






Chemical characterization and bioactive properties of a coffee-like beverage prepared from *Quercus cerris* kernels

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In the present study, a coffee-like beverage was prepared from *Quercus cerris* seeds. Seeds presented 60.4% carbohydrates (of which 26.9% is fiber), 6.3% proteins and 4.0% fat. In the coffee-like beverage, the TPC and TFC were 2070.2 mg GAE per L and 285.3 mg CEQ per L, respectively. The IC₅₀ values determined using FRAP and DPPH assays were, respectively, 203.1 mg L⁻¹ and 271.6 mg L⁻¹. Concerning the reactive species scavenging capacity, the extract was more effective against O₂^{-•}, HClO and *NO (namely, IC₅₀ = 17.24 ± 0.24 µg mL⁻¹, IC₅₀ = 9.25 ± 0.92 µg mL⁻¹ and IC₅₀ = 0.65 ± 0.06 µg mL⁻¹). Qualitative and quantitative analyses by 1D- and 2D-NMR and LC-MS indicated high levels of ellagic acid and gallotannin or ellagitannin derivatives. The optimal range of non-cytotoxic concentrations in Caco-2 and HT29-MTX cell lines was between 0.1 and 1.0 µg mL⁻¹. This is the first study that evaluates the *Q. cerris* seed extract as a beverage.

1. Introduction

Coffee is one of the most widely appreciated and consumed beverages in the world, mainly due to its stimulating effects. Depending on the country custom, this beverage is consumed in different quantities and forms, such as espresso or with milk. Likewise, coffee's attributes are widely appreciated by consumers, such as taste and aroma, being related to the roasting process. Due to this global interest, a current and highly discussed topic pertains the positive and negative effects of coffee consumption on human health as well as the possible alternatives to address the health related disadvantages. Modern consumers are much aware of potential health benefits of food and food ingredients,

demanding products with functional properties. In fact, different varieties of coffee-like substitutes such as chicory, malt or soluble cereal mixtures are available on the market.¹ Their consumption is mainly related to the absence of caffeine and their richness in diverse bioactive compounds (such as antioxidants), being appropriate for consumption by consumers of all ages as well as by consumers with medical issues.

Quercus spp. is an evergreen or deciduous tree that comprises around 450 species, belonging to the Fagaceae family and representing an important group of temperate and tropical climatic areas.² These species produce a widely known fruit, frequently identified as acorn, with several uses, especially in the Mediterranean region. In Serbia, the *Quercus cerris* seeds are considered to be a resource with low value, being mainly used for making bread or to prepare a drink that can substitute traditional coffee.³ This traditional use as a coffee substitute began during the war periods, when coffee deprivation was most pronounced and oak kernels were cheap and easily available.⁴ Nevertheless, to the best of our knowledge, the information related to the bioactive compounds present in this coffee-like beverage, their scavenging capacity or their effects on intestinal cells is scarce. The aim of this paper is to evaluate the roasted kernel nutritional composition as well as the antioxidant activity, phenolic composition, cell viability and toxic effects of the coffee-like beverage prepared with this ingredient in order to stimulate the valorisation of this under-explored natural resource as a new active ingredient by the food industry.

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2. Materials & methods

2.1. Plant material

The plant material was collected on Kosmaj Mountain in the Sumadija region, Serbia, from autochthone sources during October and November 2016. The plant was identified as *Quercus cerris* at the Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia, by standard botanical determination keys: Javorka and Csapody, Flora of the Republic of Serbia and Flora Europaea. Plant seeds were liberated from the shell and left in draft to dry. Afterwards, they were shortly exposed to heat (200 °C) for 30 minutes and left to cool until being milled to powder (2–3 mm).

2.2. Macronutrient analysis

The moisture content was instrumentally determined using a moisture analyzer (SMO 01, Scaltec Instruments, Germany). The ash content was evaluated by incinerating the sample in a muffle furnace at 550 °C, according to the AOAC 923.03 method.⁵ The protein content (nitrogen conversion factor 6.25) was quantified using the Kjeldahl procedure. Total fat was determined by the Soxhlet extraction method.⁶ The carbohydrate amount was obtained by difference.⁷ All analyses were performed in triplicate and the results are expressed as g per 100 g.

2.3. Total vitamin E and vitamer quantification

The lipid fraction for vitamin E quantification was obtained by Soxhlet extraction with petroleum ether (4 h). The chromatographic separation of the compounds was achieved on a normal phase Supelcosil™ LC-SI (3 µm; 75 × 3.0 mm; Supelco, Bellefonte, PA, USA) according to the study by Alves *et al.*⁸ Chromatographic data were analyzed using JASCO Chrom NAV Chromatography Software (Jasco, Japan). The results are expressed in mg per 100 g of the sample. Analyses were performed in triplicate.

2.4. *Quercus cerris* beverage preparation

Powdered samples (a tablespoon with ~8.8 g) were subjected to extraction with 250 mL of water at 100° C for 5 min. Extracts were filtered through Whatman no. 1 filter paper and kept under refrigeration (4 °C) prior to use. For the reactive oxygen and nitrogen scavenging assays (ROS and RNS, respectively) and cell viability assays, the extracts were lyophilized (Telstar, Cryodos, Spain) and stored at –20 °C until analysis.

2.5. Determination of the total phenolic content (TPC)

The Total Phenolic Content (TPC) was spectrophotometrically determined according to the Folin–Ciocalteu procedure⁹ with minor modifications.¹⁰ A calibration curve with gallic acid was used as the reference (linearity range = 5–100 mg L⁻¹, R² > 0.999) to obtain a correlation between absorbance and standard concentration. The results were expressed as mg of gallic acid equivalents per litre of solution (mg GAE per L). The assay was carried out in triplicate.

2.6. Determination of the total flavonoid content (TFC)

The Total Flavonoid Content (TFC) was determined by a colorimetric method based on the formation of flavonoid-aluminum compounds, according to the method described by de Francisco *et al.*,¹¹ with minor modifications. A calibration curve was prepared with catechin (linearity range = 2.5–500 µg mL⁻¹, R² > 0.996). The results were expressed as mg of catechin equivalents per litre of solution (mg CAE per L). This parameter was evaluated in triplicate.

