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Antioxidant and Anticancer Activity of Pericarp Water Extracts of Mediterranean Ancient Chestnut Accessions

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Abstract: The residue of chestnut processing generates a large amount of waste material, a resource not adequately exploited. The antioxidant and antitumoral properties of cold and hot water extracts from discarded pericarp of four chestnut Sardinian accessions and one marron variety were studied. The antioxidant capacity of the extracts was determined by spectrophotometric and electrochemical tests. The 1,1-diphenyl-2-pic-rylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) results were highly correlated with each other; likewise, a good correlation was found between Ferric Reducing Antioxidant Power (FRAP) and cyclic voltammetry (CV) values, both based on the direct transfer of electrons. The antiproliferative effect on normal cells (fibroblasts), and on colon (RKO and SW48) and breast (MCF7) cancer cells was evaluated. Additionally, this paper marks the first application of chestnut extracts to investigate their effects on melanoma (B16F10) cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test demonstrated that temperature and different extraction times significantly influenced the growth of cells, both normal and tumor. The fibroblast growth was significantly inhibited by moderate doses of cold extracts, while the GI_{50} values calculated for hot extracts were high, regardless of the accession or cultivar. An even more marked inhibitory action of the cold extracts was observed both on the growth of RKO and SW48 cells and on B16F10 melanoma cells. Otherwise, an extract concentration, both cold and hot, of no less than 243 μg mL^{-1} is required to achieve a 50% inhibition of MCF7 cell growth.

Keywords: chestnut biodiversity; pericarp; water extraction; antioxidant activity; cancer cells

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1. Introduction

The sweet chestnut tree (*Castanea sativa* Mill.), which belongs to the Fagaceae family, is widely distributed in European countries, Italy, Spain, France, Greece, Portugal and Turkey: it is a source of wood, fruit, honey and waste material for tannins [1,2]. Over the last decade, the chestnut sector has constantly grown in Europe, since fruits, mainly consumed boiled or roasted, are an excellent source of energy, due to their high starch content, minerals (K, Mg, Mn, and Cu), unsaturated fatty acids (omega-3), fiber and vitamins E and C [3,4]. A variety of preparations have been added to the traditional use of the fruit: marron glacé, purées, frozen nuts, and jams, and, more recently, different gluten-free products based on chestnut flour for celiac patients [5,6].

Burrs, leaves and the residue of chestnut processing generates a large amount of waste material, about 15% of the total production. Inner and outer shell resulting from chestnut peeling, represents about 20% of the total fruit weight [5]. This residue, habitually burned as fuel in factories, is the target of new strategies of value assessment and reuse: heavy metal absorbents or wood adhesives, raw materials for lignin and bioethanol production

and pigment source for dyeing [7,8]. At present, there are new attractive applications for utilizing chestnut waste as a source of bioactive compounds with health effects [9]; the chestnut pericarp (CP) (outer shell; husk) and integument (inner shell; pellicle) are a rich source of nutraceuticals, ascorbic acid, carotenoids, phenolics like gallic and ellagic acid, flavonoids and condensed tannins, with antioxidant, antimicrobial, and anticancer properties [4,10–12]. The *green* recovery of these bioactive compounds from chestnut shell is important, both to preserve their health properties for food, cosmetics and pharmaceutical industries and, to reduce the environmental impact of organic solvents [6]. Hence the decision to use, in this work, only deionized water as an extractant of phenolic compounds. Cold and hot water, alone or in combinations with organic solvents, has been employed by our group, with different purposes and on different matrices, since 2010 [13–17], but this work is the first on chestnut. Other researchers used cold water with Na_2SO_3 and NaOH to extract phenolic compounds, tannins and ellagitannins from Portuguese chestnut peels [18]; bur and shell extracts of *Castanea sativa* were obtained using water and hydroalcoholic solutions at 25, 50 and 75 °C to make the extraction faster, selective, and more effective [19]; Jung et al. [20] used water and a variety of organic solvents, at 60 °C, to extract polyphenols from inner shells of Japanese chestnut (*Castanea crenata*). Other authors used boiling water to maximize the recovery of bioactive compounds from inner and outer shells of Italian chestnut [21,22].

The use of water alone as a solvent is expected to provide extracts with a lower yield of phenolic compounds than organic solvents or hydroalcoholic mixtures [19]. The chemical composition of these extracts, their antioxidant and antitumoral properties, that are the subject of this research, are also expected to change [19,20,23].

In this work, the total phenol content (TP) and the antioxidant capacity of chestnut extracts have been determined by Folin–Ciocalteu and DPPH, ABTS and FRAP assays. Furthermore, according to recent studies on outer shell extracts [24] and other plant and fruit extracts [25,26], a direct electrochemical determination of TP and antioxidant capacity was also provided, since the method is particularly effective in the particular case of polyphenols extracts [27]. The redox properties of single molecules, such as gallic, ellagic and tannic acid, were also investigated [28,29]. Since the phenolic compounds can be easily oxidized on carbon electrodes [30–32], and their ionization potential determines their efficiency as antioxidants [33,34], the cyclic voltammetry (CV) was used to show the potential at which the oxidation starts and to provide a measure of antioxidant capacity [16].

Various researches, over the last 20 years, have provided a characterization of the phenolic compounds present in chestnut shell extracts, attributing them to their antioxidant and anti-tumor properties [35–37]. Although there is a general opinion on the anti-tumor action of polyphenolic fractions from various chestnut organs [12,21,38], even in this case it is yet to be established which molecules are actually responsible and which are not.

Moreover, there is some evidence that the same molecules that are responsible for their antioxidant activity at certain concentrations act as prooxidants at others [16]. Chestnut extracts, derived from flesh and the inner and outer shell, induced apoptosis in human gastric cancer cells [35]; apoptosis was also induced by chestnut honey on prostate cancer cells [39], by bark extracts on neuroblastoma cells [40], and by shell extracts on prostate, breast and hepatocellular cancer cells [12]. In this work, we tried to understand how the extraction method could affect the extraction yield of some compounds with presumed anti-tumor activity in different chestnut accessions of Sardinian biodiversity, and then we focused on the ability of the different extracts to inhibit the growth of cells belonging to different tumor cell lines.

2. Materials and Methods

Samples collection was carried out in “Barbagia” (Nu), an area historically devoted to chestnut cultivation, in the vast mountainous region of central Sardinia which extends along the sides of the “Gennargentu” massif. Samples were collected from four chestnut (*Castanea sativa* Miller) accessions of local biodiversity and one Marron variety (Table 1).

MURG, LOCG, ILDP, COEV and MARRV were all harvested in situ in the municipality of Belvì, Sardinia, Italy (39°57′38.19″ N 9°11′02.91″ E).

Table 1. Origin, elevation and harvest time of the chestnut accession and of the marron.

Accession/Variety	Origin	Elevation (m a.s.l)	Harvest Time	Acronym
MIGHELI URRU G	Belvì	822	October, 2021	MURG
LOCCHEDDU G	Belvì	812	October, 2021	LOCG
ILDUBBA P	Belvì	849	October, 2021	ILDP
COESERRA V	Belvì	686	October, 2021	COEV
MARRONE di Marradi V	Belvì	780	October, 2021	MARRV

2.1. Plant Material and Fruit Sampling

Chestnuts were collected at commercial maturity in October 2021. Fruits, free of defects and mechanical damage, were divided into three replicates of 30 chestnuts for each accession. The CP was manually separated and, in order to reduce and standardize the moisture content, oven-dried at 40 °C (thermostatic chambers BICASA B.E. 78). Subsequent weighing was carried out until a constant weight was reached (the process lasted approximately 36 h). The dried samples were ground in a mill (Micro Impact Mill Culatti, Steinen, Germany) to a fine powder (particle sizes 1 mm), and stored under vacuum in total darkness, until further analyses.

2.2. Extraction of Phenolic Compounds

Two different extractions, using water as a solvent, were carried out for the recovery of the bioactive molecules, according to [12] with some modification: (i) a conventional liquid extraction (CLE) in cold water at 20 °C for 4 h. 2.5 g of CP powder was added to 25 mL of milli-Q water in test tubes which were placed in a thermostatic bath (Lauda E100 Circulation Thermostat Bath, Lauda, Zevenhuizen, The Netherlands). The liquid and solid phases were separated by centrifugation at 3220× g for 15 min (ALC Refrigerated Centrifuge 4227 R, ALC, Milan, Italy). The supernatant was recovered by filtration through a filter paper (Whatman n. 1) under vacuum. The extraction procedure was repeated twice: the solvent/sample final ratio was 20:1 (v/w). The aqueous extract was stored at −20 °C until analysis.

(ii) A CLE in hot water at 95 °C for 1 h. A total of 2.5 g of dried sample was extracted with 50 mL milli-Q water (solvent/sample ratio 20:1 (v/w)), for 1 h, under continuous stirring in a thermostatic bath; then the suspension was cooled on ice and centrifuged (15 min at 3220× g). The supernatant was recovered by filtration through a filter paper (Whatman n. 1) under vacuum and stored at −20 °C until analysis.

2.3. Analytical Tests

Water extracts were analyzed for TP, total flavonoids (TF), proanthocyanidins (PA) also known as condensed tannins [41], individual phenolic compounds and antioxidant capacity.

2.3.1. Determination of Total Phenolic Content

The TP was assessed by Folin–Ciocalteu assay according to previous procedure [17]. Aliquots of the diluted samples were mixed in a 25 mL volumetric flask with Folin–Ciocalteu reagent (Carlo Erba, Milan, Italy) (1:1) and a 10 mL sodium carbonate solution 7.5% (Carlo Erba, Milan, Italy), and incubated for 2 h at room temperature. TP was determined by spectrophotometric analysis (8453 UVVisible Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) at an absorbance of 750 nm. The same procedure was applied for standard solutions of gallic acid. Final results were expressed as milligrams of gallic acid (Sigma–Aldrich, Milan, Italy) equivalents (GAE) per g of dried matter (DM) referring to the gallic acid calibration curve (10–100 mg L^{-1}, r^2 = 0.990). Samples were analyzed in triplicate.

2.3.2. Determination of Total Flavonoid Content

The TF was quantified by aluminum chloride assay following a previously reported procedure [17]. Briefly, 1 mL of sample properly diluted was added into a 10 mL volumetric flask containing 4 mL of milli-Q water. A total of 0.3 mL of 5% $NaNO_2$ (Sigma–Aldrich, Milan, Italy) was added at time zero and, after 5 min, 0.3 mL of 10% $AlCl_3$ (Sigma–Aldrich, Milan, Italy). After 6 min, 2 mL of 1 M NaOH (Carlo Erba, Milan, Italy) was added to the mixture. Finally, the solution was adjusted to 10 mL with milli-Q water and carefully mixed. Absorbance of the complex flavonoid–aluminum mixture was determined at 510 nm. Quantification was carried using a catechin (C) (Sigma–Aldrich, Milan, Italy) calibration curve (2.5–20 μg mL^{-1}, r^2 = 0.995). The same procedure was applied for standard solutions of C. Results were expressed as mg of catechin equivalent (CE) × g^{-1} of dried matter (mg CE g^{-1} DM). Samples were analyzed in triplicate.

2.3.3. Determination of Proanthocyanidins Content

The acidified vanillin method was used to quantify the extractable PA content, according to [17]. Briefly, 4 mL of diluted samples were mixed in a 10 mL volumetric flask containing 2 mL of ethanol (Carlo Erba, Milan, Italy) and 4 mL of vanillin (Sigma–Aldrich, Milan, Italy) solution (1% vanillin in 70% sulfuric acid (Carlo Erba, Milan, Italy)). The samples were compared to a blank with 4 mL of 70% sulfuric acid instead of vanillin solution. The absorbance of vanillin–tannin adducts was spectrophotometrically detected at 500 nm. Concentrations were calculated referring to a C calibration curve (1–6 μg mL^{-1}, r^2 = 0.998). The same procedure was applied for standard solutions of C. Results were expressed as mg CE g^{-1} DM. Samples were analyzed in triplicate.

2.3.4. Determination of Antioxidant Capacity

The antioxidant capacity of CP extracts was evaluated by DPPH and ABTS (Sigma–Aldrich, Milan, Italy) assays, according to the procedures reported in [42]. Briefly, for each assay, 0.1 mL of appropriately diluted sample was mixed with 3.9 mL of 60 μM DPPH or 7 mM ABTS, and then stored in the dark for 120 or 6 min, respectively. The absorbance was spectrophotometrically detected at 515 nm for DPPH and at 734 nm for ABTS. Concentrations were calculated referring to a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma–Aldrich, Milan, Italy) calibration curve (2–12 μmol L^{-1} of trolox; r^2 = 0.997 for the DPPH assay and r^2 = 0.998 for the ABTS assay). The same procedure was applied for Trolox standard solutions. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC), as mmol trolox equivalents·100 g^{-1} dry matter (mmol TEAC·100 g^{-1} DM). Samples were analyzed in triplicate.

