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Phenolic profile, antioxidant and antibacterial properties of *Juglans regia* L. (walnut) leaves from the Northeast of Portugal

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ABSTRACT

Juglans regia L. (walnut tree) is a recognized source of bioactive compounds with potential health benefits. In this work, hydroethanolic extracts of *J. regia* leaves were obtained by heat assisted extraction from different Portuguese samples in two phenological stages (green and yellow leaves) aiming to assess the impact of seasonal variations. The samples were compared regarding their phenolic composition and bioactivity. Seventeen phenolic compounds were identified by liquid chromatography combined with a diode array detector and electrospray ionization mass spectrometer (LC-DAD-ESI/MS^n): six phenolic acids, ten flavonoids and one tetralone derivative. The green leaves extracts presented a higher amount of total phenolic compounds (29.70 ± 0.03 mg/g extract) compared with the yellow leaves (23.26 ± 0.06 mg/g extract). In particular, yellow samples were richer in flavonoids (17.4 ± 0.2 mg/g extract; mainly quercetin-3-*O*-glucoside: 3.64 ± 0.01 mg/g extract), while the green ones presented higher phenolic acids content (16.7 ± 0.2 mg/g extract; mainly *trans* 3-*p*-coumaroylquinic acid: 6.9 ± 0.5 mg/g extract). Green leaves extract also presented higher antioxidant potential, achieving IC_{50} values around 32 ± 2 μg/mL and 26.8 ± 0.2 μg/mL for the oxidative haemolysis inhibition and the thiobarbituric acid reactive substances assays, respectively. Furthermore, only green leaves samples showed anti-inflammatory potential. The cytotoxic evaluations revealed similar anti-proliferative action of both extracts against the tumor cell lines tested. Also, an analogous anti-bacterial potential of the extracts was observed, with preferential action against Gram-positive clinical isolated bacteria, with lower minimum inhibitory concentration (MIC) values for *Enterococcus faecalis* and *Listeria monocytogenes* (MIC = 2.5 mg/mL). Therefore, the present study suggests the use of walnut leaves as a source of active ingredients without hepatotoxic effects to be used in different applications in the food or pharmaceutical areas.

1. Introduction

Medicinal plants have been used for centuries by different civilizations as therapeutic agents due to their preventive and curative properties (Tasneem et al., 2019). *Juglans regia* L. is spread across the globe, being walnut leaves widely used by the folk medicine as antiseptic, anti-inflammatory, antidiabetic, anti-helminthic and antidiarrheic agents. Furthermore, they are also traditionally used for the treatment of hyperhidrosis, ulcers and dandruff (Carvalho et al., 2010a; Cosmulescu et al., 2014). Moreover, *J. regia* leaves extract is an inventoried ingredient for cosmetic products, with reported astringent, soothing, cleansing, abrasive, bulking, skin conditioning, and masking functions (European Commission, 2006).

Many studies reported the bioactivity of walnut leaves by several *in vitro* (cell cultures) and *in vivo* (rats/mice) assays regarding their anti-diabetic (Asgary et al., 2008; Hosseini et al., 2014; Javidanpour et al., 2012; Mohammadi et al., 2012; Mollica et al., 2017; Pitschmann et al., 2014; Teimori et al., 2009), anti-inflammatory (Erdemoglu et al., 2003; Hosseinzadeh et al., 2011), anti-proliferative (Salimi et al., 2014; Santos et al., 2013), anti-septic/biofilm/bacterial (Dolatabadi et al., 2018; Pereira et al., 2007), hepatoprotective (Eidi et al., 2013), sedative and analgesic (Erdemoglu et al., 2003; Gîrzu et al., 1998; Hosseinzadeh et al., 2011), vasorelaxant (Perusquía et al., 1995), and neuroprotective (Orhan et al., 2011) properties. The study performed by Wang et al.

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(2015) evaluated the anti-hyperuricemia potential of *J. regia* leaves by determining the xanthine oxidase inhibitory activity. Moreover, the extensive evidences on the anti-diabetic activity of walnut leaves encouraged Abdoli et al. (2017) to carry out a human trial using walnut leaves-based preparations in Type II diabetic patients, obtaining positive results for the reduction of the glycemic index and HbA1c (glycated hemoglobin) values.

The secondary metabolites of *J. regia* leaves have been related to its therapeutic effects, such compounds including phenolic acids, flavonoids, organic acids, tocopherols, triterpenic acids, terpenes, terpenoids, tetralone derivatives, megastigmane derivatives, hydroxy-1,4-naphthoquinone (juglone) derivatives, among others (Cosmulescu et al., 2011; Forino et al., 2016; Panth et al., 2016; Rather et al., 2012; Salimi et al., 2014; Santos et al., 2013; Schwindl and Kraus, 2017). Phenolic compounds are the main fraction found in the plant material. They are natural occurring antioxidants, largely recognized because of their health benefits due to their radical scavenging activity, reducing oxidative stress, which is the main cause of several disorders. In fact, these exogenous antioxidant source play an important role for the oxidative stress balance, providing additional antioxidant potential for living organisms, since there is an insufficient action of our endogenous antioxidant system (Carocho and Ferreira, 2013). In this context, the inclusion of walnut leaves preparations (food and supplements) in our diet could act as a preventive medicine with several health benefits (Pires et al., 2018).

The use of *J. regia* leaves goes back from centuries, and in Portugal, especially in the northeast region (Bragança) they are traditionally used as an external antiseptic, anti-inflammatory and antidiabetic agent (Carvalho et al., 2010a). In this context, the study of walnut leaves from this region, characterizing their main constituents and potential bioactivities, will contribute to support their traditional medicinal uses with scientific evidences, valorizing this species and its origin. In this work, Portuguese walnut leaves in two phenological stages (green and yellow leaves) were evaluated aiming to assess the impact of seasonal variations on the phenolic compounds composition and the bioactive potential of the extracts obtained. The seasonal variation of the phytochemical profile of this plant material has been previously established by Cosmulescu et al. (2014) using Romanian samples. Along that study, samples were collected during each fortnight from 1 st of June to 15th of August 2012 and were only compared in terms of their phytochemical abundancy (ellagic acid, rutin, myricetin and juglone).

In order to achieve the aims proposed in this study, a hydroethanolic extract was evaluated regarding its antioxidant, cytotoxic and antibacterial activities, and the phenolic profile was obtained by LC-DAD-ESI/MS^n.

