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RESEARCH PAPER

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THE COMPARISON OF ANTIOXIDANT CAPACITY AND HPTLC POLYPHENOLIC PROFILE OF GREEN FRUITS AND LEAVES OF WALNUT (*Juglans regia* L.) CULTIVARS GROWN IN POLAND

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ABSTRACT

Walnut (*Juglans regia* L.) has a wide range of applications in food, pharmaceutical and cosmetics industries, however both unripe fruits and leaves have not been widely evaluated for varietal variation so far. Due to, leaves and immature fruits of seven walnut cultivars (Lake, Mars, Franquette, U02, SK04/10, Broadview, and Resovia) grown in Poland were compared for the first time in terms of antioxidant capacity (DPPH and FRAP), total phenolic content and qualitative polyphenol profile (HPTLC). The highest phenolic content was found for the Franquette ($p < 0.05$), both for leaves and nuts. In general, lower variability was observed for leaves, in the case of green nuts the results were more diversified by cultivar. The rapid and cost-saving HPTLC method was used to compare the polyphenolic profiles of the extracts. Cultivar-dependent differences in phenolic acids (mainly syringic) and flavonoids (mainly derivatives of quercetin) presence were found. Despite of observed differences between cultivars, it was found that both part of walnut are abundant in polyphenols. However it has been confirmed that the selection of a phytochemically appropriate cultivar may be important in providing a particularly valuable raw material for the food, pharmaceutical and cosmetic applications, thus the further quantitative studies are required.

Keywords: phenolic acids, flavonoids, DPPH, FRAP, raw material

INTRODUCTION

Walnut (*Juglans regia* L.) is a popular tree, widely cultivated in Europe, including Poland. It is used because of its tasty nuts, which have a lot of culinary uses. Moreover, the leaves, flowers, bark, and unripe fruit are also valuable herbal raw materials [Britton et al. 2008]. Walnut leaves and green nuts are the abundant source of antioxidants, mainly flavonoids and phenolic acids, as well as compounds from the group of naphthoquinones [Santos et al. 2013, Schwindl et al. 2017]. Antimicrobial, antiparasitic and hypoglycemic properties have been demonstrated for the leaves and green nuts, which also have an antiproliferative effect on selected cancerous cell lines [Oliveira et al. 2008, Carvalho et al. 2010, Santos et al. 2013, Sharma et al. 2013, Wang et al. 2016, Rabiei et al. 2018, Vieira et al. 2019].

There are many cultivars of walnut cultivated in the world, varied in terms of yield, morphology and

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growing conditions. Widely used commercial cultivars include: Franquette – an old French variety, Mars – native to the Czech Republic, Broadview – bred in Canada, and the American cultivar Lake. U02 is a Polish walnut cultivar, whose mother tree originated from the remains of historical orchards at the Łańcut Palace in the Podkarpacie region. This cultivar is characterized by high frost tolerance, and is highly fertile. In turn, SK04/10 is a cultivar with nuts of a pink colored skin on the kernel. It originates from Silesia. Although it exhibits slightly lower frost resistance, it is highly fertile and enters the fruiting phase early. The red kernels additionally enhance the decorative value of the fruits [http://szczepionyorzech.pl]. The Resovia cultivar also comes from Podkarpacie, and was named after Rzeszów, the capital of this region. It is an early cultivar, resistant to frost and diseases [Zdyb 2003].

The wide genotypic diversity of walnuts translates into a diverse phenotype. In many regions of the world, selection for genotypes with specific characteristics, including high kernel quality, fat, protein, and phenolic compound content, as well as resistance to climatic conditions has been conducted [Cosmulescu 2013, Keles et al. 2014, Sarikhani et al. 2021, Balapanov and Artykhova 2021, Hamidirad et al. 2025]. The most important biochemical properties include the content of bioactive substances in nuts, especially polyphenolic compounds, tocopherols and fatty acids [Sarikhani et al. 2021, Temizyürek et al. 2025]. The selection of superior genotypes, and their inclusion in walnut breeding program is possible thanks to modern biotechnological and bioinformatic tools [Vahdati et al. 2020, Temizyürek et al. 2025].

