# 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.

**Keywords** *Castanea sativa*; Antitumor; Antibacterial; Antifungal; Infusion; Decoction

## 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 towards tumours; 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 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 (northeastern 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 1,95 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 ºC, in a humidified air incubator containing 5% $CO_2$. For each sample, the cell line was plated with a density of $7.5\times10^3$ cells/well for MCF7 and HCT15 or $1.0\times10^4$ 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 $1.0\times10^4$ 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 35210), *Pseudomonas aeruginosa* (ATCC (American type culture collection) 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 $1\times10^5$ 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 24h 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 $1.0 \times 10^5$ 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, wih 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 further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, which indicated that 99.5% of the original inoculum was killed. DMSO at 5% was used as a negative control, while bionazole and ketokonazole were used as positive controls.

### 2.6. Statistical analysis

All the assays were carried out in triplicate for both decoctions and infusions of each cultivar. A Tukey's honestly significant difference post hoc test with $\alpha = 0.05$ preceded of a one-way analysis of variance (ANOVA) result in the different letters seen in the tables, and the results were expressed as mean values ± standard deviations (SD). The statistical analysis was carried out using the SPSS v. 20.0 program.

## 3. Results and Discussion

### 3.1. Antitumor activity

The values of the $GI_{50}$ of the antitumor activity of the samples are detailed in **Table 1**. Ellipticine, a very strong antitumor compound which intercalates with DNA and inhibits topoisomerase II was used as a positive control. None of the extracts showed inhibition towards the non-tumor liver primary culture (PLP2), corroborating the security of the consumption of these flowers. The tested human tumor cell lines of breast, colon, cervical, and liver carcinoma are some of the most used for primary antitumor screening. No effect was detected at the highest concentration in any sample towards the HeLa cervical cancer cell line. For the MCF7, breast cancer, all the samples had the same effect with no statistical differences, while for the HCT15 colon carcinoma, decoction of Judia (DJ) was the most effective. For the cell line HepG2, the activity of Longal was significantly lower than the one observed for Judia, regardless of the extraction method. The *in vitro* antitumor activity observed could be related to the presence of trigalloyl-HHDP-glucoside, followed by pentagalloyl glucoside, the two main polyphenols detected in the flowers, belonging to the ellagitannins family, which has shown antitumor activity *in vitro*. Pentagalloyl glucoside can act as a proapoptotic, anti-proliferative, anti-angiogenic and anti-metastasic compound and has been used in prostate, lung, breast cancer as well as sarcoma (Zhang et al., 2009). Although the antitumor potential was not as low as ellipticine, the results were very close to the $GI_{50}$ of other natural plant extracts (Guimarães et al., 2013), and in some cases even better (Santos et al., 2013). Moreover and contrarily to ellipticine, the samples did not show any toxicity for non-tumor cells.

## 3.2. Antibacterial activity

The antibacterial activity towards different Gram-positive and Gram-negative bacteria, of the different samples is displayed in **Table 2**. Regarding the MIC's, which represent the minimum concentration to inhibit the growth of a colony overnight, all the samples had lower values than the positive control ampicillin, which is quite satisfactory. For *E. coli* and *E. cloacae*, all the samples also had lower values that streptomycin. For *S. typhimurium,* infusion of Judia (IJ), DJ, and infusion of Longal (IL) displayed lower values than streptomycin, while DL had the same value. For *B. cereus*, the minimum inhibition level of IJ was lower than the antibiotic, while in DJ and IL the values were the same. For *M. flavus*, IL was the only sample with higher values that streptomycin. In all other cases, the antibiotic had lower inhibition concentrations. Overall, the decoctions proved better antibacterial effects with eight lowest MIC values, while infusions only displayed five. In terms of MBC, the bactericidal effects of the samples were quite interesting. Except IJ in *S. aereus* and IL in *M. flavus*, all the other extracts had lower MBC's than ampicillin. Furthermore, for S. *typhimurium*, *E. coli* and *E. cloacae*, all the samples also had lower values than streptomycin. DJ and IL displayed the same MBC as streptomycin for *S. aureus* and *B. cereus*, along with decoction of Longal (DL) in the latter. The lowest values of MBC were quite well spread throughout the four samples, with each one having the same number of lowest concentrations for different bacteria. The better results found in the MIC's of decoctions could be related to the higher quantity of trigalloyl-HHDP-glucoside, quercetin-3-O-glucuronide, the higher amounts of organic acids and better antioxidant results (Carocho et al., 2014).

### 3.3. Antifungal activity

The antifungal activity of the samples is shown in **Table 3**. Bifonazole had lower MIC's and MFC's values when compared to ketoconazole for all species, with exception of *P. funiculosum*, in which both synthetic antifungal drugs had MIC=0.2 mg/mL. Regarding the MIC's, for A. *fumigatus*, all but IL had higher values than both the positive controls, which had the same value as ketoconazole. For *A. versicolor*, IJ, DJ and DL displayed the same MIC as ketoconazole. All samples had better results than ketoconazole for *A.*

*ochraceus*, but only IJ had lower values than bifonazole. The best sample for *A. niger* was IL, along with *T. viride* and *P. funiculosum*. For *P. verrucosum*, all the samples had higher concentrations when compared to both positive controls. Overall, the best sample was IL, with the lower concentration needed to inhibit a greater number of fungi. Concerning MFC's, the samples had lower fungicidal concentrations than ketoconazole for *A. fumigatus*, *A. ochraceus*, *A. niger, T. viride*, and *P. ochrochloron*, while in *A. versicolor*, and *P. verrucosum* all samples had higher concentrations. For bifonazole, the samples also displayed higher concentrations in *A. fumigatus*, *A. versicolor*, and *P. verrucosum*. Generally, the infusions, mainly of Longal, had the lower fungicidal concentration in most of the analysed fungi. While the decoctions had better effect as antibacterial agents, the infusions proved to be more effective against fungi, especially the cultivar Longal. In terms of phenolic composition, antioxidant activity and presence of organic acids, the infusions were quite poor when compared to the decoctions (Carocho et al., 2014), which means the fungicidal compounds could be sensitive to heat extraction (lower periods of heat for infusions) and other studies should be carried out with cold extractions. These results are in line with other natural plant matrices, like *Laurus nobilis*, which had very similar activity against the same species of fungi (Dias et al., 2014). In terms of cultivars, Longal seems to have higher antimicrobial activity than Judia, regardless of the extraction method, especially against fungi.

## 4. Conclusion

Overall, the decoctions seemed to be better against HCT15 and HepG2 cancer cell lines as well as against bacteria. The infusions seemed more suitable against fungi. This manuscript brings some more proof and further corroborates the claims of the benefits of consumption of chestnut flowers for some illnesses. The antitumor activity, and lack of toxicity towards normal cells reassures the safety of consumption while the antimicrobial activity confers some applicability of these flowers in the food processing chain to be used as natural antimicrobials. Further research of individual compounds could be carried out to determine their individual contribution for each activity.

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## 5. Conflict of interest

The authors declare that they have no conflict of interest.

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