Leaves and decoction of *Juglans regia* L.: different performances regarding bioactive compounds and *in vitro* antioxidant and antitumor effects

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ABSTRACT

Juglans regia L. (walnut) leaves are considered a source of healthcare compounds, and have been widely used in traditional medicine. Furthermore, they have been reported as a source of bioactive molecules such as the antioxidant phenolic compounds. In the present study, organic acids, mono and oligosaccharides, and tocopherols were analyzed in leaves and decoction (widely used for topical applications). Furthermore, the phenolic composition and bioactivity of walnut leaves was determined in its decoction and methanol extract. Malic acid, sucrose, α-tocopherol and 3-*O*-caffeoylquinic acids and quercetin *O*-pentoside were the most abundant organic acid, disaccharide, tocopherol isomer and phenolic compounds, respectively. Methanol extract presented higher antioxidant and antitumor potential than decoction; both samples did not show toxicity for non-tumor liver primary cells. This study provides a more complete characterization of phenolic compounds of walnut leaves, and as far as we know, this is the first time that the presence of procyanidins and taxifolin derivatives, as also tocopherols were reported in *J. regia* leaves.

Keywords: *Juglans regia* leaves; Chemical characterization; Decoction/Methanol extract; Antioxidant properties; Antitumor effects

1. Introduction

In Portugal, as well as in many other European countries, especially in rural areas, walnut (*Juglans regia* L.) leaves are often used in decoctions and other preparations for the treatment of skin inflammations and ulcers, as also for their antidiarrheal, anti-anthelmintic, antiseptic and astringent properties. In traditional medicine, the walnut leaves are considered essential disinfectants and anti-inflammatory used in the washing of wounds and burns (Carvalho and Morales 2010). Other authors also refer their use in preparations for topical use in the relief of anti-rheumatic pain, and for the treatment of acne, warts and pharyngitis (Guarrera 2005; González et al., 2010).

The anti-inflammatory activity of ethanol extracts from *J. regia* leaves was demonstrated against carrageenan-induced hind paw edema model in mice, without inducing any gastric damage; it was as potent as indomethacin (anti-inflammatory drug) (Erdemoglu et al., 2003). The aqueous and ethanol extracts also showed significant antinociceptive activity (Erdemoglu et al., 2003). Furthermore, experiments using human renal cancer cell lines A-498 and 769-P and the colon cancer cell line Caco-2, demonstrated antiproliferative efficiency of walnut leaves methanol extracts (Carvalho et al., 2010). The *in vitro* antioxidant activity of aqueous (boiling water), methanol and petroleum ether extracts was also reported by different authors (Pereira et al., 2007; Carvalho et al., 2010).

Walnut medicinal properties have been related to the presence of bioactive compounds such as tocopherols or phenolic compounds (Anderson et al., 2001). Nevertheless, most of the studies regarding these phytochemicals were performed in fruits (Fukuda et al., 2003; Amaral et al., 2005; Kornsteiner et al., 2006).

A few reports about phenolic composition of walnut leaves are available. Amaral et al. (2004) identified, in methanol extracts, 3-caffeoylquinic, 3-*p*-coumaroylquinic and 4-*p*-

coumaroylquinic acids, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and also two partially identified compounds (quercetin 3-pentoside and kaempferol 3-pentoside derivatives). Pereira et al. (2007) identified another two hydroxycinnamic acid derivatives, the 5-*O*-caffeoylquinic and *p*-coumaric acids, but in aqueous extracts. Previously, Wichtl and Anton (1999) had reported the existence of phenolic acids in walnut leaves, namely caffeic, ferulic, *p*-coumaric, *p*-hydroxyphenylacetic, gallic, salicylic, 5- and 3-caffeoylquinic acids.

The main objectives of the present work were to determine the composition of walnut leaves (dry material and decoction, widely used for topical applications) in organic acids, mono and oligosaccharides, and tocopherols, as also the phenolic composition, antioxidant and antitumor effects of their methanol extract and decoction.

