Optimization of antioxidants – Extraction from *Castanea sativa* leaves

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**A B S T R A C T**

The extraction of *Castanea sativa* leaves (CsLs) with selected solvents (96% ethanol, methanol and acidified water) was assessed by means of a set of experiments following a Box–Wilson central composite circumscribed (CCC) design. CsL extraction was assessed on the basis of experimental results determined for the yield in soluble compounds, the yield in phenolics and the antioxidant activity of extracts. The *in vitro* antioxidant activity was measured in terms of the scavenging abilities for α,α-diphenyl-β-picrylhydrazyl (DPPH) radical. Selected extracts were also assayed for scavenging activity against reactive oxygen species (ROSs) and reactive nitrogen species (RNSs). The highest extraction yields were obtained using methanol, whereas water allowed a selective extraction (with yields in phenolics up to 9 mg Gallic Acid Equivalent/100 mg CsL) and provided the most concentrated (54 mg GAE/100 mg solid) and active extracts. The major compounds identified in extracts were gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, rutin, quercetin and apigenin.

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1. Introduction

*Castanea sativa* Mill. is a species of the flowering plant family *Fagaceae*, mainly cultivated in temperate regions (Asia, Southern Europe and North of Africa). The seasonal fruit is a nut, commonly named sweet chestnut or marron, which is collected in autumn. It is a traditional basic foodstuff and can be processed into different elaborated and progressively diversified food products. Recently, the nuts became important in human health owing to their applications as a component of gluten-free diets and as a source of essential fatty acids.

*C. sativa* leaves (CsL) are used in folk medicine to treat cough, diarrhea and rheumatic conditions, lower back pain, and stiff joints or muscles. As in other medicinal plants, some of the active substances have been identified as phenolic compounds. The most abundant of them in CsL are ellagic acid and gallic acid derivatives [1]. These compounds present biological activities, including ability for protecting against oxidative stress-related diseases and antimicrobial activity. The capacity of aqueous, methanol, and ethyl acetate extracts from CsL to scavenge α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, superoxide radical (*O*₂⁻), and hydroxyl radical (*HO*•) was already reported [2–5]. Water:acetone CsL extracts were further washed with ethyl acetate to separate phenolic compounds from tannins. This fraction showed antioxidant activity comparable to the ones of reference pure antioxidants, and higher than the ones determined for solvent purified fractions [3]. Ethanol:water extracts of CsL showed scavenging activities against hydrogen peroxide (*H*₂*O*₂) and singlet oxygen (*¹O*₂) comparable to that of ascorbic acid, and were more active against *O*₂⁻ than Trol-ox. In comparative terms, extracts were more active for scavenging reactive nitrogen species (RNSs) than for reactive oxygen species (ROSs) [6]. Water extracts of CsL also showed reducing power and ability to cause the inhibition of β-carotene bleaching, lipid...
peroxidation and hemolysis [4,7]. The same properties were confirmed for extracts obtained with 50% ethanol [5], which also showed ability to protect against DNA damage in rat pancreatic β-cells [5].

Csl extracts have been reported to prevent changes related to the fluidity and integrity of the erythrocyte membranes [1]. The ethyl acetate soluble fraction of the Csl aqueous extracts displayed potent antibacterial action against Gram-positive and Gram-negative bacteria, which was ascribed to the presence of flavonoids [8]; whereas ethanol extracts showed strong antibacterial activity against Gram-positive bacteria [9].

The utilization of solvent extracts from Csl in cosmetic compositions has been claimed [10]. A Csl extract obtained with ethanol–water (containing rutin, ellagic acid, hyperoside, isoorientin and chlorogenic acid) was considered safe and suitable for preventing and treating oxidative stress-related diseases and photoageing [11]. Flavonoid glycosides obtained from Csl have been considered useful for the treatment and prevention of insulin-resistance diseases [12].

