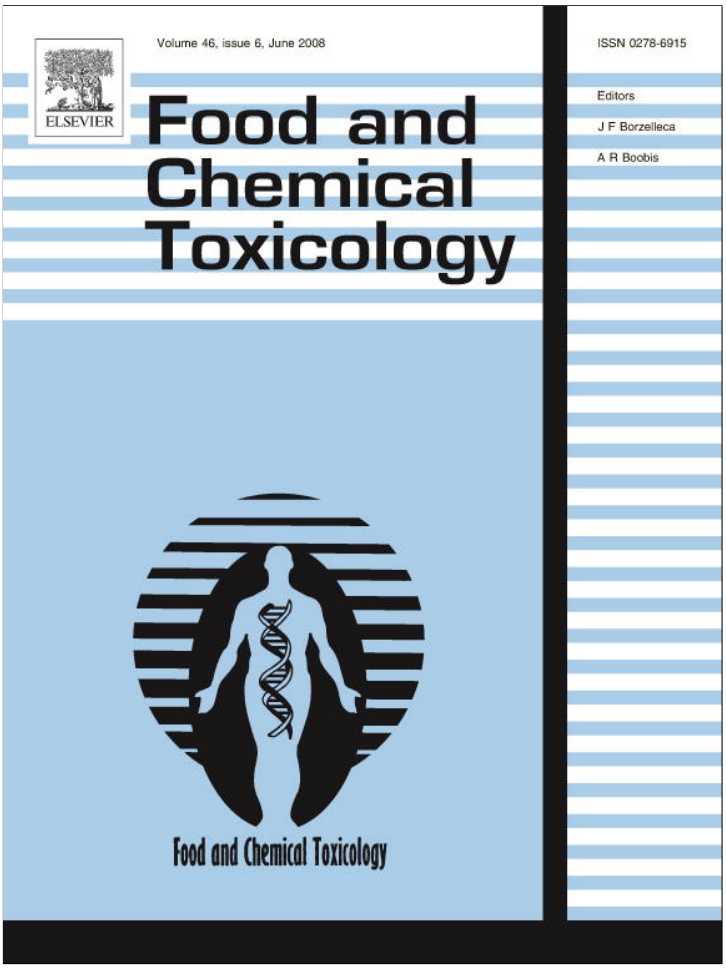

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Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars

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Abstract

The chemical composition, antioxidant potential and antimicrobial activity were studied in six walnuts (*Juglans regia* L.) cultivars (*cv.* Franquette, Lara, Marbot, Mayette, Mellanaise and Parisienne) produced in Portugal. Concerning their chemical composition the main constituent of fruits was fat ranging from 78.83% to 82.14%, being the nutritional value around 720 kcal per 100 g of fruits. Linoleic acid was the major fatty acid reaching the maximum value of 60.30% (*cv.* Lara) followed by oleic, linolenic and palmitic acids. The aqueous extracts of walnut cultivars were investigated by the reducing power assay, the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and β-carotene linoleate model system. All the walnut extracts exhibited antioxidant capacity in a concentration-dependent manner being the lowest EC_{50} values obtained with extracts of *cv.* Parisienne. Their antimicrobial capacity was also checked against gram positive (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*) and gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) and fungi (*Candida albicans*, *Cryptococcus neoformans*), revealing activity against the different tested microorganisms.

Keywords: *Juglans regia* L.; Fatty acids; Nutritional value; Antioxidant properties; Antimicrobial activity

1. Introduction

Nuts are important components of the Mediterranean diet. Walnuts (*Juglans regia* L.) are widely distributed all over the world, and in Portugal, these species are common along the country. These fruits are receiving increasing interest as a healthy foodstuff because their regular consumption has been reported to decrease the risk of coronary heart disease (Blomhoff et al., 2006; Davis et al., 2007). The health benefits of walnuts are usually attributed to their chemical composition. Walnuts are a good source of essential fatty acids and tocopherols (Amaral et al., 2003, 2005). Linoleic acid is the major fatty acid, followed by oleic, linolenic, palmitic, and stearic (Amaral et al., 2003; Ruggeri et al., 1998; Savage et al., 1999); its high content of PUFA has been suggested to reduce the risk of heart disease by decreasing total and LDL-cholesterol and increasing HDL-cholesterol (Davis et al., 2007; Tapsell et al., 2004). In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fibber, melatonin (Reiter et al., 2005), plant sterols (Amaral et al., 2003), folate, tannins, and polyphenols (Anderson et al., 2001; Li et al., 2006). The chemical composition, namely the oil content and the fatty acid and tocopherols have been found to vary significantly among different walnut cultivars and environmental condition (Amaral et al., 2005).