2. Materials and methods

2.1. Plant material and decoction preparation

Juglans regia L. (Fagales, Jugladanceae), a tree species naturalized and widely spread in Europe, has 25–40 cm long pinnately compound leaves, with 5–9 leaflets, paired alternately along the main vain with one terminal leaflet (odd-pinnate). In early summer (July 2010), the fully expanded leaves were randomly gathered from the external canopy of ten mature trees, cultivated, for a long period ago, in the edge of an extensive forest in the north-eastern Portuguese region of Trás-os-Montes. Plants selection and gathering methods followed traditional knowledge and folk medicine practices, provided by previous ethnobotanical surveys (Carvalho and Morales 2010). Such walnut trees are from an old regional variety of *J. regia* grown for timber and for their seeds (nuts). They used to be propagated from seeds and therefore the trees do not have the same genotype and chemotype. Then, the plant material was mixed to obtain a

representative sample (which reproduces local practices for medicinal uses), freeze-dried and stored in the deep-freezer at -20ºC for subsequent analysis.

For decoction preparation the lyophilized plant material (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained decoction was frozen and lyophilized.

2.2. Standards and Reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA), as also L-ascorbic acid, tocopherol, sugar and organic acid standards. Phenolic compound standards were from Extrasynthese (Genay, France). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediamine tetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Bioactive compounds

2.3.1. Organic acids. Organic acids were determined in the decoction and in leaves dry material, in this case after extraction with 4% metaphosphoric acid as previously described by the authors (Guimarães et al., 2013a). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Detection was carried out in a photodiode array detector (PDA), using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 and 245 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry plant material or decoction (dw).

2.3.2. Mono and oligosaccharides. Free sugars were determined in the decoction and in leaves dry material, in this case after extraction with 80% ethanol as previously described by the authors (Guimarães et al, 2013a). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Brelin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard (melezitose) method and sugar contents were further expressed in g per 100 g of dry plant material or decoction (dw).

2.3.3. Tocopherols. Tocopherols were determined in the decoction and in leaves dry material, in this case after extraction with methanol and n-hexane as previously described by the authors (Guimarães et al, 2013a). The analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco)

programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry plant material or decoction (dw).

2.3.4. Phenolic compounds. Phenolic compounds were determined in the decoction and in leaves dry material, in this case after extraction with methanol as previously described by the authors (Guimarães et al., 2013a). The analysis was performed by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA), with double online detection carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in mg per g of dry plant material or decoction (dw).

2.4. Bioactivity of the methanol extract and decoction

Methanol extract (prepared according with the previous section) and lyophilized decoction were re-dissolved in *i)* methanol and water, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, or *ii)* water (final concentration 8 mg/mL) for antitumor potential evaluation. The final solutions were further diluted to

different concentrations to be submitted to distinct bioactivity evaluation *in vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumor potential. Trolox and ellipticine were used as positive controls in antioxidant and antitumor activity evaluation assays, respectively.

2.4.1. Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Rafael et al., 2011; Guimarães et al., 2013b).

2.4.2. Antitumor potential and cytotoxicity in non-tumor liver primary cells. Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF-7, NCI-H460 and HCT-15) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. Sulforhodamine B assay was performed according to a procedure previously described by the authors (Guimarães et al., 2013b).

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure established by the authors (Guimarães et al., 2013b); it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

2.5. Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by

Tukey's HSD Test with α = 0.05. This treatment was carried out using SPSS v. 18.0 program.

3. Results

3.1. Identification and quantification of organic acids, mono and oligosaccharides and tocopherols

Five different organic acids were identified, being malic acid the most abundant one, mainly in the leaves (1.04 g/100 g dw, **Table 1**), followed by oxalic and citric acids. The dry material from the leaves presented the highest content of individual and total organic acids (3.16 g/100 g dw). Ascorbic acid was not detected in the decoction, probably due to the heating process that can lead to its degradation.

Two monosaccharides, fructose and glucose, and two oligosaccharides, sucrose and trehalose were identified and quantified (**Table 1**). Sucrose was the most abundant free sugar found in both in leaf extract and decoction (5.79 and 10.10 g/100 g dw, respectively). Decoction presented a higher content in total and individual sugars with exception of trehalose. The higher levels of mono and oligosaccharides observed in decoction can be a consequence of hydrolysis of polysaccharides related to heat process.

