

MINERAL COMPOSITION, ANTIOXIDANT, ANTI-UREASE, AND ANTIBIOFILM POTENTIAL OF *JUGLANS REGIA* LEAVES AND UNRIPE FRUITS

– Research paper –

Michał MIŁEK^{1,*}, Ewa CISZKOWICZ², Katarzyna LECKA-SZLACHTA², Anna MIŁOŚ³, Grzegorz ZAGUŁA⁴, Anna PASTERNAKIEWICZ¹, Małgorzata DŻUGAN¹

¹ Department of Chemistry and Food Toxicology, Institute of Food Technology and Nutrition, University of Rzeszów, Ćwiklinskiej 1a, 35-601 Rzeszów, Poland, e-mail: mmilek@ur.edu.pl (M.M.), apast@ur.edu.pl (A.P.), mdzugan@ur.edu.pl (M.D.)

² Department of Biotechnology and Bioinformatics, Faculty of Chemistry, Rzeszow University of Technology, Faculty of Chemistry, Powstańców Warszawy 6, 35-959 Rzeszów, Poland, email: eciszkow@prz.edu.pl (E.C.), szlachta@prz.edu.pl (K.L-Sz.)

³ Doctoral School of Engineering and Technical Sciences at the Rzeszow University of Technology, Department of Biotechnology and Bioinformatics, Faculty of Chemistry, Powstańców Warszawy 6, 35-959 Rzeszów, Poland, e-mail: d520@stud.prz.edu.pl

⁴ Department of Bioenergy, Food Analysis and Microbiology, Institute of Food Technology and Nutrition, University of Rzeszów, Ćwiklinskiej 1, 35-601 Rzeszów, Poland, e-mail: gzagula@ur.edu.pl

Abstract: The leaves and unripe fruits from 5 walnut clones were tested in terms of mineral composition, antioxidant activity, and the inhibitory effect on model urease. It has been shown that the leaves are richer in minerals and also have higher antioxidant potential than unripe fruits. HPTLC polyphenolic profiles of leaves extract were found more abundant in flavonoids regardless of the sample origin. Only green walnut extracts showed an effect as urease inhibitors. Higher antibacterial activity was observed against *Staphylococcus aureus* in comparison with *Staphylococcus epidermidis*. Both leaf and fruit extracts showed antibiofilm activity, up to 95% of biofilm inhibition. It was shown that all tested *Juglans regia* extracts can serve as a valuable antibacterial agent against staphylococci infections.

Keywords: *Juglans regia*, green walnut, antioxidant potential, antibiofilm activity, HPTLC, urease

INTRODUCTION

Walnut (*Juglans regia* L.) is a popular tree from Juglandaceae family, distributed all over the world, however, most likely it comes from southern Europe, Asia Minor or China (Beer et al., 2008). Walnuts have been cultivated for centuries, especially in the temperate zone, mainly used for fruit (nuts), but also for wood. Ripe walnuts are used in the food industry as an ingredient in desserts, they are also used to obtain the oil. In addition, green walnuts, shells, husks, bark, and leaves are used in the pharmaceutical and cosmetic industries (Britton et al., 2008).

Green (unripe) walnuts are especially interesting and are used in many countries as an ingredient in traditional liqueurs and tinctures. They are also used in the preparation of comfitures and pickles (Nour et al., 2016). There are also some attempts to use of green nut husks as an additive to functional food, e.g. for sausages in which this additive provided better microbiological stability of the product (Salejda et al., 2016). These types of products, made of walnuts with green husk and just before the hardening of the endocarp, show bioactivity, especially strong antioxidant potential (Alamprese et al., 2005; Stampar et al., 2006; Gawlik-Dziki et al., 2014; Cosmulescu et al., 2014).

Green walnuts, apart from their antioxidant properties, also have antimicrobial, antiparasitic, antihyperuricemic, and antiproliferative properties

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* Corresponding author.

E-Mail address: mmilek@ur.edu.pl

Phone: +48 178721730

against cancerous cell lines (Oliveira et al., 2008; Carvalho et al., 2010; Sharma et al. 2013; Wang et al., 2016). In turn, walnut leaves also show a broad spectrum of bioactivity. Their antioxidant, antimicrobial and anthelmintic properties have been demonstrated (Carvalho et al., 2010; Santos et al., 2013; Sytykiewicz et al., 2015; Khan et al., 2018).

Among the main phytochemicals found in green nuts, naphthoquinones, steroid compounds, phenolic acids, tannins, and flavonoids were found. The most important and characteristic naphthoquinones of green walnuts included juglone, juglanone A and B, regiolone, 4-hydroxy- α -tetralone and their derivatives. Among phenolic acids, chlorogenic, caffeic, p-coumaric, ferulic, ellagic or sinapic acid were usually determined. The major flavonoids are (+)-catechin and (-)-epicatechin, myricetin, apigenin, quercetin, kaempferol, and their glycosylated forms (Jakopic et al., 2008; Cosmulescu et al., 2010; Panth et al.,

2016; Trandafir et al., 2016; Tsasi et al., 2015; Tsasi et al., 2016; Jahanban-Esfahlan et al., 2019). The leaves show a very similar phytochemical composition to green nuts (Nour et al., 2013; Schwindl et al., 2017). Among the polyphenolic compounds present in the leaves, flavonoids (quercetin, kaempferol, and myricetin glycosides), as well as phenolic acids predominate (Amaral et al., 2004; Zhao et al., 2014). There is also an essential oil in the whole plant, containing anthraquinones and terpenes (Panth et al., 2016). Green husks also contain vitamins, especially ascorbic acid (vitamin C) and α -tocopherol (vitamin E) (Jahanban-Esfahlan et al., 2019).

The aim of the study was to compare the mineral composition and antioxidant activity of leaves and green fruit of several clones of walnut. The ability of the tested extracts to inhibit the model urease *in vitro* was also assessed. Moreover, the effect of the extracts on *Staphylococcus* sp. with different biofilm formation capacity was also tested.