2.7. *In vitro* antioxidant activity

The antioxidant activity was evaluated by DPPH· radical-scavenging activity and ferric reducing antioxidant power (FRAP) as detailed in the following sections.

2.7.1. DPPH free radical scavenging assay. The reaction mixture was prepared directly on a 96 well microplate, according to the study by Almeida *et al.*¹² The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{\text{DPPH}} - A_s)/A_{\text{DPPH}}] \times 100$, 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% of radical scavenging activity (EC₅₀) was calculated from the graph of RSA percentage against the extract concentration.

2.7.2. Ferric reducing antioxidant power (FRAP) assay. FRAP assay was carried out according to the method described by Benzie and Strain,¹³ with minor modifications. A calibration curve was prepared with ferrous sulphate (FeSO₄·7H₂O) as the standard (linearity range: 25–500 µM, R² > 0.985). The radical scavenging activity (RSA) was calculated as a percentage. The extract concentration providing 50% of radical scavenging activity (EC₅₀) was calculated from the graph of RSA percentage against the extract concentration. This methodology was performed in triplicate.

2.8. Identification of phenolic compounds by qualitative and quantitative analyses by ¹H NMR and LC-DAD-MS

In order to assess the composition of the obtained beverages, ¹H-NMR and 2D-NMR experiments were performed. Extracts were prepared with CDCl₃ and deuterated methanol, in order to observe the presence of constituents with different polarities. A beverage sample was prepared in deuterated water to assess the composition. Briefly, 100 mg of powder was weighed in an Eppendorf tube and 2 mL of solvent was added for the extract analysis at different polarities. The Eppendorf tube was sonicated for 10 minutes, and centrifuged (18 900 ref) and the liquid was transferred to a NMR tube for analysis. For the analysis of the beverage powdered sample, 500 mg were subjected to solvent extraction by maceration with 10 mL of deuterated water at 100° C for 5 min. The liquid was cooled and filtered and transferred to a NMR tube for analysis. NMR analysis was performed on a Bruker AVANCE III spectrometer operating at 400.13 MHz for ¹H-NMR. For solvent suppression, a noesy1d Bruker sequence was used. For 2D spectra,

Heteronuclear Single Quantum Coherence–Distortionless Enhanced Polarization Transfer (HSQC–DEPT), Heteronuclear Multiple Bond Correlation (HMBC), Correlation Spectroscopy (COSY) and Total Correlation Spectroscopy (TOCSY) were performed using presaturation on the water signal for the identification of the class of compounds occurring in mixtures.

Afterwards, the quantitative analysis of phenolic derivatives was performed by LC-DAD-MS. An Agilent 1260 chromatograph (Santa Clara, CA, USA) equipped with a 1260 diode array (DAD) and a Varian MS-500 ion trap mass spectrometer was used. As the stationary phase, an Agilent Eclipse XDB C-18 (3.0 × 150 mm) 3.5 μm was used. The mobile phases were water 0.1% formic acid (A) and acetonitrile (B). The elution gradient started at 90% A and then decreased to 0% over 30 min. The flow rate was 0.5 mL min⁻¹ and the temperature of the column was set at 30 °C. At the end of the column, a “T connector” split the flow rate to DAD and MS-500. The DAD detector was used to quantify galloyl derivatives. Gallic acid was used as the reference compound. The chromatograms were monitored at 280 nm and UV-Vis spectra were acquired in the range of 200–650 nm. The sample injection volume was 20 μL. MS spectra were recorded in the 50–2000 *m/z* range, using an ESI ion source operating in the negative ion mode. Fragmentation of the main ionic species was obtained by the turbo data depending scanning (TDDS) instrument function. The compound identification was performed based on the fragmentation spectra as well as in comparison with the literature.¹⁴ The phenolic compound quantification was performed by the calibration curve method by using the curve $y = 38.387x + 202.53$ ($R^2 = 0.996$) for gallic acid.

2.9. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) scavenging assays

A Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) was employed to evaluate the reactive species scavenging capacity. This plate was equipped with a thermostat and capable of measuring chemiluminescence, fluorescence and UV/Vis. Standards and samples were prepared according to the study by Marangi *et al.*¹⁵ and the same positive controls were used.

2.9.1. Superoxide radical scavenging assay. The superoxide anion radical (O₂^{•-}) assay was performed according to the study by Marangi *et al.*¹⁵

2.9.2. Hydrogen peroxide scavenging assay. The hydrogen peroxide (H₂O₂) scavenging assay was also performed according to the study by Marangi *et al.*¹⁵

2.9.3. Hypochlorous acid scavenging assay. The hypochlorous acid (HOCl) scavenging assay was based on a fluorescence methodology.¹⁵

2.9.4. Nitric oxide scavenging assay. The nitric oxide (•NO) scavenging assay used quercetin as the positive control, expressing the results as the inhibition (in percentage) of NO-induced oxidation of DAF-2, according to the study by Marangi *et al.*¹⁵

2.9.5. Peroxyl radical scavenging assay. The peroxyl radical (ROO•) scavenging assay was performed according to the study

by Ou *et al.*¹⁶ based on the monitoring effect of the tested extracts on the fluorescence decay resulting from ROO-induced oxidation of fluorescein. The standard control used was Trolox and the results were expressed as ROO-induced oxidation of fluorescein.

2.10. Cell viability assays

2.10.1. Cell lines, primary cell isolation and culture conditions. Caco-2 clone type C2BBE1 (passage 76–79) and HT29-MTX (passage 33–35) cells are grown separately in tissue culture flasks (Orange Scientific, Belgium) in a complete medium, consisting of DMEM supplemented with 10% (v/v) inactivated FBS, 1% (v/v) L-glutamine, 1% (v/v) non-essential amino acids and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U mL⁻¹ penicillin and 100 U mL⁻¹ streptomycin) at 37 °C and 5% CO₂ under a water saturated atmosphere in an incubator (CellCulture® CO₂ Incubator, ESCO GB Ltd, UK). At 90–95% confluence, both cell lines were harvested using trypsin (Invitrogen) and re-plated in T125 flasks.

