Article

Using HPLC–MS/MS to Assess the Quality of Beet, Mizuna, Lettuce and Corn Salad after Juglone and Walnut Leaf Extract Treatments

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Abstract: The present study was carried out to investigate the yield, quality, and metabolomic responses of four different vegetable crops to treatments with pure juglone standard and walnut (*Juglans regia* L.) leaf extract at soil concentrations found in walnut orchards. A total of 60 phenolic compounds were identified and quantified, some for the first time in these crop vegetables. *Beta vulgaris* L. and *Lactuca sativa* L. were less susceptible to juglone. For crop quality, *B. vulgaris* showed the least effects of the different treatments. Both *Brassica rapa* L. var. *japonica* and *Valerianella locusta* Laterr. showed lower yields, even at the lower juglone concentration, and reduced quality, so their cultivation in juglone-containing soils should be avoided. This study also investigated leaf quality at different ages and the quality and yield of these crop vegetables grown under the influence of allelochemicals, to determine the influence of allelochemicals on metabolomics and, thus, on the uptake of phenolic compounds considered to be beneficial to human health.

Keywords: allopathy; *Beta vulgaris* L.; *Brassica rapa* L. var. *japonica*; *Lactuca sativa* L.; phenolic compounds; *Valerianella locusta* Laterr.

1. Introduction

Consumption of fresh or processed vegetables is an essential part of the human diet and has been associated with many health benefits (e.g., reduced diet-related diseases and risk of obesity) [1]. The most important factors that contribute to the nutritional and health benefits of vegetables are vitamins and phytochemicals. To date, over 5000 phytochemicals have been identified. Phytochemicals are usually classified into three major classes: (i) phenols, (ii) terpenes, and (iii) nitrogen-containing compounds [2]. Of these phytochemicals, phenols, or phenolic compounds, are the best-studied and most abundant group of phytochemicals and are associated with various health benefits [3,4]. Phenolic compounds also have important roles in the quality of vegetables, as they affect their taste, appearance, and stability [5].

The concentration and types of phenolic compounds vary within different vegetables and plant tissues [6]. Phenolic compounds can be unique and found only in one crop or cultivar, or they can be present across several varieties. They also have important roles in plant defence against pathogens, predators, and biotic and abiotic stresses [7,8]. If a plant is under stress, its content of phenolic compounds increases as a response to the stress [8], and therefore, the plant uses energy and nutrients meant for growth and other primary functions to produce these defensive compounds [9–11]. As higher levels of phenolic compounds are associated with higher vegetable quality, plant stress is considered beneficial to some degree [9]. The problem is that the higher the levels of phenolic compounds, the lower the growth of the plants and, therefore, the lower the yield, so there is the need for a balance between quality and yield. While some stress factors can be controlled through agronomic

practices (e.g., irrigation, pest and disease control, fertilisation), others are more difficult or are not possible to control (e.g., plant residues and fungi in the soil) [9].

Plant residues can release allelochemicals into the soil, some of which have positive effects on plant growth, while others have negative effects. In some plants, allelochemicals can cause deformity, chlorosis, and wilting, thus reducing vitality, slowing down or preventing germination, hindering growth and development, and increasing susceptibility to disease, which can lead to their collapse [12]. In agriculture, this is especially problematic when one crop follows another. The greatest problems occur when walnut (*Juglans regia* L.) orchards are replaced by other crops because walnuts contain one of the first and most studied allelochemicals—juglone [13]. Juglone is released into the soil and affects the growth of surrounding crops and of crops planted after the walnut trees are cut down, with effects lasting for years [12].

Although clearing an old orchard to make way for new varieties or crop fields is common practice, little is known about the short- and long-term effects of allelochemicals that might still be present in the soil, and how they might affect the quality and yield of future crops [12]. There have been some studies on the effects of juglone, but most have focused on seed germination rather than mature crop yield or quality [11,13,14].

Therefore, the aim of the present study was to investigate the yield, quality, and molecular responses to known concentrations of juglone alone and in walnut leaf extract with concentrations of juglone normally found in the soil of walnut orchards [15], based on four different vegetable crops: beet (*Beta vulgaris* L.), mizuna (*Brassica rapa* L. var. *japonica*), lettuce (*Lactuca sativa* L.), and corn salad (*Valerianella locusta* Laterr.). The goal was also to determine whether juglone is really the crucial and only allelochemical in walnut, or whether there are other allelochemicals that have remained hidden in the shadow of juglone, as suggested more recently [13]. The data obtained represent an important basis for determining which crop varieties are susceptible to juglone and/or walnut leaf allelochemicals, and how these allelochemicals affect the yield and metabolomics of selected crops that influence human health and nutrition.

2. Materials and Methods

2.1. Plant Material

To determine whether pure juglone and a juglone-containing leaf extract have the same effects on different crop vegetables, or whether the effects are specific, four commonly cultivated crop vegetables were used: beet (*Beta vulgaris* L.), mizuna (*Brassica rapa* L. var. *japonica*), lettuce (*Lactuca sativa* L.), and corn salad (*Valerianella locusta* Laterr.). The juglone concentrations used were based on our previous germination study [13]. The plants were treated using (i) two control treatments, with K1 as the juglone extraction medium and vehicle control (0.17% dimethylsulphoxide (DMSO), 0.17% ethanol in H_2O) and K2 as the water control; (ii) positive control pure juglone treatments, prepared for final juglone concentrations of 1 mM and 10 μM in extraction medium; (iii) leaf juglone extract, prepared for the final juglone concentration of 10 μM in extraction medium. The juglone was dissolved in the extraction medium, as it is only partially soluble in water (52 mg/L), and thus, the required concentration of 1 mM control juglone cannot be achieved in water alone. As previously noted [13], studies that have used >100 μM juglone dissolved in water are questionable at best.

2.2. Growing Conditions

The experiment was conducted using nutrient film technique (NFT) hydroponics systems in a greenhouse, to better control the environment (especially to control the soil as a medium) and the juglone concentrations in the water. Five NFT systems were used, one for each treatment. Each NFT system had 4 rows with 10 plants of the same crop vegetable grown in each row, for a total of 5 biological repetitions per measure (5 for metabolomics studies and 5 for yield determination).

The plants were grown from seed in a greenhouse. After the appearance of the third leaf, the roots of the seedlings were washed to remove the growth substrate, and the seedlings were placed in plastic pots filled with rockwool. Overall, 50 evenly grown plants per crop vegetable were transferred to an NFT system, where they were grown for 3 weeks with added nutrients, as reported previously [16]. After this acclimatisation in the NFT system for 3 weeks, the treatments were added. When the crops reached technological maturity, they were collected and further analysed.

2.3. Chemicals and Plant Material

The plants were grown from seeds obtained as follows: *Beta vulgaris* L. 'Delta'; *Brassica rapa* L. var. *japonica* 'Mizuna grun'; *Lactuca sativa* L. 'Grazer Krauthäuptel 2 Treibstamm'; *Valerianella locusta* Laterr. 'Verte de Cambrai' (Austrosaat AG, Wien, Austria).

