliwal 2017). Crab apples are used in the preparation of jellies, jams and beverages (Aladedunye and Matthäus 2014; Kumari and Dhaliwal 2017). Fruits are consumed in fresh state or dried during winter. Moreover, the juice from the crab apple demonstrated a strong antiproliferative activity of toward human leukemic HL-60 cells (Yoshizawa et al. 2004). Relatively little is known about the chemical composition of the crab apples, especially of ornamental trees growing in Europe. To the best of our knowledge, the chemical composition of *M. baccata* and its nutritional properties were not evaluated in details. Therefore, the aim of the present study was to characterize ripen fruits of *M. baccata* and to evaluate their nutritional and antioxidant potential.

Materials and Methods

All reagents were analytical grade.

Plant material. Randomly chosen fruits of M. *baccata* in their fully ripen stage were collected during October, 2017 from ornamental plants growing in the different parts of London city, The United Kingdom. The samples of ripen fruits and plants during the blooming period were characterized by botanists from Department of Plant and Fungal Diversity and Resources (Bulgarian Academy of Sciences) and University of Forestry, Sofia, Bulgaria. After removal of damaged fruits, the fresh fruits were washed with distilled water and were kept at –18°C until further uses.

Chemical analysis. Moisture and ash content were done according to AOAC methods (AOAC 2007 a, b). For the moisture content fruits were dried at 105 ± 1ºC to the constant weight. For determination of ash content the pulverized samples were placed in a crucible, ignited in a muffle furnace at 550ºC to the constant weight. Then, it was cooled in a desiccator and weighed at room temperature to get the weight of the ash. For pH measurement, fresh fruits (5g) were homogenized with 25 mL of distilled water and the extract obtained was filtered. pH of the extract was measured using pH meter 7110 WTW (Germany) according to AOAC (2007) initially

calibrated with pH 4 and 7 buffers. Total acidity was measured by potentiometric titration with 0.1 M NaOH to the pH value of 8.1 and the results were expressed as malic acid (ISO 750:1998(E)). Lipid content was determined according to the AOAC methods (2012). The crude protein content was evaluated by the micro-Kjeldahl method (Bradstreet 1965). Acetylacetone-formaldehyde colorimetric method using ammonium sulfate as a standard (GB 5009.5-2010) was used for determination of nitrogen as ammonia content in the digested sample. The crude protein was calculated using 6.25 as a conversion factor. Total carbohydrate was evaluated by the difference: Total carbohydrates, % = 100 – (moisture, % + ash, % + protein, % + lipids, %). Nutritional value of crab apple fruits was calculated as previously described (Petkova et al. 2017).

Preparation of fruit extracts. The extraction from homogenized crap apple fruits was carried out with two different solvents (95% ethanol and distilled water) in solid to liquid ratio 1:5 (w/v). The extraction procedure was performed in an ultrasonic bath (VWR, Malaysia) with frequency 45 kHz and 30 W power (Petkova et al. 2014a) at 45ºC. The ultrasound-assisted extraction was performed in triplicate. Each extract was filtered, combined and used for further analysis.

HPLC analysis of sugars. Chromatographic separations and quantification of presenting sugars in the water extract were performed on an HPLC instrument Elite Chrome Hitachi, coupled with a Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.) with Pb^{2+} and a guard column Shodex SP - G (5 µm, 6 × 50 mm) operating at 85 °C and refractive index detector (RID) Chromaster 5450, operating at 35 ºC. The mobile phase was d. H_2O with a flow rate 1.0 mL/min and the sample injection volume was 20 µl (Petkova et al. 2014b).

Uronic acid content. The uronic acid content of AIS fruit material was estimated as described (Ahmed and Labavitch 1978). In brief, the AIS sample was dispersed in 72% (w/w) H_2SO_4 for 1 h at 30°C, followed by a hydrolysis step with 1 M H_2SO_4 for 3 h at 100°C. An aliquot of hydrolyzate was used for analysis by m-hydroxydiphenyl assay using galacturonic acid (12.5-100.0 µg/mL) for calibration curve construction (Blumenkrantz and Asboe-Hansen 1973).

