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Original article

Detection and quantification of some phenolic compounds in kernel of selected Hungarian and Bulgarian Persian walnut cultivars

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Summary

The Persian walnut (*Juglans regia* L.) is the most grown nutritional crop among the shell fruit species in Central and Eastern Europe. Some phenolic compounds of kernel of sixteen Persian walnut cultivars derived from Hungary, Bulgaria, and France were examined during two vegetation seasons in this paper. The vanillic acid, catechin, pyrocatechin, epicatechin, rutin, cinnamic acid, gallic acid, syringic acid, and juglone were detected in the samples. If the phenolic compounds are ranked into increasing order, the vanillic acid was in the first place because of its high concentration, followed by catechin, pyrocatechin, epicatechin, rutin, cinnamic acid, gallic acid, syringic acid, and juglone. The Hungarian-bred cultivars, collected in Hungary, produced higher quantity of the examined compounds than the Bulgarian-bred cultivars, derived from Bulgaria, in this study.

Keywords
concentration, geographic differences, HPLC, kernel, Persian walnut, phenolic compounds, state approved cultivars

Significance of this study

What is already known on this subject?

- Increasing order of the examined phenolic compounds is the following: vanillic acid, catechin, pyrocatechin, epicatechin, rutin, cinnamic acid, gallic acid, syringic acid, and juglone.

What are the new findings?

- In some cultivars the detected compounds were higher than the data found in the literature.
- Among the examined Hungarian-bred walnut cultivars the 'Alsószentiváni 117', 'Alsószentiváni kései', and 'Milotai 10' had outstanding inner content value; the highest compound concentration was detected in 'Sheinovo' among the Bulgarian-bred cultivars.
- This study further confirms that the environment plays a major role to influence the ratio of these compounds of kernels annually.

What is the expected impact on horticulture?

- According to our results the Persian walnut cultivars grown in the Carpathian Basin possess the potential to create an outstanding phenolic acid content useful for nutritive and medical applications.

Introduction

The Persian walnut (*Juglans regia* L.) is the most grown nutritional crop among the shell fruit species in Central and Eastern Europe. The global walnut production shows an increasing tendency producing 3,458,046 dried shelled metric tons annually. Today there is a good walnut market situation in the world; the growers sell their fruits on a high and stabile price year on year, and therefore there is a keen interest in the walnut production. Among the Eastern European countries, Bulgaria produces 5,020 metric tons dried fruits; the Hungarian production is approx. 3,500 metric tons yearly. In both countries the Persian walnut production is supported by local breeding programs, innovations in the nursery, and growing technology (Gandev and Arnaudov, 2011).

The walnut is an excellent fruit especially during winter time, because of its high oil content (45–72% of the kernel weight), that consists mainly of unsaturated fatty acids. In addition to oil, the kernel contains proteins (13–25% of the kernel weight), available carbohydrate (4–15% of the kernel weight), fibre (4.6–7.5% of the kernel weight), and minerals (1.65–2.4% of the kernel weight) (Germain et al., 1999; Bujdosó et al., 2010). Among the nut fruit species, Persian walnut contains the highest phenolic acid content (Alasalvar and Bolling, 2015), and the walnut has the lowest amounts of fatty acids (Mazinani et al., 2012). Studies of Slovene and Hungarian research teams on walnut fruit quality concluded that the environment plays a major role to influence the ratio of these compounds in the kernels annually (Solar, 2006; Bujdosó, 2014).