The leaf extract was prepared in the extraction medium, with HPLC–mass spectrometry (MS) used to determine the juglone content as accurately as possible. The control juglone and leaf extract dilutions were prepared as previously described [13].

The following standards were used: *p*-coumaric acid, ferulic acid, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, apigenin-7-glucoside (Fluka Chemie GmbH, Buchs, Switzerland); neochlorogenic acid (3-caffeoylquinic acid), chlorogenic acid (*trans*-5-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid), sinapic acid, caffeic acid, gallic acid, luteolin-7-glucoside, juglone (5-hydroxy-1,4-naphthoquinone) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); gluconapin and isorhamnetin-3-*O*-glucoside (Extrasynthese, Genay, France).

A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to bi-distil and purify the water used in the preparation of the samples. The acetonitrile and formic acid used for the mobile phases for MS analysis were HPLC–MS grade (Fluka Chemie GmbH, Buchs, Switzerland). The methanol used for the phenolic compound extraction was HPLC–MS grade (Sigma-Aldrich, Steinheim, Germany).

2.4. Sampling of the Plants

When the plants had reached technological maturity, they were collected and analysed. First, all of the roots were cut from the plants. Once removed from the plants, the roots and leaves were weighed to determine the yield per plant for the different treatments. To determine the dry weights, five sets of roots and leaves per crop vegetable and treatment were placed in an oven at 105 °C, to constant mass. The remaining five leaves per crop vegetable and treatment were divided into three categories: (i) young leaves (undeveloped leaves); (ii) semi-old leaves (the remaining fully developed leaves); (iii) old leaves (four outer fully developed leaves). Thereafter, the fresh leaves were immediately frozen with liquid nitrogen and stored at −20 °C until further analysis.

2.5. Extraction of the Phenolic Compounds

The protocol for extraction of the individual phenolic compounds was as previously described [13]. Briefly, 200 mg of previously lyophilised samples of *B. vulgaris*, *B. rapa* var. *japonica*, *L. sativa*, and *V. locusta* were extracted at a tissue:solution ratio of 1:100 (*w/v*). The phenolics extraction medium used was 80% methanol and 3% formic acid in water.

2.6. Preparation of J. regia Leaf Extract

Leaves for the leaf extract were obtained on 10 September 2020, from a 24-year-old *J. regia* tree grown at a planting density of 10 m × 10 m. It belonged to the French cultivar 'Franquette', which has been most frequently studied and used in research. Leaves were collected from the south side of the tree, from the middle part of the canopy, placed in a paper bag, frozen with liquid nitrogen, and then lyophilised. After lyophilisation, the leaves were ground with an automatic grounder (IKA A11 Basic, IKA-Werke GmbH & Co., KG, Köln, Germany) and added to the extraction medium containing 0.17% DMSO and 0.17% ethanol in H_2O. The extraction was performed in an ultrasonic bath filled with ice

(Sonis 4; Iskra Pio, Sentjernej, Slovenia) for 60 min. The extraction was then centrifuged at $10,000 \times g$ for 10 min at 4 °C (5810 R; Eppendorf, Hamburg, Germany). A sample was taken from the extract (i.e., supernatant) for quantification of juglone by HPLC–MS. The samples were then diluted to prepare the required leaf extract containing 10 µM juglone (referred to here as 'leaf juglone'). Parallel juglone solutions were prepared from the juglone dissolved in the extraction medium (0.17% DMSO, 0.17% ethanol in H_2O) at the required final juglone concentrations (1 mM and 10 µM), referred to here as 'control juglone'.

2.7. HPLC–Mass Spectrometry Analysis of Individual Phenolic Compounds

The individual phenolic compounds were analysed on a UHPLC system (Vanquish; Thermo Scientific, Waltham, MA, USA). The diode detector used was at 350 nm for flavonols and 280 nm for the other phenolic compounds. The spectra were recorded between 200 nm and 600 nm. A C18 column (Gemini 150 × 4.60 mm; 3 µm; Phenomenex, Torrance, CA, USA) operated at 25 °C was used to separate the phenolic compounds. Solvent A was 0.1% formic acid with 3% acetonitrile in bi-distilled water ($v/v/v$), and solvent B was 0.1% formic acid with 3% bi-distilled water in acetonitrile ($v/v/v$). The flow rate of elution was 0.6 mL/min. The gradient, washing, and reconditioning of the column between samples were similar to those described previously [17], with minor modifications. The gradient used was as follows: 0–15 min, 5–20% B; 15–20 min, 20–30% B; 20–25 min, 30–50% B; 25–30 min, 50–90% B; 30–35 min, 90–100% B; 35–45 min, 100–5% B; 46–50 min, 5% B.

Identification of the phenolic compounds was achieved by tandem MS (MS/MS; LCQ Deca XP MAX; Thermo Scientific, Waltham, MA, USA), with heated electrospray ionisation operated in negative ion mode. The parameters were as follows: sheath temperature, 320 °C; sheath gas, 50 arb; auxiliary gas, 20 arb; ion spray voltage, 3.5 kV; capillary temperature, 320 °C; capillary voltage, 10.0 V; tube lens, -68 V. Scans were performed from m/z 50 to 2000. The collision energy was 35 eV, with helium used as the collision gas to achieve collision-induced dissociation. The Xcalibur 2.2 software (Thermo Fischer Scientific Institute, Waltham, MA, USA) was used for data acquisition.

Known compounds were identified and quantified using external standards, with the literature data and MS fragmentation used for identification of the unknown compounds. The quantification of unknown compounds was based on a similar standard. Total flavonols, total flavones, total hydroxycinnamic acids, and total analysed phenolics content (TAPC), which represents the sum of all of the identified phenolics, are expressed as g/kg fresh weight, while individual phenolic compounds are expressed as mg/kg fresh weight.

2.8. Statistical Analysis

The data were collated using Microsoft Excel 2016 and R commander (Package Rcmdr) version 2.7.1. (Team, R.D.C., 2008, Stanford, CA, USA). For each methodology, five biological repetitions were performed. Data are expressed as means $\pm$ standard error (SE). One-way analysis of variance (ANOVA) with Tukey tests was used to determine significant differences between treatments, and statistical means were calculated at the 95% confidence level to determine the significance of the differences.

3. Results and Discussion

3.1. Identification of Individual Phenolic Compounds in the Crop Vegetables

A total of 60 phenolic compounds were identified based on the previous literature data: 15 for *B. vulgaris*; 15 for *B. rapa* var. *japonica*; 17 for *L. sativa*; 13 for *V. locusta*. Some of these were identified for the first time in these crop vegetables. Of these 60 compounds, 9 were identified, and their fragmentation was confirmed using standards. The remaining compounds were tentatively identified according to their pseudo-molecular ions (i.e., [M-H]$^-$) and specific fragmentation patterns (i.e., MS^2, MS^3). The phenolic compounds identified, their fragmentation, and the standards used to express them, are given in Table 1. Representative chromatograms of the phenolic compounds identified can also be seen in the Supplementary Materials.

