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Repurposing Hazelnut Waste Products for a Sustainable Economy: A Metabolomic Analysis of Cuticles and Shells to Highlight Their Antioxidant Potential and Inhibitory Activity against Verocytotoxic *Escherichia coli*

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Abstract: This study evaluated the functional components of hazelnut waste products (cuticles and shells) for potential use in the circular economy. HPLC-HRMS and ABTS assay were used to determine the molecules of waste products with functional properties and antioxidant capacity, respectively. The antioxidant capacity of hazelnut cuticles and shells was tested using two different methods of extraction (EtOH 50/50 and acetone 40/70) by ABTS radical cation decolorization assay. The growth inhibition effect of different extracts against porcine O138 *E. coli* F18^{+} was evaluated by the microdilution bacterial growth method and confirmed with the determination of minimal inhibitory concentration (MIC). The results showed that acetone extraction produced a higher yield of metabolites compared to ethanol extraction, and the cuticles exhibited higher antioxidant and antimicrobial potential. The acetone/water extraction led to the identification of 738 signals compared to 453 detected after EtOH/water extraction. Through metabolomic analysis, it was found that the cuticles presented a much higher quantitative and qualitative polyphenolic profile than did the shells. In addition, the ABTS assay revealed that the cuticles had a higher antioxidant capacity than did the shells. Thus, we believe that hazelnut-derived waste products show a high potential for use in a circular and sustainable economy.

Keywords: hazelnut; cuticles; shell; waste product; by-product; circular economy; antioxidant; antimicrobial; polyphenols

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1. Introduction

The increasing global population has led to the need to maximize natural resources. Reducing waste and using food by-products have therefore become key aspects of the circular economy. The circular economy is defined as "a model of economics that is restorative and regenerative by design, and aims to maintain products, components, and materials at their maximum utility and value at all times, distinguishing between technical and biological cycles." It is seen as a new business model that offers tools to improve and optimize sustainability within the Western food system [1].

The agribusiness sector offers many opportunities, and all stages of production possess the great potential to improve the sustainability and efficiency of the supply chain by minimizing upstream inputs and circulating residues/by-products downstream, where possible, using technological pathways that maximize the use of natural resources [2]. The recycling of food processing by-products, in particular, into animal feed, has long

been performed to increase the sustainability of production systems. Many studies have focused on using agri-food leftovers as functional ingredients in animal diets [3,4] in order to promote the health status of animals, reduce the use of antibiotics, and reduce the environmental impact of livestock production [5–7]. In addition, food by-products can also be used as natural additives to increase the shelf life of food products. In fact, these by-products contain several antimicrobial compounds that can potentially be used as preservatives, ranging from enzymes, bacteriocins, fungicides, and salts to essential oils and other components [8]. The potential use of antioxidant compounds as food preservatives has also been explored, thus reducing the use of food additives such as nitrates and nitrites in the meat industry. The use of by-products can play a pivotal role in enhancing the competitiveness of the food industry for sustainable purposes, as well as from a circular economy perspective [9–11].

Among the possible agri-food products, hazelnuts exhibit great potential, as they are abundant and cheap, and high-added value ingredients can be obtained from their by-products. The hazelnut (*Corylus avellana* L.), which belongs to the Betulaceae family, is one of the most popular tree nuts consumed worldwide, ranking second in tree nut production after almonds. Hazelnuts contain several nutrients (macro and micro) with a high nutritional and functional profile. Hazelnuts are composed of seeds—the main component used in the food industry—as well as skins, or cuticles, and shells—the main waste products resulting from the hazelnut industry [12]. Hazelnut skin, which is the residual biomass of roasting, is usually used in animal feed and fertilizer or as raw material for biogas plants, while the hazelnut shell is used as a heating source during combustion. However, in recent years, from a circular economy perspective and considering that the shell represents more than 50% of the total weight of the nut, whereas the skin makes up only around 2%, these by-products have become the subject of several studies [13–15]. Hazelnut skin contains about 7.5% moisture, 8% protein, 14.5% fat, 1.7% ash, and 67.7% dietary fiber, which is its main constituent [16]. However, the (poly)phenolic content of the skin has gained increasing interest, especially due to its potential use in different sectors, with high-value returns.

The hazelnut shell, on the other hand, is known to contain, on average, 30.4% hemicelluloses, 26.8% cellulose, 42.9% lignin, and 3.3% other extractive matter [17]. The use of hazelnuts in agri-food industries produces tons of by-products, which given the properties just described, are far from being considered waste products. In fact, the hazelnut husk is rich in monomeric and oligomeric flavanols [18], which are a class of (poly)phenols recognized for their beneficial effects on cardiovascular and cognitive functions, which could therefore, make them a valuable dietary supplement for the nutraceutical market.

Given the current need to find food sources that are functionally rich, as well as the divided public opinion concerning the use of waste products from the food industry, the characterization of hazelnut by-products could shed light on the possible reuse of this waste product. The objective of the present study was to carry out a comprehensive chemical and functional characterization of hazelnut cuticles and shells in order to identify the metabolites they contain and to determine their feasible use in feed and food chains.

2. Materials and Methods

2.1. Sample Preparation

The extraction procedure was conducted as outlined by Yuan et al. [19], with slight modifications. Briefly, 1 g of hazelnut shells or cuticles were separately suspended in 100 mL of (1) water/acetone mixture (40/60), and (2) water/ethanol mixture (50/50). The samples were homogenized in an ultrasound bath for 2 h. After ultrasound treatment, the extracts were centrifuged for 5 min at 5000× *g* and 4 °C. The supernatants were collected and an aliquot was filtered and diluted (1:10) in 1 mL of initial HPLC mobile phase (95% water acidified with 0.1% HCOOH and 5% methanol). The remaining part was filtered through a 0.45 µm syringe filter and stored at −20 °C until the analysis. All samples were processed in two technical replicates.

2.2. HPLC-High Resolution Mass Spectrometry (HPLC-HRMS)

HPLC-high resolution mass spectrometry was carried out, according to the methods in a previous study [20], setting the instrumental conditions to achieve the best separation of the most important polyphenolic compounds. Briefly, a Vanquish HPLC instrument (Thermo Fisher Scientific, San Jose, CA, USA) was used for the chromatographical separation using an Restek RP column, with a programed gradient flow of 0.1% HCOOH, in water and methanol. The Exploris HRMS-Orbitrap (Thermo Scientific, San Jose, CA, USA) device was operated simultaneously in both positive mode and negative mode, using predetermined acquisition parameters for each mode. The full scan (FS) with a resolving power of 120,000 (two scan ranges of m/z 70–800 and 800–2500) was used for the screening and statistical evaluation of the chromatographic profiles. Full scan data-dependent acquisition (FS-dd-MS2) with resolving power 60,000 and 17,500 for FS and dd-MS2, respectively, was employed for the fragmentation of pseudo-molecular ions detected in the FS mode. The fragmentation of precursors was executed with stepped, normalized collision energy (NCE) set at 20, 30, and 40 eV.

