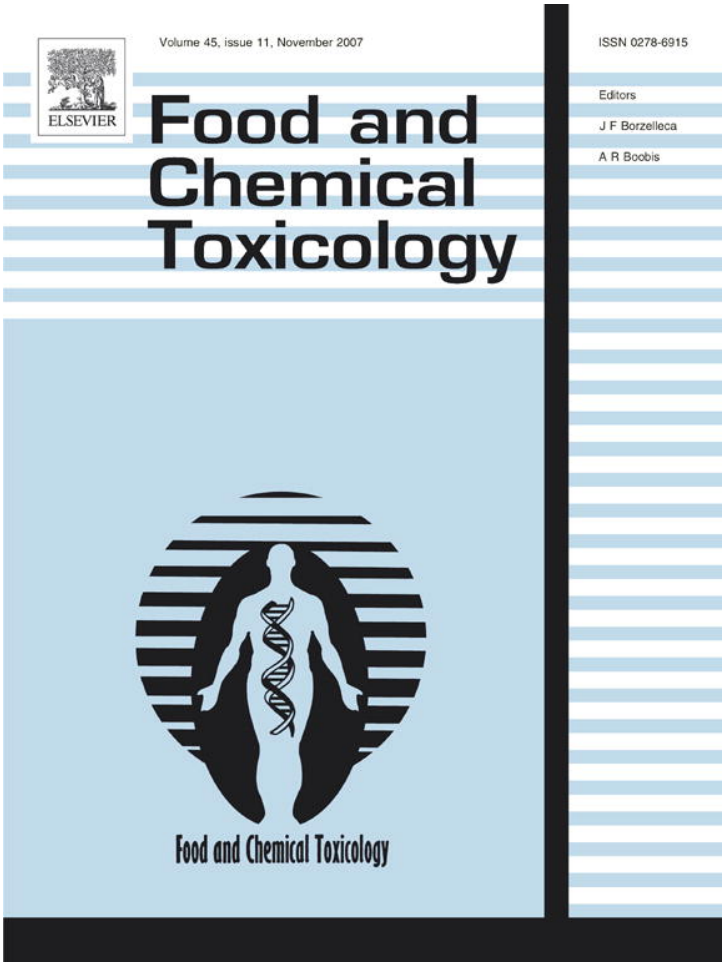

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Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars

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Abstract

Different cultivars of walnut (*Juglans regia* L.) leaves (*Cv.* Lara, Franquette, Mayette, Marbot, Mellanaise and Parisienne) grown in Portugal, were investigated in what concerns phenolic compounds and antimicrobial and antioxidant properties. Phenolics analysis was performed by reversed-phase HPLC/DAD and 10 compounds were identified and quantified: 3- and 5-caffeoylquinic acids, 3- and 4-*p*-coumaroylquinic acids, *p*-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3-rhamnoside. The antimicrobial capacity was screened against Gram positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*) and Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) and fungi (*Candida albicans*, *Cryptococcus neoformans*). Walnut leaves selectively inhibited the growth of Gram positive bacteria, being *B. cereus* the most susceptible one (MIC 0.1 mg/mL). Gram negative bacteria and fungi were resistant to the extracts at 100 mg/mL. Lara walnut leaves were also submitted to antibacterial assays using 18 clinical isolates of *Staphylococcus* sp. Antioxidant activity was accessed by the reducing power assay, the scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and β-carotene linoleate model system. In a general way, all of the studied walnut leaves cultivars presented high antioxidant activity (EC_{50} values lower than 1 mg/mL), being *Cv.* Lara the most effective one.

Keywords: Walnut leaves; Phenolics; Antimicrobial activity; Antioxidant properties

1. Introduction

The *Juglans* genus (family Juglandaceae) comprises several species and is widely distributed throughout the world. The Persian or common walnut (*Juglans regia* L.) is its best-known member, constituting an important species of deciduous trees found primarily in the temperate areas and commercially cultivated in the United States, western South America, Asia, and central and southern Europe. In Portugal, this species is common in all over the country (Anonimous, 1999). Green walnuts, shells, kernels and seeds, bark and leaves have been used in the pharmaceutical and cosmetic industries (Stampar et al., 2006). Leaves are easily available and in abundant amounts, while tree bark is scarce and its collection compromise the plant life.

Walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional

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medicine for treatment of venous insufficiency and haemorrhoidal symptomatology, and for its antidiarrheic, antihelmintic, depurative and astringent properties (Van Hellemont, 1986; Bruneton, 1993; Wichtl and Anton, 1999). Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities have also been described (Valnet, 1992; Gîrzu et al., 1998). In Portugal, as in some other European countries, especially in rural areas, dry walnut leaves are frequently used as an infusion.

