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Infusions and decoctions of *Castanea sativa* flowers as effective antitumor and antimicrobial matrices

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ABSTRACT

Chestnut trees are one of the most important crops in the north-eastern part of Portugal, representing millions of euros of yearly income. There are many ancestral claims of the health benefits of the consumption of chestnut flowers in infusions that remain unproven. In this manuscript, the antitumor and antimicrobial potential of chestnut flowers from two cultivars, Judia and Longal, extracted through infusions and decoctions are reported. In terms of antitumor activity, the most sensitive cell lines were HepG2 and HCT15 with the cultivar Judia showing higher activity for HCT15 and Longal for HepG2, regardless of the extraction methods. Regarding the antibacterial activity of the extracts, decoctions proved to be more effective with lower minimum inhibition concentrations, while infusions were better in terms of antifungal activity. The good overall antimicrobial activity could justify the inclusion of the flowers in food chain processing to act as a natural antimicrobial. Furthermore, the results corroborate some of the ancestral claims of the consumption of these flowers.

1. Introduction

Chestnut is one of the most important source of income for the north-eastern section of Portugal, where almost 85% of total chestnut trees are grown, being chestnut fruits almost entirely exported to European markets. The chestnut honey made from flowers is highly appreciated, although the most appreciated commodity is the nut of this tree. Ancestral claims report that "teas" of chestnut leaves and flowers are used for medicinal purposes, namely as mucolytic, antispasmodic and anti-dysenteric treatments, among others (Neves et al., 2009). Our research group has been working with chestnut fruits (Carocho et al., 2012), flowers (Barros et al., 2010) and leaves, with very good results regarding antioxidant (Carocho et al., 2014) and anti-candida (Barros et al., 2013) activity of the flowers. Recently, we have also reported the profile in phenolic compounds and antioxidant properties of flower decoctions and infusions, in which the most prevalent molecules were trigalloyl-HHDP-glucoside and pentagalloyl glucoside (Carocho et al., 2014).

The pressing search for new treatments and drugs for diseases has been increasing in recent years. Today, more than 60% of cancer drugs derive from natural products, which turn the spotlight on plants and other natural resources (Gordaliza, 2007). Of these compounds, many come from the secondary metabolism of plants, like terpenes, alcaloids and phenolic compounds. There are many effects that these compounds display toward tumors; namely the complete inhibition of growth, apoptosis induction, suppression of the secretion of matrix metalloproteinases and reduction of tumor invasive behavior (Carocho and Ferreira, 2013). The same issue occurs in the search for antimicrobial drugs, with resistance to antibiotics and antifungals rising in recent years along with a decrease in the supply of new drugs for these microorganisms. More interest has been placed on extracts from natural matrices for their chemical diversity and, in some cases, very potent actions against pathogens with known resistance to specific drugs (Saleem et al., 2010). Although some gallotannins have displayed some *in vitro* antitumor and antiproliferative activities (Zhang et al., 2009), as well as effective inhibition of some bacterial pathogens (Engels et al., 2009), to the authors knowledge, this is the first report

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on the antitumor and antimicrobial activities of decoctions and infusions of chestnut flowers of the most important cultivars in the European market, Judia and Longal.

2. Materials and methods

2.1. Standards and reagents

Fetal bovine serum (FBS), L-glutamine, nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and Dulbecco's Modified Eagle Medium (DMEM) media were acquired from Thermo Fischer Scientific (Waltham, MA, USA). Ellipticine and sulforhodamine B were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). All other chemicals were purchased at specialized laboratory retailers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Chestnut flower samples and preparation of decoctions and infusions

Castanea sativa Mill. flowers were collected in June 2013 near Oleiros, Bragança (north-eastern Portugal) (41°51′02″N, 6°49′54″W). Two cultivars were chosen, Longal and Judia, for the representative expression of their fruits (chestnuts) in the Portuguese and European market. The samples were then lyophilized (FreeZone 4.5, Labconco, Kansas, KS, USA) and subsequently milled down to a fine powder and mixed to obtain a homogenate sample.

