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Bio-Recycling Hazelnut Shells to Improve Antioxidant Properties of *Lentinus tigrinus* Sporophore

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Abstract: *Lentinus tigrinus* is a wood-decay fungus known for its nutritional, culinary, and medicinal benefits. It contains bioactive compounds like polyphenols, terpenes, and flavonoids that exhibit antioxidant, anti-microbial, and anti-inflammatory effects. These natural antioxidants are increasingly recognized for their potential to prevent oxidative damage linked to aging and chronic diseases. This study investigates the antioxidant activity of hydroalcoholic extracts obtained from *L. tigrinus* sporophores cultivated on hazelnut shells (Lt1_HS), a waste material rich in phenolic compounds, and sporophores cultivated on sawdust (Lt1_S). Bioactivity tests, including DPPH, TBARS, MTT, and DCFH-DA assays, were performed to assess the hydroalcoholic extracts' efficiency. The results showed that all the extracts contained various bioactive compounds, primarily polyphenols. Notably, the caffeoylquinic acids present in HS and Lt1_HS are linked to anti-peroxidant effects. Biological analyses demonstrated that the Lt1_HS extract has higher anti-peroxidant activity (IC_{50} 0.77 ± 0.01 mg/mL) compared to Lt1_S (IC_{50} 1.36 ± 0.01 mg/mL) and reduces the accumulation of reactive oxygen species in HaCaT cells by 80%. However, the specific bioactive compounds responsible for these antioxidant effects are still unclear, and further analysis will be conducted. Additionally, this study promotes recycling hazelnut shells as a valuable substrate for fungal cultivation, supporting sustainable waste management.

Keywords: medicinal mushrooms; biological effects; cultivation; waste substrate; bioactive compounds

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1. Introduction

Lentinus tigrinus (Bull.) Fr. is a wood-decay fungus (WDF), belonging to the white-rot fungi group, growing as a saprophytic organism, solitarily or in clusters, on logs and stumps of mainly *Salix* spp. and *Populus* spp. [1]. It is known in literature for its role in breaking down lignocellulosic material, by the production of laccases, peroxidases and other lignocellulosic enzymes [2,3]. *L. tigrinus* is largely spread from tropical to temperate and boreal regions; it is reported in 17 countries belonging to different regions, including Central Asia, South America, Europe, and Africa, generally fruiting between the months of May and October [2,4]. Morphologically, *L. tigrinus* is recognized by its solid, firm, round pileus adorned with light brown to black scales and toothed edges; the stipe exhibits a

white, scaly appearance. The hymenophore features lamellae with nuances of white or white-cream, and basidiospores range from ellipsoidal to cylindrical [5]. This WDF is an edible mushroom with a pleasant aroma, and unique taste, suitable for gourmet dishes; it is also known for its officinal properties [6–8]. The study of Dulay et al. [9] specifically described the nutritional composition. The pileus of *L. tigrinus* contained higher amounts of protein (25.9%), fat (2.1%), ash (7.4%), and energetic value (142.1 kcal/100 g) than the stipe, while the stipe contained higher amounts of total carbohydrates (67.7%) than the pileus. As concerns polysaccharides, fruiting bodies of *L. tigrinus* contained soluble polysaccharides ranging from 30.1% (in the pileus) to 38.3% (in the stipe) [9,10]. In addition, *L. tigrinus* contains different bioactive compounds such as polyphenols, terpenes, flavonoids, and polyketides [11,12]. All these molecules exhibit different biological effects, such as antioxidant, anti-microbial, anti-inflammatory, anti-obesity, and hypoglycemic effects [13–15]. Moreover, these compounds help to prevent oxidative stress, a pathological human process linked mainly to aging and chronic diseases [16]. Oxidative stress is caused by an imbalance between the production of oxygen-reactive species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), and the failure of the antioxidant defenses. ROS are highly reactive molecules, typically neutralized by enzymes that protect cellular health [17]. However, when this natural defense mechanism fails, ROS increase, which can cause significant cellular damage, contributing to chronic diseases such as cancer, cardiovascular disease, neurodegenerative disorders, and age-related diseases [18,19]. For this reason, nowadays, it is crucial to find bioactive compounds extracted from natural products that can inhibit the development of ROS. Biological tests investigating antioxidant activity are crucial for understanding how different substances can protect cells and tissues from oxidative damage. Today, many tests can be used to evaluate the antioxidant efficiency of an extract; among these, DPPH (1,1-diphenyl-2-picryl-hydrazil) and DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) are extensively used [20–22]. It is mandatory to underline that antioxidant testing helps in developing functional foods and nutraceuticals designed to enhance health and wellness, support preventive healthcare, and guide the development of therapeutic agents aimed at reducing oxidative damage in the body [23]. Foods rich in antioxidant compounds are hazelnuts, mainly rich in polyphenols, and mushrooms, such as *Hericium erinaceus*, that are rich in antioxidant compounds, such as flavonoids, vitamin E, and ergothioneine [24]. Polyphenols are known in the literature to neutralize ROS and free radicals by donating an electron or hydrogen atom. In addition to their antioxidant and radical scavenging capacity, polyphenols are also known as metal chelators [25]. Researchers are increasingly focusing on medicinal mushrooms to investigate their potential preventive effects against oxidative stress, as well as to explore new opportunities for dietary supplements and nutraceutical products [26,27]. Additionally, waste materials represent a promising yet underutilized source of bioactive molecules. For example, hazelnut shells (HS) contain a high concentration of phenolic compounds and other bioactive molecules [28,29]. Unfortunately, due to its high lignin content, this waste is not recycled but used as fuel or bio-pellets [30,31]. So, hazelnut shells should no longer be treated solely as fuel; they are a valuable resource that can be utilized as a substrate for fungal cultivation too [32].