MATERIALS AND METHODS

Plant material and extract preparation

Unripe fruits, as well as leaves of *Juglans regia*, were collected in June 2019 from five trees growing in home gardens in the Podkarpackie Province (Poland). The leaves were air-dried, without direct exposure to light, and the fruits were freeze-dried using FreeZone 2.5 lyophilizer (Labconco, Kansas City, MO, USA). The extracts were prepared using aqueous ethanol (80%) as solvent. One gram of pulverized plant material (unripe nuts and leaves) was flooded with 20 mL of solvent and extracted in an ultrasonic bath (Sonic-10, Polsonic, Warszawa, Poland) for 20 minutes. The extracts were then filtered and stored frozen until further analysis. For testing the antibacterial activity, the crude extracts were evaporated to dryness with the use of refrigerated CentriVap Concentrator (Labconco, Kansas City, USA) in 50°C for 1.5h and dissolved in 80% ethanol (2.5 mg/mL) to obtain equal concentrations for all tested extracts.

Mineral composition

The contents of 17 elements including toxic metals (Cd, Pb) and the beneficial elements (K, Mg, Ca, Mn, Fe, Zn, Cu, Se) in dried leaf and freeze-dried fruit were determined by optical emission spectrometry with inductively coupled plasma (ICP-OES) using a Thermo iCAP 6500 spectrophotometer (Thermo Fisher Scientific Inc., Bridgewater, USA). Before the determination, all tested samples were subjected to wet

mineralization, which was performed using the microwave mineralizer Milestone Ethos Ultrawave-One (Milestone SRL, Sorisole, Italy) for approximately 45 min under pressure in Teflon containers. After cooling (approximately 45 min), the samples were transferred to sterile flasks with a capacity of 50 mL and supplemented with redistilled water to the mark. The detection threshold obtained for each element was no less than 0.01 mg/kg (with the assumed detection capacity of the measuring apparatus at a level exceeding 1 ppb). A curve fit factor for the elements studied was above 0.99. All the analyses were made in three independent replications for each sample. The targeted repeatability expressed as the relative standard deviation (RSD), and the targeted recovery were 20 and 97 to 102%, respectively. The method was validated using certified reference material (NIST –1515). To identify the relevant measurement lines and avoid possible interferences, the method of adding an internal standard was applied (yttrium and ytterbium ions at concentrations of 2 and 5 mg/dm³, respectively). The results were expressed as mg/kg of dry weight (DW).

Total phenolic content

The total phenolic content in obtained extracts was determined according to Singleton and Rossi (1965) method, modified for use with a microplate reader. Briefly, 20 μ L of properly diluted extracts were

mixed with 100 μ L of Folin-Ciocalteu reagent (10%) and 80 μ L of 7.5% Na_2CO_3 solution in a microplate well. The samples were incubated in the absence of light for one hour and then the absorbance at 760 nm was measured using EPOCH2 microplate reader (Biotek, Winooski, VT, USA). The results were expressed in milligrams of gallic acid equivalents per gram of plant DW based on the standard curve ($y = 0.336x$, $R^2 = 0.9914$) prepared in the concentration range of 0 to 125 mg/mL.

Antioxidant capacity

DPPH scavenging ability

The ability to scavenge the synthetic DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical was measured according to Blois (1958) method, modified for use with a microplate reader. Twenty microliters of diluted sample were mixed with 180 μ L of 0.1M DPPH methanolic solution. The samples were incubated in the dark for 30 minutes and then the absorbance at 517 nm was measured against methanol using EPOCH2 microplate reader (Biotek). The results are expressed in Trolox equivalents (μ mol TE per gram of plant DW) converting the percent radical inhibition compared to the control sample based on the standard curve ($y=1.5553x$, $R^2 = 0.9970$) prepared in the concentration range of 0.25 to 3 mmol/mL.

Ferric Reducing Antioxidant Power (FRAP)

The ability to reduce iron(III) ions using the FRAP method was tested according to Benzie and Strain (1996), with slight modification. Briefly, 20 μ L of diluted sample was mixed with 180 μ L of FRAP reagent (containing 2.5 mL of a 10 mM solution of 2,4,6-tri-pyridyltriazine (TPTZ) in 40 mM HCl, 2.5 mL of 20 mM FeCl_3 and 25 mL of 0.3 M acetate buffer (pH 3.6)). After 10 min incubation at 37°C the absorbance was measured at 593 nm using EPOCH2 microplate reader (Biotek). The results are expressed in Trolox equivalents (μ mol TE per gram of plant DW) using standard curve ($y = 0.152x$, $R^2 = 0.999$) prepared in the concentration range of 0.25 to 3 mmol/mL.

Photochemiluminescence method

Antioxidant activity was determined by photochemiluminescence (PCL) using the Photochem[®] instrument (AG, Jena, Germany). The activity of the hydrophilic (PCL-ACW) antioxidant fractions was determined according to the procedures provided by the manufacturer's instruction. The results were expressed as mmol ascorbic acid equivalents (AA) per kilogram of DW (mM AA/kg). The study was performed using a

commercial reagent kit ACW (Analytik Jena AG, Jena, Germany). Leaf and fruit sample aqueous extracts (50 μ g/mL) were used. The analysis of the results was carried out using PCLSoft 5.1 software.

Polyphenol profile by HPTLC

The extracts were analyzed using HPTLC Silica Gel 60 F254 plates (20 cm $\times$ 10 cm) (Merck, Darmstadt, Germany). Five microliters of each extract were applied to the plate as 10 mm bands from the lower edge of the plate at the rate of 200 nL/s using a semi-automated HPTLC application device (Linomat 5, CAMAG, Muttentz, Switzerland). The separation was performed in an automatic developing chamber (ADC 2, CAMAG, Muttentz, Switzerland) with the mobile phase composed of chloroform:ethyl acetate:formic acid [5:4:1 v:v:v], and developed to a distance of 85 mm. The obtained results were documented using an HPTLC imaging device (TLC Visualizer, CAMAG, Muttentz, Switzerland) under white light, 254 and 366 nm. The plate was derivatized with p-anisaldehyde reagent using an automated TLC derivatizer (CAMAG Derivatizer, Muttentz, Switzerland). After derivatization, the plates were imaged under white light and 366 nm. The obtained chromatographic images were analyzed using HPTLC software (Vision CATS, CAMAG, Muttentz, Switzerland).

Urease inhibition assay

A model plant urease inhibition assay was performed according to Tanaka et al (2003) with slight modification. Jack bean (*Canavalia ensiformis*) urease purchased from Merck was used. Briefly, 25 μ L of urease solution (1 U) was mixed with 25 μ L of tested extract and preincubated for 30 min. at room temperature. After preincubation 200 μ L of 100 mM phosphate buffer (pH 6.8) containing 500 mM urea and 0.002% phenol red was added to each well and the absorbance was read at 570 nm using EPOCH2 microplate reader (Biotek) after 1 h incubation at room temperature. Percentage inhibition of urease was calculated from the formula:

$$I\% = (1 - (A/A_C)) \times 100\%$$

where A is the absorbance of the tested sample and A_C is the absorbance of the control sample, without inhibitor addition.