The aim of the study was to compare seven walnut cultivars grown in the same soil and climate condition in terms of antioxidant capacity and phenolic profile of unripe fruits and leaves. To obtain qualitative polyphenol profiles, a rarely used method of high-performance thin-layer chromatography (HPTLC) was used, which allows for quick, easy and cheap comparison (screening) of many samples simultaneously. The tested cultivars were evaluated for the usefulness of these parts of the plant as raw materials for the preparation of tinctures or some pharmaceutical preparations and also in terms of indicating cultivars valuable for walnut cultivation.

MATERIALS AND METHODS

Plant material and extracts preparation

Leaves and unripe walnuts were obtained in May–June 2020 from the walnut plantation in Urzejowice (Podkarpackie, Poland, 50°0'49" N, 22°27'23" E). Research material of seven walnut cultivars: Lake, Mars, Franquette, U02, SK04/10, Broadview and Resovia was collected and deposited in the collection of Department of Chemistry and Food Toxicology, University of Rzeszów. After harvesting, the leaves were dried at a temperature below 40 °C, while the nuts were cut into quarters and freeze-dried using the Alpha 1–2 LD plus freeze dryer (Martin Christ, Osterode am Harz, Germany). The dried material was ground into powder using a grinder (MK-06M, MPM, Milanówek, Poland). Two grams of each sample were extracted using 40 mL of 80% (v/v) ethanol, using an ultrasound-assisted method (700 W, 40 kHz; SONIC-10, Polsonic, Warszawa, Poland). The extracts were filtered through a paper filter and stored in a freezer until further analyses.

Total phenolic content and antioxidant capacity

The total phenolic content was measured using Folin-Ciocâlteu method, as described by Dżugan et al. [2021]. Extracts were diluted 100-fold and aliquotes of 0.02 mL were placed into a 96-well plate. Then, 0.1 mL of 10% Folin-Ciocâlteu reagent followed by 0.08 mL of 7.5% (w/v) of Na_2CO_3 solution were added. Samples were kept in the dark for 60 min, and then the absorbance was measured against blank at 760 nm using a microplate reader (EPOCH 2, BioTek, Vermont, USA). The results were expressed as mg of gallic acid (GAE) equivalents per gram of dry mass (mg GAE g^{-1}) based on calibration curve prepared for GAE standard solutions in the range 0–250 μg mL^{-1} ($y = 0.0555x$, $R^2 = 0.9976$).

The FRAP assay (Ferric Reducing Antioxidant Power) was carried out according to Dżugan et al. [2021], as follows: to 0.02 mL of diluted plant extract, 0.18 mL FRAP reagent – 2.5 mL of a 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer (pH 3.6) – was added and after 10 min incubation at 37 °C the absorbance of the reaction mixture was measured using a microplate reader (EPOCH 2)

against blank at 593 nm. The results were expressed as μmol of Trolox (TE) equivalents per gram of dry mass (μmol TE g^{-1}) based on calibration curve (y = 0.026x, R^2 = 0.9989).

The DPPH test was performed also according to Dżugan et al. [2021], as follows: to 0.02 mL of diluted plant extract 0.18 mL of DPPH radical methanolic solution (0.1 mM) was added and kept in the dark for 30 min. Then, the absorbance of tested (As) and control (Ao) samples was measured at 517 nm against methanol using microplate reader (EPOCH 2). The reduction of DPPH radical was calculated according to equation: DPPH% = [(Ao − As) / Ao] × 100. The results were calculated for Trolox equivalents using a calibration curve in the range 0.5–6 μmol of Trolox per sample (y = 15.553x, R^2 = 0.9970).

HPTLC polyphenols detection

The analysis of polyphenol profile was performed using a HPTLC set (Camag, Muttenz, Switzerland) consisted of the semi-automated application device (Linomat 5, CAMAG), automatic developing chamber (ADC2, CAMAG), the imaging device (TLC Visualizer, CAMAG) and the automated derivatizer of TLC plates (CAMAG Derivatizer). Extracts were applied to plates (HPTLC Silica Gel 60 F254, 20 × 10 cm, Merck Darmstadt, Germany) in a volume of 3 μL and the plates were developed with the mobile phase composed of chloroform, ethyl acetate and formic acid (5:4:1, v/v/v) to a distance 70 mm. Obtained results were documented using UV light (366 nm). Additionally, plates were derivatized with p-anisaldehyde sulfuric acid reagent. After derivatization, plates were heated at 110 °C for 10 min and imaged under UV 366 nm. Obtained chromatographic images were analyzed using the HPTLC software (Vision CATS 3.2, CAMAG).