The aim of this study was to evaluate whether the antioxidant activity of *L. tigrinus* sporophores grown on hazelnut shells was better than those grown on sawdust. To compare sporophores' hydroalcoholic extracts, bioactivity tests and biological assays were performed. Furthermore, this project aimed to identify molecules responsible for antioxidant effects, focusing on phenolic compounds. In conclusion, this study enhances the potential of *L. tigrinus* grown on hazelnut shells as innovative food products useful for

decreasing oxidative stress, and at the same time, it points out a good practice for recycling this recalcitrant waste.

2. Material and Methods

2.1. Lentinus tigrinus Strain

The sporophore of *L. tigrinus* was collected in San Genesio ed Uniti (Pavia, Italy), and the mycelium was isolated in pure culture in Petri dishes containing 2%$_{w/v}$ Malt Extract Agar (MEA). The strain was identified by molecular analysis. The total genomic DNA was extracted from lyophilized mycelium using Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany). The primer pair ITS1-ITS4 was used to amplify the Internal Transcribed Spacer (ITS) region by Polymerase Chain Reaction (PCR) as reported by Girometta et al. [33]. The strain (Lt1) was maintained in the Fungal Research Culture Collection (MicUNIPV) at the Department of Earth and Environmental Sciences (University of Pavia, Pavia, Italy).

2.2. Cultivation of L. tigrinus on Sawdust (S) and on Hazelnut Shells (HS)

The protocol used for *L. tigrinus* sporophore cultivation both on sawdust (S) and on hazelnut shells (HS) followed the guidelines proposed by Lechner and Albertò [7] and Stamets [34]. Spawn production occurred in an inoculated jar (SacO2, Deinze, Belgium) containing 150 g of sterilized millet. Sterile portions of Lt1 mycelium grown on MEA were transferred to the jar containing the cereal, and 10 mL of distilled water was added. The jar was closed with a filter cap and stored at 25 °C in the dark until the complete millet colonization (about 15 days). The cultivation of Lt1 sporophores was conducted in parallel on sawdust, a substrate most like natural conditions, and on hazelnut shells (to enhance this recalcitrant agricultural waste). Each substrate was inserted in autoclavable polypropylene (PP) transparent filter bags (20 × 30, SacO2, Deinze, Belgium) and sterilized; this process was repeated twice. Subsequently, bags were inoculated with 10% spawn, sealed, and stored at 25 °C in the dark, until the complete colonization of the substrate. After 1 month, the substrate was completely colonized by the mycelium, and the bags were moved to the climatic chamber, at 19–24 °C, 85% humidity, and 8 h of light. The collected sporophores were weighed fresh and then subjected to slow drying at low temperature (T = 35 °C). After drying, the sporophores were weighed again and stored in the freezer for further analysis.

Substrate S was composed of 75% oak sawdust, 10% wheat flour, 10% oatmeal, and finally 5% $CaCO_3$ (Table 1). For each bag, 150 g of vegetable substrate and 150 mL of distilled water were added, resulting in a final weight of 300 g. A total of 10 filter bags were prepared. The *L. tigrinus* sporophore grown on sawdust was referred to as Lt1_S.

Table 1. Composition of the two growing substrates, sawdust and hazelnut shells, for *L. tigrinus* sporophore cultivation.

Composition (%)	Code	Total Dry Weight of Substrates (g)
75% oak sawdust, 10% wheat flour, 10% oatmeal 5% $CaCO_3$	S	1500 g
100% Hazelnut shells	HS	3000 g

Hazelnut shells were provided by the farm "Le Nocciole di Greta" located in Bascapè, Pavia. Initially, a meticulous selection of shells occurred, and subsequently, they were ground using a jaw foil until a "coarse" grain size was obtained. The substrate used for the cultivation of Lt1 was composed of 100 g of dry weight of hazelnut shells (Table 1). A total of 30 filter bags were prepared. The collected sporophores were weighed fresh and then

subjected to slow drying at low temperature (T = 35 °C). After drying, the sporophores were weighed again and stored in the freezer for further analysis. The *L. tigrinus* sporophore grown on hazelnut shells was referred to as Lt1_HS.