2.3. HRMS Workflow Untargeted Metabolomics Approach

The detailed untargeted metabolomic workflow applied in this study is described in our recent publication [21]. Briefly, the Exploris Orbitrap raw data were submitted to Compound Discoverer (CD) 3.3 software (Thermo Fisher, Waltham, MA, USA), which enabled programmed compound identification and statistical evaluation. The criteria for the putative identification of the metabolites identified by the CD workflow were chosen as a combination of different assets, including an mzCloud match score higher than 70% and the same identification proposed by at least one external web database: the Human Metabolome platform HMDB (https://hmdb.ca/, accessed on 25 November 2022), the Kyoto Encyclopedia of Genes and Genomes (KEGG), (https://www.genome.jp/kegg, accessed on 25 November 2022), Pubchem (www.pubchem.com, accessed on 25 November 2022), the Small Molecule Pathway Database (SMPDB) (http://smpdb.ca, accessed on 28 November 2022), and Lipid Maps (https://www.lipidmaps.org, accessed on 28 November 2022). If the mass fragmentation pattern did not correspond to any of the databases, the fragmentation pattern was manually verified using ChemDrow software or by comparing it with recent publications [22]. Differential analysis was performed as part of the CD workflow, and the statistical evaluation was expressed in hierarchical cluster processing and a volcano plot graph.

2.4. Evaluation of Antioxidant Properties (ABTS Assay)

The antioxidant activity was tested using an ABTS assay, according to the methods of Dell'Anno et al. [23]. Briefly, 10 µL of the diluted sample were added to 1 mL of $ABTS^{\bullet+}$ working solution. The absorbance was recorded after 6 min of incubation in the dark, and all determinations were performed in triplicate.

In order to obtain different dilutions for testing, the extracts were diluted in their solvent, the water/acetone mixture (40/60), or the water/ethanol mixture (50/50), respectively. Concerning the shell extracts, the samples were tested, i.e., as is, 1:2, 1:4, and 1:8. On the other hand, for the cuticle extracts, the samples were tested in the following ratios: 1:16, 1:32, 1:64, and 1:100.

The total antioxidant capacity was expressed as the percentage of the inhibition of radical scavenging activity (PI%), according to the following equation:

$$\text{PI}\ (\%) = \left[\left(\text{AbsABTS}^{\bullet+} - \text{Abs Sample}/\text{AbsABTS}^{\bullet+}\right)\right] * 100 \quad (1)$$

$\text{AbsABTS}^{\bullet+}$ denotes the initial absorbance of diluted $ABTS^{\bullet+}$, and the Abs sample denotes the absorbance of the sample after 6 min of reaction. All assays were performed in technical triplicate and with two biological replicates aimed at verifying the replicability

of the experiment using the same procedures, which included repeating the experiment starting with the sample extraction and repeating the test on different days.

2.5. Growth Inhibition Assay against Escherichia coli

A growth inhibition assay was performed against the O138 *E. coli* strain. Briefly, extracts of hazelnut shells and hazelnut cuticles were filtered with a 0.22 µm syringe filter and stored at −20 °C until the analysis. According to the methods of Reggi et al. [24], a liquid culture-based growth inhibition assay against *E. coli* O138 was performed to evaluate the ability of hazelnut shell and hazelnut cuticle extracts to inhibit bacterial growth. Briefly, samples were diluted in Luria–Bertani (LB) broth in order to obtain four different concentrations (1:4; 1:8; 1:16; 1:32). A total of 100 µL of the diluted extracts was added in co-culture with 30 µL of *E. coli* inoculum. The negative controls were used to correct the background color. All samples were then incubated at 37 °C in a shaking incubator for 6 h. The growth rate of *E. coli* was estimated every hour for 6 h by measuring the absorbance with a microplate reader spectrophotometer (ScanReady P-800, Life Real, Hangzhou, China) at an optical density (OD) of 620 nm. The measured OD was converted into log10 of the number of cells/mL, considering 1 OD = 1×10^9 cells/mL [25]. All assays were performed in technical quadruplicate and with three biological replicates.

2.6. Determination of Minimal Inhibitory Concentration (MICs)

Minimum inhibitory concentrations (MICs) were determined through broth microdilution method. Briefly, different concentrations (35 mg/mL, 25 mg/mL, 10 mg/mL, 5 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, and 0 mg/mL) of hazelnut cuticle and shell extracts were prepared by diluting the dried extracts with LB broth. A total of 100 µL of the different concentrations were plated in a 96-well microplate, and 10 µL of an overnight culture of *E. coli* O138 in Luria–Bertani (LB) broth (approximately 10^6 CFU/mL) was inoculated in each well of the plate, except for the blank and negative control ($CTRL^-$) wells, and incubated at 37 °C for 20 h. Bacterial growth was determined by the change in absorbance after reading the microplates at 620 nm in a microplate reader spectrophotometer (ScanReady P-800, Life Real, Hangzhou, China). The inhibition rate was estimated by the following formula:

$$Inhibition\ Rate\ (\%) = 100 * \left(\frac{ODCTRL^- - ODsample}{ODCTRL^- - ODBlank} \right) \quad (2)$$

The MIC was defined as the lowest extract concentration that did not produce turbidity when compared with positive control (0 mg of extract/mL) [26]. The experiment was performed in technical triplicate and with two biological replicates.

2.7. Statistical Analysis

All the data were analyzed using GraphPad Prism software (Version 9.0.0). Statistical analysis was performed after the evaluation of the normal distribution of data through Shapiro–Wilk and D'Agostino–Pearson tests. Concerning data obtained from the assays of antioxidant activity and bacterial growth inhibition, a two-way analysis of variance (ANOVA) was performed, evaluating the effect of treatment, time, and their interaction with each other. Post hoc pairwise comparisons were performed using the Bonferroni–Sidak test. Data were reported as mean ± standard error, and differences were considered statistically significant for $p < 0.05$.

3. Results and Discussion

Given the current importance of the use of agro-industrial by-products in economic terms, enabling industries to reduce the disposal costs of waste biomass, and livestock farmers to mitigate animal feed costs, this study focused on the metabolomic characterization and in vitro evaluation of the functional properties of hazelnut shells and cuticles in order to establish their further use as functional additives.

3.1. Evaluation of Molecules with Antioxidant Properties

In this study, an HRMS-based metabolomics investigation was conducted for the first time in both polarization modalities (positive and negative) synchronously, within the same analytical run. A reliable, sensitive, and double-confirmed identification of key polyphenolic metabolites in complex matrices of hazelnut shells and cuticles was thus achieved. For the acetone/water extract, a total of 738 signals were obtained (Figure 1a). The most abundant polyphenolic/antioxidant/natural products (165 structures), detected with a high level of confirmation certainty, are presented in Figure 2a and Table 1. On the other hand, the ethanol/water extract showed a reduced metabolomic profile, with 453 signals (Figure 1b), of which only 67 were considered as important differentiators (Figure 2b and Table 2).