Foods of plant origin, such as fruits and vegetables and whole grain products have been suggested as a natural source for antioxidants. Antioxidants can play an important role in disease prevention and health maintenance. Plant-derived products can be used either as a source of antioxidants in industry or for medicinal purposes

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(Ramadan and Moersel, 2006). The antioxidant effect showed by these products proceeds from phenolic compounds and phytochemicals, which protect from harmful effects of free radicals (Fukuda et al., 2003; Kornsteiner et al., 2006). Walnut possess a high content of α-tocopherol, (Amaral et al., 2005) a vitamin E family compound, which has antioxidant activity, mainly in the prevention of lipid oxidation process (Amaral et al., 2005; Koksak et al., 2006).

Plant-derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest (Cowan, 1999). The increased resistance to antibiotics and the problems presented by antimicrobial agents added in food (resistance, mutagenesis and carcinogenesis effects, for example) and public's pressure on the food industry to avoid chemical preservatives are the main factors justifying the search and development of new antimicrobial agents, especially those of natural origin (Rauha et al., 2000; Puupponen-Pimiä et al., 2001; Proestos et al., 2005). Antimicrobial activity of phenols as already been reported (Fernández et al., 1996; Oliveira et al., 2007; Pereira et al., 2006, 2007; Proestos et al., 2005; Sousa et al., 2006).

In previous works the chemical composition of walnut fruits produced in Portugal was presented (Amaral et al., 2003, 2004). Recently, our research group evaluated also the antioxidant activity and antimicrobial potential of walnut leaves (Pereira et al., 2007). Nevertheless, information about aqueous walnut kernel extracts antioxidant activity is not extended and is inexistent concerning their antimicrobial activity.

In the present work, six walnut cultivars (*cv.* Franquette, Lara, Marbot, Mayette, Mellanaise and Parisienne) grown in Portugal, were characterized in respect to their chemical composition, antioxidant potential and antimicrobial activity. The samples were analyzed for proximate constituents (moisture, fat, crude protein, ash), nutritional value and fatty acids profile by GC/FID. Antioxidant potential was accessed by the reducing power assay, the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and β-carotene linoleate model system. We also demonstrate for the first time, as far as we know, the antimicrobial activity of walnut extracts studying their antimicrobial capacity against gram positive (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*) and gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*), and fungi (*Candida albicans*, *Cryptococcus neoformans*).

2. Materials and methods

2.1. Samples

Walnut fruits were obtained from six *J. regia* L. cultivars (Franquette, Lara, Marbot, Mayette, Mellanaise and Parisienne), and were collected on September 2006 in Bragança, northeast of Portugal (41°47′47.50918″N, 6°46′5.71990″W, 744.341 m). The orchard has a planting density of 3.5 × 7 m. The trees are ten years old, being pruned when necessary. No phytosanitary treatments were applied. The fruits, ≈2 kg per cultivar, were handpicked from the soil, dried in a stove (Memmert Schwabach 854, 1994) at 30 °C, in the dark, for five days. The fruits were put in plastic bags and frozen to −20 °C until the analyses.

2.2. Samples preparation

Before each kind of analysis (chemical, antioxidant and antimicrobial) the walnuts were manually cracked and shelled, and then chopped in an appliance mill (model A327R1, Moulinex, Spain). For fatty acid analysis, crude oil was obtained from finely chopped nuts (ca. 15 g) extracted with light petroleum ether (b.p. 40–60 °C) in a Soxhlet apparatus; the remaining solvent was removed by vacuum distillation.

2.3. Chemical analysis

Analyses of moisture, total fat, ash, and protein contents were carried out in duplicate according to AOAC Official Methods (AOAC, 1995). Moisture was determined at 100 (±2 °C) (5 g test sample) in a stove until constant weight. Total fat was determined by extracting a known weight of sample (5 g) with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. The crude protein content ($N \times 4.38$) of the samples was estimated by the macroKjeldahl method. Carbohydrate content was estimated by difference of the other components using the following formula: carbohydrate content = 100% − (%moisture + protein + %fat + %ash). Energy was expressed as kilocalories, using the factors mentioned in the Portuguese Legislation (Decreto-Lei no. 167/2004): Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g lipid).

2.4. Fatty acid composition

Fatty acids were determined by gas chromatography with flame ionization detection (GC–FID) capillary column based on the following transesterification procedure: fatty acids were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v/v), during at least 12 h in a bath at 50 °C and 160 rpm; then the 5 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 5 mL of diethyl ether by shaking in a vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Milipore. The fatty acid profile was analyzed with a DAN1 model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 μm df). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. For each analysis 1 μL of the sample was injected in GC. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area. Fatty acids were identified by comparing the relative retention times of FAMEs peaks from samples with standards.

