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Graphical Abstract

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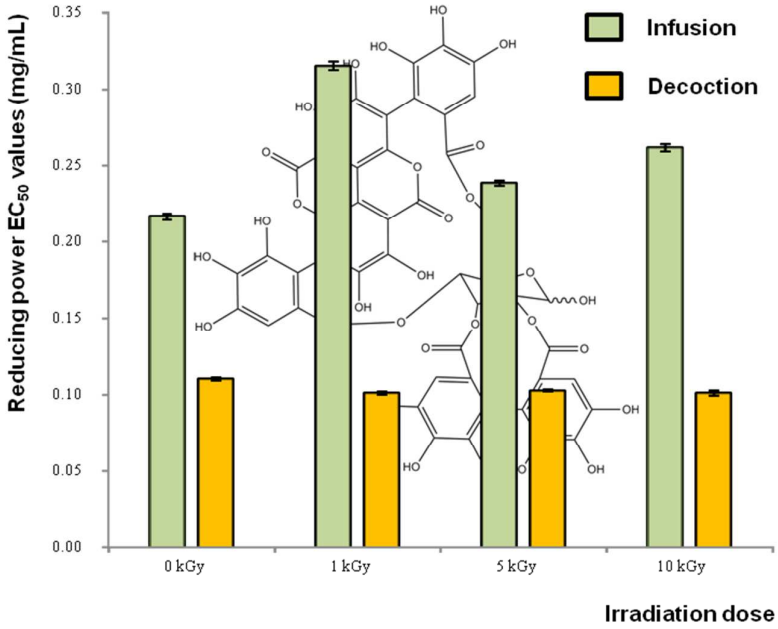

The preparation method had higher influence on bioactive properties than the processing treatment, being decoctions preferable over infusions, as indicated by the higher antioxidant activity and levels of phenolic compounds.

Combined effects of gamma-irradiation and preparation method on antioxidant activity and phenolic composition of *Tuberaria lignosa*

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Abstract

In this study, the effect of different doses of gamma-irradiation (0, 1, 5 and 10 kGy) on colour, antioxidant activity and phenolic compounds of shade-and freeze-dried samples of *Tuberaria lignosa* were evaluated and compared. The last two parameters were performed on decoctions and infusions in order to investigate the influence of the preparation method as well. In general, gamma-irradiation has no influence on colour parameter; changes caused by this technology were only identifiable on the lipid peroxidation inhibition capacity of the shade-dried samples and also on a few phenolic compounds. Differences among preparation method were significant for all assayed parameters, being decoctions preferable over infusions, as indicated by the higher antioxidant activity and levels of phenolic compounds. Overall, the gamma-irradiation treatment (up to 10 kGy) did not significantly affect the analyzed parameters. Nevertheless, other studies are of interest to evaluate the preservation effectiveness of this technology.

Keywords: *Tuberaria lignosa*; decoction/infusion; gamma-irradiation/drying; colour; antioxidant activity; phenolic compounds.

Introduction

During the last decades, medicinal and aromatic plants have been extensively studied and found to be excellent sources of bioactive and health-promoting compounds.[1,2] Actually, many plant extracts rich in phenolic compounds are used as food complements or can be integrated into cosmetic or pharmaceutical formulations.[3,4] For this reason, the industry is strongly interested in bioactive molecules from natural sources.

Based on ethnobotanical surveys conducted in western regions of the Iberian Peninsula, *Tuberaria lignosa* (Sweet) Samp. (Fam. Cistaceae) arises as one of the most quoted medicinal plants.[5,6] After being dried, this plant is used in herbal preparations (infusion and decoction) for treating various diseases and ailments, such as gastrointestinal and hepato-depurative disorders and skin inflammations.[6] These local practices are supported by documented biological effects, namely anti-inflammatory and antiulcerogenic (cytoprotective) properties,[7] as well as *in vitro* antioxidant[8] and antiviral activities.[9] Additionally, the phenolic fraction of this plant, mainly composed of ellagitannins and flavonoids, may be linked to the above mentioned effects.[8,9]

During the entire production process (from harvesting and drying to packaging and storage), raw medicinal plants are prone to chemical and microbial contaminations and insect infestation, which can lead to spoilage, quality deterioration and consequent economic loss.[10,11] Besides being a health hazard to consumers, contaminated medicinal plants can also adversely affect the efficacy and stability of their bioactive compounds, especially during storage,[12] and lead to spoilage of pharmaceuticals and food items to which they are added.[13] Therefore, an effective and sustainable decontamination process must be followed to ensure the hygienic quality of these products, making them suitable for human consumption and commercialization.