Apart from the main compounds mentioned it was detected that twenty-seven phenolic compounds derived from hydrolysable tannins polyphenolic subclass occur in the kernel (Slatnar et al., 2015), seventeen compounds from the green fruits using an alcoholic extract (Cosmulescu et al., 2014), and thirteen phenolic compounds were identified by Stampar et al. (2006) in walnut husks (chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone). Six compounds (ferulic acid, vanillic acid, coumaric acid, elagic acid, myricetin and juglone) were detected in the walnut leaves (Nour et al., 2012; Cosmulescu et al., 2011). The total phenolic content of the kernel was 154-fold higher than phenolics in the walnut oil (Slatnar et al., 2015). The hull contained a higher phenolic content than the kernel (Labuckas et al., 2007). The total phenolic content was in a descending sequence in the study made by Wang and his team (Wang et al., 2014): the shell extract had the highest content, followed by leaf extract, stem extract, defatted walnut kernel extract, and green husk extract. In the form of liquor made of green walnut fruits, a solution having 40% alcohol and added sugar showed the highest concentration of phenolic compounds (Jakopic et al., 2008). The phenolic content (ellagic acid, rutin, myricetin and juglone) of walnut leaves increased until early August in the vegetative season (Cosmulescu et al., 2013).

Among the shell fruit species different organs of walnut had the highest tannin fractions content (Matok et al., 2009; Kalogeropoulos et al., 2013). Its catechin content was equal to 550 mg eq g^{-1} (Kalogeropoulos et al., 2013; Karamac, 2009). Juglon is the predominant compound in the green husk (Cosmulescu et al., 2011). The phenolic compound content decreased by enzyme polyphenol oxidase, which occurred with changes in the metabolism of phenolics and their derivatives (e.g., catechin), although their quantity was regulated by chemicals (Araji et al., 2014).

Phenolic compounds of the walnut organs had inhibitory effects on weeds (Matok et al., 2009), grain aphid (*Sitobion avenae* F.) development (Chrzanowski et al., 2012), and antifungal activity (Sytykiewicz et al., 2015). There is a negative correlation between the total amount of phenolics present in the fruit tissues and blight severity, indicating the role of these compounds in the fruit-*Xanthomonas* interactions. Seasonal fluctuations were typical for the quantity of phenolic components. Among the compounds, juglone and maybe the gallic acid seemed to have a dominant and important role in the negative correlation with disease development during defence mechanism in the fruit-bacteria interactions (Solar et al., 2012a, b; Matławska et al., 2015).

If the phenolic compounds of the examined cultivars are ranked based on their quantity into increasing relative order, Greek research team detected outstanding gallic acid content followed by catechin, epicatechin, syringic acid in the walnut samples derived from Northern, Central and Southern Greece (Kalogeropoulos et al., 2013). The Spanish research team (Gómez Caravaca et al., 2008) measured very small gallic acid and gallotannins content in the Persian walnut. Juglone and 1,4-naphthoquinone are detected in all walnut parts (defatted kernel, green husk and leaves of walnut) (Hama et al., 2016).

The five major phenolic compounds in the walnut kernel were gallic and cinnamic acid, catechin, juglone and rutin. Gallic acid is the most readily bio available compound for the human body, and is followed by catechin (Williamson and Manach, 2007). Cinnamic acid appears to improve glycaemic parameters in diabetic patients suffering from type two diabetes inconsistently (Rudkowska, 2009). Some studies show that juglone has anti-cancer activity against human tumour lines and has anti-cancer effect against chemo/radio resistant melanomas among in vivo conditions (Babich and Stern, 1993; Bonifazi et al., 2010). Rutin has many physiological functions and is known for its anti-oxidant activity (Harborne and Williams, 2000). Other bioactive phenols include vanillic acid, epicatechin, cinnamic acid, which are characteristic for higher plants.

The phenolic compounds in the seed coat were higher compared to the walnut flesh. For example, total phenolic content of a dehulled 'Chandler' kernel was 16.3 mg g^{-1} in the kernel and 403 mg g^{-1} in the seed coat (Labuckas et al., 2008). In addition, 38-fold higher phenolic content was measured in the pellicle of 'Alsószentiváni 117' (727 mg g^{-1}) than in the walnut kernel (19.16 mg g^{-1}). Thus the pellicle is a much better source of phenolic compounds than the kernel. This research group detected more juglone content in the kernel of 'Alsószentiváni 117' than in the other nine examined cultivars ('Adams', 'Cisco', 'Chandler', 'Elit', 'Fernette', 'Fernor', 'Franquette', 'Lara', 'Rasna') (Colaric et al., 2005).