Table 1. Tentative identification of the 60 phenolic compounds from *B. vulgaris*, *B. rapa* var. *japonica*, *L. sativa*, and *V. locusta*, and the standards used.

Source	Compound	Rt (min)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Expressed as
Beta vulgaris L.	*p*-Coumaroylcaffeic acid	11.09	337	119 (100), 179 (42), 163 (12)		*p*-Coumaric acid
	p-Coumaric acid hexoside	12.17	325	163 (100), 145 (83), 119 (8), 235 (8)		*p*-Coumaric acid
	Ferulic acid hexoside	13.12	355	193 (100), 217 (54), 175 (29)		Ferulic acid
	Vitexin hexoside	14.85	593	311 (100), 341 (25)	283 (100), 191 (3)	Apigenin-7-glucoside
	Ferulic acid	15.13	193	149 (100), 178 (72), 134 (48)		Ferulic acid
	Ferulic acid derivative 1	17.48	443	267 (100), 193 (9)	249 (100), 113 (72), 175 (14)	Ferulic acid
	Ferulic acid derivative 2	17.79	639	443 (100)	267 (100), 193 (10)	Ferulic acid
	Vitexin hexoside	18.23	577	413 (100)	293 (100)	Apigenin-7-glucoside
	Luteolin dihexoside	18.89	609	285 (100), 257 (4)	257 (100), 241 (45), 229 (37), 151 (32) 213 (26)	Luteolin-7-glucoside
	Vitexin pentoside	18.89	563	413 (100)	293 (100)	Apigenin-7-glucoside
	Vitexin (apigenin-*C*-hexoside isomer)	19.47	431	311 (100)	283 (100), 191 (3)	Apigenin-7-glucoside
	Isorhamnetin dihexoside	19.93	639	315 (100), 300 (17)	300 (100), 287 (4)	Isorhamnetin-3-glucoside
	Vitexin hexoside derivative	20.19	651	607 (100)	457 (100), 293 (3)	Apigenin-7-glucoside
	Isorhamnetin rutinoside	20.68	609	315 (100), 300 (13)	300 (100), 287 (5)	Isorhamnetin-3-glucoside
	Malonyl pentosylvitexin	21.59	649	605 (100)	455 (100), 293 (5)	Apigenin-7-glucoside
Brassica rapa L. var. *japonica*	Neochlorogenic acid (3-caffeoylquinic acid)	9.27	353	191 (100), 179 (46), 135 (7)		Neochlorogenic acid
	Kaempferol-3-*O*-diglucoside-7-*O*-glucoside	10.34	771	609 (100)	285 (100), 284 (100), 429 (98), 257 (10), 179 (3)	Kaempferol-3-glucoside
	Gluconapin	11.57	372	259 (100), 275 (29), 194 (20), 130 (7)	139 (100), 97 (39), 199 (13), 241 (7)	Gluconapin
	Kaempferol-3-*O*-caffeoyldiglucoside-7-*O*-glucoside	12.19	933	771 (100)	609 (100)	Kaempferol-3-glucoside
	Kaempferol-3-*O*-sinapoyldiglucoside-7-*O*-glucoside	13.42	977	815 (100)	609 (100)	Kaempferol-3-glucoside
	Sinapoyl glycoside	13.42	385	223 (100), 247 (48), 205 (40)	164 (100), 208 (44), 179 (35)	Sinapic acid
	Kaempferol diglucoside	13.57	609	447 (100), 285 (13), 284 (2)	284 (100), 285 (46), 151 (5)	Kaempferol-3-glucoside
	Kaempferol-3-*O*-feruoylglucoside-7-*O*-glucoside	14.07	947	785 (100)	623 (100), 609 (92), 591 (43)	Kaempferol-3-glucoside
	Isorhamnetin-3-*O*-glucoside-7-*O*-glucoside	14.07	639	477 (100), 315 (9), 300 (1)		Isorhamnetin-3-glucoside

Table 1. *Cont.*

Source	Compound	Rt (min)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Expressed as
	Caffeoyl malate	17.50	295	179 (100), 133 (1)		Caffeic acid
	Hydroxyferuoyl malate	17.89	325	209 (100), 133 (23), 165 (5)		Ferulic acid
	Kaempferol hexoside derivative	19.92	567	447 (100)	285 (100), 284 (31)	Kaempferol-3-glucoside
	Coumaroyl malate	21.41	279	163 (100), 133 (19)		*p*-Coumaric acid
	Sinapoyl malate	22.06	339	223 (100)	164 (100), 208 (79), 179 (53)	Sinapic acid
	Feruloyl malate	22.24	309	193 (100), 133 (7)		Ferulic acid
Lactuca sativa L.	Dihydroxybenzoic acid hexoside	8.69	315	153 (100), 108 (19)		Gallic acid
	Esculetin glucoside	9.87	339	177 (100)		Gallic acid
	Chlorogenic acid (5-caffeoylquinic acid)	12.47	353	191 (100), 179 (4), 135 (1)		Chlorogenic acid
	Galloyl hexoside	13.13	331	313 (100), 168 (61), 125 (19), 169 (24)		Gallic acid
	Cryptochlorogenic acid (4-caffeoylquinic acid)	14.21	353	191 (100), 179 (3), 135 (1)		Cryptochlorogenic acid
	Sinapoyl hexoside derivative	15.38	431	385 (100)	223 (100), 179 (41), 208 (27)	Sinapic acid
	cis 5-*O*-*p*-Coumaroylquinic acid	15.91	337	191 (100), 163 (8)		*p*-Coumaric acid
	trans 5-*O*-*p*-Coumaroylquinic acid	17.24	337	191 (100), 163 (7)		*p*-Coumaric acid
	Caffeoyl malate	17.60	295	179 (100), 133 (53)		Caffeic acid
	Quercetin-3-*O*-galactoside	19.34	463	301 (100), 300 (3)		Quercetin-3-*O*-galactoside
	Quercetin-3-*O*-glucoside	20.68	463	301 (100), 300 (19)		Quercetin-3-*O*-glucoside
	Kaempferol-3-*O*-glucuronide	21.42	463	285 (100), 284 (41)		Kaempferol-3-glucoside
	Quercetin-3-*O*-glucuronide	21.96	477	301 (100), 300 (4)		Quercetin-3-*O*-glucoside
	Quercetin 3-(6″-malonylglucoside)	22.55	549	505 (100)	301 (100), 300 (57)	Quercetin-3-*O*-glucoside
	Quercetin-3-(6″-acetylglucoside)	23.70	505	301 (100), 300 (64)		Quercetin-3-*O*-glucoside
	Caffeoyltartaric acid hexoside 1	26.63	473	293 (100), 311 (99)		Caffeic acid
	Caffeoyltartaric acid hexoside 2	29.21	473	311 (100), 293 (87)		Caffeic acid
Valerianella locusta Laterr.	4-Hydroxyphenylaoyl glucoside derivative	7.16	359	313 (100)	151 (100), 269 (2), 185 (1)	Gallic acid
	Chlorogenic acid (5-caffeoylquinic acid)	12.37	353	191 (100), 179 (4), 135 (1)		Chlorogenic acid
	Cryptochlorogenic acid (4-caffeoylquinic acid)	14.10	353	191 (100), 179 (3), 135 (1)		Cryptochlorogenic acid
	cis 5-*O*-*p*-Coumaroylquinic acid	15.89	337	191 (100), 163 (8)		*p*-Coumaric acid
	cis 5-*O*-Feruoylquinic acid	16.88	367	191 (100), 173 (3)		Ferulic acid
	trans 5-*O*-*p*-Coumaroylquinic acid	17.22	337	191 (100), 163 (7)		*p*-Coumaric acid
	trans 5-*O*-Feruoylquinic acid	18.02	367	191 (100), 173 (2)		Ferulic acid