Chemical fumigants have been used to decontaminate plant products, being now prohibited or increasingly restricted in several countries due to health, environmental or occupational safety issues.[14] Furthermore, once conventional thermal treatments can damage many plant properties, either chemical or physical,[15] new and emerging non-thermal technologies are being investigated and applied. Among them, irradiation processing with gamma-rays is in an exceptional position. This physical method, considered safe and effective by several international authorities (namely FAO, IAEA and WHO),[16] has been used for insect disinfestations and parasite inactivation (with low doses up to 1 kGy), reduction of non-spore forming pathogens and spoilage microorganisms (with medium doses from 1 to 10 kGy), and reduction of microorganisms to the point of sterility (achieved at high doses above 10 kGy).[13,17] Likewise, the gamma-irradiation treatment provides an alternative way to eliminate pesticide residues from plant products.[18] In the European Union, the maximum dose of gamma-irradiation approved to sanitize dried herbs is 10 kGy,[19] whereas in USA the maximum is 30 kGy.[20]

Meanwhile, there is a growing scientific interest in irradiation-induced modifications on antioxidant activity and the compounds responsible for such activity. It is known that during the irradiation process, free radicals and other reactive species are generated due to the interaction with water molecules, capable of breaking chemical bonds and modify various molecules.[12] A previous study conducted by our team on *T. lignosa* showed that it has strong antioxidant activity;[8] however the effects of gamma-irradiation on the chemical and physical properties of this plant have never been studied. Therefore, the present study was undertaken to explore the effect of different doses of gamma-irradiation (0, 1, 5 and 10 kGy) on the antioxidant activity, phenolic compounds and colour parameters of shade- and freeze-dried *T. lignosa* samples. The first two

parameters were performed on decoctions and infusions, forms traditionally used for therapeutic applications, in order to investigate the influence of the preparation method as well.

1. Materials and methods

1.1. Standards and reagents

1.1.1. *For irradiation.* The dose rate of irradiation was estimated by Fricke dosimetry, using a chemical solution sensitive to ionizing radiation prepared in the lab following the standards.[21] The irradiation dose was estimated during the process using Amber Perspex routine dosimeters (batch V, from Harwell Company, UK). To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

1.1.2. *For antioxidant activity evaluation.* 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system.

1.1.3. *For phenolic compounds analysis.* Acetonitrile (99.9%, HPLC grade) was from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (apigenin-6-*C*-glucoside, *p*-coumaric acid, ellagic acid, gallic acid, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-C-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system.

115 1.2. Samples

116 *Tuberaria lignosa* (Sweet) Samp. (synonym of *Xolantha tuberaria* (L.) Gallego, Munoz
117 Garm & C. Navarro) was collected in the flowering season in Miranda do Douro (Trás-
118 os-Montes, north-eastern Portugal), considering the local medicinal uses as well as
119 healers' and selected consumers' criteria, which are related to particular gathering sites
120 and requirements for safe herbal dosages forms, such as infusion and decoction.
121 The option for wild samples, instead of ones from commercial origin, was supported by
122 a previous work of our research team[8] that highlighted wild *T. lignosa* samples as
123 having higher phenolics content and antioxidant activity than those obtained in a local
124 herbal shop available as dried rosettes of leaves and inflorescences. While the plant
125 material collected in the field is fresh, the commercial one from herbal shops may have
126 been stored for a long period of time or dried differently, which leads to quality loss.
127 Voucher specimens were deposited in the Herbarium of the Escola Superior Agrária de
128 Bragança, Portugal.
129
130 1.3. Samples drying
131 *Tuberaria lignosa* flowering aerial parts (*e.g.*, basal leaves, stems and inflorescences)
132 were submitted to two different drying methods: freeze-drying (7750031 Free Zone 4.5,
133 Labconco, Kansas City, MO, USA) immediately after being gathered; and shade-
134 drying, being stored in a dark and dry place in cellophane or paper bags kept at room
135 temperature (~21 ºC and 50% relative humidity) for 30 days, simulating informants'
136 general conditions of traditional plant-use.
137
138 1.4. Samples irradiation

Freeze- and shade-dried samples were divided into four groups (conveniently packaged in sterilized polyethylene bags): control (non-irradiated, 0 kGy), sample irradiated at 1 kGy, sample irradiated at 5 kGy, and sample irradiated at 10 kGy, where 1, 5 and 10 kGy were the predicted doses.

The samples irradiation was performed in a ^{60}Co experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources, total activity 177 TBq (4.78 kCi), in January 2014. The estimated dose rate for the irradiation position was obtained with a Fricke dosimeter. During irradiation process, the dose was estimated using Amber Perspex routine dosimeters, following the procedure previously described by Fernandes et al.[22] The estimated doses after irradiation were: 0.92 ± 0.01 kGy, 4.63 ± 0.28 kGy and 8.97 ± 0.35 kGy for the freeze-dried samples irradiated at 1, 5 and 10 kGy, respectively; and 1.00 ± 0.04 kGy, 5.07 ± 0.27 kGy and 9.66 ± 0.90 kGy for the shade-dried samples irradiated at 1, 5 and 10 kGy, respectively. The dose rate was 1.9 kGy.h^{-1} and the dose uniformity ratio (D_{max}/D_{min}) was 1.1 for the freeze- and shade-dried sample irradiated at 1 kGy, and 1.2 for the freeze- and shade-dried sample irradiated at 5 and 10 kGy. For simplicity, in the text, tables and figures, the values 0, 1, 5 and 10 kGy were considered for the doses.