2.5. Extract preparation

For each cultivar, three powdered sub samples (∼5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman no. 4 paper. The aqueous extracts were frozen, lyophilized and redissolved in water at concentrations of 100 mg/mL and 10 mg/mL for antimicrobial and antioxidant activities assays, respectively.

2.6. Determination of total phenol content

Phenolic compounds concentration in the obtained extracts was estimated by a colorimetric assay based on procedures described by Singleton

and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytik Jena 200-2004 spectrophotometer). Gallic acid was used for constructing the standard curve (0.01–0.4 mM). The results are expressed as mg of gallic acid equivalents/g of extract (GAEs).

2.7. Antioxidant activity

Reagents. BHA (2-*tert*-butyl-4-methoxyphenol), TBHQ (*tert*-butylhydroquinone) and α-tocopherol were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol was obtained from Pronalab (Lisboa, Portugal). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Reducing power assay. The reducing power was determined according to a described procedure (Oyaizu, 1986; Ferreira et al., 2007). Various concentrations of sample extracts (2.5 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After incubation 2.5 mL of 10% trichloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge (Centorion K24OR-2003), for 8 min. The upper layer (5 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration. BHA and α-tocopherol were used as reference compounds.

Scavenging effect assay. The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Hatano et al., 1988). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discolouration using the equation: % scavenging effect $= [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 25% inhibition (EC_{25}) was calculated from the graph of scavenging effect percentage against extract concentration. BHA and α-tocopherol were used as reference compounds.

β-Carotene linoleate model system. The antioxidant activity of walnut kernel extracts was evaluated according to a described procedure (Mi-Yae et al., 2003). β-Carotene solution was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. Two millilitres of this solution were placed in a 100 mL round-bottom flask. After chloroform removal, at 40 °C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier, and 100 mL of distilled water were added to the flask under vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing 0.2 mL of different concentrations of walnut extracts. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm was measured. Absorbance readings were then recorded until the control sample had changed colour. A blank assay, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity was calculated using the following equation: Antioxidant activity = (β-carotene content after 2 h of assay/initial β-carotene content) × 100. The assays were carried out in triplicate and the results were expressed as mean values ± standard deviations. The extract concentration providing 25% antioxidant activity (EC_{25}) was calculated from the graph of antioxidant percentage against extract concentration. TBHQ was used as reference compound.

2.8. Antimicrobial activity

Reagents. Ampicillin and cycloheximide were of the highest available quality, and purchased from Merck (Darmstadt, Germany). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Microorganisms and culture conditions. Microorganisms CECT were obtained from the Spanish type culture collection (CECT) of Valencia University, while microorganisms ESA were clinically isolated strains identified in Microbiology Laboratory of Escola Superior Agrária de Bragança. Gram + (*B. cereus* CECT 148, *B. subtilis* CECT 498 and *S. aureus* ESA 7 isolated from pus) and gram − (*E. coli* CECT 101, *P. aeruginosa* CECT 108 and *K. pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*C. albicans* CECT 1394 and *C. neoformans* ESA 3 isolated from vaginal fluid) were used to screen antimicrobial activity of the three walnut cultivars. Microorganisms were cultured aerobically at 37 °C (Scientific 222 oven model, 2003) in nutrient agar medium for bacteria, and at 30 °C (Scientific 222 oven model, 2003) in sabouraud dextrose agar medium for fungi.

Test assays for antimicrobial activity. The screening of antibacterial activities against gram + and gram − bacteria and fungi and the determination of the minimal inhibitory concentration (MIC) were achieved by an adaptation of the agar streak dilution method based on radial diffusion (Hawkey and Lewis, 1994; Ferreira et al., 2004; Sousa et al., 2006). Suspensions of the microorganism were prepared to contain approximately 10^8 cfu/mL, and the plates containing agar medium were inoculated (100 μL; spread on the surface). Each sample (50 μL) was placed in a hole (3 mm depth, 4 mm diameter) made in the centre of the agar. Under the same conditions, different solutions of ampicillin (antibacterial) and cycloheximide (antifungal) were used as standards. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria after 24 h and fungi after 48 h. The diameters of the inhibition zones corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

3. Results and discussion

3.1. Chemical analysis and fatty acid composition

The proximate composition of the different analyzed walnut varieties is shown in Table 1. Fat was the highest constitute in all samples, ranging from 68.83% in *cv.* Marbot to 72.14% in *cv.* Franquette. Moisture and ash presented the lower values, with mean values of 4%. Moisture content was higher in *cv.* Mellanaise (4.50%) and lower in *cv.* Lara (3.85%), while ash content was higher in *cv.* Mellanaise (4.26%) and lower in *cv.* Parisienne (3.31%). The results indicate that walnut consumption conduce to a high input level of energetic value, ranged from 711 kcal in *cv.* Mayette and 727.4 kcal in *cv.* Franquette (Table 1). If the obtained results were compared with the ones previously obtained for fruits collected in the same cultivars and in the same orchard by Amaral et al. (2003), some differences can be observed namely ash, fat content (both higher on the present work) and carbohydrates (lower in the present work). Its differences could be attributed to the harvesting year and environmental condition, with different temperatures, rainfall and light, which can influence the chemical composition of fruits (Parcerisa et al., 1995).