Cellulose content. The cellulose content was evaluated according to Kürschner-Hoffer gravimetrical method (Kürschner and Hoffer 1931). Briefly, dry sample (0.5 g) was boiled (30 min) with 25 mL acetic-nitric reagent (acetic acid:H_2O:HNO_3 = 8:2:1 v/v/v) in round-bottom flask. After cooling the insoluble residue was filtrated through a filter paper under vacuum, and washed successively with hot acetic-nitric reagent, then with deionized water to a neutral pH, ethanol (95% v/v) and finally with an excess of petroleum ether. The obtained residue was dried in a laboratory ventilated oven at 50ºC to a constant weight.

Natural pigments. Total chlorophylls and total carotenoids were evaluated spectrophotometrically with 95 % ethanol as a solvent and calculated according to equations reported by Lichtenthaler and Wellburn (1983). Total anthocyanins content was determined using the pH differential method (Lee et al. 2005) at two wavelengths 520 and 700 nm. The results were presented as cyanidin-3-glycoside per 100 g fresh fruits.

Total phenolic contents. Folin-Ciocalteu reagent was used for determination of total phenolic content. Briefly, 1 mL of five times diluted Folin-Ciocalteu reagent was mixed with 0.2 mL extract and then 0.8 mL 7.5% Na_2CO_3 was added to the sample. The reaction was performed for 20 min at 25°C in darkness. Then the absorbance was measured at 765 nm against the blank. The results were expressed as mg equivalent gallic acid (GAE) per g sample (Ivanov et al. 2014).

The total flavonoids content. The total flavonoids content was analyzed by $Al(NO_3)_3$ reagents (Kivrak et al. 2009). Absorbance was measured at 415 nm against blank sample. The results were presented as mg quercetin equivalents (QE) per g fw (or dry weight) according to the calibration curve (Ivanov et al. 2014).

The DPPH radical-scavenging ability. Freshly prepared 0.1mM solution of DPPH in methanol (2.85 mL) was added to 95% ethanol or water extracts (0.15 mL). The samples were incubated for 15 min at 37°C in darkness. The reduction of absorbance was measured at 517 nm in a comparison to the blank containing methanol and % inhibition was calculated (Ivanov et al. 2014).

Ferric reducing antioxidant power (FRAP). The FRAP reagent was freshly prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and part 20 mM $FeCl_3 \times 6H_2O$ in d. H_2O in ratio 10:1:1 (v/v/v). The reaction was initiated by mixing 3.0 mL FRAP reagent with 0.1 mL of crab apple extract. The reaction time was performed for 10 min at 37°C in darkness and the absorbance was measured at 593 nm against blank prepared with water. Antioxidant activity was expressed as mM Trolox® equivalents (TE) per g fw (or dw) (Ivanov et al. 2014).

Statistical analysis. All analyses were performed in triplicate (n=3). The data were presented as mean values ± standard deviation (SD). Statistical analysis was performed using MS Excel 2010. A difference was considered statistically significant, when $p < 0.05$.

Results and Discussion

The fruit and flesh color was observed as yellow with round shape for fresh fruits. The air dried fruits were orange to pale brown color (Figure 1). The mean diameter of fruits was 2.8±0.4 cm and the length was 3.2±0.3 cm, as 100 g fruits were used for analysis.

The detailed phytochemical characteristics and nutritional properties of crab apple fruits were summarized in Table 1. The moisture content in *Malus baccata* fruits was 75.53±0.34%, while the total dry weight was 24.47%. Our values for dry matter were higher than finding of Hallmann et al. (2011) and it was in accordance with reported values for cultivated varieties (approximately 24%) and lower than naturally growing Siberian representatives (approximately 30% of the raw mass) (Rudikovskaya et al. 2014). In our study, moisture content in *Malus baccata* fruits collected from UK was close to values reported for Indian representatives – 78.53% (Kumari and Dhaliwal 2017). Ash content in fruits was relatively low - only 0.91%. The ash content and pH values were higher than that demonstrated values in *Malus baccata* fruits from India (Kumari and Dhaliwal 2017).