2.10.2. MTT assay. The sample effect on cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) conversion assay. For that, Caco-2 and HT29-MTX cells were seeded separately in 96-well microplates at 25 × 10³ cells per well in supplemented DMEM and incubated for 24 h at 37 °C in a 5% CO₂ environment. The sample and negative (DMEM) and positive controls (1% (v/v) Triton X-100) were added in triplicate to the cell culture. The medium was changed and the cells were treated with sample/controls for 24 h. Each treatment was tested in six individual wells. The supernatant was removed and the MTT solution was added to each well and incubated for 3 h at 37 °C to allow the formation of formazan crystals. After that, the medium was removed and the blue formazan crystals were eluted from cells using 150 μL of DMSO. The absorbance was measured at 590 nm with background subtraction at 630 nm. Experiments were carried out in triplicate.

2.11. Statistical analysis

All experimental analyses were performed in triplicate and means and standard deviations were calculated. Statistical analysis of the data was carried out using IBM SPSS Statistics Version 24.0 (SPSS, Inc. Chicago, IL). The IC₅₀ values of ROS and RNS scavenging activity were calculated from the curves of percentage of inhibition *versus* antioxidant concentration, using the GraphPad Prism 5 software.

3. Results and discussion

3.1. Macronutrient analysis

The nutritional profile of the kernels analysed in this study is described in Table 1. These seeds are composed of a high amount of carbohydrates (60.4%), of which 26.9% is fiber. Kernels also present a considerable source of proteins (6.3%), being poor in lipids (4.0%).

Table 1 Nutritional composition of *Q. cerris* seeds (mean \pm standard deviation). Values are an average of three individual samples ($n = 3$). nd – not detectable

	<i>Q. cerris</i> seeds
Moisture	6.7 \pm 0.13
Carbohydrates ^a	60.4 \pm 0.1
Of which fiber ^a	26.9 \pm 0.2
Proteins ^a	6.3 \pm 0.2
Lipids ^a	4.0 \pm 0.06
Ash ^a	2.4 \pm 0.00
Vitamins (mg per 100 g)	
α -Tocopherol	7.670 \pm 0.100
β -Tocopherol	nd
γ -Tocopherol	5.765 \pm 0.075
δ -Tocopherol	0.397 \pm 0.02
α -Tocotrienol	1.616 \pm 0.095
β -Tocotrienol	nd
γ -Tocotrienol	nd
δ -Tocotrienol	nd
Total	15.448 \pm 0.19

^a % in dry weight.

The results are in accordance with those published by Vinha *et al.*, who evaluated *Q. suber*, *Q. faginea*, *Q. nigra* and *Q. ilex* kernels, describing a high amount of carbohydrates (ranging between 86 and 89 g per 100 g dry weight (dw)), followed by proteins (between 5 and 7 g per 100 g dw).² Similarly, the lipidic content is low.¹⁰ In another study, Nieto *et al.* also reported a low protein content for Spanish acorns produced by *Q. rotundifolia* (holm oak) (4.8 g per 100 g dw) and *Q. suber* (6.3 g per 100 g dw).¹⁷ In 2015, Li *et al.* stated that *Q. glandulifera* seeds had a low fat value (4.20 g per 100 g dw) and a protein content of 10.16 g per 100 g dw,¹⁸ being in line with the present results.

The vitamin E content of kernels is also summarized in Table 1. It is well known that Vitamin E is the major lipid-soluble antioxidant in the cell defence system, being composed of eight naturally occurring and structurally related vitamers, namely four tocopherols (α -, β -, γ -, and δ -) and four tocotrienols (α -, β -, γ -, and δ -). α -Tocopherol is the most biologically active form and the biological effects are generally due to its antioxidant properties. The main vitamer present in kernels was α -tocopherol (7.670 mg per 100 g), followed by γ -tocopherol (5.765 mg per 100 g) and α -tocotrienol (1.616 mg per 100 g). δ -Tocopherol was the minor one (0.397 mg per 100 g). β -Tocopherol, β -tocotrienol, γ -tocotrienol and δ -tocotrienol were not detected. Once again, these results are in line with those of Vinha *et al.*, who reported that α -tocopherol and γ -tocopherol were the predominant vitamers in *Q. suber*, *Q. faginea*, *Q. nigra* and *Q. ilex* acorns.² Comparatively to *Q. glandulifera* seeds, the total vitamin E is higher. Li *et al.* reported a content of 10.78 mg per 100 g for this species.¹⁸

3.2. Total phenolic and total flavonoid contents and antioxidant activity

The TPC, TFC and antioxidant activity of the coffee-like beverage were evaluated. The TPC was 2070.21 \pm 69.02 mg GAE per

L, while the TFC was 285.27 \pm 10.96 mg CAE per L. To screen the antioxidant properties, two different assays were employed: scavenging activity on DPPH[•] radicals and the ferric reducing antioxidant power (FRAP). In what concerns DPPH, the IC₅₀ value was 271.61 \pm 24.61 μ g mL⁻¹, while for FRAP the result was 203.11 \pm 3.56 μ g mL⁻¹. To the best of our knowledge, this is the first study that evaluates the antioxidant content of a coffee-like beverage prepared from kernels. Compared with coffee silverskin for example, the TPC and TFC are considerably higher.¹⁸ Costa *et al.* reported that the higher TPC obtained was 310 mg GAE per L, using a hydroalcoholic mixture (50 : 50) at 50 °C or 60 °C for 90 min.¹⁹ In what concerns the TFC, Costa *et al.* stated that the highest value (144 mg CAE per L) was achieved with the hydroalcoholic extract (25% water : 75% ethanol) at 60 °C for 90 min.¹⁹ The results reported in the present study are considerably higher. Also, it should be highlighted that in the present study the solvent used was water.