1.5. Colour measurement

A colorimeter (model CR-400; Konica Minolta Sensing, Inc., Japan) with an adapter for granular materials (model CR-A50) was used to measure the colour of the samples. Using illuminant C and the diaphragm opening of 8 mm, the CIE L^*, a^* and b^* colour space values were registered through the computerized system using a colour data software "Spectra Magic Nx" (version CM-S100W 2.03.0006). The instrument was calibrated using the standard white plate before analysis.

164 The colour of the shade-and freeze-dried irradiated and non-irradiated samples was
165 measured in three different points on each set of samples, being considered the average
166 value to determine the colour coordinates L^* (lightness ↔ darkness), a^* (redness ↔
167 greenness), and b^* (yellowness ↔ blueness).
168
169 1.6. Preparation of decoctions and infusions
170 To prepare decoctions, each sample (1 g) was added to 200 mL of distilled water,
171 heated on a heating plate (VELP Scientific, Usmate, Italy) and boiled for 5 min. The
172 mixture was left to stand at room temperature for 5 min more, and then filtered through
173 Whatman No. 4 paper.
174 To prepare infusions, each sample (1 g) was added to 200 mL of boiling distilled water
175 and left to stand at room temperature for 5 min, and then filtered through Whatman No.
176 4 paper.
177 A portion of the obtained decoctions and infusions was frozen and lyophilized (Free
178 Zone 4.5, Labconco, Kansas City, MO, USA) for subsequent analysis of phenolic
179 compounds. The antioxidant properties were evaluated directly on the
180 decoctions/infusions.
181
182 1.7. *In vitro* antioxidant properties
183 1.7.1. *General.* The decoctions and infusions were redissolved in water (final
184 concentration 1 mg/mL) and further diluted to different concentrations to be submitted
185 to distinct in vitro assays[8] to evaluate their antioxidant properties. The extract
186 concentration providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were
187 calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene
188 bleaching inhibition and TBARS formation inhibition assays) or absorbance at 690 nm

(reducing power assay) against extract concentrations. Trolox was used as standard.

1.7.2. *DPPH radical-scavenging activity.* This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Potton, UK). The reaction mixture in each one of the 96-wells consisted of one of the different solution concentrations (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} M). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: RSA (%) = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, 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 D_{PPH} solution.

1.7.3. *Reducing power.* This methodology was performed using the microplate reader described above. The different solution concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in 48-well plates, with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

1.7.4. *Inhibition of β-carotene bleaching.* A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water

(100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany). β-carotene bleaching inhibition (CBI) was calculated using the following equation: CBI (%) = (Abs after 2 h of assay/ initial Abs) × 100

1.7.5. *Inhibition of thiobarbituric acid reactive substances (TBARS) formation.* Porcine *(*Sus scrofa) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100, where A and B were the absorbance of the control and the compound solution, respectively.

1.8. Phenolic compounds

Each lyophilised decoction/infusion (1 mg) was dissolved in water:methanol (80:20 v/v), filtered through 0.2 μm nylon filters from Whatman, and analysed by HPLC (Hewlett–Packard 1100 chromatograph, Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP ChemStation (Rev. A.05.04) data-processing station. A Spherisorb S3 ODS-2 C18 (Waters, Dinslaken, Germany), 3 μm (4.6 mm × 150 mm) column thermostatted at 35 ºC was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15-25% B over 5 min, 25-35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by Analyst 5.1 software. Zero grade air served as the nebuliser gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive scan modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to obtain full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10 V. Spectra were recorded in negative ion mode between *m/z* 100 and 1500. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) of the

previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the decoctions/infusions were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations (2.5-100 μg/mL) of different standard compounds: apigenin-6-*C*-glucoside (y = 246.05 x - 309.66; R^2 = 0.9994); *p*-coumaric acid (y = 321.99x + 98.308; R^2 = 0.9984); ellagic acid (y = 35.695x - 265.7; R^2 = 0.9991); gallic acid (y = 556.94x + 738.37; R^2 = 0.9968); kaempferol-3-*O*-glucoside (y = 190.75x - 36.158; R^2 = 1); kaempferol-3-*O*-rutinoside (y = 17 5.02x - 43.877; R^2 = 0.9999); luteolin-6-*C*-glucoside (y = 365.93x + 17.836; R^2 = 0.9997); quercetin-3-*O*-glucoside (y = 316.48x + 2.9142; R^2 = 1), and quercetin-3-*O*-rutinoside (y = 222.79x + 243.11; R^2 = 0.9998). The results were expressed in mg per g of lyophilised decoction/infusion.

1.9. Statistical analyses

In all cases, analyses were carried out using three samples separately processed, each of which was further measured three times. Data were expressed as mean±standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors “irradiation dose” (ID) and “preparation method” (PM). When a statistically significant interaction (ID×PM) was detected, the two factors were evaluated simultaneously by the estimated marginal

means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means were compared using Tukey's honestly significant difference (HSD), or other multiple comparison test (*t*-test).