Phytochemicals, such as phenolic compounds, are considered beneficial for human health, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation (Silva et al., 2004; Pulido et al., 2000; Tseng et al., 1997). They have been shown to possess free radical-scavenging and metal-chelating activity in addition to their reported anticarcinogenic properties (Middleton, 1998).

In walnut leaves, naphtoquinones and flavonoids are considered as major phenolic compounds (Wichtl and Anton, 1999). Juglone (5-hydroxy-1,4-naphthoquinone) is known as being the characteristic compound of *Juglans* spp. and is reported to occur in fresh walnut leaves (Bruneton, 1993; Wichtl and Anton, 1999; Gîrzu et al., 1998; Solar et al., 2006). Nevertheless, because of polymerization phenomena, juglone only occurs in dry leaves at vestigial amounts (Wichtl and Anton, 1999). Several hydroxycinnamic acids (3-caffeoylquinic, 3-*p*-coumaroylquinic and 4-*p*-coumaroylquinic acids) and flavonoids (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified quercetin 3-pentoside and kaempferol 3-pentoside derivatives) of different walnut cultivars collected at different times were studied by our group in a previous work (Amaral et al., 2004). In addition, the existence of 5-caffeoylquinic acid was also reported (Wichtl and Anton, 1999).

Some studies have demonstrated the antimicrobial activity of walnut products, particularly of bark (Alkhawajah, 1997), and the specific compound juglone (Clark et al., 1990), but information about the leaf is almost inexistent (Qa'dan et al., 2005). On the other hand, antioxidant potential of walnut leaves was not studied.

The aim of the present work was to determine the phenolic compounds and to evaluate the antimicrobial and antioxidant capacity of different cultivars of walnut leaves (*Cv.* Lara, Franquette, Mayette, Marbot, Mellanaise and Parisienne) grown in Portugal. For this purpose phenolics were determined by reversed-phase HPLC/DAD. The antimicrobial activity was screened using different microorganisms, namely Gram positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*) and Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella penumoniae*) bacteria and fungi (*Candida albicans*, *Cryptococcus neoformans*) and also 18 *Staphylococcus* sp. strains provided by clinical isolates. The evaluation of the antioxidant properties involved several assays: reducing power, scavenging effects on DPPH radicals and β-carotene linoleate model system.

2. Experimental

2.1. Walnut leaf sample

Walnut leaves were obtained form six *Juglans regia* L. cultivars: Franquette, Marbot, Mayette, Mellanaise, Lara and Parisienne, and were collected at 31st May 2006 in Bragança, northeast of Portugal (6°46′W, 41°49′N, 670 m a.s.l.). The orchard has a planting density of 7 × 7 m. The trees have 22 years old, being pruned when necessary. No phytosanitary treatments were applied. The leaves were collected from the middle third of branches exposed to sunlight, put in plastic bags and immediately frozen at −20°. The plant material was then freeze dried.

2.2. Identification and quantification of phenolic compounds

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

Phenolic compounds analysis. *Standards*. The standards used were from Sigma (St. Louis, MO, USA) or Extrasynthèse (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use.

HPLC-DAD system for analysis of phenolic compounds. Chromatographic separation was achieved as previously reported (Amaral et al., 2004) with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (250_4.6 mm, 5 lm particle size, Merck, Darmstadt, Germany) column. The solvent system used was a gradient of water/formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 20% B at 5 min, 25% B at 12 min, 30% B at 15 min, 40% B at 20 min, 45% B at 30 min, 50% B at 40 min, 70% B 45 min and 0% B at 46 min. The flow rate was 1 mL min^{-1}, and the injection volume was 20 μL. Detection was accomplished with a diode array detector (DAD) (Gilson), and chromatograms were recorded at 320 and 350 nm. Spectral data from all peaks were accumulated in the 200–400 nm range. Data were processed on an Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France).

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, with detection at 320 nm for phenolic acids and at 350 nm for flavonoids. 3-Caffeoylquinic acid was quantified as 5-caffeoylquinic acid, 3- and 4-*p*-coumaroylquinic acids were quantified as *p*-coumaric acid; the quercetin 3-pentoside derivative and quercetin 3-xyloside were quantified as quercetin 3-galactoside. The other compounds were quantified as themselves.