Table 1. *Cont.*

Source	Compound	Rt (min)	[M-H]⁻ (*m/z*)	MS² (*m/z*)	MS³ (*m/z*)	Expressed as
	Luteolin-7-rutinoside	19.62	593	285 (100)	285 (100), 241 (24), 217 (14), 199 (16), 175 (17), 151 (6)	Luteolin-7-glucoside
	Diosmetin apiosylglucoside	21.74	593	299 (100), 284 (17)	284 (100)	Luteolin-7-glucoside
	Diosmin (diosmetin-7-*O*-rutinoside)	22.07	607	299 (100), 284 (24)		Luteolin-7-glucoside
	Dicaffeoylquinic acid	22.58	515	353 (100), 179 (2)		Caffeic acid
	Apigenin-rutinoside	24.75	577	531 (100), 269 (98)		Apigenin-7-glucoside
	Caffeic acid hexoside derivative	25.48	637	535 (100), 341 (23)	161 (100), 179 (57), 341 (57)	Caffeic acid

Rt, retention time; [M-H]⁻, pseudo-molecular ion identified in negative ion mode; (), relative abundance of fragment ions.

For the 25 hydroxycinnamic acids, 4 were identified through the use of standards and their fragmentation: neochlorogenic acid (3-caffeoylquinic acid); cryptochlorogenic acid (4-caffeoylquinic acid); chlorogenic acid (*trans*-5-caffeoylquinic acid); ferulic acid. The remaining 21 hydroxycinnamic acids were identified through their typical fragmentation patterns. *p*-coumaric acid and caffeic acid derivatives were identified through their fragmentation patterns of MS^n ion *m/z* 163 and 119 and *m/z* 179, as reported by Liu et al. [18]. *cis* 5-*O*-*p*-coumaroylquinic acid, *trans* 5-*O*-*p*-coumaroylquinic acid, caffeoyl-tartaric acid hexoside, and dicaffeoylquinic acid were identified through their fragmentation patterns as previously reported for *V. locusta* and *L. sativa* by Hernández et al. [19] and Abu-Reidah et al. [20]. Coumaroyl malate, sinapoyl malate, sinapoyl glycoside, feruloyl malate, and caffeoyl malate were identified by their specific fragmentation patterns as reported for pak choi (*Brassica campestris* L. ssp. *chinensis* var. *comunis*) by Harbaum et al. [21], which are described here for the first time for *B. rapa* var. *japonica*. Ferulic acid derivatives were identified through their fragmentation patterns of MS^n ion *m/z* 193 and 175, as reported by Vieira et al. [22], and *cis* 5-*O*-feruoylquinic acid and *trans* 5-*O*-feruoylquinic acid were identified through their fragmentation patterns as reported by Hernández et al. [19]. There were only two hydroxybenzoic acids identified, dyhydroxybenzoic acid hexoside and galloyl hexoside, both of which were previously identified by Abu-Reidah et al. [20].

All of the 11 flavones identified were in *B. vulgaris* and *V. locusta*. Four flavones (luteolin-7-rutinoside, diosmetin apiosylglucoside, diosmin, and apigenin-rutinoside) were identified in *V. locusta*, all of which were previously reported by Hernández et al. [19], and seven (vitexin hexoside 1 and 2, vitexin pentoside, luteolin dihexoside, vitexin, vitexin hexoside derivate, and malonyl pentosylvitexin) in *B. vulgaris*. Vitexin, vitexin hexoside, vitexin pentoside, and malonyl pentosylvitexin were previously reported in *B. vulgaris* by Vissers et al. [23] and AbdEl-Ghffar et al. [24], while, luteolin dihexoside and vitexin hexoside derivatives are reported here for the first time in *B. vulgaris*.

There were 13 flavonols identified, 2 in *B. vulgaris*, 7 in *B. rapa* var. *japonica*, and 6 in *L. sativa*. In *B. vulgaris*, both of the flavonols identified (isorhamnetin dihexoside, rutinoside) are reported here for the first time in *B. vulgaris*, and these followed the typical fragmentation pattern MS^n *m/z* 315 and 300, MS^{n+1} *m/z* 300, 287 for isorhamnetin. In *B. rapa* var. *japonica*, of the seven flavonols identified, only kaempferol hexoside derivative is reported for the first time, which followed the kaempferol typical fragmentation pattern MS^n *m/z* 285 and 284. The other flavanols identified in *B. rapa* var. *japonica* were previously identified in pak choi (*Brassica campestris* L. ssp. *chinensis* var. *comunis*) and curly kale (*Brassica oleracea* L. Convar. *acephala* var. *sabellica*) by Harbaum et al. [21] and Olsen et al. [25], and here for the first time in *B. rapa* var. *japonica*. In *L. sativa*, all six flavonols identified were previously reported by Medic et al. [13].

3.2. Effects of the Juglone Treatments on the Crop Vegetable Yields

The control effects of juglone (1 mM, 10 μM) and the leaf extract with 10 μM juglone were not the same across these different crop vegetables, as was also seen previously in germination studies by Medic et al. [13]. The effects of the different treatments on the crop yields and root weights are shown in Figure 1 and in Supplementary Materials.