Principal components analysis (PCA) was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

2. Results and discussion

2.1. Colour assessment

The results for CIE colour L^* (lightness), a^* (redness) and b^* (yellowness) are presented in **Table 1**. The colour coordinate L^* measures the lightness of the sample surface and ranges from black at 0 to white at 100. The chromaticity coordinate a^* measures red when positive and green when negative, and chromaticity coordinate b^* measures yellow when positive and blue when negative.[23] The reported values are given as the mean value of each irradiation dose (ID), including results from shade-or freeze-dried samples, as well as the mean value of each drying method (DM), considering all irradiation doses in each case. The significance of the effect of DM was evaluated using a *t*-test for equality of means (after checking the equality of variances through a Levene's test), since there were fewer than three groups. The interaction among factors (ID×DM) was never significant ($p > 0.05$), allowing to compare the effects of each factor *per se*. As it can be concluded from **Table 1**, the effect of ID was not significant in any case, indicating that these physical parameters are not strongly affected by

312 gamma-irradiation. On the other hand, the effect of the DM was always significant,
313 showing that samples dried under shade are prone to present lower lightness and redness
314 and higher yellowness. According to the literature, higher L^* values and lower a^*/b^*
315 values are desirable in dried products.[24] Therefore, freeze-drying may be indicated as
316 the most suitable DM for *T. lignosa* samples. Additionally, the lack of significant
317 changes observed in irradiated samples might be an advantageous feature, since the
318 colour parameters are of great importance in food and cosmetics industry.[25] In fact, the
319 colour of dried medicinal and aromatic plants is considered as a primary quality
320 criterion[23] and is directly related to consumers' appreciation of a product as they tend to
321 associate product colour with its taste, hygienic security, shelf-life and personal
322 satisfaction.[26]
323
324 2.2. Antioxidant activity
325 The EC_{50} values obtained for each antioxidant assay are presented in **Table 2**, both for
326 shade-dried and freeze-dried samples. The reported values are given as the mean value
327 of each irradiation dose (ID), including results from samples submitted to infusion or
328 decoction, as well as the mean value of each preparation method (PM), containing the
329 results for all assayed doses in each case. The significance of the effect of PM was
330 evaluated using a *t*-test for equality of means (after checking the equality of variances
331 through a Levene's test), since there were fewer than three groups. The interaction
332 among factors (ID×PM) was significant ($p < 0.05$) in all cases, acting as a source of
333 variability. Thereby, no multiple comparison tests could be performed. However, some
334 conclusions could be drawn after analysing the estimated marginal mean (EMM) plots.
335 For instance, shade-dried (**Figure 1A)** and freeze-dried (**Figure 1B)** samples, further
336 extracted by decoction, gave greater antioxidant activity than infusion ones. Concerning

the effect of ID, the only identifiable tendency was the apparently negative effect of the 5 kGy dose on the lipid peroxidation inhibition capacity in shade-dried samples.

The interest of decoctions and infusions from shade- and freeze-dried samples of *T. lignosa* was already highlighted as a source of bioactive compounds and having appreciable antioxidant properties.[8] The same work also highlighted wild *T. lignosa* samples as having higher phenolics content and bioactivity than those obtained in a local herbal shop available as dried rosettes of leaves and inflorescences. That is why wild samples were chosen for this study instead of ones from commercial origin.

Although the antioxidant activities of different medicinal and aromatic plants have already been studied,[1-3] nothing has been reported on the effect of gamma-irradiation on the antioxidant activity of *T. lignosa*. However, some research studies report different effects of the gamma-irradiation treatment on the antioxidant properties of other plant materials. A study conducted by Pereira et al.[11] indicated that, in general, the antioxidant properties were increased in borututu (a folk medicine obtained from the African tree *Cochlospermum angolense*) infusions and methanolic extracts with the irradiation dose of 10 kGy. Carocho et al.[27] found that the antioxidant potential of Portuguese chestnuts was increased at 3 kGy. As well, Hussain et al.[28] reported a significant decrease in EC_{50} values (corresponding to a higher antioxidant activity) of sun-dried irradiated (3 kGy) apricots. According to Pérez et al.,[29] a 30 kGy dose applied to dry sage and oregano for sanitization did not significantly affect the capacity to inhibit the DPPH radical or the reducing power, nor did it affect the total phenolic content of the methanolic and aqueous extract. Similarly, Mustapha et al.[30] observed no significant changes in the free radical scavenging activity of irradiate millet flour up to 5 kGy. In contrast, Kim and Yook[31] observed that irradiation of kiwifruit up to 3 kGy had negative effects on vitamin C content and antioxidant activity.