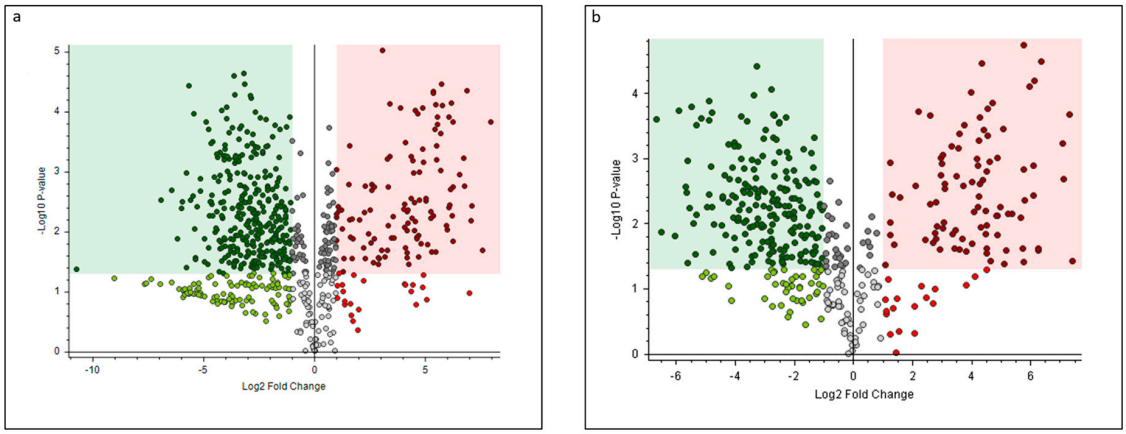

Figure 1. Volcano plot comparison between the relative intensity revealed in the hazelnut shell and cuticle extracts: (**a**) chromatographic peak of 738 compounds in acetone/water solvent; (**b**) chromatographic peak of 453 compounds in ethanol/water solvent. The right region contains an upregulated signal with significantly higher intensities obtained from the shells than from the cuticles, and these intensities were greater than those in the upper fold-change (FC) threshold. The left region includes downregulated peaks, in which the intensities from the shells were significantly lower than those from cuticles, and less than those in the lower FC threshold; *p*-value (PV) = 0.05.

Table 1. Compounds from hierarchical cluster analysis of acetone/H_2O extracts identified as the most significant differentiators between shells and cuticles, with statistical evaluation and regulation trend.

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Lignan	Lariciresinol 4-o-glucoside	−2.76	0.0002	down
	Cycloolivil	5.55	0.0002	up
	Dihydroconiferin	−2.91	0.0225	down
Pyranones	Dihydrokavain	0.85	0.0032	up
Amino acids and metabolites	Citrulline	−3.27	0.0034	down
	Glutamic acid	−2.66	0.0004	down
	Isoleucine	−2.59	0.0020	down
	Tryptophan	−4	0.0004	down
	Arginine	−4.64	0.0549	down
	Aspartic acid	−1.12	0.1216	down
	Asparagine	−4.08	0.1099	down
	Alpha-ketoglutaric acid	−0.3	0.1150	ns
	O-heptanoylcarnitine	−4.38	0.1412	down
	Propionylcarnitine	−4.56	0.1450	down
	Kynurenic acid	−2.76	0.0398	down

Table 1. *Cont.*

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Anisoles	2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	−0.22	0.1818	ns
	Vanillin	4.21	0.0224	up
	Homovanillic acid	4.22	0.0073	up
Anthracenecarboxylic acids	Carminic acid	−6.92	0.0030	down
Aurone flavonoids	Aureusidin 6-glucuronide	−5.77	0.0010	down
	Bractein	−2.77	0.0048	down
	2,6,3′,4′-Tetrahydroxy-2-benzylcoumaranone	−4.17	0.0185	down
	Nigrescin	−1.9	0.0392	down
	Castillene B	2.7	0.0018	up
	Amaronol A	2.31	0.0017	up
Azaspirodecane derivatives	Petasitenine	0.66	0.0502	ns
Benzoquinones	5-o-methylembelin	−0.75	0.4685	ns
Carbohydrates and derivatives	Trehalose	−4.19	0.0310	down
	Hexose	1.22	0.0043	up
	Saccharic acid	−0.8	0.0117	down
	Gluconic acid	−0.71	0.0098	down
	Mannitol	−0.96	0.0133	down
	Xylitol	−0.35	0.1614	ns
	Paeonolide	−3.06	0.0144	down
Chalcones and dihydrochalcones	Pseudosindorin	−3.49	0.0007	down
	Lusianin	2.25	0.0662	ns
	Desmosdumotin C	−0.3	0.0740	ns
	Hamilcone	4.73	0.0264	up
	2′,6′-Dihydroxy-4′-methoxy-3′-prenyldihydrochalcone	−3.45	0.0057	down
	2′-Hydroxy-4′,6′-dimethoxy-3′-methyldihydrochalcone	4.4	0.0029	up
	2′,4′,4-Trihydroxy-3′,3-methoxychalcone	0.99	0.0303	up
	Derricin	−3.61	0.0000	down
	Pseudosindorin	−0.56	0.1130	ns
	Brosimacutin H	6.8	0.0017	up
	Grandiflorone	−1.38	0.0280	down
	Phloretin	−2.84	0.0006	down
	Trilobatin	−2.73	0.0064	down
	Kanzonol B	−5.66	0.0000	down
	4,2′,6′-Trihydroxy-4′-methoxy-3′,5′-dimethyldihydrochalcone	4.75	0.0030	up
	Isoneobavachalcone	−1.61	0.0056	down
	2′,3′,4′,6′-Tetramethoxychalcone	1.66	0.0125	up
	Okanin	−2.72	0.0174	down
	Aspalathin	0.25	0.0391	up
	Okanin 3,4,3′,4′-tetramethyl ether	4.11	0.0003	up
	Chalconaringenin 2′-xyloside	5.45	0.0002	up
	4-Hydroxy-2′,4′-dimethoxydihydrochalcone	4.23	0.0044	up
	Purpuritenin B	−3.08	0.0033	down
	Phloretin 3′,5′-Di-C-glucoside	−1.36	0.0499	down
Chromenes	Sec-o-glucosylhamaudol	2.47	0.0108	up
	2-Methyl-5-acetonyl-7-hydroxychromone	4.84	0.0161	up
	Eugenitin	−2.25	0.0261	down