2.3. 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. CECT microorganisms were obtained from the Spanish type culture collection of Valencia University. ESA microorganisms were isolated in the Northeast Hospital Centre (Bragança-Portugal) from different biological fluids, and deposited 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 six walnut leaves cultivars. Also 18 *Staphylococcus* sp. strains clinically isolated from different biological fluids were used to additionally evaluate the antibacterial activity of Lara cultivar. 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; 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). 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 assays with the standards were carried out using DMSO solutions, which was chosen as the best solvent. After comparative toxicity assays this solvent showed to be not toxic. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria or fungi, after 24 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.

2.4. Antimicrobial 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, 1896). 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% triclhoroacetic 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 50% inhibition (EC_{50}) 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 leaf 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 leaf 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 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant percentage against extract concentration. TBHQ was used as reference compound.

3. Results and discussion

3.1. Phenolic compounds analysis

The HPLC-DAD analysis of walnut leaves aqueous extracts revealed the presence of several hydroxycinnamic acid and flavonoid derivatives. By this means, in the six analysed cultivars, it was possible to identify ten phenolic compounds: 3- and 5-caffeoylquinic acids, 3- and 4-*p*-coumaroylquinic acids, *p*-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3-rhamnoside (Figs. 1 and 2). 5-Caffeoylquinic and *p*-coumaric acids were not described before (Amaral et al., 2004) in these cultivars, being detected now.

Juglone was not found in the samples, which is not surprising considering its slight solubility in hot water and that it is volatile by steam (Anonimous, 1989).

The quantification of the phenolics present in the different cultivars extracts revealed a high amount of these compounds, ranging from ca. 65 to 73 g/kg, dry basis. Flavonols were always the major compounds, varying between 54.8% and 62.9% of total phenolics (Table 1). Mayette and Franquette cultivars showed the highest content of compounds, while Lara presented the lowest one (Table 1).

All samples exhibited the same phenolic profile, in which quercetin 3-galactoside was the major compound, corresponding to ca. 26.8% of total phenolics, followed by 3-caffeoylquinic acid (ca. 19.7% of total compounds) (Fig. 3). *p*-Coumaric acid was the minor compound, representing ca. 1.4% of total phenolics (Fig. 3).

In a previous work of our research group (Amaral et al., 2004) we study the evolution of phenolic compounds in the leaves of different walnut varieties from May to September. In that study no significant variation were observed according the sampling time. However, in the paper herein we have used the samples collected in May, the collection time with the highest quantity of phenolic compounds. When comparing the results with those previously obtained (Amaral et al., 2004) it could be noticed that the total phenolics content found now is considerably higher, mainly due to an increase phenolic acids derivatives contents. Considering that the analysed leaves were collected in the same period and from the same trees of the previous work, this