The extraction and purification of biologically active compounds from natural sources is becoming a growing research field, finding applications in the formulation of ingredients and additives for food, pharmaceuticals and cosmetics. The yields in bioactive compounds obtained from different plant materials are strongly influenced by the extraction and purification procedures. The type of solvent is an influential variable: when Csl phenolics are the target compounds, water [7], methanol [10], acetone, ethylacetate [3], ethanol–aqueous solutions [1,6,9,11] are suitable agents for extraction.

Recent literature has been reported on the extraction of antioxidant phenolic compounds from Csl, with emphasis on the type of solvent, characterization of the extracts, and comparative evaluation of different parts of the plant. Despite the increased extraction yields and antioxidant potential of phenolics from skins [1,7], leaves can be easily collected and possess a traditional background. To our knowledge, the combined effects of the major operational variables (temperature, contact time and liquid–solid ratio) on the extraction of phenolics from Csl has not been reported before.

This study provides an experimental assessment of the effects of selected operational variables on the extraction of phenolic compounds with antioxidant activity from Csl. The composition of Csl was measured, and the extraction of target fractions was assessed using a set of experiments with a Box–Wilson central composite circumscribed (CCC) design. In experiments, the yields in soluble solids and phenolics, as well as the antioxidant activities of extracts, were measured. The in vitro antioxidant activity was characterized using the DPPH radical scavenging test. Selected extracts were also assayed for scavenging activity against ROS (O2, H2O2, peroxyl radical ROO·, hypochlorous acid HOCl, and 1O2) and RNS (nitric oxide ‘NO and peroxynitrite ONOO−).

2. Materials and methods

2.1. Materials

Chestnut Csl from the 2005 harvest were locally collected. Air-dried to 9.7% average moisture, ground, sieved to pass 1 mm, and stored in sealed plastic bags in a dry and dark. All the chemicals and reagents were of analytical grade. Ethanol, hexane and methanol were purchased from Panreac (Drogallega, S.L, Spain). Butylhydroxy anisol (BHA) and butyl hydroxytoluene (BHT) were purchased from Analema (Drogallega, S.L, Spain). Gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, quercetin, and apigenin were obtained from Sigma–Aldrich (St. Louis, USA). a,a′-Azodisobutyramidine dihydrochloride (AAPH), histidine and Trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt was obtained from Aldrich (Milwaukee, USA).

2.2. Solid–liquid extraction

Ground Csl samples were in contact for the desired time with the selected solvent (aqueous ethanol (96%) denoted as ES, methanol denoted as MS, or acidified water denoted as AWS) at the selected liquid to solid ratio and temperature in capped Erlenmeyer flasks kept in an orbital shaker at 120 rpm. Phase separation was accomplished by filtration. The liquid phase was vacuum evaporated and freeze-dried to obtain a crude extract. The extraction yield was determined gravimetrically.

2.3. Experimental plan

The experimental plan followed a three-factor, central composite circumscribed (CCC) involving 20 experiments with six replicates in the central point of the experimental domain. The independent variables, their nomenclatures in dimensional and dimensionless terms and their variation ranges were as follows: contact time (t, X1, 30–90 min); temperature (T, X2, 25–50 °C) and liquid to solid ratio (L/S, X3, 15–25 v/w). The particle size distribution (in the range 0.25–1 mm) was constant in all experiments. Three solvents (96% ethanol, methanol, acidified water) were employed in individual sets of experiments. The range selected for the extraction time covered the conditions of practical interest, whereas the temperature range was fixed keeping in mind a compromise between extraction yield, extraction rate and limitation of thermal denaturation. The values assayed for the liquid to solid ratio was in a wide interval where mass transfer is not limited. Table 2 lists the operational conditions assayed for individual experiments.

The measured effects were the Soluble Extraction Yield (SEY, mg soluble solids/100 mg dry Csl), the phenolic extraction yield (PEY, mg GAE/100 mg dry Csl), and the DPPH radical scavenging capacity (DPPH RS), which was selected to assess the antioxidant activity.