Table 1
Proximate chemical composition (grams per 100 g of fresh weight) of walnut cultivars grown in Portugal (mean ± SD)

Cultivar	Moisture	Crude protein	Total fat	Carbohydrates	Ash	Energy (kcal)
Franquette	4.07 ± 0.03	15.65 ± 0.22	72.14 ± 0.27	3.88 ± 0.10	4.23 ± 0.02	727.4
Lara	3.85 ± 0.05	14.38 ± 0.27	70.59 ± 0.59	7.16 ± 0.36	4.03 ± 0.01	721.4
Marbot	4.10 ± 0.14	18.03 ± 0.29	68.83 ± 2.00	4.86 ± 2.3	4.18 ± 0.13	711.0
Mayette	3.99 ± 0.06	16.33 ± 0.02	71.89 ± 1.08	3.75 ± 1.49	4.04 ± 0.45	727.3
Mellanaise	4.50 ± 0.22	15.42 ± 0.14	69.75 ± 0.37	6.10 + 0.31	4.26 ± 0.02	713.8
Parisienne	4.28 ± 0.21	15.82 ± 0.12	71.51 ± 0.04	5.09 ± 0.59	3.31 + 0.31	727.2

Mean values and standard deviations of the fatty acid composition for the six walnut varieties are shown in Table 2. The fatty acids profile is constituted by 19 fatty acids, and all the studied varieties presented similar profile in constitution with some variations among fatty acids (Table 2). Linoleic acid was the most abundant one in all varieties ranging from 55.51% in *cv.* Franquette to 60.30% in *cv.* Lara. Oleic acid was the second in order of importance ranging from 14.92% (*cv.* Franquette) to 20.22% (*cv.* Lara) followed by linolenic acid, from 13.2% (*cv.* Parisienne) to 17.61% (*cv.* Mayette). In the remaining fatty acids only palmitic (5.95–6.61%) and stearic acids (2.70–3.07%) showed considerable amounts. In general terms, the obtained results were in agreement with the observed in Portuguese samples (Amaral et al., 2003) and other geographical origin such us Italy (Ruggeri et al., 1998), Canada (Li et al., 2007), and New Zealand (Zwarts et al., 1999).

Fatty acid analysis allowed the estimation of the different nutritional fractions: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). PUFA (due to the high content of linoleic acid) were the main component of total fat extracted from the studied cultivars, representing values higher than 69.55% of total fat. MUFA were present in percentages ranging from 15.16% (*cv.* Lara) to 20.53% (*cv.* Franquette). SFA content were the minor group, with lower than 10% (Table 2). Walnut fat is mostly unsaturated, and unsaturated fatty acids have been associated with beneficial effects on serum lipids (Feldman, 2002). Walnuts have high amounts of omega−6 and omega−3 PUFA, which are essential dietary fatty acids. Epidemiological and clinical studies suggest that omega−3 PUFA may have a significant role in the prevention of coronary heart disease and showed that the inclusion of walnut consumption in the diet had a significant protective benefit with respect to fatal and nonfatal coronary heart diseases events (Davis et al., 2007).

The study of phenolic content present in the different walnut cultivars aqueous extracts (Table 3) revealed values between 58.78 mg GAES/g in *cv.* Lara and 95.06 mg GAES/g in *cv.* Mayette. Comparing our results with the results obtained by Kornsteiner et al. (2006), that registered 1025 mg GAE/100 g, we concluded that all the extracts prepared by us showed high amounts in a ratio from 3.6

Table 2
Fatty acid composition (percent) of oil extracted from analyzed walnut samples (mean ± SD)

