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Hydroethanolic extract of *Juglans regia* L. green husks: A source of bioactive phytochemicals

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ABSTRACT

Juglans regia L. (walnut) green husks are an important fraction of waste resulting from the walnut production, thus representing an interesting natural matrix to explore as a source of bioactive compounds. In this work, the hydroethanolic extract of walnut green husks was studied considering the phytochemical composition and the biological activity using different cell model assays, most of them evaluated for the first time for this matrix.

From the HPLC-DAD-ESI/MS^n analysis, sixteen compounds were identified, being the extract mostly composed of naphthalene derivatives (including tetralone derivatives) and less abundant in phenolic compounds (hydroxycinnamic acids and flavonols). The cytotoxic potential of the extract was assessed against tumour (MCF-7, NCI–H460, HeLa and HepG2) and non-tumour (PLP2) cell lines. Moreover, the antioxidant activity of the extract was evaluated by inhibition of the oxidative haemolysis (OxHLIA) and the formation of thiobarbituric acid reactive substances (TBARS), and the anti-inflammatory potential by the inhibition of the NO production by the RAW264.7 cell culture. The antibacterial effects of the extract were also evaluated against Gram-negative and Gram-positive bacteria. The results obtained represent a stepping stone for the development of future applications using walnut green husks as a source of added value compounds with bioactive potential.

1. Introduction

Natural products are recognised sources of bioactive compounds that can find many applications as natural antioxidants, antimicrobial, anti-inflammatory, cytotoxic, and colouring agents, among other functions (Carocho and Ferreira, 2013; Lockowandt et al., 2019; Pinela et al., 2019; Pires et al., 2018; Rostagno and Prado, 2013). Among them, by-products generated in the food and agricultural processing industries can be valorised as natural sources of antioxidants (Balasundram et al., 2006). That is the case of the green husks of walnut trees (*Juglans regia* L.), a common species in Portugal (Pereira et al., 2007) and the most widespread nut tree in the world (Martínez et al., 2010). They are part of the resulting waste from walnut (fruits) production and their extracts were already proposed as a natural source of dyeing and antimicrobial agents for cosmetic products (Beiki et al., 2018) or the reducing and stabilizing agents in the biosynthesis of gold nanoparticles (Izadiyan et al., 2018). In the food area, walnut green husks were studied as additives with functional properties in meat processing (Salejda et al., 2016) and their extracts were applied to preserve the quality of fresh walnuts during storage (Chatrabnous et al., 2018). Finally, a more traditional application is the use of walnut husks to produce a Slovenian walnut liqueur, which is extremely rich is in phenolic compounds and vitamins (Stampar et al., 2006).

To assist the development of new applications in the food, pharmaceutical, and cosmetic areas, among others, it is important to have a detailed characterization of the phytochemical profile of the extracts and, also, of their bioactivity, as this by-product is a source of molecules of potential pharmaceutical interest (Alshatwi et al., 2012; Bagheri et al., 2012; Jahanban-Esfahlan et al., 2019; Oliveira et al., 2008; Sharma et al., 2013; Soto-Maldonado et al., 2019; Zhou et al., 2015).

A few studies can be found for *J. regia* L. in which the phytochemical composition of green husks has been determined by HPLC coupled to a UV/DAD detector (Akbari et al., 2012; Chatrabnous et al., 2018; Cosmulescu et al., 2011, 2010; Liu et al., 2008; Soto-Maldonado et al.,

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2019; Stampar et al., 2006), reporting the amounts of juglone, phenolic acids, flavonoids and/or tetralone derivatives in the biomass. In another study, phenolic acids and tetralone derivatives were the main compounds identified in samples collected in China, using spectroscopic analysis (UV–Vis, 1H NMR, 13C NMR, 2D NMR, HR-ESI-MS) (Du et al., 2014). Other authors identified phenolic acids and flavonoids during the characterization performed this time by LC-MS (Gawlik-Dziki et al., 2014).

Regarding the bioactivity studies of the walnut green husks, remarkable results regarding its antioxidant activity have been reported, mainly because of their free-radical scavenger and metal chelator capacities (Akbari et al., 2012; Fernández-Agulló et al., 2013; Meshkini and Tahmasbi, 2017). However, only a few of studies (Bagheri et al., 2012; Carvalho et al., 2010; Soto-Maldonado et al., 2019) provided cellular antioxidant activity models which are considered better representatives of the complexity of biological systems (Gupta, 2015). Cellular models have been also used to evaluate the antimicrobial and anti-tumour potentials in previous works (Alshatwi et al., 2012; Carvalho et al., 2010; Fernández-Agulló et al., 2013; Keskin et al., 2012; Oliveira et al., 2008; Sharma et al., 2013; Soto-Maldonado et al., 2019; Zhang et al., 2014).

