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Identification and Quantification of Naphthoquinones and Other Phenolic Compounds in Leaves, Petioles, Bark, Roots, and Buds of *Juglans regia* L., Using HPLC-MS/MS

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Abstract: The present study was designed to identify and quantify the major phenolic compounds in different *Juglans regia* L. (common walnut) tissues (leaves, petioles, bark, roots, buds), to define the compositions and contents of phenolic compounds between these tissues. A total of 91 individual phenolic compounds were identified and quantified, which comprised 8 hydroxycinnamic acids, 28 hydroxybenzoic acids, 11 flavanols, 20 flavonols, 22 napthoquinones, and 2 coumarins. Naphthoquinones were the major phenolic group in leaves, petioles, bark, and buds, as >60% of those identified, while hydroxybenzoic acids were the major phenolic group in side roots, as ~50% of those identified. The highest content of phenolic compounds was in the *J. regia* main root, followed by side roots and buds, leaves, and 1-year-old bark; the lowest content was in petioles and 2-year-old bark. Leaves, roots, and buds of *J. regia* represent a valuable source of these agro-residues.

Keywords: *Juglans regia* L.; walnut; leaf; petiole; bark; root; bud; phenolic compounds; naphthoquinones

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1. Introduction

The Persian, English, or common walnut (*Juglans regia* L.) is a valuable tree nut and a well-known member of the Juglandaceae family. Walnuts are the third most consumed nut in the world, and they are known for their high content of phenolic compounds [1,2]. Over the last two decades, much attention has been paid to characterizing the contents of phenolic compounds in various plant materials, as these can have beneficial effects on human health. For example, phenolic compounds can reduce the risk of cardiovascular and degenerative diseases by preventing oxidative stress and oxidation of biological macromolecules [2]. Numerous studies have also demonstrated human health benefits of such bioactive compounds, in terms of potential protection against cancers, diabetes, and cardiovascular diseases, as well as showing anti-allergen, antimicrobial, anti-inflammatory, and antioxidant activities, among others [2,3]. Phenolic compounds can also be used effectively as functional ingredients in foods, as they prevent lipid oxidation, and mold and bacterial growth [4].

Juglans regia is recognized as a rich source of phenolic compounds. The kernel, fresh green fruit, husks, shell skins, leaves, bark, and roots have been comprehensively studied for use in the food, cosmetic, and pharmaceutical industries [2,5,6]. Leaves of *J. regia* are known to contain considerable amounts of phenolic compounds, which are mainly attributed with the excellent pharmacological and therapeutic properties associated with these leaves [2,3,7]. Leaves and petioles are easily available in large quantities, while the other parts of the tree, such as bark, roots, and buds, are not abundant, and whole plants would have to be cut down to obtain them.

Walnut leaves have historically served as a source of health-promoting compounds, and have been used extensively in conventional medicine due to their anthelmintic, purga-

tive, antidiarrheal, astringent properties, and for the treatment of hemorrhoidal symptoms and venous insufficiency [2,8]. Extracts of walnut leaves are also reported to have antiscrofulous, hypotensive, antifungal, keratolytic, hypoglycemic, and sedative activities [9–12]. While the leaves have been extensively studied and the contents of their phenolic compounds quantified, there have been no studies that have identified or quantified the phenolic compounds in the petioles. Like the leaves, the petioles are easily abundant, and might serve as a good source of phenolic compounds.

Leaves and petioles are the most easily available of the plant tissues, although bark, roots, and buds might also be good sources of phenolic compounds [2]. However, these cannot be harvested from the trees in the same way as leaves and petioles. Alternatively, these plant tissues can be considered as agro-residues when the trees are cut for timber or when an orchard is too old to be economically sustainable. The efficient use of these walnut agro-residues would be a strategy to simultaneously help to increase the economic return for farmers and companies while protecting the environment, as the efficient use and recovery of such secondary metabolites might be used to generate functional ingredients to substitute for synthetic chemicals, thus also adding more value to the walnut industry [13,14]. To effectively recover and use the phenolic compounds in walnut leaves, petioles, bark, roots, and buds, the chemical profiles of each of these agro-residues need to be defined, especially with respect to the individual phenolic compounds that they contain. Nonedible tissues of *J. regia* are indeed considered as good sources of naphthoquinones and flavonoids. Naphthoquinones have significant toxicity due to their nonspecific mechanisms of action, which can be observed for juglone and its allopathic effects. Due to these properties, many studies have explored the biological and toxicological activities of naphthoquinones, to potentially discover and develop new drugs [15].

The aim of the present study was to determine the phytochemical compositions of walnut leaves, petioles, 1-year-old and 2-year-old bark, side roots and main roots, and buds, and to thus extend the discussion on the possible uses of these bioactive molecules from *J. regia*. As only leaves, buds, and bark have been studied in particular [5], the present study also provides interesting insights into the biochemical compositions of walnut roots and petioles, for which scientific information on their chemical constituents is scarce. Quantification of phenolic compounds across these different plant parts will also provide valuable data on their contents, and will demonstrate where the extraction of individual phenolic compounds might be meaningful. This study thus defines the many phenols that can be identified in the agro-residues of these different walnut tree tissues, and proposes a new direction for future studies for the agro-food, cosmetic, and pharmaceutical industries.

2. Materials and Methods

2.1. Plant Materials

Samples of walnut leaves, petioles, bark, roots, and buds were obtained from 2-year-old plants of *J. regia* (n = 10). The plants were grown in Slovenia from mixed seeds of known and unknown cultivars, as commonly used for rootstock for seedling production of *J. regia*. As older plants cannot be dug up whole without damaging the main and lateral roots, 2-year-old plants were used. This also provided more accurate results on the basis of the phenolic compounds in the whole of each plant tissue, rather than just for a part of the tissue. All of the plants were grown and collected from the Experimental Field of the Biotechnical Faculty of Ljubljana University (Slovenia; 46°2′54″ N; 14°28′22″ E; 295 m a.s.l.). The samples were obtained from a total of 10 plants, with two plants used as one replicate for analysis, defining a total of five replicates per tissue. Two plants were used for each replicate because there would have been insufficient material for the bud and petiole analyses if a single plant was used. The plants were transported to the laboratory of the Department of Agronomy in the Biotechnical Faculty, where the tissues were carefully separated. The roots and bark were further subdivided: the bark according to 1 year or 2 years of plant stem growth, and the roots according to the main root and side roots, as shown in Figure 1.

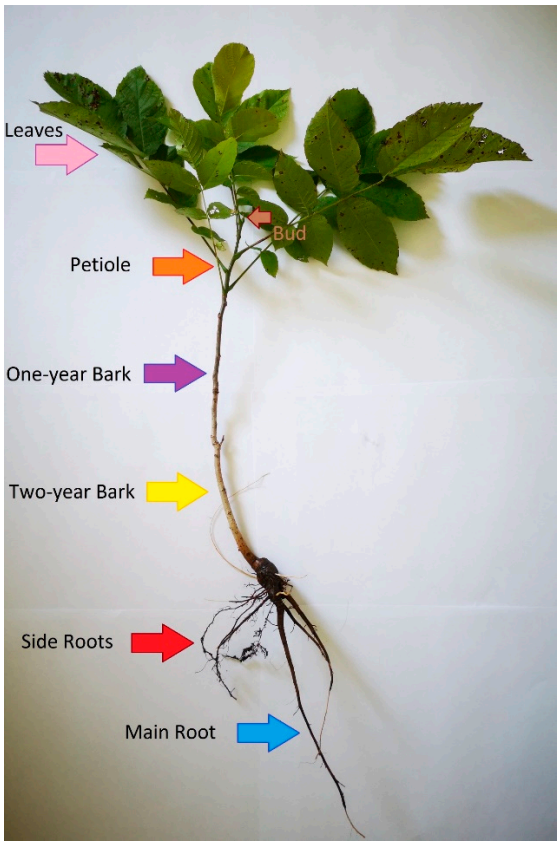

Figure 1. The different tissues of the 2-year-old walnut plants included in the analyses.