The four isoforms of tocopherols were present either in leaves or decoction (**Table 1**). The leaf extract presented higher concentration in total (282.20 mg/100 g dw) and individual tocopherols than the decoction. This could be explained by tocopherols oxidation induced by heat in the decoction process. α-Tocopherol was the most abundant isoform (199.99 mg/100 g dw).

3.2. Identification and quantification of phenolic compounds

Twenty-five compounds were detected (**Figure 1** and **Table 2**), five of which were phenolic acid derivatives (hydroxycinnamic acid derivatives). Among them, two compounds (peaks 1 and 4) were caffeoylquinic acid derivatives identified according to their UV spectra and pseudomolecular ions. Peak 1 ($[M-H]^-$ at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity >70% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford et al. (2003) and Guimarães et al. (2013b). Peak 4 was easily distinguished from the other two isomers by its base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 97% abundance of base peak, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003) and Guimarães et al. (2013b). Three signals at *m/z* 337 were also detected (peak 3, 6 and 7); these compounds were tentatively identified according to their MS^2 fragmentation as different isomers of *p*-coumaroylquinic acid. Identities were assigned based on the patterns reported for the caffeoylquinic acid isomers. Peak 3 was identified as 3-*p*-coumaroylquinic acid, yielding the base peak at *m/z* 191, as reported by Clifford et al. (2003) and Guimarães et al. (2013b). Fragmentation of peaks 6 and 7, yield a majority MS^2 product ion at *m/z* 173, coherent with 4-*p*-coumaroylquinic acid, being peak 7 associated to the *trans* isomer, whereas peak 6 with similar mass spectral characteristics was assigned to *cis* 4-*p*-coumaroylquinic acid. The assignment was made based on their relative order of elution, as hydroxycinnamoyl *cis* derivatives would be expected to elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory.

Procyanidin oligomers were also detected, corresponding to peaks 2, 5 and 8, which showed UV spectra with λ_{max} 279-280 nm, characteristic of proanthocyanidins. Peaks 2

and 8 presented a pseudomolecular ion corresponding to procyanidin dimers ($[M-H]^-$ at *m/z* 577), and peak 5 to a procyanidin trimer ($[M-H]^-$ at *m/z* 865).

Peaks 9, 10, 13, 14 and 17 were assigned to taxifolin derivatives based on their UV spectra and pseudo molecular ion $[M-H]^-$ at *m/z* 435, releasing a fragment at *m/z* 303 $[taxifolin-H]^-$ (-132 mu, loss of a pentosyl moiety). They were tentatively identified as taxifolin *O*-pentosides that could either correspond to different steroisomers given the asymmetric nature of C-2 and C-3 of taxifolin (i.e., dihydroquercetin) or possess different pattern of sugar substitution. ESI/MS analysis does not allow obtaining information about the nature and position of the sugar moieties.

The remaining phenolic compounds corresponded to flavonol derivatives, derived from quercetin (λ_{max} around 350 nm and an MS^2 fragment at *m/z* 301), kaempferol (λ_{max} around 348 nm, MS^2 fragment at *m/z* 285), myricetin (λ_{max} around 354 nm, MS^2 fragment at *m/z* 317) and 3'-*O*-methylmyricetin (laricitrin; λ_{max} at 354 nm, MS^2 fragment at *m/z* 331) (**Table 2**). Myricetin 3-*O*-glucoside (peak 11) and quercetin 3-*O*-glucoside (peak 18) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Peaks 19 and 20 ($[M-H]^-$ at *m/z* 463), and 21 and 22 ($[M-H]^-$ at *m/z* 433 and 447, respectively) were assigned to quercetin derivatives; peaks 23 and 24 ($[M-H]^-$ at *m/z* 417), and 25 ($[M-H]^-$ at *m/z* 431) were assigned to kaempferol derivatives, and peaks 15 and 16 ($[M-H]^-$ at *m/z* 449 and 463, respectively) were assigned to myricetin derivatives. They presented MS^2 fragments corresponding to distinct losses of hexosyl (-162 mu), pentosyl (-132 mu) and rhamnosyl (-146 mu) moieties, and an elution order coherent with the type of substituent sugars, according to their expected polarity, although the position and nature of the sugar moieties could not be identified, because their retention times did not correspond to any of the standards available. Peak 12 was assigned to a laricitrin *O*-

hexoside according to its UV and mass spectra; the presence of myricetin and quercetin 3-*O*-glucosides as majority flavonoid hexosides would point to peak 12 being also a 3-*O*-glucoside.