The present work aims to further characterize the phytochemical composition of *Juglans regia* L. green husks by liquid chromatography combined with a diode array detector and electrospray ionization mass spectrometer (LC-DAD-ESI/MS^n), and to extend the study of the bioactive potential of their hydroethanolic extracts using different *in vitro* cellular approaches. Therefore, the bioactivity of the extract was studied by evaluating the antioxidant (the thiobarbituric acid reactive substances and the oxidative haemolysis inhibition assays), anti-inflammatory (inhibition of the nitric oxide production by macrophages), cytotoxicity (tumour and non-tumour cell lines), and antibacterial (Gram-negative and Gram-positive bacteria) properties. To the best of our knowledge, it is the first time that the selected cell models are applied to evaluate the anti-inflammatory and cytotoxic activities of this extract.

2. Material and methods

2.1. Plant material and extract preparation

Juglans regia L. green husks were collected in Bragança, Northeast of Portugal, during October of 2018. The samples were dried to a constant weight in an incubator at 35 °C. After, the plant material was ground (≈40 mesh), and the homogeneous sample was stored in a desiccator protected from light for subsequent assays.

The extract was obtained by stirring the raw material (1 g) in an aqueous ethanolic solution (80% ethanol, v/v; 30 mL) at room temperature (25 °C) for 60 min. After filtration (Whatman no. 4), the extraction procedure was repeated once. Then, the solvent was recovered in order to obtain a dry extract: first, evaporation at 40 °C and reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland), and then freeze-drying (Telstar Cryodos-80, Terrassa, Barcelona).

2.2. Phytochemical characterization

The dry extract was re-suspended at 10 mg/mL using aqueous ethanol (80% ethanol, v/v) and filtered through a 0.2 μm disposable LC filter disk (30 mm, nylon). The phytochemical characterization of walnut green husks was traced by liquid chromatography with diode-array detection (280, 330 and 370 nm wavelengths) coupled to electrospray mass ionization operating in negative mode (Dionex Ultimate 3000 UPLC and Linear Ion Trap LTQ XL, Thermo Scientific, San Jose, CA, USA) by using a Waters Spherisorb S3 ODS-2 C_{18} (3 μm, 4.6 mm × 150 mm), as previously described by the authors (Bessada et al., 2016). The compounds were identified according to their retention time, UV–vis and mass spectra by comparison with those obtained using standard compounds, as well as with literature data. Calibration curves of appropriate standards (*p*-coumaric acid, *α*-tetralone, juglone and quercetin-3-*O*-glucoside, HPLC grade, Sigma-Aldrich) were obtained in the range between 200 and 5 μg/mL, for the quantitative analysis. The results were expressed as milligrams of each compound per gram of extract (mg/g). Triplicates were analysed with two independent injections.

2.3. In vitro antioxidant assays

The antioxidant activity was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) and by the oxidative haemolysis inhibition (OxHLIA) assays. The extract was diluted at a concentration of 10 mg/mL (distilled water and PBS solution, respectively, for the TBARS and OxHLIA assays) and then, further dilutions were carried out (500–6.25 μg/mL).

The TBARS assay was performed by measuring the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) at 532 nm, following a methodology described by Barreira et al. (2013), and the results were expressed as IC_{50} values (sample concentration providing 50% of antioxidant activity).

The OxHLIA assay was carried out by evaluating the delay effect of the extract on the erythrocyte haemolysis at 690 nm, as previously described by Lockowandt et al. (2019). The results were presented as IC_{50} values (extract concentration that delayed the haemolysis time for 60 min, with 50% of intact erythrocytes). Trolox was used as positive control for both antioxidant activity evaluations. Triplicates were used and three independent assays were performed.

2.4. Anti-inflammatory activity

The walnut green husks extract was re-suspended in water at a concentration of 10 mg/mL and then diluted in the range between 500 and 7.8 μg/mL. To perform the assay, the nitric oxide production by a mouse macrophage-like cell line (RAW264.7) was measured, using the Griess Reagent System (GRS) kit. The inhibition of the NO production was performed according to the methodology described by Corrêa et al. (2015), with measurements at 515 nm (EL*x*800 microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). Results were expressed as EC_{50} values (sample concentration providing 50% of inhibition of NO production) and dexamethasone was used as a positive control, while for the negative control, no lipopolysaccharide (LPS) was added. For the assay, triplicates were used in three independent assays.

2.5. Cytotoxicity assays

The hydroethanolic extract of walnut green husks was re-suspended in water at 10 mg/mL and, then, further diluted in the range between 500 and 7.8 μg/mL. The cytotoxic properties were assessed using four human tumour cell lines: MCF-7 (breast adenocarcinoma), NCI–H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma). A non-tumour cell line (porcine liver primary cells, PLP2) was also evaluated using a procedure described by Abreu et al. (2011). The sulforhodamine B assay was performed according to a protocol described elsewhere (Barros et al., 2013). Ellipticine was used as a positive control, while the negative control was represented by each suspension of cells. The results were expressed in GI_{50} values (concentration that inhibited 50% of the cell proliferation). Three independent assays were performed using triplicates.