After separation of the tissues, they were immediately frozen in liquid nitrogen, lyophilized, and stored at −20 °C prior to further analysis, to avoid oxidation of the compounds that they contained.

2.2. Extraction of the Individual Phenolic Compounds

The protocol for extraction of the individual phenolic compounds was as described by Medic et al. [5]. Briefly, 0.25 g of leaves, petioles, bark and roots, and 0.1 g of buds, were extracted using 80% methanol, in water, at a tissue–solution ratio of 1:20 (*w*/*v*). The samples were vortexed (TOP-MIX 94,500 vortex mixer; Heidolph, Schwabach, Germany), then sonicated in iced water (Sonis 4 ultrasonic bath; Iskra pio, Sentjernej, Slovenia) for 60 min and centrifugated (5810 R; Eppendorf, Hamburg, Germany) at 10,000× *g* for 10 min at 4 ∘C. The samples were filtered through 0.2-µm polyamide filters (Chromafil AO-20/25; Macherey- Nagel, Düren, Germany), and transferred to vials and stored at −20° until further analysis.

2.3. HPLC–Mass Spectrometry Analysis of the Individual Phenolic Compounds

Analysis of the phenolic compounds was carried out on an UHPLC system (Vanquish; Thermo Scientific, Waltham, MA, USA), with a diode array detector at 350 nm to detect flavonols, and at 280 nm to detect hydroxycinnamic acids, hydroxybenzoic acids, flavanols, napthoquinones, and coumarins. A C18 column (Gemini; 150 × 4.60 mm, 3 µm; Phenomenex, Torrance, CA, USA) was used to separate the phenolic compounds. The spectra were recorded from 200 nm to 600 nm, and the other parameters were as described by Medic et al. [6].

Tandem mass spectrometry (MS/MS; LTQ XL; Thermo Scientific, Waltham, MA, USA) with heated electrospray ionization operating in negative ion mode was used for identification of the phenolic compounds. The parameters were as described by Medic et al. [6]. The data were processed using the Xcalibur 2.2 software (Thermo Fisher Scientific Institute,

Waltham, MA, USA). For identification and quantification of known compounds, external standards were used. For identification of unknown compounds, MS fragmentation and literature data were used, with quantification using the most relevant similar standards. As the contents of juglone, hydrojuglone, and 1,4-naphthoquinone are usually very low and other compounds can interfere with their quantification on HPLC, more accurate content quantification was obtained using MS/MS (as above). Hydrojuglone-β-D-glucopyranoside was quantified by both UHPLC and MS/MS to compare the accuracy of the UHPLC and MS quantification for the compounds that were present at higher levels. The rest of the compounds were quantified using the UHPLC system. The contents of the individual phenolic compounds are given as grams per kilogram dry weight.

2.4. Chemicals

For the identification and quantification of the phenolic compounds, the following standards were used: procyanidin B1, *p*-coumaric acid, quercetin-3-glucoside, and kaempferol-3-glucoside (Fluka Chemie GmbH, Buchs, Switzerland); (+)catechin (Roth, Karlsruhe, Germany); chlorogenic acid (*trans*-5-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid), neochlorogenic acid (3-caffeoylquinic acid), myricetin-3-galactoside, quercetin-3-galactoside, quercetin-3-rhamnoside, juglone (5-hydroxy-1,4-naphthoquinone), 1,4-naphthoquinone, caffeic acid, gallic acid, ellagic acid, and (−)epicatechin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); and myricetin-3-rhamnoside, quercetin-3-arabinofuranoside, quercetin-3-arabinopyranoside, and quercetin-3-xyloside (Apin Chemicals, Abingdon, UK).

Acetonitrile and formic acid for the mobile phases were of HPLC-MS grade (Fluka Chemie GmbH, Buchs, Switzerland). The water used for all sample preparation, solutions, and analyses was bi-distilled and purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.5. Statistical Analysis

The data were collated using Microsoft Excel 2016, and analyzed using R commander. For each methodology (tissue), five repetitions were performed. The data are expressed as means ± standard error (SE), and one-way analysis of variance (ANOVA) with Tukey's test was used to determine significant differences between the data. Statistical means at a 95% confidence level were calculated, to determine the significance of the differences.

3. Results and Discussion

3.1. Identification of Individual Phenolic Compounds in Walnut

A total of 91 phenolic compounds were tentatively identified in these *J. regia* plant tissues, based on the literature and the use of standard compounds. Of these 91 phenolic compounds, 21 were identified using standards. Fragmentation of both the standards and addition of external standards to the samples were used to confirm the identities. The remaining 70 phenolic compounds were tentatively identified according to their specific fragmentation patterns and pseudo molecular ions $[MH]^-$. The identified phenolic compounds are shown in Table 1. HPLC-MS full scans, along with the compounds identified, are included in the Supplementary Materials, as Figures S1–S7.

Table 1. Tentative identification of the 91 phenolic compounds from the leaves, petiole, bark, roots and buds of *Juglans regia* L.

Compound	Rt	$[M\text{-}H]^-$	MS^2	MS^3	MS^4	Plant Tissue				
	(min)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	Leaves	Petioles	Bark	Roots	Buds
Bis-HHDP-glucose 1	8.45	783	**301**, 481, 275, 257	257, 229, 185					x	
			301, 481, **275**, 257	257, 231, 203, 247	185, 229, 213, 20, 157					
Epigallocatechin	8.46	305	179, 221, 125	165, 151, 137, 109		x	x	x		x
(epi)Catechin derivative 1	9.04	593	425, 289, 407	289	245, 205, 179, 125			x		
Procyanidin dimer 1	9.58	577	425, 407, 289			x	x			
1-O-(4-Hydroxy-3,5-dimetoxybenzoyl)-D-glucopyranoside	9.69	359	197, 239, 299	153, 181, 121				x	x	