The radical scavenging activity (RSA) was determined as a percentage of DPPH and ABTS discoloration, according to [19]. $EC_{50,}$ (mg mL^{-1}) is the extract concentration necessary to achieve a 50% radical DPPH or ABTS inhibition, calculated from the graph of RSA percentage against the extract concentration (from 0.01 to 0.1 mg mL^{-1}) (Figures S3 and S4 in Supplementary Materials).

The FRAP assay was carried out according to [19]. An aliquot (100 μL) of each extract was transferred to a test tube and 3.0 mL of freshly prepared FRAP reagent (25 mL of 300 mM acetate buffer pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ, Sigma–Aldrich, Milan, Italy) in 40 mM HCl (Carlo Erba, Milan, Italy) and 2.5 mL of 20 mM $FeCl_3 \times 6H_2O$, (Sigma–Aldrich, Milan, Italy) was added. The absorbance was recorded after 5 min at 593 nm. The antioxidant activities were calculated from the calibration curve of L-ascorbic acid (Sigma–Aldrich, Milan, Italy) (0.1– 1 mM, r^2 = 0.989). The same procedure was applied for L-ascorbic acid standard solutions. The results expressed as mg of L-ascorbic acid equivalents (AAeq) g^{-1} DM. Samples were analyzed in triplicate.

2.3.5. Determination of Antioxidant Capacity by Electrochemical Method

The antioxidant capacity (AAox) determination of the CP extracts was also performed by CV as previously reported [16,43] with some modifications. Measures were carried out

by screen-printed sensors [44] purchased by GSI Technologies (Burr Ridge, IL, USA), consisting of a 4 mm carbon working electrode (WE), an Ag/AgCl pseudo reference electrode (RE), and a carbon auxiliary electrode (AE). Currents were recorded by Quadstat, a commercial four-channel potentiostat (eDaQ Quadstat, e-Corder 410 and Echem software 2.1.0., eDAQ Europe Poland, Warsaw Poland). Cyclic voltammograms (CVs) were performed from −0.2 V to +0.8 V (vs. Ag/AgCl pseudo-RE) at a scan rate of 0.1 V/s. A first aliquot of 70 µL, containing only PBS (used as a supporting electrolyte), was deposited on the screen-printed WE with a graduated micropipette in order to obtain a baseline. Once the baseline current was recorded, the PBS drop was dried with absorbent paper without touching the surface of the sensor, and 70 µL aliquot of 2 mg mL^{-1} CP extract solution was deposited on the sensor surface, thus obtaining the corresponding CV pattern (the experiment was performed in triplicate). In order to provide a quantitative comparison among the CV patterns of extracts of different origin, the voltammograms were integrated and the area under curve (AUC) was calculated at +0.5 V and +0.8 V and expressed in microcoulombs (µC). In accordance with previous studies [16], the redox potential of +0.5 V is used as a threshold to detect the antioxidant activity of chestnut extracts, while +0.8 V as a measure of TP. As already reported [45], oxidation potentials higher than +0.5 V refer to polyphenols with low reducing power which, in this work, were not accounted as antioxidants.

2.4. HPLC Analysis of Phenolic Compounds

Chromatographic separation of phenolic compounds was carried out with reverse-phase HPLC method using an Agilent 1100 Liquid Chromatography (LC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (G1311A), degasser, column thermostat, auto-sampler (G1313A), and a diode array detector (G1315 B, DAD). The column was a Luna C18 column (250 mm × 4.6 mm, 5 µm) from Phenomenex (Torrance, CA, USA) with a security guard cartridge (4 mm × 2 mm). The flow rate was set at 0.8 mL min^{-1}, and the column temperature was set to 30 °C. Elution was carried out with a binary mobile phase of solvent A (water and 0.1% trifluoracetic acid, Sigma–Aldrich, Milan, Italy) and solvent B (acetonitrile, (Carlo Erba, Milan, Italy)) and the gradient elution program was according to [17]: 0 min, 99% A; 5 min, 95% A; 6 min, 93% A; 10 min, 85% A; 15 min, 75% A; 20 min, 10% A; 25 min, 99% A, with a post-time of 3 min. Detection was performed at 254, 280, 350, and 360 nm. Phenolic molecules were identified according to the retention time of a mixture of standards, selected from the literature concerning CP phenolic, their UV absorption spectra, as well as by adding standard solutions to the sample composition and quantified using the respective calibration curves. Samples were appropriately diluted before injection. The results are presented as milligrams per gram of dry matter (mg g^{-1} DM).

2.5. Cell Culture and Biological Assays

Five different cell lines, obtained from American Type Culture Collection (ATCC), have been selected to be tested with cold and hot water CP extracts: (1) Human fibroblast (normal cells); (2) B16F10 (murine melanoma cells; N-ras, Braf and p53 wt); (3) MCF7 (human metastatic breast cancer cells, estrogen receptor (ER) +, progesterone receptor (PR)+, epithelial receptor 2 (HER2)−, BRCA1 wt); (4) RKO (human metastatic colon cancer cells; P53, K-ras and PTEN wt, Braf p.V600E, PIK3CA p.H1047R); and (5) SW48 (human metastatic colon cancer cells; P53, K-ras and PTEN wt, Braf p.R347X[c], PIK3CA p.G914R[c]).

Human fibroblasts, B16F10 and MCF7 were grown in DMEM, RKO cells in EMEM and SW48 in RPMI1640 at 37 °C under humidified 5% CO_2/air. All media were completed with 10% FBS, 1% penicillin (100 U mL^{-1})/streptomycin (100 µg mL^{-1}), and 1% L-glutamine. For experimental studies, cells were plated in 96 well plates at a density of about 2 × 10^4 mL^{-1} and 24 h post seeding, exposed to increasing concentrations of hot and cold extract (10–50–100–250 µg mL^{-1}) for 24 h.

MTT Assay

The MTT assay was used to assess the cell viability as previously reported [16]: cells were incubated with 100 µL (0.05 mg mL^{-1}) of MTT, and the cultures were allowed to incubate at 37 °C for 3 h. The MTT was removed and the formazan crystals were dissolved in 100 µL of isopropanol. The color was read at 570 nm using a microplate reader (EMax® Plus Microplate Reader, Molecular Devices, LLC, San Jose, CA, USA). The percentage of cell growth and metabolic activity was calculated by normalizing the absorbance of the treated cells to corresponding control. All the experiments were done in quadruplicate and repeated at least three times.

2.6. Statistical Analysis

The statistical analysis was performed by GraphPad Prism 8.0 for Windows software (Graph-Pad Software, Inc., La Jolla, CA, USA). A two-way ANOVA was used to estimate how the chemical parameters (TP, TF, PA, HPLC quantification of phenols and antioxidant capacity) of the different accessions changed according to the temperature of extraction (20 °C and 95 °C). Data were reported as mean ± standard deviation (SD). In order to compare means obtained at 20 °C and 95 °C, a Student's *t*-test was performed for every parameter within each chestnut accession and for the marron. The level of significance was fixed at $p \leq 0.05$. Pearson correlation coefficients (R^2) were calculated using the same software and used to determine the relationship between the content of studied chemical parameters (TP, TF, PA, phenolics identified by HPLC) and their antioxidant capacity of cold and hot extracts (DPPH, ABTS, FRAP and CV). In this work, we considered that two parameters are highly correlated when $R^2 > 0.900$; they are in good correlation if the R^2 value is between 0.900 and 0.800; the correlation is moderate when R^2 is between 0.700 and 0.800. If the R^2 is < 0.700 we consider there is no correlation.

For each accession, a Student's *t*-test was used to compare the effects of different extract concentration on fibroblasts, setting the significance level at $p \leq 0.01$ vs. Ctrl. Then, a one-way ANOVA was performed to compare the effects of different extract concentration on cancer cells, setting the significance level at $p \leq 0.01$ vs. fibroblasts. The mean value ± SE is reported in the figures. The concentration of extract that results in inhibition of cell growth by 50% (GI_{50}) was calculated using nonlinear regression analysis in GraphPad Prism 8.0.

3. Results

The ability to recover bioactive molecules of two extraction techniques, one with cold water (20 °C), the other with hot water (95 °C), from the pericarp of different accessions and varieties of chestnut was investigated. It has been demonstrated that, at the same extraction time, hot water is a more effective extractant [19]. So, in this work, the contact time between matrix and solvent was diversified: 4 h with cold water and 1 h with hot water. The results of this experimental plan are reported in Tables 2 and 3, where it has been shown that the two extraction techniques sometimes lead to the same result, and other times to significant and even important quantitative and qualitative differences.

Table 2. Total phenolics (TP) assessed by Folin–Ciocalteu (F-C) and cyclic voltammetry (CV) at +0.8 V, total flavonoids (TF) and proanthocyanidins (PA) in the extracts of the pericarp chestnut samples.

Accessions or Variety	T °C	Total Phenolics		Total Flavonoids	Proanthocyanidins
		F-C (mg GAE g^{-1} DM)	CV $AUC_{+0.8}$ (µC)	mg CE g^{-1} DM	mg CE g^{-1} DM
MURG	20	39.58 ± 1.69	6.79 ± 0.25 *	18.69 ± 0.42 *	9.10 ± 0.72
	95	37.35 ± 0.64	4.92 ± 0.18 *	16.52 ± 0.14 *	9.20 ± 0.20
LOCG	20	44.33 ± 2.89	6.44 ± 0.11	12.92 ± 1.70 *	6.37 ± 1.17
	95	40.10 ± 2.16	7.37 ± 0.39	16.44 ± 2.3 *	7.55 ± 1.38
ILDP	20	42.96 ± 1.25	7.82 ± 0.25	11.37 ± 1.14 *	7.05 ± 1.25
	95	38.92 ± 2.89	7.48 ± 0.33	16.26 ± 1.62 *	7.99 ± 0.96

Table 2. *Cont.*

Accessions or Variety	T °C	Total Phenolics		Total Flavonoids	Proanthocyanidins
		F-C (mg GAE g^{-1} DM)	CV $AUC_{+0.8}$ (μC)	mg CE g^{-1} DM	mg CE g^{-1} DM
COEV	20	25.06 ± 0.46	4.55 ± 0.26	6.38 ± 0.21 *	4.73 ± 0.59
	95	22.70 ± 0.43	5.03 ± 0.34	9.29 ± 1.66 *	4.02 ± 0.51
MARRV	20	15.04 ± 3.38 *	6.89 ± 0.22 *	4.33 ± 1.73	1.28 ± 0.11 *
	95	31.47 ± 1.58 *	9.03 ± 0.27 *	3.47 ± 0.68	3.53 ± 0.09 *
A × T		#	#	#	n.s.

# indicates the presence of interaction between accessions and temperature of extraction; n.s. = not significant. * indicates that means significantly differ at $p \leq 0.05$.

Table 3. Antioxidant capacity determined by DPPH, ABTS and FRAP tests, and by cyclic voltammetry, in the extracts of the pericarp chestnut samples.

Accessions or Variety	T °C	DPPH	ABTS	FRAP	CV	EC_{50} (DPPH)	EC_{50} (ABTS)
		mmol TEAC 100 g^{-1} DM		mg AAeq g^{-1} DM	$AUC_{+0.5}$ (μC)	mg mL^{-1}	mg mL^{-1}
MURG	20	24.29 ± 1.16 *	33.33 ± 1.24	3.28 ± 0.27	3.02 ± 0.12 *	0.042 *	0.028
	95	31.77 ± 2.40 *	34.02 ± 0.67	3.20 ± 0.16	2.04 ± 0.07 *	0.034 *	0.029
LOCG	20	23.91 ± 1.34	31.17 ± 0.31	3.10 ± 0.25	2.16 ± 0.13 *	0.032	0.031
	95	27.52 ± 2.84	29.57 ± 0.92	3.33 ± 0.34	2.97 ± 0.19 *	0.039	0.034
ILDP	20	27.20 ± 2.89	35.71 ± 1.50	3.55 ± 0.93	3.15 ± 0.15	0.035	0.031
	95	28.17 ± 2.39	34.51 ± 0.91	3.35 ± 0.45	3.17 ± 0.31	0.029	0.032
COEV	20	15.31 ± 0.36 *	21.03 ± 0.37	2.18 ±0.09	1.52 ± 0.37	0.058	0.046
	95	20.07 ± 2.94 *	25.11 ± 2.49	2.54 ± 0.29	1.87 ± 0.28	0.051	0.037
MARRV	20	9.12 ± 1.74 *	11.60 ± 0.20 *	2.61 ± 0.21 *	2.61 ± 0.20 *	0.057 *	0.040 *
	95	14.22 ± 0.24 *	18.59 ± 0.05 *	3.53 ± 0.11 *	3.78 ± 0.25 *	0.038 *	0.026 *
A × T		#	#	#	#	n.s.	n.s.