Interestingly, the highest crop yields and root weights were obtained for the 10 μM leaf juglone treatments for *B. rapa* var. *japonica* and *L. sativa*, and for the K2 control as the second-highest for *B. vulgaris* and *V. locusta*. This might be because the leaf extracts contain other nutrients and beneficial allelochemicals besides juglone that can stimulate plant growth and increase crop yields. In contrast to our previous seed germination study [13], where the 10 μM juglone leaf extract was not different from the control treatments, here, the 10 μM leaf juglone even showed higher yields, compared with the other treatments. Indeed, this would appear logical considering that most biostimulants are produced from plant waste [26]. However, higher concentrations of juglone in the leaf extract might have inhibitory effects also on crop yields, as observed in the germination trial by Medic et al. [13],

where 1 mM juglone leaf extracts significantly inhibited seed germination and seedling growth. Therefore, further studies are needed before a conclusion can be drawn.
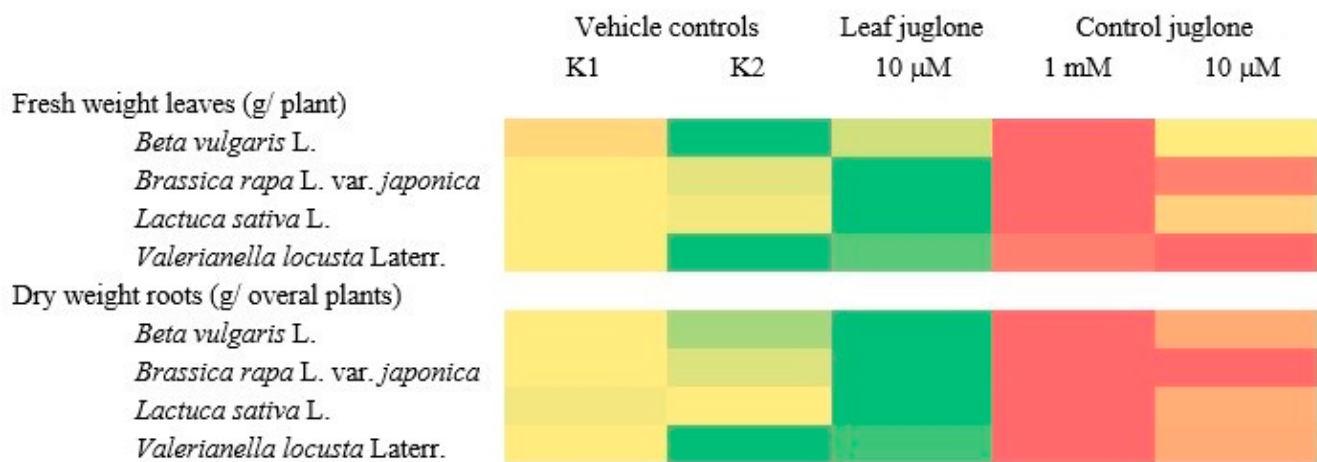

Figure 1. Heat map showing leaf fresh weights and root dry weights, from highest (green) to lowest (red) values between treatments (columns). K1: extraction medium control (0.17% DMSO, 0.17% ethanol in H_2O); K2: water control.

It is known that juglone is absorbed from the soil through the roots and, therefore, acts first on the roots of the plants. It penetrates the plasma membrane of the root cells and induces depolarisation by blocking the K^+ channels, which inhibits root and, consequently, shoot, nutrient uptake, and growth [27]. This explains the lowest yields for all of these crop vegetables, as well as the lowest root weights, which were seen for 1 mM, followed by 10 μM, control juglone treatments. Overall, juglone showed allelopathic effects on the yields of all of these crop vegetables. However, the yields of *B. vulgaris* and *L. sativa* appeared to be less affected by the lower concentration of the control juglone (10 μM) than that of *B. rapa* var. *japonica* and *V. locusta* and would, therefore, be a better choice for cultivation in soils where juglone is still present.