Regarding the DPPH and FRAP assay, our results are shown as IC₅₀. Once again, compared with those of Costa *et al.*, the present results are considerably better. The highest DPPH scavenging ability reported by the authors was 416 \pm 4.2 mg Trolox Equivalents (TE) per L, obtained with a hydroalcoholic extract (25% water : 75% ethanol) at 30 °C during 60 min. In what concerns the FRAP assay, the best value determined by Costa *et al.* was 1700 mg ferrous sulphate equivalents (FSE) per L, once again with the hydroalcoholic extract. The authors did not achieve the IC₅₀ results. In another study, Rocha-Guzmán *et al.* evaluated water infusions of mature and fresh *Q. resinosa* leaves, reporting higher IC₅₀ values for the DPPH assay, namely 507 and 548 μ g mL⁻¹ for fresh and mature leaves.²⁰

When a regression analysis was performed between the values of μ mol Trolox equivalents per L and the IC₅₀ determined for the FRAP assay, a positive correlation is achieved ($R^2 = 0.570$; $p < 0.01$). Nevertheless, when the regression analysis is performed between the values of μ mol Trolox equivalents per L and the IC₅₀ determined for the DPPH assay, a high positive correlation is obtained ($R^2 = 0.811$; $p < 0.001$). Also, the correlation between the mg Catechin equivalents per L and the IC₅₀ determined in the DPPH assay is extremely positive ($R^2 = 0.969$; $p < 0.01$), while for FRAP the Pearson correlation is negative ($R^2 = -0.262$; $p < 0.01$). Thus, according to these results, the antioxidant activity observed could be due to the presence of phenolic compounds, particularly flavonoids. Nevertheless, the interpretation of results needs to be performed with precaution, as the final effects of a complex extract result from the combinatory and synergic actions of all its constituents. In that way, the identification and quantification of phenolic compounds are extremely important.

3.3. ¹H-NMR and LC-MS analysis

As a preliminary investigation of the kernel composition, a ¹H-NMR analysis was performed to assess the presence of different classes of constituents. For this reason, a vegetal material was extracted with deuterated chloroform (Fig. 1(a)) and deuterated methanol in order to evaluate the most lipo-

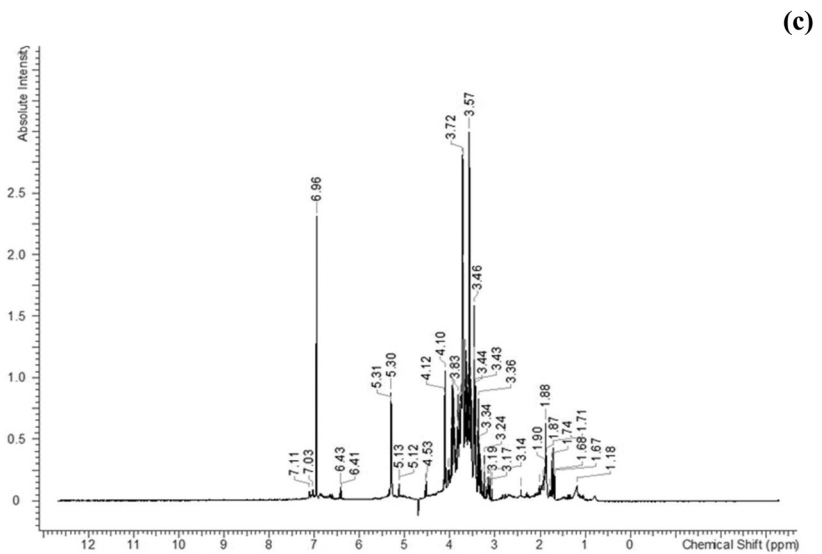
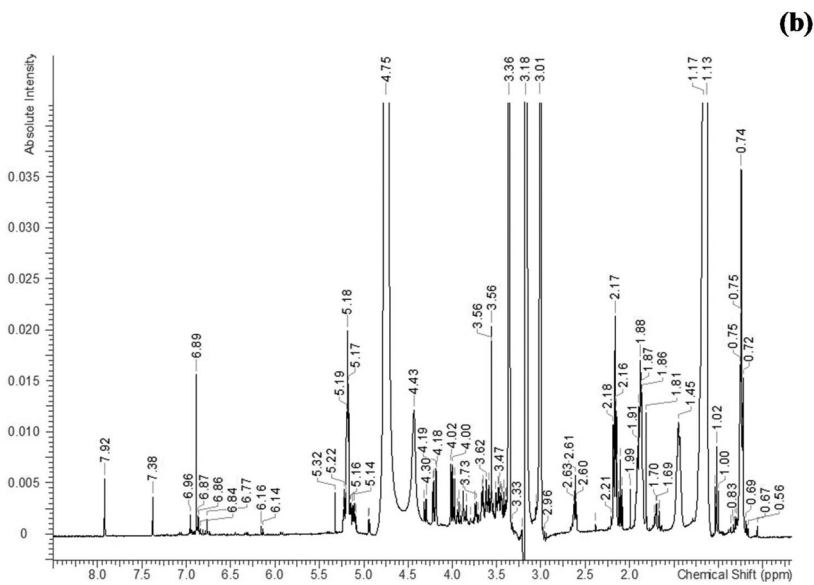
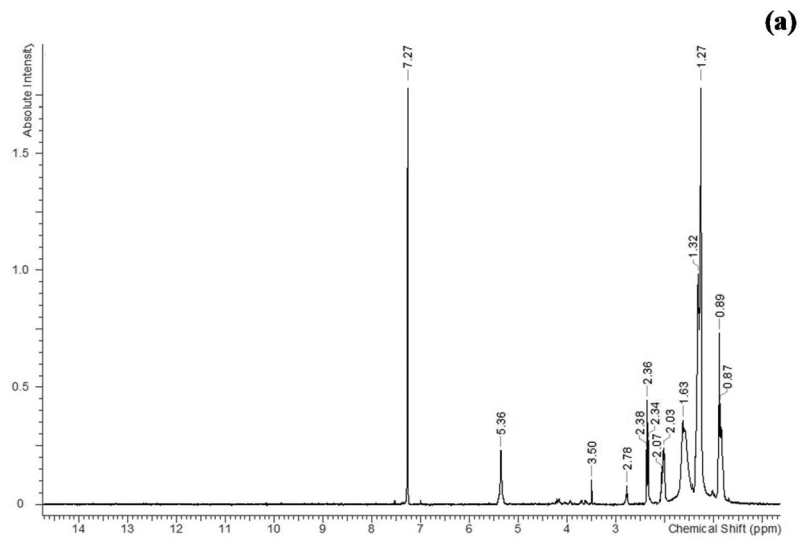


Fig. 1 $^1\text{H-NMR}$ of the extract obtained in deuterated chloroform (a), in deuterated methanol (b) and in deuterated water with presaturation of the water solvent (c).

philic constituents as well as the compounds with intermediate polarity. Additionally, a sample was prepared using deuterated water (Fig. 1(b)) in order to analyse the final product of the trial.