2.6. Antibacterial activity

The hydroethanolic extract of walnut green husks was dissolved in a mixture of dimethyl sulfoxide (DMSO) + Mueller-Hinton Broth (MHB)/Tryptic Soy Broth (TSB) (5 + 95%, v/v) to give a final concentration of 100 mg/mL for the stock solution and, then, successive dilutions were

carried out ranging from 20 to 1.25 mg/mL. The antimicrobial potential was assessed using five Gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Morganella morganii*) and three Gram-positive bacteria (*Enterococcus faecalis*, *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* - MRSA). These strains were clinical isolates donated by hospitalized patients (Local Health Unit of Bragança and Hospital Centre of Trás-os-Montes and Alto-Douro, Vila Real, Northeast of Portugal) with multi-resistant profile, previously characterized by Alves et al. (2014). For each bacteria, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using a colorimetric assay as described by Kuete et al. (2011) and Pires et al. (2018). Duplicates were used and three independent assays were performed.

3. Results and discussion

3.1. Phytochemical composition of walnut husks

The phytochemical composition of the hydroethanolic extracts of *J. regia* green husks was characterized for samples at the mature stage of the fruit (October 2018) and the main molecules identified are presented in Table 1 with their quantification. The tentative identification was carried out according to their retention time (*Rt*), maximum absorbance wavelength (λ_{max}), pseudomolecular ion ($[M-H]^-$), the correspondent fragmentation pattern (MS^2), and also using literature information. It was possible to identify sixteen compounds as presented in Fig. 1.

The extract was composed of three organic acids, of which two phenolic acids (*p*-hydroxycinnamic acid derivatives), six tetralone derivatives, three naphthalene derivatives, and four flavonoids (flavonols).

The first compound presented a fragment at *m/z* 133 ($[M-H]^-$) and maximum UV absorption at 212 nm, and additional MS^2 fragments at *m/z* 115, 89 and 71; therefore, compound 1 was tentatively identified as malic acid. The presence of this organic acid in *Juglans* spp., as well as the MS/MS fragmentation pattern, was previously reported by Wang et al. (2017) in *J. mandshurica* Maxim. branches.

The two phenolic acids identified in *J. regia* green husks, peaks 2 and 6 ($[M-H]^-$ at *m/z* 337), were assigned as 3- and 4-*p*-coumaroylquinic acid, respectively. The MS^2 fragments at *m/z* 191, 173, 163, 155 and 119 are in accordance to the hierarchical fragmentation pattern described by Clifford et al. (2003), with base peak at *m/z* 163 for the 3-*p*-coumaroylquinic acid and *m/z* 173 for the 4-*p*-coumaroylquinic acid. These assumptions were also in agreement with the observations of Gawlik-Dziki et al. (2014) using the same plant material, reporting similar mass fragmentations and maximum UV–Vis absorption (307 and 310 nm, respectively). The amounts of both hydroxycinnamic acids were very similar (1.07 ± 0.03 mg/g dry extract and 1.356 ± 0.001 mg/g dry extract, for 3-*p*-coumaroylquinic and 4-*p*-coumaroylquinic acids, respectively), yielding a total amount of phenolic acids of 2.42 ± 0.03 mg/g dry extract.

Peaks 11 and 12 ($[M-H]^-$ at *m/z* 463), 14 ($[M-H]^-$ at *m/z* 433) and 15 ($[M-H]^-$ at *m/z* 447) yielded the same base peak at *m/z* 301. These compounds were identified as quercetin glycoside derivatives, more precisely: quercetin-3-*O*-glucoside (11), quercetin-*O*-hexoside (12), quercetin-*O*-pentoside (14) and quercetin-*O*-deoxyhexoside (15). The presence of these flavonols are in agreement with the results reported by Gawlik-Dziki et al. (2014), as well as the mass fragments and UV–vis maximum absorptions for the same plant material. Regarding the amounts found for the quercetin derivatives, they ranged from 0.265 ± 0.005 mg/g dry extract for the *O*-hexoside form to 0.3822 ± 0.0003 mg/g dry extract for the *O*-pentoside one, yielding a total amount of flavonoids of 1.293 ± 0.004 mg/g dry extract. These compounds were the least abundant class of molecules found.

The following group of compounds, tetralone derivatives, are

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, n = 6) of the tentatively identified compounds in green husks extracts of *J. regia*.