According to many authors [35–37], the production of sporophores was evaluated by calculating the biological efficiency (BE):

$$\mathrm{BE} = \frac{\text{total fresh weight of sporophores (g)}}{\text{total dry weight of substrates (g)}} \times 100$$

2.3. Biological Activity

2.3.1. Preparation of the Hydroalcoholic Extracts

The extracts from *L. tigrinus* sporophores were obtained through a dynamic maceration process. Briefly, 1.5 g of dried sample was weighed and extracted with EtOH/H_2O (80:20, *v*/*v*, 30 mL), with a magnetic agitation, and left to macerate for one hour at room temperature on a stirring plate. Afterward, the extract was filtered through a paper filter (Whatman n° 4, Merck KGaA, Darmstadt, Germany), the residue was recovered, and the same process was repeated for a re-extraction purpose. The liquid part was filtered while the residue was removed, and the extract was concentrated using a rotary vacuum evaporator (≤40 °C, 100 rpm, Büchi, R-210, Flawil, Switzerland) to promote the complete evaporation of the ethanol. The aqueous phase was then lyophilized (FeeeZone 4.5, Labconco, Kansas City, MO, USA).

2.3.2. DPPH$^{\bullet}$ (1,1-Diphenyl-2-picryl-hydrazil) Radical Scavenging Assay

The DPPH assay was conducted according to the procedure described by Souilem et al. [38]. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Santa Clara, CA, USA). The reaction mixture in each of the 48 wells consisted of 30 µL of the successive dilutions from the mother solution from the extracts (20 mg/mL) and 270 µL of the methanolic solution of DPPH radicals (6×10^5 mol/L). The mixture was left to stand for 60 min in the dark for the reaction to occur. The reduction of the DPPH radical was determined by measuring the absorbance at a wavelength of 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation: % RSA = [(ADPPH − AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution (control). The results were expressed as IC_{50} values mg/mL. In this way, it was possible to calculate the extract concentration required to scavenge the free radical and to find the antioxidant activity of 50% of the extract. Ascorbic acid (Farmacia Fapa, Pavia, Italy) was used as positive control in the concentration range of 500 µM to 1 µM.

2.3.3. TBARS (Thiobarbituric Acid Reactive Substances) Assay

The protocol used for the analysis was the same as that reported in [38]. Mushroom hydroethanolic extract was re-dissolved in water and subjected to dilutions from 20 mg/mL to 0.625 mg/mL. Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 *w*/*v* solution which was centrifuged at 3000× *g* for 10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the sample solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37.5 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w*/*v*, 500 µL), followed by thiobarbituric acid (TBA, 2%, *w*/*v*, 380 µL), and the mixture was then incubated at 80 °C for 20 min. Then, the samples were centrifugated at 3000× *g* for 10 min. The color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was

measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = [(A − B)/A] × 100%, where A and B are the absorbance of the control and the sample solution, respectively. The results were expressed as IC_{50} values mg/mL. In this way, the extract concentration required to keep 50% of lipid peroxidation was able to be calculated. Trolox was used (Sigma Chemical Co., St. Louis, MO, USA) as a positive control.

2.4. In Vitro Biological Assay

2.4.1. Cell Culture—HaCaT

The HaCaT cell line is a spontaneously immortalized human keratinocyte line purchased from Cell Line Service (Eppelheim, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cls) supplemented with 10% fetal bovine serum (FBS, Gibco—Life Technologies Europe BV, Bleiswijk, The Netherlands) and 1% penicillin–streptomycin antibiotics (Gibco—Life Technologies Europe BV, Bleiswijk, The Netherlands). HaCaT cells were grown in a 37 °C incubator with a humidified atmosphere and 5% CO_2. The medium was changed every 2–3 days, and after reaching ~90% confluence, the cells were detached with TrypLE Express (Gibco—Life Technologies Europe BV, Bleiswijk, The Netherlands) and resuspended in fresh medium.

2.4.2. Cell Viability Determination Using the MTT Assay

The protocol used for the analysis was the same as that reported by Brandalise et al. [39].

Human skin cell (HaCaT) viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay at 24–48 h.

In summary, cells were cultured in a 25 cm^2 flask and then seeded into a 96-well plate at a density of 10,000 cells per well (0.2 mL per well). The plates were incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2. The following day, the culture medium was replaced with a fresh medium supplemented with various extract concentrations (ranging from 0.0125 to 1 mg/mL). For the control condition, cells were incubated with the culture medium alone. After 24 h of exposure, MTT solution (20 µL per well) was added in darkness, and the plates were subsequently incubated for approximately three hours at 37 °C. Then, the formazan crystals were solubilized using DMSO. Next, the quantification was conducted by measuring the absorbance of the samples at 550 nm using the ELx808TM Absorbance Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The same steps were repeated after 48 h of treatment. The data were expressed as the percentage of cell viability, and IC_{25} was calculated after 24 to 48 h of treatment. In this way, it was possible to calculate the extract concentration required to inhibit 25% of cell viability.

The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = [(A − B)/A] × 100%, where A and B are the absorbance of the control and the sample solution, respectively. The results were expressed as IC_{25} values mg/mL. In this way, it was possible to calculate the extract concentration that inhibits 25% of cell viability.