Antibacterial activity

In order to explore the antibacterial properties of the obtained extracts, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 35984 (a strain with high biofilm formation capacity) were used as a part of Department of Biotechnology and Bioinformatics (Faculty of Chemistry at Rzeszow

University of Technology) collection. The *Staphylococcus epidermidis* ATCC 12228, a control strain with a very low biofilm formation capacity, was kindly provided by the Chair and Department of Medical Microbiology Medical University of Lublin.

Minimum Inhibitory Concentration (MIC)

The antibacterial activity of extracts was evaluated by the determination of the minimum inhibitory concentration (MIC) with the use of the microdilution method (Pusz et al. 2018) with minor modifications. The experiments were carried out in triplicate in the range of concentration between 0.039 mg/mL and 1.25 mg/mL. Appropriate controls were applied: positive control of culture growth (MHB medium with no extract added), a negative control (MHB medium with extracts and no bacterial cultures added), and solvent control (serial dilutions of 80% ethanol).

Inhibition of biofilm formation

The influence of the tested extracts on the degree of biofilm inhibition by *S. aureus* and *S. epidermidis* was assessed using the method of vital staining using 0.3% MTT solution in PBS according to Bocian et al. (2020). Exact changes in the amount of formazan formed were assessed by performing spectrophotometric measurements at 450 nm. The tests were carried out in triplicate for each extract against *S. aureus* ATCC 6538, high biofilm-producing *S. epidermidis* ATCC 35984. No biofilm-producing *S. epidermidis* ATCC 12228 was used as a negative control. Serial dilutions were performed to determine the degree of biofilm inhibition of the test extracts in the range from 0.039 mg/mL to 1.25 mg/mL. The amount of biofilm inhibition was calculated relative to the amount of biofilm grown in the absence of an anti-biofilm agent (defined as 100%) and the media sterility control (defined as 0%). Results from at least three separate biological replicates were averaged.

RESULTS

Mineral composition

The mineral composition of *Juglans regia* leaves and green fruits determined by the ICP-OES method is presented in Table 1. Walnut leaves were generally characterized by a much higher content of individual elements compared to nuts, excluding P, Zn, Cu, Mo, Se. Macronutrients present in the tested nuts and leaves were ranked from the highest content as: K > Ca > Mg > P > S. A very low Na content in leaves and complete its absence in the nuts was surprising. The walnut leaves were particularly rich in calcium, manganese, aluminum, and sulfur. Among the micronutrients, these raw materials contained significant amounts of zinc, strontium, copper, and iron. Higher amounts of selenium were found in green nuts. Low contamination with heavy metals was observed, however, in the case of sample 1, slightly higher levels of cadmium, chromium, and lead were recorded. The specific accumulation of aluminum in leaves was observed (0.12% of total elements). Statistically significant differences between individual clones were noted in the case of aluminum, calcium, copper (in leaves), potassium, manganese, phosphorus, sulfur, strontium, and zinc (Table 1). In some cases, the scatter of the results was so large that the differences in the content of the elements were statistically insignificant.

Antioxidant activity

The antioxidant effect of tested extracts was evaluated using three methods: DPPH radical inhibition, ferric ion reduction power (FRAP), and photochemiluminescent method using Photochem device. These results as well as determined total phenolic content are summarized in Table 2.

In general, the better antioxidant properties for unripe walnuts than for leaves were observed regardless of the used method, however, due to the great variability of tested samples, the significant differences were found for DPPH results only. The total content of phenolic compounds varied in the tested samples between 25.6 to 64.78 mg GAE/g for leaves and 38.39 to 86.21 mg GAE/g for fruits.

A strong positive correlation was observed between the polyphenol content (TPC) in the samples and their antioxidant potential ($r = 0.925$ for TPC-FRAP and 0.865 for TPC-DPPH assays). The results obtained with the PCL ACW method were less closely correlated ($r = 0.511$ for TPC, 0.554 for FRAP, and 0.505 for DPPH), but it should be remembered that this method takes into account only the hydrophilic fraction of antioxidants. Comparing the averaged data for all tested leaves and fruits, we can observe a higher content of polyphenols and a stronger antioxidant potential of green nuts than leaves, except for the data from the PCL ACW method, which are at a comparable level. This difference can be related to the variable content of water-insoluble antioxidants which was not included in PCL measurements.