Alcohol insoluble solids represented 29.4 % of crap apple fruits on the base of dry matter (Table 1), that means 71 % of dry solids are alcohol soluble part. Protein content was (2.8 % dw) higher than *Malus baccata* fruits from India (Kumari and Dhaliwal 2017).

Figure 1. *Malus baccata* (L.) Borkh leaves and fruits in a dried state (authors` picture)

Lipid content in crab apple fruits was quite low - only 0.55 % dw. Our data were in an agreement with the report of Kumari and Dhaliwal (2017) – 0.36%

Even though lower lipid content, Dadwal et al. (2018) found that extracts obtained from pulp or seeds of a Himalayan crab apple (M. baccata) fruits contained moderate concentrations of palmitic acid, ethyl palmitate, methyl petroselinate and linolein.

This finding evaluated Himalayan crab apples as a good source of fatty acids at low cost providing adequate nutritive values.

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Table 1. Phytochemical and nutritional characteristics of *Malus baccata* fruits

Characteristics	Fresh weight	Dry weight
Yield of Alcohol insoluble solids, % dw	1.20	29.43
Moisture, %	75.53±0.34	
Dry matter, %	24.47±0.34	
Ash, %	0.91±0.01	2.22±0.01
Titrable Acidity	0.08±0.03	1.96±0.03
pH	3.37±0.03	3.37±0.03
Protein, %	0.67±0.10	2.80±0.10
Lipids, %	0.13±0.05	0.55±0.05
Total Carbohydrate, %	4.06±0.20	19.0±0.20
Glucose	0.64±0.10	2.68±0.10
Fructose	0.85±0.13	3.53±0.13
Sucrose	0.01±0.01	0.05±0.01
Uronic acids	0.43±0.10	1.80±0.10
Cellulose	1.50 ±0.10	6.20±0.10
Nutritional value, kcal/100 g	22	92

Data were expressed as Mean ± SD (n = 3)

Carbohydrate analysis. The individual sugar composition present in water extracts of *Malus baccata* fruits was analyzed by HPLC-RID method (Figure 2). Only three sugars (sucrose, glucose and fructose) were detected, as glucose and fructose were the main constituents.

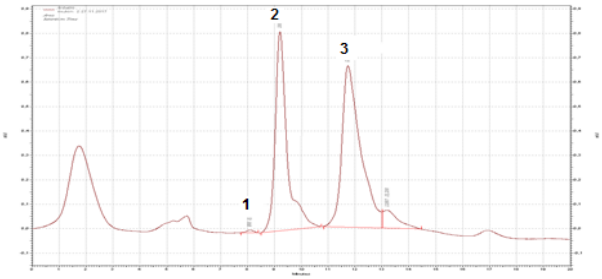

Figure 2. HPLC-RID chromatogram of aqueous extracts from Malus baccata fruits, where 1 – sucrose, 2 – glucose and 3 – fructose

From disaccharides, only sucrose was detected in a very low amount (0.01% fw). Together with Cornelian cherry fruit (Petkova and Ognyanov 2018), crab apple can be evaluated as fruits with low sucrose content. The sum of detecting sugars (glucose, fructose and sucrose) did not exceed 3% fw. In our case, their content was lower than the values detected in Siberian and Indian Malus baccata fruits in the range from 6.3 to 8.6 % fresh plant material (Rudikovskaya et al. 2014; Rudikovskaya et al. 2015; Kumari and Dhaliwal 2017). The sum of glucose and fructose was higher than the content of reducing sugars in Indian Malus baccata fruits (3.12%) reported by Kumari and Dhaliwal (2017). In comparison, the previous reports showed that the dominating carbohydrates in the pulp and seeds of a Himalayan crab apple (*Malus baccata*) fruits were sucrose, D-glucose, D-fructose, arabinose and inositol (Dadwal et al. 2018). Contrary to the statement that fructose and sucrose were found in a fair amount of 21 and 17.3 mg/g in pulp, in our study, we detected fructose (0.85%) and glucose (0.64%) as dominating sugars for whole fresh fruits. Fructose content was higher than reported data (Dadwal et al. 2018) but in our case sucrose was significantly low. Probably, this could be explained with low pH of the sample and possible hydrolysis of sucrose under acidic condition. Special attention was paid to the polysaccharide composition of crab apple fruits due to their beneficial effect. Dietary fiber comprising soluble and insoluble dietary fiber possessed proven beneficial effect on the human health and nutrition. Uronic acid composes the main homogalacturonan fragments in pectin polymer. Because of this the evaluation of uronic acid content is a representable feature of pectin presence in crab apple fruit cell walls. Pectin (as calcium pectate) – 5.57 % and fibers 1.26 % were found in crab apple (*Malus baccata*) growing in India (Kumari and Dhaliwal 2017). In our study, cellulose (6% dw) dominated in fruits (Table 1), whereas their uronic acid content did not exceed 1.8 %. This is the first report for crab apple fruits that evaluated the presence of one of the most distributed dietary fibers (cellulose and pectic