2. Material and methods

2.1. Plant material and extract preparation

Juglans regia L. (walnut) leaves were collected in Bragança, Northeast of Portugal, in May (green leaves) and November (yellow leaves) of 2018. The samples were dried until constant weight in an incubator at 35 °C. Then, the plant material was ground to approximately 40 mesh, and the homogeneous samples were stored in a desiccator protected from light.

The hydroethanolic extract was obtained by heat assisted extraction, using the previous optimized conditions for the extraction of phenolic compounds reported by the authors (Vieira et al., 2017): aqueous ethanolic solution (50.4%, v/v; 30 g/mL) at 61.3 °C for 116 min. After filtration (Whatman no. 4 filter), the solvent was first evaporated at 40 °C, under reduced pressure, in a rotary evaporator (Büchi R-210, Flawil, Switzerland) and the residual solvent was removed in a freeze drier (Telstar Cryodos-80, Terrassa, Barcelona).

2.2. Phenolic compounds characterization

The dry extracts were re-suspended at a concentration of 10 mg/mL using aqueous ethanol (20%, v/v) and filtered (0.2 µm disposable LC filter disk, 30 mm, nylon). Afterwards, the phenolic profile of walnut leaves was found by liquid chromatography with diode-array detector (280, 330, and 370 nm wavelengths) coupled to an electrospray ionization mass spectrometry operating in negative mode (Dionex Ultimate 3000 UPLC and Linear Ion Trap LTQ XL, Thermo Scientific, San Jose, CA, USA), as previously described by the authors (Bessada et al., 2016). The phenolic compounds were identified according to their chromatographic characteristics by comparison to those obtained with standard compounds and with literature. Calibration curves of appropriate standards were obtained in the range 200-5 µg/mL, for the quantitative analysis. The results were expressed in mg per g of extract (mg/g).

2.3. In vitro antioxidant assays

The lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated through the thiobarbituric acid reactive substances (TBARS) assay. The ethanolic extracts were prepared at a concentration of 10 mg/mL in distilled water and further successive dilutions were performed to obtain the working concentrations ranging from 400 to 6.25 µg/mL. The decrease of the TBARS production was performed by measuring the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) at 532 nm. The methodology followed was previously reported by Barreira et al. (2013) with slight modifications. Briefly, dissected porcine brain was homogenized with Tris−HCl buffer (20 mM, pH 7.4) at a ratio of 1:2 (w/v). The homogenate was centrifuged (3000 rpm for 10 min) and an aliquot (100 µL) of the supernatant was incubated with the stock solutions of extract (200 µL), $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37 °C for 60 min. Then, the reaction was stopped by adding trichloroacetic acid (28%, w/v, 500 µL), thiobarbituric acid (2%, w/v, 380 µL), and heating the mixture at 80 °C for 20 min. After that, the mixtures were centrifuged (3000 rmp) for 10 min and the absorbances of the supernatants were measured. The inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, being A and B the absorbance of the control and the sample solution, respectively. The EC_{50} value defines the concentration providing 50% of antioxidant activity, and was calculated by interpolation from the graph of TBARS formation inhibition percentage against sample concentration.

The anti-haemolytic activity of walnut leaves samples was evaluated by the oxidative haemolysis inhibition assay (OxHLIA). The adopted methodology was previously described by Takebayashi et al. (2012); however, slight modifications were implemented. Erythrocytes were obtained from sheep blood collection. Healthy animals were selected for the purpose and the full blood was centrifuged at 1000*g* at 10 °C for 5 min. Then, plasma and buffy coats were discarded and erythrocytes were once washed with NaCl (150 nM) and three times with phosphate buffer solution (PBS, pH 7.4) (Evans et al., 2013), being the erythrocyte fraction resuspended in PBS (2.8%, v/v). The extract was dissolved in PBS (pH 7.4) to obtain a stock solution of 10 mg/mL, and successive solutions were prepared, ranging from 400 to 6.25 µg/mL. About 400 µL of each solution was transferred for a 48 well plate (duplicates). Water and PBS (pH 7.4) were used as controls of haemolysis (plasmolytic and isotonic media, respectively) and Trolox as positive control. Then, the suspension of erythrocytes (200 µL) was mixed with the previous solutions/controls and the 48-well plate was incubated for 10 min at 37.5 °C with agitation. After that, a solution of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS, 200 µL) was added and the absorbance of the mixtures was measured at 690 nm (BioTek ELx800). The microplate was incubated under the same conditions and absorbances were measured every 10 min during approximately 400 min. The percentage of the erythrocyte population that remained intact (*P*) was calculated using the following equation:

$$P\ (\%) = (S_t - CH_0/S_0 - CH_0) \times 100$$

where S_t and S_0 correspond to the absorbance of the sample at time t and 0 min, respectively, and CH_0 is the absorbance of the complete haemolysis at 0 min. The results were, then, expressed as delayed time of haemolysis (Δt), which was calculated according to the following equation:

$$\Delta t\ (\text{min}) = Ht_{50}\ (\text{sample}) - Ht_{50}\ (\text{control})$$

where Ht_{50} is the haemolytic time (minutes) obtained by graphical representation of the haemolysis curve of each sample concentration. Then, the Δt values were correlated to the antioxidant sample concentrations promoting the desirable Δt haemolysis delay (Takebayashi et al., 2012). The results were expressed as IC_{50} values (μg/mL) at 60 min, meaning the extract concentration required to keep 50% of the erythrocytes intact for 60 min.

2.4. Anti-inflammatory activity

The walnut leaves extracts were re-dissolved in water at a concentration of 8 mg/mL and then diluted in the range of 400 to 6.25 μg/mL using supplemented DMEM (10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10% MEM non-essential amino acids solution, 100 U/mL penicillin and 100 μg/mL streptomycin). A mouse macrophage-like cell line RAW264.7 was used in this study and maintained at 37 °C under 5% of CO_2. Briefly, a suspension of cells (5×10^5 cells/mL) was allowed to sediment on the 96-well plate (1.5×10^5 cells/well) overnight. Then, cells were treated with the different concentrations of extract for 60 min. Thereafter, the cells were stimulated with LPS (1 μm g/mL, in supplemented DMEM) during 18 h. Besides the GRS, the kit contains sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride (NED) and nitrite solutions. The cell culture supernatant (100 L) was transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, 5 and 10 min each, at room temperature. The nitrite produced was determined by measuring the optical density at 515 nm, using the ELx800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) following a methodology previously reported (Correa et al. 2015). The results were expressed in EC_{50} values (sample concentration providing 50% of inhibition of NO production) and dexamethasone was used as a positive control, while in negative controls no LPS was added.