Table 1. Mineral composition of *Juglans regia* leaves and green fruits

	Leaves						Green walnuts					
Element content [mg/kg DW]	LI	L II	L III	L IV	L V	Average	GW I	GW II	GW III	GW IV	GWV	Average
Al	36.08±0.72 ^a	64.00±2.29 ^b	41.44±0.34 ^{ac}	68.15±2.34 ^{bd}	42.63±3.03 ^{cd}	50.46±14.54*	0±0 ^A	3.55±1.25 ^B	0±0 ^A	1.68±0.31 ^B	0±0 ^A	1.05±1.58*
Ca	18465±96 ^a	15777±96 ^b	12315±67 ^c	16995±160 ^d	13284±191 ^e	15367±2552*	3762±59 ^A	3061±43 ^B	1832±11 ^C	3838±57 ^A	2427±19 ^D	2984±863*
Cd	0.09±0.00 ^a	0.03±0.05 ^a	0.11±0.02 ^a	0.08±0.07 ^a	0.06±0.02 ^a	0.07±0.03	0.05±0.08 ^A	0.02±0.02 ^A	0.05±0.05 ^A	0±0 ^A	0.06±0.02 ^A	0.03±0.02
Cr	1.50±1.07 ^a	0.06±0.10 ^b	0±0 ^b	0±0 ^b	0±0 ^b	0.31±0.67	0.15±0.26 ^A	0.33±0.57 ^A	0±0 ^A	0±0 ^A	0±0 ^A	0.10±0.15
Cu	6.71±0.34 ^a	9.25±0.07 ^b	19.15±0.21 ^c	10.91±0.39 ^d	8.25±0.29 ^e	10.85±4.88	7.09±0.47 ^A	8.65±0.41 ^B	7.52±0.39 ^A	9.12±0.35 ^B	7.74±0.27 ^{AB}	8.02±0.84
Fe	82.95±36.87 ^a	98.53±23.74 ^a	138.28±9.25 ^a	100.97±30.53 ^a	73.58±13.90 ^a	98.86±24.75*	12.26±15.50 ^A	88.09±20.37 ^B	28.29±9.79 ^{AB}	23.41±40.54 ^A	43.10±14.53 ^{AB}	39.03±29.58*
K	14097±86 ^a	11147±9 ^b	26401±207 ^c	15054±12 ^d	18447±106 ^e	17029±5851	14444±90 ^A	10525±109 ^B	9579±17 ^C	9991±112 ^D	12846±40 ^E	11477±2085
Mg	5294±11 ^a	4975±50 ^b	4973±44 ^c	5255±11 ^a	4333±23 ^d	4966±385*	1549±10 ^A	1229±12 ^B	794±1 ^C	984±11 ^D	1193±2 ^E	1150±283*
Mn	46.02±0.74 ^a	227.15±2.80 ^b	53.69±0.45 ^c	81.52±2.20 ^d	95.22±3.64 ^e	100.72±73.46*	13.36±0.87 ^A	35.06±0.83 ^B	14.33±1.90 ^A	14.98±0.22 ^A	14.78±1.07 ^A	18.50±9.28*
Mo	0.36±0.09 ^a	0.03±0.05 ^b	0.26±0.09 ^{ac}	0.12±0.11 ^{bc}	0.21±0.00 ^{abc}	0.20±0.13	0.23±0.18 ^A	0.03±0.05 ^A	0.09±0.05 ^A	0±0 ^A	0.08±0.07 ^A	0.09±0.09
Na	19.84±7.53 ^a	0±0 ^b	0±0 ^b	0±0 ^b	20.00±10.71 ^a	7.97±10.91	0±0 ^A	0±0 ^A	0±0 ^A	0±0 ^A	0±0 ^A	0±0
P	1659±4 ^a	1996±2 ^b	3674±4 ^c	1953±5 ^d	2888±20 ^e	2434±832	2170±6 ^A	1934±14 ^B	1672±11 ^C	1978±8 ^D	2334±5 ^E	2017±251
Pb	0.09±0.09 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0.02±0.04	0.03±0.05 ^A	0±0 ^A	0±0 ^A	0±0 ^A	0±0 ^A	0.01±0.01
S	1486±2 ^a	1516±8 ^b	2302±9 ^c	1482±4 ^a	1400±7 ^d	1637±374*	1189±4 ^A	842±11 ^B	660±7 ^C	813±6 ^D	931±1 ^E	887±195*
Se	1.09±1.89 ^a	0.14±0.24 ^a	0±0 ^a	0±0 ^a	0±0 ^a	0.25±0.47	0.64±0.55 ^A	0±0 ^A	0.56±0.82 ^A	0.09±0.15 ^A	0.74±0.36 ^A	0.40±0.34
Sr	42.77±0.33 ^a	50.49±0.21 ^b	28.28±0.30 ^c	37.23±0.08 ^d	25.94±0.21 ^e	36.94±10.17*	10.59±0.12 ^A	9.00±0.12 ^B	6.88±0.03 ^C	6.47±0.09 ^D	5.07±0.05 ^E	7.58±2.19*
Zn	25.00±0.08 ^a	30.03±0.25 ^b	36.86±0.28 ^c	20.14±0.05 ^d	17.47±0.06 ^e	25.90±7.78	19.47±0.03 ^A	21.21±0.20 ^B	15.71±0.05 ^C	19.36±0.16 ^A	14.75±0.08 ^D	18.10±2.74
TOTAL	41263±173 ^a	35891±118 ^b	49985±158 ^c	41061±149 ^a	40637±43 ^d	41767±5101*	23178±101 ^A	17757±186 ^B	14612±30 ^C	17680±153 ^B	19817±62 ^D	18608±3158*

a,b,c,d,e —Means for leaves marked with the different superscript letter within the column are significantly different (Tukey's honest significant difference test. $p < 0.05$). a,b,c,d,e—Means for fruits marked with the different superscript letter within the column are significantly different (Tukey's honest significant difference test. $p < 0.05$).

*—Means marked with an asterisk for average values differ significantly (for leaves and green walnuts) (t-test, $p < 0.05$).

Table 2. Total phenolic content and antioxidant activity of tested samples

	Sample	Total phenolic content [mg GAE/g]	DPPH [$\mu\text{mol TE/g}$]	FRAP [$\mu\text{mol TE/g}$]	PCL ACW [$\mu\text{mol AA/g}$]
Leaves	L I	64.78 $\pm$ 4.98 ^a	262.84 $\pm$ 36.40 ^a	122.15 $\pm$ 2.11 ^a	30.20 $\pm$ 4.60 ^a
	LII	63.69 $\pm$ 0.89 ^a	250.12 $\pm$ 20.97 ^a	128.29 $\pm$ 2.87 ^a	40.00 $\pm$ 5.00 ^a
	L III	25.60 $\pm$ 0.60 ^b	72.07 $\pm$ 11.06 ^b	53.29 $\pm$ 14.25 ^b	28.20 $\pm$ 4.00 ^a
	L IV	35.12 $\pm$ 1.30 ^c	117.29 $\pm$ 18.69 ^b	65.57 $\pm$ 2.31 ^b	26.60 $\pm$ 3.10 ^a
	L V	37.80 $\pm$ 2.32 ^c	104.57 $\pm$ 11.28 ^b	59.43 $\pm$ 2.66 ^b	28.00 $\pm$ 3.60 ^a
	Average	45.40$\pm$17.79	161.38$\pm$88.48*	85.75$\pm$36.36	30.60$\pm$5.41
Green walnuts	GW I	62.60 $\pm$ 2.09 ^A	289.69 $\pm$ 31.80 ^A	171.93 $\pm$ 10.07 ^A	38.10 $\pm$ 4.20 ^A
	GW II	71.73 $\pm$ 0.30 ^B	269.90 $\pm$ 23.71 ^A	171.05 $\pm$ 6.68 ^A	27.20 $\pm$ 4.00 ^{AB}
	GW III	51.29 $\pm$ 0.17 ^C	275.56 $\pm$ 50.76 ^A	87.28 $\pm$ 1.52 ^B	28.20 $\pm$ 2.20 ^B
	GW IV	38.39 $\pm$ 1.81 ^D	216.21 $\pm$ 40.84 ^A	102.63 $\pm$ 6.28 ^B	28.80 $\pm$ 4.20 ^{AB}
	GW V	86.21 $\pm$ 5.21 ^E	299.58 $\pm$ 40.35 ^A	194.52 $\pm$ 0.76 ^C	34.00 $\pm$ 3.80 ^{AB}
	Average	62.04$\pm$18.39	270.19$\pm$32.35*	145.48$\pm$47.39	30.58$\pm$14.36