2.4.3. Trypan Blue Assay

The protocol used for the analysis was the same as that reported by Giammello et al. [40]. For the Trypan Blue assay, 250,000 HaCaT cells were seeded into each well of a 6-well plate and incubated overnight to allow attachment. Then, the cells were treated with IC_{25} concentrations of extracts, previously determined by an MTT assay. After 24 h of treatment, the cells were washed with 1× phosphate-buffered saline (PBS) and detached using TrypLE™ Select Enzyme (1×) (Gibco) (Life Technologies Europe BV, Bleiswijk, The Netherlands) for 10 min.

The cell suspension was centrifuged at 800 rpm for 7 min, the supernatant was carefully removed, and the cell pellet was resuspended in 1 mL of fresh medium. To assess cell

viability, 10 µL of the resuspended cells was mixed with 10 µL of 0.4% Trypan Blue solution. The mixture was loaded into the chamber of the Countess™ 3 Automated Cell Counter (Thermo Fisher Scientifics Inc., Life Technologies Corporation, Washington, DC, USA) for cell counting. Viable cells (unstained) and non-viable cells (stained) were quantified, and the percentage of viable cells was calculated.

2.4.4. DCFH–DA (2′,7′-Dichlorodihydrofluorescein Diacetate) Assay

The protocol used for the analysis was the same as that reported by Kim and Xue [41]. The fluorogenic dye DCFH-DA was used to assess the ability of the Lt1_S and Lt1_HS extracts to reduce intracellular levels of reactive oxygen species in HaCaT cells. Once passively diffused into cells, DCFH-DA is deacetylated by intracellular esterases to produce a non-fluorescent compound, which is subsequently oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF), which can be quantified by fluorescence detection. In total, 150,000 HaCaT cells were seeded on coverslips placed in 6-well plates incubated overnight, at 37 °C with 5% CO_2, to allow for cell adhesion. Thereafter, the cells were treated with IC_{25} concentrations of the extracts, calculated at 24 h.

The next day, two experimental conditions were established: a basal condition and an oxidative stress condition. For the basal condition, cells were incubated with 10 µM DCFH-DA diluted in a medium for 30 min at 37 °C, in the dark. For the oxidative stress condition, cells were first exposed to 500 µM hydrogen peroxide (H_2O_2) for 30 min to induce oxidative stress. Following H_2O_2 treatment, cells were washed with PBS and then incubated with 10 µM DCFH-DA as described above. After incubation with DCFH-DA for 30 min in both basal and H_2O_2-induced conditions, the cells were washed twice with 1× PBS to remove excess DCFH-DA. Fluorescence intensity, which correlates with ROS production, was measured using an Olympus BX51 optical microscope (Olympus Italia S.r.l., Segrate, Italy) equipped with a 100 W mercury lamp at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Images were recorded with an Olympus MagnaFire camera, and the results were processed with Olympus Cell F software, version 3.1. In both conditions, fluorescence intensity correlates with ROS production, and the antioxidant effects of Lt1_S and Lt1_HS extracts were evaluated by comparing the fluorescence.

2.5. Characterization of the Extracts: Phenolic Compound Analysis by HPLC-DAD-(ESI)MS/MS

Extracts were analyzed by a High-Performance Liquid Chromatography (HPLC—Dionex UltiMate 3000 series, Thermo Fisher Scientific—San Jose, CA, USA) instrument equipped with a diode array detector (DAD) and connected in series to an Ion Trap mass spectrometer (MS, LinearIon Trap LTQ XL, Thermo Fisher Scientific—San Jose, CA, USA). Compounds were separated in a Spherisorb S3 ODS-2 C18 column (3 µm, 4.6 × 150 mm, Waters—Milford, MA, USA) kept at 35 °C, under a gradient of 0.1% (*v*/*v*) formic acid in ultrapure water (A) and acetonitrile (B). UV-Vis spectra were acquired between 180 and 700 nm, and the chromatograms were processed at 280, 330, and 370 nm for the different classes of phenolic compounds. The HPLC eluate was analyzed by high-resolution, tandem mass spectrometry, and the compounds were ionized using an electrospray ion source (ESI) source operating in negative mode. Full MS and MS/MS spectra were acquired in the range from 110 to 1800 charge-to-mass ratio (*m*/*z*). The full MS scan and MS n scans were acquired by applying a collision energy of 35 (arbitrary units). When needed, a dynamic exclusion strategy was employed. Data acquisition and processing were conducted with the Xcalibur® software 4.3 (Thermo Fisher Scientific, San Jose, CA, USA). For compound identification, the elution order on the C 18 column and characteristics of the UV-Vis and mass spectra (molecular ion ($[M-H]^-$), and MS/MS fragments) were interpreted and compared with standards when available, the literature data, and the libraries available. Quantification

was performed using 9-point external calibration curves of authentic standards. The results of phenolic compounds were expressed as mg per g of freeze-dried extract ($mg \cdot g^{-1}$, dry weight, dw).