The experimental data determined for each of the three solvents were adjusted according to the following second order equation:

\[ Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} X_i X_j \]  

(1)

where \( X_i, X_2, \ldots, X_k \) are the independent, dimensionless variables affecting the responses (\( Y \)); \( b_0, b_i (i = 1, 2, \ldots, k), b_{ij} (i = 1, 2, \ldots, k, j = 1, 2, \ldots, k) \) are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; \( k \) is the number of variables. The Statsoft vs 5.0 software was used in calculations.

2.4. Analytical methods

Ash content was determined by calcination at 550 °C. Extractives (in hexane, 80% ethanol, and toluene:ethanol) were determined gravimetrically after Soxhlet extraction. Ground Csl samples were subjected to moisture determination and to quantitative acid hydrolysis with 72% sulfuric acid, following standard methods [15]. The solid residue after hydrolysis was considered
as Klasson lignin. Monosaccharides and acetic acid were determined by HPLC using a Hewlett-Packard chromatograph fitted with a refractive index detector (temp., 40 °C). Other analysis conditions were: column, ION-300 (Transgenomic Inc., USA); mobile phase, 3 mM H2SO4; flow, 0.4 mL/min. The yield in soluble compounds was determined gravimetrically. The phenolic content was determined spectrophotometrically [16] as galacturonic acid equivalents (GAEs).

Other analyses were carried out using an Agilent HPLC 1100 instrument equipped with a Waters Spherosorb ODS-2 column and DAD detector, operating at a flow rate of 1 mL/min. Elution followed the following nonlinear gradient: solvent A (acetonitrile/5%(v/v) formic acid in water, 10:90); solvent B (acetonitrile/5%(v/v) formic acid in water, 90:10); elution periods: 0 min, 100% A, 0% B; 40 min, 85% A, 15% B; 45 min, 0% A, 100% B; 55 min, 0% A, 100% B; 60 min, 100% A, 0% B; 65 min, 100% A, 0% B.

2.5. Antioxidant activity assays

In vitro antioxidant assays were performed using a microplate reader (Synergy HT, BIO-TEK), for fluorescence, absorbance in UV/vis and luminescence measurements, equipped with a thermo-stat. Control assays with commercial synthetic antioxidants were run in parallel for comparative purposes.

2.5.1. DPPH (2,2-Diphenyl-1-pircrylhydrazyl) radical scavenging assay

Two milliliters of a 3.6 × 10−6 M methanolic solution of DPPH were added to 50 μL of a methanolic solution of the considered extract. The decrease in absorbance at 515 nm was recorded after 16 min. The Half Maximal Effective Concentration (EC50) was calculated as the amount of methanolic extract causing a 50% inhibition of the DPPH radical.

2.5.2. Trolox equivalent antioxidant capacity (TEAC)

ABTS radical cation (ABTS•+) was produced according as described previously [17]. Assays were run with solvent blanks and extracts, and the percentage inhibition of absorbance was referred to the concentration of extracts and Trolox.

2.5.3. Superoxide radical (O2−) scavenging assay

The O2− scavenging activity was determined by monitoring the effects of the tested extracts on the O2−-induced reduction of nitroblue tetrizolium (NBT) at 560 nm [18]. O2− was generated by the β-nicotinamide adenine dinucleotide/phenaione methosulfate/oxygen (NADH/PMS/O2) system. The results were expressed in terms of inhibition percentage of NBT reduction to diformazan. Each result corresponds to four experiments, performed in triplicate.

2.5.4. Hydrogen peroxide (H2O2) scavenging assay

The H2O2 scavenging activity measured the inhibition (in percentage) of the H2O2-induced oxidation of lucigenin [18]. Each study corresponds to four experiments, performed in triplicate.

2.5.5. Hypochlorous acid (HOCl) scavenging assay

The HOCl scavenging activity measured the inhibition percentage of HOCl-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123 [18]. HOCl was prepared by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with dropwise addition of 10%H2SO4. Each study corresponds to four experiments, performed in triplicate.

2.5.6. Singlet oxygen (1O2) scavenging assay

The 1O2 scavenging activity was measured by monitoring the inhibition (in percentage) of 1O2-induced oxidation of non-fluorescent DHR [18]. 1O2 was generated by the thermal decomposition of endoperoxide dioxidum 3,3′-(1,4-naphthalene) bispropionate (NDPO2) [19]. Each study corresponds to four experiments, performed in triplicate.