Regarding the use of gamma-irradiation for preservation purposes, its suitability for the hygienization of *T. lignosa* is unknown; nevertheless some studies support its effectiveness in similar doses for comparable natural matrices, including other dried medicinal and aromatic plants, without affecting their bioactive properties. Chiang et al.[32] demonstrated that 2 kGy is sufficient for the inactivation of enterobacteria and 6 kGy for elimination of yeasts and fungi in *Polygonum multiflorum* Thunb. (an herb used in traditional Chinese medicine), without adversely compromising the total phenols content or the antioxidant potential. Likewise, Kumar et al.[33] concluded that an irradiation dose up to 10 kGy is adequate to ensure the microbiological decontamination of Indian herbs retaining their antioxidant properties. Furthermore, in the European Union, the maximum dose of gamma-irradiation approved to sanitize dried herbs is 10 kGy, assuring its decontamination.[19]

2.3. Phenolic compounds

Table 3a and **b** shows the quantified amounts of phenolic compounds in non-irradiated and irradiated samples of *T. lignosa* previously freeze- or shade-dried, respectively. The results are expressed as mean value of each ID for different PM, as well as the mean value of each PM, comprising results for all the assayed ID. In general, despite slight quantitative differences, the phenolic profiles described herein were coherent to those previously characterized in *T. lignosa*.[8] The most abundant compounds were punicalagin isomers (compounds 1 and 3) and punicalagin gallate isomers (compounds 2 and 4) (**Figure 2**), which accounted for more than 90% of the quantified phenolic compounds. In fact, *T. lignosa* was previously reported as an important source of this type of compounds.[9] The interaction among factors (ID×PM) was again significant ($p < 0.05$) in all cases; thus, no multiple comparison tests could be performed. Nevertheless,

some observations can be made. In general, shade-dried samples contained lower levels of phenolic compounds than freeze-dried ones. Also a tendency to a decrease in the concentrations of phenolic compounds, especially ellagitannins, was observed in the irradiated samples in relation to non-irradiated ones, which was more accused in the shade-dried samples, although the changes were not statistically significant. Significant differences existed, however, in the levels of compounds depending on the preparation procedure (**Figure 2**). With no exception, higher contents of ellagitannins, flavones and flavonols were found in samples extracted by decoction than by infusion, both in shade- and freeze-dried products.

According to Khattak et al.[34] the effects of gamma-irradiation on the phenolic content and antioxidant activity would be influenced by plant type and composition, state of the sample (fresh or dry), extraction solvent and procedures, and dose of gamma-irradiation. Furthermore, the irradiation treatment of plant products previously dehydrated under a selected drying method may be a strategy to maintain or improve some chemical or physical parameters.

In general, from the obtained results, it might be concluded that the decoction methodology is preferable to infusion, as indicated by the higher antioxidant activity and levels of phenolic compounds. This finding may be linked to the higher extraction yield achieved with the longer extraction time of decoction compared to infusion. However, local medicinal uses as well as healers' or selected consumers' criteria should be taken into account during the preparation and use of these herbal beverages. Indeed, infusions are commonly used for internal use while decoctions are used for external and topical application. Furthermore, *T. lignosa* preparations should be avoided during long-term treatments in order to prevent possible side effects or toxicity, which can vary considerably according to the preparation method, doses and physical condition of the

individual.[6] That is why the folk medicine recommends specific dosages and controlled periods of intake with ritual healing practices.[6]

The obtained results are in agreement with those of Martins et al.,[35,36] who concluded that decoction preparations are preferable over infusions or even hydroalcoholic extracts to achieve higher concentration of flavonoids and total phenolic compounds, as well as greater antioxidant activity, from oregano and thyme plants. Vergara-Salinas et al.[37] reported that for extracting phenolics from thyme with water, 100 °C and 5 min are appropriate operating conditions, whereas antioxidant-active non-phenolic compounds were favored at higher temperatures and exposure times. Another recent study, conducted by Martínez-Las Heras et al.,[38] concluded that the drying method (including shade- and freeze-drying) and preparation procedures have a great influence on the stability and extractability of bioactive compounds from persimmon leaves. The authors showed that increasing the extraction time (up to 60 min) and temperature (from 70 ºC to 90 ºC) during water extraction of the herbal beverage increases the concentration of flavonoids and phenolic compounds. Similarly, He et al.[39] studied the subcritical water extraction of phenolic compounds from pomegranate seed residues and showed that increasing the same variables (extraction time up to 30 min and temperature up to 220 ºC) increases the content of these compounds.

2.4. Principal component analysis (PCA)

In sections 3.2. and 3.3., the effects resulting from ID or PM were compared considering antioxidant properties and phenolic composition separately. Despite, some statistically significant changes were observed in both cases, the true effects of the evaluated factors were not completely clear. Accordingly, the results for those parameters were evaluated simultaneously through principal components analysis

(PCA). Chromatic parameters were not considered in this analysis since they were measured before the preparation of extracts; furthermore, colour parameters after decoction or infusion of herbs are less relevant.