2.6. Statistical Analyses

Statistical analyses were performed with GraphPad Prism 10.2 software (GraphPad Software Inc., La Jolla, CA, USA). Data are reported as mean ± standard error of the mean (SEM). To verify statistically significant differences, we used a one-way analysis of variance (ANOVA) followed by Bonferroni's test. The differences are considered statistically significant for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

3. Results

3.1. Production of Sporophores Grown on Sawdust and Hazelnut Shells

The identity of *L. tigrinus* strain (Lt1) was confirmed by molecular analysis. The accession number of the ITS sequences of this Italian strain has been deposited in GenBank [42] as PQ136439. Sporophores were cultivated on sawdust (Lt1_S) or hazelnut shells (Lt1_HS) as described in Section 2 (Figure 1). *L. tigrinus* sporophores grown on sawdust (Lt1_S) were collected (fresh weight total yield 621.8 g), giving 58.81 g dry weight, with a biological efficiency (BE) of 40.6% (Table 2). *L. tigrinus* sporophores grown on hazelnut shells (Lt1_HS) were collected (fresh weight total yield 432 g), giving 34.82 g dry weight, with a BE of 14.5% (Table 2). The BE was statistically different between the two samples (*p*-value: **** 0.0001).

Figure 1. *L. tigrinus* sporophore cultivation on sawdust (Lt1_S) and hazelnut shells (Lt1_HS). Photos by A. Desiderio.

Table 2. Values are expressed as: total fresh weight, total dried weight, and mean biological efficiency ± SEM of *L. tigrinus* sporophores grown in different substrates. The comparison between the BE mean values of Lt1_S and Lt1_HS is statistically significant. Student's *t*-test was performed using Prism GraphPad 10.2 software; *p*-value: *p* **** < 0.0001.

Complete Name of the Sample	Code Samples	Fresh Weight (g)	Dry Weight (g)	Mean BE%
L. tigrinus sporophores cultivated on sawdust	Lt1_S	621.8	58.81	40.6 ± 6.14 ****
L. tigrinus sporophores cultivated on hazelnut shells	Lt1_HS	432	34.82	14.5 ± 1.82

3.2. Similar Antioxidant, but Different Anti-Peroxidant Effect of Lt1_S and Lt1_HS Hydroalcoholic Extracts

Hydroalcoholic extracts of the Lt1_S and Lt1_HS and of the hazelnut shells (HS) were prepared as described (see Section 2). Antioxidant and anti-peroxidant effects of the three extracts were evaluated using DPPH and TBARS assays, respectively.

The results obtained from the DPPH assay showed that all the extracts exhibited an antioxidant effect. Specifically, the HS extract exhibited an IC_{50} of 0.098 ± 0.011 mg/mL, which is in line with previously published data by Esposito et al. [30]. The hydroalcoholic extracts of sporophores grown on either substrate displayed a similar antioxidant activity, lower than the HS sample (IC_{50} 0.339 ± 0.009 mg/mL and 0.352 ± 0.001 mg/mL for Lt1-S and Lt1-HS, respectively; $p < 0.0001$ for both the samples compared to HS) (Figure 2A). The difference between the antioxidant activity of the two sporophores (Lt1_S and Lt1_HS) was not statistically significant (Figure 2A). This result suggests that the two substrates, sawdust and hazelnut, had a similar effect on the antioxidant properties of *L. tigrinus* in the DPPH assay.

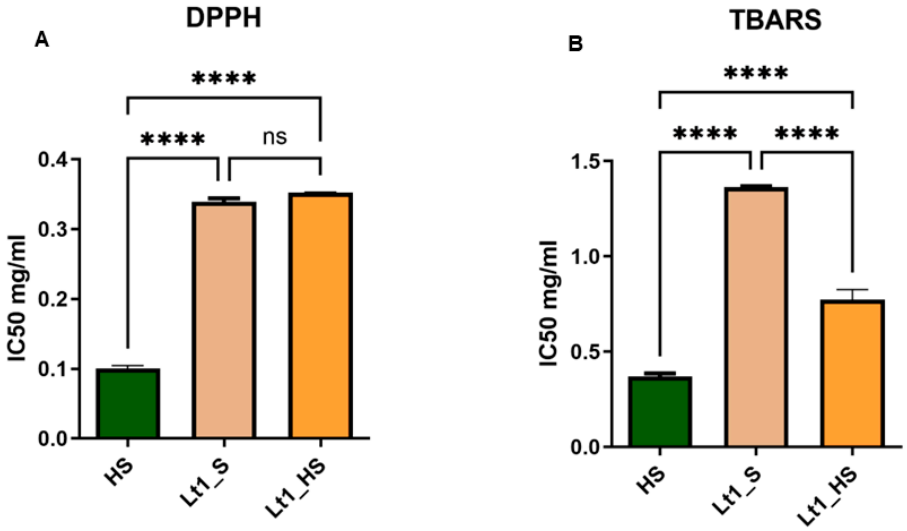

Figure 2. The Lt1_S and Lt1_HS extracts have similar antioxidant properties but differing anti-peroxidant effects. IC_{50} values (mg/mL) obtained with DPPH (**A**) and TBARS (**B**) for the three extracts: (HS) hazelnut shell, (Lt1_S) *L. tigrinus* sporophore cultivated on sawdust, and (Lt1_HS) *L. tigrinus* sporophore cultivated on hazelnut shells. One-way ANOVA was performed using Prism GraphPad 10.2 software; *p*-value: p **** < 0.0001; ns, not statistically significant.