a,b,c,d,e —Means for leaves marked with the different superscript letter within the column are significantly different (Tukey's honest significant difference test. $p < 0.05$). A,B,C,D,E—Means for fruits marked with the different superscript letter within the column are significantly different (Tukey's honest significant difference test. $p < 0.05$). *—Means marked with an asterisk for average values differ significantly (for leaves and green walnuts) (t-test, $p < 0.05$).

Polyphenolic profile by HPTLC

Tested extracts were separated on HPTLC plates simultaneously with selected standards of polyphenolic compounds. Figure 1 shows the image of the plate seen under UV light (366 nm) before and after derivatization and in visible light seen after p-anisaldehyde derivatization.

The analysis of the profiles obtained on HPTLC chromatograms allows the conclusion the profiles obtained for the leaves and unripe nuts of the clones are generally similar, differing in the presence or intensity of some selected bands. In the original chromatogram visualized under 366 nm UV light (Figure 1A) bands corresponding to different groups of compounds (based on colors) can be distinguished: orange bands can be assigned to chlorophylls and carotenoids, blue bands to phenolic acids, and yellow or yellow-green bands to flavonoids. Chlorophylls and carotenoids were present mainly in leaf extracts (tracks 6 – 10), they were visible in the R_f range of 0.65 – 0.85. For clones IV, V and VI, additional orange bands were present at $R_f = 0.42$, 0.3, and 0.29. After derivatization (Figure 1B) these bands turned purple. The presence of juglone ($R_f = 0.62$) and 1,4-naphthoquinone ($R_f = 0.40$) used as references were not determined by HPTLC analysis in both leaves

and green walnuts. There were numerous flavonoids in the tested extracts, including the identified (+)-catechin ($R_f = 0.09$), taxifolin ($R_f = 0.22$) and avicularin ($R_f = 0.03$). Their bands after p-anisaldehyde derivatization were particularly visible as yellow in visible light (Figure 1B) and blue (avicularin) brown (taxifolin) or black (catechin) in UV light (Figure 1C). These compounds were identified in the leaf extracts, in the case of green fruit they are not present, but a weakly intense blue band is visible at $R_f = 0.04$ (Figure 1C). The presence of gallic acid ($R_f = 0.11$) was also found in green nuts. Ellagic acid ($R_f = 0.05$), manifested as a pale blue band in UV 366 prior derivatization is also most likely present in all samples. In the leaf extracts, there was an intense blue band at $R_f = 0.07$, which probably corresponds to another compound from the flavonoids group.

Urease inhibition assay

The percentage inhibition of a model plant urease (from *Canavalia ensiformis*) by leaves and fruit extracts is shown in the graph (Figure 2). Average inhibition ranging from 49.72 to 69.92% was observed only when the enzyme was incubated with green walnut extracts. Leaf extracts showed no potential as urease inhibitors.