Statistical analysis

All numerical data were obtained in triplicates. The mean values and standard deviations were calculated. Significance of differences was checked using ANOVA one-way analysis of variance followed by a Tukey's (HSD) test (p = 0.05). In order to demonstrate the similarity of the tested varieties, a cluster analysis was performed based on the results obtained. All calculations and analyses were performed using Statistica 13.1 software (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Interest in unripe walnuts as a culinary raw material with valuable nutritional and therapeutic properties is constantly growing [Mukarram et al. 2024]. On the other hand, walnut leaves are less known and less frequently used. However, both raw materials can be a source of bioactive substances for food fortification and the development of new therapeutic and cosmetic preparations.

Samples of leaves and unripe fruit of the tested walnut cultivars were assessed for the content of phenolic compounds and antioxidant activity. Data on total phenolic content are presented in Figure 1.

The obtained results showed considerable differentiation. Both raw materials are a valuable source of polyphenolic compounds, and the difference between leaves and nuts in each case reaches max. 20%. In some cultivars (Mars, U02, SK04/10), higher levels of phenols were recorded in the leaves, for others in green nuts. The Franquette containing over 90 mg GAE g^{-1} definitely stood out among the green fruits samples. Obtained results are in line with Jakopič et al. [2009] findings, who investigated the content of phenols in the unripe fruits of two *J. regia* cultivars, namely: Franquette and Elit. In cited study the amount of 135.27 mg GAE g^{-1} and 126.20 mg GAE g^{-1} were found, respectively. In turn, Pycia et al. [2019] determined lower polyphenol content (at the level of 7.15 mg GAE g^{-1} for Resovia and 21.49 mg GAE g^{-1} for Leopold) in nuts harvested in July, and further decrease was noted during maturation. Other authors give data for the green husk of walnut at the level of up to 74.08 mg g^{-1} [Oliveira et al. 2008]. Also in these studies Franquette turned out to be the richest in phenolic compounds among the cultivars tested by the authors, which is consistent with the results obtained in the present study.

In the case of leaves, the results were slightly less differentiated. The highest content of phenols (in the range of 65–70 mg GAE g^{-1}) was recorded for the Lake, Franquette and SK 04/10 cultivars. The obtained results are consistent with Einali et al. [2018] findings who provide the value of 52.48 mg GAE g^{-1}.

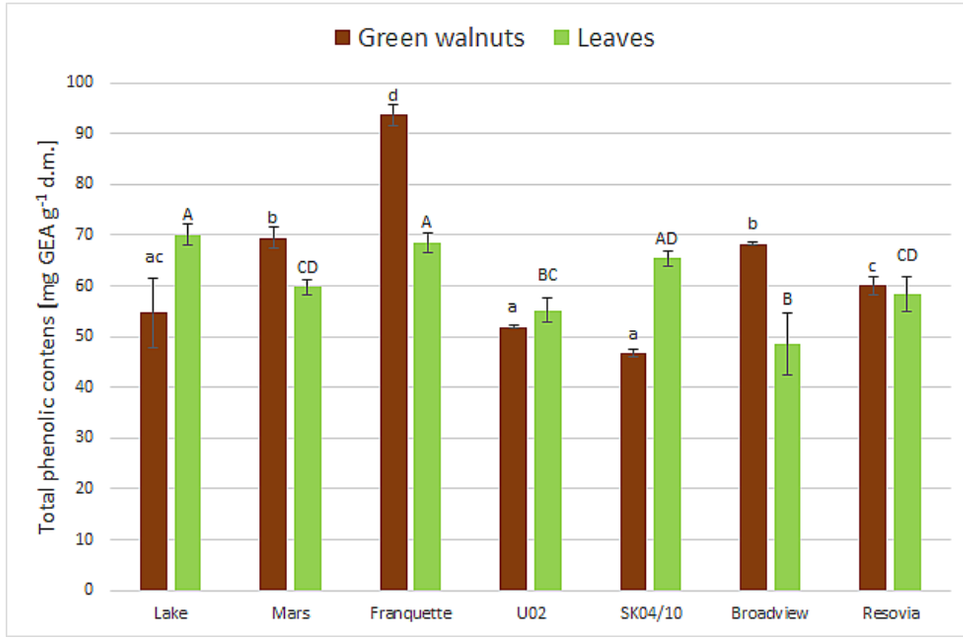

Fig. 1. The comparison of total phenolic content in extracts of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