On the contrary, the TBARS assay showed different IC_{50} values among the three samples. Higher anti-peroxidant activity was present in the HS extract (IC_{50} 0.36 ± 0.04 mg/mL), but between the two sporophores, the Lt1_HS extract displayed a higher anti-peroxidant activity (IC_{50} 0.77 ± 0.01 mg/mL) compared to Lt1_S extract (IC_{50} 1.36 ± 0.01 mg/mL) (Figure 2B). The extract HS is statistically significantly different compared to Lt1_S and Lt1_HS (Figure 2B), but interestingly, the Lt1_HS extract exhibited a twice higher anti-peroxidant activity compared to Lt1_S. From the results of the one-way ANOVA analysis, a statistically significant difference of $p < 0.0001$ between Lt1_S and Lt1_HS was exhibited.

3.3. Lt1_S and Lt1_HS Extracts' Cell Viability

To evaluate the antioxidant effect in a biological model, we studied the antioxidant effects in a cell culture. In particular, because dermal skin cells are a well-established model for studying oxidative stress, a spontaneously immortalized human keratinocyte line was chosen, the dermal skin cell line (HaCaT). First, we tested the suitable dose of the extract that did not cause cytotoxicity in HaCaT cells, utilizing the MTT assay. HaCaT cells

were exposed for 24 h to different concentrations of Lt1_S and Lt1_HS extracts, ranging from 12.5 µg/mL to 1 mg/mL, and cell viability was assessed (Figure 3). All data were statistically significant except the control compared with the following concentrations: 1.25×10^{-2} (Lt1_S and Lt1_HS) and 6.25×10^{-2} of Lt1_HS.

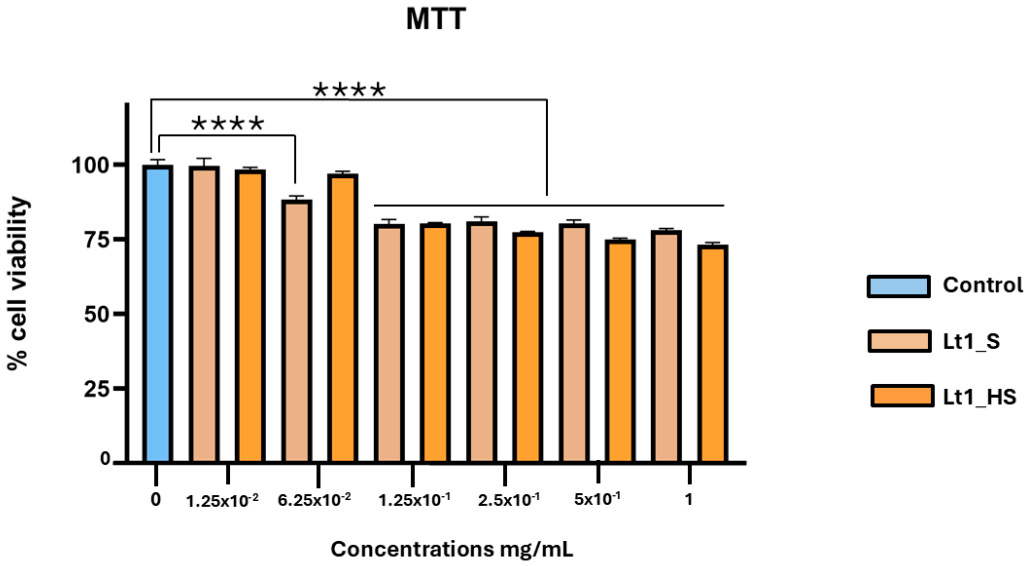

Figure 3. Cell viability of HaCaT cells exposed to different concentrations of Lt1_S (brown) and Lt1_HS extract (orange) for 24 h (mean ± SEM, n = 4 replicates), compared with the control (blue). One-way ANOVA was performed using Prism GraphPad 10.2 software; *p*-value: *p* **** < 0.0001.

IC_{25} was calculated to evaluate the concentration at which the extract inhibits cell viability of 25% after 24 h of exposure. Lt1_S did not exhibit a cell viability of 25% at the highest concentration tested (1 mg/mL), whereas the Lt1_HS extract inhibits cell viability of 25% at a concentration of 0.531 ± 0.015 mg/mL.

To confirm the MTT assay results, we performed the Trypan Blue assay. This assay allows for determining the percentage of live cells after a specific treatment. Based on the MTT results, the IC_{25} concentrations of Lt1_S (1 mg/mL) and Lt1_HS (0.531 ± 0.01 mg/mL) were tested after 24 h of exposure. The results showed that the viability of the HaCaT cells after Lt1_S and Lt1_HS extract exposure was not statistically significant and was equal to 75% at the concentrations tested (Figure 4). The results for Lt1_S and Lt1_HS extracts were similar, and no difference was present between the two extracts.