Peak	*Rt* (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Walnut green husks (mg/g extract)
1	5.2	212	133	115(100), 89(10), 71(8)	Malic acid[a]	n.q.
2	6.0	307	337	191(10), 173(10), 163(100), 155(5), 119(7)	3-*p*-Coumaroylquinic acid[b]	1.07 ± 0.03
3	7.7	262, 320	339	159(100), 115(52)	Dihydroxytetralone hexoside[a,c]	0.42 ± 0.02
4	8.4	222, 263	409	337(28), 247(100), 203(77), 175(84), 131(5)	Trihydroxynaphthalene hexoside derivative[c]	4.2 ± 0.2
5	10.9	263, 320	177	159(100), 131(15), 115(5)	Dihydroxytetralone[a,c]	7.1 ± 0.4
6	11.9	310	337	191(5), 173(100), 163(5), 119(3)	4-*p*-Coumaroylquinic acid[b]	1.356 ± 0.001
7	13.0	223, 264	193	175(100), 157(5), 147(3), 131(3)	Trihydroxytetralone[a,c]	1.174 ± 0.005
8	15.7	260, 320	491	331(92), 271(100), 211(100), 169(10)	Dihydroxytetralone galloyl-hexoside isomer 1[a,c]	2.8 ± 0.1
9	16.6	220, 264	491	331(94), 271(100), 211(5), 169(3)	Dihydroxytetralone galloyl-hexoside isomer 2[a,c]	1.486 ± 0.003
10	17.7	213, 261, 330	507	331(100), 271(42), 211(5), 169(3)	Trihydroxytetralone galloyl-hexoside[a]	1.23 ± 0.04
11	18.5	350	463	301 (100)	Quercetin 3-*O*-glucoside[d]	0.3255 ± 0.0003
12	18.8	348	463	301 (100)	Quercetin *O*-hexoside[d]	0.265 ± 0.005
13	20.3	222, 273	489	313(61), 301(56), 271(100), 211(15), 169(8)	Trihydroxynaphthalene galloyl-hexoside[c]	4.7 ± 0.3
14	21.4	350	433	301 (100)	Quercetin *O*-pentoside[d]	0.3822 ± 0.0003
15	22.4	350	447	301 (100)	Quercetin *O*-deoxyhexoside[f]	0.321 ± 0.001
16	26.2	223, 263	487	325(100), 307(10)	Jugnaphthalenoside A[c]	0.76 ± 0.02
					Total phenolic acids	2.42 ± 0.03
					Total flavonoids	1.293 ± 0.004
					Total phenolic compounds	3.72 ± 0.03
					Total tetralone derivatives	14.2 ± 0.2
					Total naphthalene derivatives	9.68 ± 0.04

Calibration curves: Peaks 2 and 6: *p*-coumaric acid (y = 301950x + 6966.7; r^2 = 0.999; LOD = 0.78 μg/mL; LOQ = 1.97 μg/mL); peaks 3, 5, 7, 9, 10 and 11: α-tetralone (y = 6173.3x + 58913; r^2 = 0.9991; LOD = 3.1 μg/mL; LOQ = 9.5 μg/mL); peaks 4, 14: juglone (y = 3754.1x + 45119; r^2 = 0.9992; LOD = 2.9 μg/mL; LOQ = 8.8 μg/mL); peaks 12, 13, 15 and 16: 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 – Wang et al. (2017); b –Clifford et al. (2003), Clifford et al. (2005); c – Jin-Hai et al. (2018); d – Santos et al. (2013); f – Gawlik-Dziki et al. (2014). n.q.: not quantified.

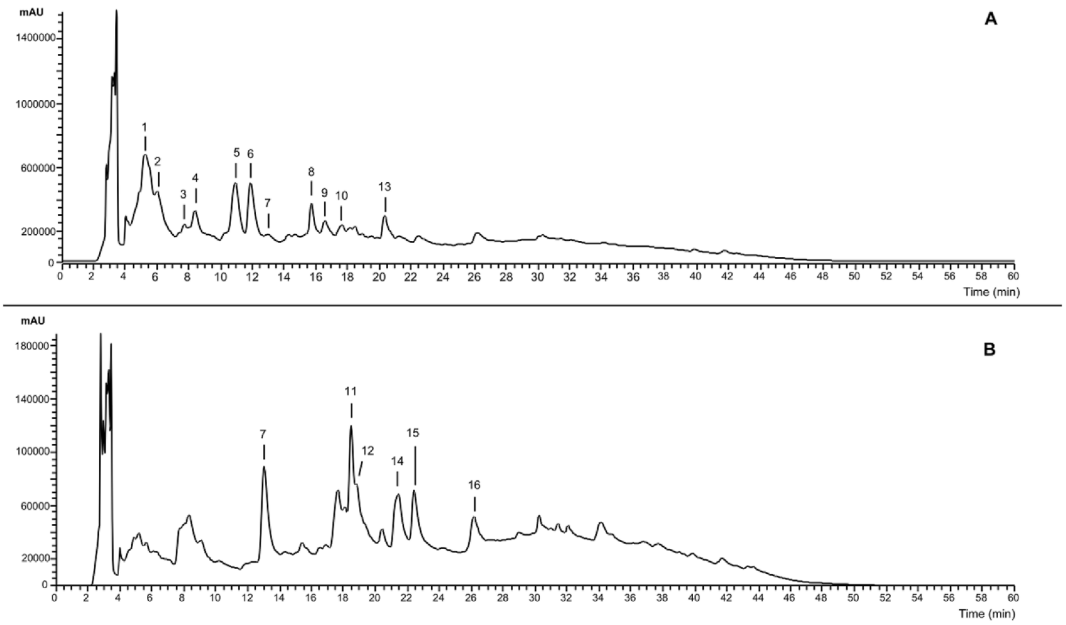

Fig. 1. HPLC phytochemical profile of *J. regia* L. green husks at 280 nm (A) and 370 nm (B) wavelengths. The peaks identification and quantification are presented in Table 1.