2.5.7. Peroxy radical (ROO•) scavenging assay

The ROO• scavenging activity measured the effects of the tested extracts on the fluorescence decay resulting from ROO•-induced oxidation of fluorescein, and the results were expressed in terms of the “Oxygen Radical Absorbance Capacity” (ORAC) [18]. Each study corresponds to four experiments, performed in triplicate.

2.5.8. Nitric oxide (NO) scavenging assay

The NO scavenging activity measured the inhibition percentage of NO-induced oxidation of non-fluorescent 4,5-diminofluorescein (DAF-2) to the fluorescent triazolofluorescein (DAF-2T) [18]. Each study corresponds to four experiments, performed in triplicate.

2.5.9. Peroxynitrite (ONOO−) scavenging assay

The ONOO− scavenging activity measured the effects of the tested extracts on the inhibition percentage of the ONOO−-induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123 [18]. ONOO− was synthesized as described before [20]. Parallel assays were performed in the presence of 25 mM NaHCO3 in order to simulate the physiological CO2 concentrations, where the reaction between ONOO− and bicarbonate is predominant, with a very fast rate constant (k2 = 3–5.8 × 108 M−1 s−1) [21]. Each study corresponds to four experiments, performed in triplicate.

3. Results and discussion

3.1. Composition

Table 1 lists compositional data of CsL. Lignin was the major structural fraction, followed by hemicelluloses, which were mainly constituted of xylose and uronic acids. In comparison with the data reported for green leaves from South West England [22], the results determined for CsL showed similar cellulose content and a significantly higher lignin content. Extractives in 80% ethanol accounted for 13.5% of the dry material, below the ranges reported for 80% methanol (17–20%) [10] and hot water (20.9%) [7].

Extractables in the ethanol solution (80% v/v) yield an extract with a phenolic concentration intermediate between those reported for hot water [3,7], methanol [3] and aqueous ethanol [9,11] and 80% ethanol was a solvent more selective for phenolic compounds than toluene:ethanol and hexane. The yield in soluble solids, phenolic content and scavenging capacity for pro-oxidant species obtained from less polar solvents than acidified water solvent (AWS) was lower, corroborating previously reported findings [3]. Reduced yields and activities have been reported for extracts obtained after sequential extractions with hexane, chloroform, ethyl acetate, methanol, and water [8], although ethyl acetate extracts eluted by medium-pressure liquid chromatography with methanol were highly active [3]. The general trend of antioxidant activity is closely dependent on the solvent polarity, the antioxidant potential and the sequence found in this work was BHA > 80% ethanol > toluene:ethanol (2:1) ≫ BHT ≫ hexane.

3.2. Optimization of the extraction conditions

In order to achieve maximum yields of active extracts, the joint optimization of the most influential variables was addressed. The liquid phases for extraction were selected on the basis of previous data and in practical aspects (including availability, cost, toxicity,
and extracting capacity). Ethanol was chosen owing to its frequent food applications [6,9]. Methanol is a reference solvent with high extracting ability [10] and leads to fractions of high radical scavenging activity [3]. Csl were extracted with each solvent under the experimental conditions assayed, expressed in terms of dimensional and dimensionless independent variables.

The statistical optimization procedure applied in the present study enables the evaluation of the effects caused by both individual independent variables and interactions among them. Related studies have been reported on the maximization of the extraction of phenolic compounds from berries [24], evening primrose meal [25], black currants [13], grape pomace [26] or wheat [14].

3.2.1. Solubles extraction yield (SEY)

The maximum extraction yield (18.2%) was reached using water as a solvent under the conditions of experiment 6 (operation for 90 min at 50 °C using 25 mL AWS/g Csl). The yield was close to the value reported for extracts from leaves of C. sativa grown in a near geographical area, obtained with boiling water for 30 min [7]. Ethanol (ES) and methanol (MS) provided lower yields than AWS, with maximum values in experiment 8 (performed for 90 min at 50 °C using 15 mL extracting agent/g Csl). The lowest yield (6.00 mg soluble solids/mg dry Csl) corresponded to operation with ES for 9.6 min at 37.5 °C using 25 mL ethanol/g Csl, and for 90 min at 25 °C using 25 mL ethanol/g Csl.