substances). The results for cellulose and uronic acids content clearly showed that crap apple fruits are rich sources of dietary fibers.

Natural pigments. Carotenoids are natural coloring substance widely distributed in vegetal raw materials. They find enormous application in food, pharmaceuticals, cosmetics, and animal feed industries, not only as colorants, but also as bioactive substances in food fortification as provitamin A. Their biological activity includes different health benefit, such as strengthening the immune system, reducing the risk of degenerative diseases, antioxidant properties and antiobesity/hypolipidemic activities (Mezzomo and Ferreira 2016) The results from this study on the quantity of natural pigments found in crap apple fruits were presented in Table 2.

Table 2. Natural pigments in 95 % ethanol extract from fruits of *Malus baccata* (L.) Borkh, μg/g

	Fresh weight	Dry weight
Total Chlorophylls	6.51±0.11	27.13±0.11
Chlorophyll a	4.38±0.12	18.25±0.12
Chlorophyll b	2.12±0.09	8.80±0.09
Chlorophyll a/b ratio	2.07	2.07
Total carotenoids	4.80±0.07	19.60±0.07
Total monomeric antocyanidins	Not detected	Not detected

Data were expressed as Mean ± SD (n = 3)

Table 3. Total phenolic content, total flavonoids and antioxidant activity in *Malus baccata* fruits

Extracts	**Total phenolic content, mg GAE/g**	**Total flavonoids content, mg QE/g**	**Antioxidant activity, mM TE/g**	
			DPPH	**FRAP**
95% ethanol	2.67±0.82[a]	0.13±0.08[a]	17.27±0.54[a]	14.34±0.20[a]
	11.00±0.82[b]	0.53±0.08[b]	70.50±0.54[b]	58.50±0.20[b]
water	1.99±0.54[a]	0.10±0.05[a]	16.85±2.96[a]	10.37±1.45[a]
	8.12±0.54[b]	0.41±0.05[b]	68.80±2.96[b]	42.30±1.45[b]

Data were expressed as Mean ± SD (n = 3), where a-fresh weight, b-dry weight

Total chlorophylls 6.51 μg/g dominated in fresh fruits. The chlorophyll a/b ratio was 2.07. The total carotenoid content was also high 4.80 μg/g fw. However, in our samples total monomeric anthocyanins could not be evaluated, because of the trace amount and low sensitivity of the method. Total pigments content in crap apple did not exceed 12 μg/g of fresh plant material and 50 μg/g dw that was near to report for total pigment in domestic apple cultivars for the peel (58.72–1510.77 μg/g dw) and in the flesh (14.80–71.57 μg/g dw) (Delgado-Pelayo et al. 2014). The fresh fruits of *Malus bacata* were also reported to contain 86.7 μg/g lutein, 10.5 μg/g lycopene and 1.7 μg/g β-carotene (Hallmann et al. 2011).

Total phenolic compounds and antioxidant activity. Phenolic compounds are of particular interest to consumers health and nutrition because they prevent from oxidation processes. The results for total phenolic and total flavonoids content, as well as antioxidant activity in 95 % ethanol and water extracts from *Malus baccata* fruits were summarized in Table 3.