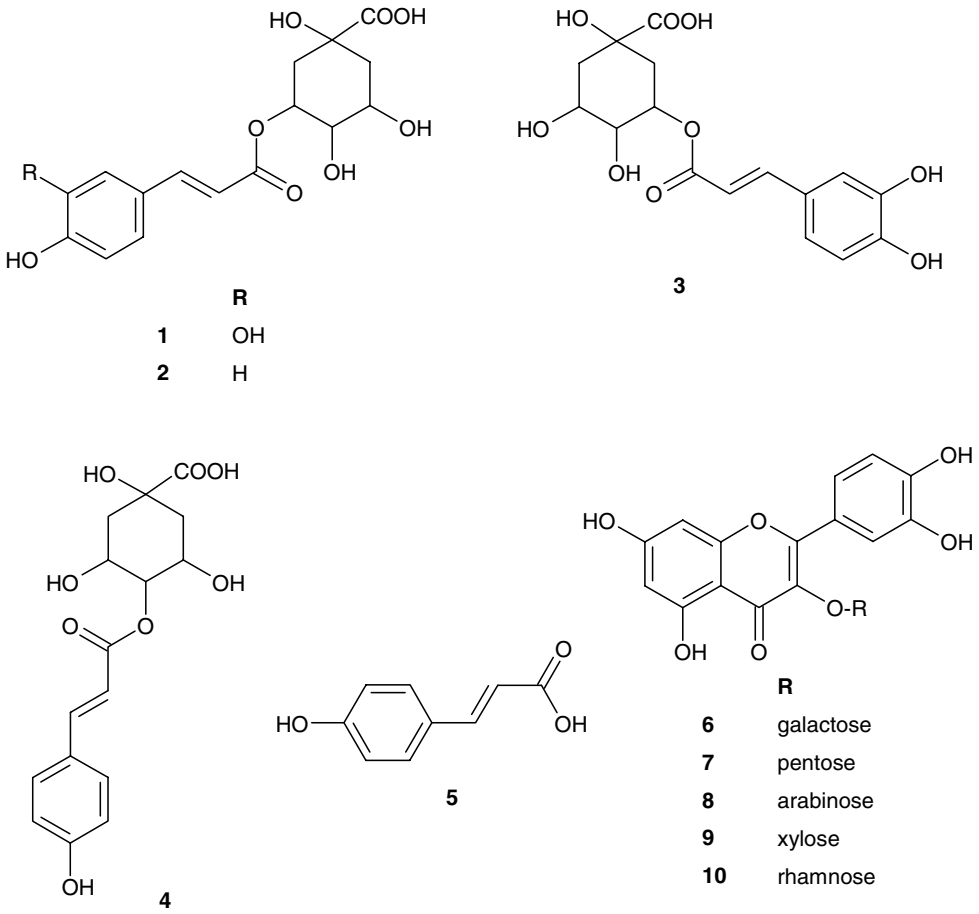

Fig. 1. Chemical structures of the phenolic compounds identified in walnut leaves. (1) 3-caffeoylquinic acid; (2) 3-*p*-coumaroylquinic acid; (3) 5-caffeoylquinic acid; (4) 4-*p*-coumaroylquinic acid; (5) *p*-coumaric acid; (6) quercetin 3-galactoside; (7) quercetin 3-pentoside derivative; (8) quercetin 3-arabinoside; (9) quercetin 3-xyloside; (10) quercetin 3-rhamnoside.

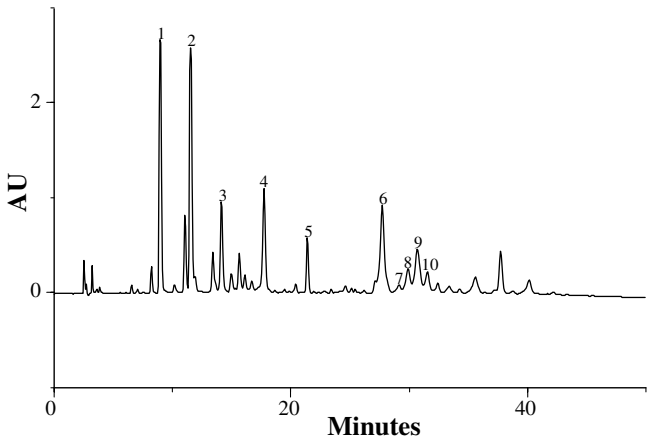

Fig. 2. HPLC-DAD of phenolic compounds in walnut leaves (*Cv.* Mayette). Detection at 320 nm. Peaks: (1) 3-caffeoylquinic acid; (2) 3-*p*-coumaroylquinic acid; (3) 5-caffeoylquinic acid; (4) 4-*p*-coumaroylquinic acid; (5) *p*-coumaric acid; (6) quercetin 3-galactoside; (7) quercetin 3-pentoside derivative; (8) quercetin 3-arabinoside; (9) quercetin 3-xyloside; (10) quercetin 3-rhamnoside.

rise could be attributed to the drying procedure to which they were subjected: in this work the leaves were freeze-dried, which is a faster and less drastic drying process than that of ventilated stove at 30 °C for five days used before (Amaral et al., 2004), that allows enzymatic reactions, with possible alteration and loss of compounds.

Nevertheless, the influence of environmental factors cannot be excluded. As observed before, quercetin 3-galactoside is the main compound, but a decrease in its relative amount was now observed (24.3–30.4%). In addition, *p*-coumaric acid, not identified before, is now the minor compound, as above mentioned.

3.2. Antimicrobial activity

The walnut leaves aqueous extracts were screened for their antimicrobial properties 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 (Table 2) were determined as an evaluation of the antimicrobial activity of the samples.

Despite all the cultivars revealed antimicrobial activity, the response for each microorganism tested was different. The extracts presented similar antimicrobial capacity,

Table 1
Phenolic compounds in walnut leaves samples (mg/kg, dry basis)[a]