In experiments with ethanol, increasing the contact time while keeping temperature and liquid to solid ratio constant doubled the extraction yield (see experiments 15–20, performed in the central point of the experimental domain). Although a variety of compounds are soluble in all the liquid phases assayed in this study, both monomeric sugars (glucose, xylose, arabinose) and sugar oligomers were preferentially solubilized by AWS (data not shown).

The experimental results in Table 3 were adjusted to Eq. (1) by minimization of the deviation squares, enabling the calculation of the results for given operation conditions. For comparative purposes, the data calculated for the considered effects are included in Table 3 together with the experimental results. Only the model coefficients significant at a confidence level > 95% (based on a t-test) were considered for model predictions. The model coefficients are listed in Table 6, as well as the results of the ANOVA.

The equations employed to predict the extraction yield of soluble compounds (SEY), expressed in terms of the dimensional independent variables, and including just the significant terms, were as follows:

\[
\text{SEY}_{\text{ES}} = 11.6 - 0.904 t^2
\]

\[
\text{SEY}_{\text{MS}} = 9.81 + 0.932 \cdot T + 0.854 \cdot t \cdot T - 0.981 \cdot t \cdot L/S + 0.931 \cdot L/S^2
\]

\[
\text{SEY}_{\text{AWS}} = 11.9 + 1.14 \cdot t + 1.34 \cdot T + 1.31 \cdot t \cdot T + 1.12 t \cdot L/S
\]
In the studied operational range, the maximum value of PEY (9.16 mg GAE/100 mg CsL) achieved with AWS corresponded to 60 min at 50 °C using 25 mL of methanol/g CsL. In comparison with results reported in related studies, the phenolic yields achieved in ES extractions were lower than those reported for ultrasound-assisted ethanol extraction of leaves from the Italian “marrone” cultivar [9], and when the solvent was methanol the yields achieved were also lower than those reported for solvent extraction from a commercial powder plant [3].

The ratio between the yields in phenolics and soluble solids (PEY/S), which measured the phenolic content in terms of mass ratio respect the total weight of extract, was higher than 50% for aqueous extracts, doubling the maximal values reached with alcohols. So, under optimal conditions, water was more selective than ethanol for phenolics extraction than the rest solvents employed in the present work, as well as in literature studies with the same raw material [6,11], including extracts obtained with boiling water [7] and with a sequence of solvents [3]. As a general trend, the phenolic yields obtained with water were between 2–4 or 3–10 times higher than those obtained with methanol or ethanol, respectively (Table 4).

Starting from the results listed in Table 6, it can be inferred that the empirical equations derived from PEY with ES and AWS solvents (considering only the coefficients significant at the 95% confidence level) are as follows:

\[
PEY_{ES} = 0.938 + 0.300 \cdot t + 0.296 \cdot T + 0.116 \cdot L/S + 0.197 \cdot t \cdot T 
\]

(5)

\[
PEY_{AWS} = 3.91 + 0.788 \cdot T + 0.682 \cdot L/S + 0.908 \cdot t \cdot L/S 
\]

(6)

Both equations were significant at the 95% confidence level, whereas no significant dependence of PEY on the independent variables was observed in extractions performed with methanol.

Besides the significant terms shown in Eqs. (5) and (6), the contribution of the interaction term time to temperature was significant at the 90% level in ethanol extractions. Fig. 1B shows the response surfaces calculated for PEY as a function of the extraction time and temperature (at the optimal L/S) and as a function of temperature and L/S (at the optimal contact time) for ES and AWS extractions. The amount of solubilized phenolics increased with temperature in both solvents, reaching maximal values (around 6 mg GAE/100 mg dry CsL) operating with AWS at 58.5 °C for 9.54 min. The solubilized phenolics with AWS decreased with time in such a way that prolonged contact times provided the lowest PEY values. This behavior is possibly due to a thermal decomposition of phenolic compounds in acidified water at high temperatures. A similar behavior during conventional solvent extraction was reported for compounds from wheat [14], black currants [13] or from berries [23].