# indicates the presence of interaction between accessions and temperature of extraction; n.s. = not significant. * indicates that means significantly differ at $p \leq 0.05$.

3.1. Phytochemical Content of Cold and Hot Water Pericarp Extracts

The phytochemical content of water extracts at 20 and 95 °C of pericarp of four ancient Sardinian chestnut accessions and of one commercial marron cultivar was determined, together with their antioxidant capacity. TP, measured both with Folin–Ciocalteu and CV, TF and PA varied considerably among chestnut accessions and the marron (Table 2) since they were cultivated in different pedoclimatic conditions, at different altitudes and harvested at different times, as specified in the Material and Methods section. MURG, ILDP and LOCG accessions were characterized by the highest level of TP, TF, and PA, both when they were extracted at 20 °C and 95 °C. COEV had the lowest TP, TF and PA content of all the accessions. The commercial MARRV cultivar was characterized by a lower content of phenolic compounds than the four accessions. Despite this fact, in this work, we did not take into account the differences between the accessions or between them and the marron, but we mainly focused on the differences between treatments (20 °C vs. 95 °C) within the same accession or variety.

The content of TP and PA of MURG, ILDP, LOCG and COEV was not influenced by the extraction temperature, while flavonoids content was. The exact opposite was observed for marron: hot water extraction strongly increased the yield of TP and PA but not that of TF. Statistical analysis indicated the presence of interaction between accessions and extraction temperature (A × T) for all measured parameters, but this was more evident in the case of flavonoid content, where the difference between extraction at 20 and 95 °C was always significant. No correlation was found between $TP_{F\text{-}C}$ and $AUC_{0.8}$ neither for the cold nor for the hot extracts (Tables S1 and S2 in Supplementary Materials).

3.2. Antioxidant Capacity Determination

The antioxidant capacity of pericarp chestnut samples, determined by DPPH, ABTS, FRAP and CV tests, are reported in Table 3 together with the chestnut extract concentrations necessary to achieve a 50% radical DPPH or ABTS inhibition (EC_{50}).

The three different spectrophotometric tests seem to indicate that the antioxidant activity of MURG, LOCG, COEV and ILDP accessions was not particularly influenced by the solvent temperature, albeit with the exception of the DPPH tests on MURG and COEV. Differently, the antioxidant activity of the MARRV extract at 95 °C was definitely higher than those at 20 °C. The results of the electrochemical tests slightly differed from those of the other assays, but this was expected since the radicals (DPPH* and ABTS*+) react with almost all the compounds present in the extracts able of giving up electrons, while the AUC values refer only to compounds that release electrons at an applied potential (E_{app}) of +0.5 V; AUC was higher at 20 °C in MURG, at 95 °C in LOCG and MARRV, and not statistically different in ILDP and COEV. The difference among the results obtained with different methods will be discussed, with appropriate bibliographical references, in the "Discussion" chapter.

EC_{50} values ranged from 0.029 and 0.058 mg mL^{-1} when calculated with DPPH, and from 0.026 and 0.046 when calculated with ABTS method. Slight statistically significant differences in EC_{50} were observed for MURG only when determined with DPPH, while a marked difference was observed between hot and cold extracts of MARRV according to both methods.

The relationship among the parameters shown in Table 2 and the antioxidant capacities displayed in Table 3 were determined by Pearson correlation analysis and reported in Tables S1 and S2 in Supplementary Materials. For cold extracts: TP was highly correlated with DPPH (R^2 = 0.979) and ABTS (R^2 = 0.971) values but moderately with FRAP (R^2 = 0.787); the PA was highly correlated with DPPH (R^2 = 0.904) and ABTS (R^2 = 0.933); TF values were in good correlation with DPPH (R^2 = 0.807) and ABTS (R^2 = 0.831) and moderately with FRAP (R^2 = 0.714). DPPH and ABTS spectrophotometric assays were highly correlated with each other and in good correlation with FRAP; and FRAP was in good correlation with $AUC_{0.5}$ and $AUC_{0.8}$, while DPPH and ABTS were not. For hot extracts: the correlation coefficients between TP and spectrophotometric tests for antioxidant activity were lower than for cold extracts (R^2 = 0.758 vs. DPPH, 0.733 vs. ABTS and 0.743 vs. FRAP); and TF and PA were highly correlated with DPPH and ABTS but not correlated with FRAP. DPPH and ABTS values were highly correlated with each other (R^2 = 0.995) but not with FRAP; also in this case, as for the cold extracts, FRAP resulted in good correlation with $AUC_{0.5}$ and in moderate correlation with $AUC_{0.8}$. Finally, all the EC_{50} values were negatively correlated to the studied parameters, thus indicating that an increase in bioactive compounds corresponded to a lower extract concentration necessary to achieve 50% of DPPH or ABTS radicals' inhibition.

3.3. Electrochemical Characterization of CP Extracts

The cyclic voltammetric patterns recorded for CP samples are displayed in Figure 1.

The voltammograms showed that the oxidation in the extracts started from +0.06 to +0.2 V, both at 20 °C and 95 °C. The shapes of the voltammograms were similar but the currents, recorded at +0.5 V, by different accessions, varied from 8 to 14.5 μA at 20 °C, and from 7.5 to 17 μA at 95 °C. These differences should be ascribed to the activity of compounds with different redox potentials (see also AUC values in Tables 2 and 3). If we consider cold extraction first, and we refer the voltammetries to a gallic acid calibration curve (Figure S1a,b in Supplementary Materials), COEV had the lowest quantity of compounds that oxidize at +0.5 V. LOCG and MURG had similar voltammograms but while MURG had a higher concentration of compounds that oxidize at a potential lower than +0.5 V, the molecules that oxidize at an E_{app} greater than this threshold appear more represented in LOCG. ILDP had the highest redox peak of all the other cold extracts, and also had the highest polyphenol component that oxidizes at less than +0.5 V. MARRV had

a voltammogram comparable to MURG. Differently, when we consider the hot extracts, the COEV and MURG voltammograms were superimposable. They indicated that the two samples had the lowest concentration of compounds, no matter whether they oxidize at a potential lower or higher than +0.5 V. The voltammograms of ILDP and LOCG were also very similar to each other, but the extracts had a concentration of antioxidant compounds higher than the other two Sardinian accessions. Finally, the marron MARRV had a higher antioxidant capacity than all four accessions.

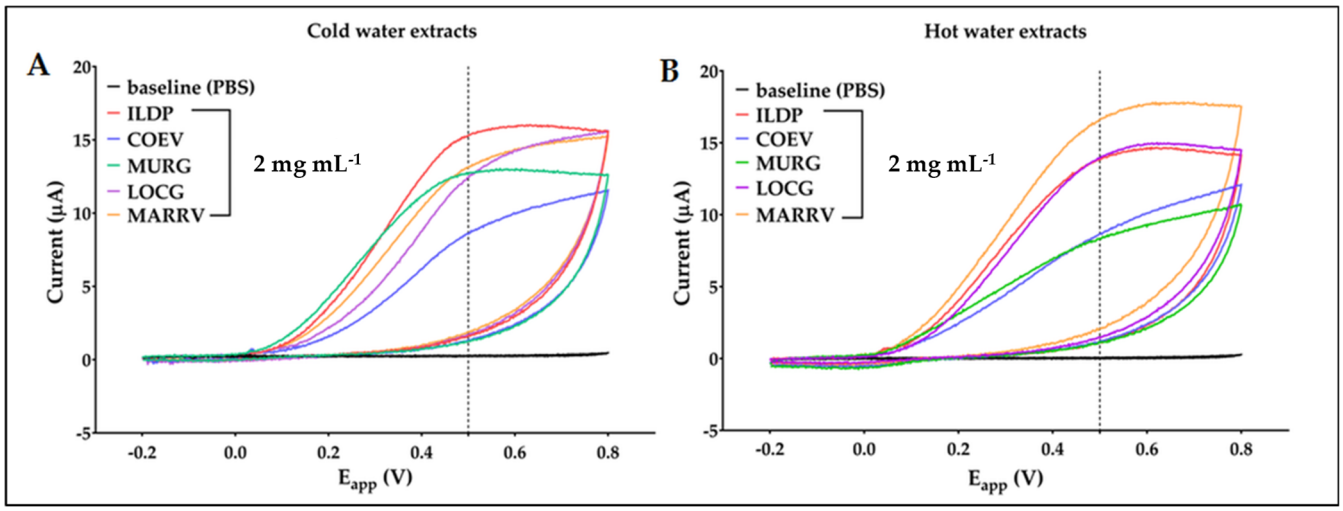

Figure 1. Cyclic voltammetry, with a scanned potential range (E_{app}) comprised between −0.2 V and +0.8 V vs. carbon pseudoreference, in the absence (PBS black line) and in the presence of 2 mg mL^{-1} of MURG (green line), LOCG (purple line), ILDP (red line), COEV (blue line) and MARRV (yellow line) CP cold water extracts (**A**) and hot water extracts (**B**).

3.4. Chemical Characterization of CP Extracts

The concentration of the main phenolic compounds in the water extracts of CP was determined by High Performance Liquid Chromatography (HPLC). The research was focused on those phenolic compounds, present in the pericarp of chestnut, which were reported to have cytoprotective effects towards normal cells and/or antiproliferative effects on cancer cells. Molecules like gallic acid and ellagic acid, two phenolics of low molecular weight, the ellagitannin isomers castalagin and vescalagin, and flavanols represented by catechin, epicatechin and epigallocatechin [4,38,46] were identified and quantified, and the results are reported in Table 4; the chromatogram of ILDP can be found, as an example, in Figure S2 in Supplementary Materials.

Gallic acid, ellagic acid, vescalagin, castalagin, catechin, epicatechin and epigallocatechin were the seven main phenolic compounds identified in the four accessions and in the marron. Water at 20 °C was more efficient than hot water at extracting gallic and ellagic acid from the epicarp of all the accessions, but less efficient in the marron. The ellagic acid was the most represented compound in all the samples and its content was 5-fold higher when the extraction was done with cold water. Ellagic acid aside, each accession is distinguished by the prevalence of certain compounds: vescalagin, castalagin and epicatechin characterized the MURG extracts, regardless of the temperature used for extraction.

LOCG cold extracts were characterized by gallic acid, vescalagin and castalagin, while the hot ones have a high content only of vescalagin, castalagin and, particularly, of epicatechin. ILDP cold extracts could be distinguished by their content of gallic acid, catechin and epicatechin, whereas the hot extracts by vescalagin, castalagin and epicatechin. COEV was distinguished by a low content of all the compounds (thus confirming the results of the spectrophotometric assay) if the extraction took place in cold water, and by a prevalence of castalagin and vescalagin when the extractant was hot water.

Table 4. Main phenolic compounds (mg g^{-1} DM) quantified by HPLC analysis in the pericarp extracts of the four chestnut accessions and of the marron variety.

Phenolic Compound	T °C	MURG	LOCG	ILDP	COEV	MARRV
Gallic acid	20	0.402 ± 0.003 *	0.553 ± 0.001 *	0.929 ± 0.125 *	0.299 ± 0.022	0.131 ± 0.040 *
	95	0.300 ± 0.007 *	0.390 ± 0.027 *	0.349 ± 0.025 *	0.280 ± 0.029	0.255 ± 0.009 *
Ellagic acid	20	2.864 ± 0.032 *	4.732 ± 0.190 *	4.469 ± 0.492 *	3.395 ± 0.434 *	1.659 ± 0.068 *
	95	0.573 ± 0.019 *	0.727 ± 0.090 *	0.684 ± 0.075 *	0.363 ± 0.050 *	0.869 ± 0.033 *
Vescalagin	20	0.525 ± 0.058 *	0.406 ± 0.013 *	0.364 ± 0.037 *	0.237 ± 0.001 *	0.168 ± 0.029 *
	95	0.768 ± 0.007 *	0.998 ± 0.069 *	0.629 ± 0.012 *	0.409 ± 0.025 *	0.463 ± 0.007 *
Castalagin	20	0.631 ± 0.041	0.449 ± 0.021 *	0.364 ± 0.037 *	0.246 ± 0.012 *	0.238 ±0.060
	95	0.714 ± 0.015	0.980 ± 0.167 *	0.629 ± 0.012 *	0.419 ± 0.064 *	0.374 ± 0.050
Catechin	20	0.219 ± 0.008	0.261 ± 0.022	0.564 ± 0.028 *	0.161 ± 0.005	0.199 ± 0.027 *
	90	0.231 ± 0.009	0.268 ± 0.016	0.248 ± 0.002 *	0.180 ± 0.017	0.670 ± 0.013 *
Epicatechin	20	0.595 ± 0.077	0.271 ± 0.033 *	0.732 ± 0.067	0.287 ± 0.044	0.204 ±0.011 *
	95	0.665 ± 0.016	2.232 ± 0.279 *	0.900 ± 0.009	0.285 ± 0.065	0.521 ± 0.008 *
Epigallocatechin	20	0.067 ± 0.012	0.081 ± 0.011	0.091 ± 0.020	0.055 ± 0.001	0.046 ± 0.004
	95	0.052 ± 0.015	0.063 ± 0.001	0.075 ± 0.003	0.041 ± 0.000	0.056 ± 0.006

* indicates that means significantly differ at $p \leq 0.05$.