3.3. Effects of the Juglone Treatments on the Crop Vegetable Quality

Looking at the effects of the juglone treatments with the same 10 μM concentration of control juglone and leaf juglone on the crop quality in terms of TAPC, it can be seen that these were not the same across the crop vegetables (Figures 2–5), as also previously reported by Medic et al. [13] and Ercisli and Turkkal [28]. While *L. sativa* and *V. locusta* showed similar responses, the responses of *B. vulgaris* and *B. rapa* var. *japonica* were almost contrary. All of the data that showed significant differences are further detailed in the Supplementary Materials.
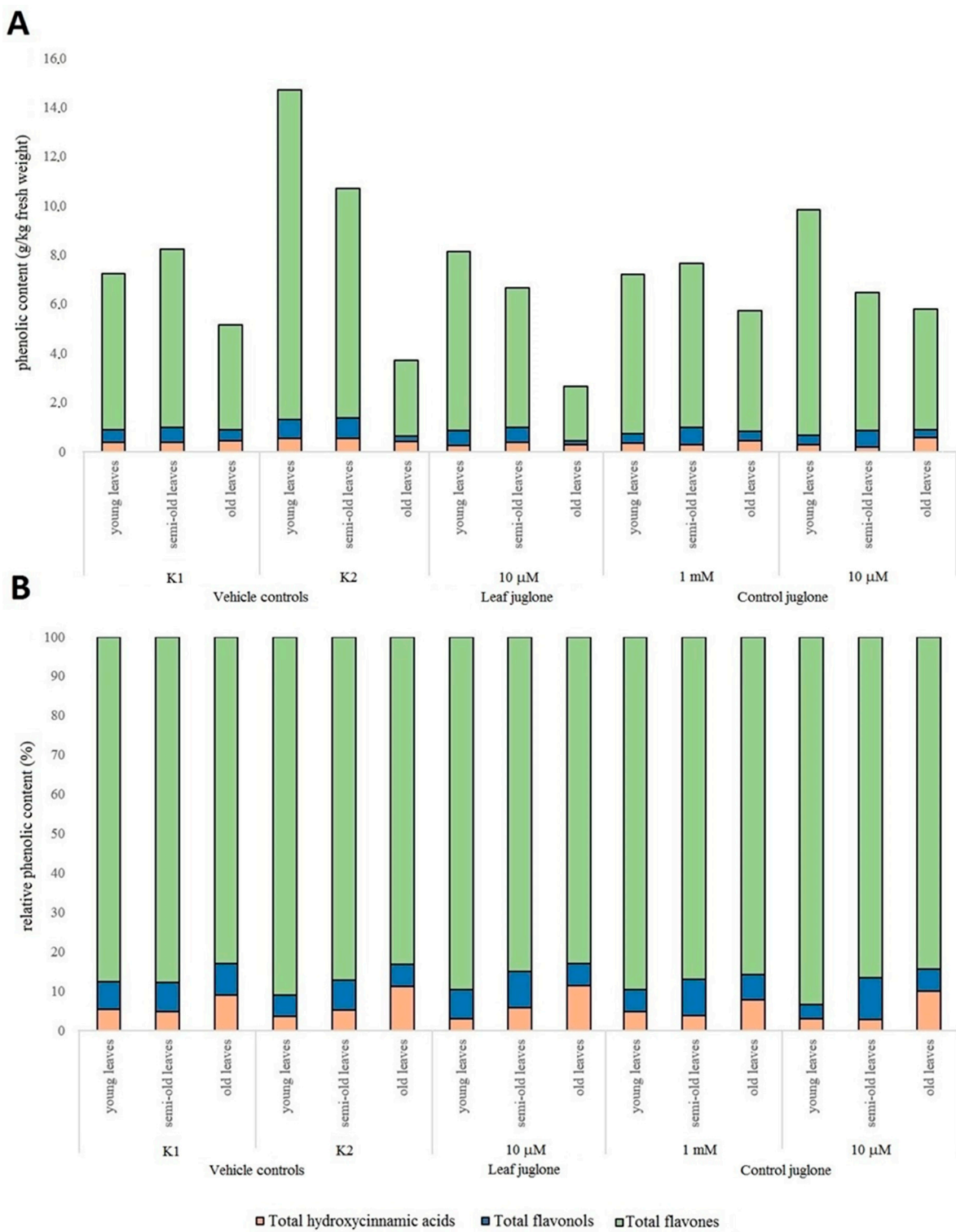
Figure 2. Contents of the total phenolic groups identified in *B. vulgaris* expressed relative to fresh weight (**A**) and as