	Cultivar					
	Franquette	Lara	Marbot	Mayette	Mellanaise	Parisienne
$C_{14:0}$	0.03 ± 0.00	0.04 + 0.00	0.03 ± 0.00	0.02 + 0.00	0.02 + 0.00	0.03 + 0.00
$C_{15:0}$	0.01 ± 0.00	0.02 + 0.00	0.01 ± 0.00	0.01 + 0.00	0.01 + 0.00	0.02 + 0.00
$C_{16:0}$	6.61 ± 0.08	6.28 + 0.01	6.35 ± 0.01	5.95 + 0.05	6.31 + 0.04	6.16 + 0.05
$C_{16:1}$	0.10 ± 0.00	0.06 + 0.00	0.07 ± 0.00	0.07 + 0.00	0.07 + 0.00	0.08 + 0.00
$C_{17:0}$	0.04 ± 0.00	0.04 + 0.00	0.05 ± 0.00	0.04 + 0.00	0.05 + 0.00	0.04 + 0.00
$C_{17:1}c$-10	0.03 ± 0.00	0.02 + 0.00	0.02 ± 0.00	0.02 + 0.00	0.03 + 0.00	0.03 + 0.00
$C_{18:0}$	3.07 ± 0.02	2.80 + 0.03	2.78 ± 0.01	2.70 + 0.03	2.80 + 0.02	2.90 + 0.01
$C_{18:1}$n9c + t	20.22 ± 0.02	14.92 + 0.11	16.34 ± 0.11	16.08 + 0.08	17.09 + 0.16	19.50 + 0.12
$C_{18:2}$n6c	55.51 ± 0.03	60.30 + 0.34	59.66 ± 0.25	57.14 + 0.33	58.75 + 0.34	57.68 + 0.37
$C_{18:3}$n3	14.01 ± 0.00	15.20 + 0.17	14.33 ± 0.12	17.61 + 0.15	14.51 + 0.11	13.20 + 0.18
$C_{20:0}$	0.11 ± 0.00	0.10 + 0.00	0.09 ± 0.00	0.10 + 0.00	0.09 + 0.00	0.10 + 0.00
$C_{20:1}c$-11	0.18 ± 0.00	0.16 + 0.01	0.18 ± 0.00	0.18 + 0.00	0.17 + 0.00	0.18 + 0.00
$C_{20:2}c$-11.14	0.02 ± 0.00	0.03 + 0.00	0.03 ± 0.00	0.03 + 0.00	0.02 + 0.00	0.02 + 0.00
$C_{20:4}$n6	0.01 ± 0.00	0.00 + 0.00	0.00 ± 0.00	0.00 + 0.00	0.00 + 0.00	0.00 + 0.00
$C_{20:3}$n3 + $C_{21:0}$	0.01 ± 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00
$C_{22:0}$	0.03 ± 0.00	0.02 + 0.00	0.03 + 0.00	0.03 + 0.00	0.03 + 0.00	0.03 + 0.00
$C_{22:1}$n9	0.01 ± 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00
$C_{23:0}$	0.01 ± 0.00	0.01 + 0.00	0.01 + 0.00	0.00 + 0.00	0.01 + 0.00	0.01 + 0.00
$C_{24:0}$	0.01 ± 0.00	0.00 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00	0.01 + 0.00
SFA	9.91 ± 0.10	9.30 ± 0.04	9.35 ± 0.02	8.86 ± 0.08	9.33 ± 0.06	9.29 ± 0.06
MUFA	20.53 ± 0.02	15.16 ± 0.12	16.62 ± 0.11	16.35 ± 0.08	17.37 ± 0.16	19.79 ± 0.12
PUFA	69.55 ± 0.03	75.54 ± 0.51	74.03 ± 0.37	74.79 ± 0.48	73.29 ± 0.45	70.92 ± 0.55

Table 3
Total phenolic contents (mg GAES/g) and EC_{50} values (mg/mL) of walnut aqueous extracts

Cultivar	Total phenols contents	Reducing power (EC_{50})	DPPH (EC_{50})	β-Carotene bleaching (EC_{25})
Franquette	78.56 ± 0.01	0.19	0.15	1.63
Lara	58.78 ± 0.00	0.26	0.22	3.96
Marbot	60.83 ± 0.01	0.23	0.20	4.09
Mayette	95.06 ± 0.03	0.17	0.16	>50
Mellanaise	75.87 ± 0.01	0.25	0.22	3.02
Parisienne	91.09 ± 0.02	0.16	0.16	1.56

to 5.8 folds more. However, those authors also observed that walnut fruits showed the highest total phenolic contents when 10 nuts (almonds, Brazil nuts, cashews, hazelnuts, macadamias, peanuts, pecans, pines, pistachios and walnuts) were studied. Probably, the differences in the results could be explained by the different extraction methodologies. In the present study water at boiling temperature was use as extraction solvent whereas Kornsteiner et al. (2006) extracted the phenolic fraction with a solution of 75% acetone and 25% of 526 μmol/L sodium metabisulfite.

3.2. Antioxidant activity

In the present work, the antioxidant potential of walnut kernel samples was measured by three different assays: reducing power, scavenging activity on DPPH radicals and lipid peroxidation inhibition by β-carotene linoleate system.