For the infusions, the lyophilized samples (1 g) were added to 200 mL of boiling distilled water and left to stand for 5 min, and finally filtered through a Whatman filter paper No. 4. For the decoctions, the lyophilized samples (1 g) were added to 200 mL of distilled water, heated to boiling point and maintained at this state for 5 min. The mixture was then left to stand at room temperature for 5 min more, and then filtered through a Whatman filter paper No. 4. The obtained infusions and decoctions were then frozen, lyophilized and dissolved in water or 5% DMSO (final concentration 10 mg/mL) for *in vitro* evaluation of antitumor and antimicrobial activities, respectively. The final solutions were further diluted to different concentrations, ranging from 500 to 195 μg/mL, 50 to 450 μg/mL and 30 to 1000 μg/mL for antitumor, antibacterial and antifungal activities evaluation, respectively.

2.3. Antitumor activity and hepatotoxicity

The antitumor activity was evaluated by the Sulphorhodamine B assay that has been previously described by Guimarães et al. (2013), in which four human tumor cell lines were tested: MCF7 (breast adenocarcinoma), HCT15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The positive control used was ellipticine. These cells were maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine (MCF7 and HCT15) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. For each sample, the cell line was plated with a density of 7.5×10^3 cells/well for MCF7 and HCT15 or 1.0×10^4 cells/well for HeLa and HepG2 in 96-well plates.

Furthermore, a hepatotoxicity evaluation was conducted, using a cell culture prepared from fresh liver of a pig, which was slaughtered in a certified facility. This methodology was conducted, according to the authors Abreu et al. (2011). Before confluence was reached, and by monitoring the growth with a phase-contrast microscope every day, the cell culture was sub-cultured and also plated in a 96 well plat, using a density of 1.0×10^4 cells/well. DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin was added, and once again ellipticine was used as positive control. Finally, the results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth).

2.4. Antibacterial activity

For the antibacterial activity, the procedure previously described by Espinel-Ingroff (2001) was followed. The following Gram-negative bacteria were used: *Escherichia coli* (ATCC (American type culture collection) 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method. The bacterial colony forming unites (CFU)/mL were cultured overnight and adjusted at a concentration of 1×10^5 CFU/mL, using a spectrophotometer at 625 nm. In order to determine the absence of contamination, the inocula were cultured on a solid medium. The sample solutions were pipetted into the wells containing 100 μL of Tryptic Soy Broth (TSB) and afterwards, 10 μL of inoculum was added to all the wells. After incubating the microplates at 37 °C for 24 h the MIC were determined by adding 40 μL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) with posterior incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The MICs obtained from the susceptibility of the various bacteria were also tested with a colorimetric assay, which is based on the reduction of a INT color and compared with positive control for each bacterial strains (CLSI, 2009). Finally, the lowest concentration with no growth was regarded as the MBC and was determined by serial sub-cultivation of 10 μL into microplates containing 100 μL of TSB. Streptomycin and ampicillin were used a positive controls, while 5% dimethyl sulfoxide (DMSO) was used as negative control.

2.5. Antifungal activity

For the antifungal activity, the procedure previously described by Booth (1971) was followed, using the following microfungi: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM (Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan) 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum var. cyclopium* (food isolate). The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-cultured once a month.

Initially, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL/well, and the inocula were stored at 4 °C for further use. To verify the absence of contamination, dilutions of the inocula were cultured on solid MA. To determine the MICs, serial dilutions were used, with 96-well microplates. Furthermore, the sample solutions were added to broth malt medium with the fungal inoculum. The microplates were incubated for 72 h at 28 °C. The MICs were defined as the lowest concentrations without visible growth, determined through a binocular microscope. The minimum fungicidal concentrations (MFC) were determined by serial sub-cultivation of 2 μL in microtitre plates containing 100 μL of malt broth per well and