2.5. Cytotoxic assays

The extracts were re-dissolved in water at 8 mg/mL concentration and further diluted in the range 400 to 6.25 μg/mL. The four human tumor cell lines assessed (MCF-7, breast adenocarcinoma; NCI-H460, non-small cell lung cancer; HeLa, cervical carcinoma; and HepG2, hepatocellular carcinoma) were maintained as adherent cell cultures in RPMI-1640 medium containing heat-inactivated fetal bovine serum (FBS, 10%), glutamine (2 mM), MEM non-essential amino acids solution (10%), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C, in a humidified air incubator with CO_2 (5%). For evaluation of the cytotoxicity in non-tumor cells, a cell culture designated as PLP2, was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by Abreu et al. (2011). The cell proliferation of each cell culture was monitored every two to three days using a phase contrast microscope and, before confluence was reached, the cells were subcultured in the supplemented RPMI-1640 medium mentioned above. Then, the cells were distributed in 96-well-plates (190 μL) according to their appropriate density (1.0×10^4 cells/well for all cell cultures) and tested with the solutions of extracts (10 μL) during 48 h. Ellipticine was used as positive control. Hereinafter, the adherent cells were fixed by adding trichloroacetic acid (TCA, 10%, 100 μL) and incubation at 4 °C for 60 min. After the incubation period, the TCA was removed and plates were washed (distilled water, three times) and dried. The sulforhodamine B solution (SRB, 0.1% in 0.1% acetic acid, 100 μL) was then added and allowed to bound at room temperature for 30 min. The excess of SRB was removed by washing the 96-well plates with acetic acid (1%, three times) and allowed to dry. The bound SRB was solubilized by adding Tris buffer solution (10 mM, 200 μL) and the absorbances were measured at 540 nm using the microplate reader mentioned above. The results were expressed in GI_{50} values (concentration that inhibited 50% of the cell proliferation), being the negative control constituted by each suspension of cells (Barros et al., 2013).

2.6. Antibacterial activity

The antimicrobial potential of the walnut husks was assessed for bacterial strains obtained from patients hospitalized in different departments at the Northeastern local health unit (Bragança, Portugal) and Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli,* isolated from urine; *Proteus mirabilis,* isolated from wound exudate; *Klebsiella pneumoniae,* isolated from urine; *Pseudomonas aeruginosa,* isolated from expectoration; and *Morganella morganii,* isolated from urine), three Gram-positive bacteria (*Enterococcus faecalis,* isolated from urine; *Listeria monocytogenes,* isolated from cerebrospinal fluid; methicillin-resistant *Staphylococcus aureus* (*MRSA*), isolated from expectoration) were tested. All these microorganisms were incubated at 37 °C in appropriate fresh medium for 24 h before analysis to maintain the exponential growth phase. The minimum inhibitory concentration (MIC) determinations on all bacteria were performed using a colorimetric assay described by Pires et al. (2018).

The extracts were dissolved in a mixture of dimethyl sulfoxide (DMSO) + Mueller-Hinton Broth (MHB; *E. coli, P. mirabilis, K. pneumoniae, P. aeruginosa, M. morganii* and MRSA)/ Tryptic Soy Broth (TSB, *E. faecalis* and *L. monocytogenes*) (5 + 95%, v/v) to give a final concentration of 100 mg/mL for the stock solution and successive dilutions were further carried out ranging from 20 to 1.25 mg/mL. Then, 90 μL of each concentration and 10 μL of each inoculum (standardized at 1.5×10^8 Colony Forming Unit (CFU)/mL) were pipetted (duplicates) for a 96-well microplate. Three negative controls were prepared (1: MHB/ TSB; 2: extract; 3: medium + antibiotic + bacteria), whereas, the positive control was prepared with MHB/TSB and each inoculum. The Ampicillin and Imipenem antibiotics were used as negative control for all Gram-negative bacteria tested and *Listeria monocytogenes*, while Ampicillin and Vancomycin were selected for *Enterococcus faecalis* and *MRSA*. The microplates were covered and incubated at 37 °C for 24 h. The MIC of samples was detected following addition of *p*-iodonitrotetrazolium chloride (INT) (0.2 mg/mL, 40 μL) and further incubation at 37 °C for 30 min. MIC was defined as the lowest concentration that inhibits the bacterial growth, determined visually by change of the coloration from yellow to pink (viable microorganism) as previously described by Kuete et al. (2011). To determine the minimum bactericidal concentration (MBC), 10 μL of liquid from each well that showed no change in colour was inoculated on Blood agar solid medium (7% sheep blood) and incubated at 37 °C for 24 h. The MBC was determined according to the lowest concentration that yielded no growth. Meanwhile, MBC was established as the lowest concentration required to kill bacteria.

2.7. Statistical analysis

Triplicates of green and yellow samples were assayed and three repetitions of each methodology were performed, being the results expressed as mean values and standard deviations (SD). The significant differences between the two samples were established by applying a Student´s *t*-test, with $p = 0.05$ (SPSS v. 23.0 program).

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification, and quantification estimation (mean ± SD) of the tentatively identified phenolic compounds in green and yellow leaves extracts of *J. regia*.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Green leaves(mg/g extract)	Yellow leaves (mg/g extract)	*t*-Students test *p*-value
1	4.48	324	341	179(100)	Caffeic acid hexoside[a]	1.80 ± 0.08	0.44 ± 0.01	< 0.001
2	5.14	324	355	191(100),179(46),173(7),161(5),135(11)	3-*O*-Caffeoylquinic acid[a]	3.5 ± 0.4	0.191 ± 0.005	< 0.001
3	5.92	312	337	191(12),173(6),163(100),155(5),119(6)	*cis* 3-*p*-Coumaroylquinic acid[a]	2.34 ± 0.08	0.99 ± 0.01	< 0.001
4	6.34	312	337	191(19),163(100),155(5),119(6)	*trans* 3-*p*-Coumaroylquinic acid[a]	6.9 ± 0.5	2.87 ± 0.09	< 0.001
5	8.46	310	339	159(100),115(52)	Dihydroxytetralone hexoside[b,c]	nq	nq	–
6	9.28	320	355	193(100),175(20)	Ferulic acid hexoside[d]	0.73 ± 0.06	0.46 ± 0.01	< 0.001
7	9.85	312	325	163(100)	*p*-Coumaric acid hexoside[a]	1.43 ± 0.03	0.91 ± 0.03	< 0.001
8	15.3	350	479	317(100)	Myricetin-3-*O*-glucoside[d,f]	0.97 ± 0.05	0.87 ± 0.01	0.010
9	17.87	350	463	317(100)	Myricetin-*O*-rhamnoside[f]	0.78 ± 0.01	0.83 ± 0.03	0.038
10	18.73	353	463	301(100)	Quercetin-3-*O*-glucoside[d,f]	2.79 ± 0.02	3.64 ± 0.01	< 0.001
11	19.2	353	463	301(100)	Quercetin-*O*-hexoside[f]	2.28 ± 0.2	2.75 ± 0.03	0.005
12	20.75	350	433	301(100)	Quercetin-*O*-pentoside[f]	0.67 ± 0.02	0.857 ± 0.005	< 0.001
13	21.47	347	447	285(100)	Kaempferol-3-*O*-glucoside[d]	1.12 ± 0.01	1.27 ± 0.02	< 0.001
14	21.8	351	433	301(100)	Quercetin-*O*-pentoside[f]	1.43 ± 0.02	3.21 ± 0.03	< 0.001
15	22.78	350	447	301(100)	Quercetin-*O*-rhamnoside[f]	1.6 ± 0.1	2.13 ± 0.05	0.001
16	24.62	348	417	285(100)	Kaempferol-O-pentoside[f]	0.721 ± 0.002	1.03 ± 0.04	< 0.001
17	28.86	340	489	447(33),301(100)	Acetylquercetin *O*-rhamnoside[d]	0.627 ± 0.005	0.81 ± 0.01	< 0.001
					Total phenolic acids	16.7 ± 0.2	5.9 ± 0.1	< 0.001
					Total flavonoids	13.0 ± 0.2	17.4 ± 0.2	< 0.001
					Total phenolic compounds	29.70 ± 0.03	23.26 ± 0.06	< 0.001