Table 1. *Cont.*

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Coumarins	Scopoletin acetate	6.9	0.0000	up
	5,6,7-Trimethoxy-2H-chromen-2-one	6.12	0.0037	up
	Glabrocoumarone A	−3.91	0.0010	down
Diarylheptanoid	1,7-bis(4-hydroxyphenyl)-3,5-heptanediol	−0.96	0.1334	ns
Dipeptide	Leucylproline	0.34	0.0195	up
Flavanones and conjugates	Eriodictyol	−1.82	0.0257	down
	Pectolinarigenin 7-rhamnoside	−1.57	0.2553	ns
	Protofarrerol	5.55	0.0006	up
	Naringenin	−3.39	0.0048	down
	5,6,7,3′,4′-Pentamethoxyflavanone	7.6	0.0207	up
	Fustin	−4.01	0.0136	down
	Astilbin	−3.13	0.0018	down
	Hesperetin	5.37	0.0000	up
	Brosimacutin A	4.88	0.0004	up
	5,4′-Dihydroxy-6-C-prenylflavanone 4′-xylosyl-(1->2)-rhamnoside	2.9	0.0276	up
	Naringin dihydrochalcone	−2.32	0.0038	down
Flavanonol	Dihydromorin	−3.38	0.0007	down
Flavans and conjugates	3,4,7-Trihydroxy-5,4′-dimethoxy-6,8-dimethylflavan	4.55	0.0069	up
	Epiafzelechin 3-O-gallate	−3.88	0.0021	down
	Nitenin	−3.33	0.0251	down
	Vitexin 2″-O-p-coumarate	−2.45	0.0075	down
	Myricitrin	−2.57	0.0347	down
	Tricetin	−6.41	0.0021	down
	Tangeritin	4.4	0.0061	up
	Baohuoside 1	6.08	0.0001	up
	Luteolin	−3.88	0.0249	down
	5,7,3′-Trihydroxy-6,4′,5′-trimethoxyflavanone	5.7	0.0002	up
	Barbatoflavan	−2.76	0.0153	down
	8-Hydroxytricetin 7-glucuronide	−1.76	0.0002	down
	6-Methoxyluteolin 7-glucuronide	−1.53	0.0212	down
Flavonols and conjugates	Kaempferol-3-glucoside	−3.2	0.0031	down
	Quercetin	−3.42	0.0039	down
	Miquelianin	−4.81	0.0353	down
	Myricetin	−4.32	0.0015	down
	Ampelopsin 3′-methyl ether 4′-rhamnoside	−4.49	0.0012	down
	Kaempferol 3-(2″-p-coumaryl-alpha-L-arabinopyranoside)	−1.89	0.0773	down
	Quercetin 3-(2″-galloylgalactoside)	−2.35	0.0225	down
	Diffutin	−2.22	0.0013	down
Glycinol derivate	(6αs,11αs)-4-dimethylallyl-3,6α,9-trihydroxypterocarpan	5.79	0.0001	down
Hydrolyzable tannins	3,4-di-o-methylellagic acid	−3.12	0.0023	down
	Gallic acid	−0.47	0.0176	down
	Robinetinidol 3-O-gallate	−6.16	0.0131	down
	3,4,3′-Tri-O-methylellagic acid	−1.27	0.0157	down
	Propyl galiate	4.09	0.0085	up
	Epigallocatechin	−2.79	0.0165	down
	Ellagic acid	−1.64	0.0746	down
	Quinate	2.03	0.1993	ns

Table 1. *Cont.*

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Organic acid	2-methylcitric acid	−1.15	0.0145	down
	Malic acid	−0.52	0.0249	down
	2,4-Dihydroxybenzoic acid	−1.92	0.0356	down
	Citric acid	−0.43	0.1457	down
Isoflavone	Genistein	−1.79	0.0229	down
Isoprenoid	Abscisic acid	−1.69	0.0018	down
Lipids and derivates	Choline	−1.55	0.0037	down
	Monoolein	−3.08	0.0268	down
	Oleic acid	−3.59	0.0312	down
	1-Linoleoyl glycerol	−2.58	0.0068	down
	9(Z),11(E)-Conjugated linoleic acid	−3.81	0.0694	down
	2-methoxyestrone 3-glucosiduronic acid	−2.06	0.0031	down
	Palmitoleic Acid	2.29	0.0049	down
	Ethyl myristate	−1.09	0.1308	ns
	Stearic acid	−0.22	0.3352	down
	5-Hydroxy-2-furoic acid	−0.39	0.0504	down
	2,3-Dihydroxypropyl stearate	1.12	0.0500	up
	Stearidonic acid	−1.15	0.0484	down
Nucleotide/nucleoside	Adenosine	−4.11	0.0059	down
	Cytidine	−3.45	0.0198	down
Phenolic acid and derivates	4-Methoxycinnamic acid	3.31	0.0205	up
	Coniferyl ferulate	7.07	0.0066	up
	2-Protocatechuoyl phloroglucinol carboxylic acid	−4.37	0.0006	down
	Chlorogenic acid	−5.13	0.0021	down
	N-feruloylglycine	−4.94	0.0321	down
	Sinapinic acid	−3.66	0.0134	down
	Scutellarioside II	−4.63	0.0024	down
Phenolic glycosides	Avenein	−5.76	0.0393	down
Piperazines	2-piperazinecarboxamide	−4.47	0.0103	down
Piperidinecarboxylic acid	Nipecotic acid	1.78	0.0532	up
Proanthocyanidins/ tannins	Catechin	−0.37	0.0471	down
	Epicatechin	−0.5	0.0025	down
	Cinnamtannin A2	−3.54	0.0117	down
	Cinnamtannin D2	−3.83	0.0325	down
	Fisetinidol	−0.98	0.0234	down
	4′-methyl-epigallocatechin-3′-glucuronide	−1.58	0.0013	down
	Gallocatechin gallate	−4.37	0.0005	down
	Epicatechin 5-O-beta-D-glucopyranoside-3-benzoate	−2.56	0.0395	down
Pterocarpans	Phaseollin	−1.78	0.0025	down
Quinazolines	Fumiquinazoline D	3.59	0.0127	up
Sesquiterpene	Cnicin	5.13	0.0165	up
	Spicatin	4.18	0.0047	up
Shikimic acid derivate	4-coumaroylshikimic acid	−0.91	0.0266	down
	Chorismic acid	4.65	0.0122	up
Stilbenoid	4-prenyloxyresveratrol	0.87	0.0135	up
	Vanillyl mandelic acid	−2.89	0.0001	down
	Ampelopsin 3′-methyl ether 4′-rhamnoside	−1.8	0.0132	down

Table 1. *Cont.*

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Terpenoids	Genipin	1.32	0.0462	up
	Loganin	−4.01	0.0023	down
Triterpene	Bruceine D	1.56	0.0016	up
Unclassified	5,6,7-Trimethoxy-2-(2,3,4-trimethoxybenzylidene)indan-1-one	4.4	0.0006	up
	(4E)-1,7-Bis(4-hydroxyphenyl)-4-hepten-3-one	−4.11	0.0005	down
	3-[3-(beta-D-Glucopyranosyloxy)-2-methoxyphenyl]propanoic acid	−3.56	0.0007	down
	2-[(5Z)-5-tetradecenyl]cyclobutanone	0.87	0.1403	ns
	1,7-bis(4-hydroxyphenyl)heptan-3-one	−3.74	0.0179	down
	4-(4-Hydroxyphenyl)-2-butanyl 6-O-[(4ξ)-α-L-threo-pentofuranosyl]-β-D-glucopyranoside	−3.48	0.0192	down

Table 2. Compounds from hierarchical cluster analysis of EtOH/H_2O extracts identified as the most relevant differentiators between shells and cuticles, with statistical evaluation and regulation trend.

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Lignan	Dihydroconiferin	3.02	0.0012	up
Amino acids and metabolites	Tryptophan	−2.07	0.0129	down
	Citrulline	−2.7	0.0266	down
	Phenylalanine	−1.62	0.0387	down
Anisoles	Vanillin	−2.09	0.0041	down
Anthracenecarboxylic acids	Carminic acid	−0.98	0.0054	down
Aurone flavonoids	Castillene B	−1.37	0.0276	down
Carbohydrates and derivatives	Saccharic acid	−1.3	0.0507	ns
	Raffinose	−5.12	0.0650	ns
	Trehalose	−4.8	0.0706	ns
Chalcones and dihydrochalcones	Phlorizin	−3.35	0.0015	down
	Brosimacutin H	−3.52	0.0029	down
	Phloretin	−3.32	0.0029	down
	Okanin 3′-glucoside	−3.89	0.0081	down
	2′,3′,4′,6′-Tetramethoxychalcone	0.5	0.0189	up
	Okanin 3,4,3′,4′-tetramethyl ether	6.28	0.0244	up
	Lusianin	−4.97	0.0561	ns
	4-Hydroxy-2′,4′-dimethoxydihydrochalcone	−2.62	0.0848	ns
	Okanin	−0.31	0.3281	ns
Coumarins	Glabrocoumarone A	5.99	0.0001	up
	Scopoletin acetate	−1.84	0.0027	down
Diarylheptanoid	1,7-Bis(4-hydroxyphenyl)-3,5-heptanediol	−1.43	0.0008	down
Dipeptide	Leucylproline	−0.43	0.0322	down
Flavanones and conjugates	Fustin	−2.77	0.0001	down
	Naringin dihydrochalcone	5.78	0.0015	up
	Naringenin	−3.88	0.0090	down
	5,6,7,3′,4′-Pentamethoxyflavanone	7.44	0.0382	up