3-*O*-Caffeoylquinic acid and quercetin *O*-pentoside were the most abundant phenolic compounds in the methanol extract and decoction. In general, the decoction preparation showed a lower concentration of the identified phenolic compounds, even though the decrease shown in the total amount is not relevant (**Table 3**).

3.3. Bioactivity

Methanol extract presented lower EC_{50} values (higher antioxidant activity) than decoction, in all the assays (**Table 4**). Both samples gave higher DPPH radical scavenging activity and reducing power than methanol extracts obtained from different Portuguese cultivars of *J. regia* (EC_{50}=0.151 to 0.202 mg/mL for DPPH scavenging activity; $EC_{0.5}$=0.192 to 0.229 for reducing power, Pereira et al., 2007; EC_{50}=0.199 mg/mL for DPPH scavenging activity, Carvalho et al., 2010) and from different Iranian cultivars of microshoots (EC_{50}=0.312 to 0.631 mg/mL for DPPH scavenging activity; $EC_{0.5}$=0.374 to 0.668 mg/mL for reducing power, Cheniany et al., 2013). For lipid peroxidation inhibition measured by β-carotene/linoleate assay, the EC_{50} values obtained in our study were also lower (EC_{50}=189.92 and 269.27 µg/mL for methanol extract and decoction, respectively) than the values reported by Pereira et al. (2007) (EC_{50}= 0.742 to 1.645 mg/mL).

Both samples showed similar potential against breast (MCF-7), colon (HCT-15) and hepatocellular (HepG2) carcinoma cell lines (**Table 4**). The methanol extract was most potent against cervical carcinoma cell line (HeLa, GI_{50}=294.87 µg/mL), while decoction

did not show any effect. Both extracts did not present toxicity for non-tumor liver primary cells (PLP2).

Carvalho et al. (2010) described antiproliferative activity of *J. regia* leaves methanol extract, using two human renal cell lines (A-498 and 769-P) and a colon cancer cell line (Caco-2). Even though the cell line for colon cancer is different from the one used in this study (HCT-15), the results presented by Carvalho et al. (2010) (IC_{50}=229 µg/mL) were similar to the ones obtained herein (GI_{50}=258.93 and 215.58 µg/mL for methanol extract and decoction, respectively).

4. Discussion

The organic acids found in *J. regia* leaves might play a protective role against various diseases due to their antioxidant properties, as is the case of ascorbic acid. Malic and citric acids also behave as antioxidants because they have the ability to chelate metals. Oxalic acid is the simplest dicarboxylic acid and its most striking chemical impact is the strong chelating ability for multivalent cations (Oliveira et al., 2008). Some organic acids, namely alpha-hydroxy acids (AHAs), are used in dermatological and cosmetic products, whose functions are to increase exfoliation and moisturization on the skin. AHAs most commonly used in cosmetic applications are typically derived from food products, including glycolic acid (from sugar cane), lactic acid (from sour milk), malic acid (from apples), citric acid (from citrus fruits) and tartaric acid (from grape wine) (Lamberg, 1998; Silva et al., 2001). Therefore, *J. regia* leaves that present some of the mentioned organic acids could be used in this perspective.

Other compounds that coul also exhibit antioxidant potential are mono- and oligosaccharides that depending on their structure, can possess reducing activity (Paulus

and Klockow 1996); fructose and glucose are examples found in *J. regia* leaves that show the mentioned activity.