In general, 95 % ethanol extract from fruit samples showed the highest value of total phenols and antioxidant potential. In our study, the amounts of total phenolic content were found to be higher than flavonoid content in comparison to the data earlier reports on *Malus domestica* fruit species during maturation and Himalayan crab apple fruits (*Malus baccata*) (Dadwal et al. 2018). The total phenolic content in Malus baccata fruits was 2.67 mg GAE/g fw. Our values were higher than the reported quantity of total polyphenols for two Siberian ecological forms (tall and dwarf) of *M. baccata* - 1.01-1.56 mg GAE/g raw matter (Rudikovskaya et al. 2014), higher than 1.17 ± 0.03 mg GAE/g values found in the pulp of crab apple fruits (Sharma and Nath 2016) and in the range of 1-5 mg/g (Tsao et al. 2003). In addition, Rudikovskaya et al. (2015), reported lower content of total phenols in the flesh than in the peel of Siberian crab apple (305.32 ± 18.78 and 5841.81 ± 309.62 μg/g fw, respectively) Moreover, Hallmann et al. (2011) did not find any phenolic acids, only flavonoids in crab apple fruits. Phenolic content in collecting purified fraction with organic solvents (n-butanol, ethyl acetate and etc.)

varied in the range from 183 to 761 mg GAE/g dry extract. The ethyl acetate extracts of crab apple (*M. baccata*) showed potential as natural antioxidants (Aladedunye and Matthäus 2014). The level of total flavonoids content in *Malus baccata* fruits reached 0.13 mg QE/g fw. The low levels of quercetin glycosides were also reported in *M. baccata* -from 256.5 to 465.7 µg/g of raw mass (Rudikovskaya et al. 2014). The obtained value was consistent with the study of Hallmann et al. (2011) who reported 15.3 mg/100 g fw, but higher that Padewska (2008), who determined value of 6.7 mg/100 g fw for fruits gathered from the territory of Poland. In the present study, the antioxidant activity of *Malus baccata* fruits was evaluated by two different methods DPPH and FRAP, based on different mechanisms (Table 3). The highest antioxidant activity was obtained using DPPH method based on hydrogen transfer. Water and 95% ethanol crap apple extracts demonstrated insignificantly different values for antioxidant activity. The antioxidant potential of fruits was in the range of 10 - 17 mM TE/g fw and 42 - 70 mMTE/g dw. Dadwal et al. (2018) successfully applied DPPH, ABTS and Fe^{2+} chelating ability to evaluate the antioxidant potential of *M. baccata* fruits and seeds. Our results were higher than the highest values reported for radical-scavenging activity 3789.25 µmol TE/100 g fw (DPPH assay) of ethanol extracts from different crap apple varieties (Li et al. 2014). Ferric reducing antioxidant power (FRAP Assay) of *Malus rockii* Rehder ethanol extract (9356.14 µmol TE/100 g) (Li et al. 2014) was also lower than reported by us antioxidant activity of *Malus baccata* ethanol extract. The high antioxidant activity by DPPH method for crap apple fruit could be explained with the higher number of –OH groups and those –OH group present in ortho-position in the aromatic ring usually quenches more DPPH molecules on the molar basis (Dadwal et al. 2018). In the present study, the antioxidant activity of *Malus baccata* fruits was much higher than the reported value of apple peels and wild crap apple varieties from China (Li et al. 2014). Therefore, *Malus baccata* fruits are a rich source of phenolic compounds with high antioxidant activities for preparing extracts that could be applied in foods and cosmetics.

Conclusions

This is the first study showing the phytochemical composition of the crab apple fruits grown as ornamental plants in the United Kingdom. Underutilized *Malus baccata* fruits could be consumed as a new food ingredient containing proteins, dietary fibers (cellulose and pectins), sugars (glucose, fructose and sucrose), natural pigments and phenolic compounds. The results of the current study demonstrated that fruits contained low sucrose levels, high dietary fiber content and moderate total phenolic content. Together with their antioxidant potential the fruits of *Malus baccata* were evaluated as a source of phytonutrients. Therefore its utilization in food and feed technologies present a perspective supplement.

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