Skin of the walnut is a good free radical scavenger and could be effective in reducing oxidative stress among other beneficial health effects, which could be exploited for product development (Samaranayaka et al., 2008).

Stronger antioxidant capacity was found in the yellow pellicles compared to red pellicles, however the genotypes with red pellicles had a larger amount of anthocyanins (Trandrafir et al., 2016).

Dried shelled fruits mixed with dried fruits such as figs and apricot, reached a lower total phenolics content than the fruits and nut fruits alone because of their antagonistic effect (Kamiloglu et al., 2014).

Materials and methods

Fruit production conditions

The fruit samples were collected in the fall of 2012 and 2014. Fruits of the eight state-registered Hungarian-bred Persian walnut cultivars ('Alsószentiváni 117', 'Milotai 10', 'Tiszacsécsi 83', 'Milotai bőtermő', 'Milotai kései', 'Milotai intenzív', 'Bonifác', 'Alsószentiváni kései') and one selected genotype (BD06) were examined in the trial. Fruits were collected from experimental orchards of the National Agricultural Research and Innovation Centre Fruitculture Research Institute (GIS coordinates of the location: 47°20'14.78"N; 18°51'47.05"E, average annual sunshine hours are 2,094 h, average annual temperatures are 11.1°C, average annual precipitation is 560 mm, type of the soil is Chernozem soil $K_A = 40$, pH = 8, lime content 5%, humus content 2.3%).

Fruit samples of the French-bred 'Fernor' cultivar were collected at Juglans Hungaria Ltd. in Lengyeltóti (Hungary) (GIS coordinates of the location: 46°42'15.24"N; 17°38'22.47"E, average annual sunshine hours are 2,098 h, average annual temperatures are 11.2°C, average annual precipitation is 550 mm, type of the soil is Chernozem soil $K_A = 38$, pH = 8, lime content 5%, humus content 1.9%). The Bulgarian-bred cultivars, 'Izvor 10', 'Dryanovski', 'Perustinski', 'Sheinovo', 'Silistrenski' (Nedev et al., 1983) were grown in Plovdiv (Bulgaria) (GIS coordinates of the location: 42°66'45.10"N; 24°24'34,70"E, average annual sunshine hours are 2,550 h, average annual temperatures are 13.7°C, average annual precipitation is 570 mm, type of the soil is alluvial meadow with pH = 7.2).

In order to analyze the chemical contents of the fruits, the husks were removed immediately after the harvest. The samples were washed and dried up to 10% of moisture content and were stored as a walnut in its shell at +8°C until further processed in the laboratory. The harvest time was when 50% of husks had been opened.

Chemicals

Analytical HPLC grade standards of different phenolic compounds such as juglone (PubChem CID: 3806), gallic acid (PubChem CID: 370), chlorogenic acid (PubChem CID:

1794427), pyrocatechin (PubChem CID: 161125), catechin (PubChem CID: 73160), syringic acid (PubChem CID: 10742), rutin (PubChem CID: 5280805), cinnamic acid (PubChem CID: 44539), epicatechin (PubChem CID: 72276), vanillic acid (PubChem CID: 8468), and the solvents phosphoric acid and methanol (MeOH), were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The standards (0.5 g mL^{-1}) were dissolved in methanol and a 100× dilution was used as the working standard for HPLC.

Sample preparation

Whole kernels with their seed coats (1,000 g) were used as starter material and 1 g-samples were extracted in 10 mL methanol for 12 hours in the dark at 4°C, using an Edmund Bühler SM 30 control shaker (200 rpm min^{-1}). The supernatant was decanted and centrifuged in Eppendorf tubes in a Hettich Mikro 22R centrifuge (15,000 rpm min^{-1} for 5 min). The supernatant was filtered on a 0.45 µm MILLEX® HV Syringe Driven Filter Unit (SLHV 013 NL, PVDF Durapore), purchased from Millipore Co. (Bedford, MA, USA), and injected into the HPLC system. The quantities of the individual phenolic compounds are given in mg g^{-1}.