Table 2. *Cont.*

Class	Name	Shells vs. Cuticles		
		Log_2 Fold Change	***p*-Value**	**Regulation**
Flavans and conjugates	5,7-Dihydroxy-3-methoxy-4′-prenyloxyflavone	4.43	0.0004	up
	Myricitrin	−3.23	0.0011	down
	3,4,7-Trihydroxy-5,4′-dimethoxy-6,8-dimethylflavan	−2.18	0.0054	down
	6-Methoxyluteolin 7-glucuronide	5.78	0.0387	up
	8-Hydroxytricetin 7-glucuronide	−2.33	0.0518	ns
	4′,5,6,7-Tetramethoxyflavanone	−0.08	0.3485	ns
	Tricetin	0.11	0.7318	ns
	Myricetin	−3.09	0.0029	down
	Quercitrin	−4.06	0.0082	down
	Kaempferol-3-glucoside	0.34	0.0199	up
Hydrolyzable tannins	3,4,3′-Tri-O-methylellagic acid	5	0.0057	up
	Quinate	2.82	0.0175	up
	Propyl galiate	−1.6	0.0270	down
	Robinetinidol 3-O-gallate	−4.22	0.0914	ns
	Gallic acid	−0.67	0.1800	ns
Isoflavone	Genistein	0.3	0.0903	up
Isoprenoid	Abscisic acid	−1.11	0.0051	down
Lipids and derivates	2-Arachidonoyl glycerol	−2.57	0.0051	down
	Choline	−2.19	0.0077	down
	Oleic acid	−2.1	0.0921	ns
	9,12,13-Trihydroxy-15-octadecenoic acid	−1.1	0.2899	ns
	13-Hydroperoxylinoleic acid	−0.15	0.2955	ns
Nucleotide/nucleoside	Adenine	−1.73	0.0150	down
	Adenosine	−2.05	0.1434	ns
Organic acid	2,4-Dihydroxybenzoic acid	−1.17	0.0160	down
	Malic acid	−1.13	0.0855	ns
Phenolic acid and derivates	4-Methoxycinnamic acid	7.11	0.0006	up
	Chlorogenic acid	−0.7	0.0145	down
	Coniferyl ferulate	0.57	0.0226	up
	2-Protocatechuoyl phloroglucinol carboxylic acid	2.94	0.0253	up
Proanthocyanidins/ tannins	Catechin	−5.32	0.0073	down
	Epicatechin	−0.52	0.0937	ns
Pterocarpans	Medicocarpin	1.58	0.0040	up
	Phaseollin	0.74	0.0534	ns
Shikimic acid derivate	4-coumaroylshikimic acid	−1.71	0.0660	ns
Unclassified	3-hydroxy-3,4-bis[(4-hydroxy-3-methoxyphenyl)methyl]oxolan-2-one	7.32	0.0002	up
	3-[3-(beta-D-Glucopyranosyloxy)-2-methoxyphenyl]propanoic acid	−3.76	0.0051	down
	(4E)-1,7-Bis(4-hydroxyphenyl)-4-hepten-3-one	−3.84	0.0063	down
	5,6,7-Trimethoxy-2-(2,3,4-trimethoxybenzylidene)indan-1-one	4.52	0.0108	up
	Didodecyl-3,3-thiodipropionate (DLTDP)	−0.35	0.0455	down

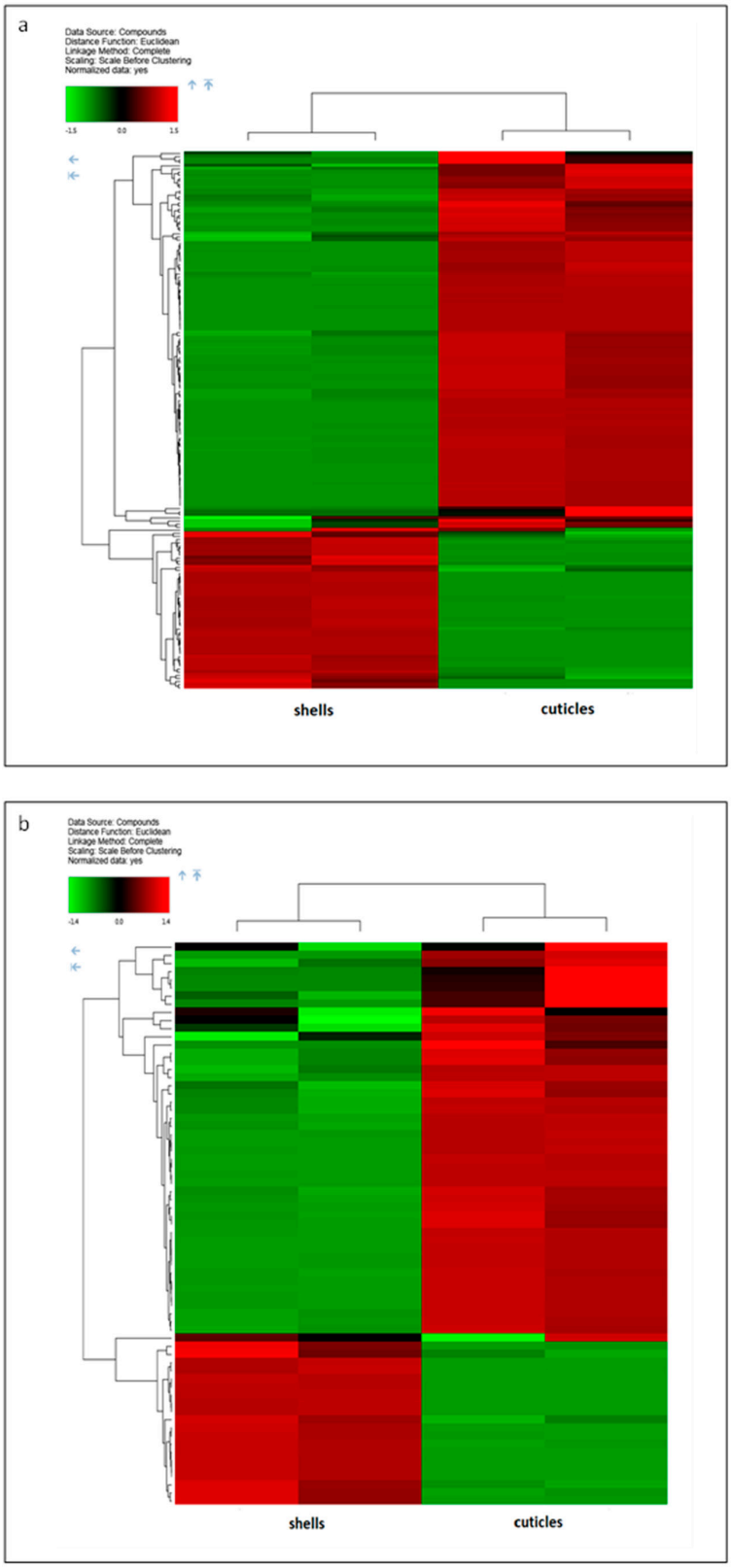

Figure 2. Hierarchical cluster analysis for (**a**) the 165 most abundant species identified in the water/acetone extracts, (**b**) the 67 most abundant species identified in the water/ethanol extracts. The heatmap reflects the differences between the relative amounts through normalized chromatographic peak areas in relation to the sample type; the z-color scale indicates normalized peak area value: red and green indicate more and less abundant, respectively.