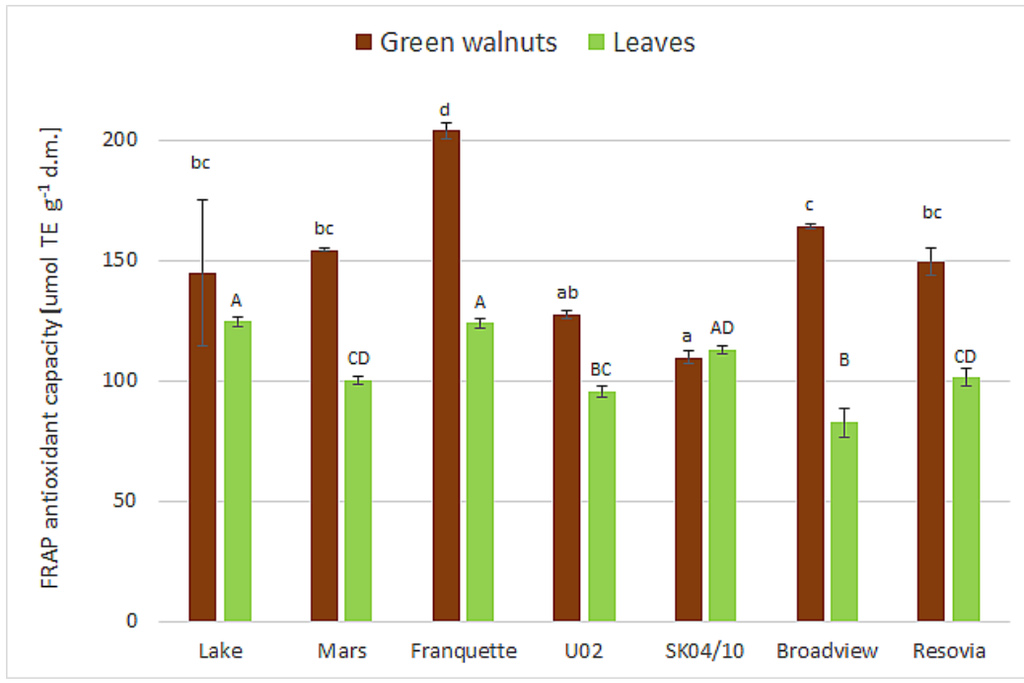

Fig. 2. The comparison of FRAP antioxidant capacity of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

However, the values reported in the literature show a large dispersion, from 25.3 mg GAE g^{-1} [Santos et al. 2013] to 410 mg GAE g^{-1} [Sharafati-Chalestori et al. 2011]. Such huge differentiation of the data may result from different sampling times or growing conditions as well as sample preparation and storage [Pakrah et al. 2020]. Cosmulescu and Trandafir [2011] observed seasonal variability of the phenol content in walnut leaves of several cultivars.

Literature data confirm strong correlation between total polyphenols content and antioxidant potential of both unripe fruits [Pycia et al. 2019, Cosmulescu et al. 2014] and leaves [Vieira et al. 2019, Pereira et al. 2008, Masek et al. 2019] of *J. regia*. Thus, the antioxidant capacity of the extracts was tested using two methods: FRAP (Fig. 2) and DPPH (Fig. 3).

According to both methods, the Franquette showed the highest activity among the samples of green nuts. Slightly different results were obtained by DPPH method, where Mars and Resovia did not differ significantly from the Franquette in terms of ability to scavenge free radicals. In the case of leaves, the differences were smaller The Lake, Franquette and SK 04/10 showed the strongest reducing abilities, and the Lake stood out in the free radical scavenging method.

There was a positive correlation coefficient between both methods (r = 0.773). The results for both assays were also strongly positively correlated with the total content of phenols: r = 0.761 and 0.832 for FRAP and DPPH, respectively. The correlation coefficients examined separately for leaves and green nuts are even higher (Table 1). High values of correlation coefficients indicate the dominant share of phenolic compounds determined by the Folin-Ciocâlteu method in shaping the overall antioxidant capacity of the tested extracts.