Furthermore, α-Tocopherol, the most abundant isoform found in the studied leaves, is also the most biologically active form of the four tocopherols. It is the major soluble antioxidant found in plasma, red blood cells and tissues stratum corneum, which allows protecting the integrity of the lipid structures (Burton and Traber 1990; Pinnell 2003; Carocho and Ferreira 2013). Its role as free radical scavenger, may protect our body from various degenerative disorders, including cancer and cardiovascular disease (Fang et al., 2002).

A more complete characterization of phenolic compounds in walnut leaves was also provided in this study, as previous reports only described nine (Amaral et al., 2004) and ten (Pereira et al., 2007) compounds. Wichtl and Anton (1999) and Cheniany et al. (2013) reported the presence of other phenolic acids such as caffeic, ferulic, *p*-coumaric, *p*-hydroxyphenylacetic, gallic and salicylic acids, in leaves and microshoots of *J. regia*. Pereira et al. (2007) and Amaral et al. (2004) presented 3-*O*-caffeoylquinic acid and quercetin 3-galactoside as the major phenolic compounds present. As far as we know, this is the first time that the presence of procyanidins, taxifolin and myricetin derivatives was reported in walnut leaves. Furthermore there are no results of phenolic determination in decoctions, the most used procedure for walnut leaves in ethnopharmacology.

The characterization of phenolic compounds is quite important because these molecules have been described as having multiple biological effects including antioxidant, antitumoral, antibacterial and antimutagenic (Shui and Leong 2002). Some phenolic compounds have the ability to eliminate the products of lipid peroxidation, prevent oxidative damage of DNA and block ROS such as superoxide, hydrogen peroxide and

hydroxyl radicals (Kähkönen et al., 1999). Flavonoids are the most abundant phenolic compounds in plants (Mladenka et al., 2010) and have been related to different processes of health promotion for their antioxidant, anti-allergic, anticancer, antispasmodic, antiviral, antibacterial, anti-inflammatory and anti-estrogenic and hepatoprotective properties (Middleton et al., 2000; Zhang et al., 2005).

Overall, five organic acids, two monosaccharaides, two disaccharides and the four isoforms of tocopherols were identified in *J. regia* leaves, being malic acid, sucrose and α-tocopherol the most abundant compounds of each call either in dry plant material or in decoction. Furthermore, twenty-five phenolic compounds were identified and quantified: five phenolic acid derivatives (caffeoylquinic and *p*-coumaroylquinic acid derivatives), two dimers and one trimer of procyanidins, twelve flavonols (quercetin, myricetin and kaempferol derivatives), and five taxifolin *O*-pentoside isomers. 3-*O*-caffeoylquinic acids and quercetin *O*-pentoside were the main phenolic compounds in *J. regia* leaves. Methanol extract presented higher *in vitro* antioxidant and antitumor potential than decoction; both preparations did not show toxicity for non-tumor liver primary cells.

This study provides a more complete characterization of phenolic compounds in walnut leaves and, as far as we know, it is the first study reporting phenolic compounds composition of the decoction (widely used for topical applications), as also the presence of procyanidins, taxifolin derivatives, and tocopherols in *J. regia* leaves.

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Table 1. Organic acids, mono and oligosaccharides and tocopherols in lyophilized leaves of *Juglans regia* and in lyophilized decoction prepared from the former plant material (mean±SD).

	Leaves	Decoction
Organic acids (g/100 g, dw)		
Oxalic acid	0.93 ± 0.07^{a}	0.85 ± 0.00^{b}
Malic acid	1.04 ± 0.02^{a}	0.83 ± 0.01^{b}
Ascorbic acid	0.20 ± 0.01	nd
Shikimic acid	0.10 ± 0.00^{a}	0.06 ± 0.00^{b}
Citric acid	0.89 ± 0.01^{a}	0.71 ± 0.00^{b}
Total organic acids	3.16 ± 0.09^{a}	2.44 ± 0.01^{b}
Mono and oligosaccharides (g/100 g, dw)		
Fructose	nd	2.08 ± 0.03
Glucose	0.79 ± 0.01^{b}	3.47 ± 0.19^{a}
Sucrose	5.79 ± 0.35^{b}	10.10 ± 0.23^{a}
Trehalose	2.69 ± 0.05^{a}	2.12 ± 0.21^{a}
Total sugars	9.27 ± 0.31^{b}	17.17 ± 0.61^{a}
Tocopherols (mg/100 g, dw)		
α – tocopherol	199.99 ± 1.48^{a}	3.99 ± 0.01^{b}
β – tocoferol	2.63 ± 0.20^{a}	0.35 ± 0.01^{b}
γ – tocoferol	120.16 ± 5.87^{a}	1.24 ± 0.01^{b}
δ – tocoferol	67.56 ± 0.36^{a}	20.26 ± 0.11^{b}
Total tocopherols	282.20 ± 1.90^{a}	25.84 ± 0.10^{b}