The marron MARRV showed a different phenolic composition from the Sardinian accessions: in cold water extracts, low quantities of all seven molecules were detected, except for ellagic acid, whose value was the lowest among the samples tested. Differently, the hot extracts were characterized by moderate quantities of vescalagin and epicatechin and by a high quantity of catechin, higher than that found in the four accessions.

3.5. Contribution of Main Polyphenols to the Total Antioxidant Activity of CP Extracts

The electrochemical and redox properties of the identified compounds were investigated based on a comparison with previous studies of our group [43,47] and the specific literature on chestnut [24]. The redox potentials of all the molecules listed in Table 4 are reported in Table 5.

Table 5. Redox potential values, reported in the literature, of standards of phenolic compounds detected in chestnut pericarp extracts.

Phenolic Compound	Redox Potential (V)	Reference
Gallic acid	+0.391	[24]
Ellagic acid	+0.367	[24]
Vescalagin	+0.384	[24]
Castalagin	+0.384	[24]
Catechin	+0.391 +0.300	[24,43]
Epicatechin	+0.120	[43]
Epigallocatechin	+0.080	[43]

The oxidation potential of a molecule may have small variations as a function of several variables: the working electrode, the pseudo-reference [45], the supporting electrolyte [43] and various other differences in experimental protocols; furthermore, sometimes the main oxidation peak is taken into consideration [24], and sometimes the point at which the molecule's voltammogram separates from the baseline is considered (i.e., the moment in which a molecule begins to give up its electrons). Due to these differences, different authors may record different values. The oxidation potential of the seven compounds in Table 4 are all in within the range of +0.1 and +0.4 V, which is the range of interest for the most active molecules according to the parameters reported for antioxidants phenolic compounds [30–32].

The quantities of the seven phenolic compounds detected in the four Sardinian accessions and in the marron were correlated with the antioxidant activity values determined with the different methods, and the results are reported in Tables S3 and S4 in Supplementary Materials. In the cold extracts, the highest correlation coefficients were calculated between epicatechin and epigallocatechin content and DPPH (R^2 = 0.982 and 0.930, respectively) and ABTS (R^2 = 0.970 and 0.901, respectively) values. For all the other molecules, it can be stated that, with the sole exception of catechin, there was a good or at least moderate correlation. Correlations with the FRAP test were slightly lower for all molecules compared to the two previously mentioned methods, while no correlation was found with $AUC_{0.5}$. In the case of hot extracts, finding high correlations was more difficult: the contents of gallic acid, castalagin and vescalagin were well correlated with DPPH and ABTS, while ellagic acid was the only molecule to be highly correlated with FRAP and $AUC_{0.5}$ (R^2 = 0.965 and 0.932, respectively).

3.6. Antiproliferative Activity of CP Extracts on Normal and Cancer Cells

The antiproliferative effects of the five CP extracts on the four cancer cell lines are reported in Figures 2–6, where they are compared to the effect on normal cells. The concentration of extracts inhibiting cells' growth by 50% (GI_{50}) is instead reported in Table 6.

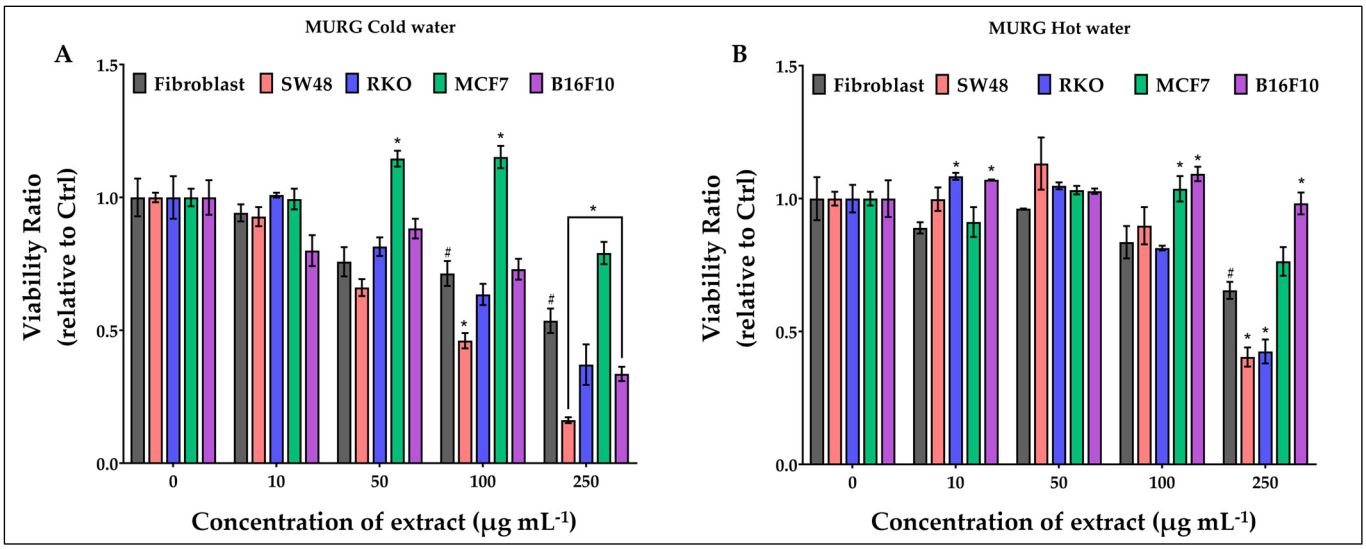

Figure 2. Effect of growing concentrations (from 10 to 250 μg mL^{-1}) of MURG cold (**A**) and hot (**B**) CP extract on viability of normal cells (fibroblasts), and of colon (SW48 and RKO), breast (MCF7) and melanoma (B16F10) cancer cells. # = $p \leq 0.01$ vs. Ctrl; * = $p \leq 0.01$ vs. fibroblasts.

The MURG cold water extract (Figure 2A) significantly reduced the viability of fibroblasts starting from 100 μg mL^{-1} up to a reduction of 46% at 250 μg mL^{-1}. Treatments became effective reducing the viability of SW48 at 100 μg mL^{-1} and, at the highest concentration, reduced the viability of RKO and B216F10; differently they increased the viability of MCF7 starting from 50 μg mL^{-1}. The hot water extract (Figure 2B) appeared less effective both on fibroblast and cancer cells but increased the viability of B16F10 at 100 and 250 μg mL^{-1}.

The LOCG cold water extract (Figure 3A) reduced the viability of fibroblasts starting from 50 μg mL^{-1}. The viability of SW48 and RKO was progressively reduced from 50 to 250 μg mL^{-1}, and that of B16F10 from 100 to 250 μg mL^{-1}. The viability of MCF7 was significantly higher than fibroblasts at 50, 100 and 250 μg mL^{-1}. The hot water extracts were less effective than the cold ones (Figure 3B) on fibroblasts, since a 26% significant reduction of the viability was observed only at the highest concentration. A significant

effect on cancer cells was observed on RKO (−21% vs. fibroblasts) and on MCF7 (+36% vs. fibroblasts).

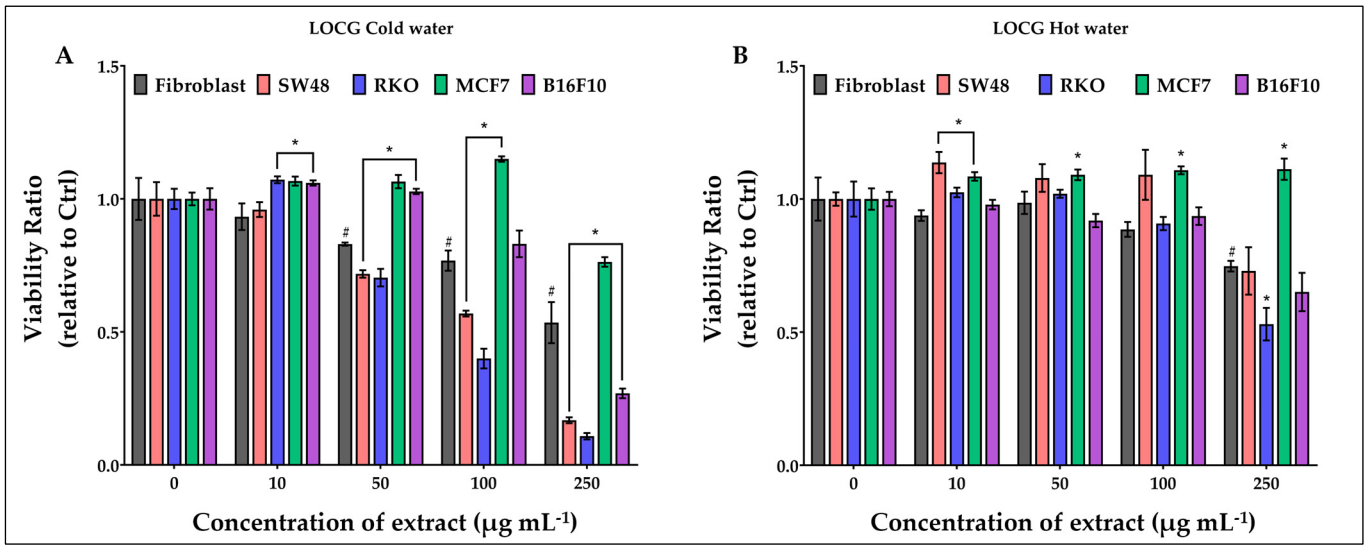

Figure 3. Effect of growing concentrations (from 10 to 250 μg mL^{-1}) of LOCG cold (**A**) and hot (**B**) CP extract on viability of normal cells (fibroblasts), and of colon (SW48 and RKO), breast (MCF7) and melanoma (B16F10) cancer cells. # = $p \leq 0.01$ vs. Ctrl; * = $p \leq 0.01$ vs. fibroblasts.

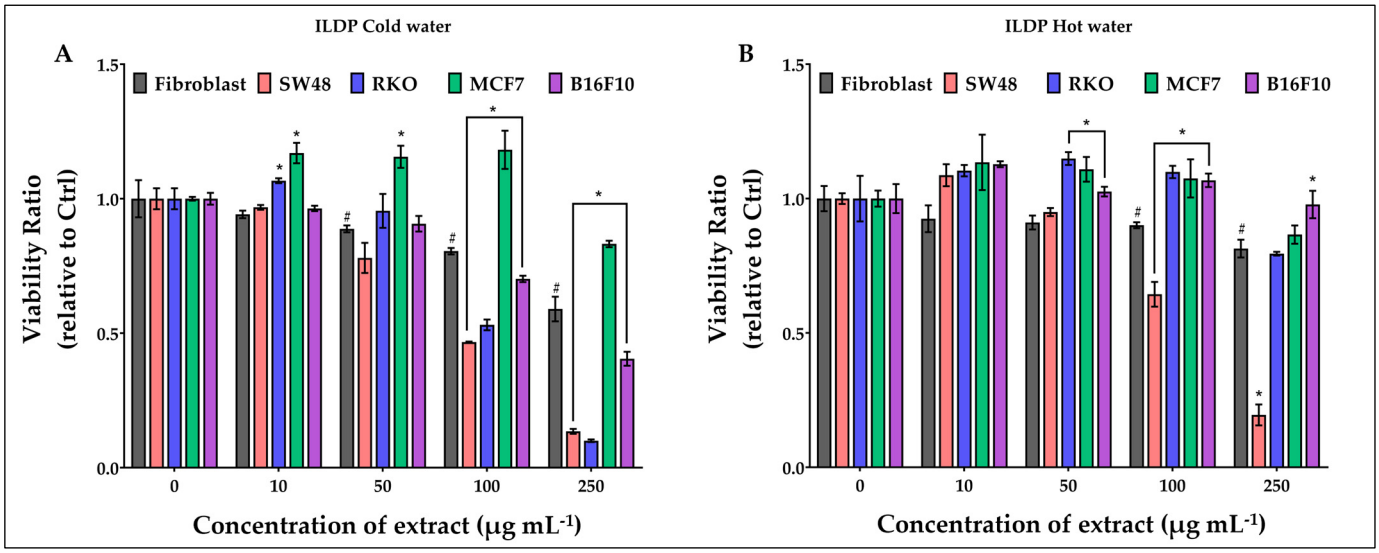

Figure 4. Effect of growing concentrations (from 10 to 250 μg mL^{-1}) of ILDP cold (**A**) and hot (**B**) CP extract on viability of normal cells (fibroblasts), and of colon (SW48 and RKO), breast (MCF7) and melanoma (B16F10) cancer cells. # = $p \leq 0.01$ vs. Ctrl; * = $p \leq 0.01$ vs. fibroblasts.