Calibration curves: Peaks 1 and 2: caffeic acid (y = 388345x + 406369; r^2 = 0.994; LOD = 0.78 μg/mL; LOQ = 1.97 μg/mL); peaks 3, 4 and 7: *p*-coumaric acid (y = 301950x + 6966.7; r^2 = 0.999; LOD = 0.68 μg/mL; LOQ = 1.61 μg/mL); peak 6: ferulic acid (y = 63326x – 185462; r^2 = 0.999 LOD = 0.20 μg/mL; 1.01 μg/mL); peaks 8 to 17: quercetin 3-*O*-glucoside (y = 34843x – 160173; r^2 = 0.9998; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL).
References applied for the tentative identification: a – Clifford et al. (2003), Clifford et al. (2005); b –Wang et al. (2017), c –Jin-Hai et al. (2018); d- standard, DAD and MS fragmentation pattern; f – Santos et al. (2013).

3. Results and discussion

3.1. Composition in phenolic compounds

The phenolic composition of the extracts of *J. regia* leaves was assessed for samples in two phenological stages (green and yellow samples, collected in May and November, respectively). The identified molecules and their quantification are presented in Table 1. In this context, phenolic compounds were tentatively identified according to their retention time (*Rt*), maximum absorbance wavelength (λ_{max}), pseudomolecular ion ([M − H]–), and respective fragmentation pattern (MS^2). For both samples, it was possible to identify seventeen phenolic compounds: six phenolic acids (*p*-hydroxycinnamic acid derivatives), ten flavonoids (flavonols) and one tetralone derivative (Fig. 1).

Regarding the six phenolic acids identified in *J. regia*, peak 1 ([M − H]– at *m/z* 341), 6 ([M − H]– at *m/z* 355), and 7 ([M − H]– at *m/z* 325) were tentatively identified as caffeic, ferulic, and *p*-coumaric acid hexosides, based on the signal MS^2 fragment at *m/z* 179 [caffeic acid – H]–, 193 [ferulic acid – H]–, and 163 [*p*-coumaric acid – H]–, respectively, revealing the loss of a hexosyl moiety (−162 u). The first two compounds were previously identified byGawlik-Dziki et al. (2014), however, to the best of our knowledge, ferulic acid hexoside has not been previously identified in *J. regia* leaves, hence the free forms of these phenolic acids have also been previously identified (Wichtl and Anton, 1999). Peak 2 ([M − H]– at *m/z* 353) and peaks 3 and 4 ([M − H]– at *m/z* 337) were assigned as *trans* 3-*O*-caffeoylquinic acid and *cis* and *trans* 3-*p*-coumaroylquinic acid, respectively. These assumptions were taken into account, due to the hierarchical fragmentation pattern described by Clifford et al. (2003), but also by using literature data described by other authors, that identified these compounds in *J. regia* leaves (Amaral et al., 2004; Santos et al., 2013; Wichtl and Anton, 1999).

Peak 5 ([M−H]– at *m/z* 353) belongs to a different group of phenolic molecules, the tetralone derivatives. This type of molecules was already reported in *Juglans* species. The fragmentation yielded the ion at *m/z* 159 ([M−H]– - H_2O – 180, loss of a hexosyl unit and a neutral loss of water) corresponding to the dihydroxytetralone. This molecule frequently occurs in branches, fruit and pericarps/husks of *J. mandshurica* samples (Bi et al., 2016; Gawlik-Dziki et al., 2014; Jin-Hai et al., 2018; Liu et al., 2004; Wang et al., 2017; Zhou et al., 2015) and *J. sigillata* pericarps (Liu et al., 2010). To the best of our knowledge, it is the first time that the presence of dihydroxytetralone hexoside is reported as a phenolic constituent in *J. regia* leaves. Thus, a similar fragmentation pattern ([M−H]– at *m/z* 339, with a fragment at 159, UV–vis maximum absorption, 258, 313 nm) was previously found by Gawlik-Dziki et al. (2014) in *J. regia* leaves and pericarps. Nevertheless, the authors stated the molecule as an unknown phenolic compound (Gawlik-Dziki et al., 2014).