nd-not detected. In each row different letters (a and b) mean significant (p<0.05) differences between the two samples (leaves and decoction); a corresponds to the highest value and b the lowest one.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and tentative identification of phenolic compounds in lyophilized leaves of *Juglans regia* and in lyophilized decoction prepared from the former plant material.

Peak	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	323	353	191(100),179(89),173(20),135(78)	3-*O*-Caffeoylquinic acid
2	280	577	451(34),425(55),407(92),289(75),287(16)	Procyanidin dimer
3	310	337	191(100),173(29),163(98),155(17),137(2)	3-*p*-Coumaroylquinic acid
4	331	353	191(76),179(97),173(100),135(96)	4-*O*-Caffeoylquinic acid
5	280	865	739(8),713(17), 695(9), 577(4), 575(6), 425(3),407(2), 289(100)	Procyanidin trimer
6	310	337	191(23), 173(100), 163(2), 155(10), 137(9)	*cis* 4-*p*-Coumaroylquinic acid
7	311	337	191(5), 173(100), 163(62), 155(14), 137(19)	*trans* 4-*p*-Coumaroylquinic acid
8	279	577	451(90),425(73),407(100),289(80),287(17)	Procyanidin dimer
9	291	435	303(20),285(100)	Taxifolin *O*-pentoside isomer
10	291	435	303(9),285(100)	Taxifolin *O*-pentoside isomer
11	353	479	317(100)	Myricetin 3-*O*-glucoside
12	354	493	331(100)	Laricitrin *O*-hexoside
13	292	435	303(41),285(100)	Taxifolin *O*-pentoside isomer
14	294	435	303(56),285(100)	Taxifolin *O*-pentoside isomer
15	356	449	317(100)	Myricetin *O*-pentoside
16	350	463	317(100)	Myricetin *O*-rhamnoside
17	294	435	303(35),285(100)	Taxifolin *O*-pentoside isomer
18	354	463	301(100)	Quercetin 3-*O*-glucoside
19	354	463	301(100)	Quercetin *O*-hexoside
20	354	433	301(100)	Quercetin *O*-pentoside
21	353	433	301(100)	Quercetin *O*-pentoside

22	350	447	301(100)	Quercetin *O*-rhamnoside
23	348	417	285(100)	Kaempferol *O*-pentoside
24	349	417	285(100)	Kaempferol *O*-pentoside
25	346	431	285(100)	Kaempferol *O*-rhamnoside

Table 3. Phenolic compounds quantification in methanol extract and decoction lyophilized leaves of *Juglans regia* (mean±SD).