Table 1. *Cont.*

Compound	Rt	[M-H]$^-$	MS^2	MS^3	MS^4	Plant Tissue				
	(min)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	Leaves	Petioles	Bark	Roots	Buds
Neochlorogenic acid (3-Caffeoylquinic acid)	9.74	353	191, 179, 135			x	x			x
Procyanidin dimer 2	10.69	577	425, 407, 289			x	x			
Bis-HHDP-glucose 2	10.73	783	257, 231, 203, 247					x	x	x
(epi)Catechin isomer	10.76	289	245, 205, 179, 125							x
Gallic acid derivative 1	11.01	483	313, 271, 169						x	
Gallic acid derivative 2	11.19	411	169, 241	169, 125					x	
Gallic acid derivative 3	11.56	483	313, 271, 169					x		
Ellagic acid derivative 1	11.68	533	511, 420, 442	**502**, 275, 301 502, **275**, 301	420, 442, 275, 301 257, 231, 203, 247				x	
Tellimagrandin isomer (digalloyl-HHDP-glucose) 1	11.95	785	301, 275	257, 229, 185					x	
Procyanidin dimer 3	12.01	577	425, 407, 289			x	x	x		x
3-p-Coumaroylquinic acid	12.36	337	163, 191, 119, 173			x	x			x
Strictin/isostrictin isomer (galloyl-HHDP-glucose)	12.47	633	301, 257, 275, 229, 185					x	x	
Cryptochlorogenic acid (4-caffeoylquinic acid)	12.58	353	191, 179, 135			x	x			x
(+)Catechin	12.88	289	245, 205, 179, 125			x	x	x	x	x
Ellagic acid derivative 2	13.25	467	**458**, 391, 275, 169 458, 391, **275**, 169	382, 299, 169 257, 231, 203, 247					x	
Dihydroxytetralone hexoside	13.27	339	159, 177			x	x	x		x
Hydrojuglone derivative 1	13.59	401	355, 193	193	175	x	x	x	x	x
Chlorogenic acid (5-caffeoylquinic acid)	13.86	353	191, 179, 135			x				x
(epi)Catechin dihexoside	14.23	613	603, 458, 301, 289	289	245, 205, 179, 125			x		
Hydrojuglone dihexoside	14.46	499	175, 337	131, 157, 147, 103						x
Hydrojuglone hexoside derivative	14.70	355	193, 319, 175	175	131, 157, 147, 103	x	x			
Ellagic acid derivative 3	14.75	482	445, 467, 275, 301					x	x	
5-Hydroxy-2,3-dihydro-1,4-napthalenedione	14.92	175	131, 157, 147, 103			x	x			
(-)Epicatechin	15.32	289	245, 205, 179, 125				x			
Ellagic acid derivative 4	15.54	661	301, 275	257, 229, 185					x	
Ellagic acid derivative 5	15.63	467	391, 301	301, 275	257, 229, 185			x	x	x
Procyanidin dimer 4	15.64	577	425, 407, 289				x			
Tellimagrandin isomer (digalloyl-HHDP-glucose) 2	15.88	785	301, 275	257, 229, 185					x	
p-Coumaroylquinic acid	16.01	337	163, 191, 119, 173				x	x		x
Myricetin galloyl hexoside	16.25	631	479	316, 317						x
Trigalloyl-glucose isomer	16.47	635	465, 313	313, 295, 169, 271					x	
Hydrojuglone-β-D-glucopyranoside	17.39	337	175	131, 157, 147, 103		x	x	x	x	x
Myricetin-3-galactoside	17.73	479	316, 317	179, 151		x	x			x
Hydrojuglone derivative 2	18.00	451	**301**, 325, 319, 193 301, **325**, 319, 193	215, 257, 283, **175** 192, 235, 177	147, 131, 103, 157	x	x			x
Ellagic acid pentoside	18.08	433	301	257, 229, 185					x	
Hydrojuglone derivative 3	18.17	465	301, 193, 151, 319	**215**, 257, 283, 175 215, 257, 283, **175**	186, 171, 143 147, 131, 103, 157			x		x
Myricetin-3-glucoside	18.21	479	316, 317	179, 151			x			
Gallic acid derivative 4	18.50	491	271, 331							x
Gallic acid derivative 5	18.50	469	393, 169, 301, 275					x		
Gallic acid derivative 6	18.51	475	313, 271, 169	169, 125					x	
Hydrojuglone derivative pentoside 1	18.66	435	303, 285			x	x			
Quercetin galloyl hexoside	18.93	615	463, 301	301	179, 151					x
Hydrojuglone derivative 4	19.21	465	301, 193, 151, 319					x		
Tetralone hexoside	19.48	491	271, 331			x	x	x	x	x
Gallic acid derivative 7	19.94	475	313, 271, 169					x	x	x
Myricetin pentoside	19.96	449	317, 316	179, 151			x			
Myricetin-3-rhamnoside	20.12	463	316, 317	179, 151		x	x	x		x
Quercetin-3-galactoside	20.36	463	301	179, 151		x	x	x		x
Ellagic acid	20.54	301	257, 229, 185					x	x	
Quercetin-3-glucoside	20.59	463	301	179, 151		x	x			x
(epi)Catechin galloyl	20.73	441	289	245, 205, 179, 125				x		x
Gallic acid derivative 8	20.94	469	393, 169	317, 169, 125					x	
Trihydroxytetralone galloyl hexoside	20.97	507	331, 271				x			x
Digalloylgallate	21.18	473	313, 271, 169	169, 125					x	
Gallic acid derivative 9	21.23	489	271, 313, 169						x	
Hydrojuglone derivative rhamnoside	21.26	449	303, 285					x	x	x
Gallic acid derivative 10	21.43	489	271, 313, 169					x	x	x
Quercetin-3-xyloside	21.43	433	301	179, 151			x			
Hydrojuglone derivative pentoside 2	21.56	435	303, 285			x	x	x	x	x
Kaempferol-3-glucoside	21.67	447	284, 285	255, 227, 151		x	x			
Quercetin-3-arabinopyranoside	21.77	433	301	179, 151		x	x	x		x
3-O-Methylellagic acid-4-O-β-D-arabinopyranoside	22.01	447	315	300				x	x	

Table 1. *Cont.*

Compound	Rt	[M-H]$^-$	MS^2	MS^3	MS^4	Plant Tissue				
	(min)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	(*m*/*z*)	Leaves	Petioles	Bark	Roots	Buds
Quercetin-3-arabinofuranoside	22.18	433	301	179, 151		x	x			
Gallic acid derivative 11	22.28	489	271, 313, 169					x	x	x
Quercetin-3-rhamnoside	22.42	447	301	179, 151		x	x	x		x
Kaempferol-7-hexoside 1	22.78	447	285	165, 119				x		
Kaempferol-pentoside 1	22.91	417	284, 285	255, 227, 151		x	x			
Kaempferol-7-hexoside 2	23.12	447	285	165, 119				x		
p-Coumaric acid hexoside derivative 1	23.13	487	325	307	145, 163, 235				x	x
Gallic acid derivative 12	23.31	489	337, 301, 313, 271	317, 229, 187, 247					x	
Dihydrokaempferol pentoside 1	23.21	419	287, 269, 259, 179	259, 243, 201, 125		x				
Dihydrokaempferol pentoside 2	23.56	419	287, 269, 259, 179	259, 243, 201, 125		x				
1-β-D-Glucopyranosyloxy-4,8-dihydroxy-2-napthoic acid	23.64	381	**218**, 229, 247, 175 218, 229, 247, **175**	173 131, 103, 147, 157					x	
Kaempferol-pentoside 2	23.84	417	285, 284	257, 267, 241, 229, 151		x	x			
Kaempferol-3-rhamnoside	24.15	431	285, 284	257, 267, 241, 229, 151		x				
1,4,8-trihydroxynapthalene-1-D-glucopyranoside	24.34	503	327, 285, 217, 229, **175**	131, 103, 147, 157					x	
Isofraxidin	24.50	221	206, 191	191, 177	163, 135				x	
Isofraxidin derivative	24.64	265	221	206, 191					x	
Caffeic acid hexoside derivative	25.06	517	341, 371, 281, 209, 251			x	x			
Hydrojuglone derivative 5	25.51	601	285, 303	241, 175				x	x	
p-Coumaric acid hexoside derivative 2	26.25	485	325	235, 163		x				
Hydrojuglone derivative 6	26.73	517	175	131, 157, 103, 147				x	x	
Hydrojuglone dihexoside derivative	27.08	515	355, 193	193	175	x				
1,4-Napthoquinone	28.33	173	111, 155, 129, 145			x	x	x	x	x
Hydrojuglone	28.33	175	131, 147, 157, 115, 103			x	x	x	x	x
Juglone (5-hydroxy-1,4-napthoquinone)	29.58	189	161	117, 133		x	x	x	x	x