The fraction soluble in deuterated chloroform presents a $^1\text{H-NMR}$ spectrum that clearly shows the presence of fatty acid and lipid constituent fractions, namely the triplet at δ 0.74 ascribable to terminal methyl groups, the large signals at δ 1.27 and 1.63 ppm due to CH_2 chains, the multiplet at δ 2.03 supporting the CH_2 vicinal to double bonds and the triplet at δ 2.36 ascribable to CH_2 vicinal to the carboxyl function. A broad signal at δ 5.36 supports the presence of double bonds suggesting the presence of an unsaturated lipid fraction. The spectral region of aromatic and lower fields (6–8 ppm) did not reveal the presence of other constituents in a significant amount.

Regarding the methanolic soluble fraction, it was possible to observe the presence of fatty acid signals, such as in the aqueous extract, but also a crowded region of signals in the ppm ranging from δ 3.0 to 5.0 supporting the presence of

sugar residues. In particular, the doublets at δ 4.93 and δ 4.29 may be assigned to anomeric proton signals of sugars. Furthermore in the spectral region from δ 5.8 to 7.8, signals ascribable to olefinic and aromatic protons can be observed, as well as singlets at δ 9.4 and 9.3 suggesting the presence of aldehyde groups.

The sample prepared as a coffee-like beverage but using deuterated water (Fig. 1(c)) presents a different $^1\text{H-NMR}$ spectrum, showing almost the absence of fatty acid signals, while the crowded region at δ 3–5 ppm supports the presence of sugar derivatives.

An intense signal in the aromatic region was a singlet at δ 6.96 that in the HSQC spectrum acquired with water pre-saturation correlates with carbon resonance at δ 108.3. Other significant H–C direct connections were observed in the HSQC between signals respectively at δ 3.50–60.0 and δ 3.52–62.5; from signals in the range δ 3.65–3.75 with carbon resonances 74.1–79.0 and from δ 3.50–3.56 with carbon resonances at δ 84.0 (Fig. 2). Further signals ascribable to anomeric protons are detected as doublets at δ 5.27–91.1 and δ 5.23–99.2 (Fig. 2(a)).

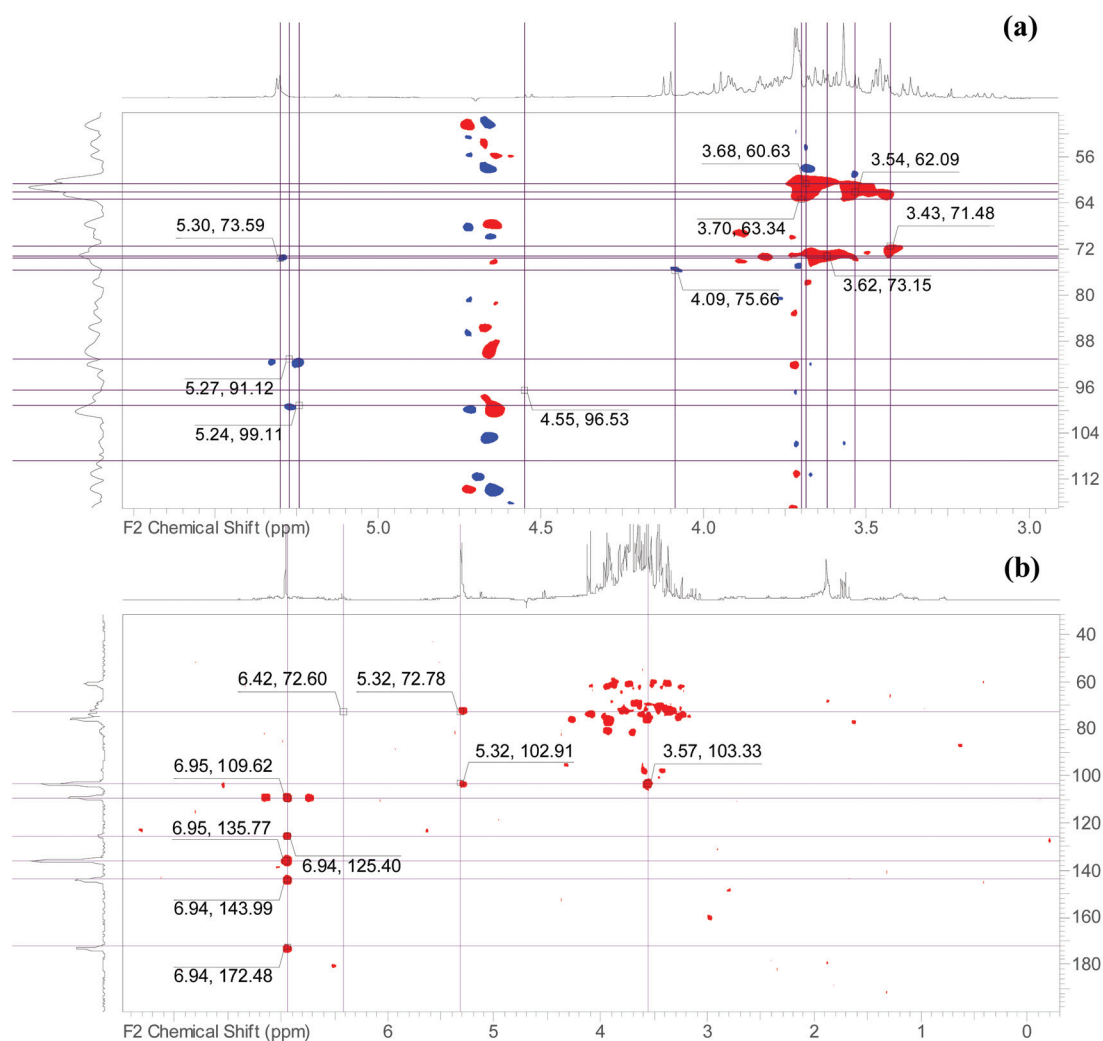


Fig. 2 HSQC (a) and HMBC (b) of the extract obtained in deuterated water.