proportions of the total phenolic groups identified (**B**). K1: extraction medium control (0.17% DMSO, 0.17% ethanol in H_2O); K2: water control. Young leaves, undeveloped leaves; semi-old leaves, remaining fully developed leaves; old leaves, four outer fully developed leaves.
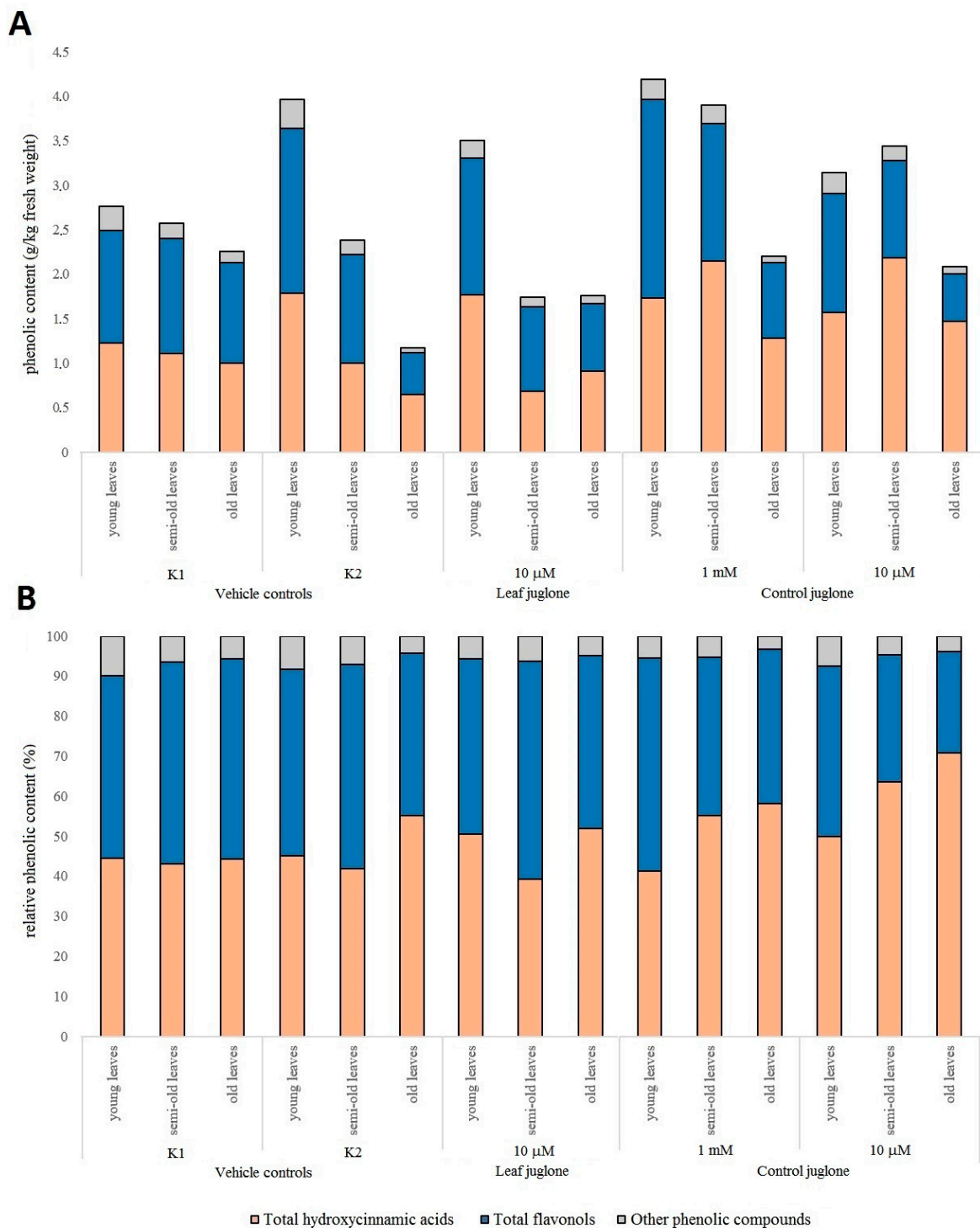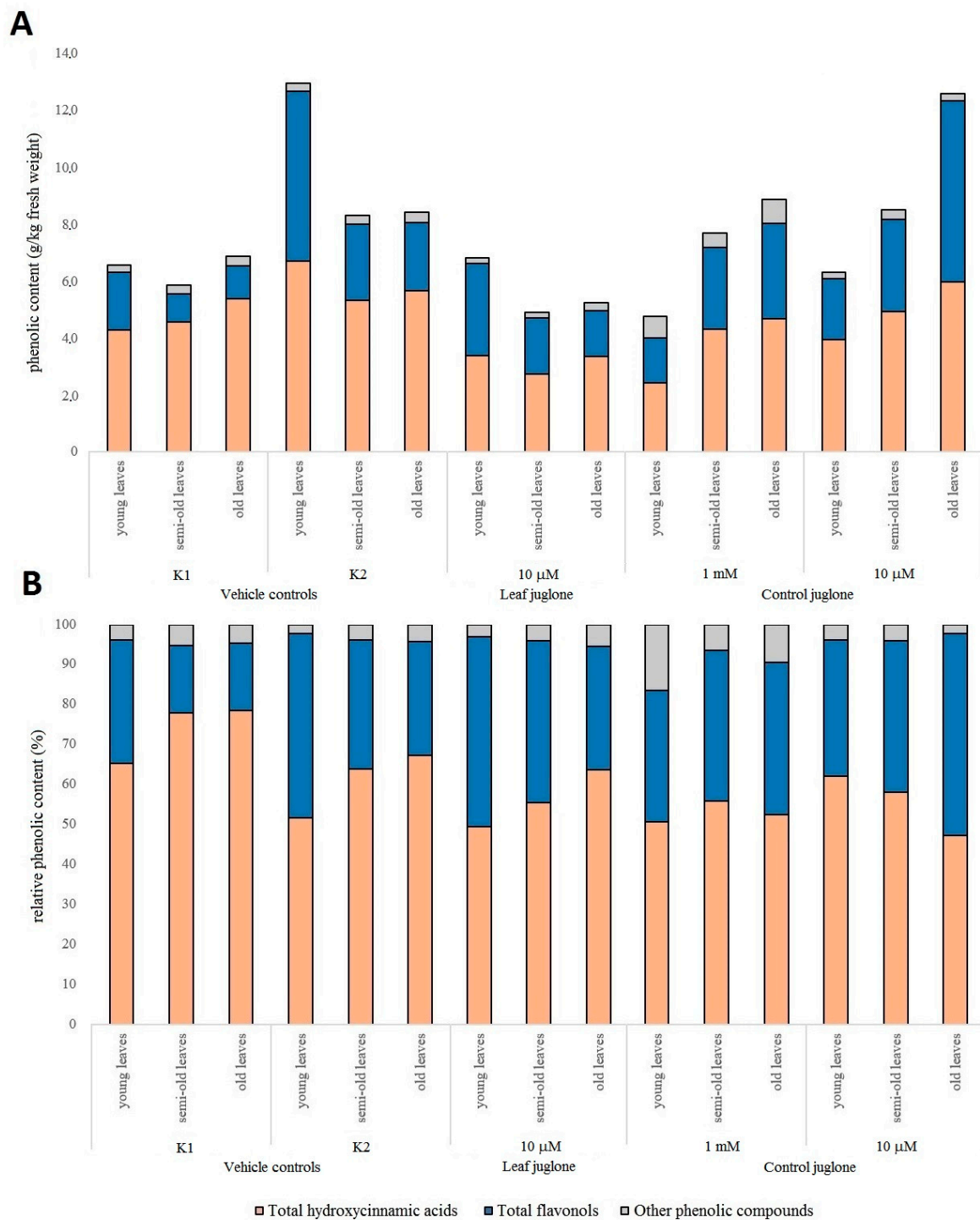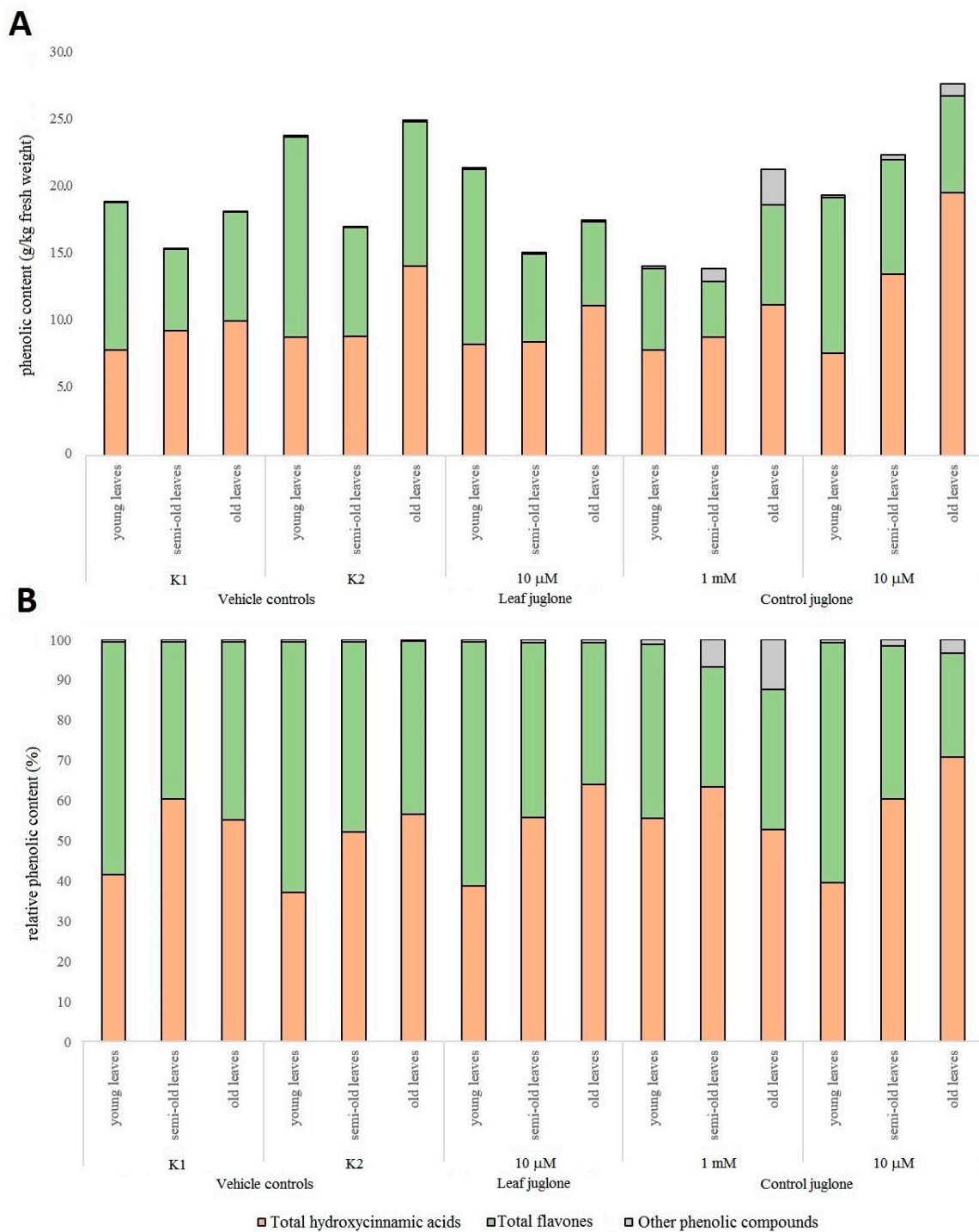
Figure 3. Contents of the total phenolic groups identified in *B. rapa* var. *japonica* expressed relative to fresh weight (**A**) and as proportions of the total phenolic groups identified (**B**). K1: extraction medium control (0.17% DMSO, 0.17% ethanol in H_2O); K2: water control. Young leaves, undeveloped leaves; semi-old leaves, remaining fully developed leaves; old leaves, four outer fully developed leaves.