Phenolic compounds	Methanol extract	Decoction
3-*O*-Caffeoylquinic acid	6.41 ± 0.17	5.08 ± 0.06
Procyanidin dimer	0.34 ± 0.01	0.30 ± 0.03
3-*p*-Coumaroylquinic acid	1.20 ± 0.04	0.92 ± 0.01
4-*O*-Caffeoylquinic acid	0.95 ± 0.05	1.76 ± 0.06
Procyanidin trimer	1.86 ± 0.08	1.61 ± 0.01
cis 4-*p*-Coumaroylquinic acid	0.22 ± 0.02	0.27 ± 0.00
trans 4-*p*-Coumaroylquinic acid	0.19 ± 0.01	0.27 ± 0.00
Procyanidin dimer	0.17 ± 0.01	0.06 ± 0.00
Taxifolin *O*-pentoside isomer	tr	tr
Taxifolin *O*-pentoside isomer	0.06 ± 0.00	0.03 ± 0.00
Myricetin 3-*O*-glucoside	0.13 ± 0.01	0.13 ± 0.00
Laricitrin O-hexoside	0.12 ± 0.01	0.11 ± 0.01
Taxifolin *O*-pentoside isomer	tr	0.25 ± 0.00
Taxifolin *O*-pentoside isomer	0.05 ± 0.00	0.06 ± 0.00
Myricetin *O*-pentoside	0.08 ± 0.01	0.07 ± 0.01
Myricetin *O*-rhamnoside	0.12 ± 0.00	0.10 ± 0.00
Taxifolin *O*-pentoside isomer	1.50 ± 0.01	0.72 ± 0.01
Quercetin 3-*O*-glucoside	2.37 ± 0.09	2.56 ± 0.03
Quercetin *O*-hexoside	0.98 ± 0.03	0.69 ± 0.06
Quercetin *O*-pentoside	0.38 ± 0.00	0.40 ± 0.00
Quercetin *O*-pentoside	5.04 ± 0.09	4.87 ± 0.01
Quecetin *O*-rhamnoside	1.77 ± 0.02	1.58 ± 0.03
Kaempferol *O*-pentoside	0.38 ± 0.01	0.32 ± 0.01
Kaempferol *O*-pentoside	0.74 ± 0.02	0.69 ± 0.03
Kaempferol *O*-rhamnoside	0.25 ± 0.01	0.22 ± 0.00
Total phenolic acids (mg/g, dw)	8.95 ± 0.27[a]	8.29 ± 0.01[b]
Total flavan-3-ols (mg/g, dw)	2.37 ± 0.08[a]	1.98 ± 0.02[b]
Total flavonols/flavanonols (mg/g, dw)	13.98 ± 0.20[a]	12.55 ± 0.10[b]
Total phenolic compounds (mg/g, dw)	25.30 ± 0.39[a]	22.82 ± 0.13[b]

tr- traces. In each row different letters (a and b) mean significant (p<0.05) differences betw
(methanol extract and decoction); a corresponds to the highest value and b the lowest one.

Table 4. Bioactivity of methanol extract and decoction prepared from lyophilized leaves of *Juglans regia* (mean±SD).

	Methanol extract	Decoction	Positive control
Antioxidant activity (EC_{50} values, µg/mL extract or decoction)			
DPPH scavenging activity	65.91 ± 6.47[b]	78.97 ± 5.61[a]	43.03 ± 1.71
Reducing power	75.87 ± 2.41[b]	83.71 ± 1.15[a]	29.62 ± 3.15
β-carotene bleaching inhibition	189.92 ± 12.61[b]	269.27 ± 2.33[a]	2.63 ± 0.14
TBARS inhibition	20.36 ± 0.82[b]	114.68 ± 16.41[a]	3.73 ± 0.74
Antitumor activity (GI_{50} values, µg/mL extract or decoction)	Methanol extract	Decoction	Positive control
MCF-7 (breast carcinoma)	209.28±8.83[b]	242.14±10.14[a]	0.91±0.04
NCI-H460 (non-small lung cancer)	> 400	> 400	1.42±0.00
HCT-15 (colon carcinoma)	215.58±0.58[b]	258.93±11.02[a]	1.91±0.06
HeLa (cervical carcinoma)	294.87±9.36	> 400	1.14±0.21
HepG2 (hepatocellular carcinoma)	240.67±7.13[b]	285.20±0.25[a]	3.22±0.67
Hepatotoxicity (GI_{50} value, µg/mL extract or decoction)			
PLP2	> 400	> 400	2.06±0.03

Trolox and ellipticine were used as positive controls for antioxidant and antitumor activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters (a and b) mean significant (p<0.05) differences between the two samples (methanol extract and decoction); a corresponds to the highest value and b the lowest one.