Analytical conditions

The WATERS High Performance Liquid Chromatograph (purchased from Waters Co., 34 Maple Street, Milford, MA, USA) was equipped with 2487 Dual λ Absorbance Detector, a 1525 binary HPLC pump , and in-line degasser, a column thermostat (set at 40°C) and an 717plus auto sampler (set at 5°C) and was controlled using EMPOWER TM[2] software. A KINETEX C_{18} 2.6 µm 150×4.6 mm column (Phenomenex, 411 Madrid Avenue, Torrance, CA, USA) was installed. The gradient mobile phase was A: H_2O:MeOH:H_3PO_4 = 940:50:1; B: MeOH (0–30 min: A 100%–10%, 30–30.1 min: 10%–100%, 30.1–31: A 100%) with a flow rate 1 cm^3 min^{-1}, the pressure in the column was 4200 ± 10 psi at a column temperature of 30°C. The running time was 25 minutes. Each injected volume was 20 µL. The sampling rate was 10 pt sec^{-1}, and the phenolic components were monitored at a wavelength of 280 nm (gallic acid, pyrocatechin, catechin, syringic acid, rutin, cinnamic acid, vanillic acid, epicatechin) and 350 nm (juglone). The retention times of the standards were gallic acid (7.7 min), pyrocatechin (7.5 min), catechin (9.6 min), chlorogenic acid (11.4 min), vanillic acid (11.8 min), epicatechin (12.7 min), syringic acid (12.9 min), rutin (18.5 min), juglone (20.6 min) and cinnamic acid (21.6 min) respectively.

Statistical analysis

Three replicates were made in all cases. The statistical analysis was performed using IBM SPSS Statistics 22. One-way ANOVA was carried out to separate the homogeneous groups using Tukey's test. The RSD value of the determination was 5% (n = 5). The results were expressed as Rm of 1 g of dried weight kernel.

Results

The phenolic compound profiles of the fifteen tested walnut cultivars are shown in Figures 1 to 8. All compounds except epicatechin were detected in all cultivars examined in this trial.

The vanillic acid concentration was the highest in 'Alsószentiváni kései' (16.76 mg g^{-1}), 'Milotai kései' (14.85 mg g^{-1}), and 'Milotai 10' (12.76 mg g^{-1}), the lowest amount was detected in 'Perustinski' (2.12 mg g^{-1}), 'Silistrenski' (2.57 mg g^{-1}), 'Dryanovski' (4.02 mg g^{-1}) and 'Izvor 10' (4.08 mg g^{-1}) (Figure 1).

The catechin content was outstanding in all samples; the highest concentration was in 'Alsószentiváni kései' (5.10 mg g^{-1}), 'Milotai kései' (4.43 mg g^{-1}) and 'Milotai intenzív' (4.16 mg g^{-1}). The Bulgarian-bred 'Perustinski' (1.11 mg g^{-1}), 'Izvor 10' (1.34 mg g^{-1}), and 'Sheinovo' (2.35 mg g^{-1}) produced the lowest concentration of catechin (Figure 2).

The pyrocatechin had the second largest concentration after catechin. 'Alsószentiváni kései' (3.14 mg g^{-1}), 'Milotai 10' (2.75 mg g^{-1}), and 'Milotai kései' (2.67 mg g^{-1}) produced the highest concentration of pyrocathecin; the lowest was produced by 'Perustinski' (0.68 mg g^{-1}), 'Izvor 10' (0.82 mg g^{-1}), 'Sheinovo' (1.12 mg g^{-1}) (Figure 3).