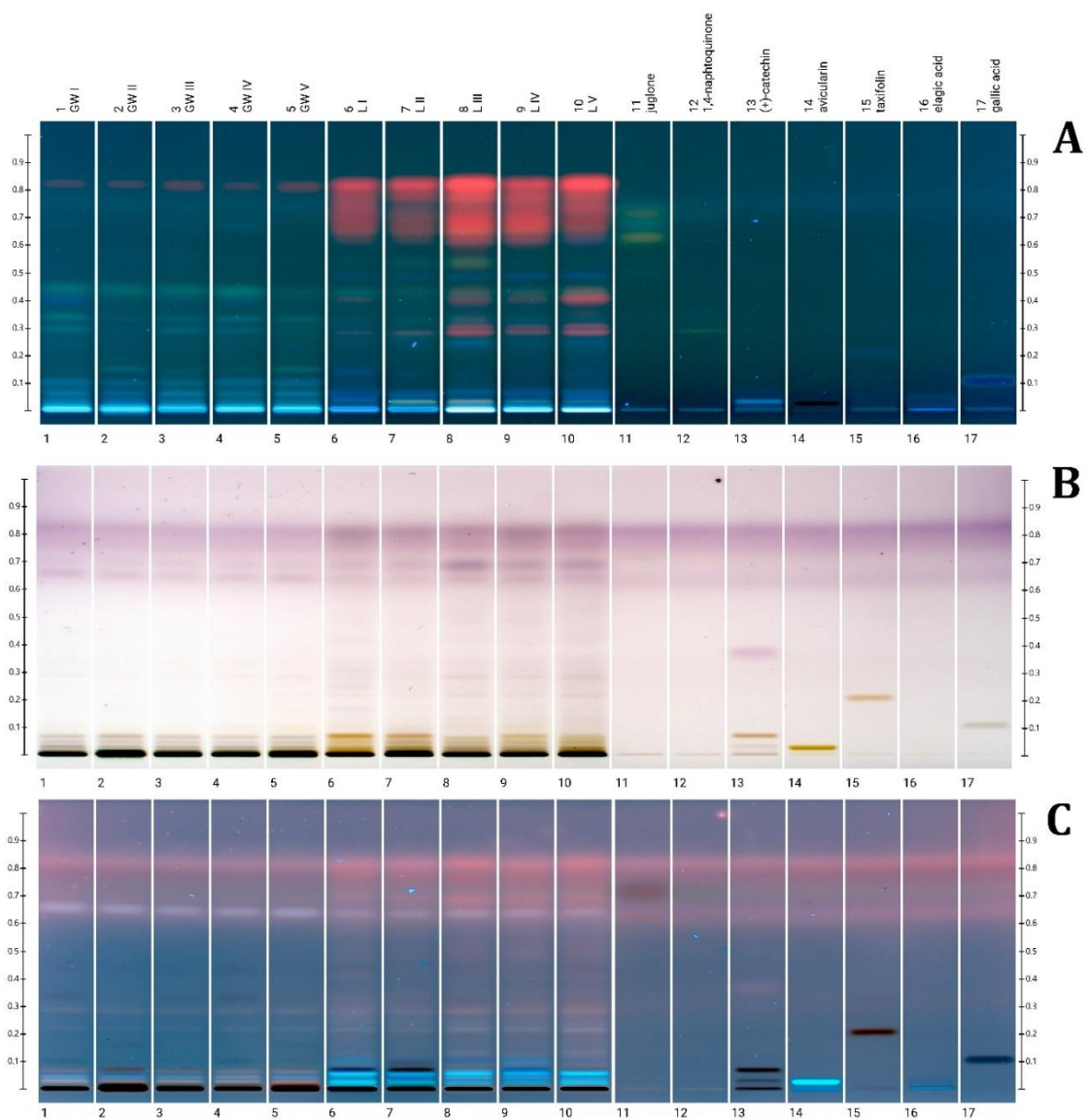


Figure 1. HPTLC plate images: A – in UV 366 nm before derivatization, B – in white light after derivatization, C – in UV 366 nm after derivatization

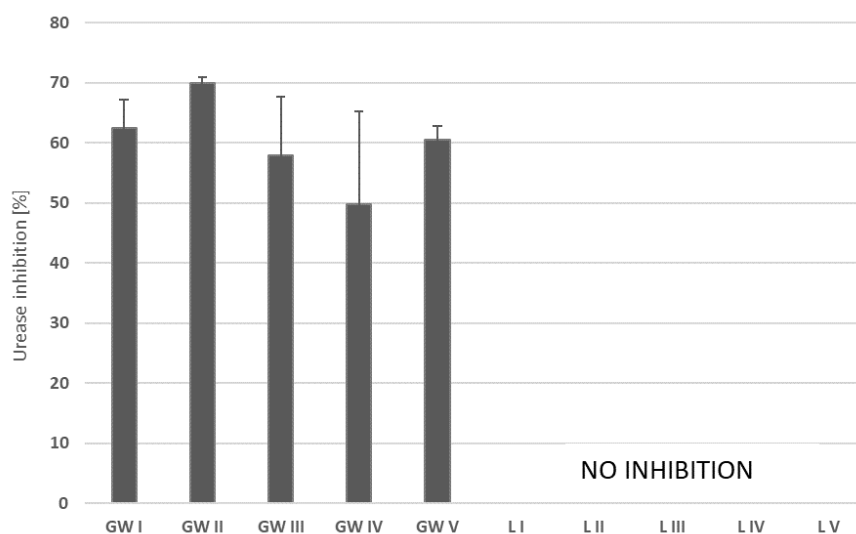


Figure 2. Inhibition of *C. ensiformis* urease by tested green walnuts (GW) and leaves (L) extracts. The error bars represent the standard deviation values. The results show no statistically significant differences.

Antibacterial activity

Minimum Inhibitory Concentration (MIC) Determination

Higher antibacterial activity was observed for all tested extracts against *Staphylococcus aureus* (0.078 – 0.156 mg/mL) in comparison with both *Staphylococcus epidermidis* strains (0.156 – 0.625 mg/mL) (Table 3).

Inhibition of biofilm formation

The effects of *Juglans regia* L. extracts prepared from crude samples on the inhibition of biofilm formation were investigated. The biofilm was grown in the presence of a decreasing concentration of fruit and leaf extracts. The results revealed that the amount of biofilm formed by *S. aureus* and *S. epidermidis* was differently affected upon the exposure of fruit and leaf extracts (Figure 2 and Figure 3). Both leaf and fruit extracts, at 1.25

mg/mL concentration, inhibited biofilm formation by *S. aureus* and *S. epidermidis* in the ranges of 81 – 92% and 59 – 94%, respectively. No statistically significant differences were observed between leaf and green walnut extracts, however, a clear dose-dependency for individual samples was observed. The strongest effect against *S. epidermidis* was observed for the leaves of clones I and II, which extracts show a strong inhibitory effect even at lower concentrations (0.3125 mg/mL) (Figure 3). Also, fruit extracts from these clones show a stronger effect at lower concentrations than the others. L IV and L V extracts showed the weakest capacity to inhibit the *S. epidermidis* biofilm formation in the range of tested concentrations. In opposite, clone 4 and 5 extracts, both leaf (L IV and L V) and green fruit (GW IV and GW V), proved to be more effective against *S. aureus* than others.

Table 3. MIC values of leaf and green walnut extracts against *Staphylococcus sp.* strains.

	MIC (mg/mL)		
	<i>S. aureus</i>	<i>S. epidermidis</i> 35984	<i>S. epidermidis</i> 12228
L I	0.078	0.156	0.313
L II	0.078	0.625	0.625
L III	0.078	0.625	0.313
L IV	0.156	0.625	0.625
L V	0.156	0.625	0.625
GW I	0.078	0.313	0.156
GW II	0.156	0.156	0.156
GW III	0.078	0.625	0.156
GW IV	0.156	0.313	0.156
GW V	0.078	0.313	0.156