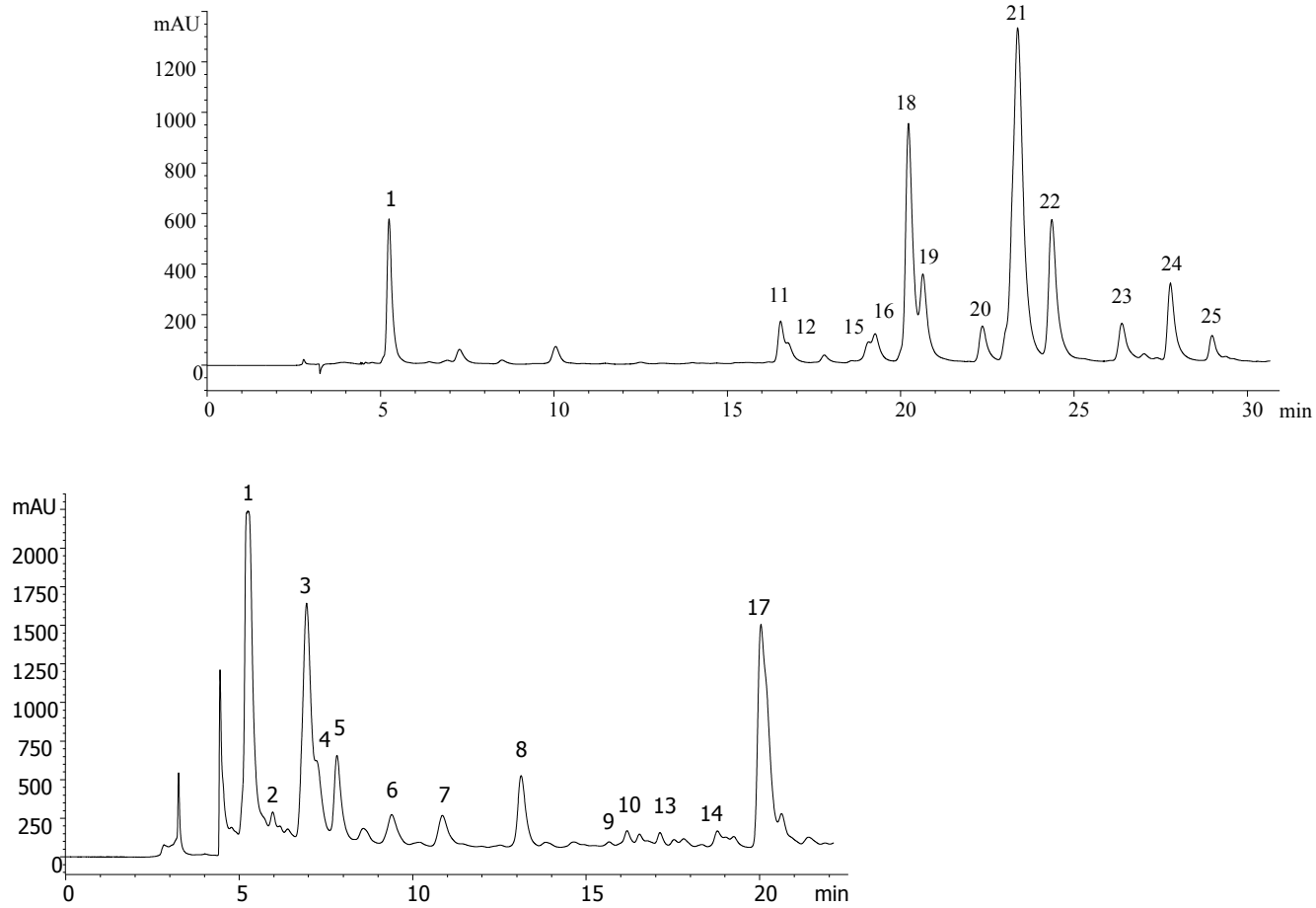

Figure 1. HPLC phenolic profile of the methanol extract prepared from *Juglans regia* lyophilized leaves, obtained at 370 nm (A) and 280 nm (B) for flavonoids and phenolic acids, respectively. The identification of the peaks 1-25 is presented in Table 2.