ES showed an inverse behavior, with maximal values of solubilized phenolics at temperatures higher than 50 °C and 90 min. In this case, as expected, increased temperatures resulted in higher PEY, particularly, where the enhanced solubility and diffusivity of solutes play a major role.
Table 6

Regression coefficients and ANOVA of the adjusted models for the objective functions (SEY, PEY, radical scavenging, EC 50).

<table>
<thead>
<tr>
<th>Model parameters</th>
<th>SEY (mg/100 mg dry CsL)</th>
<th>PEY (mg GAE/100 mg dry CsL)</th>
<th>EC 50 (mg extract/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (Linear)</td>
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<td>0.517</td>
<td>0.396</td>
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<tr>
<td>Linear Coefficients</td>
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<td>0.162</td>
<td>0.099</td>
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<tr>
<td>Quadratic Coefficients</td>
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<tr>
<td>Interaction Coefficients</td>
<td>0.052</td>
<td>0.087</td>
<td>0.285</td>
</tr>
</tbody>
</table>

Table 5

3.2.3. DPPH radical scavenging activity

Extracts with high radical scavenging capacity (DPPH RS), measured as EC50, were obtained in AWS extractions performed under the conditions of experiments 5 (in which the phases were in contact for 90 min at 25 °C using 25 mL of acidified water/g CsL), 9 (9.6 min, 37.5 °C, 20 mL of acidified water/g CsL) and 14 (60 min, 37.5 °C, 28.4 mL of acidified water/g CsL) with an half maximal Effective Concentration of 0.31 and 0.29 mg aqueous extract/mL (Table 5). These experiments resulted in extracts with the highest phenolic concentrations (0.338, 0.448 and 0.529 mg GAE/mL soluble solids, respectively). AWS extraction under the conditions of experiment 14 led to the extract with the highest concentration of phenolics, which also showed the highest antioxidant activity.

The lowest EC50 of the extracts, indicative of optimal antioxidant activity, was below the one determined for BHA (EC50 = 0.25 mg/mL). The most active alcoholic extracts were produced in experiment 6 (performed for 90 min at 50 °C using 25 mL of alcohol/g CsL), with EC50 = 0.45 mg ethanolic extract/mL and 0.36 mg methanolic extract/mL. It can be noted that the temperature needed to obtain extracts with high antioxidant activity was lower for water extractions (25–37.5 °C) than for alcohols (50 °C). The dependence of the radical scavenger capacity of extracts obtained with the three solvents studied on the operational variables are given by the following equations:

\[
\text{DPPH RS}_{\text{ES}} = 1.52 - 0.566 \cdot t - 0.230 \cdot C - 0.223 \cdot L/S + 0.288 \cdot t^2
\]

(7)

\[
\text{DPPH RS}_{\text{AWS}} = 0.517 - 0.264 \cdot t - 0.155 \cdot L/S
\]

(8)

\[
\text{DPPH RS}_{\text{DPPH}} = 0.396 - 0.046 \cdot L/S - 0.052 \cdot t - 0.062 \cdot T \cdot L/S
\]

(9)

The equations developed for methanol and ethanol extracts were significant at the 95% confidence level, whereas the one deduced for water extracts was significant at the 90% confidence level. The major contributions of the dependent variables (and interactions among them) can be identified in Eqs. (7)–(9).

Activities of commercial antioxidants, such as ascorbic acid (EC50 = 0.12 mg/mL) and rutin (EC50 = 0.16 mg/mL) determined in our laboratory, were slightly higher than the best extracts obtained in this work. Higher DPPH radical scavenging capacities were reported for an ethanolic extract produced by a series of five extractions stages of 10 min (EC50 = 0.012 mg/mL) [11], for crude extracts and fractions obtained with a sequence of solvents (EC50 = 0.017–0.071 mg/mL) [3], and slightly higher for a boiling water extract from chestnut leaves (EC50 = 0.17 mg/mL) [7].