widely distributed in *Juglans* spp. plant material (*e.g.* pericarps, green husks, stem-bark, branches, etc.) (Du et al., 2014; Li et al., 2013, 2008a; Liu et al., 2010; Talapatra et al., 1988; Wang et al., 2017). Particularly, the tetralone derivatives found in green husks of *J. regia* usually occur as hydroxyl, hexosyl and hydroxybenzoyl derivatives, with several isomeric alternatives (Zhou et al., 2015). In this work, peak 3 ($[M-H]^-$ at *m/z* 339), peak 5 ($[M-H]^-$ at *m/z* 177) and peak 7 ($[M-H]^-$ at *m/z* 193) were identified as dihydroxytetralone hexoside, dihydroxytetralone and trihydroxytetralone, respectively. These identifications were possible by comparison with literature data, describing the different fragmentation patterns of individual compounds found in *J. mandshurica* samples (Jin-Hai et al., 2018; Wang et al., 2017). Peaks 8 and 9 ($[M-H]^-$ at *m/z* 491), and peak 10 ($[M-H]^-$ at *m/z* 507) showed similar MS^2 fragments at *m/z* 271, 211 and 169. Following the observations of Jin-Hai et al. (2018) and Wang et al. (2017), the compounds were identified as tetralone galloyl hexoside derivatives, specifically, dihydroxytetralone galloyl hexoside isomers (peaks 8 and 9) and trihydroxytetralone galloyl hexoside (peak 10). Dihydroxytetralone was the most abundant compound (7.1 ± 0.4 mg/g dry extract), about half of the total amount of tetralone derivatives (14.2 ± 0.2 mg/g dry extract). Dihydroxytetralone galloyl-hexoside isomers were the second most abundant compounds (2.8 ± 0.1 mg/g dry extract and 1.486 ± 0.003 mg/g dry extract, for isomers 1 and 2, respectively), and dihydroxytetralone hexoside was the least abundant tetralone derivative (0.42 ± 0.02 mg/g dry extract).

Peaks 4, 13 and 16 correspond to naphthalene derivatives. This group of molecules was already identified in *J. mandshurica* fruits (Jin-Hai et al., 2018), husks (Jin-Hai et al., 2018; Zhou et al., 2015) and branches (Wang et al., 2017). As with tetralone derivatives, natural naphthalene also occurs with hydroxyl, hexosyl and hydroxybenzoyl substitutions (Sun et al., 2012). Peak 4 ($[M-H]^-$ at *m/z* 409) was identified due to its MS^2 fragments, which complies with the previous fragmentation pattern descriptions of Jin-Hai et al. (2018) and Wang et al. (2017) for a trihydroxynaphthalene hexoside; thus, the loss of −72u was unknown and, therefore, peak 4 was assigned to a trihydroxynaphthalene hexoside derivative. Peak 13 ($[M-H]^-$ at *m/z* 489) was identified as trihydroxynaphthalene galloyl-hexoside, while peak 16 ($[M-H]^-$ at *m/z* 487) was identified as jugnaphthalenoside A, taking into account the fragmentation patterns reported by Jin-Hai et al. (2018). Regarding their quantification, these molecules were present in similar amounts (4.2 ± 0.2 mg/g dry extract and 4.7 ± 0.3 mg/g dry extract, respectively), values that are considerably higher than the yield obtained for jugnaphthalenoside A (0.76 ± 0.02 mg/g dry extract). Naphthalene derivatives were the second most abundant group of compounds.

The phytochemical profile of *Juglans regia* green husks is quite variable; however, our findings are in good agreement with the most abundant groups of compounds found in literature for *Juglans* spp. samples such as phenolic acids and flavonoids (Cosmulescu et al., 2010; Gawlik-Dziki et al., 2014; Stampar et al., 2006), as well as tetralone and naphthalene derivatives (Du et al., 2014; Jin-Hai et al., 2018; Wang et al., 2017). As phenolic compounds (*e.g.* phenolic acids and flavonols) are widely distributed throughout the plant kingdom (Crozier et al., 2006), their presence in walnut green husks was expected. However, their lower abundancy compared to other compounds such as tetralones and naphthalene derivatives in walnut green husks is little discussed in the literature. In fact, this type of comparisons were only carried out for the presence of another naphthalene derivative, juglone (naphthoquinone), being the most abundant molecule in walnut green husks previously studied by Cosmulescu et al. (2010) and Stampar et al. (2006), but not in walnut leaves (Cosmulescu et al., 2014a, 2014b). However, in the present work, juglone was not detected in walnut green husks samples.

The bioactive potential of this extract was further evaluated, aiming to complement the phytochemical characterization, and support its potential application in several areas including food, cosmetic and pharmaceutical industries (Balasundram et al., 2006; Butler et al., 2014; Newman and Cragg, 2016).

3.2. Bioactivity of the hydroethanolic extracts

The antioxidant activity of the extracts was evaluated by two *in vitro* assays using cells and tissues. The extract potential for the inhibition of the lipid peroxidation (evaluated by the thiobarbituric acid reactive substances – TBARS assay) was assessed using porcine brain tissues, while the maintenance of the erythrocyte integrity by the extract action was performed by the oxidative haemolysis inhibition (OxHLIA) assay. The results are presented in Table 2, and the anti-haemolytic action of the extracts is presented in Fig. 2.