Rt, retention time; [M-H]$^-$, pseudomolecular ion identified in negative ion mode; x, presence of the compound identified. HHDP, hexahydroxydiphenoyl; bold numbers, fragments further fragmented; first fragment number, fragment that was further fragmented if no bold numbers are given.

Most of the phenolic compounds identified in these *J. regia* tissues were in the roots (41), followed by petioles, bark, and buds (38), with the least identified in the leaves (37). The majority of hydroxycinnamic acids were detected in leaves (6), hydroxybenzoic acids in roots (25), flavanols in petioles (7), and flavonols in leaves and petioles (13). Naptho-quinones were similar in leaves, petioles, bark, and buds (13), and in roots (12). The only two coumarins identified were in the roots. Interestingly, of the 28 hydroxybenzoic acids, none were identified in leaves and petioles; conversely, of the 20 flavonols, none were identified in roots.

Only seven phenolic compounds were identified as present in all of these plant tissues; these were all of the naphthoquinones: juglone (5-hydroxy-1,4-napthoquinone); 1,4-napthoquinone; hydrojuglone; hydrojuglone-β-D-glucopyranoside; hydrojuglone derivative pentoside 2; hydrojuglone derivative 1; and tetralone hexoside.

For the eight hydroxycinnamic acids: neochlorogenic acid (3-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid), and chlorogenic acid (trans-5-caffeoylquinic acid) were identified through their fragmentation in addition to an external standard; 3-*p*-coumaroylquinic acid was identified through its fragmentation pattern of MS *m/z* 337 and MS^2 *m/z* 163, 191, and 173, and by the retention time previously reported by Medic et al. [5] in *J. regia*; *p*-coumaroylquinic acid was identified through its fragmentation pattern; caffeic acid derivatives were identified through their fragmentation patterns of MS^n *m/z* 179; and *p*-coumaric acid derivatives were identified through their fragmentation patterns of MS^n *m/z* 163 and 119, as reported previously by Medic et al. [5].

The hydroxybenzoic acids included the identification of 28 phenolic compounds. Here, bis-HHDP-glucose 1 and 2, tellimagrandin isomers (digalloyl-HHDP-glucose) 1 and 2, and strictin/isostrictin isomer (galloyl-HHDP-glucose) were previously identified and quantified in *J. regia* pellicle [1], and are here reported for the first time in the other tissues of

J. regia. Ellagic acid and its derivatives were identified through their fragmentation patterns of MS^n *m/z* 301 and MS^{n+1} *m/z* 257, 229, and 185, and gallic acid derivatives through their fragmentation patterns of MS^n *m/z* 169 and 125, as reported by Medic et al. [1]. Many of the ellagic and gallic acid derivatives had also been previously identified in *J. regia* pellicle [1], and bark and buds [5]. 3-*O*-Methylellagic acid-4-*O*-β-D-arabinopyranoside was identified in bark and roots through its fragmentation pattern of MS *m/z* 447 and MS^2 *m/z* 315 and 300, as previously reported for *Caesalpinia ferrea* bark by Wyrepkowski et al. [16], and here for the first time in *J. regia*. 1-*O*-(4-Hydroxy-3,5-dimetoxybenzoyl)-D-glucopyranoside was also identified in bark and roots, through its fragmentation pattern of MS *m/z* 359, MS^2 *m/z* 299, 239, and 197 and MS^3 *m/z* 153 and 181, as previously reported by Huo et al. [17] for *Juglans mandshurica*, and here for the first time in *J. regia*.

Of the 11 flavanols, (+)catchin and (−)epicatechin were identified through their fragmentation in addition to external standards. (epi)Catechin derivatives were identified through their fragmentation patterns of MS^n *m/z* 245, 205, and 179, and procyanidin derivatives through their fragmentation patterns of MS^n *m/z* 577 and MS^{n+1} *m/z* 425, 407, and 289, as previously reported by Medic et al. [5]. Epigallocatechin was identified through its fragmentation pattern of MS^n *m/z* 179, 221, and 125, and MS^{n+1} *m/z* 165, 151, 137, and 109, as previously reported by Ambigaipalan et al. [18]. Epigallocatechin was reported here for the first time in *J. regia*.

There were 20 flavonols identified here, with many previously reported by Medic et al. [5,6]. The flavonols included the identification of three groups of compounds: (i) quercetin glycosides, through their fragmentation patterns of MS^n *m/z* 301 and MS^{n+1} *m/z* 179 and 151; (ii) kaempferol glycosides, through their fragmentation patterns of MS^n *m/z* 284 and 285, and MS^{n+1} *m/z* 255 and 227; and (iii) myricetin glycosides, through their fragmentation patterns of MS^n *m/z* 316 and 317, and MS^{n+1} *m/z* 179 and 151, as reported by Viera et al. [19], Santos et al. [20], and Medic et al. [5].

The two coumarins identified in the roots were isofraxidin and isofraxidin derivative, and these were identified through their fragmentation patterns of MS^n *m/z* 221, MS^{n+1} *m/z* 206 and 191, and MS^{n+2} *m/z* 177, 163, and 135, according to Tsugawa et al. [21]. These compounds are reported here for the first time in *J. regia*, or any other *Juglans* species.

For the naphthoquinones, many had been identified and quantified previously in the bark, buds, and husk of *J. regia* by Medic et al. [5,6]. Of those that had previously not been identified, the hydrojuglone derivatives were identified by their fragmentation patterns of MS^n *m/z* 175 and MS^{n+1} *m/z* 131, 157, 103, 147, and 115, as previously reported by Medic et al. [5], and 1-β-D-glucopyranosyloxy-4,8-dihydroxy-2-napthoic acid by its fragmentation pattern MS^n *m/z* 381 and MS^{n+1} *m/z* 218 $[M\text{-}H\text{-}C_6H_{11}O_5]^-$ and 175 $[M\text{-}H\text{-}C_6H_{10}O_5\text{-}CO_2]^-$, as reported by Huo et al. [17] in *J. mandshurica*.

Fragmentation patterns were seen for all of the groups of phenolic compounds except the coumarins, with the loss of pentosyl (−132), rhamnosyl (−146), galoyll (−152), and hexosyl (−162) residues seen, as previously reported by Medic et al. [5] and Vieira et al. [19].