The obtained results were used to perform a cluster analysis, and construct the classification tree (Fig. 4). Based on the results, the closest relationships can be found between the Resovia and Mars, as well as

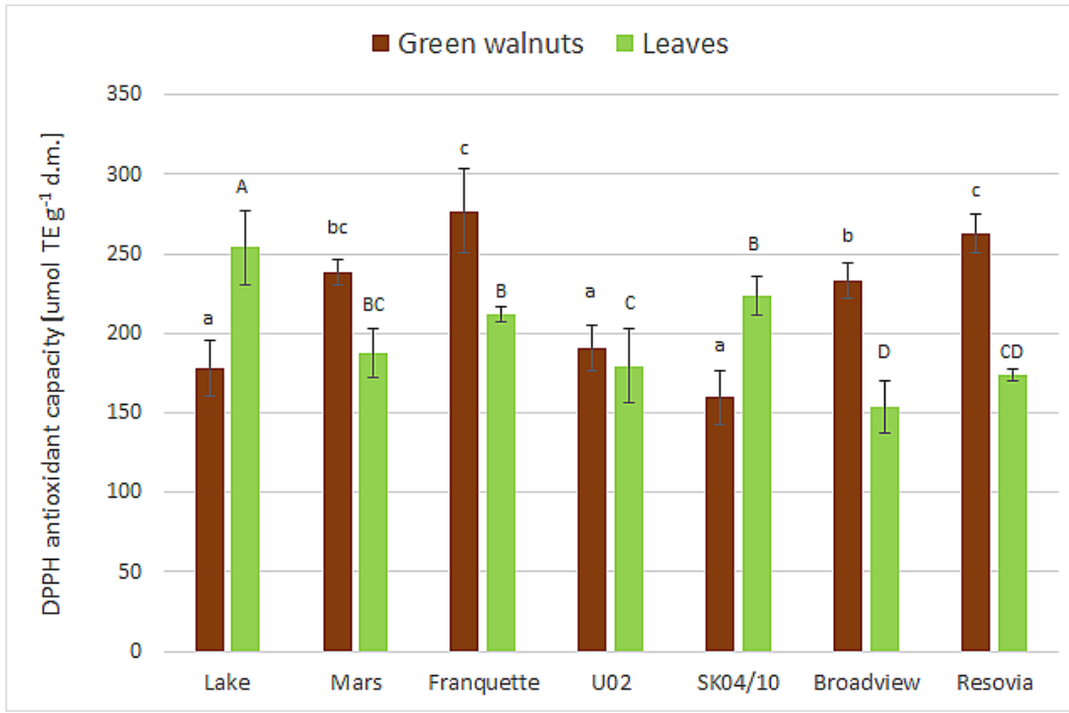

Fig. 3. The comparison of DPPH antioxidant capacity of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