Treatments with cold and hot ILDP extracts (Figure 4) did not appear to have a particular efficacy on fibroblasts, except for the cold ones at 250 μg mL^{-1} (−41% of viability). Differently, the cold water extracts (Figure 4A) seriously reduced the viability of SW48 and RKO starting from 100 μg mL^{-1}, while significantly increased that of MCF7 starting from 10 μg mL^{-1}. The effect of hot water extracts was limited to SW48 cells (Figure 4B).

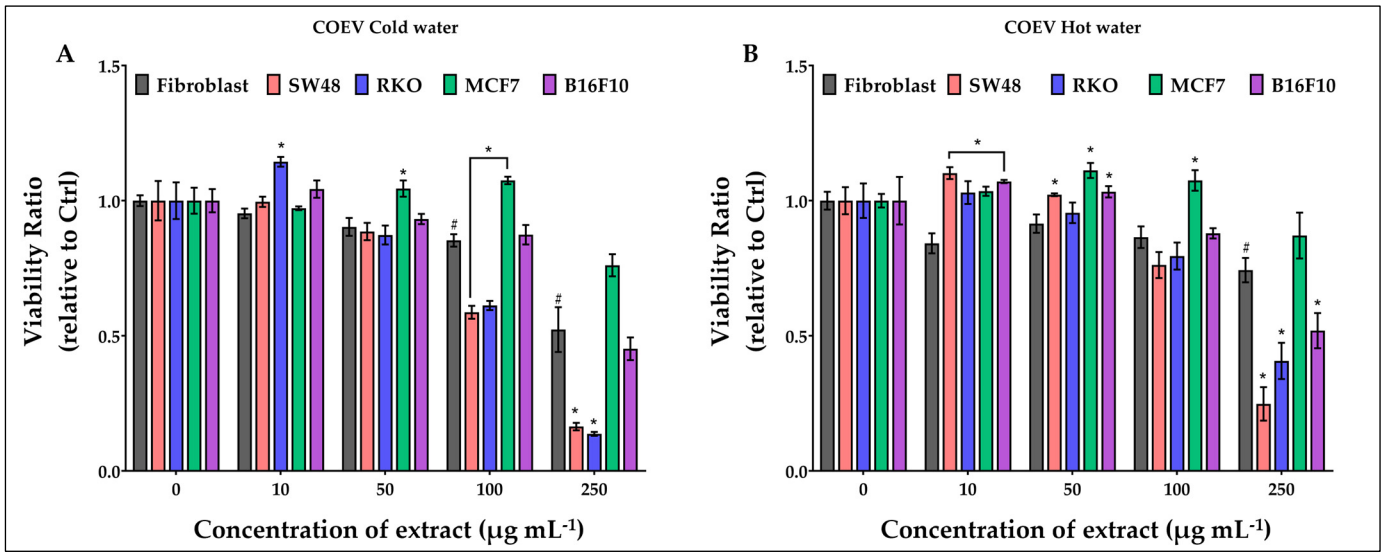

Figure 5. Effect of growing concentrations (from 10 to 250 μg mL^{-1}) of COEV cold (**A**) and hot (**B**) CP extract on viability of normal cells (fibroblasts), and of colon (SW48 and RKO), breast (MCF7) and melanoma (B16F10) cancer cells. # = $p \leq 0.01$ vs. Ctrl; * = $p \leq 0.01$ vs. fibroblasts.

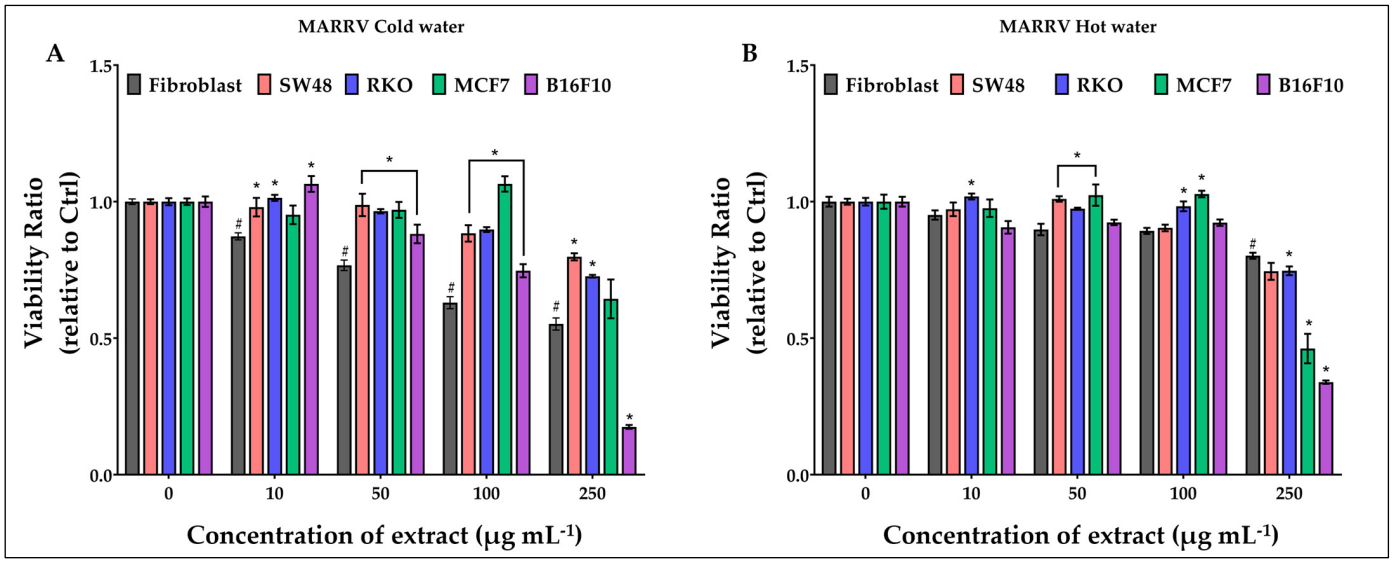

Figure 6. Effect of growing concentrations (from 10 to 250 μg mL^{-1}) of MARRV cold (**A**) and hot (**B**) CP extract on viability of normal cells (fibroblasts), and of colon (SW48 and RKO), breast (MCF7) and melanoma (B16F10) cancer cells. # = $p \leq 0.01$ vs. Ctrl; * = $p \leq 0.01$ vs. fibroblasts.

Treatments with cold and hot water COEV extracts affected the viability of fibroblasts at 100 and 250 μg mL^{-1}, respectively (Figure 5). The same concentrations of cold water extract significantly reduced the viability of colon cancer cells (Figure 5A) and slightly increased that of MCF7; the hot water extract was effective also on B16F10 (Figure 5B).

The marron MARRV extract had different effects, both on fibroblasts and cancer cells, compared to the four Sardinian chestnut accessions (Figure 6). The viability of fibroblasts was significantly and progressively reduced, starting from 10 μg mL^{-1}, by treatments with cold water extracts; no significant reduction, except for melanoma cells at 250 mg mL^{-1}, was observed on cancer cells compared to fibroblasts (Figure 6A). Hot extract treatments reduced the viability of normal cells only at 250 μg mL^{-1}. Instead, unlike all Sardinian accessions, hot extracts of MARRV were the only ones to have shown significant antiproliferative activity on breast cancer MCF7 cells (Figure 6B).

Table 6. GI_{50} values for ILDP, MURG, COEV, LOCG and MARRV cold and hot water extracts in fibroblasts, SW48, RKO, MCF7 and B16F10 cells.

	Cold Water Extracts				
	MURG	**LOCG**	**ILDP**	**COEV**	**MARRV**
Fibroblasts	367.5 (−0.096–1.000)	244.7 (0.598–1.067)	177.3 (0.370–0.978)	261.0 (0.049–0.971)	493.3 (0.562–0.999)
SW48	105.7 (−0.132–0.998)	132.6 (−0.153–0.996)	84.59 (0.100–0.985)	117.8 (0.003–1.002)	293.0 (0.535–0.997)
RKO	274.7 (−0.385–1.019)	82.59 (−0.024–1.002)	99.9 (0.034–1.04)	121.5 (−0.017–1.000)	155.4 (0.624–1.005)
MCF7	249.5 (0.523–1.073)	244.7 (0.560–1.067)	249.3 (0.523–1.166)	252.8 (0.435–1.023)	245.9 (0.401–0.990)
B16F10	190.8 (−0.077–0.994)	153.9 (0.065–1.062)	124.4 (0.265–0.989)	243.8 (−0.071–1.011)	147.7 (−0.040–1.015)
	Hot Water Extracts				
	MURG	**LOCG**	**ILDP**	**COEV**	**MARRV**
Fibroblasts	118.1 (0.586–1.001)	>500	>500	>500	>500
SW48	215.4 (0.014–1.000)	249.3 (0.423–1.084)	112.4 (0.119–1.015)	129.1 (0.097–1.102)	345.8 (0.296–0.995)
RKO	151.3 (0.220–1.061)	199.8 (0.256–1.200)	264.4 (0.383–1.106)	183.5 (0.071–1.019)	253.1 (0.482–1.001)
MCF7	259.6 (0.276–1.009)	>500	>500	264.8 (0.424–1.055)	243.7 (0.125–1.008)
B16F10	268.4 (0.787–1.048)	209.5 (0.458–0.983)	249.1 (0.912–1.052)	182.6 (0.256–1.046)	231.4 (0.195–0.981)

The data obtained through the viability test were also used to calculate the GI_{50} value for each extract on different cancer cell lines (see also Figures S5 and S6 in Supplementary Materials). Table 6 clearly indicates that (i) with the sole exception of MURG, much higher concentrations of hot extract than cold ones were required to inhibit fibroblast growth by 50%; (ii) the concentration of cold extracts inhibiting the growth of SW48 was always lower than the concentration of hot extracts; (iii) with the sole exception of MURG, all GI_{50} values obtained on RKO cells with cold extracts were lower than those obtained with hot extracts; (iv) a concentration of extract, both cold and hot, of no less than 243 μg mL^{-1} was required to obtain 50% inhibition of the growth of MCF7 cells; and (v) even in the case of B16F10 melanoma cells, and with the sole exception of COEV, the GI_{50} of cold extracts were lower than the hot ones.

4. Discussion

For the worldwide steady-rate growing chestnut industry, the upgrade provided by this research at sustainably recovering bioactive phyto-complexes from waste/by-products of downstream applications is of great significance: green recovery of bioactive compounds from chestnut shells not only preserves their health properties for food, cosmetics, and pharmaceutical industries but also, importantly, reduces the environmental impact of organic solvents. According to this scientific report, chestnut waste is a valuable economic resource supporting the circular economy concept.

This work aimed to investigate some health properties of polyphenolic waste extracts obtained through sustainable and eco-friendly methods for industrial applications.

The research focused on water extracts of the outer shell of four chestnut accessions and one marron variety, with emphasis on their phenolic composition, antioxidant activity and antiproliferative effect on normal cells (fibroblasts), and on two colon (RKO and SW48), one breast (MCF7) and one melanoma (B16F10) cancer cell lines.