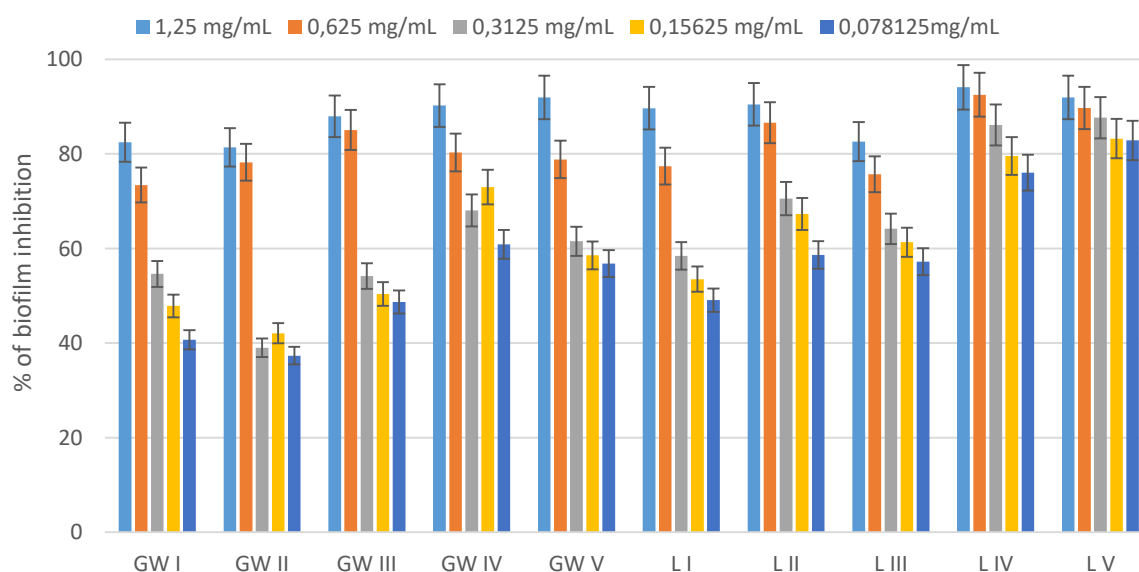


Figure 3. Antibiofilm activity of *Juglans regia* L. green walnut (GW) and leaf (L) extracts against *S. aureus* ATCC 6538. The error bars represent the standard errors.

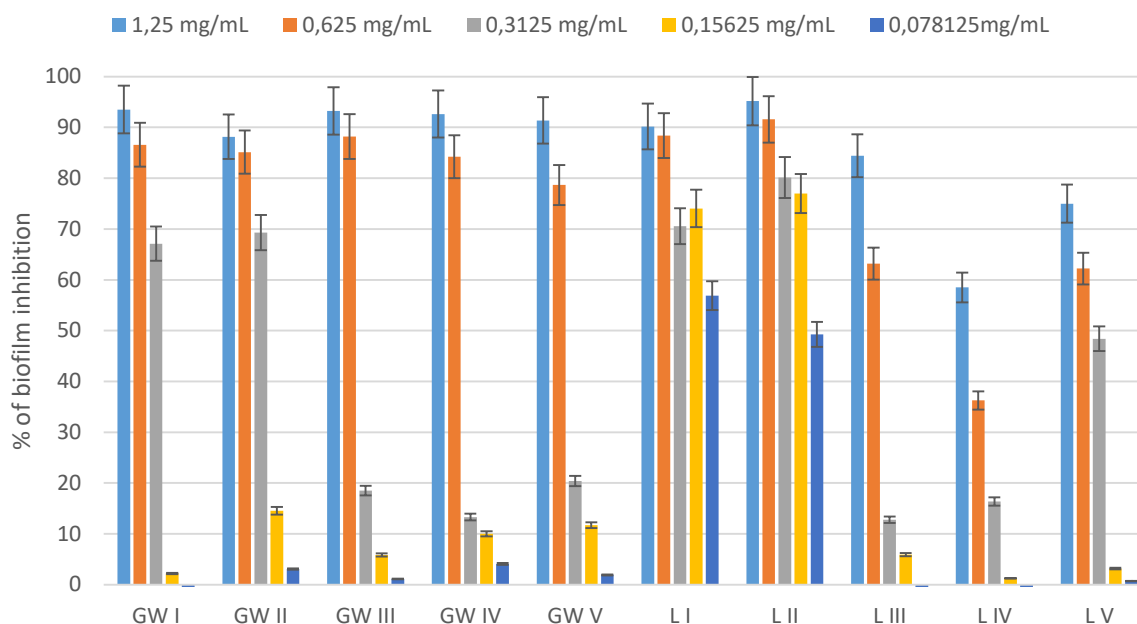


Figure 4. Antibiofilm activity of *Juglans regia* L. green walnut (GW) and leaf (L) extracts against *S. epidermidis* ATCC 35984. The error bars represent the standard errors.

DISCUSSION

Mineral composition

Due to, literature data on the mineral composition of green walnuts are rarely reported, the occurrence of 17 minerals in green walnuts and leaves was studied using ICP-OES method for the first time. The total determined average content of minerals in leaves over 2 times higher than in green walnuts was found. Also in the case of individual elements, a similar tendency between leaves and unripe fruits was observed. Potassium accounted for the highest percentage of all elements (40.77% and 61.68%), followed by calcium (36.79% and 16.03%), magnesium (11.89% and 6.18%), and phosphorus (10.84% and 5.83% in leaves) for leaves and fruits, respectively.

An interesting observation was the great content of selenium in leaves (0.25 mg/kg on average, ie. 25 µg/100 g) and green nuts (0.4 mg / kg, ie. 40 µg/100 g on average). This element with antioxidant properties was previously found in large amounts in some species of nuts, especially Brazil nuts (Tinggi, 2008). In ripe walnuts, the selenium content is given at the level of 3 – 4.6 µg/100 g (Feldman, 2002; Poon & Baldwin, 2014). The specific feature of the studied material was the low content of sodium which was not detected in green fruits whereas a small amount in leaves was found (about 10 mg/kg). The mineral composition of the leaves of several varieties of walnut was investigated only by Ughini et al. (1990). As in our case, they did not notice the presence of sodium or determined it in a small amount (in the order of several dozen ppm). Their

findings are also in line with obtained results regarding a high content of potassium, phosphorus, and sulfur. Among the micronutrients, they determined a significant amount of iron and manganese, as well as boron, which was not included in our study.

Antioxidant activity

The high content of polyphenols in *J. regia* leaves have been reported in available literature. Our data obtained for leaves are in line with other authors' reported values: 46.47 (Shah et al., 2018) and 52.82 mg GAE/g (Einali et al., 2018). Sharafati-Chaleshtori et al. (2011) found a higher level of phenolics, up to 410 mg GAE/g for Iranian *J. regia* leaves. In turn, the values reported for green husk were in the range of 6.27 - 36.10 mg GAE/g DW (Jahanban-Esfahlan et al., 2019) and between 32.61 and 74.08 mg GAE/g (Oliveira et al. 2008, Rahimipanaah et al., 2010) which are in agreement with our study. For whole green nuts, the total content of phenolic compounds was determined by Jakopić et al. (2009) who obtained for two varieties the results of 126.20 and 135.27 mg GAE/g. respectively. However, these results were expressed for fresh weight, which makes it impossible for the direct comparison of data.