3.2. Quantification of Individual Phenolic Compounds in Walnut

The highest contents of the phenolic compounds were in the *J. regia* main roots, followed by the side roots and buds, then leaves and 1-year-old bark, with the lowest in the petioles and 2-year-old bark, as shown in Figure 2A.

The reason why the roots showed the highest content of phenolic compounds compared to other tissues was mainly because of their higher content of hydroxybenzoic acids and naphthoquinones, which are known for their defense mechanisms against pathogens [22,23]. As the soil contains more pathogens then are present above ground [23], higher phenolic content, especially content of hydroxybenzoic acids and naphthoquinones, was expected to be found in underground tissues as observed. Therefore, the roots represent a particularly good source of hydroxybenzoic acids, while, the leaves, roots, and buds all contained high levels of naphthoquinones. On the other hand, the contents of flavanols and flavonols, which are known to have health-promoting effects [6], were the

highest in leaves and buds. Both flavanols and flavonols are typically found in leaves and are considered to have a defensive role against viral and bacterial infections that usually affect leaves [6], therefore the highest content was expected to be present in the leaves as observed. Both petioles and 2-year-old bark were less suitable sources of phenolic compounds compared to the other tissues. Previously, buds have been suggested as the best source of phenolic compounds [5]; however, as shown here, the roots contained almost twice the levels of the buds. Roots are also more abundant, and thus the extraction of phenolic compounds would be more meaningful for roots, rather than buds, especially when old walnut orchards are dug up. The results of these total analyzed phenolic compounds in bark and buds of juvenile plants was in agreement with the content of total analyzed phenolic compounds in bark and buds of 24-year-old *J. regia* plants. The compositions were, however, slightly different, as juvenile plants contained higher levels of naphthoquinones and hydroxybenzoic acids and lower levels of flavanols, compared to the 24-year-old plants reported by Medic et al. [5].

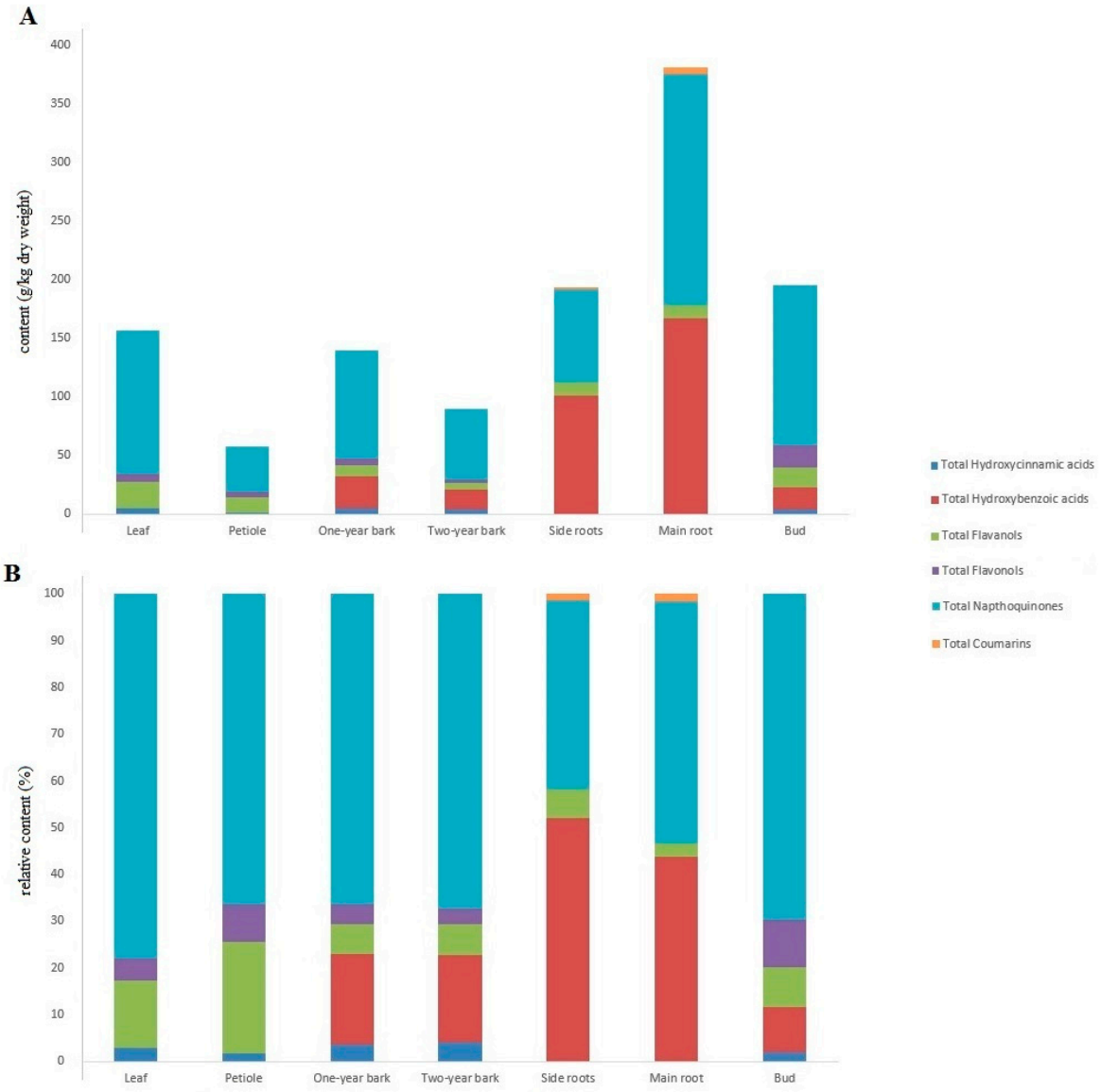

Figure 2. Content of the various phenolic groups identified in the different walnut tissues, as g/kg dry weight (**A**) and as relative contents as a proportion of the total phenolic compounds identified (**B**).

The highest relative contents of naphthoquinones were seen for leaves, flavanols for petioles, hydroxycinnamic acids for bark, hydroxybenzoic acids and coumarins for roots,

and flavonols for buds (Figure 2B). The total naphthoquinones were the major phenolic group in leaves, petioles, bark, and buds, where they represented >60% of the phenolic compounds identified; conversely, hydroxybenzoic acids were the major phenolic group in side roots, at ~50% of the phenolic compounds identified. The total phenolic compounds in roots was higher than any previously reported for *J. regia* kernel [1], husk [5], leaves [22], shoots [24], bark [5], or buds [5], which further justifies the use of roots as a valuable source of phenolic compounds; instead, the use of petioles cannot be justified. The contents of the total and individual phenolic compounds in each of the tissues analyzed here are given in Table 2.

Table 2. Contents of the 91 phenolic compounds identified in the *J. regia* leaves, petioles, bark, roots, and buds.