The mean concentration leading to half of the malondialdehyde

Table 2
Antioxidant activity, NO formation inhibition and cytotoxicity of *J. regia* green husks (mean ± SD, n = 9).

	Hydroethanolic extract	Positive control
Antioxidant activity (EC_{50} values, μg/mL)		
TBARS inhibition	101 ± 4	5.8 ± 0.6
OxHLIA	80 ± 4	19.6 ± 0.6
Anti-inflammatory activity (EC_{50} values, μg/mL)		
Nitric oxide (NO) production	56 ± 3	16 ± 1
Cytotoxicity (GI_{50} values, μg/mL)		
MCF-7 (breast carcinoma)	26 ± 1	1.21 ± 0.02
NCI–H460 (non-small lung cancer)	41 ± 2	0.9 ± 0.1
HeLa (cervical carcinoma)	34 ± 2	1.03 ± 0.09
HepG2 (hepatocellular carcinoma)	24 ± 2	1.1 ± 0.09
PLP2 (porcine liver primary cells)	87 ± 4	2.3 ± 0.2

Trolox was used as positive control in the antioxidant activity assays, dexamethasone in the anti-inflammatory activity assay, while ellipticine was used in the cytotoxicity assays.

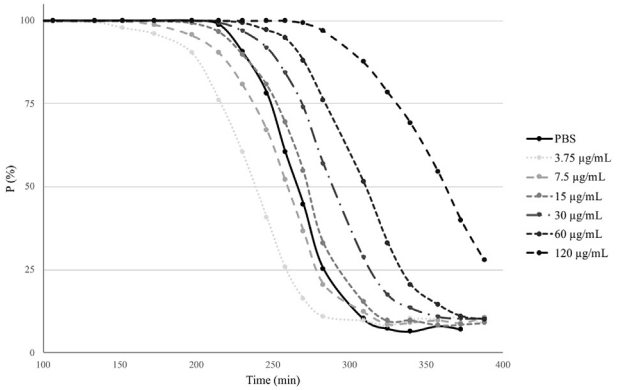

Fig. 2. Kinetic behaviour of *J. regia* green husks extract during the erythrocyte heamolysis (OxHLIA assay).

formed during the peroxidation of the unsaturated fatty acids present in the brain tissues, in the TBARS assay, was IC_{50} = 101 ± 4 μg/mL. In the OxHLIA assay, the inhibition of the erythrocyte's membrane damage promoted by the free radical AAPH was achieved at IC_{50} = 80 ± 4 μg/mL. The IC_{50} values obtained by the OxHLIA assay were lower than the ones for the TBARS, contrarily to the results found for the positive control, with IC_{50} = 19 ± 0.6 μg/mL and 5.8 ± 0.6 μg/mL, respectively.

To our knowledge, the effect of the walnut green husks extract against the lipid oxidation through the TBARS assay was only reported by Bagheri et al. (2012). The hydroalcoholic walnut green husk extract was able to inhibit the LDL oxidation with a dose-dependency from 0.2 to 20 μg/mL. Regarding the anti-haemolytic potential, Carvalho et al. (2010) also evaluated the dose-dependency between different concentrations of the methanolic extract and the time of the AAPH free radical exposure. The IC_{50} mean value was 127 ± 14 μg/mL after 3 h of reaction ($\Delta t \approx 60$ min), which is in agreement with the results obtained in this work.

The anti-inflammatory potential was assessed using macrophages cells (Table 2). After recognition of a pathogen, macrophages release a series of toxic molecules as an immune defence, being one of them nitric oxide (NO). In this context, NO is a recognised intercellular marker in the immune system, being involved in immunologically mediated diseases and inflammation (Tripathi et al., 2007). An extract concentration of 56 ± 3 μg/mL inhibited half of the NO produced by the macrophage's cultures (EC_{50}). This result was 3.5-fold higher than the pure standard used for the positive control, dexamethasone, with EC_{50} = 16 ± 1 μg/mL. To the best of our knowledge, this assay was applied for the first time to the extracts of walnut husks. The inhibition of the NO production is frequently carried out by simple chemical reactions using sodium nitroprusside, but in those cases only the antioxidant activity is evaluated (Akbari et al., 2012; Fernández-Agulló et al., 2013; Ghasemi et al., 2011; Sharma et al., 2013). It is usually applied as an antiradical indicator because the source of NO is not provided by cells (*e.g.* macrophages). In this context, some studies about the NO scavenging activity of walnut green husks can be found. Akbari et al. (2012) showed that the methanolic extracts of six green husks genotypes were able to capture, on average, about 69.18 ± 1.57% of the NO free radicals. The results for the NO inhibition in sample extracts from India (obtained with different organic solvents) were all > 500 μg/mL (Sharma et al., 2013). Ghasemi et al. (2011) obtained IC_{50} values ranging from 141 to 2890 μg/mL for methanolic extracts depending on the *J. regia* cultivar. Regarding the NO scavenging activity of other samples from Portuguese origin, the aqueous extract of a *Mellanaise* walnut husk variety resulted in EC_{50} values of 960 ± 130 μg/mL (Fernández-Agulló et al., 2013). Though the results cannot be directly compared, as they were obtained in different assays, the concentration values from the chemical assay were all higher than the ones obtained in this study with the cellular model.