The extraction of phenolic compounds from plant materials has been carried out with organic solvents (methanol, ethanol, acetone, etc.) for years, and it was the same for the chestnut [48,49]. Vázquez et al. [50] compared the yields resulting from different extraction methods, with organic solvents (96% n-hexane, acetone, ethyl acetate, ethanol and methanol), with water or with a 2.5% Na_2SO_3 aqueous solution heated to 90 °C for one hour: the yield of extractable compounds increased with the polarity of the solvent and the highest yield corresponded to water. The same authors, studying the influence of extraction conditions (water and different alkaline solutions at 70 and 90 °C), observed an increase of total phenols and antioxidant activity when the temperature increased. The use of water, alone or combined in hydroalcoholic solutions, is more recent [18,19,21,22], and there are green technologies, like subcritical water extraction, used to maximize the extraction yield and polyphenol content [51], which offer a more economically sustainable

and environmentally friendly alternative for large-scale industrial applications. In this work, we took into account previous results which demonstrated that the use of hot water, for the same extraction time, increased the extraction capacity of the solvent [19]. The use of hot water has two important critical issues: first, heating the water has a cost for industries; second, the thermolabile phenolic component is affected [51]. We showed that this is not always true, at least in terms of the yield of total polyphenols, flavonoids and proanthocyanidins. After 4 h of extraction in cold water or one hour of extraction in hot water at 95 °C, the extracts from the four Sardinian accessions contained statistically non-different quantities of TP and PA. Differently, for the marron, hot water was able to extract a greater quantity of total polyphenols and proanthocyanidins than cold water. In regard to the flavonoid content, exactly the opposite was observed.

The DPPH, ABTS and FRAP tests for antioxidant capacity determination showed that the antioxidant activity of the Sardinian accessions was not influenced by the temperature of the aqueous solution. No significant differences were observed by the extraction at different temperatures for MURG, LOCG and ILDP accessions, while a higher antioxidant activity was measured for COEV and MARRV when the extraction was performed at 95 °C. The extract concentration of MURG, LOCG and ILDP that provide a 50% radical inhibition (EC_{50}) were lower than that of COEV and MARV, and both if calculated with DPPH and ABTS. The extraction temperature did not affect the EC_{50} of the Sardinian accessions, but affected the marron one, suggesting a higher antioxidant capacity of the hot extract of MARRV. Our results are of the same order of magnitude as those obtained by Rodrigues et al. [52] who carried out the extraction with an ethanol/water mixture at 50 °C; they are also in line with those of Fernández-Agulló et al. [19] who, with extraction in water at 75 °C, obtained EC_{50} values of 0.031 mg mL^{-1} (DPPH method). Two other research groups, following extraction in hot water, obtained EC_{50} average values higher than ours: 0.061 mg mL^{-1} using water at 90 °C [23], and 0.027–0.078 mg mL^{-1} by extracting with boiling water [22].

The electrochemical tests, however, showed a different trend: the antioxidant capacity of MURG extracts was higher at 20 °C, the one of LOCG and MARRV was higher at 95 °C, and ILDP and COEV did not statistically differ. A comparison between DPPH assay and CV cyclic voltammetry can be found in a previous study [24]: what the two works have in common, and what gives us a valid term of comparison, is the threshold of +0.5 V established a priori in order to discriminate the real antioxidant capacity of the extracts. Ricci's group attributes two important limitations to the DPPH assay, long incubation times and difficulties with colored samples: we completely agree and, furthermore, we also believe that the DPPH and ABTS assays tend to overestimate the antioxidant capacity because they take into account species in solution, which oxidize at a potential greater than +0.5 V [33]. Fadda and Sanna [53], in a dissertation on advantages and pitfalls of methods for antioxidant activity evaluation, affirmed that the capacity of antioxidants to reduce the radical cation $ABTS^{\bullet+}$ to ABTS is directly related to the reduction potential of the antioxidants, and only those with E^0 values lower than that of the couple $ABTS^{\bullet+}$/ABTS (E^0 = 0.68 V) are able to perform this reduction. Obtaining a good correlation between spectrophotometric and electrochemical methods is possible if similar trends are observed, but this does not mean that comparable antioxidant capacity values are obtained. This reasoning is supported by the correlation values found between the various methods for determining antioxidant capacity: DPPH and ABTS values were highly correlated with each other but not with FRAP or AUC values. Differently, FRAP and AUC values, especially $AUC_{0.5}$, are in good correlation with each other since both are based on direct electron transfer, and this is in accordance with previous studies [54–56]. The antioxidant capacity of the outer shell extracts is based on the contribution of all the identified and unidentified complex structures [27], but is primarily linked to the concentration of phenolic compounds that are capable of being oxidized at an E_{app} lower than +0.5 V. In the absence of a mass spectrometric analysis, a lot of unknown molecules occurring in the investigated accessions cannot be considered, nor their specific reducing power. According to our HPLC

analysis, we can state that all seven compounds identified and quantified have a redox potential lower than +0.4 V and that all of them contributed to the antioxidant capacity according to their concentration in the extracts. Even though the obtained qualitative phenolic profile was similar to that reported by other authors, differences in quantitative content was found [4,38,46]: TP values similar to our MARVV and lower than those of the Sardinian accessions, but with shorter extraction times, were recorded by De Vasconcelos et al. [4], while condensed tannins values were comparable to our results. A content of TP approximately ten-fold higher than ours was found in chestnut waste consisting of inner and outer shell water extract [23]. This high variability assessed in the literature could be attributed to varietal differences, although a wide number of factors influence the phytochemical levels in vegetal matrices, such as geographical origin, pedoclimatic conditions, irrigation, seasonality, etc. Beyond all this, the role of temperature must be considered. The HPLC analysis showed that the temperature discriminates between the different molecules: the ellagic acid showed the highest concentration in the cold extracts, ellagitannins in the hot ones and, for the other molecules, specific cultivar-dependent trends were recorded. Vella et al. [22] found a TP and flavonoids content much lower than ours in water extract at boiling temperature (for 40 min) of outer shell chestnut Campania cultivars. An increase in the castalagin and gallic acid concentration, consequence of an increase in the extraction temperature, was reported by De Vasconcelos et al. [4] as for our cultivar MARRV. The thermal stability at 60, 80 and 100 °C of water solutions of gallic acid, catechin, and vanillic acid was studied: it was demonstrated that the degradation of the phenolic compounds in grape seeds and spruce bark vegetal extracts was lower for all the temperatures than individual standard solutions, confirming that the complex chemical composition of vegetal extracts has to take into account to evaluate the thermal stability of phenolic compounds [57]. It was reported that high temperature and long extraction times can promote phenols oxidations and reduce antioxidant properties [7], that temperatures up to 90 °C increase the extraction of phenolic compounds [4,22,58], and that this increase is not always associated with the maximum antioxidant capacity [19] due to additive and synergic effects of individual phytochemicals.

All the considerations made so far are based on in vitro studies, chemical and electrochemical tests, but to better understand the role of phenolic compounds, their biological activity on normal and cancer cells has been investigated. It is known that polyphenols decrease DNA damage by inducing various carcinogens acting as ROS scavengers, chelating transition metals, or modulating the expression and the activity of the enzymes related to oxidative stress [59]. The antioxidant activity of polyphenols could be essential but not sufficient for chemoprevention [60], and there is evidence that naturally occurring antioxidants elicit different redox responses according to a dose–response mechanism and the intracellular redox state [61–63].

Like many other species, the chestnut phenolic compounds have been correlated with anticancer activity. Unfortunately, studies on the anticancer properties of chestnut are still limited to a few articles: one tumor (DU 145) and one normal prostate epithelial (PNT2) cell line were exposed to increasing concentration of shell polyphenols extracts, thus showing an increase of apoptotic cells after 48 and 72 h treatments [12]; anticancer activity vs. prostate cancer cells (DU 145 and PC3) was attributed to a quinoline-pyrrolidine alkaloid present in chestnut honey, and the inhibition of clonogenic activity was congruent with apoptotic events [39]. Apoptotic-induced effects were also observed on neuroblastoma cells (SH-SY5Y) when exposed to increasing concentration extracts obtained by bark of *Castanea sativa* containing high amounts of vescalagin, castalagin gallic and ellagic acid [40].

In our work, normal cells and four cancer cell lines, SW48, RKO, MCF7 and B16F10, were exposed to cold and hot water pericarp extracts of four Sardinian accessions and of one marron cultivar. The temperature and different extraction times significantly influenced the growth of cells, both normal and tumor. The fibroblast viability was significantly reduced, at least by 20%, by treatments with cold extracts starting from 100 μg mL^{-1} of MURG and COEV, 50 μg mL^{-1} of LOCG and ILDP, and even 10 μg mL^{-1} of MARRV. According to

Floris et al. [64], a 20% reduction in viability is the threshold to consider a treatment as being effective. Such a reduction, with hot extracts, was observed only with doses greater than 250 μg mL^{-1}, regardless of the accession or cultivar to which they belong, and were confirmed by GI_{50} values obtained by nonlinear regression analysis. This clearly indicates that the pool of molecules extracted at 95 °C exerts a cytotoxic effect significantly lower than that of cold extracts.

Even on cancer cells, the most evident cytotoxic effects were observed when the cells were treated with cold extracts. The viability of metastatic colon cancer cells, SW48 and RKO, was strongly reduced by 100 μg mL^{-1} (if not even 50 μg mL^{-1} of LOCG) of all the Sardinian accessions extracts, while MARRV had no effect; also in this case, the cytotoxic action of the hot extracts was unquestionably lower, and only at the highest doses. Furthermore, it was observed that the GI_{50} values calculated on colon cancer cells in this work, are lower than those reported for infusions and decoctions of *Castanea sativa* flowers on HCT15 (colon carcinoma) cells [65]. The activity against colon cancer cells of chestnut extracts was also investigated by other research groups. The capacity of inner shell extracts of Japanese chestnut (*Castanea crenata* Siebold & Zucc., 1846), to inhibit the viability of LoVo and HT29, two human colon adenocarcinoma cell lines, was explored [20]: the water extracts (24 h at 60 °C), containing 2.78 mg g^{-1} of gallic acid (higher than ours) and 1.07 mg g^{-1} of ellagic acid (lower than ours), induced a viability reduction, varying between 20 and 60%, starting from 125 μg mL^{-1}, a result comparable to those obtained by our group on RKO and SW48. On the other hand, the bioactive compounds of shells of Chinese chestnut (*Castanea mollissima* Blume) seemed to have a moderate anti-cancer activity on human COLO 320 DM colon cancer cells, but results cannot be compared with ours since a 90:10 (*v*/*v*) ethanol–water solution was used as extractant [66].

Treatments on B16F10 melanoma cells were less effective: a decrease in viability by cold MURG extracts was induced only by the maximum dose, while the same dose of hot extract increased cell growth. The cold extracts of LOCG, ILDP and COEV also had mild effects, while the hot extracts appeared almost ineffective. The same goes for MARRV, with reduction in cell viability only at the highest dose of hot and cold extracts. Unfortunately, we were not able to compare our data with the literature since we did not find any other pertinent study concerning melanoma cancer cells and chestnut derivatives.

A separate discussion must be made for breast cancer MCF7 cells: cold extracts of MURG, LOCG, ILDP and COEV induced a significant increase in cell viability starting from moderate doses (50 μg mL^{-1}); hot extracts had a similar, but less evident effect compared to reference fibroblasts. What was surprising, compared with the Sardinian accessions, was the effect induced by MARRV hot extracts, which induced a reduction in cell viability of approximately 50% at the highest treatment dose. This is confirmed by the GI_{50} values calculated on MCF7 for all the Sardinian accessions and for MARRV, and is in agreement with the values reported, on the same cell line, for infusions and decoctions of chestnut flowers [65]. Cacciola et al. [12], worked with chestnut shell water extract, rich in gallic acid but with a much lower ellagic acid content than that found in our extracts; they observed that treatments with 100 μg mL^{-1} of extract induced a significant reduction in the viability of MCF7 cells, but less than 20%, and no effect on MDA-MB-231, another breast cancer cell line. This is in agreement with our results, since MARRV hot extracts were not effective at 100 μg mL^{-1} and became effective only at 250. A similar result, 18% inhibition of cell viability, on the MDA-MB-231 line was obtained by treating the cells for 24 h with doses of 200 μg mL^{-1} of chestnut powder [35]. Finally, a study worth reporting suggests that chestnut leaf extracts of *Castanea crenata* could increase the susceptibility of MCF7-derived cancer stem cells to paclitaxel, an anticancer drug [67].