Furthermore, a signal supporting the presence of an esterified CHO group is revealed at δ 5.31–73.3. In the aromatic region, signals are observed at δ 6.98–125.3, 6.79–96.5 and δ 6.90–108.3; no proton signals in the region from δ 7.5 to 10.0 ppm were observed. HMBC correlations were observed from the singlet at 6.93 with carbon resonances at δ 109.6, 125.4, 135.7, 143.9 and 172.5 supporting the presence of an ester derivative of gallic acid with saccharides as glucose (Fig. 2(b)).

The preliminary analysis by NMR in seeds revealed the presence of a series of aromatic derivatives, fatty acids and sugars. In what concerns the coffee-like beverage, the main classes of extracted compounds were phenolics and sugars. Sugar residues can be ascribed to hexose as glucose or epimers as well as 6-deoxy hexose characterized by the signals at δ 1.70–1.73 that are correlated in the HSQC with resonances at δ 32.6, while in the HMBC those signals correlate with carbon resonances at δ 79.5 and 68.0.

Further data were obtained using LC-DAD-ESI-MSⁿ in the negative ionization mode. Two prevalent peaks at 280 nm and some minor peaks presenting the same UV spectrum, mainly ellagic acid and gallotannin or ellagitannin derivatives (Table 2), were identified on the basis of their MSⁿ spectra and comparison with the literature. The most abundant observed *m/z* and relatively low energy collision induced dissociations occurring in the ion trap are summarised in Fig. 3 and Table 2.

Quantification was performed using a diode array detector and gallic acid as the reference compound. Ellagic acid was co-eluted with tetragalloyl glucose derivatives and its quantification was not possible by LC-DAD. The most abundant constituents are tetragalloyl hexose and dihydroxymethylbenzoyl tetragalloylglucose. On the dried extracts, the amount of the phenolic constituents is about 11% (w/w dry weight).

The most abundant constituents were tetragalloyl hexose (6.79 w/w) and dihydroxymethylbenzoyl tetragalloylglucose (1.93 w/w), while trigalloyl hexose was the less abundant (0.05 w/w).

3.4. Scavenging of ROS and RNS

The results of ROS and RNS scavenging activity are presented in Table 3.

Currently, the consumer concerns related to aging and health have generated deep interest in the food composition, namely bioactive compounds that could increase not only the nutritional value but also the health status.¹¹ ROS and RNS are involved in important physiological processes, such as the inflammatory response, cellular signaling systems or even organism defense. When an imbalance occurs between the antioxidant system and the ROS/RNS production, an “oxidative stress” stage occurs, affecting major cellular components such as lipids, proteins or DNA and leading to the development of different diseases. Different radicals normally occur in humans. The oxygen molecule is the most reactive. As it is possible to observe in Table 2, the coffee-like beverage showed a notable capacity to scavenge all the tested ROS and RNS in a concentration-dependent manner. The IC₅₀ values determined were found at low concentrations, particularly for the superoxide anion radical and the nitric oxide assays. O₂^{•−} is an important precursor of a variety of powerful oxidants. The IC₅₀ determined for this ROS was 17.24 μg mL^{−1}. This value is considerably lower than the one reported for the positive control used. H₂O₂, together with O₂^{•−}, is another species responsible for generating reactive and potent species, such as HO[•] and HOCl. In fact, H₂O₂ may cross cell membranes and react with transition metals by the Fenton reaction, producing [•]OH, which is considered one of the most reactive species. The IC₅₀ determined for H₂O₂ was 275.70 μg mL^{−1}. This result is higher than the positive control used (93.84 μg mL^{−1}). Regarding HClO, which is formed in neutrophils from the reaction between H₂O₂ and chloride ions catalysed by myeloperoxidase enzyme, the IC₅₀ determined was 9.25 μg mL^{−1}. This species is extremely reactive with biomolecules particularly during the inflammatory process. In what concerns nitric oxide, this reactive nitrogen species is the conversion result of L-arginine to L-citrulline by nitric oxide synthase, being in high concentrations associated with inflammatory processes and cell effects. The IC₅₀ determined for this species was 0.65 μg mL^{−1}, higher than that for quercetin. Finally, the peroxy radical is generated during the lipid oxidation chain reactions having the ability to react with contiguous molecules. The results

Table 2 Phytochemical compounds tentatively identified, observed *m/z* and main fragments in the negative ion mode for the coffee-like beverage by LC-DAD-ESI-MSⁿ

No.	<i>t</i> _R (min)	Compound	MS [M – H] [−]	Fragments	% (w/w)
1	1.5	Galloyl hexose	341.5	179	1.02
2	3.2	Galloyl derivative	493	313	0.62
3	12.4	Trigalloyl hexose	635	465, 483, 313	0.20
4	15.2	Trigalloyl hexose	635	465, 483, 313	0.05
5	15.8	Trigalloyl hexose	635	465, 483, 313	0.16
6	18.5	Tetragalloyl hexose	787	617, 573, 465, 447, 313, 170	0.47
7	19.9	Tetragalloyl hexose	788	617, 573, 465, 447, 313, 171	0.47
8	19.6	Tetragalloyl hexose	788	617, 573, 465, 447, 313, 171	6.79
9	19.7	Ellagic acid	301	229, 201, 186, 174	^a
10	17.1	Dihydroxymethylbenzoyl tetragalloylglucose	937	857, 785, 767, 741, 617, 599, 589, 465	0.16
11	19.5	Dihydroxymethylbenzoyl tetragalloylglucose	938	857, 785, 767, 741, 617, 599, 589, 466	1.93
Total content					11.87

^a Ellagic acid was not quantified by DAD because it overlapped with a larger peak of tetragalloyl hexose.