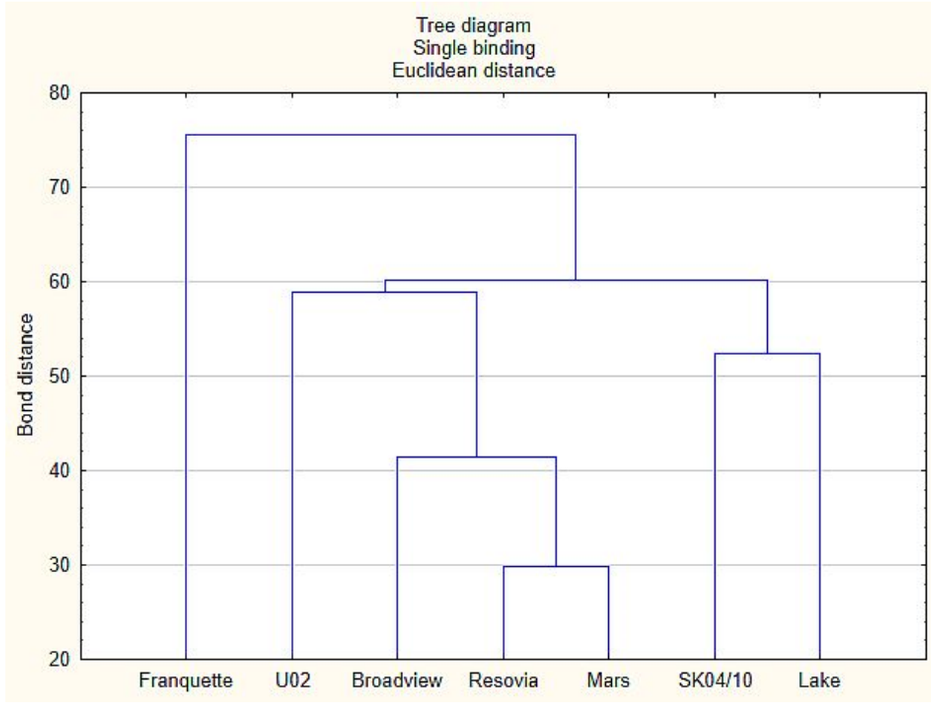

Fig. 4. Classification tree constructed on the basis of the results of the content of phenolic compounds and the antioxidant properties of leaves and green walnuts

Table 1. Pearson's correlation coefficients for tested parameters

Parameter	Leaves			Green walnuts			Overall		
	TPC	FRAP	DPPH	TPC	FRAP	DPPH	TPC	FRAP	DPPH
TPC	1	–	–	1	–	–	1	–	–
FRAP	0.969	1	–	0.990	1	–	0.761	1	–
DPPH	0.829	0.838	1	0.927	0.908	1	0.832	0.773	1

Broadview, which were characterized by similar antioxidant properties. The Franquette, for which the most favorable properties have been shown, differs significantly from the others.

The demonstrated differences between cultivars in the content of bioactive compounds and antioxidant properties may have a genetic basis, but the influence of environmental conditions cannot be excluded, although the tested raw materials came from trees grown in one location. It has been repeatedly shown that the genotypic variability of walnuts translates not only into phenotypic, but also phytochemical traits, including the content of important nutritional and bioactive components [Vahdati et al. 2020, Sarikhani et al. 2021].

In the present study, leaf and green nut extracts were analyzed for polyphenol profiles using the HPTLC method for the first time. This method is particularly useful for preliminary screening of multiple samples that are separated simultaneously, and the results obtained provide a direct comparison of component profiles under the same conditions [Choma et al. 2019]. Based on the set of obtained bands, a qualitative comparison of the fingerprints of each nut culti-

var can be made (number, color, intensity). Moreover, having Rf values for the expected standard substances determined under the same chromatographic separation conditions, it is possible to identify bands showing the same Rf value (Table 2). Images of HPTLC plates before and after derivatization with p-anisaldehyde are shown in Figure 5.

The compounds detected in HPTLC chromatograms include phenolic acids (blue bands in UV 366 nm) and flavonoids (yellow-green bands). Table 2 summarizes the presence of several reference substances, which were expected to occur based on the literature [Santos et al. 2013, Shi et al. 2018, Fernández-Agulló et al. 2020, Medic et al. 2021]. There was no confirmed presence of (–)-epicatechin, gallic acid, taxifolin, 1,4-naphtoquinone, and juglone in any of the tested samples using this method. Orange bands at the top of the plate indicate the presence of carotenoids and chlorophylls, especially in leaf extracts. The leaves showed more diversified phenolic profiles than unripe fruits which was manifested by a larger number of bands. Among the compounds distinguishing individual cultivars, (+)-catechin should be mentioned, present only in Lake leaves, the pattern of orange bands of photosynthetic pigments present in particular cultivars is also different. In the case of leaf extracts, the SK04/10, Franquette, Mars and Broadview have a clear blue band at Rf = 0.1, corresponding to an unidentified compound, most likely from the flavonoid group. The predicted presence of applied standard metabolites has not been confirmed in any case in green walnut extracts. The lack of detection of juglone, which is a characteristic metabolite, is probably related to the non-optimal extraction system used in the research. The 80% ethanol extraction used is more suitable for the recovery of polar phenolic compounds whereas non-polar solvents, such as petroleum ether, hexane, or chloroform, are optimal for juglone extraction [Strugstad and Despotovski, 2012]. There are also known examples of juglone extraction from green walnuts using pure ethanol or methanol [Jakopič et al. 2009, Cosmulescu et al. 2011], but not their mixtures with water. Juglone was also not detected in tinctures made of green nuts of the Mars with 40% ethanol [Miłek et al. 2022b], as well as using the analogous extraction system and HPTLC detection method in extracts of leaves and unripe walnuts extracts [Miłek et al. 2022a].