5. Conclusions

This paper deals with the antioxidant and anticancer properties of cold and hot water pericarp extracts from chestnut accessions belonging to the Sardinian biodiversity. The study showed that the extracts contain bioactive compounds whose properties are at-

tributable, at least in part, to molecules that are extracted in different quantities, depending on temperature and contact times with the solvent. Cold extraction gives rise to mixtures of molecules that are apparently more effective than hot extracts in reducing the cellular viability of tumor cells but which, at the same time, have a greater impact on the viability of fibroblasts; for this reason, doses and duration of treatments must be carefully evaluated, regardless of the type of extract. As far as we know, there are only a few studies reporting the use of chestnut extracts as potential antitumor agents in the treatment of colon and breast cancer, and this is the first time that chestnut extracts were tested on melanoma cells. The reported results are preliminary, but preparatory for further studies (i) to distinguish the different role of each of the main phenolic classes and, (ii) to combine the extracts with clinically standardized anti-tumor therapies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14041422/s1, Table S1: Triangular matrix of Pearson correlation coefficients calculated considering TP, TF and PA content of cold CP extracts vs. the antioxidant capacity values measured by spectrophotometric and electrochemical tests; Table S2: Triangular matrix of Pearson correlation coefficients calculated considering TP, TF and PA content of hot CP extracts vs. the antioxidant capacity values measured by spectrophotometric and electrochemical tests; Table S3: Matrix of Pearson correlation coefficients calculated between the phenolic compounds quantified by HPLC analysis in the cold CP extracts, and their antioxidants capacities; Table S4: Matrix of Pearson correlation coefficients calculated between the phenolic compounds quantified by HPLC analysis in the hot CP extracts, and their antioxidants capacities; Figure S1: Cyclic voltammograms of growing concentration of gallic acid (a) and relative calibration curve (b); Figure S2: Chromatographic profile of ILDP pericarp cold (A) and hot (B) water extracts; Figure S3: Scavenging activity on DPPH radical (%) of cold (a) and hot (b) CP extracts; Figure S4: Scavenging activity on ABTS radical (%) of cold (a) and hot (b) CP extracts; Figure S5: Dose–response curves, obtained by non-linear regression analysis (inhibitor vs. response), used to calculate the concentration (μg mL^{-1}) of chestnut cold water extracts that results in inhibition of fibroblasts', SW48, RKO, MCF7 and B16F10 cancer cells' growth by 50% (GI50); Figure S6: Dose–response curves, obtained by non-linear regression analysis (inhibitor vs. response), used to calculate the concentration (μg mL^{-1}) of hot chestnut water extracts that results in inhibition of fibroblasts', SW48, RKO, MCF7 and B16F10 cancer cells' growth by 50% (GI50).

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References

1. Poljak, I.; Idžojtić, M.; Šatović, Z.; Ježić, M.; Ćurković-Perica, M.; Simovski, B.; Acevski, J.; Liber, Z. Genetic Diversity of the Sweet Chestnut (*Castanea sativa* Mill.) in Central Europe and the Western Part of the Balkan Peninsula and Evidence of Marron Genotype Introgression into Wild Populations. *Tree Genet. Genomes* **2017**, *13*, 18. [CrossRef]
2. Massantini, R.; Moscetti, R.; Frangipane, M.T. Evaluating Progress of Chestnut Quality: A Review of Recent Developments. *Trends Food Sci. Technol.* **2021**, *113*, 245–254. [CrossRef]
3. Piccolo, E.L.; Landi, M.; Ceccanti, C.; Mininni, A.N.; Marchetti, L.; Massai, R.; Guidi, L.; Remorini, D. Nutritional and Nutraceutical Properties of Raw and Traditionally Obtained Flour from Chestnut Fruit Grown in Tuscany. *Eur. Food Res. Technol.* **2020**, *246*, 1867–1876. [CrossRef]
4. De Vasconcelos, M.D.C.B.M.; Bennett, R.N.; Quideau, S.; Jacquet, R.; Rosa, E.A.S.; Ferreira-Cardoso, J.V. Evaluating the Potential of Chestnut (*Castanea sativa* Mill.) Fruit Pericarp and Integument as a Source of Tocopherols, Pigments and Polyphenols. *Ind. Crops Prod.* **2010**, *31*, 301–311. [CrossRef]
5. Cerulli, A.; Napolitano, A.; Masullo, M.; Hošek, J.; Pizza, C.; Piacente, S. Chestnut Shells (Italian Cultivar "Marrone Di Roccadaspide" PGI): Antioxidant Activity and Chemical Investigation with in Depth LC-HRMS/MSn Rationalization of Tannins. *Food Res. Int.* **2020**, *129*, 108787. [CrossRef]
6. Braga, N.; Rodrigues, F.; Oliveira, M.B.P.P. *Castanea sativa* by-Products: A Review on Added Value and Sustainable Application. *Nat. Prod. Res.* **2015**, *29*, 1–18. [CrossRef]
7. Hu, M.; Yang, X.; Chang, X. Bioactive Phenolic Components and Potential Health Effects of Chestnut Shell: A Review. *J. Food Biochem.* **2021**, *45*, e13696. [CrossRef]
8. Zhu, T.; Shen, Q.; Xu, Y.; Li, C. Ionic Liquid and Ultrasound-Assisted Extraction of Chestnut Shell Pigment with Good Hair Dyeing Capability. *J. Clean. Prod.* **2022**, *335*, 130195. [CrossRef]
9. Santos, M.J.; Pinto, T.; Vilela, A. Sweet Chestnut (*Castanea sativa* Mill.) Nutritional and Phenolic Composition Interactions with Chestnut Flavor Physiology. *Foods* **2022**, *11*, 4052. [CrossRef] [PubMed]
10. Pinto, D.; Moreira, M.M.; Vieira, E.F.; Švarc-Gajić, J.; Vallverdú-Queralt, A.; Brezo-Borjan, T.; Delerue-Matos, C.; Rodrigues, F. Development and Characterization of Functional Cookies Enriched with Chestnut Shells Extract as Source of Bioactive Phenolic Compounds. *Foods* **2023**, *12*, 640. [CrossRef] [PubMed]
11. Silva, V.; Falco, V.; Dias, M.I.; Barros, L.; Silva, A.; Capita, R.; Alonso-Calleja, C.; Amaral, J.S.; Igrejas, G.; Ferreira, I.C.F.R.; et al. Evaluation of the Phenolic Profile of *Castanea sativa* Mill. By-Products and Their Antioxidant and Antimicrobial Activity against Multiresistant Bacteria. *Antioxidants* **2020**, *9*, 87. [CrossRef]
12. Cacciola, N.A.; Squillaci, G.; D'Apolito, M.; Petillo, O.; Veraldi, F.; La Cara, F.; Peluso, G.; Margarucci, S.; Morana, A. *Castanea sativa* Mill. Shells Aqueous Extract Exhibits Anticancer Properties Inducing Cytotoxic and Pro-Apoptotic Effects. *Molecules* **2019**, *24*, 3401. [CrossRef]
13. Fadda, A.; Barberis, A.; D'Aquino, S.; Palma, A.; Angioni, A.; Lai, F.; Schirra, M. Residue Levels and Performance of Potassium Sorbate and Thiabendazole and Their Co-Application against Blue Mold of Apples When Applied as Water Dip Treatments at 20 or 53 °C. *Postharvest Biol. Technol.* **2015**, *106*, 33–43. [CrossRef]
14. Fadda, A.; Virdis, A.; Barberis, A.; Melito, S. Variation in Secondary Metabolites Contents of Spinoso Sardo Artichoke (*Cynara cardunculus* L.) under Different Day Lengths. *Turk. J. Agric. For.* **2018**, *42*, 372–381. [CrossRef]
15. Ramli, I.; Posadino, A.M.; Zerizer, S.; Spissu, Y.; Barberis, A.; Djeghim, H.; Azara, E.; Bensouici, C.; Kabouche, Z.; Rebbas, K.; et al. Low Concentrations of *Ambrosia maritima* L. Phenolic Extract Protect Endothelial Cells from Oxidative Cell Death Induced by H_2O_2 and Sera from Crohn's Disease Patients. *J. Ethnopharmacol.* **2023**, *300*, 115722. [CrossRef]
16. Spissu, Y.; Gil, K.A.; Dore, A.; Sanna, G.; Palmieri, G.; Sanna, A.; Cossu, M.; Belhadj, F.; Gharbi, B.; Pinna, M.B.; et al. Anti- and Pro-Oxidant Activity of Polyphenols Extracts of Syrah and Chardonnay Grapevine Pomaces on Melanoma Cancer Cells. *Antioxidants* **2023**, *12*, 80. [CrossRef] [PubMed]
17. Salim, A.; Deiana, P.; Fancello, F.; Molinu, M.G.; Santona, M.; Zara, S. Antimicrobial and Antibiofilm Activities of Pomegranate Peel Phenolic Compounds: Varietal Screening Through a Multivariate Approach. *J. Bioresour. Bioprod.* **2023**, *8*, 146–161. [CrossRef]
18. Aires, A.; Carvalho, R.; Saavedra, M.J. Valorization of Solid Wastes from Chestnut Industry Processing: Extraction and Optimization of Polyphenols, Tannins and Ellagitannins and Its Potential for Adhesives, Cosmetic and Pharmaceutical Industry. *Waste Manag.* **2016**, *48*, 457–464. [CrossRef]
19. Fernández-Agulló, A.; Freire, M.S.; Antorrena, G.; Pereira, J.A.; González-Álvarez, J. Effect of the Extraction Technique and Operational Conditions on the Recovery of Bioactive Compounds from Chestnut (*Castanea sativa*) Bur and Shell. *Sep. Sci. Technol.* **2014**, *49*, 267–277. [CrossRef]
20. Jung, B.S.; Lee, N.-K.; Na, D.S.; Yu, H.H.; Paik, H.-D. Comparative Analysis of the Antioxidant and Anticancer Activities of Chestnut Inner Shell Extracts Prepared with Various Solvents. *J. Sci. Food Agric.* **2016**, *96*, 2097–2102. [CrossRef]