The last group of molecules were identified as flavonol glycoside derivatives, such as myricetin, quercetin and kaempferol derivatives. Compounds 8 (myricetin-3-*O*-glucoside), 10 (quercetin-3-*O*-glucoside), and 13 (kaempferol-3-*O*-glucoside) were positively identified by comparison with the corresponding commercial standards. The remaining compounds (peaks 9, 11, 12, and 14–17) were identified as quercetin (λ_{max} = 350 nm; MS^2 fragment at *m/z* 301), kaempferol (λ_{max} = 348 nm; MS^2 fragment at *m/z* 285), and myricetin (λ_{max} = 350 nm, MS^2 fragment at *m/z* 317), showing a MS^2 fragmentation pattern with different losses of hexosyl (−162 u), pentosyl (−132 u), and rhamnosyl (−146 u) residues, being assigned as myricetin-*O*-rhamnoside (peak 9), quercetin-*O*-hexoside (peak 11), quercetin-*O*-pentoside (peaks 12 and 14), quercetin-*O*-rhamnoside (peak 15), and kaempferol-*O*-pentoside (peak 16). The majority of the identified compounds have been previously identified by Santos et al. (2013). Finally, peak 17 ([M−H]– at *m/z* 489) presented 42 u (acetyl moiety) higher then compound 15 (quercetin-*O*-rhamnoside), being therefore tentatively identified as acetylquercetin-*O*-rhamnoside. In

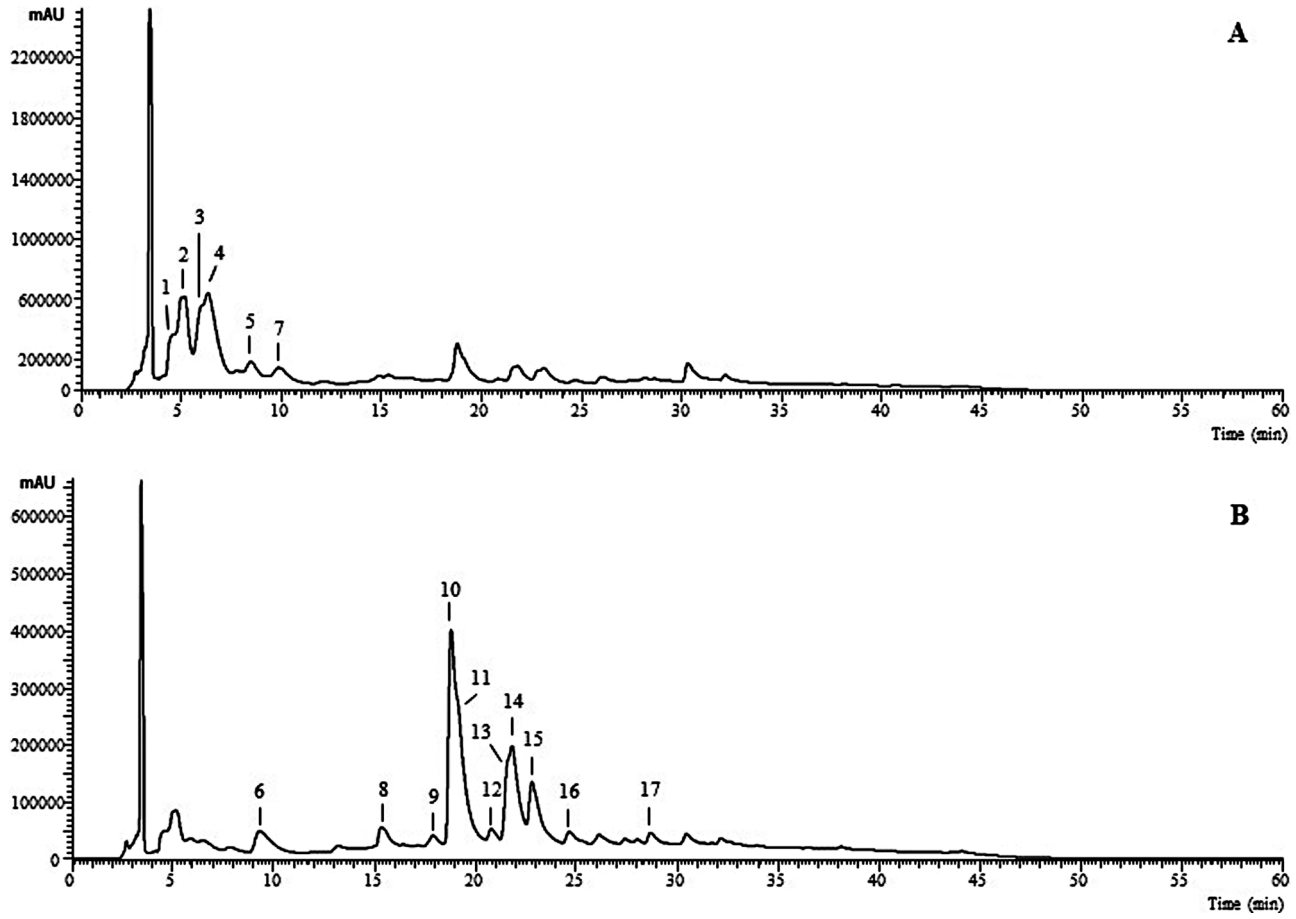

Fig. 1. HPLC phenolic profile of *J. regia* L. leaves. Graphical representation of walnut green sample, recorded at 280 nm (A) and 370 nm (B). The peaks identification and quantification are presented in Table 1.

fact, the occurrence of acetyl-quercetin derivatives (and kaempferol as well) in *J. regia* leaves has been also previously described by Gawlik-Dziki et al. (2014).

Regarding the general profile of the samples (Table 1), green leaves were mostly composed by phenolic acids (green samples: 16.7 ± 0.2 mg/g extract; yellow samples: 5.9 ± 0.1 mg/g extract), while the yellow ones by flavonoids (green samples: 13.0 ± 0.2 mg/g extract; yellow samples: 17.4 ± 0.2 mg/g extract). Overall, the green leaves of *J. regia* showed a significant higher amount of total phenolic compounds (29.70 ± 0.03 mg/g extract) in comparison to the yellow samples (23.26 ± 0.06 mg/g extract). As previous referred, phenolic acids found in walnut leaves were essentially hydroxycinnamic acids derivatives, being the *trans* 3-*p*-coumaroylquinic acid the most abundant phenolic acid for both samples (6.9 ± 0.5 and 2.87 ± 0.09 mg/g extract, for green and yellow leaves, respectively). The presence of hydroxycinnamic acid derivatives in *J. regia* leaves is common, regardless of the cultivar or their maturity stage, raising the importance of walnut leaves as a source of this type of bioactive compounds (Amaral et al., 2004; Gawlik-Dziki et al., 2014; Pereira et al., 2007; Pitschmann et al., 2014; Santos et al., 2013; Vieira et al., 2018, 2017; Wojdylo et al., 2007). Regarding the relative abundancy of 3-*p*-coumaroylquinic acid isomers as the main phenolic acids in walnut leaves samples, that is not frequent; in fact, caffeoylquinic acids have been found as the main phenolic acids. In this context, 3-*O*-caffeoylquinic acid was reported as the most abundant phenolic acid in several studies (Amaral et al., 2004; Pereira et al., 2007; Santos et al., 2013; Vieira et al., 2017, 2018), while 5-*O*-caffeoylquinic acid was found in higher concentrations in Chinese samples (Zhao et al., 2014). On the other hand, ellagic acid was the main phenolic acid for Romanian samples (Pedro cultivar: 1.345 mg/g of plant dw), and caffeic acid for Polish samples (1.48 mg/g of plant dw) (Wojdylo et al., 2007). Despite the atypical relative abundancy of 3-*p*-coumaroylquinic acid isomers, it is relevant to mention that this compound frequently occurs in walnut leaves (Gawlik-Dziki et al., 2014; Pitschmann et al., 2014). Regarding the amounts found in Portuguese samples, Amaral et al. (2004) reported concentrations about 0.38 to 2.59 mg/g of plant dw and Pereira et al. (2007) from 4.69 to 5.99 mg/g of plant dw, depending on the *J. regia* cultivar, while Santos et al. (2013) obtained from 0.92 to 1.2 mg/g of plant dw. Finally, Zhao et al. (2014) quantified lower values, reaching 0.00672 mg/g of plant dw in Chinese walnut leaves.