The metabolomic profiles of the hazelnut shells and cuticles were significantly different in both extracts. The profile of the acetone/water extract was much more varied than the profile obtained from the ethanol/water solvent. The acetone/water hazelnut shell extract showed a high amount of specific flavonoids, polyphenolic acids, and sesquiterpenes: 5,6,7,3′,4′-pentamethoxyflavanone, coniferyl ferulate, scopoletin acetate, brosimacutin H and A, 5,6,7-Trimethoxy-2H-chromen-2-one, chalconaringenin 2′-xyloside, Hesperetin, 4,2′,6′-Trihydroxy-4′-methoxy-3′,5′-dimethyldihydrochalcone, cnicin, spicatin, etc. On the other hand, the cuticles were characterized by higher levels of amino acids, upregulated compounds from the chalcone group, higher amounts of most hydrolyzable tannins, and a higher relative quantity of the entire proanthocyanidins class that belongs to the group of condensed tannins [27].

The ethanol/water treatment was not as qualitatively efficient as the acetone/water extraction, although the metabolomic profiles were similar. Some important compounds identified in the acetone/water extract (listed above) were also found in the ethanol/water extract. The particular characteristic of ethanol extract was the appearance of oxidated fatty acids (9,12,13-Trihydroxy-15-octadecenoic acid and 13-Hydroperoxylinoleic acid). Moreover, the very intensive signal of didodecyl-3,3-thiodipropionate (DLTDP) found exclusively in the ethanol/water extract of both the shells and cuticles requires further clarification.

The aqueous acetone solution extracted significantly higher amounts of phenolics, with a higher antioxidant capacity than aqueous ethanol. This may be due to the fact that the leftover hazelnut material exhibits a particular matrix due to its rigid and compact inner structure, which slowed down the solvent penetration. Acetone has a lower viscosity than ethanol, which evidently encourages the diffusion of the solvent into the shell cavitation or interior cuticles. This process notably increases the yield of the extracted polyphenolic material.

The cuticles generally presented a better qualitative and quantitative polyphenolic profile than did the shells. Special attention should therefore be paid to these hazelnut by-products in terms of the possible accumulation of antioxidant compounds in the cuticle membranes, which to date, has not been studied in detail.

3.2. Evaluation of Functional Properties

The results of the functional activity assays of the hazelnut cuticles and shells revealed the antioxidant and antimicrobial properties of both by-products. These activities are likely to be related to the high amounts of secondary compounds, especially phenols, condensed tannins, and vitamins [28,29]. Given their nutritional and functional value, by-products of the hazelnut industry could be successfully used as supplements in the field of animal nutrition. In fact, as reported in other studies, the biologically active compounds in these products can also be used to enrich animal diets, thereby improving the nutritional value and quality of meat and milk [30,31].

3.2.1. Antioxidant Activity

The antioxidant properties were evaluated after 6 min of reaction between $ABTS^{\bullet+}$ and the shell or cuticle extracts, based on the timing defined for the radical cation decolorization assay [32,33]. This study was performed using two different types of extraction solvents: water/acetone mixture (40/60) and water/ethanol mixture (50/50).

For both skins (Figure 3a) and shells (Figure 3b), significant differences ($p < 0.05$) were highlighted between the two extraction solvents for each dilution, excluding the first one. For both matrices, the extraction with acetone (40/60) was more effective, leading to an approximately 10% higher extraction of antioxidant compounds, than that obtained from the water/ethanol extraction (Supplementary Table S1). This result also been shown in previous studies, in which the acetone extraction led to a better extraction of both polyphenolic and phytosterol compounds from the plant matrix [34–36]. This is because the extraction of phenolic compounds, which are the main factors responsible for antioxidant activity, is closely related to both the solvent used and the temperature chosen for extraction [36].

In fact, extraction is the main step in the recovery and isolation of bioactive compounds in the sample under analysis. The extraction process is influenced by the nature of the compounds themselves, the methodology used, and the particle size of the sample, as well as the presence of interfering substances [37]. The solvent used plays a key role during the extraction, as it is strongly related to the solubility of the phenolic compounds [12]. For a solvent to be optimal for phenol extraction, it needs to be able to extract the maximum number of phenols and the minimum number of foreign substances [36]. Among the different solvates used for the extraction of phenolic compounds, several studies [35,36] have shown that acetone yields the best results for hazelnut shell and husk waste. This could also be partly explained by the fact that acetone has a low viscosity (0.306 cP at 25 °C), which would increase the concentration gradient, making the extraction more effective [38].

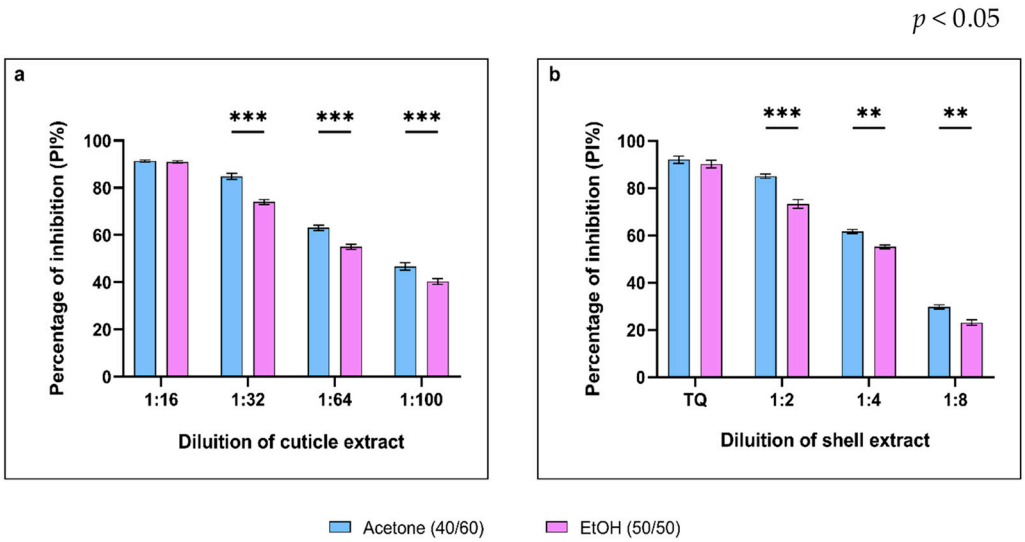

Figure 3. Percentage of inhibition (PI%) of the radical scavenging activity of hazelnut cuticles and shells. (**a**) PI% of radical scavenging activity of hazelnut cuticles at four different concentrations (1:16; 1:32; 1:64: 1:100). (**b**) PI% of radical scavenging activity of hazelnut shells at four different concentrations (as is; 1:2; 1;4; 1:8). Data are shown as means and standard error. Asterisk means (n = 3), with different superscripts, are significantly different; *** $p < 0.0001$, ** $p < 0.05$.