Cultivar	Compound[b]										Total
	1	2	3	4	5	6	7	8	9	10	
Lara	13812.8 (5.5)	4795.5 (52.0)	6036.6 (219.5)	2591.8 (63.4)	1172.1 (29.8)	15722.9 (181.8)	1473.3 (7.8)	5680.7 (51.2)	10031.2 (116.8)	3279.5 (41.6)	64596.4
Franquette	14236.7 (128.3)	4688.0 (108.3)	8131.4 (276.2)	2849.4 (50.5)	884.6 (26.5)	19449.2 (56.4)	1890.2 (68.6)	5950.4 (96.1)	10382.0 (484.1)	4209.7 (198.2)	72671.6
Mellanaise	14822.8 (534.3)	4960.6 (10.3)	6648.6 (57.0)	3328.5 (1.1)	1080.0 (11.4)	16320.0 (59.0)	1443.9 (55.8)	5294.9 (71.9)	10174.5 (147.8)	4149.8 (65.1)	68223.6
Mayette	14110.9 (131.1)	5893.5 (223.9)	5428.7 (51.4)	3419.0 (2.7)	1166.2 (3.3)	19683.9 (42.6)	1910.4 (3.2)	7005.3 (38.4)	10345.4 (71.3)	3410.7 (0.7)	72374.0
Parisienne	12057.3 (185.7)	5993.2 (359.4)	4463.9 (70.3)	3041.5 (83.2)	874.1 (11.0)	21682.1 (130.7)	2263.9 (3.6)	7679.0 (50.6)	10201.5 (79.2)	3030.0 (41.1)	71286.5
Marbot	14521.5 (12.5)	5668.3 (134.2)	7224.2 (132.8)	2784.2 (37.2)	830.3 (4.9)	17343.0 (58.6)	1606.9 (42.6)	5476.7 (59.3)	9735.8 (81.7)	3995.1 (74.1)	69186.0

[a] Results are expressed as mean (standard deviation) of three determinations.
[b] (1) 3-caffeoylquinic acid; (2) 3-*p*-coumaroylquinic acid; (3) 5-caffeoylquinic acid; (4) 4-*p*-coumaroylquinic acid; (5) *p*-coumaric acid; (6) quercetin 3-galactoside; (7) quercetin 3-pentoside derivative; (8) quercetin 3-arabinoside; (9) quercetin 3-xyloside; (10) quercetin 3-rhamnoside.

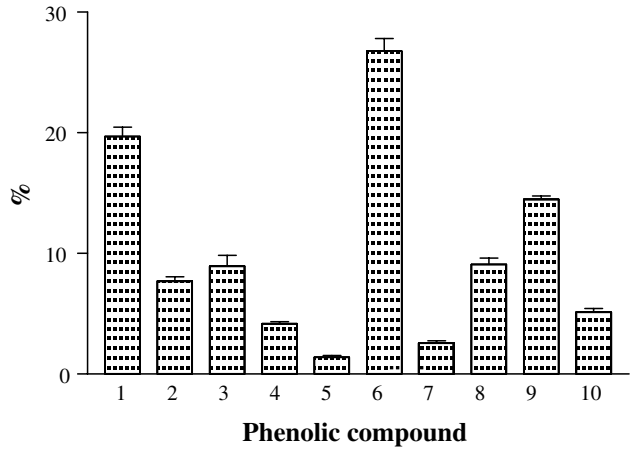

Fig. 3. Phenolic profile of walnut leaves. Values represent mean, and standard error bars are on the top of each column. Compounds: (1) 3-caffeoylquinic acid; (2) 3-*p*-coumaroylquinic acid; (3) 5-caffeoylquinic acid; (4) 4-*p*-coumaroylquinic acid; (5) *p*-coumaric acid; (6) quercetin 3-galactoside; (7) quercetin 3-pentoside derivative; (8) quercetin 3-arabinoside; (9) quercetin 3-xyloside; (10) quercetin 3-rhamnoside.

inhibiting only Gram+ bacteria and in the order *B. cereus* > *S. aureus* > >*B. subtilis*. *B. cereus* was the most susceptible microorganism, presenting MICs of 0.1 mg/mL. The tested Gram− bacteria (*E. coli*, *P. aeruginosa* and *K. peumoniae*) and fungi (*C. albicans* and *C. neoformans*) species were resistant to all cultivars. The selectivity obtained for this walnut leaves is clearly different from that of walnut bark, which revealed a broad spectrum antimicrobial activity: the bark inhibited the growth of several species of pathogenic microorganisms, representing both Gram+ (*S. aureus* and *S. mutatis*) and Gram− (*E. coli* and *P. aeruginosa*) bacteria and a pathogenic yeast (*C. albicans*) (Alkhawajah, 1997). Recently, Darmani et al. (2006) reported the growth inhibition of various cariogenic bacteria (*Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus casei* and *Actinomyces viscosus)* by walnut aqueous extracts. The most sensitive organisms were *A. viscosus*, followed by *S. mutans*, *S. salivarius*, with *L. casei* being the most resistant. All these species are Gram+ bacteria, which is consistent with our results.