In general, the amount of the individual flavonoids in *J. regia* leaves was significantly higher for the yellow samples. Quercetin-3-*O*-glucoside was the most abundant flavonoid for both samples, with yields of 3.64 ± 0.01 and 2.79 ± 0.02 mg/g of extract in the yellow and green leaves extracts, respectively. Quercetin-*O*-pentoside (peak 14) was the second main flavonoid in *J. regia* yellow leaves (3.21 ± 0.03 mg/g extract) and the third for the green samples (1.43 ± 0.02 mg/g extract). This tendency was also found by other authors (Santos et al., 2013; Vieira et al., 2017, 2018). In this regard, Santos et al. (2013) found quercetin-3-*O*-glucoside concentrations around 2.37 to 2.56 mg/g of plant dw, and, 0.38 to 5.04 mg/g of plant dw for quercetin-*O*-pentoside for the methanolic extract and decoction, respectively. Higher amounts were found in Spanish hydroethanolic extracts by Vieira et al. (2017), achieving 13.8 to 14.2 mg/g of plant dw for quercetin-3-*O*-glucoside and 11.7 to 14.0 mg/g of plant dw, depending on the extraction methodology. Later, the authors increased the extraction yields, reaching 16.0 and 14.8 mg/g of plant dw for each quercetin derivative by applying choline chloride based-deep eutectic

Table 2
Antioxidant, anti-inflammatory, and cytotoxic activities of *J. regia* leaves extracts and positive controls (mean ± SD).

	J. regia		*t*-Students test *p*-value
	Green leaves	Yellow leaves	
Antioxidant activity (IC_{50} values μg/mL)			
OxHLIA, Δt = 60 min	32 ± 2	51 ± 2	< 0.001
TBARS formation inhibition	26.8 ± 0.2	48.5 ± 0.5	< 0.001
Anti-inflammatory activity (EC_{50} values μg/mL)			
Nitric oxide (NO) production	319 ± 13	> 400	–
Cytotoxicity (GI_{50} μg/mL)			
Tumor cell lines			
MCF-7 (breast carcinoma)	268 ± 12	305 ± 5	0.143
NCI-H460 (non-small lung carcinoma)	288 ± 5	328 ± 5	< 0.001
HeLa (cervical carcinoma)	280 ± 8	312 ± 2	0.002
HepG2 (hepatocelular carcinoma)	252 ± 10	260 ± 5	0.001
Non-tumor cells			
PLP2 (porcine liver primary culture)	> 400	> 400	–

IC_{50}: extract concentration corresponding to a 50% of antioxidant activity. Trolox IC_{50} values: 19.6 ± 0.6 μg/mL (OxHLIA); 5.8 ± 0.6 μg/mL μg/mL (TBARS inhibition). GI_{50} values correspond to the sample concentration responsible for 50% inhibition of growth in a cell culture. GI_{50} values for Ellipticine (positive control): MCF-7: 1.21 ± 0.02 μg/mL; NCI-H460: 0.91 ± 0.11 μg/mL; HeLa: 1.03 ± 0.09 μg/mL; HepG2: 1.1 ± 0.09 μg/mL; PLP2: 2.29 ± 0.18 μg/mL. EC_{50} values correspond to the sample concentration responsible for 50% inhibition of NO production. EC_{50} values for dexamethasone (positive control): 16 ± 1 μg/mL.

solvents. Moreover, quercetin-3-*O*-galactoside was reported to be the main flavonoid in Portuguese samples, and Amaral et al. (2004) obtained concentrations from 5.15 to 14.90 mg/g of plant dw, while Pereira et al. (2007) found 15.72 to 21.68 mg/g of plant dw, depending on the *J. regia* cultivar. The abundancy in flavonols, specially quercetin derivatives, is in good agreement with literature data (Amaral et al., 2004; Cosmulescu et al., 2014; Pereira et al., 2007; Pitschmann et al., 2014; Santos et al., 2013; Vieira et al., 2018, 2017; Zhao et al., 2014). In addition, some walnut leaves also revealed the presence of other flavonoids, such as taxifolin (flavanonol), epicatechin, laricitrin, and procyanidin derivatives (Santos et al., 2013; Zhao et al., 2014).

3.2. Bioactivity of the hydroethanolic extracts

The antioxidant potential for the two phenological stages of the *J. regia* leaves was assessed by two *in vitro* approaches: the inhibition of the lipid peroxidation by the TBARS assay and the anti-haemolytic activity through the OxHLIA assay. The obtained results are presented in Table 2.

The kinetic behavior of the OxHLIA was similar to the previous reported by Lockowandt et al. (2019). The heamolysis profile of the assay using the green leaves extract is depicted in Fig. 2, representing the dose-dependent effect to maintain the erythrocyte integrity. As the graphical representation suggests, the heamolysis can be delayed by increasing the extract concentration.

The green leaves extract showed the highest antioxidant potential for both TBARS and OxHLIA assays, with lower IC_{50} values than the yellow leaves extract. Regarding the OxHLIA assay, the protection of a half of the erythrocyte population from the heamolysis was achieved by using 32 ± 2 and 51 ± 2 μg/mL concentrations, for green and yellow leaves extracts, respectively. The same tendency was observed for the TBARS assay, with slightly lower IC_{50} values: 26.8 ± 0.2 and 48.5 ± 0.5 μg/mL, for green and yellow samples extracts, respectively. The results of the TBARS assay for the green leaves extract is in good agreement with the previous report of *J. regia* leaves methanolic extract by Santos et al. (2013), presenting a IC_{50} of 20.36 ± 0.82 μg/mL. Moreover, the antioxidant potential of the walnut leaf essential oil was also evaluated, reaching 50% of TBARS inhibition formation with about 60 μg/mL (Rather et al., 2012).