Beyond the intrinsic characteristics of the plant material (geographic location, time of collection), the differences found in literature are also due to the selected extraction conditions (extraction technique, solvent, time), which are selective factors during the extraction of compounds from natural matrices (Sampaio et al., 2016; Zhang et al., 2018).

Regarding the cytotoxicity of walnut green husks, the results for the tumour and non-tumour cell cultures are presented in Table 2. The extract showed similar activity against the four tumour cell lines (non-small lung cancer, breast, cervical and hepatocellular carcinomas), with lower GI_{50} values (higher cytotoxic potential) against the HepG2 (hepatocellular carcinoma) and MCF-7 (breast carcinoma) cells, reaching 24 ± 2 μg/mL and 26 ± 1 μg/mL, respectively. Slightly lower activity was obtained against HeLa (cervical carcinoma) and NCI–H460 (non-small lung cancer) cell lines, with GI_{50} values of 34 ± 2 μg/mL and 41 ± 2 μg/mL, respectively. Concerning the results of the hepatotoxicity, the extract was toxic for the primary liver cells; however, at higher concentrations compared to the tumour cell lines, with GI_{50} = 87 ± 4 μg/mL.

To the best of our knowledge, it is the first time that the cytotoxic potential of *J. regia* green husks extracts is studied against the proliferation of the selected cell cultures. Nevertheless, the cytotoxic effect of pure isolated compounds from *Juglans mandshurica* Maxim extracts was evaluated for HepG2 cells (Yang et al., 2019; Zhou et al., 2015). According to Zhou et al. (2015), lower IC_{50} values were found for naphthoquinone derivatives than the ones obtained for tetralone, and the non-glycosylated forms were more active against the HepG2 cells than the glycosylated ones: dihydroxynaphthoquinone isomers (7.33 ± 0.52 μM to 15.37 ± 1.63 μM) < dihydroxy tetralone (regiolone: 56.87 ± 4.27 μM) < trihydroxy naphthoquinone hexoside isomers (78.61 ± 2.38 μM to 83.32 ± 4.54 μM), while dihydroxy- and trihydroxytetralone hexoside isomers were not active against the cell culture. Similar results were found for regiolone in *Juglans cathayensis* Dode samples against the same cell line (42.56 μM) (Li et al., 2008b). The authors also presented results about the regiolone toxicity against HeLa (30.48 μM) and HL-60 (40.21 μM) cell cultures. Furthermore, two tetralone dimers (juglanone A and B) were isolated from *J. regia* pericarps (husks) by Li et al. (2013). The cytotoxicity of the pure compounds was assessed against a series of human cell lines (A549, MCF-7, BEL-7402, HeLa, COLO205, BGC-823, and SK-OV-3). Juglanone A showed a higher inhibition rate for the MCF-7 cells (94.16 ± 0.37%), while juglanone B revealed higher activity against HeLa cells (92.62 ± 2.00%). Also, the five cyclic diarylheptanoids isolated by Yang et al. (2019) from the ethanolic (95%) extract resulted in IC_{50} values in the range of 27.72 ± 3.71 μM (Juglanin H) to

383.54 ± 29.57 μM (Juglanin J).

Regarding the anti-proliferative potential of the green husks from *J. regia*, Carvalho et al. (2010) reported IC_{50} values of 285 ± 51 μg/mL and 496 ± 71 μg/mL for A-498 and 769-P (human kidney carcinomas) cell lines, respectively. For the Caco-2 (colorectal carcinoma) cells, the IC_{50} was higher than 500 μg/mL. The increase on the TUNEL-positive apoptotic cell count of different extracts (methanol, *n*-hexane and chloroform) from this *J. regia* by-product was assessed for PC-3 (human prostate cancer) cells by Alshatwi et al. (2012), and the *n*-hexane extract was the most active. Moreover, the recent findings of Soto-Maldonado et al. (2019) showed the antiproliferative and cytotoxic potentials of ethanolic extracts against the HL-60 cells, in which the plant extract presented higher activity than pure juglone. Overall, walnut green husks have shown to be a rich natural source of bioactive compounds with anti-proliferative potential, either in terms of the final extract or of their pure components.

The antimicrobial properties of the hydroethanolic extract of walnut green husks were also studied. Five Gram-negative (*Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis,* and *Pseudomonas aeruginosa*) and three Gram-positive (*Enterococcus fecalis*, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus*) bacteria obtained from clinical isolates were used (Alves et al., 2014). The results of the antibacterial activity of the extract and positive controls are presented in Table 3.

Both type of bacteria were susceptible to the ethanolic extract, being the lowest MIC (higher anti-bacterial potential) obtained for the methicillin-resistant *Staphylococcus aureus* (MRSA) strain (MIC = 5 mg/mL). The double of inhibitory concentration (10 mg/mL) was found to be effective against one Gram-positive (*L. monocytogenes*) and one Gram-negative (*E. coli*) bacteria. However, under the experimental concentration range, the extract was not able to inhibit *P. aeruginosa* (MIC > 20 mg/mL), being the remaining species susceptible to a concentration of 20 mg/mL (*K. pneumoniae*, *M. morganii*, *P. mirabilis*, and *E. fecalis*). In all cases, the minimum bactericidal concentration was higher than 20 mg/mL.