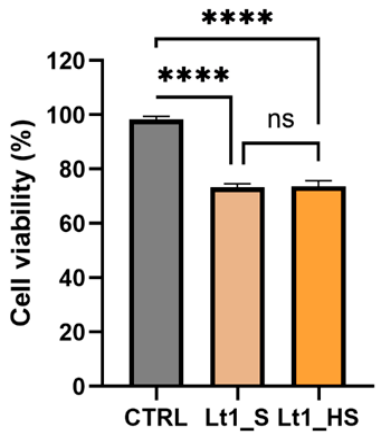

Figure 4. Cell viability percentages of HaCaT cells after 24 h treatments with the Lt1_S and Lt1_HS sporophore extracts. The two extracts showed no statistically significant difference. One-way ANOVA was performed using Prism GraphPad 10 software; *p*-value: *p* **** < 0.0001; ns, not statistically significant.

3.4. Hazelnut Shells Enriched the Antioxidant Activity of L. tigrinus Sporophore in Dermal Skin Cells

The DCFH-DA assay measures reactive oxygen species (ROS) in cells. DCFH-DA converts to a fluorescent compound (DCF) upon reacting with ROS, and the fluorescence intensity indicates oxidative stress levels. Based on the MTT and Trypan Blue results at 24 h, the IC_{25} concentrations of Lt1_S and Lt1_HS extracts were tested in HaCaT cells. Cells in the control condition (CTRL) displayed basal oxidative stress without any exposure.

The DCFH-DA analysis revealed that the Lt1_HS extract significantly inhibited oxidative stress under basal conditions, achieving an 80% reduction compared to the control. In contrast, the Lt1_S extract decreased the oxidative stress by about 30% compared to the control. The two extracts decrease the oxidative stress in a statistically significant way compared to the control ($p < 0.0001$). Between the two extracts, Lt1_HS showed a significant decrease compared to Lt1_S ($p < 0.0001$), (Figure 5A). The higher antioxidant effect of Lt1_HS compared to Lt1_S was confirmed in an induced oxidative stress condition. HaCaT cells were exposed to hydrogen peroxide which doubled the ROS production compared to basal condition. It should be noted that Lt1_HS extract decreased the oxidative stress from 200% to about 30%, reaching an oxidative stress value lower than the control condition. In comparison, Lt1_S extract reduced oxidative stress to 100%, an oxidative-stress-value-like control condition.

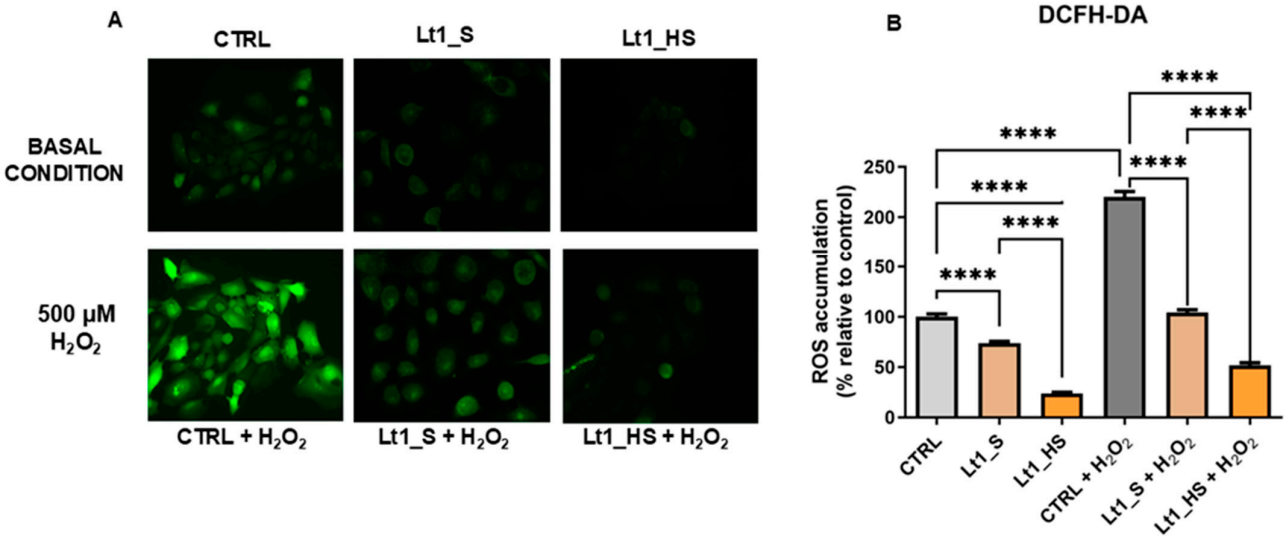

Figure 5. Different antioxidant effects of Lt1_S and Lt1_HS on HaCaT cells. (**A**) The upper panels show the basal conditions. The lower panels show the induced oxidative stress condition. (**B**) The histogram summarizes the data in the two experimental conditions. One-way ANOVA was performed using Prism GraphPad 10.2 software; *p*-value: p **** < 0.0001.

The obtained results indicated that Lt1_HS extract displayed a very high antioxidant activity both in basal and induced oxidative stress conditions, with a very high statistically significant difference compared to Lt1_S (Figure 5B). Therefore, the substrate composed of hazelnut shells enriched the antioxidant activity of *L. tigrinus* sporophore in dermal skin cells.