It was intended to verify if differences observed in each evaluated parameter were high enough to correlate with the defined principal components in a way that the geometric distribution of their loadings would lead to the individual clustering of each ID or PM. Regarding shade-dried samples, the first two dimensions (first: Cronbach's α, 17.060; eigenvalue, 0.984; second: Cronbach's α, 2.671; eigenvalue, 0.654) accounted for most of the variance of all quantified variables (74.2% and 11.6%, respectively). Groups corresponding to each gamma-irradiation dose (0 kGy, 1 kGy, 5 kGy and 10 kGy) were not individualized, as it could be hypothesized from **Tables 2**, **3a** and **b**. In fact, only the group corresponding to those samples irradiated with 10 kGy and prepared by decoction (please confront **Figure 3A** and **B**) were clearly separated from the remaining cases. The other defined groups include objects corresponding to non-irradiated and irradiated samples distributed in a random manner. This mixed grouping did not allow to define which of the assayed parameters better describe each one of applied ID, which might be considered as an indication of the lack of significant effects of gamma-irradiation at the assayed doses (except samples extracted by decoction and further submitted to a 10 kGy ID) on the antioxidant and phenolic profiles of *T. lignosa.* On the other hand, object points corresponding to each PM were clearly separated, proving that the previously highlighted significant differences were high enough to profile each of these methodologies (**Figure 3B**). In an overall analysis, it is clear that samples obtained by decoction have higher amounts of phenolic compounds as also stronger antioxidant activity, as indicated by the diametrically opposed position of their component loadings and the antioxidant activity assays object points.

462 Concerning freeze-dried samples, the first two dimensions (first: Cronbach's α, 17.383;
463 eigenvalue, 0.985; second: Cronbach's α, 1.739; eigenvalue, 0.444) also accounted for
464 most of the variance of all quantified variables (75.6% and 7.6%, respectively). The
465 obtained outcomes were quite similar, with no separation of object scores according to
466 each of the applied ID (**Figure 4A**). Curiously, a small group of objects was
467 individually clustered, as it was verified in shade-dried samples. Nevertheless, in this
468 case, this group corresponded to non-irradiated samples prepared by decoction. This
469 dissimilarity among samples dried using different methodologies is in agreement with
470 the observed significant interaction among factors (ID×PM). As it can be easily deduced
471 from **Figure 4B**, object points corresponding to each PM were clearly separated. Once
472 again, infusions showed lower levels in phenolic compounds, as also weaker antioxidant
473 activity.
474
475 In general, the preparation method (infusion or decoction) had higher influence in the
476 phenolic profile and antioxidant activity than the irradiation treatment at the applied
477 doses. In addition, CIE colour parameters were also more sensitive to the drying method
478 than irradiation. Differences among infusions and decoctions were significant for all
479 assayed parameters, while changes caused by gamma-irradiation were only significant
480 in TBARS formation inhibition, β-carotene bleaching inhibition and a few phenolic
481 compounds. Besides their effects in individual cases, when all parameters were
482 evaluated together, modifications caused by the preparation method were clearly higher
483 than those observed for gamma-irradiation. As it might be depicted from the PCA plots,
484 object points corresponding to different irradiation doses were grouped arbitrarily, while
485 those corresponding to infusions and decoctions were completely separated. The
486 obtained results indicate that the decoction should be the preferable choice to prepare

beverages from this plant, in order to obtain the higher antioxidant activity and phenolic content. Furthermore, the gamma-irradiation treatment (up to 10 kGy), if applied as a preservation technology, will not significantly affect the antioxidant properties of dried *T. lignosa* samples. Nevertheless, other studies are of interest to evaluate the preservation effectiveness of this technology.

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579 **Figure 1.** Estimated marginal mean plots representing the effect of the preparation
580 method on the antioxidant properties of shade-dried (**A**) and freeze-dried (**B**) samples of
581 *T. lignosa*. **A**- DPPH scavenging activity; **B**- reducing power; **C**- β-carotene bleaching
582 inhibition; **D**- TBARS formation inhibition.

583 **Figure 2.** HPLC profile of phenolic compounds in decoction **(A)** and infusion **(B)** of *T.*
584 *lignosa* freeze-dried samples irradiated with 1 kGy, recorded at 280 nm.

585 **Figure 3.** Biplot of object scores (gamma-irradiation doses) and component loadings
586 (evaluated parameters) using gamma-irradiation **(A)** and preparation method (**B**) as
587 labelling variables for shade-dried samples.

588 **Figure 4.** Biplot of object scores (gamma-irradiation doses) and component loadings
589 (evaluated parameters) using gamma-irradiation **(A)** and preparation method (**B**) as
590 labelling variables for freeze-dried samples.

Table 1. CIE colour *L** (lightness), *a** (redness) and *b** (yellowness) of non-irradiated and irradiated *T. lignosa* shade- or freeze-dried samples. The results are presented as mean±SD[a].

		*L**	*a**	*b**
Irradiation dose (ID)	0 kGy	47 ± 5	0 ± 3	17 ± 3
	1 kGy	46 ± 6	0 ± 3	18 ± 3
	5 kGy	45 ± 5	-2 ± 3	18 ± 2
	10 kGy	43 ± 7	-1 ± 3	18 ± 3
p-value (n = 18)	Tukey's test	0.154	0.252	0.770
Drying method (DM)	Shade-dried	41 ± 5	-2 ± 2	19 ± 2
	Freeze-dried	49 ± 4	1 ± 2	17 ± 3
p-value (n = 36)	*t*-student's test	<0.001	<0.001	<0.001
p-value (n = 72)	ID×DM	0.253	0.262	0.077

[a]Results are reported as mean values of each irradiation dose (ID), including results from shade- or freeze-dried samples, as well as the mean value of each drying method (DM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or DM), and can be higher than mean values.