The obtained results of antioxidant activity are even more difficult to compare with literature data due to different units and manner of expressing the effect. However, all the data show the strong antioxidant potential of both the leaves and the green husk of

the walnut, expressed in both antiradical activities (DPPH or ABTS assays) and reducing capacity (FRAP) (Jahanban-Esfahlan et al., 2019). The firstly used assay in the presented study was the photochemiluminescent method which is a unique sensitive technique that allows the assessment of hydrophilic (PCL-ACW) and lipophilic (PCL-ACL) antioxidant fractions. The obtained results in ACW test were similar for all the tested samples and the samples of leaves II and fruits I and V stand out positively. This may indicate that the differences between samples concern mainly the hydrophobic fraction of antioxidants, including naphthoquinones. PCL tool was earlier a source of interesting data on the composition of antioxidants both in plants and food products (Besco et al., 2007; Zielińska et al., 2007; Zayzafoon et al., 2011; Wiczowski et al., 2016; Wesołowska and Dżugan, 2016) and seems to be a valuable technique in food analysis.

Polyphenolic profile by HPTLC

The HPTLC, still not very popular although simple method was used to evaluate the polyphenol profiles of the examined extracts. The obtained results of the chromatographic analysis are generally consistent with the available data which indicate the presence of some phenolic acids (ellagic and gallic acid) and flavonoids ((+)-catechin, avicularin, taxifolin) in *J. regia* leaves. Catechin is one of the dominant polyphenols in the composition of walnut leaves (Nour et al., 2013). Taxifolin and its derivatives have been identified in the leaves of *J. regia* by Santos et al (2013). These compounds were not identified in green nuts whereas tannins and gallic acid were found in these samples. The remaining metabolites may belong to quercetin and kaempferol derivatives, the composition of which in leaves and nuts is very rich (Nour et al., 2013; Panth et al., 2016). Among the polyphenols present in the tested extracts, many other phenolic acids could be expected, e.g. syringic, vanillic, chlorogenic, sinapic, or coumaric (Nour et al., 2013), but the limited number of standards that can be used do not allow their precise identification on the chromatograms. There were also differences in the content of chlorophylls that dominated in the leaves extracts. Surprisingly, the juglone, pointed as the main specific component of *J. regia* was not detected. The reason for the absence of compounds from the group of naphthoquinones may be the extraction solvent used - aqueous ethanol, while these less polar compounds were extracted using hydrophobic solvent as chloroform (Girzu et al., 1998).

Urease inhibition

The observed difference in the action of leaf and green walnuts extracts against urease probably results from the different compositions of these extracts, as shown by HPTLC chromatograms. Ingredients responsible for the inhibition of the enzyme may be tannins and gallic acid derivatives present in large amounts in unripe fruits. The content of these compounds in leaves was lower, and other types of polyphenols predominated: phenolic acids and flavonoids (Amaral et al., 2004; Zhao et al., 2014). Nabati et al. (2012) among many tested medicinal plants showed that walnut shell septum extract showed very strong (above 93%) inhibition of urease. It is known, that it resulted from the presence of characteristic metabolites from the group of naphthoquinones, especially juglone. The inhibition mechanism of this compound is explained as a result of the arylation of thiol groups in the structure of urease (Kot et al., 2010). Urease inhibition is especially important for the treatment of *Helicobacter pylori* infections. Nariman et al. (2004) showed that extracts from several plants, including *J. regia*, are effective against this problematic bacteria. Other researchers have shown that juglone has the ability to inhibit key *H. pylori* enzymes: cystathionine γ -synthase, malonyl-CoA: acyl carrier protein transacylase, and β -hydroxyacyl-ACP dehydratase (Kong et al., 2008). In turn, the effectiveness of *J. regia* leaf extracts to inhibit of α -amylase, a key enzyme for diabetes was also reported (Rahimzadeh et al., 2014).

Antibacterial effect

Leaf and green walnut extracts exhibited antibacterial properties against planktonic and biofilm-forming bacteria of staphylococci strains, however, the level of inhibitory effect differs between *S. aureus* and *S. epidermidis*. Values of minimum inhibitory concentrations are 2-3 times lower for *S. aureus*, thus slightly higher antibacterial activity is observed for this strain. Significant inhibition of biofilm formation of *S. aureus* (94.1% for L IV) and *S. epidermidis* (95.2% for L II) was identified in a dose-dependent manner. These results are interesting due to the fact that *Staphylococcus epidermidis* is one of the opportunistic pathogens and is the leading cause of biofilm infections related to implanted medical devices (McCann et al., 2008). After biofilm formation, the bacteria are protected from the host immune system and are more resistant to antibiotics than their planktonic counterparts. Bacteria in biofilm reduce the diffusion of antibacterial agents due to interactions with the polysaccharide matrix (Lv et al., 2017). Organic compounds present in

plant extracts, including *J. regia* leaf and fruit extracts, can inhibit biofilm formation by reducing the bacterial surface adhesion (initiation step) by changing hydrophobicity and hydrophilicity, surface roughness, and/or texture of the surface (Ghosh et al., 2020). Extract's components may reflect the biofilm formation by interfering with the quorum-sensing system (Perumal & Mahmud, 2013), the bacterial communication system at the molecular level (Ghosh et al., 2020). Phenolic compounds of plant extracts could be responsible for inhibiting both planktonic form and the biofilm formation by pathogenic bacteria, including *S.*

aureus and *S. epidermidis* (Lopes et al., 2017). Obtained results may confirm a significant role of phenolic compounds in the mechanism of action on the formation of biofilm by this bacterium. The most potent extracts (from clones I and II) were characterized by the highest content of polyphenols and antioxidant activity. In the case of the test on the *S. aureus* strain, where the best effect was demonstrated for the extracts of clones IV and V, it cannot be clearly stated that polyphenolic compounds have a key effect. Another mechanism for inhibiting the bacterial biofilm is possible here.

CONCLUSION

Studies of leaves and unripe walnuts have shown the health-promoting potential of these raw materials. The elemental composition of green nuts was determined for the first time. Strong antioxidant potential for both *J. regia* samples was confirmed which was correlated with the content of polyphenolic compounds. The HPTLC analysis made it possible to visualize the differences in the qualitative composition of polyphenolic fraction that occurred in leaf and fruit extracts. The strong inhibitory effect on biofilm formation by

Staphylococcus sp., resulted from the phenolics occurrence in extracts of leaves and unripe fruit of *J. regia*, was determined for the first time. For green walnut extracts only, the potential to inhibit model urease has also been proven, which may be important for their use in the treatment of urease-positive bacterial infections. Thanks to their beneficial properties, both the leaves and unripe walnuts can be the basis for the preparation of homemade traditional recipes as well as commercial dietary supplements.

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