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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