Table 2. Antioxidant properties (EC_{50} values, mg/mL) of non-irradiated and irradiated *T. lignosa* shade- or freeze-dried samples, according to the irradiation dose (ID) and preparation method (PM). The results are presented as mean±SD[a].

		DPPH scavenging activity	Reducing power	Lipid peroxidation inhibition	
				TBARS formation inhibition	β-carotene bleaching inhibition
Shade-dried					
Irradiation dose (ID)	0 kGy	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.04 ± 0.02
	1 kGy	0.2 ± 0.1	0.17 ± 0.05	0.2 ± 0.1	0.02 ± 0.01
	5 kGy	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.07 ± 0.04
	10 kGy	0.3 ± 0.1	0.16 ± 0.04	0.3 ± 0.1	0.03 ± 0.01
p-value (n = 18)	Tukey's test	0.242	0.160	<0.001	<0.001
Preparation method (PM)	Infusion	0.39 ± 0.05	0.25 ± 0.04	0.4 ± 0.1	0.05 ± 0.04
	Decoction	0.15 ± 0.01	0.11 ± 0.01	0.2 ± 0.1	0.025 ± 0.002
p-value (n = 45)	*t*-student's test	<0.001	<0.001	<0.001	<0.001
p-value (n = 90)	ID×PM	<0.001	<0.001	<0.001	0.046
Freeze-dried					
Irradiation dose (ID)	0 kGy	0.3 ± 0.1	0.16 ± 0.05	0.02 ± 0.02	0.3 ± 0.1
	1 kGy	0.3 ± 0.2	0.2 ± 0.1	0.03 ± 0.02	0.2 ± 0.1
	5 kGy	0.3 ± 0.1	0.2 ± 0.1	0.02 ± 0.01	0.2 ± 0.1
	10 kGy	0.3 ± 0.2	0.2 ± 0.1	0.03 ± 0.01	0.2 ± 0.1
p-value (n = 18)	*p*-value (n=18)	0.861	0.386	0.430	0.528
Preparation method (PM)	Infusion	0.41 ± 0.05	0.26 ± 0.04	0.04 ± 0.01	0.35 ± 0.05
	Decoction	0.15 ± 0.02	0.10 ± 0.01	0.01 ± 0.01	0.11 ± 0.05
p-value (n = 45)	*t*-student's test	<0.001	<0.001	<0.001	<0.001
p-value (n = 90)	ID×PM	<0.001	<0.001	<0.001	<0.001

[a]Results are reported as mean values of each irradiation dose (ID), including samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values.

Table 3a. Phenolic compounds (mg/g) of non-irradiated and irradiated *T. lignosa* freeze-dried samples. The results are presented as mean±SD[a].

Compound	Irradiation dose (ID)				Tukey's test	Preparation method (PM)		*t*-student's test	ID×PM
	0 kGy	1 kGy	5 kGy	10 kGy	*p*-value (n = 18)	Infusion	Decoction	*p*-value (n = 36)	*p*-value (n = 72)
1) Punicalagin (isomer)	23 ± 2	20 ± 10	21 ± 9	22 ± 7	0.776	15 ± 4	28 ± 3	<0.001	<0.001
2) Punicalagin gallate (isomer)	28 ± 11	25 ± 14	22 ± 12	24 ± 8	0.561	14 ± 3	36 ± 3	<0.001	<0.001
3) Punicalagin (isomer)	47 ± 5	37 ± 13	43 ± 14	43 ± 11	0.058	32 ± 7	53 ± 3	<0.001	<0.001
4) Punicalagin gallate (isomer)	33 ± 13	27 ± 15	27 ± 12	28 ± 9	0.520	17 ± 3	41 ± 3	<0.001	<0.001
5) Luteolin-6-*C*-glucose-8-*C*-glucose	0.27 ± 0.05	0.25 ± 0.05	0.28 ± 0.05	0.29 ± 0.05	0.198	0.22 ± 0.02	0.33 ± 0.02	<0.001	<0.001
6) 5-*O*-*p*-Coumaroylquinic acid	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.4	<0.001	0.8 ± 0.3	0.6 ± 0.1	0.001	<0.001
7) Luteolin-8-*C*-glucoside	1.3 ± 0.4	1.5 ± 0.5	1.5 ± 0.5	1.4 ± 0.4	0.634	0.9 ± 0.1	1.9 ± 0.2	<0.001	<0.001
8) Apigenin-8-*C*-glucoside	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	0.111	1.2 ± 0.1	1.4 ± 0.1	<0.001	0.025
9) Quercetin-3-*O*-rutinoside	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.349	0.16 ± 0.01	0.30 ± 0.04	<0.001	<0.001
10) Apigenin-6-*C*-glucoside	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	0.003	1.2 ± 0.1	1.4 ± 0.1	<0.001	<0.001
11) Kaempferol-3-*O*-rutinoside	0.37 ± 0.04	0.43 ± 0.05	0.44 ± 0.05	0.41 ± 0.05	0.014	0.35 ± 0.03	0.47 ± 0.05	<0.001	<0.001
12) Luteolin-6-*C*-glucoside	0.01 ± 0.01	0.03 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	<0.001	nd	0.02 ± 0.02	-	-
13) Kaempferol-*O*-rhamnoside-*O*-rutinoside	nd	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.002	nd	0.2 ± 0.1	-	-
14) Kaempferol-*p*-coumaroylglucoside-glutarate	nd	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.001	nd	0.2 ± 0.1	-	-
15) Kaempferol-*p*-coumaroylglucoside	nd	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.001	nd	0.2 ± 0.1	-	-
Phenolic acids	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.4	<0.001	0.8 ± 0.3	0.6 ± 0.1	0.001	<0.001
Flavonols	0.6 ± 0.1	1.1 ± 0.5	1.1 ± 0.5	1.0 ± 0.5	0.005	0.51 ± 0.02	1.4 ± 0.4	<0.001	<0.001
Flavones	4 ± 1	4 ± 1	4 ± 1	4 ± 1	0.680	3.5 ± 0.2	5.0 ± 0.3	<0.001	<0.001
Ellagitannins	130 ± 30	109 ± 52	114 ± 47	118 ± 35	0.469	78 ± 17	158 ± 4	<0.001	<0.001