Lara walnut leaves proved to be the most promissory cultivar to inhibit Gram+ bacteria growth, presenting lower MICs and higher growth inhibition zones. Attending to this fact, this sample was submitted to further antibacterial assays, against 18 *Staphylococcus* sp. strains clinically isolated from sputum, pus and blood (Table 3). The results obtained suggest a broad activity of Lara walnut leaves against all the *S. aureus* strains, in a concentration-dependent manner. The strains isolated from sputum seem to be the most susceptible: they exhibited MICs of 0.1 mg/mL, while for the strains isolated from pus and blood it ranged between 0.1 and 1 mg/mL. *S. bovines*, *S. slimi*, *S. sintata* and *S. capitis* strains were also more resistant, with MICs of 1 mg/mL. *S. chromogenes* was the less susceptible *Staphylococcus* species, being the strain isolated

Table 2
Antimicrobial activity of leaf extract of different walnut cultivars

Cultivar	MIC (mg/mL)							
	B. cereus	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
Lara	0.1 (+ + + +)	10 (+ + + +)	0.1 (+ + + +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Franquette	0.1 (+ +)	10 (+ + +)	0.1 (+ +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Mellanaise	0.1 (+ +)	10 (+ + +)	0.1 (+ +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Mayette	0.1 (+ +)	10 (+ + + +)	1 (+ + + +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Parisienne	0.1 (+ +)	10 (+ +)	1 (+ + + +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)
Marbot	0.1 (+ + + +)	10 (+ +)	1 (+ + + +)	100 (−)	100 (−)	100 (−)	100 (−)	100 (−)

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.

Table 3
Antimicrobial activities (inhibition zones in mm) of different *Cv*. Lara leaf extract concentration on clinical isolates of *Staphylococcus* sp. strains

Strain	Biological fluid	*Cv* Lara leaf extract (mg/mL)		
		0.1	1	10
S. aureus ESA 11	Sputum	10.5 ± 1.29	10.3 ± 0.50	12.5 ± 1.29
S. aureus ESA 12	Sputum	8.8 ± 0.50	13.5 ± 1.29	17.0 ± 0.82
S. aureus ESA 13	Sputum	12.3 ± 0.96	16.5 ± 2.65	19.3 ± 1.71
S. aureus ESA 14	Sputum	10.8 ± 0.96	13.3 ± 0.50	19.0 ± 0.82
S. aureus ESA 16	Sputum	6.5 ± 7.51	12.5 ± 1.00	15.0 ± 0.82
S. aureus ESA 21	Sputum	12.5 ± 2.08	18.8 ± 1.89	19.8 ± 5.32
S. aureus ESA 10	Pus	0.0 ± 0.00	12.3 ± 2.63	29.0 ± 6.73
S. aureus ESA 15	Pus	10.5 ± 2.38	11.5 ± 1.00	13.3 ± 2.36
S. aureus ESA 20	Pus	6.0 ± 6.93	11.5 ± 1.73	17.0 ± 1.63
S. aureus ESA 17	Blood	11.5 ± 9.40	15.8 ± 2.5	18.3 ± 2.06
S. aureus ESA 18	Blood	0.0 ± 0.00	15.8 ± 1.71	18.8 ± 2.06
S. aureus ESA 19	Blood	13.3 ± 2.06	17.5 ± 5.07	17.3 ± 2.87
S. capitis ESA 23	Sputum	0.0 ± 0.00	9.8 ± 1.50	10.3 ± 1.26
S. sintata ESA 24	Sputum	0.0 ± 0.00	0.0 ± 0.00	11.3 ± 1.50
S. slimi ESA 26	Sputum	0.0 ± 0.00	16.5 ± 1.91	16.0 ± 2.16
S. bovines ESA 27	Pus	0.0 ± 0.00	11.3 ± 2.63	15.3 ± 0.50
S. chromogenes ESA 25	Pus	0.0 ± 0.00	0.0 ± 0.00	14.0 ± 1.63
S. chromogenes ESA 28	Blood	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