Figure 4. Contents of the total phenolic groups identified in *L. sativa* expressed relative to fresh weight (**A**) and as proportions of the total phenolic groups identified (**B**). K1: extraction medium control (0.17% DMSO, 0.17% ethanol in H_2O); K2: water control. Young leaves, undeveloped leaves; semi-old leaves, remaining fully developed leaves; old leaves, four outer fully developed leaves.

Figure 5. Contents of the total phenolic groups identified in *V. locusta* expressed relative to fresh weight (**A**) and as proportions of the total phenolic groups identified (**B**). K1: extraction medium control (0.17% DMSO, 0.17% ethanol in H_2O); K2: water control. Young leaves, undeveloped leaves; semi-old leaves, remaining fully developed leaves; old leaves, four outer fully developed leaves.

As can be seen in Figure 2A, the extraction medium control (K1; with vehicles used for the control juglone and leaf juglone treatments) affected the quality of *B. vulgaris*, compared with K2 (water control), with lower TAPC in the younger and semi-old leaves. There were no differences between the K1, 1 mM and 10 μM control juglone, and 10 μM leaf juglone treatments, except for TAPC in older leaves treated with 10 μM leaf juglone, which had lower TAPC than for the other treatments. Figure 2B shows that for *B. vulgaris*, there

were no differences in the proportions of total hydroxycinnamic acid, total flavonols, and flavones, indicating that the quality of *B. vulgaris* was not greatly affected by the 1 mM and 10 μM control juglone and 10 μM leaf juglone treatments. In terms of quality, the younger leaves tended to have the highest TAPC and, therefore, the highest quality, while the older leaves had lower TAPC, which is usually the case for all plants [29]. The higher TAPC in the younger leaves is the result of the plant defence mechanisms, as leaves with higher TAPC are better protected against bacterial infections than older leaves [30]. The TAPC of *B. vulgaris* was consistent with previous measures of Vissers et al. [23]; however, most of the phenolic compounds identified, as well as the highest contents seen in the present study, were flavones, compared with the phenolic acids reported by Vissers et al. [23].

The vehicle effect (i.e., K1 vs. K2) on quality seen for *B. vulgaris* was not seen for *B. rapa* var. *japonica*, *L. sativa*, or *V. locusta*. As shown in Figure 3A, in *B. rapa* var. *japonica*, there were no clear trends seen for TAPC, and thus, 1 mM and 10 μM control juglone and 10 μM leaf juglone did not have any effects on the crop quality. However, if the contents of hydroxycinnamic acids and flavonols are considered, it can be seen that the 1 mM and 10 μM control juglone treatments resulted in higher hydroxycinnamic acids contents in the semi-old leaves, while the semi-old leaves treated with 10 μM leaf juglone had lower hydroxycinnamic acids content, compared with both controls (K1, K2), as can be seen in Figure 3B. In addition, the treatments with 1 mM and 10 μM control juglone also affected the flavonols content, with increased total flavonols in the older leaves, compared with the young leaves, which was not seen for the control or 10 μM leaf juglone treatments. Overall, in the semi-old leaves, 10 μM leaf juglone decreased the hydroxycinnamic acids content, while the flavonols content increased, in contrast to the 1 mM and 10 μM control juglone treatments, for which in the semi-old leaves, the hydroxycinnamic acids content increased and the flavonols content decreased. This suggests that other allelochemicals are present in *J. regia* that can have actions similar to those of juglone, as also previously indicated by Medic et al. [13]. The TAPC of *B. vulgaris* was consistent with that of Harbaum et al. [21] in *Brassica campestris* L. *chinensis* var. *communis* and higher than previously reported in *B. rapa* var. *japonica* by Khanam et al. [31]. Most of the phenolic compounds identified in the present study, as well as the highest contents, were for the hydroxycinnamic acids, similar to a previous report by Khanam et al. [31].

Similar to *B. rapa* var. *japonica*, in *L. sativa* the majority of the identified and quantified phenolic compounds represented the hydroxycinnamic acids, followed by the flavonols, which is in agreement with Abu-Reidah et al. [20] and Ribas-Agustí et al. [32]. TAPC was slightly lower than previously reported by Santos et al. [33]. Figure 4 shows that the 1 mM and 10 μM control juglone treatments affected the metabolic response of *L. sativa* in the same way as for *B. rapa* var. *japonica*, while the 10 μM leaf juglone treatment had no effects on the secondary metabolites. The highest TAPCs were seen for the older leaves in the 1 mM and 10 μM control juglone treatments, compared with the younger leaves, mainly due to the higher flavonols and hydroxycinnamic acids contents. Considering the relative values of the phenolic compounds (Figure 4B), it can be seen that the treatments with 1 mM and 10 μM control juglone resulted in higher proportions of hydroxycinnamic acids and lower proportions of flavonols in the younger leaves, compared with the older leaves, contrary to other treatments (K1, K2, 10 μM leaf juglone), in which the proportions of hydroxycinnamic acids were higher in the older leaves, and proportions of flavonols were higher in the younger leaves. This has been observed previously for *B. vulgaris*, *B. rapa* var. *japonica*, and *V. locusta*, and it suggests a uniform metabolomics response of these crop vegetables to produce hydroxycinnamic acids when affected by the allelochemical juglone.

As can be seen in Figure 5, both of the 1 mM and 10 μM control juglone treatments had effects on the quality of *V. locusta*, while there were no differences between the controls and the 10 μM leaf juglone treatments. The metabolic responses of *V. locusta* were similar to those of *L. sativa*, with the older leaves showing the highest levels of TAPC, mainly due to the increase in the hydroxycinnamic acids content in the older leaves treated with juglone. Apart from this difference in the hydroxycinnamic acids in the 1 mM and 10 μM control

juglone treated plants in the old leaves, the major difference was in the contents of the other phenolic compounds, which were higher in older leaves, compared with younger leaves. The TAPC of *V. locusta* is in agreement with that reported by Hernández et al. [19]. Most of the phenolic compounds identified in the present study, as well as the highest contents, were hydroxycinnamic acids, followed by flavones, as previously reported by Hernández et al. [19].

4. Conclusions

As also previously reported by Medic et al. [13], we can confirm that each of these crop vegetables responds differently to the allelochemicals, and although juglone appears to be the most toxic of the allelochemicals, it might not be the only one in these leaf extracts of *J. regia*. Here, the treatment with the leaf extract (i.e., 10 μM leaf juglone) resulted in the highest yields and comparable qualities in terms of TAPC, compared with the other treatments. This either means that this leaf extract of *J. regia* also contains beneficial allelochemicals that can stimulate growth, or that the leaf extract is simply a good source of additional minerals and nutrients that stimulate growth without affecting the metabolic responses of the plants themselves. The possibility that different concentrations of *J. regia* leaf extracts have different biostimulatory effects on such crop yields should be further investigated.

In addition to the identification and quantification of the phenolic compounds in these crop vegetable leaves of different ages, the main objective of this study was to investigate the yields, quality, and metabolic responses of these different crop vegetables, so as to determine which of them cannot thrive in soils containing juglone or other *J. regia* allelochemicals. From the data obtained, it can be concluded that *B. vulgaris* and *L. sativa* are more suitable for planting in soils where walnuts were previously grown, since, although they were affected by the 1 mM control juglone, they showed fewer negative effects for the 10 μM control juglone in terms of crop yields. In terms of crop quality, the smallest effects of the different treatments were seen for *B. vulgaris*. Both *B. rapa* var. *japonica* and *V. locusta* showed lower yields even for the lower of the control juglone treatments (i.e., 10 μM), and the quality was also affected, so cultivation in juglone-containing soils should be avoided.

Apart from identifying and quantifying 60 compounds from *B. vulgaris*, *B. rapa* var. *japonica*, *L. sativa*, and *V. locusta*, with some reported for these crop vegetables for the first time, this study also serves as a basis for the selection of more suitable crops in the early years of planting of crops in the soils where walnuts were previously planted. The quality of the leaves of these crop vegetables at different ages and the quality and yields of these selected crop vegetables grown under the influence of allelochemicals were also investigated, to determine the influence of allelochemicals on their metabolomics and, thus, on the uptake of phenolic compounds considered to be beneficial to human health. The data obtained could be relevant for future studies on crop management in different soils and the use of allelochemicals to modify the phytochemical composition of vegetables.

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