The anti-haemolytic activity of walnut leaves was also evaluated by Carvalho et al. (2010b), achieving IC_{50} values around 60 μg/mL after 3 h of reaction ($\Delta t \approx 60$ min). Regarding the IC_{50} values for the positive control (Trolox), they were lower than the ones obtained using the extracts (19.6 ± 0.6 and 5.8 ± 0.6 μg/mL, for OxHLIA and TBARS assays, respectively). That is, Trolox shows higher anti-haemolytic and anti-peroxidation activities than the natural extracts. However, Trolox is a pure antioxidant molecule while the extracts are composed by several compounds with different bioactive actions. The extracts showed a lower IC_{50} value for the TBARS assay compared with OxHLIA, being closer to the positive control in the latter case. In fact, the IC_{50} values of the extracts are 1.6–2.6 times higher than Trolox ([sample]/[Trolox]) in the OxHLIA assay, compared with the 4.2–8.4 times difference found for the TBARS assay.

The green leaves extract was the only sample showing inhibition of the NO production up to the maximum concentrations tested 400 μg/mL (Table 2). The concentration with the ability to inhibit 50% of the nitric oxide production by the macrophages was found to be 319 ± 3 μg/mL. To the best of our knowledge, it is the first time that the anti-inflammatory activity evaluation though an immune cells approach is made for this plant material. Nevertheless, the NO inhibition production by walnut leaves extracts was previous studied as antioxidant indicators. In this context, IC_{50} values of 1.95 ± 0.29 μg/mL were obtained by measuring the NO generated by the NOC-5 (nitric oxide amine-complex donor) decomposition. Moreover, Orhan et al. (2011) quantified the radical scavenging activity of a series of extracts obtained from different solvents (dichloromethane, ethyl acetate, acetone, methanol and water), and the leaf water extract at 2000 μg/mL was able to inhibit about 24% of the NO production. On the other hand, some authors used mice models to perform the anti-inflammatory evaluations. The carrageenan-induced paw edema in mice assay was adopted by Erdemoglu et al. (2003), and showed potent anti-inflammatory activity for the ethanolic extract, achieving 67.9% of inhibitory ratios, without any gastric lesions. Later, Hosseinzadeh et al. (2011) used the xylene-induced ear edema and the chronic inflammation (cotton-plate) test for the same purpose, achieving effective doses in lower concentrations for the ethanolic extract of *J. regia* leaves than the aqueous one. Overall, the data presented in this work demonstrate the anti-inflammatory potential of ethanolic-based extracts of walnut green leaves using a different method.

The results concerning the cytotoxic potential of the samples are also presented in Table 2. Regarding the results for the tumor cell cultures, the green leaves of *J. regia* showed a significant higher anti-proliferative potential against to all the cell lines assessed. The most susceptible cell culture was the HepG2 (hepatocellular carcinoma) one, being a half of the cell proliferation inhibited by concentrations about 252 ± 10 to 260 ± 5 μg/mL (green and yellow samples, respectively), while the NCI-H460 (non-small lung carcinoma) cells were the less vulnerable ones (GI_{50} = 288 ± 5 and 328 ± 5 μg/mL, respectively, for the green and yellow samples). Interestingly, a sample previously studied from the same origin, did not present activity against these non-small lung cancer cell line (GI_{50} > 400 μg/mL) (Santos et al., 2013). This fact is easily justified by environmental changes, which contribute for phytochemical differences and then, slight variations in terms of bioactivities. Even so, these results are very close to the previously reported by the authors for methanolic and decoction extracts (Santos et al., 2013). The proliferative inhibition of human renal (A-498 and 769-P) and colon (Caco-2) tumor cell lines was prior evaluated by Carvalho et al. (2010b), achieving the walnut leaves methanolic extracts GI_{50} of 226, 352 and 229 μg/mL, respectively. Furthermore, the extracts studied by Santos et al. (2013) also presented anti-proliferative action against the human colon carcinoma (HCT-15) with GI_{50} of 215 to

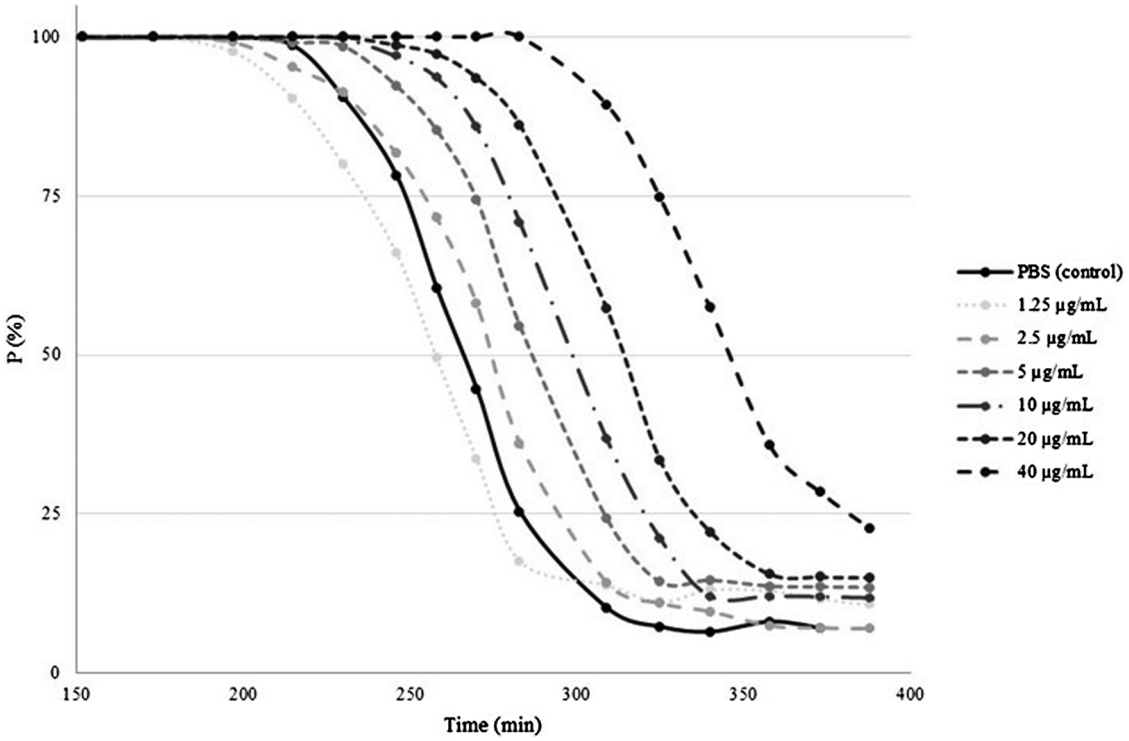

Fig. 2. Kinetic profile of the action of *J. regia* green leaves extract during the erythrocyte heamolysis (OxHLIA assay).