Just eight cultivars contained epicatechin among the ob-

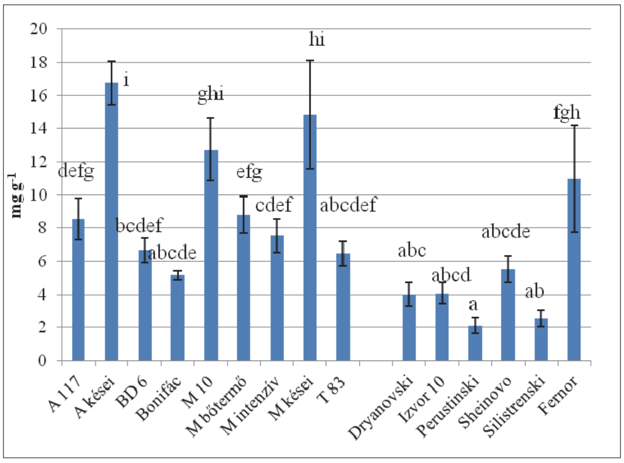

FIGURE 1. Vanillic acid content of kernels in analyzed cultivars ($SD_{5\%}$ = 446).

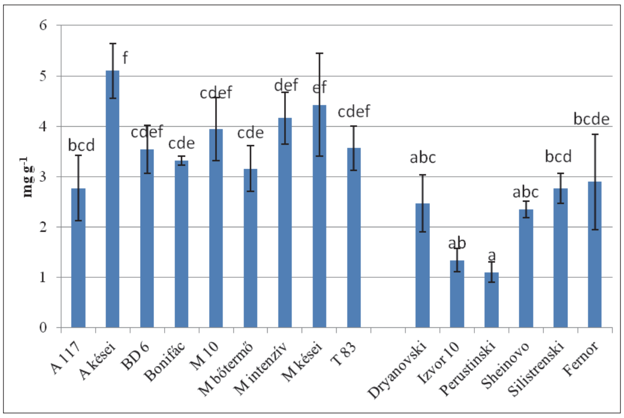

FIGURE 2. Catechin content of kernels in analyzed cultivars ($SD_{5\%}$ = 1.15).

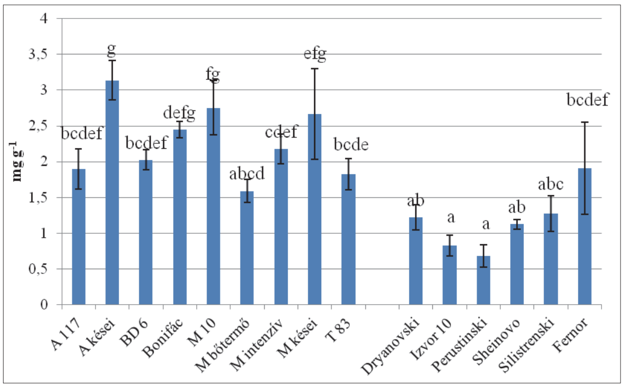

FIGURE 3. Pyrocatechin content of kernels in analyzed cultivars ($SD_{5\%}$ = 0.75).

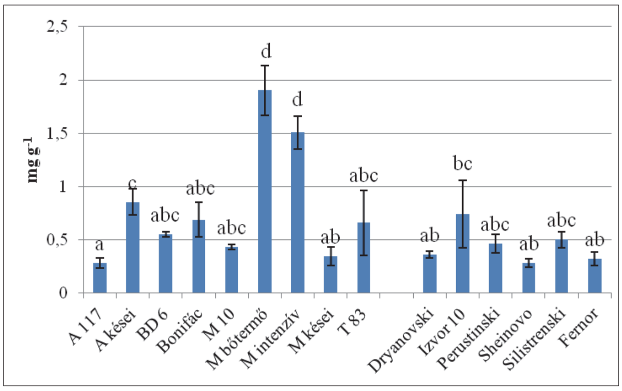

FIGURE 4. Rutin content of kernels in analyzed cultivars ($SD_{5\%} = 0.47$).

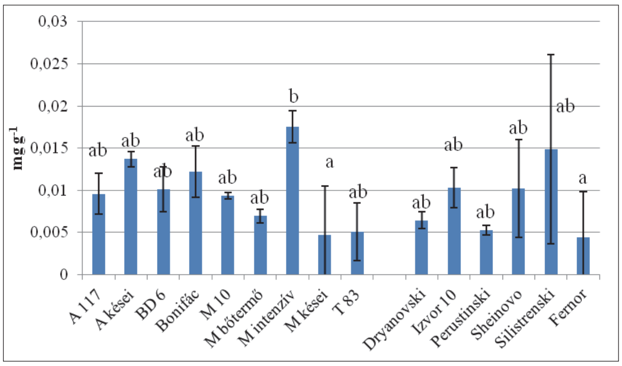

FIGURE 5. Cinnamic acid content of kernels in analyzed cultivars ($SD_{5\%} = 0.0052$).