Reducing power assay results are showed in Fig. 1. A concentration-dependent activity is observed. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration; a higher absorbance at 700 nm indicates a higher reducing power. Walnut's kernel showed high reducing power, even at concentrations below 1 mg/mL, being even more potent than BHA ($A_{700} = 0.12$ at 3.6 mg/mL) and α-tocopherol ($A_{700} = 0.13$ at 8.6 mg/mL) standards. All the six cultivars showed similar results, especially to concentrations below 0.1 mg/mL. Cultivar Parisienne presented the best results (lowest EC_{50} values), while *cv.* Lara presented the worse results (highest EC_{50} values for reducing power, Table 3).

Scavenging activity on DPPH radicals assay provides basic information about the antiradical activity of extracts, and is expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. In the present work, aqueous walnut lyophilized extracts of all the six walnut cultivars revealed a strong concentration-dependent antioxidant activity (Fig. 2). The scavenging activity on DPPH radicals were very high at low extract concentrations. At an extract concentration of 0.5 mg/mL, the activity ranged from 90.2%, for *cv.* Lara extracts, to 92.6% for *cv.* Parisienne (Fig. 2). The obtained results are much better that the ones achieved for BHA (96.0% at 3.6 mg/mL) and α-tocopherol (95.0% at 8.6 mg/mL) standards. The results were similar for all cultivars, being *cv.* Parisienne the one with lower EC_{50} value (0.16 mg/mL), and *cv.* Lara the one with the highest EC_{50} value (0.26 mg/mL) (Table 3).

The antioxidant activity of walnut lyophilized extracts measured by the bleaching of β-carotene is shown in Fig. 3. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of different

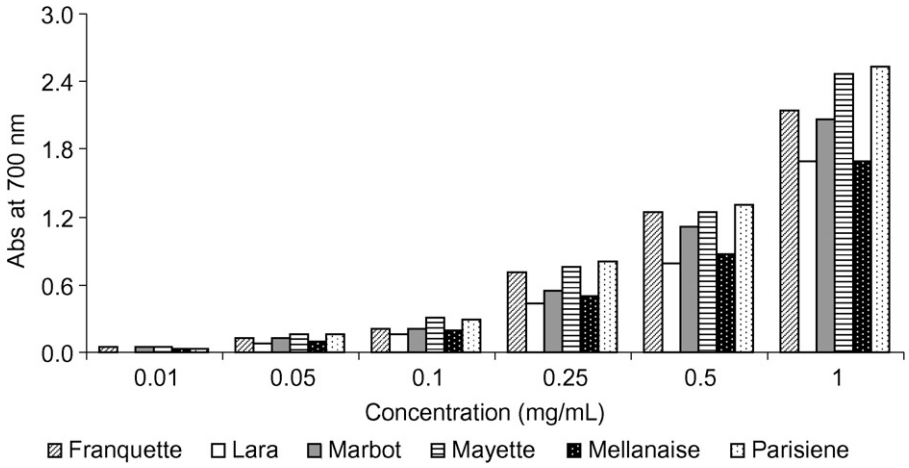

Fig. 1. Reducing power values of different walnut cultivars aqueous extracts. Each value is expressed as mean ± standard deviation.

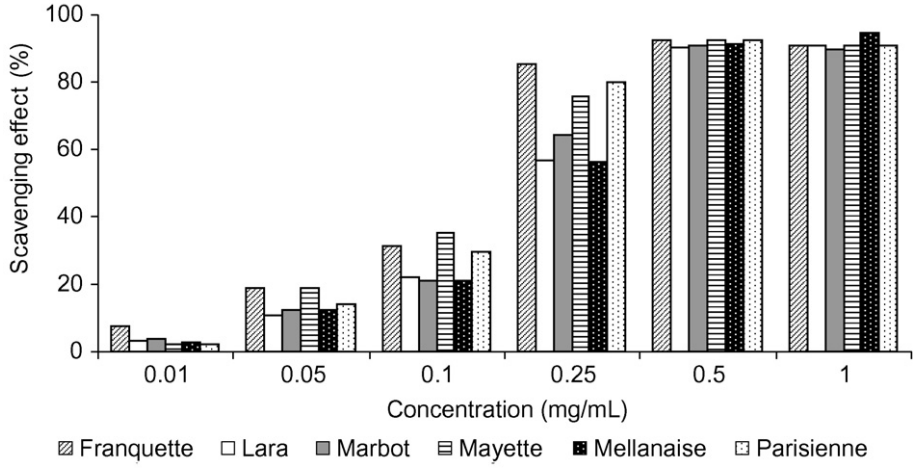

Fig. 2. Scavenging effect on DPPH of different walnut cultivars aqueous extracts. Each value is expressed as mean ± standard deviation.