[a]Results are reported as mean values of each irradiation dose (ID), including results from samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values. nd- not detected.

Table 3b. Phenolic compounds (mg/g) of non-irradiated and irradiated *T. lignosa* shade-dried samples. The results are presented as mean±SD[a].

Compound	Irradiation dose (ID)				Tukey's test	Preparation method (PM)		*t*-student's test	ID×PM
	0 kGy	1 kGy	5 kGy	10 kGy	*p*-value (n = 18)	Infusion	Decoction	*p*-value (n = 36)	*p*-value (n = 72)
1) Punicalagin (isomer)	26 ± 14	17 ± 3	17 ± 8	13 ± 13	0.003	9 ± 5	27 ± 7	<0.001	<0.001
2) Punicalagin gallate (isomer)	21 ± 13	14 ± 4	19 ± 10	13 ± 13	0.086	7 ± 4	27 ± 6	<0.001	<0.001
3) Punicalagin (isomer)	50 ± 20	32 ± 6	33 ± 14	24 ± 24	<0.001	19 ± 12	50 ± 12	<0.001	<0.001
4) Punicalagin gallate (isomer)	23 ± 15	15 ± 5	21 ± 12	15 ± 15	0.097	7 ± 4	30 ± 7	<0.001	<0.001
5) Luteolin-6-*C*-glucose-8-*C*-glucose	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.09 ± 0.02	<0.001	0.14 ± 0.04	0.3 ± 0.1	<0.001	<0.001
6) 5-*O*-*p*-Coumaroylquinic acid	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	<0.001	0.6 ± 0.2	1.0 ± 0.2	0.001	<0.001
7) Luteolin-8-*C*-glucoside	1 ± 1	1 ± 1	1 ± 1	1 ± 1	0.503	0.4 ± 0.2	2.0 ± 0.3	<0.001	<0.001
8) Apigenin-8-*C*-glucoside	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	0.056	1.1 ± 0.1	1.3 ± 0.1	<0.001	<0.001
9) Quercetin-3-*O*-rutinoside	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.479	0.08 ± 0.03	0.32 ± 0.05	<0.001	<0.001
10) Apigenin-6-*C*-glucoside	1.3 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	0.218	1.1 ± 0.1	1.5 ± 0.1	<0.001	<0.001
11) Kaempferol-3-*O*-rutinoside	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.188	0.3 ± 0.04	0.53 ± 0.05	<0.001	<0.001
12) Luteolin-6-*C*-glucoside	0.02 ± 0.02	0.002 ± 0.002	0.002 ± 0.002	0.02 ± 0.02	<0.001	nd	0.02 ± 0.02	-	-
13) Kaempferol-*O*-rhamnoside-*O*-rutinoside	0.2 ± 0.2	nd	nd	nd	-	nd	0.1 ± 0.1	-	-
14) Kaempferol-*p*-coumaroylglucoside-glutarate	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	nd	0.001	nd	0.2 ± 0.1	-	-
15) Kaempferol-*p*-coumaroylglucoside	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	nd	0.002	nd	0.2 ± 0.1	-	-
Phenolic acids	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	<0.001	0.6 ± 0.2	1.0 ± 0.2	0.001	<0.001
Flavonols	1.1 ± 0.5	0.8 ± 0.4	0.9 ± 0.4	0.6 ± 0.3	0.018	0.4 ± 0.1	1.3 ± 0.3	<0.001	<0.001
Flavones	4 ± 1	4 ± 1	4 ± 1	4 ± 2	0.360	2.7 ± 0.4	5.2 ± 0.5	<0.001	<0.001
Ellagitannins	121 ± 62	78 ± 18	90 ± 45	65 ± 65	0.012	42 ± 25	135 ± 31	<0.001	<0.001

[a]Results are reported as mean values of each irradiation dose (ID), including results from samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values. nd- not detected.