In order to precisely determine the polyphenolic profiles and estimate their content an accurate LC method coupled with MS should be used. Banding patterns on HPTLC plates may, however, be of value as an auxiliary classification and quality assessment method. Thanks to the ability to analyze up to 20 samples on a single chromatographic plate, relatively low analysis costs and time savings, the HPTLC method is particularly useful for the initial screening of samples and the qualitative comparison of their composition [Choma et al. 2019]. However, when using this technique, there are often problems with the identification

Table 2. Presence of polyphenolic compounds used as reference substances in fruit and leaf extracts

Compound	Rf	Color		Lake		SK 04/10		Franquette		Mars		Broadview		U02		Resovia	
		366 nm	366 nm after derivatization	GW	L	GW	L	GW	L	GW	L	GW	L	GW	L	GW	L
Quercetin 3-glucoside	0.01	yellow	greenish blue	–	+	–	+	–	+	–	+	–	+	–	+	–	+
Avicularin	0.02	black	blue	–	+	–	+	–	+	–	+	–	+	–	+	–	+
(–)-epicatechin	0.06	dark blue	black	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(+)-catechin	0.08	blue	black	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Gallic acid	0.11	dark blue	dark blue	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Taxifolin	0.22	blue	brown	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Syringic acid	0.50	blue	gray	–	+	–	+	–	+	–	+	–	+	–	+	–	+
1,4-naphtoquinone	0.58	gray blue	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Juglone	0.62	yellow	orange	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Rf – retardation factor, GW – green walnuts, L – leaves