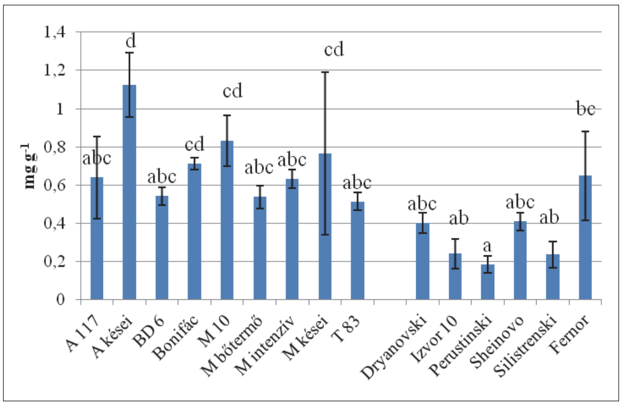

FIGURE 6. Gallic acid content of kernels in analyzed cultivars ($SD_{5\%} = 0.28$).

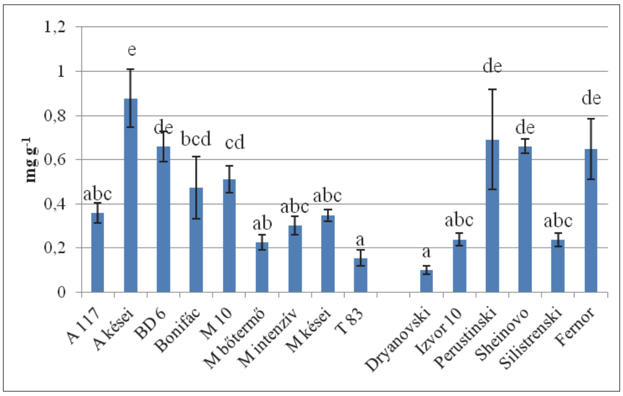

FIGURE 7. Syringic acid content of kernels in analyzed cultivars ($SD_{5\%} = 0.24$).

served ones. The highest concentration of epicatechin was produced by 'Alsószentiváni kései' (2.19 mg g-1), followed by 'Milotai 10' (1.67 mg g-1), 'Milotai kései' (1.44 mg g-1), 'Fernor' (1.2 mg g-1), 'Milotai bőtermő' (1.0 mg g-1), 'Tiszacsécsi 83' (0.88 mg g-1), 'Sheinovo' (0.31 mg g-1) and 'Silistrenski' (0.23 mg g-1). Other cultivars didn't have any epicatechin content.

The Hungarian-bred 'Milotai bőtermő' (1.90 mg g-1) had the highest rutin content, followed by 'Milotai intenzív' (1.51 mg g-1), and 'Alsószentiváni kései' (0.85 mg g-1); the lowest concentration was detected in 'Alsószentiváni 117' (0.27 mg g-1), followed by 'Sheinovo' (0.28 mg g-1), and 'Fernor' (0.31 mg g-1) (Figure 4).

The 'Milotai intenzív' (0.017 mg g-1) had the highest cinnamic acid content content, followed by 'Silistrenski' (0.015 mg g-1), and 'Alsószentiváni kései' (0.013 mg g-1); the lowest concentration was detected in 'Fernor' (0.04 mg g-1) and 'Milotai kései' (0.04 mg g-1), followed by 'Tiszacsécsi 83 (0.005 mg g-1) and 'Perustinski' (0.005 mg g-1) (Figure 5).