In any case, for both solvent mixtures, a dose-dependent effect was observed. In fact, at the highest concentration, a 90% inhibition of radical scavenging activity was found, which decreased as the concentration decreased, reaching values of around 25% at the lowest concentration tested (Figure 3).

3.2.2. Evaluation of the Growth Inhibitory Activity against Verocytotoxic *Escherichia coli* and Determination of Minimal Inhibitory Concentrations (MICs)

Due to the growing problem of antibiotic resistance, there has been increasing interest in natural antimicrobial compounds. Several studies on hazelnuts and their derivatives have been conducted aimed at evaluating their antimicrobial power [39]. In this study, antimicrobial properties were evaluated considering the inhibitory activity against an *Escherichia coli* strain from our strain library (O138 *E. coli* F18$^+$). The growth inhibition activity against O138 *E. coli* F18$^+$ was evaluated for each extract, considering different dilutions (1:4; 1:8; 1:16; 1:32). Both solvents led to the extraction of bioactive compounds showing antimicrobial activity at dilutions of 1:4, 1:8, and 1:16 (Figures 4 and 5). At a dilution of 1:4, the use of EtOH (50/50) as an extraction solvent highlighted the growth inhibition of *E. coli* for both cuticle and shell matrices (Figure 4a), while at the same dilution, the acetone (40/60) only showed the antimicrobial capacity of the shell extract (Figure 5a). For the next two dilutions, the evaluation of *E. coli* growth inhibition was significant ($p < 0.0001$) for both the cuticle and shell extracts, regardless of whether they

were produced using methanol (50/50) or acetone (40/60), particularly after 3 and 4 h of incubation (Figures 4b,c and 5b,c). In any case, the data obtained from the growth inhibition assay showed that the use of acetone led to better extraction of the bioactive compounds from the cuticles of the hazelnuts, compared to the husks. On the other hand, while allowing for more growth of *E. coli*, the use of ethanol showed more evidence of the antimicrobial capabilities of both the cuticles and the shells (Figure 4). For the latter, in fact, extraction in ethanol led to the detection of a significantly lower cell/mL (log_{10}) value, even for a dilution of 1:32 (Figure 4d). The results obtained from the growth inhibition assay showed that both cuticles and shells revealed an antimicrobial effect, probably related to some of their components, such as tannins and phenols [40,41]. Further studies are needed, however, to identify the correlation between the solvent used for extraction and the extracted matrix in order to more accurately identify the extractive power of different solvents.

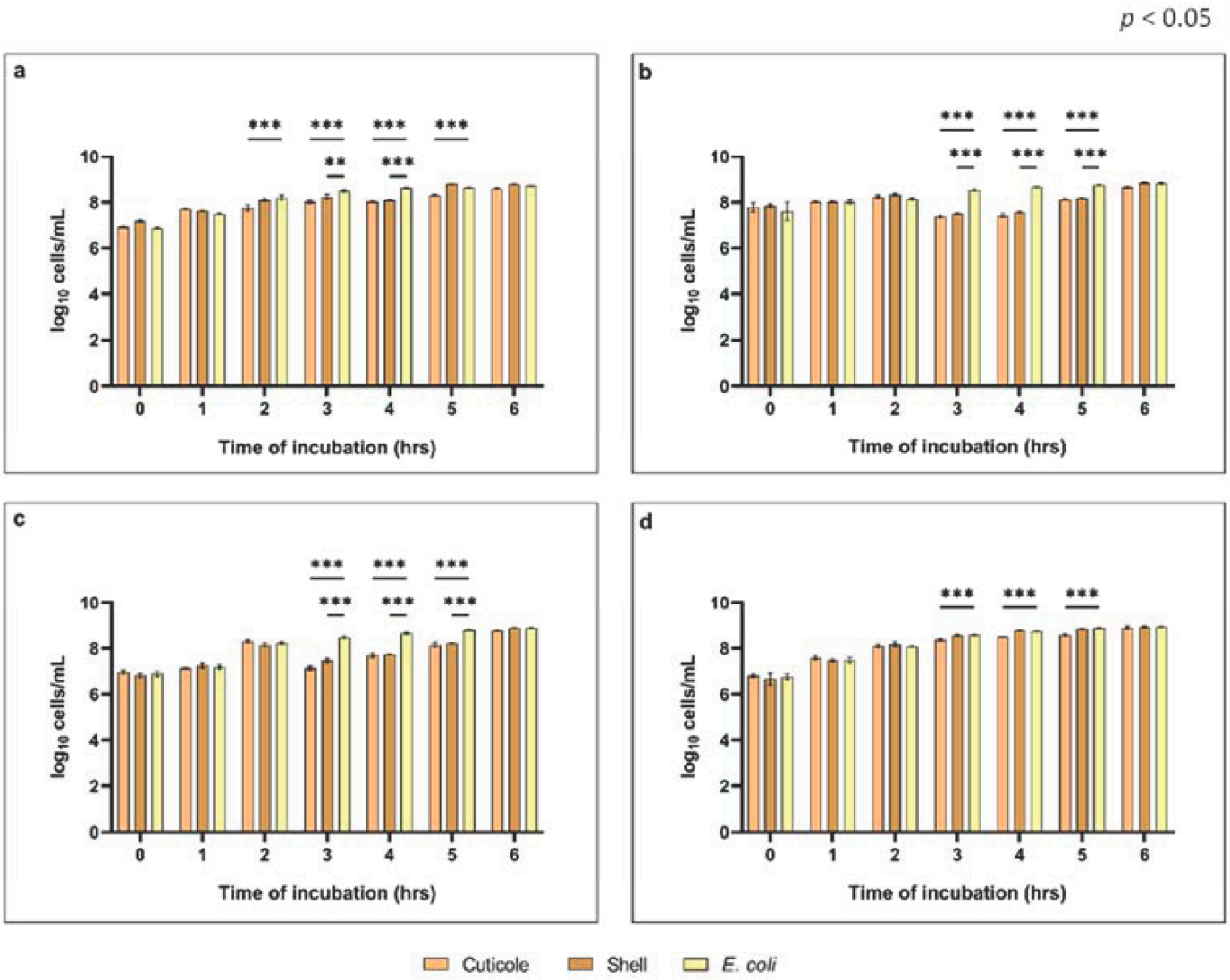

Figure 4. Evaluation of the growth inhibition of hazelnut cuticle and shell extracts with ethanol against *E. coli* F18$^+$: (**a**) growth inhibition assay at a dilution of 1:4; (**b**) growth inhibition assay at a dilution of 1:8; (**c**) growth inhibition assay at a dilution of 1:16; (**d**) growth inhibition assay at a dilution of 1:32. Data are shown as means and standard error. * Asterisk means (n = 3), with different superscripts, are significantly different; *** $p < 0.0001$, ** $p < 0.05$.