21. Squillaci, G.; Apone, F.; Sena, L.M.; Carola, A.; Tito, A.; Bimonte, M.; Lucia, A.D.; Colucci, G.; Cara, F.L.; Morana, A. Chestnut (*Castanea sativa* Mill.) Industrial Wastes as a Valued Bioresource for the Production of Active Ingredients. *Process Biochem.* **2018**, *64*, 228–236. [CrossRef]
22. Vella, F.M.; De Masi, L.; Calandrelli, R.; Morana, A.; Laratta, B. Valorization of the Agro-Forestry Wastes from Italian Chestnut Cultivars for the Recovery of Bioactive Compounds. *Eur. Food Res. Technol.* **2019**, *245*, 2679–2686. [CrossRef]
23. Galiñanes, C.; Freire, M.S.; González-Álvarez, J. Antioxidant Activity of Phenolic Extracts from Chestnut Fruit and Forest Industries Residues. *Eur. J. Wood Prod.* **2015**, *73*, 651–659. [CrossRef]
24. Ricci, A.; Parpinello, G.P.; Teslić, N.; Kilmartin, P.A.; Versari, A. Suitability of the Cyclic Voltammetry Measurements and DPPH• Spectrophotometric Assay to Determine the Antioxidant Capacity of Food-Grade Oenological Tannins. *Molecules* **2019**, *24*, 2925. [CrossRef] [PubMed]
25. Alam, M.W.; Najeeb, J.; Naeem, S.; Usman, S.M.; Nahvi, I.; Alismail, F.; Abuzir, A.; Farhan, M.; Nawaz, A. Electrochemical Methodologies for Investigating the Antioxidant Potential of Plant and Fruit Extracts: A Review. *Antioxidants* **2022**, *11*, 1205. [CrossRef] [PubMed]
26. Zheng, Y.; Karimi-Maleh, H.; Fu, L. Evaluation of Antioxidants Using Electrochemical Sensors: A Bibliometric Analysis. *Sensors* **2022**, *22*, 3238. [CrossRef] [PubMed]
27. Blasco, A.J.; González Crevillén, A.; González, M.C.; Escarpa, A. Direct Electrochemical Sensing and Detection of Natural Antioxidants and Antioxidant Capacity in Vitro Systems. *Electroanalysis* **2007**, *19*, 2275–2286. [CrossRef]
28. Raja, A.N.; Annu; Singh, K.; Jain, R. Ultrasensitive Quantification of Ellagic Acid Using Gr/Bi2O3/GCE as Voltammetric Sensor. *Int. J. Electrochem. Sci.* **2020**, *15*, 10040–10057. [CrossRef]
29. Ghoreishi, S.M.; Behpour, M.; Khayatkashani, M.; Motaghedifard, M.H. New applied method for simultaneous determination of ellagic and tannic acid by multi-wall carbon nanotube paste electrode: Application in quantification punica granatum and quercus infectoria. *Dig. J. Nanomater. Biostruct. (DJNB)* **2011**, *6*, 625–635.
30. Buratti, S.; Scampicchio, M.; Giovanelli, G.; Mannino, S. A Low-Cost and Low-Tech Electrochemical Flow System for the Evaluation of Total Phenolic Content and Antioxidant Power of Tea Infusions. *Talanta* **2008**, *75*, 312–316. [CrossRef]
31. Spissu, Y.; Barberis, A.; D'hallewin, G.; Orrù, G.; Scano, A.; Serra, G.R.; Pinna, M.; Pinna, C.; Marceddu, S.; Serra, P.A. An Ascorbate Bluetooth© Analyzer for Quality Control of Fresh-Cut Parsley Supply Chain. *Antioxidants* **2021**, *10*, 1485. [CrossRef]
32. Spissu, Y.; Barberis, A.; Bazzu, G.; D'hallewin, G.; Rocchitta, G.; Serra, P.A.; Marceddu, S.; Vineis, C.; Garroni, S.; Culeddu, N. Functionalization of Screen-Printed Sensors with a High Reactivity Carbonaceous Material for Ascorbic Acid Detection in Fresh-Cut Fruit with Low Vitamin C Content. *Chemosensors* **2021**, *9*, 354. [CrossRef]
33. Percevault, L.; Limanton, E.; Nicolas, P.; Paquin, L.; Lagrost, C. Electrochemical Determination and Antioxidant Capacity Modulation of Polyphenols in Deep Eutectic Solvents. *ACS Sustain. Chem. Eng.* **2021**, *9*, 776–784. [CrossRef]
34. Karadag, A.; Ozcelik, B.; Saner, S. Review of Methods to Determine Antioxidant Capacities. *Food Anal. Methods* **2009**, *2*, 41–60. [CrossRef]
35. Lee, H.S.; Kim, E.J.; Kim, S.H. Chestnut Extract Induces Apoptosis in AGS Human Gastric Cancer Cells. *Nutr. Res. Pract.* **2011**, *5*, 185–191. [CrossRef]
36. Lee, J.H.; Khor, T.O.; Shu, L.; Su, Z.-Y.; Fuentes, F.; Kong, A.-N.T. Dietary Phytochemicals and Cancer Prevention: Nrf2 Signaling, Epigenetics, and Cell Death Mechanisms in Blocking Cancer Initiation and Progression. *Pharmacol. Ther.* **2013**, *137*, 153–171. [CrossRef]
37. Dashwood, R.H. Frontiers in Polyphenols and Cancer Prevention. *J. Nutr.* **2007**, *137*, 267S–269S. [CrossRef]
38. Sorice, A.; Siano, F.; Capone, F.; Guerriero, E.; Picariello, G.; Budillon, A.; Ciliberto, G.; Paolucci, M.; Costantini, S.; Volpe, M. Potential Anticancer Effects of Polyphenols from Chestnut Shell Extracts: Modulation of Cell Growth, and Cytokinomic and Metabolomic Profiles. *Molecules* **2016**, *21*, 1411. [CrossRef]
39. Beretta, G.; Moretti, R.M.; Nasti, R.; Cincinelli, R.; Dallavalle, S.; Montagnani Marelli, M. Apoptosis-Mediated Anticancer Activity in Prostate Cancer Cells of a Chestnut Honey (*Castanea sativa* L.) Quinoline–Pyrrolidine Gamma-Lactam Alkaloid. *Amino Acids* **2021**, *53*, 869–880. [CrossRef]
40. Santulli, C.; Brizi, C.; Durante, M.; Micucci, M.; Budriesi, R.; Chiarini, R.; Frosini, M. Apoptotic-Induced Effects of *Castanea sativa* Bark Extract in Human SH-SY5Y Neuroblastoma Cells. *NPC-Nat. Prod. Commun.* **2018**, *13*, 887–890. [CrossRef]
41. Domínguez-Rodríguez, G.; Marina, M.L.; Plaza, M. Strategies for the Extraction and Analysis of Non-Extractable Polyphenols from Plants. *J. Chromatogr. A* **2017**, *1514*, 1–15. [CrossRef]
42. Surveswaran, S.; Cai, Y.-Z.; Corke, H.; Sun, M. Systematic Evaluation of Natural Phenolic Antioxidants from 133 Indian Medicinal Plants. *Food Chem.* **2007**, *102*, 938–953. [CrossRef]
43. Barberis, A.; Deiana, M.; Spissu, Y.; Azara, E.; Fadda, A.; Serra, P.A.; D'hallewin, G.; Pisano, M.; Serreli, G.; Orrù, G.; et al. Antioxidant, Antimicrobial, and Other Biological Properties of Pompia Juice. *Molecules* **2020**, *25*, 3186. [CrossRef]
44. Yamanaka, K.; Vestergaard, M.C.; Tamiya, E. Printable Electrochemical Biosensors: A Focus on Screen-Printed Electrodes and Their Application. *Sensors* **2016**, *16*, 1761. [CrossRef]
45. José Jara-Palacios, M.; Hernanz, D.; Luisa Escudero-Gilete, M.; Heredia, F.J. Antioxidant Potential of White Grape Pomaces: Phenolic Composition and Antioxidant Capacity Measured by Spectrophotometric and Cyclic Voltammetry Methods. *Food Res. Int.* **2014**, *66*, 150–157. [CrossRef]

46. Sangiovanni, E.; Piazza, S.; Vrhovsek, U.; Fumagalli, M.; Khalilpour, S.; Masuero, D.; Di Lorenzo, C.; Colombo, L.; Mattivi, F.; De Fabiani, E.; et al. A Bio-Guided Approach for the Development of a Chestnut-Based Proanthocyanidin-Enriched Nutraceutical with Potential Anti-Gastritis Properties. *Pharmacol. Res.* **2018**, *134*, 145–155. [CrossRef]
47. Pedotti, S.; Patti, A.; Dedola, S.; Barberis, A.; Fabbri, D.; Dettori, M.A.; Serra, P.A.; Delogu, G. Synthesis of New Ferrocenyl Dehydrozingerone Derivatives and Their Effects on Viability of PC12 Cells. *Polyhedron* **2016**, *117*, 80–89. [CrossRef]
48. Ham, J.-S.; Kim, H.-Y.; Lim, S.-T. Antioxidant and Deodorizing Activities of Phenolic Components in Chestnut Inner Shell Extracts. *Ind. Crops Prod.* **2015**, *73*, 99–105. [CrossRef]
49. Barreira, J.C.M.; Ferreira, I.C.F.R.; Oliveira, M.B.P.P. Bioactive Compounds of Chestnut (*Castanea sativa* Mill.). In *Bioactive Compounds in Underutilized Fruits and Nuts*; Murthy, H.N., Bapat, V.A., Eds.; Springer International Publishing: Cham, Switzerland, 2020; pp. 303–313, ISBN 978-3-030-30182-8.
50. Vázquez, G.; Fontenla, E.; Santos, J.; Freire, M.S.; González-Álvarez, J.; Antorrena, G. Antioxidant Activity and Phenolic Content of Chestnut (*Castanea sativa*) Shell and Eucalyptus (*Eucalyptus globulus*) Bark Extracts. *Ind. Crops Prod.* **2008**, *28*, 279–285. [CrossRef]
51. Cravotto, C.; Grillo, G.; Binello, A.; Gallina, L.; Olivares-Vicente, M.; Herranz-López, M.; Micol, V.; Barrajón-Catalán, E.; Cravotto, G. Bioactive Antioxidant Compounds from Chestnut Peels through Semi-Industrial Subcritical Water Extraction. *Antioxidants* **2022**, *11*, 988. [CrossRef] [PubMed]
52. Rodrigues, F.; Santos, J.; Pimentel, F.B.; Braga, N.; Palmeira-de-Oliveira, A.; Oliveira, M.B.P.P. Promising New Applications of *Castanea sativa* Shell: Nutritional Composition, Antioxidant Activity, Amino Acids and Vitamin E Profile. *Food Funct.* **2015**, *6*, 2854–2860. [CrossRef]
53. Fadda, A.; Sanna, D. Advantages and Pitfalls of the Methods for the Antioxidant Activity Evaluation. In *Advances in Food Analysis Research*; Haynes, A., Ed.; Nova Science Publishers: Hauppauge, NY, USA, 2015; pp. 65–88.
54. Firuzi, O.; Lacanna, A.; Petrucci, R.; Marrosu, G.; Saso, L. Evaluation of the Antioxidant Activity of Flavonoids by "Ferric Reducing Antioxidant Power" Assay and Cyclic Voltammetry. *Biochim. Biophys. Acta (BBA)-Gen. Subj.* **2005**, *1721*, 174–184. [CrossRef]
55. Zhang, D.; Chu, L.; Liu, Y.; Wang, A.; Ji, B.; Wu, W.; Zhou, F.; Wei, Y.; Cheng, Q.; Cai, S.; et al. Analysis of the Antioxidant Capacities of Flavonoids under Different Spectrophotometric Assays Using Cyclic Voltammetry and Density Functional Theory. *J. Agric. Food Chem.* **2011**, *59*, 10277–10285. [CrossRef]
56. Pastor, F.T.; Šegan, D.M.; Gorjanović, S.Ž.; Kalušević, A.M.; Sužnjević, D.Ž. Development of Voltammetric Methods for Antioxidant Activity Determination Based on Fe(III) Reduction. *Microchem. J.* **2020**, *155*, 104721. [CrossRef]
57. Volf, I.; Ignat, I.; Neamtu, M.; Popa, V. Thermal Stability, Antioxidant Activity, and Photo-Oxidation of Natural Polyphenols. *Chem. Pap.* **2014**, *68*, 121–129. [CrossRef]
58. Vázquez, G.; González-Alvarez, J.; Santos, J.; Freire, M.S.; Antorrena, G. Evaluation of Potential Applications for Chestnut (*Castanea sativa*) Shell and Eucalyptus (*Eucalyptus globulus*) Bark Extracts. *Ind. Crops Prod.* **2009**, *29*, 364–370. [CrossRef]
59. León-González, A.J.; Auger, C.; Schini-Kerth, V.B. Pro-Oxidant Activity of Polyphenols and Its Implication on Cancer Chemoprevention and Chemotherapy. *Biochem. Pharmacol.* **2015**, *98*, 371–380. [CrossRef]
60. Khan, H.Y.; Zubair, H.; Ullah, M.F.; Ahmad, A.; Hadi, S.M. A Prooxidant Mechanism for the Anticancer and Chemopreventive Properties of Plant Polyphenols. *Curr. Drug Targets* **2012**, *13*, 1738–1749. [CrossRef]
61. Pasciu, V.; Posadino, A.M.; Cossu, A.; Sanna, B.; Tadolini, B.; Gaspa, L.; Marchisio, A.; Dessole, S.; Capobianco, G.; Pintus, G. Akt Downregulation by Flavin Oxidase–Induced ROS Generation Mediates Dose-Dependent Endothelial Cell Damage Elicited by Natural Antioxidants. *Toxicol. Sci.* **2010**, *114*, 101–112. [CrossRef] [PubMed]
62. Giordo, R.; Cossu, A.; Pasciu, V.; Hoa, P.T.; Posadino, A.M.; Pintus, G. Different Redox Response Elicited by Naturally Occurring Antioxidants in Human Endothelial Cells. *Open Biochem. J.* **2013**, *7*, 44–53. [CrossRef] [PubMed]
63. Posadino, A.M.; Giordo, R.; Cossu, A.; Nasrallah, G.K.; Shaito, A.; Abou-Saleh, H.; Eid, A.H.; Pintus, G. Flavin Oxidase-Induced ROS Generation Modulates PKC Biphasic Effect of Resveratrol on Endothelial Cell Survival. *Biomolecules* **2019**, *9*, 209. [CrossRef]
64. Floris, A.; Mazarei, M.; Yang, X.; Robinson, A.E.; Zhou, J.; Barberis, A.; D'hallewin, G.; Azara, E.; Spissu, Y.; Iglesias-Ara, A.; et al. SUMOylation Protects FASN Against Proteasomal Degradation in Breast Cancer Cells Treated with Grape Leaf Extract. *Biomolecules* **2020**, *10*, 529. [CrossRef]
65. Carocho, M.; Calhelha, R.C.; Queiroz, M.-J.R.P.; Bento, A.; Morales, P.; Soković, M.; Ferreira, I.C.F.R. Infusions and Decoctions of *Castanea sativa* Flowers as Effective Antitumor and Antimicrobial Matrices. *Ind. Crops Prod.* **2014**, *62*, 42–46. [CrossRef]
66. Zhang, L.; Gao, H.; Baba, M.; Okada, Y.; Okuyama, T.; Wu, L.; Zhan, L. Extracts and Compounds with Anti-Diabetic Complications and Anti-Cancer Activity from *Castanea mollissina* Blume (Chinese Chestnut). *BMC Complement. Altern. Med.* **2014**, *14*, 422. [CrossRef]
67. Woo, Y.; Oh, J.; Kim, J.-S. Suppression of Nrf2 Activity by Chestnut Leaf Extract Increases Chemosensitivity of Breast Cancer Stem Cells to Paclitaxel. *Nutrients* **2017**, *9*, 760. [CrossRef]