258 µg/mL (methanol extract and decoction, correspondingly).

Finally, the tested extracts did not reveal cytotoxicity for the primary liver porcine cells (PLP2) up to 400 µg/mL. These results are according to the previous observations of Santos et al. (2013), for both methanolic and decoction extracts. Besides the evidences about absence of hepatotoxicity, a study performed by Eidi et al. (2013) showed a positive effect over the liver in an *in vivo* model. The authors observed a hepatoprotective effect in rats against the carbon tetrachloride (CCl_4) induced oxidation damage in rats by an aqueous-ethanolic extract.

The walnut leaves hydroethanolic extracts were also studied regarding their antimicrobial properties. To this end, five Gram-negative (*E. coli, K. pneumoniae, M. morganii, P. mirabilis,* and *P. aeruginosa*) and three Gram-positive (*E. fecalis, L monocytogenes,* and MRSA) bacteria were selected. Data regarding the antibacterial activity of the extracts are presented in Table 3. The obtained results do not vary between samples, being the antibacterial potential of walnut leaves independent of the maturity stage. Furthermore, those results suggest a selective action against the Gram-positive strains. In fact, the achieved MIC values for the Gram-negative bacteria are about 20 mg/mL (*E. coli* and *K. pneumoniae*) or higher (*M. morganii, P. mirabilis* and *P. aeruginosa*), while the results for the Gram-positive ones are considerably lower (≥ 5 mg/mL). The lowest MIC value (highest antibacterial activity) was observed in *E. faecalis* and L. *monocytogenes* (MIC = 2.5 mg/mL). Comparatively, the methicillin-resistant *S. aureus* (MRSA) was inhibited with a higher concentration (MIC = 5 mg/mL). These findings allied to the fact that none of the extracts showed MBC up to 20 mg/mL, make *J. regia* leaves extracts good candidates for applications aiming to establish the balance of natural flora caused by Gram-positive bacteria proliferation. In this regard, the provided data of the present work, endorse the proposal of Qa'dan et al. (2005) for the use of *J. regia* leaves extracts for acne treatment.

In a previous study (also using bacteria from clinical isolates), Pereira et al. (2007) also reported the preferable Gram-positive susceptibility of *J. regia* leaves aqueous extracts from different Portuguese cultivars. The authors determined the MIC according to the agar streak dilution method based on radial diffusion, reporting concentrations about 0.1 to 1 mg/mL able to inhibit the *S. aureus* growth. The extracts were active against other Gram-positive bacteria (*B. cereus* and *B.*

Table 3
Antimicrobial activity (MIC and MBC mean values) of *J. regia* leaves extracts and positive controls.

	Green leaves		Yellow leaves		Ampicillin (20 mg/mL)		Imipenem (1 mg/mL)		Vancomycin (1 mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Escherichia coli	20	> 20	20	> 20	< 0.15	< 0.15	< 0.0078	< 0.0078	nt	nt
Klebsiella pneumoniae	20	> 20	20	> 20	10	20	< 0.0078	< 0.0078	nt	nt
Morganella morganii	> 20	> 20	> 20	> 20	20	> 20	< 0.0078	< 0.0078	nt	nt
Proteus mirabilis	> 20	> 20	> 20	> 20	< 0.15	< 0.15	< 0.0078	< 0.0078	nt	nt
Pseudomonas aeruginosa	> 20	> 20	> 20	> 20	> 20	> 20	0.5	1	nt	nt
Gram-positive bacteria										
Enterococcus faecalis	2.5	> 20	2.5	> 20	< 0.15	< 0.15	nt	nt	< 0.0078	< 0.0078
Listeria monocytogenes	2.5	> 20	2.5	> 20	< 0.15	< 0.15	nt	nt	nt	nt
MRSA*	5	> 20	5	> 20	< 0.15	< 0.15	nt	nt	< 0.0078	< 0.0078

MRSA- Methicillin Resistant *Staphylococcus aureus*; MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; nt: not tested.

subtilis), but not for the studied Gram-negative ones (*P. aeruginosa, E. coli* and *K. pneumonia*). However, the study guided by Dolatabadi et al. (2018) using Iranian walnut leaves (aqueous and methanolic) extracts against clinical isolates of *P. aeruginosa*, revealed positive results. The authors used the microdilution method, determining the MIC and MBC of the samples by ELISA, achieving inhibitions with 4 and 8 mg/mL and bactericidal effect using 8 and 16 mg/mL (aqueous and methanolic extract, respectively). Iranian samples were also tested against other Gram-positive strains by Sharafati-Chaleshtori et al. (2011). The authors tested hydroethanolic extracts against face oral problematic bacteria (*S. mutans, S. salivarius, S. sanguinis* and *A. viscosus*), obtaining MIC values from 15.6 to 187.5 mg/mL and the MBC ranged from 31.25 to 250 mg/mL. The problematic bacteria *M. turbeculosis* was successfully inhibited by Mexican (walnut leaves) methanolic extract, with MIC values of 125 µg/mL. Finally, the bactericidal effect of Indian *J. regia* leaves essential oil was also an object of study by Rather et al. (2012), presenting lower MIC values against Gram-positive bacteria (*B. subtilis, S. epidermidis* and *S. aureus*) than the Gram-negative ones (*E. coli, K. pneumonia, P. aeruginosa*, *P. vulgaris, S. typhi* and *S. dyssenteriae*).

4. Conclusions

The Portuguese walnut leaves proved to be a good source of hydroxycinnamic acid derivatives and flavonols, especially *trans* 3-*p*-coumaroylquinic acid and quercetin-3-*O*-glucoside. The former compounds were abundant in green samples, while the later ones were found in higher amounts in the yellow samples. The green leaves extract showed the highest antioxidant activity, being more capable to inhibit the lipid peroxidation as well as the erythrocyte heamolysis. Similarly, in the anti-inflammatory assay, only green samples presented anti-inflammatory potential. It was also verified a cytotoxic effect against tumor cell lines, but not for the non-tumor ones. Furthermore, both extracts were active against Gram-positive bacteria. Overall, this study shows the importance of walnut leaves as a source of bioactive molecules, providing antioxidant, anti-inflammatory, anti-proliferative and antibacterial properties, with potential to be used by different industries.

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