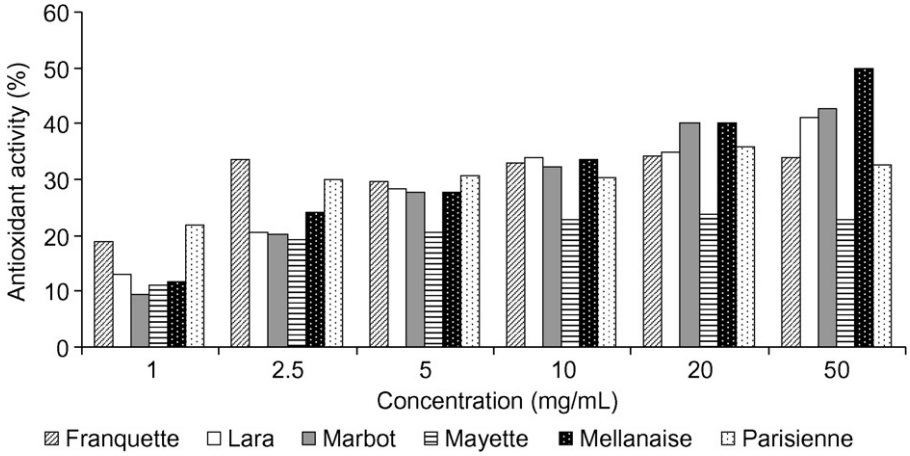

Fig. 3. Antioxidant activity (%) by β-carotene bleaching method of different walnut cultivars aqueous extracts. Each value is expressed as mean ± standard deviation.

antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001). Accordingly, the absorbance decreased rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time. Again, concentration-dependent activity is observed. The best results were obtained with *cv.* Parisienne, while *cv.* Mayette presented the worse results. In this assay, *cv.* Parisienne presented the lowest EC_{25} value (Table 3). TBHQ standard showed higher antioxidant activity than the tested samples (82.2% at 2 mg/mL).

Antioxidant activity of walnut kernels and walnut oils has already been evaluated by DPPH method (Espín et al., 2000). Nevertheless, the authors attribute the antioxidant capacity of walnut oils to the presence of tocopherols (Savage et al., 1999; Espín et al., 2000). The EC_{50} values of nuts (both walnut and heartnut) obtained from the DPPH assay were correlated significantly and inversely with the total tocopherol contents, indicating that tocopherols are the principal components responsible for the antioxidant effect. Espín et al. (2000) also suggested that the radical scavenging capacity of the lipid fraction of the different plant oils including walnut oil was mainly due to the content of all the tocopherols. Those authors used as extraction solvents methanol and ethanol (Espín et al., 2000), hexane (Li et al., 2006, 2007), ethyl acetate and different combinations of solvents (hexane/ethyl acetate/methanol), solvents that allowed the extraction of tocopherols.

The present work reports for the first time the use of water as solvent extraction, and therefore we attributed the high antioxidant activity to the high amount of total phenols (Table 3). Aqueous extracts of other matrices with high fat content, such as table olives (Pereira et al., 2006), stoned table olives (Sousa et al., 2008) and hazelnuts (Oliveira et al., in press), also showed relevant antioxidant activity. In this work, when we correlate the total phenols content and antioxidant activity of walnut aqueous extracts, in a general way an increase of total phenols content was associated with a decrease of EC_{50} values. A sig-

nificantly negative linear correlation was established between the total phenols content and EC_{50} reducing power values (determination coefficients 0.743; $p = 0.027$) and between total phenols content and EC_{25} for β-carotene bleaching system, when the *cv*. Mayette was excluded (determination coefficients 0.858; $p = 0.024$). However for DPPH scavenging effect EC_{50} values no significantly correlation was established (determination coefficients 0.547; $p = 0.093$). The negative linear correlation proves that the sample with highest phenols content shows higher antioxidant activities (*cv*. Parisienne). On walnut kernels several polyphenols have been identified, namely chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid (hydroxycinnamic acids) and ellagic acid and syringic acid (hydroxybenzoic acids) as well as syringaldehyde (hydroxybenzoic aldehyde) and juglone (quinone) (Colaric et al., 2005).

3.3. Antimicrobial activity

Table 4 shows the antimicrobial screening of six walnut aqueous extracts (from *cv*. Franquette, Lara, Marbot, Mayette, Mellanaise and Parisienne) against *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans* and *C. neoformans*. The minimal inhibitory concentration (MIC) values for the tested bacteria and fungi were determined as an evaluation of the antimicrobial activity of the samples.

All the tested extracts revealed antimicrobial activity. However the action of each walnut extract variety was different according the tested microorganism.