The 'Alsószentiváni kései' (1.12 mg g-1), 'Milotai 10' (0.83 mg g-1), and 'Milotai kései' (0.76 mg g-1) had the highest gallic acid content, and the Bulgarian-bred 'Perustinski' (0.18 mg g-1), 'Silistrenski' (0.24 mg g-1), 'Izvor 10' (0.24 mg g-1) had the lowest concentration (Figure 6).

The highest syringic acid content was detected in 'Alsószentiváni kései' (0.88 mg g-1), 'Perustinski' (0.69 mg g-1), 'Sheinovo' (0.66 mg g-1); the lowest concentration was in 'Dryanovski' (0.10 mg g-1), 'Tiszacsécsi 83' (0.15 mg g-1) and 'Milotai bőtermő' (0.22 mg g-1) (Figure 7).

The juglon had the lowest concentration among the examined compounds. We measured the highest juglon concentration in 'Alsószentiváni kései' (0.13 mg g-1), 'Milotai kései' (0.13 mg g-1), and 'Tiszacsécsi 83' (0.10 mg g-1); the lowest was in 'Dryanovski' (0.02 mg g-1), 'Izvor 10' (0.03 mg g-1), and 'Sheinovo' (0.03 mg g-1) (Figure 8).

Discussion

If the phenolic compounds are ranked into increasing order, the vanillic acid is in the first place because of its high concentration, followed by catechin, pyrocatechin, epicatechin, rutin, cinnamic acid, gallic acid, syringic acid, and juglone. In samples except 'BD6', 'Bonifác', 'Tiszacsécsi 83', and all Bulgarian-bred cultivars, there was a higher vanillic acid content than Gómez-Caravaca et al. (2008) detected.

Among the examined phenolic compounds cathechin concentration was the highest in all cultivars. This result is similar to results of Matok et al. (2009) and Gómez-Caravaca et al. (2008), and the quantity of the cultivars having high

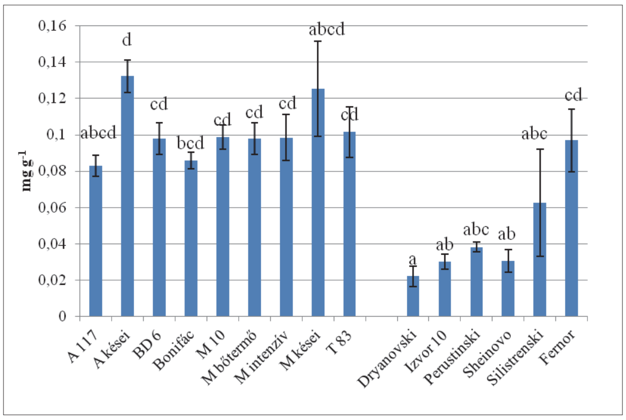

FIGURE 8. Juglone content of kernels in analyzed cultivars ($SD_{5\%} = 0.037$).

concentration was similar to data of Karamac (2009) and Kalogeropoulos et al. (2013). The top-ranked cultivars involved in our study reached 6-fold higher syringic acid content than results of Kalogeropoulos et al. (2013). It was the same tendency in the case of epicatechin. We measured 2.5-fold higher epicatechin in the cultivars ranked on the top in our study compared to Greek walnut genotypes measured by Kalogeropoulos et al. (2013). In contrast, the Greek research group detected 37-fold higher gallic acid in the Greek samples, than in the best Hungarian-bred cultivars (Kalogeropoulos et al., 2013). Gómez-Caravaca et al. (2008) detected very small (0.1 mg g^{-1} kernel) gallic acid content in their samples, which was lower than Bulgarian-bred 'Perustinski', containing the lowest value in our research.

Among the examined Hungarian-bred walnut cultivars the 'Alsószentiváni 117', 'Alsószentiváni kései', and 'Milotai 10' had outstanding inner content value; the highest compound concentration was detected in 'Sheinovo' among the Bulgarian-bred cultivars.

This study further confirms that the environment plays a major role in influencing the ratio of these compounds of kernels annually.

According to our results, the Persian walnut cultivars grown in the Carpathian Basin possess the potential to create an outstanding phenolic acid content useful for nutritive and medical applications.

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