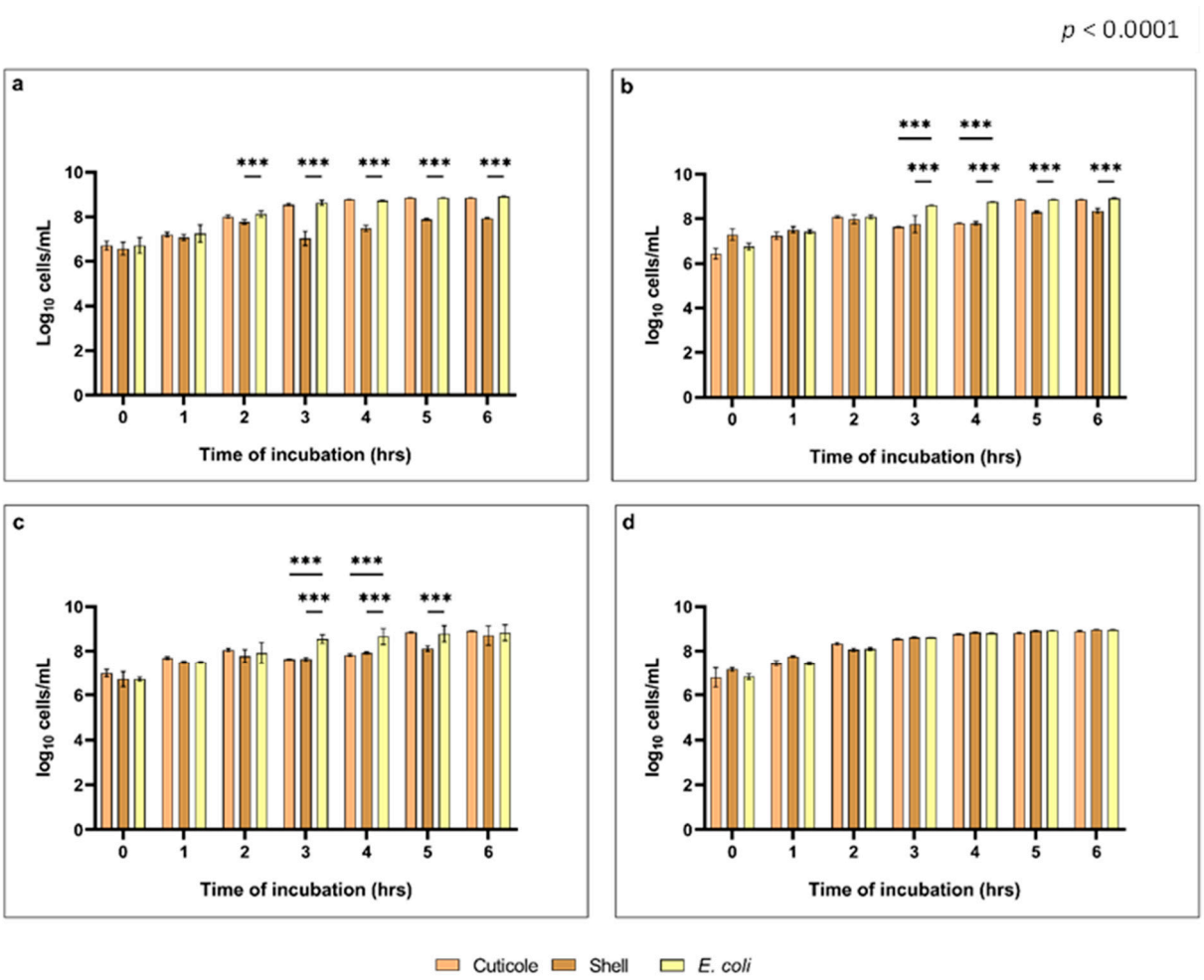

Figure 5. Evaluation of growth inhibition of hazelnut cuticle and shell extracts with acetone against *E. coli* F18$^+$; (**a**) growth inhibition assay at a dilution of 1:4; (**b**) growth inhibition assay at a dilution of 1:8; (**c**) growth inhibition assay at a dilution of 1:16; (**d**) growth inhibition assay at a dilution of 1:32. Data are shown as means and standard error. * Asterisk indicates statistically significant differences among tested compounds (treatment *** $p < 0.0001$).

In addition, a time-dependent effect in relation to inhibitory activity was observed. In fact, the assay revealed that in the first few hours, the inhibition abilities of the tested extracts were comparable to the positive control (*E. coli*). Only after at least 2 h did the difference in inhibition ability between the tested compounds and the positive control become significant, reaching a maximum inhibitory effect at 3 and 4 h of incubation time (Figures 4 and 5). For the ethanol extracts, as shown in Figure 4, regardless of the dilution, after 5 h of incubation, the inhibitory activity was similar between the tested extracts and the positive control (*E. coli*). This suggests the exhaustion of bioactivity due to the degradation of bioactive compounds within the extract tested, or due to the development of bacterial resistance [42]. In any case, it is important to consider the transit time of the feed in the gastrointestinal tract of animals, which, for example in swine lasts for 4 h, thus guaranteeing the effect throughout the digestive tract.

Data obtained by the evaluation of the growth inhibition of *E. coli* were confirmed by the determination of the minimal inhibitory concentrations (MICs). As shown in Table 3, extracts in methanol are found to show an inhibitory capacity at lower concentrations than are the extracts in acetone. The lowest inhibitory concentrations determined reveal an inhibition rate of 96% for the extracts derived from hazelnut cuticles (96.56% for the extract in methanol and 95.57% for the extract in acetone), while the shell extracts show an inhibition rate of 53% (54.86% and 52.21%, respectively).

Table 3. Minimal inhibitory concentration (MIC) of hazelnut cuticles and shells on *E. coli* F18$^+$.

Extraction Solvent	MIC (mg/mL)	
	Cuticle	Shell
Acetone (40/60)	5	10
Methanol (50/50)	1	5

4. Conclusions

Given the growing need to maximize the use of natural resources, waste products are currently playing a prominent role. However, given the wide variety of waste products created within the food industry, it is necessary to evaluate them nutritionally and functionally in order to determine which ones can be efficiently introduced within a circular economy approach, for example, employing them as functional ingredients in both food and feed, but also employing them as useful substances for extending the shelf life of food products. Agro-food waste derived from hazelnuts has been shown to have important potential for multiple applications, given its high nutritional value. In this study metabolomic analyses highlighted the richness of the cuticles and shells in terms of biologically active compounds, thus making them a rich source of natural antioxidants and antimicrobial compounds. The cuticles presented a qualitatively and quantitatively higher polyphenolic profile than did the shells. This was also reflected in the in vitro evaluation of both their antioxidant and antimicrobial activities. In fact, although both cuticles and shells showed both antioxidant and antimicrobial capacities, these was significantly greater in the cuticles than in the shells. Special attention should thus be paid to these hazelnut by-products in relation to the possible accumulation of antioxidant compounds in the cuticle membrane. This study also highlights the role of the extraction solvent used. The aqueous acetone solution extracted significantly higher amounts of phenolics, with higher antioxidant and antimicrobial capacities, than did the aqueous ethanol. Additional in vitro studies will be required to further investigate the use of different extraction solvents. Moreover, preliminary studies should also investigate their possible in vivo use for animal and human nutrition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15043268/s1, Table S1: Percentage inhibition of the radical scavenging activity of hazelnut cuticles and shells.

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