Gram positive bacteria (*B. cereus*, *B. subtilis*, *S. aureus*) were inhibited by *cv.* Lara aqueous extract at very low concentrations, presenting MICs of 1 mg/mL for *B. cereus*, and 0.1 mg/mL for *B. subtilis* and *S. aureus*. *S. aureus* was also inhibited for *cv*. Franquette but at higher extract concentration (100 mg/mL) (Table 4).

Gram negative bacteria (*P. aeruginosa*, *E. coli* and *K. Pneumoniae*) were also sensible for some walnut extracts. *cv*. Lara inhibited the growth of *K pneumoniae* (MIC of 100 mg/mL), *cv. Mayette* inhibited the development of *P. aeruginosa* and *E. coli* with MICs of 50 and 10 mg/mL, respectively, and *cv.* Mellanaise inhibited the growth of *E. coli* and *K. pneumoniae* at concentration of 100 mg/mL.

All the walnut varieties exhibited antifungi activity against the tested species (*C. albicans* and *C. neoformans*). *C. albicans* was susceptible to five walnut varieties, only being resistant to *cv.* Mellanaise extract. The higher inhibition was observed with *cv*. Lara extract (MIC of 1 mg/mL). On the other hand *C. neoformans* was resistant to *cv*. Mellanaise extract at the tested concentrations (maximum of 100 mg/mL) and was very sensitive to *cv*. Franquette extract (MIC of 0.1 mg/mL). As expected, the standards ampiciline (antibacterial) and cycloheximide (antifungal) presented lower MICs than the walnut extracts. Usually, pure active compounds reveal more activity than crude extracts.

Similarly to the results observed in walnut leaves (Pereira et al., 2007), *cv*. Lara fruit extracts proved to be the most promissory cultivar. The extracts of this cultivar inhibited five of the eight microorganisms tested, and was effective against gram positive and gram negative bacteria and fungi, which increase their potentiality.

Some authors demonstrated that phenolic compounds possess antimicrobial activity (Rauha et al., 2000; Puupponen-Pimiä et al., 2001; Proestos et al., 2005; Pereira et al., 2006, 2007) that varied in a dependent manner with a total phenolic content of the tested extracts (Vaquero et al., 2007). But in this study no phenolic concentration dependence was observed. The *cv.* Lara extract, with lower total phenols (58.78 mg GAES/g) was the most effective against microorganisms. This situation could be related with the nature of the fenolic fraction that constitutes the extracts. In fact, studies with pure phenolic compounds showed distinct antimicrobial activity according to the studied compounds (Vaquero et al., 2007).

Table 4
Antimicrobial activity [MIC (mg/mL)] of walnut aqueous extracts

	Cultivar						Standards	
	Franquette	Lara	Marbot	Mayette	Mellanaise	Parisienne	Ampicillin	Cycloheximide
B. cereus	100 (−)	1 (++)	100 (−)	100 (−)	100 (−)	100 (−)	0.00313 (++++)	NT
B. subtilis	100 (−)	0.1 (+++)	100 (−)	100 (−)	100 (−)	100 (−)	0.0125 (++++)	NT
S. aureus	100 (+++)	0.1 (+++)	100 (−)	100 (−)	100 (−)	100 (−)	0.00625 (++++)	NT
P. aeruginosa	100 (−)	100 (−)	100 (−)	50 (++++)	100 (−)	100 (−)	0.00625 (++++)	NT
E. coli	100 (−)	100 (−)	100 (−)	10 (+++)	100 (+++)	100 (−)	0.00625 (++++)	NT
K. pneumoniae	100 (−)	100 (+++)	100 (−)	100 (−)	100 (++++)	100 (−)	0.00625 (++++)	NT
C. albicans	10 (++++)	1 (++++)	10 (+)	50 (+++)	100 (−)	50 (+++)	NT	0.0125 (++)
C. neoformans	0.1 (++++)	100 (−)	10 (+++)	50 (+)	10 (+)	50 (++)	NT	0.00625 (++++)

No antimicrobial activity (−), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone > 9 mm. Standard deviation ± 0.5 mm. NT – not tested.

In some cases, walnut extracts, with higher MICs, showed similar antimicrobial activity to walnut leaf extracts for the same cultivars (Pereira et al., 2007) and other plant extracts like hazel leaves (Oliveira et al., 2007), hazelnut fruits (Oliveira et al., in press) and olive fruits (Pereira et al., 2006; Sousa et al., 2006). Walnut fruit extracts proved to have antifungi action against *C. albicans* and *C. neoformans*, two important yeasts that cause human diseases, and which was not observed in the referred matrices (walnut leaves, hazel leaves, hazelnut fruits and table olives).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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