The effects of the most influential variables on the extract concentration needed to scavenge 50% of DPPH (EC50) are shown in Fig. 1c. Optimal conditions for antioxidant activity of the aqueous extracts are defined by contact times in the range of 50–90 min and temperatures below 30 °C, whereas alcoholic extracts manufactured at increased temperatures (up to 50 °C) showed increased activity.

The ABTS+ radical scavenging capacity of extracts produced under selected conditions showed similar potency regardless the extracting solvent. The most active extracts were obtained with ES (EC50 = 0.34 mg/mL). This activity was near the ones determined for AWS (0.38 mg/mL) and MS (0.37 mg/mL) extracts.

The phenolic content of extracts, measured by the ratio PEY/SEY showed a close dependence on the DPPH radical scavenging capacity of the corresponding extracts (see Fig. 2). This finding suggests that the total phenolic content of different extracts was a major factor to explain their antioxidant activity, as it has been proposed for polyphenols both in polar solvents [3] and in hot water extracts, but not for flavonoids [7].
It can be noted that temperature caused different effects on the PEY and on the antioxidant activity: PEY increased with temperature, whereas an intermediate temperature led to maximal antioxidant activity. It can be noted that increased temperatures enhance extraction by improving both solubilities and diffusion coefficients. However, intermediate temperatures can be more favorable for extracting active phenolics, due to the limited degradation.

3.3. Antioxidant properties characterization

CsL aqueous extracts obtained under optimal conditions described above were assayed for their ability to scavenge ROS ($O_2^{-}$, $H_2O_2$, ROO$, HOCl$, and $O_2^*$) and RNS (NO and ONOO$^-$, in the presence and absence of $HCO_3^-$). Limited effects were observed in experiments with $H_2O_2$ and HOCl, whereas the scavenging activi-
ties against O$_2^\cdot$. $^{1}$O$_2$ and ROS were remarkable (Fig. 3), presenting IC$_{50}$ in low mg/mL range (Table 7). The scavenging effects observed for the studied RNS were much higher than those obtained for ROS, a finding in agreement with literature [6].

Sonication-assisted ethanol extraction of chestnut leaves was reported by Živković et al. [5]. In the present work, this strategy enhanced SEY and the phenolic content of extracts by a factor of 1.2, without compromising the extraction selectivity significantly (EC$_{50}$ = 0.36 mg/mL).

3.4. Composition of the CS extracts

The literature reported on the phenolic compounds present in CS lists a number of phenolic acids ($p$-hydroxyl, vanillyl, syringil, gallic, ellagic, $p$-coumaric, chlorogenic and ferulic acids) [1,2] and flavonoids (rutin, hesperidin, quercetin, hyperoside, isorquercitrin, apigenin, morin, naringin, narcissin, astragalin, galangin and kaempferol) [6,8,10]. In the aqueous extracts produced according to the optimized process of the present work, the major phenolic compounds identified by HPLC were gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, rutin, quercetin and apigenin.

4. Conclusions

An experimental design was employed to develop mathematical equations suitable for assessing the yield in soluble compounds, the yield in phenolics and the antioxidant activity of extracts produced from C. sativa leaves (CSL) with water, ethanol and methanol. This feedstock is bio-renewable material of huge availability, employed in traditional medicine, and a potential source of radical scavengers. Both extraction time and temperature affected the yields of soluble solids and phenolics strongly. Prolonged extraction periods (>90 min) at high temperatures (50 °C) led to optimal extraction of phenolics with water and ethanol; whereas limited effects were associated to the contact time in extractions with methanol at high temperatures. Acidified water was the best solvent for phenolic extractions, which yield extracts of high purity. Under selected conditions, extracts containing up to 54 wt% of phenolic acids and flavonoids were produced, with a radical scavenging capacity similar to that of BHA. The process proposed in this work is simple, flexible, and causes low environmental impact.

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