The antibacterial potential of Portuguese walnut green husks was previously evaluated by Oliveira et al. (2008). The authors evaluated the decoction extracts of several *J. regia* cultivars against different Gram-negative and Gram-positive bacteria with positive inhibitions against *S. aureus* (MIC = 0.1 mg/mL), *Bacillus cereus* (MIC = 0.1–1 mg/mL), *Bacillus subtilis* (MIC = 0.1–10 mg/mL) and *P. aeruginosa* (MIC = 100 mg/mL). Later, Keskin et al. (2012)evaluated the activity of aqueous extracts by the disk diffusion method, obtaining higher inhibition zones against *Pseudomonas fluorescens* (15 mm) and lower for *B. subtilis* and *P. aeruginosa* (8 mm). The water extracts, studied by Fernández-Agulló et al. (2013), have shown higher potential against *B. cereus* (MIC = 20 mg/mL), but lower for the Gram-negative *P. aeruginosa* and *E. coli* (MIC = 100 mg/mL). On the other hand, Sharma et al. (2013) evaluated the antibacterial activity of walnut green husks extracts obtained by different solvents (ethanol, ethyl acetate and water). Generally, the ethanolic extract showed higher diameter of inhibition against the studied bacteria (*E. coli*, *K. pneumoniae* and *S. aureus*), while the ethyl acetate extract was most effective against *B. subtilis*. Finally, the results obtained by Zhang et al. (2014) for the chloroform fraction of Chinese samples showed the lowest MIC values against *E. coli*, *P. aeruginosa, B. subtilis* and *S. aureus* (6.25, 1.56, 3.13 and 3.13 mg/mL, respectively). In general, the MIC values obtained in the present work were higher in comparison with those reported in literature. These differences can be caused by several factors, such as the extraction methodology and solvents applied, and other factors related to the plant material, as previously discussed. Moreover, the selected microorganisms can also be a determinant factor, because the strains used in this work are multi-resistant clinical isolates, which may demand higher extract concentrations to inhibit their growth.

4. Conclusions

Walnut green husks presented a diverse phytochemical profile with different classes of phenolic compounds and naphthalene derivatives, including tetralone derivatives. The extract was richer in tetralone derivatives (14.2 ± 0.2 mg/g) compared to the other bioactive phytochemicals such as phenolic acids and flavonoids (3.72 ± 0.03 mg/g). The hydroethanolic extract presented relevant antioxidant, anti-inflammatory, cytotoxic and antibacterial potentials, being all these assays carried out using *in vitro* cell models. In this regard, the anti-proliferative potential of walnut green husks hydroethanolic extract was assessed for the first time for the selected cell cultures (MCF-7, NCI–H460, HeLa, HepG2 and PLP2). Moreover, it was also the first time that the anti-inflammatory properties of the extract were studied using a specific cell model (RAW264.7). Thus, this study contributes to increase the knowledge about *J. regia* walnut green husks since, as far as we know, it was the first time that this set of combined results are presented, showing in general consistent results with the few studies available in literature.

CRediT authorship contribution statement

Vanessa Vieira: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Carla Pereira:** Methodology, Investigation, Formal analysis. **Rui M.V. Abreu:** Methodology. **Ricardo C. Calhelha:** Methodology, Formal analysis. **Maria José Alves:** Methodology. **João A.P. Coutinho:** Writing - review & editing. **Olga Ferreira:** Writing - original draft, Writing - review & editing, Funding acquisition. **Lillian Barros:** Methodology, Investigation, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Formal analysis,

Table 3
Antibacterial properties of J. regia green husks against Gram-positive and Gram-negative bacteria (mean, n = 6).

	Ethanolic extract		Ampicillin		Imipenem		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria								
Escherichia coli	10	> 20	< 0.15	< 0.15	< 0.0078	< 0.0078	n.t.	n.t.
Klebsiella pneumoniae	20	> 20	10	20	< 0.0078	< 0.0078	n.t.	n.t.
Morganella morganii	20	> 20	20	> 20	< 0.0078	< 0.0078	n.t.	n.t.
Proteus mirabilis	20	> 20	< 015	< 0.15	< 0.0078	< 0.0078	n.t.	n.t.
Pseudomonas aeruginosa	> 20	> 20	> 20	> 20	0.5	1	n.t.	n.t.
Gram-positive bacteria								
Enterococcus faecalis	20	> 20	< 0.15	< 0.15	n.t.	n.t.	< 0.0078	< 0.0078
Listeria monocytogenes	10	> 20	< 0.15	< 0.15	< 0.0078	< 0.0078	n.t.	n.t.
Methicillin-resistant *Staphylococcus aureus* (MRSA)	5	> 20	< 0.15	< 0.15	n.t.	n.t.	0.25	0.5

n.t. not tested.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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