Compound	Content in Plant Tissue (g/kg Dry Weight)						
	Leaves	Petioles	Bark		Roots		Bud
			One-year	Two-Year	Side	Main	
Hydroxycinnamic acids							
Neochlorogenic acid (3-caffeoylquinic acid) [1]	2.01 ± 0.14 b	0.41 ± 0.03 a	nd	nd	nd	nd	0.76 ± 0.05 a
Cryptochlorogenic acid (4-caffeoylquinic acid) [2]	1.56 ± 0.13 b	0.23 ± 0.01 a	4.81 ± 0.11 d	3.48 ± 0.22 c	nd	nd	1.67 ± 0.07 b
Chlorogenic acid (5-caffeoylquinic acid) [3]	0.17 ± 0.01	nd	nd	nd	nd	nd	nd
3-*p*-Coumaroylquinic acid [4]	0.48 ± 0.04 b	0.12 ± 0.01 a	nd	nd	nd	nd	0.39 ± 0.03 c
p-Coumaroylquinic acid [4]	nd	0.12 ± 0.01 a	0.16 ± 0.04 a	0.09 ± 0.01 a	nd	nd	0.38 ± 0.02 b
p-Coumaric acid hexoside derivative 1 [4]	nd	nd	nd	nd	0.26 ± 0.01 b	0.28 ± 0.03 b	0.09 ± 0.01 a
p-Coumaric acid hexoside derivative 2 [4]	0.11 ± 0.00	nd	nd	nd	nd	nd	nd
Caffeic acid hexoside derivative [5]	0.16 ± 0.01 b	0.01 ± 0.00 a	nd	nd	nd	nd	nd
Hydroxybenzoic acids							
bis-HHDP-glucose 1 [6]	nd	nd	nd	nd	3.20 ± 0.06 a	3.54 ± 0.24 a	nd
bis-HHDP-glucose 2 [6]	nd	nd	0.57 ± 0.01 ab	0.35 ± 0.02 a	4.57 ± 0.06 c	4.80 ± 0.18 c	0.82 ± 0.03 b
Tellimagrandin isomer (digalloyl-HHDP-glucose) 1 [6]	nd	nd	nd	nd	2.37 ± 0.03 a	3.77 ± 0.27 b	nd
Tellimagrandin isomer (digalloyl-HHDP-glucose) 2 [6]	nd	nd	nd	nd	2.03 ± 0.09 a	5.39 ± 0.46 b	nd
Strictin/isostrictin isomer (galloyl-HHDP-glucose) [6]	nd	nd	0.35 ± 0.05 a	0.23 ± 0.02 a	1.22 ± 0.03 b	1.80 ± 0.03 c	nd
Digalloylgallate [6]	nd	nd	nd	nd	0.87 ± 0.04 a	1.16 ± 0.17 a	nd
Trigalloyl-glucose isomer [6]	nd	nd	nd	nd	1.90 ± 0.07 a	4.28 ± 0.51 b	nd
Gallic acid derivative 1 [6]	nd	nd	nd	nd	2.27 ± 0.05 b	1.88 ± 0.04 a	nd
Gallic acid derivative 2 [6]	nd	nd	nd	nd	4.88 ± 0.07 b	4.04 ± 0.09 a	nd
Gallic acid derivative 3 [6]	nd	nd	0.78 ± 0.04 b	0.54 ± 0.04 a	nd	nd	nd
Gallic acid derivative 4 [6]	nd	nd	nd	nd	nd	nd	1.08 ± 0.11
Gallic acid derivative 5 [6]	nd	nd	1.22 ± 0.13 b	0.73 ± 0.04 a	nd	nd	nd
Gallic acid derivative 6 [6]	nd	nd	nd	nd	2.19 ± 0.04 a	3.65 ± 0.12 b	nd
Gallic acid derivative 7 [6]	nd	nd	1.40 ± 0.03 bc	0.69 ± 0.05 a	4.05 ± 0.08 d	1.64 ± 0.12 c	1.11 ± 0.02 b
Gallic acid derivative 8 [6]	nd	nd	nd	nd	0.65 ± 0.03 a	0.82 ± 0.06 b	nd
Gallic acid derivative 9 [6]	nd	nd	nd	nd	7.29 ± 0.18 a	8.96 ± 0.43 b	nd
Gallic acid derivative 10 [6]	nd	nd	12.49 ± 6.51 d	9.04 ± 0.57 d	1.77 ± 0.05 b	3.38 ± 0.10 c	0.47 ± 0.02 a
Gallic acid derivative 11 [6]	nd	nd	2.86 ± 0.08 a	1.52 ± 0.09 a	5.60 ± 0.06 b	16.95 ± 0.95 d	13.30 ± 0.36 c
Gallic acid derivative 12 [6]	nd	nd	nd	nd	1.57 ± 0.06 a	2.68 ± 0.03 b	nd
Ellagic acid [7]	nd	nd	1.02 ± 0.14 a	0.45 ± 0.05 a	6.13 ± 0.07 b	8.66 ± 0.74 c	nd
Ellagic acid pentoside [7]	nd	nd	nd	nd	13.65 ± 0.11 a	33.66 ± 2.14 b	nd
Ellagic acid derivative 1 [7]	nd	nd	nd	nd	6.68 ± 0.10 b	5.22 ± 0.16 a	nd
Ellagic acid derivative 2 [7]	nd	nd	nd	nd	5.03 ± 0.09 a	5.57 ± 0.37 a	nd
Ellagic acid derivative 3 [7]	nd	nd	1.97 ± 0.20 a	0.93 ± 0.09 a	5.41 ± 0.30 b	9.53 ± 0.74 c	nd
Ellagic acid derivative 4 [7]	nd	nd	nd	nd	4.95 ± 0.19 a	10.59 ± 0.59 b	nd
Ellagic acid derivative 5 [7]	nd	nd	2.06 ± 0.28 a	0.88 ± 0.10 a	5.01 ± 0.23 b	11.82 ± 0.99 c	2.24 ± 0.03 a
3-*O*-Methylellagic acid-4-*O*-β-D-arabinopyranoside [7]	nd	nd	1.54 ± 0.12 a	0.99 ± 0.06 a	3.20 ± 0.05 b	7.40 ± 0.63 c	nd
1-*O*-(4-Hydroxy-3,5-dimetoxybenzoyl)-D-glucopyranoside [6]	nd	nd	0.78 ± 0.07 a	0.51 ± 0.03 a	4.18 ± 0.10 b	5.58 ± 0.69 b	nd
Flavanols							
(+)Catechin [8]	3.81 ± 0.26 a	3.98 ± 0.28 a	nd	nd	11.73 ± 0.23 c	11.06 ± 0.33 c	9.30 ± 0.08 b
(-)Epicatechin [9]	nd	0.71 ± 0.06	nd	nd	nd	nd	nd
(epi)Catechin galloyl [8]	nd	nd	2.58 ± 0.08 b	1.49 ± 0.07 a	nd	nd	1.74 ± 0.06 a
(epi)Catechin dihexoside [8]	nd	nd	2.55 ± 0.20 b	1.58 ± 0.20 a	nd	nd	nd
(epi)Catechin isomer [8]	nd	nd	nd	nd	nd	nd	1.14 ± 0.02
(epi)Catechin derivative 1 [8]	nd	nd	1.69 ± 0.23 b	1.06 ± 0.07 a	nd	nd	nd