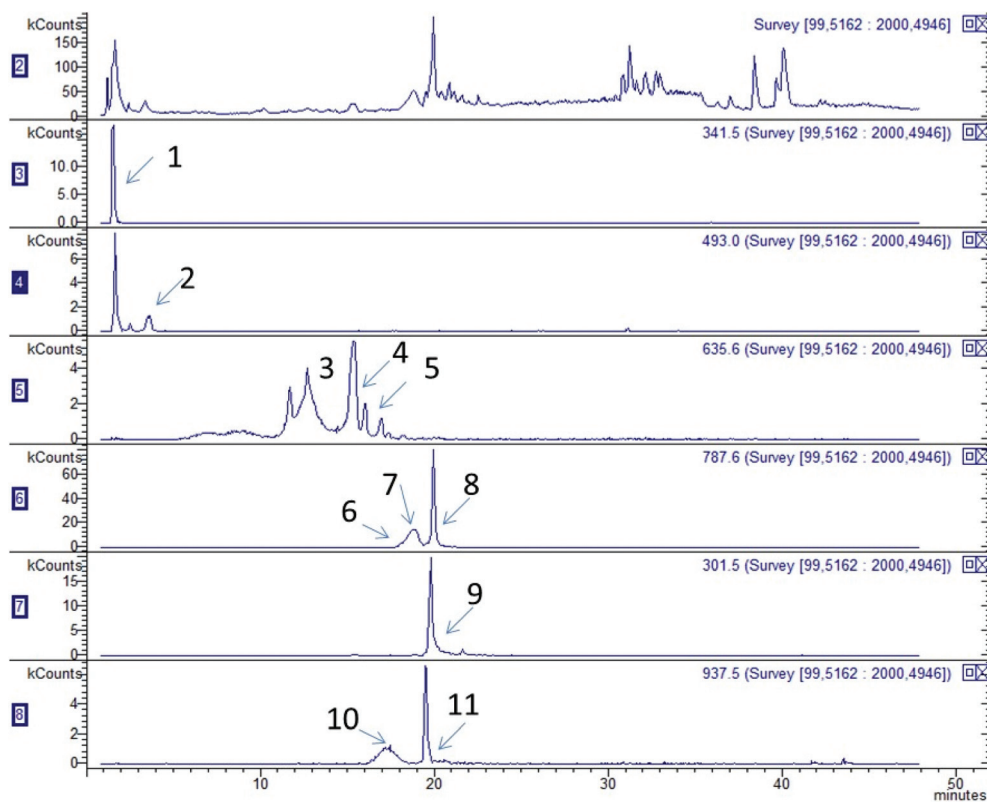


Fig. 3 LC-MS of the extract obtained with water. Chromatograms corresponding to main ionic species namely m/z 341, 493, 365, 787, 301 and 937 are shown. Peaks: see Table 2.

Table 3 Superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxy radical (ROO^{\cdot}), nitric oxide ($\cdot NO$) and peroxynitrite ($ONOO^-$) scavenging capacities of the extract. Values are expressed as mean \pm standard deviation ($n = 3$). Different letters in the same column indicate significant differences between mean values ($p < 0.05$)

	Reactive oxygen species			Reactive nitrogen species	
	IC_{50} ($\mu g\ mL^{-1}$) ($n = 4$)				
	$O_2^{\cdot-}$	H_2O_2	HOCl	$\cdot NO$	ROO^{\cdot} (S_{sample}/S_{Trolox}) ^a
Sample	17.24 \pm 0.24	275.70 \pm 20.25	9.25 \pm 0.92	0.65 \pm 0.06	NA
Positive controls					
Ascorbic acid	370.10 \pm 27.98	93.84 \pm 4.59	2.23 \pm 0.05	—	0.53 \pm 0.06
Tiron	—	—	—	—	—
Quercetin	—	—	0.21 \pm 0.01	0.06 \pm 0.007	4.09 \pm 0.52

IC_{50} = *in vitro* concentration required to decrease in 50% the reactivity of the studied reactive species in the tested media (mean \pm standard error of the mean; $n = 3$); NA: No activity was determined up to the highest tested concentration ($1\ mg\ mL^{-1}$). ^a S_{sample} = slope of extract curves. S_{Trolox} = slope of the trolox curve.

obtained in the present study showed that the coffee-like beverage is not able to scavenge ROO^{\cdot} in *in vitro* systems.

The activity observed for all species results is a consequence of the action of the several bioactive molecules present on the extract. The notable scavenging effect of the coffee-like beverage, especially against $O_2^{\cdot-}$ and $\cdot NO$, was probably due to the high content of bioactive compounds, particularly phenolic compounds. Recently Gamboa-Gómez *et al.* assessed the *in vitro* and *in vivo* antioxidant effects of oak leaf (*Q. convallata*

and *Q. arizonica*) infusions and fermented beverages.²¹ The authors reported a high antioxidant potential (ranging between 11.02 and 53.31 μM Trolox equivalents per μg dry sample, respectively for the *Q. arizonica* fermented beverage and *Q. convallata* leaves infusion, respectively) and scavenging activity for radicals such as peroxy (between 0.10 and 0.45 μM Trolox equivalents per μg dry sample) and peroxynitrite anions (from 8.40 to 15.98 μM Trolox equivalents per μg dry sample). Nevertheless, compared with results in Table 2, the values

reported for the scavenging activity were lower than the ones obtained in this study. The authors did not achieve the IC_{50} . In another study, Rodrigues *et al.* evaluated the *in vitro* antioxidant and anti-inflammatory properties of *Limonium algarvense* flower infusions and decoctions.²² The IC_{50} reported for the hydroxyl radical was 2.00 mg mL^{-1} for the *L. algarvense* infusion while for the decoction it was 1.82 mg mL^{-1} . In what concerns the superoxide radical, the values ranged from 0.30 to 0.32 mg mL^{-1} , respectively, for the decoction and the infusion. All results were considerably higher than the ones presented for the coffee-like beverage (Table 2).

3.5. Effects of extracts towards Caco-2 and HT29-MTX cells

In order to evaluate the effects of the coffee-like beverage on intestinal cells, the extract was added at different concentrations (0.1 – $1000 \text{ } \mu\text{g mL}^{-1}$) to Caco-2 and HT29-MTX cells (Fig. 4). Both cell lines were selected to mimic the intestinal model, particularly the small intestine, since it is in this location that the majority of the absorption process occurs.