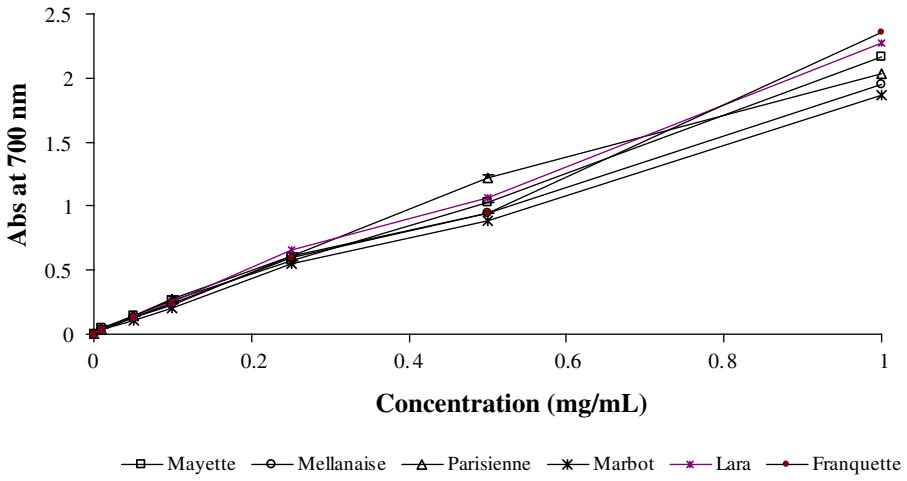

Fig. 4. Reducing power values of different cultivars of walnut leaf extracts. Each value is expressed as mean ± standard deviation.

from blood the most resistant one. These results are important considering that *S. aureus* can produce several types of enterotoxins that cause gastroenteritis, which is a major food-borne disease in most countries (Halpin-Dohnalek and Marth, 1989). Natural products have been a particularly rich source of anti-infective agents. Flavonoids

showed posses antimicrobial activity, and quercetin and other related compounds acts essentially by enzyme inhibition of DNA gyrase (Cushnie and Lamb, 2005).

3.3. Antioxidant activity

In the reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each extract. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} concentration can be monitorized by measuring the formation of Perl's Prussian blue at 700 nm. The reducing power of the walnut leaves extracts increased in a concentration-dependent way, as shown in Fig. 4. Walnut leaves showed high reducing powers at very low concentrations (<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. The reducing power of the different cultivars was very similar and followed the order Lara > Parsienne ~ Mellanaise ~ Franquette > Mayette > Marbot (Table 4).

The radical scavenging activity assay constitutes a screening method currently used to provide basic information on the antiradical activity of extracts. The walnut leaves extracts displayed an effective concentration-dependent scavenging capacity, for concentrations below 0.5 mg/mL (Fig. 5). Lara and Marbot cultivars showed the highest and the lowest activities, respectively (Table 4). These results are much better than those obtained for BHA (96.0% at 3.6 mg/mL) and α-tocopherol (95.0% at 8.6 mg/mL).

Table 4
EC_{50} values (mg/mL) of different walnut leaf samples

Cultivar	Reducing power (EC_{50})	DPPH (EC_{50})	β-carotene bleaching (EC_{50})
Lara	0.192	0.151	0.742
Franquette	0.208	0.156	0.894
Mellanaise	0.206	0.195	1.645
Mayette	0.215	0.187	0.444
Parisienne	0.201	0.170	0.764
Marbot	0.229	0.202	0.819

The antioxidant activity of walnut leaves extracts measured by the bleaching of β-carotene is shown in Fig. 6. In the tested system linoleic acid free radical attacks the highly unsaturated β-carotene. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Denyer and Stewart, 1998). In the absence of antioxidants the absorbance at 470 nm decreases rapidly, whereas in their presence, the colour, and thus absorbance, is retained for a longer time. The results obtained with walnut leaf extracts indicated a concentration-dependent antioxidant capacity (Fig. 6), following the order Mayette > Lara > Parisienne > Marbot > Franquette > Mellanaise (Table 4). However, the protection of β-carotene bleaching provided by the samples was lower than that TBHQ standard (82.2% at 2 mg/mL).

The antioxidant capacity of walnut polyphenols has already been described. Anderson et al. (2001) reported the in vitro inhibition of human plasma and low density lipoproteins (LDL) oxidation by a walnut extract containing ellagic acid, gallic acid and flavonoids.

Fukuda et al. (2003) described the remarkable superoxide dismutase-like activity and radical scavenging effect of 14 walnut polyphenols and recently examined the *in vivo* antioxidative effect of a polyphenol-rich walnut extract on oxidative stress in mice with type 2 diabetes (Fukuda et al., 2004). In walnut leaves we have found a considerable amount of quercetin heterosides. Quercetin as other flavonoids are able to protect against chemically induced DNA damage in human lymphocytes and increase the total antioxidant capacity of plasma (Wilms et al.,