Table 2. *Cont.*

Compound	Content in Plant Tissue (g/kg Dry Weight)						
	Leaves	Petioles	Bark		Roots		Bud
			One-year	Two-Year	Side	Main	
Epigallocatechin [8]	0.48 ± 0.04 c	0.29 ± 0.02 ab	0.38 ± 0.01 ac	0.27 ± 0.02 a	nd	nd	0.40 ± 0.02 bc
Procyanidin dimer 1 [10]	10.73 ± 0.70 b	1.81 ± 0.13 a	nd	nd	nd	nd	nd
Procyanidin dimer 2 [10]	2.14 ± 0.18 a	2.92 ± 0.19 a	nd	nd	nd	nd	nd
Procyanidin dimer 3 [10]	5.33 ± 0.35 c	2.24 ± 0.22 a	1.95 ± 0.04 a	1.57 ± 0.10 a	nd	nd	3.90 ± 0.40 b
Procyanidin dimer 4 [10]	nd	1.80 ± 0.21	nd	nd	nd	nd	nd
Flavonols							
Myricetin-3-galactoside [11]	0.19 ± 0.02 a	0.81 ± 0.08 b	nd	nd	nd	nd	2.64 ± 0.07 c
Myricetin-3-glucoside [11]	nd	0.26 ± 0.03	nd	nd	nd	nd	nd
Myricetin-3-rhamnoside [12]	0.57 ± 0.03 bc	0.41 ± 0.03 a	0.65 ± 0.02 c	0.46 ± 0.04 ab	nd	nd	3.90 ± 0.06 d
Myricetin pentoside [11]	nd	0.15 ± 0.01	nd	nd	nd	nd	nd
Myricetin galloyl hexoside [11]	nd	nd	nd	nd	nd	nd	3.44 ± 0.09
Quercetin-3-galactoside [13]	0.66 ± 0.02 a	0.70 ± 0.05 a	1.47 ± 0.04 b	0.53 ± 0.08 a	nd	nd	2.08 ± 0.05 c
Quercetin-3-glucoside [14]	0.49 ± 0.01 b	0.29 ± 0.02 a	nd	nd	nd	nd	0.75 ± 0.01 c
Quercetin-3-xyloside [15]	nd	0.23 ± 0.01	nd	nd	nd	nd	nd
Quercetin-3-arabinopyranoside [16]	0.24 ± 0.01 a	0.20 ± 0.01 a	1.20 ± 0.10 c	0.62 ± 0.02 b	nd	nd	0.90 ± 0.19 bc
Quercetin-3-arabinofuranoside [17]	0.64 ± 0.01 a	0.59 ± 0.04 a	nd	nd	nd	nd	nd
Quercetin-3-rhamnoside [18]	0.64 ± 0.02 b	0.54 ± 0.04 b	0.68 ± 0.04 b	0.34 ± 0.02 a	nd	nd	4.03 ± 0.08 c
Quercetin galloyl hexoside [14]	nd	nd	nd	nd	nd	nd	1.87 ± 0.05
Kaempferol-3-glucoside [19]	0.24 ± 0.02 a	0.25 ± 0.02 a	nd	nd	nd	nd	nd
Kaempferol-3-rhamnoside [19]	0.28 ± 0.01	nd	nd	nd	nd	nd	nd
Kaempferol-pentoside 1 [19]	0.58 ± 0.04 b	0.11 ± 0.01 a	nd	nd	nd	nd	nd
Kaempferol-pentoside 2 [19]	0.54 ± 0.06 b	0.08 ± 0.01 a	nd	nd	nd	nd	nd
Kaempferol-7-hexoside 1 [19]	nd	nd	1.47 ± 0.05 b	0.92 ± 0.05 a	nd	nd	nd
Kaempferol-7-hexoside 2 [19]	nd	nd	0.33 ± 0.02 b	0.17 ± 0.01 a	nd	nd	nd
Dihydrokaempferol pentoside 1 [19]	1.30 ± 0.09	nd	nd	nd	nd	nd	nd
Dihydrokaempferol pentoside 2 [19]	1.02 ± 0.25	nd	nd	nd	nd	nd	nd
Napthoquinones							
Juglone (5-hydroxy-1,4-napthoquinone) [20]	0.13 ± 0.06 a	0.70 ± 0.02 c	0.32 ± 0.02 b	0.65 ± 0.01 c	0.20 ± 0.00 ab	0.14 ± 0.01 a	0.22 ± 0.01 ab
1,4-Napthoquinone [21]	0.06 ± 0.01 a	0.03 ± 0.00 a	0.01 ± 0.00 a	0.02 ± 0.00 a	0.23 ± 0.02 c	0.15 ± 0.01 b	0.02 ± 0.00 a
Hydrojuglone [20]	0.14 ± 0.01 c	0.01 ± 0.00 a	0.02 ± 0.00 ab	0.02 ± 0.00 ab	0.03 ± 0.00 b	0.02 ± 0.00 a	0.01 ± 0.00 a
Hydrojuglone-β-D-glucopyranoside [20]	74.72 ± 2.86 e	19.63 ± 0.28 ab	43.89 ± 0.87 c	27.68 ± 0.59 b	15.84 ± 0.82 a	54.00 ± 1.26 d	54.88 ± 4.81 d
Hydrojuglone dihexoside [20]	nd	nd	nd	nd	nd	nd	13.48 ± 0.21
Hydrojuglone dihexoside derivative [20]	0.64 ± 0.04	nd	nd	nd	nd	nd	nd
Hydrojuglone derivative rhamnoside [20]	nd	nd	22.59 ± 0.32 d	12.81 ± 0.67 c	3.39 ± 0.09 a	10.76 ± 0.88 bc	9.88 ± 0.17 b
Hydrojuglone derivative pentoside 1 [20]	2.89 ± 0.17 b	1.87 ± 0.12 a	nd	nd	nd	nd	nd
Hydrojuglone derivative pentoside 2 [20]	31.90 ± 1.84 e	5.08 ± 0.33 cd	0.76 ± 0.13 ab	0.35 ± 0.03 a	3.94 ± 0.29 bc	7.66 ± 0.30 d	8.97 ± 0.36 d
Hydrojuglone hexoside derivative [20]	2.18 ± 0.09 b	0.98 ± 0.10 a	nd	nd	nd	nd	nd
Hydrojuglone derivative 1 [20]	1.59 ± 0.08 a	1.45 ± 0.11 a	3.32 ± 0.27 a	1.82 ± 0.12 a	16.14 ± 0.51 b	25.78 ± 1.90 c	2.46 ± 0.12 a
Hydrojuglone derivative 2 [20]	1.02 ± 0.05 a	2.60 ± 0.21 b	nd	nd	nd	nd	2.92 ± 0.21 b
Hydrojuglone derivative 3 [20]	nd	nd	7.16 ± 0.79 b	3.82 ± 0.33 a	nd	nd	10.16 ± 0.45 c
Hydrojuglone derivative 4 [20]	nd	nd	7.36 ± 1.45 a	9.27 ± 0.47 a	nd	nd	nd
Hydrojuglone derivative 5 [20]	nd	nd	1.48 ± 0.29 b	0.66 ± 0.09 a	3.44 ± 0.05 c	5.52 ± 0.24 d	nd
Hydrojuglone derivative 6 [20]	nd	nd	1.82 ± 0.17 a	0.63 ± 0.13 a	17.7 ± 0.21 b	66.34 ± 1.75 c	nd
Tetralone hexoside [20]	1.89 ± 0.16 a	2.06 ± 0.15 a	2.96 ± 0.25 a	2.01 ± 0.14 a	8.02 ± 0.22 b	14.25 ± 1.79 c	24.82 ± 0.33 d
Dihydroxytetralone hexoside [20]	1.91 ± 0.10 c	1.33 ± 0.12 b	1.25 ± 0.04 b	0.74 ± 0.04 a	nd	nd	3.58 ± 0.27 d
Trihydroxytetralone galloyl hexoside [20]	nd	0.19 ± 0.03 a	nd	nd	nd	nd	4.83 ± 0.10 b
5-Hydroxy-2,3-dihydro-1,4-napthalenedione [20]	3.16 ± 0.16 b	2.03 ± 0.16 a	nd	nd	nd	nd	nd
1-β-D-Glucopyranosyloxy-4,8-dihydroxy-2-napthoic acid [20]	nd	nd	nd	nd	4.69 ± 0.27 a	7.55 ± 0.82 b	nd
1,4,8-trihydroxynapthalene-1-D-glucopyranoside [20]	nd	nd	nd	nd	4.81 ± 0.35 a	4.85 ± 0.53 a	nd