According to the obtained results, the coffee-like beverage caused a decrease in Caco-2 cell viability. The concentration of 10 and $100 \text{ } \mu\text{g mL}^{-1}$ leads to a cell viability of, respectively, 64.66 and 67.32% while the concentration of $1000 \text{ } \mu\text{g mL}^{-1}$ leads to a cell viability of 47.07% . On the other hand, the concentrations of 0.1 and $1.0 \text{ } \mu\text{g mL}^{-1}$ did not decrease the cell viability. In contrast, the HT29-MTX viability decreased with all concentrations tested in a similar way. It was around 70% for all concentrations, except $1000 \text{ } \mu\text{g mL}^{-1}$ (in this case, the cell viability was 58.51%). These results support the evidence reported by Almeida *et al.* as well as by de Francisco *et al.* that Caco-2 cells are more sensitive cells than HT29-MTX.^{11,12} Both authors evaluated the extract effects on these two cell lines, reporting that Caco-2 cells are more sensitive. Taking into account the results observed for both cell lines, the optimal range of non-cytotoxic concentrations of the coffee-like beverage should be between 0.1 and $1.0 \text{ } \mu\text{g mL}^{-1}$ for further experiments. Recently, Martins-Gomes *et al.* tested decoctions and

hydroethanolic extracts of *Thymus carnosus* Boiss in two breast cancer cell lines (MCF-7 and BT-474).²³ After 24 hours of contact, the IC_{50} values for the decoction were 533.87 and $841.28 \text{ } \mu\text{g mL}^{-1}$, respectively, for MCF-7 and BT-474 cells, while for the infusion these values were almost 10 times lower (respectively 86.87 and $39.91 \text{ } \mu\text{g mL}^{-1}$ for MCF-7 and BT-474 cells).²³ Thus, the coffee-like beverage was considerably safer than these beverages.

In fact, growing interest in the relationship between food and health has been observed in the last decade in order to achieve a healthy and longer life, changing the food consumers' behaviour and leading to the development of functional foods. These functional food characterization studies are firstly based on the identification of bioactive compounds and then on evidence of their benefits to human health. Besides the role played by some bioactive compounds already reported in the previous sections, their antioxidant properties against reactive oxygen/nitrogen species may be associated with the reduction of the risk of developing several diseases such as cancer or neurodegenerative disorders. The employment of *in vitro* assays to observe potential benefits is an option. To exert their biological functions, these compounds must be released from foods and become available to absorption by the human body which can be observed performing intestinal assays using 3D models, for example.²⁴ Considering the obtained results and other studies, absorption is expected. Further studies are being developed to conclude about that.

4. Conclusion

In the present study, a coffee-like beverage from *Q. cerris* kernels was successfully produced and chemically characterized. In what concerns the kernel composition, a high content of fiber and carbohydrates (60.4%) and the prevalence of α -tocopherol as a principal vitamer deserve attention. On the other hand, the main constituents of *Q. cerris* kernels are lipids that are not extracted during the coffee-like preparation. Preliminary analysis by NMR spectroscopy revealed the presence, in the prepared beverage, of sugars as well as phenolic constituents, mainly tetragalloyl hexose and dihydroxymethylbenzoyl tetragalloylglucose. The coffee-like beverage revealed a high amount of total phenolics ($2070.21 \text{ mg GAE per L}$) and flavonoid contents (285.27 CAE per L) as well as antioxidant activity (the IC_{50} determined for the DPPH assay was $271.61 \text{ } \mu\text{g mL}^{-1}$, while for FRAP the value was $203.11 \text{ } \mu\text{g mL}^{-1}$). A high efficiency in the scavenging activity of ROS and RNS, especially O_2 , H_2O_2 , $HClO$ and $\cdot NO$, was observed. The extract was also screened for cell viability assays using two different intestinal cell lines (HT29-MTX and Caco-2), showing that concentrations between 0.1 and $1.0 \text{ } \mu\text{g mL}^{-1}$ are safe and should be employed in further studies. Altogether, this study suggests that the coffee-like beverage prepared from *Q. cerris* kernels could be further explored as potentially health-promoting. Additionally, it is interesting to note that this plant material can be considered as a natural source of biocompounds with

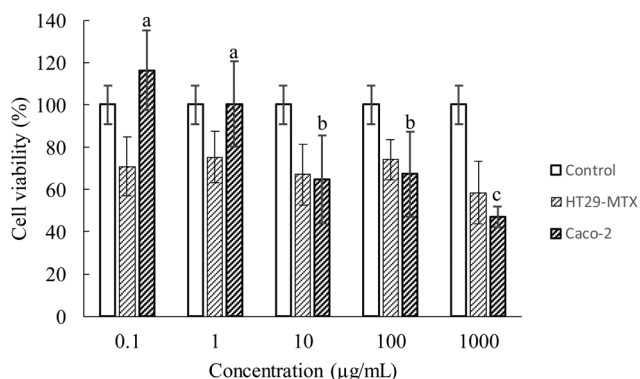


Fig. 4 Effect of coffee-like beverage exposure on the viability of HT29-MTX and Caco-2 cells at different concentrations, as measured by the MTT assay. Values are expressed as means \pm SD ($n = 6$). Different letters on the same cell line mean statistical differences between concentrations ($p < 0.05$).

antioxidant potential and can be consumed as a beverage. Nevertheless, animal studies should be performed to ensure these optimistic results and definitive studies demonstrating direct health benefits on consumers should be conducted.

Author contributions

Conceived and designed the experiments: Diana Pinto, Santiago Diaz Franco, Ana Margarida Silva, Sónia Soares, Stefania Sut, and Francisca Rodrigues. Drafted or revised the manuscript: Sónia Soares, Francisca Rodrigues, Marijana Koskovic, Ksenija Kojicic, Stefano Dall'Acqua, and M. Beatriz P. P. Oliveira. Contributed reagents/materials/analysis tools: Snezana Cupara, Stefano Dall'Acqua, and M. Beatriz P. P. Oliveira.

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