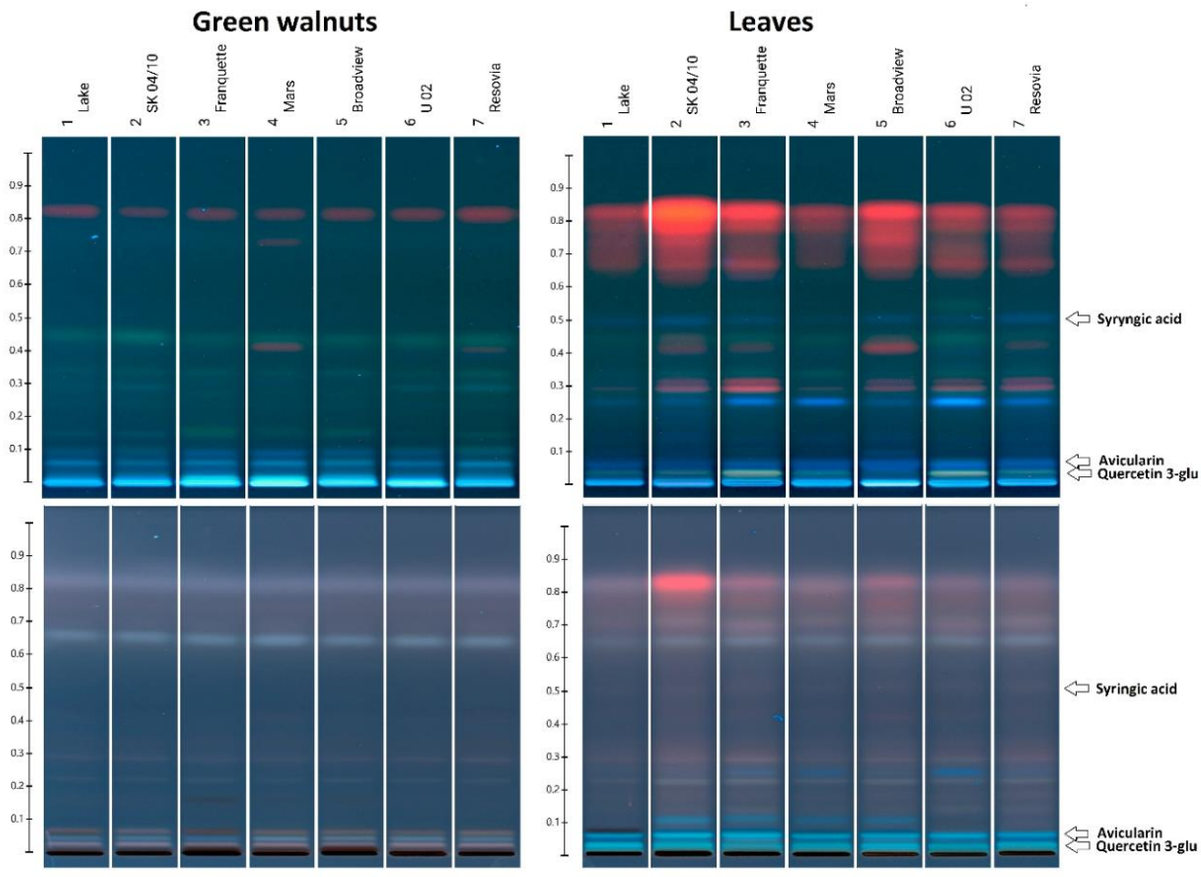

Fig. 5. HPTLC polyphenolic profiles for tested extracts with selected identified compounds marked, viewed in UV 366 nm, before (top) and after p-anisaldehyde reagent derivatization (bottom)

of compounds resulting from the need to use standards that are not always available and also from the frequent overlap of bands.

Previous studies of polyphenol profiles for various walnuts cultivars (using the UPLC-PDA-MS/MS method) showed that the qualitative composition is basically constant, however, thanks to the sensitive method, quantitative differences between cultivars were found, e.g. the Leopold was characterized by an early stage of ripeness nuts with a particularly high content of many compounds, e.g. procyanidins or quinic acid and its derivatives, so for this cultivar the total polyphenol content was significantly higher [Pycia et al. 2019]. In green walnut extracts, (+)-catechin and (–)-epicatechin as well as numerous phenolic acids were previously determined to be dominant [Cosmulescu et al. 2014]. Optimized extraction of green walnut shells with 50% ethanol allowed to identify polyphenolic compounds, as well as organic acids, lipids, terpenes and quinones using UHPLC–ESI–MS/MS [Savić and Savić Gajić, 2025]. Among the compounds identified in *J. regia* leaf extracts, caffeoylquinic and coumaroylquinic acids, as well as quercetin and kaempferol derivatives dominated [Amaral et al. 2004, Santos et al. 2013]. Hamdi et al. [2025] additionally identified numerous myricetin derivatives, juglone and its derivatives in ethanol extracts of leaves of Gran Jefe. Also for leaf extracts, differences between varieties were demonstrated by Nour et al. [2013] who concerned both compounds belonging to the class of

flavonoids, phenolic acids and naphthoquinones. The results of previous studies confirm that the composition of secondary metabolites depends on the plant genotype [Nour et al. 2013].

Illustrating differences in the qualitative composition of the tested extracts by HPTLC may help explain trends in their antioxidant activity. The similar total phenolic compound contents are reflected in similar band intensities in the chromatograms for leaves and green walnuts. The presence of pigments (chlorophylls and carotenoids), abundant in the leaf extracts, apparently does not affect the antioxidant activity. Therefore, it can be concluded that this activity is primarily due to the presence of polyphenolic compounds. The detected metabolites, e.g. flavonoid glycosides and especially syringic acid are known for their strong antioxidant properties [Srinivasulu et al. 2018] which confirms the observed correlation between the total phenols content and antioxidant capacity.

CONCLUSIONS

The preliminary analyzes carried out for selected seven cultivars of walnut grown in Poland confirm the high pro-health potential of both leaves and unripe fruits of these trees. The Franquette old cultivar, traditionally grown in Europe, turned out to be the best source of antioxidants. The HPTLC analysis of polyphenolic profiles allowed to create characteristic fingerprints for each cultivar and can be applied for appropriate and valuable walnut selection which can be introduced into wider cultivation. However, due to seasonal and climatic variability of the content of polyphenolic compounds in plant material, further long term research across growing season is needed to describe the effect of season on polyphenolic profiles of leaves and unripe fruits of *J. regia*. It is also necessary to conduct an in-depth phytochemical analysis of the tested raw materials using advanced extraction and sensitive analytical method as LC-MS/MS. Such detailed phytochemical analysis of raw materials is crucial for their use in various industries.

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The Authors declare no conflict of interest.

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