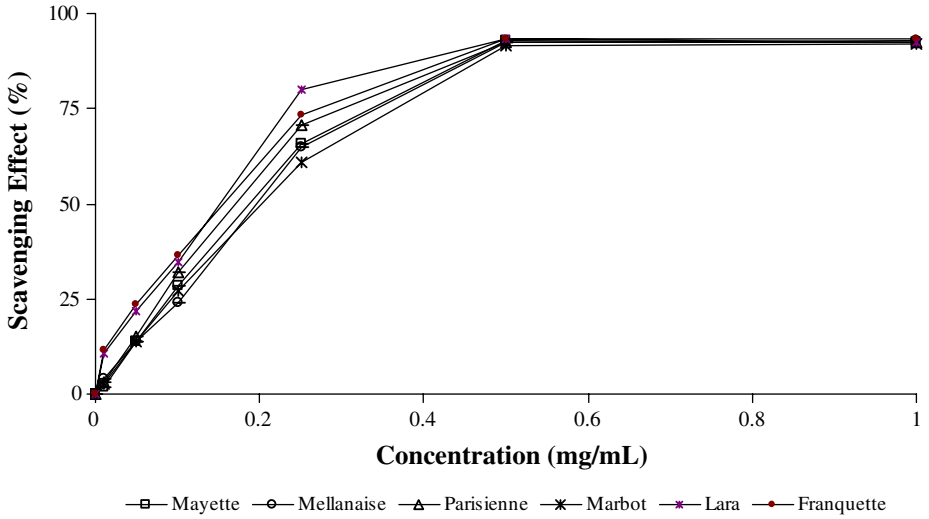

Fig. 5. Scavenging effect on DPPH of different cultivars of walnut leaf extracts. Each value is expressed as mean ± standard deviation.

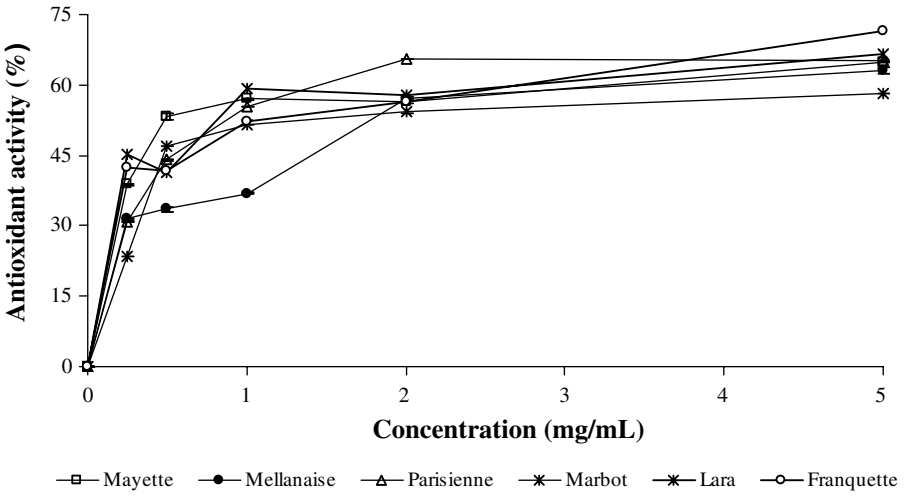

Fig. 6. Antioxidant activity (%) by β-carotene bleaching method of different cultivars of walnut leaf extracts. Each value is expressed as mean ± standard deviation.

2005; Tieppo et al., 2007), increased genomic stability in cirrhotic rats, suggesting beneficial effects, probably by its antioxidant properties. Flavonoids can also protect cells by acting as free radical scavengers, inhibiting DNA damage and mutagenicity (Edenharder and Grünhage, 2003; Salter et al., 2004). Otherwise, the hydroxycinnamic acid derivatives, such as 5-caffeoylquinic acid and caffeic acid present an antioxidant activity upon low density lipoprotein peroxidation (Laranjinha et al., 1994). So, a synergistic effect between quercetin heterosides and hydroxycinnamic derivatives can explain the walnut leaves extract antioxidant activity (Liu, 2003). Despite these studies, this is the first time that the antioxidant potential of walnut leaves is reported.

In conclusion, the results obtained in this study demonstrate that walnut leaves may be a good candidate for employment as antimicrobial agent against bacteria responsible for human gastrointestinal and respiratory tract infections. These results are particularly important against *S. aureus*, due to its ability to produce enterotoxins and exceptionally resistance to a number of phytochemicals. Walnut leaves may also constitute a good source of healthy compounds, namely phenolics, suggesting that it could be useful in the prevention of diseases in which free radicals are implicated. Despite some studies performed with walnut fruits, as far as we know, this is the first report considering the antioxidant and antimicrobial potential of walnut leaves.

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