Table 2. *Cont.*

Compound	Content in Plant Tissue (g/kg Dry Weight)						
	Leaves	Petioles	Bark		Roots		Bud
			One-year	Two-Year	Side	Main	
Coumarins							
Isofraxidin [6]	nd	nd	nd	nd	1.61 ± 0.05 a	4.23 ± 0.05 b	nd
Isofraxidin derivative [6]	nd	nd	nd	nd	0.92 ± 0.02 a	1.72 ± 0.15 b	nd
Total Hydroxycinnamic acids	4.49 ± 0.30 cd	0.89 ± 0.05 a	4.97 ± 0.14 d	3.57 ± 0.23 b	0.26 ± 0.01 a	0.28 ± 0.03 a	3.84 ± 0.08 bc
Total Hydroxybenzoic acids	nd	nd	27.05 ± 6.09 a	16.86 ± 1.10 a	100.67 ± 1.24 b	166.79 ± 9.93 d	19.02 ± 0.46 a
Total Flavanols	22.49 ± 1.39 e	13.75 ± 1.09 cd	9.14 ± 0.43 a	5.97 ± 0.43 b	11.73 ± 0.23 bc	11.06 ± 0.33 bc	16.49 ± 0.44 d
Total Flavonols	7.40 ± 0.29 d	4.61 ± 0.34 b	5.81 ± 0.17 c	3.04 ± 0.21 a	nd	nd	19.60 ± 0.44 e
Total Napthoquinones	122.22 ± 4.60 d	37.96 ± 1.57 a	92.92 ± 3.77 c	60.46 ± 1.33 b	78.41 ± 1.16 c	197.01 ± 5.83 e	136.22 ± 5.71 d
Total Coumarins	nd	nd	nd	nd	2.53 ± 0.06 a	5.94 ± 0.17 b	nd
Total Analysed Phenolic Content	156.60 ± 4.69 bc	57.21 ± 3.03 a	139.89 ± 2.38 b	89.90 ± 3.22 a	193.60 ± 2.61 d	381.08 ± 16.21 e	195.16 ± 7.08 cd

Data are means ±standard error. Means followed by different letters across the tissues (within rows) are significantly different ($p < 0.05$). nd, not detected; HHDP, hexahydroxydiphenoyl; [1] expressed as Neochlorogenic acid; [2] expressed as Cryptochlorogenic acid; [3] expressed as Chlorogenic acid; [4] expressed as *p*-Coumaric acid; [5] expressed as Caffeic acid; [6] expressed as Gallic acid; [7] expressed as Ellagic acid; [8] expressed as (+)Catechin; [9] expressed as (−)Epicatechin; [10] expressed as Procyanidin B1; [11] expressed as Myricetin-3-galactoside; [12] expressed as Myricetin-3-rhamnoside; [13] expressed as Quercetin-3-galactoside; [14] expressed as Quercetin-3-glucoside; [15] expressed as; Quercetin-3-xyloside [16] expressed as Quercetin-3-arabinopyranoside; [17] expressed as Quercetin-3-arabinofuranoside; [18] expressed as Quercetin-3-rhamnoside; [19] expressed as Kaempferol-3-glucoside; [20] expressed as Juglone; [21] expressed as 1,4-Napthoquinone.

As indicated above, the seven individual phenolic compounds that were present in all of these plant tissues were naphthoquinones. Of these, hydrojuglone derivative pentoside 2 and hydrojuglone-β-D-glucopyranoside were highest in the leaves, hydrojuglone derivative 1 was highest in the main root, and tetralone hexoside was highest in the buds. The hydrojuglone-β-D-glucopyranoside contents in the bark and buds were a little higher than those previously reported for bark and buds by Medic et al. [5]. The juglone, 1,4-napthoquinone and hydrojuglone contents were up to one tenth of those previously reported by Medic et al. [5], and lower by up to a factor of 1000 compared to those previously reported by Niculina et al. [22]. This clearly demonstrates that when comparing the contents of compounds that are present at such low levels, mass spectrometry quantification is necessary, as the errors can be very large.

The quantification of the compounds that are present at higher levels can, however, be quantified on HPLC, as there were no differences in the quantification of hydrojuglone-β-D-glucopyranoside, which was present at higher levels in all of these tissues. To the best of our knowledge, this is the first study to quantify the contents of juglone, 1,4-napthoquinone, and hydrojuglone using mass spectrometry instead of the usual HPLC quantification, thereby providing very accurate determination of the contents of these compounds in the *J. regia* tissues.

As indicated, of these individual phenolic compounds that were identified and quantified in the *J. regia* leaves, petioles, bark, roots and buds, there were 8 hydroxycinnamic acids, 28 hydroxybenzoic acids, 11 flavanols, 20 flavonols, 22 napthoquinones, and 2 coumarins. Many of these phenolic compounds are reported here for *J. regia* for the first time, and some of them for the first time in the Juglandaceae family. To the best of our knowledge, this is the most complete analysis and description of the levels of the many phenolic compounds in the different walnut tissues. Furthermore, this is the first report to provide detailed characterization and quantification of these phenolic compounds in the roots and petioles of *J. regia*.

Furthermore, considering the quantification of juglone, 1,4-napthoquine, hydrojuglone, and hydrojuglone-β-D-glucopyranoside in particular, this study has provided the most accurate quantification of these compounds to date, as we used mass spectrometry instead of HPLC, which has been used previously for such quantification. The present study provides useful information on the contents of the various phenolic compounds in these different tissues of *J. regia* which can now be further investigated to determine their potential use for the cosmetic, pharmaceutical, and agro-food industries.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7090326/s1, Figure S1: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* leaves, Figure S2: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* side roots, Figure S3: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* buds, Figure S4: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* one-year old bark, Figure S5: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* petiole, Figure S6: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* main root, Figure S7: Full scan on a HPLC-MS, and the phenolic compounds identified for *J. regia* two-year old bark.

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Data Availability Statement: Part of the data presented in this study are available in Supplementary Material here. The remaining data presented in this study are available on request from the corresponding author. The remaining data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

HHDP	hexahydroxydiphenoyl
(U)HPLC	(ultra)high performance liquid chromatography
MS(/MS)	(tandem) mass spectrometer

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