3.5. Characterization of Phenolic Compound Profiles

The higher antioxidant activity of Lt1_HS could be due to the presence of phenolic compounds that are present in hazelnut shells. We hypothesize that polyphenolic compounds could be absorbed by the *L. tigrinus* sporophore during its growth. The richness of polyphenols in hazelnut shells can be attributed to their natural role in the plant's defense system. Polyphenols act as antioxidants and protective agents, shielding the hazelnut

kernel from oxidative damage, microbial attacks, and environmental stressors [43]. To test the hypothesis of the presence of phenolic compounds, HPLC-DAD chromatograms were performed on hydroalcoholic extracts HS, Lt1_S, and Lt1_HS (Figure 6). The identified molecules were reported (Tables 3–5) for each sample.

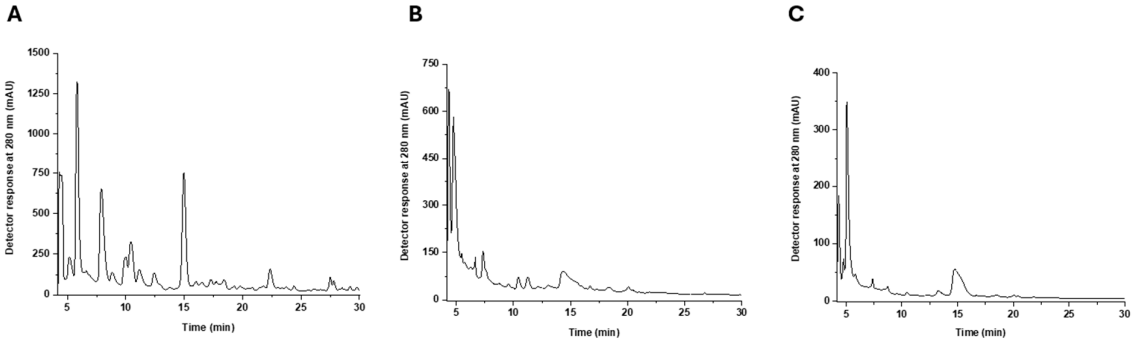

Figure 6. Chromatograms obtained by HPLC-DAD for (**A**) HS, (**B**) Lt1_S, (**C**) Lt1_HS. Peak identification is given in Tables 3–5.

Table 3. Identification (chromatographic and spectroscopic characteristics) and quantification of compounds in hazelnut shell (HS) extract.

Peak [a]	Rt (min) [b]	$[M-H]^-$ (*m*/*z*)	MS^2 Fragments (*m*/*z*)	Tentative Identification	Quantification (mg/g Extract dw) [c]
1	4.32	169	125	Gallic acid	11.42 ± 0.06
2	5.18	153	nd	Hydroxytyrosol	2.12 ± 0.14
3a	5.86	153	109	Protocatechuic acid	9.74 ± 0.27
3b		341	nd	Caffeic acid-*O*-hexoside	
4	6.48	211	nd	*C*-veratroylglycol	0.11 ± 0.01
5	7.9	137	109	Tyrosol	15.47 ± 0.05
6	8.83	137	nd	4-Hydroxybenzoic acid	0.50 ± 0.06
7a	9.98	195	nd	β-hydroxypropiovanillone	2.34 ± 0.01
7b		353	nd	Caffeoylquinic acid	
8a	10.44	365 (adduct)	319	Threo-1,2-bis(4-hydroxy-3-methoxyphenyl)-1,3-propandiol	2.17 ± 0.03
8b		167	nd	Vanillic acid	
9	11.16	197	182, 153	Syringic acid	0.53 ± 0.05
10	13.86	361	343, 331, 301, 271, 241	Giffonin P	tr
11a	14.97	359	197	Syringic acid-*O*-hexoside	23.33 ± 0.09
11b		151	nd	Vanillin	
12	17.29	345	327, 315	ent-Cedrusin	0.45 ± 0.03
13	18.4	541	541, 377, 292, 190	2-(3-hydroxy-2-oxoindolin-3-yl)acetic acid 3-*O*-6′galactopyranosyl-2″-(2″oxoindolin-3″yl) acetate	1.92 ± 0.15

Table 3. *Cont.*

Peak [a]	Rt (min) [b]	[M−H]$^-$ (*m*/*z*)	MS^2 Fragments (*m*/*z*)	Tentative Identification	Quantification (mg/g Extract dw) [c]
14a	21.83	419	373	Erythro-(7*S*,8*R*)-guaiacylglycerol-β-coniferyl aldehyde ether	0.32 ± 0.01
14b		187	125	Azelaic acid	
15	22.37	343	315, 301, 283, 269, 255	Carpinontriol B	3.44 ± 0.06
16	22.93	471 (adduct)	435, 273	Phlorizin (Phloretin-2-*O*-glucoside)	0.69 ± 0.03
17	24.4	345	125	Ceplignan	0.24 ± 0.01

[a] Peaks numbered according to the chromatogram shown in Figure 3. [b] Retention time on C_{18} column. [c] Quantitative data expressed as mg/g of the freeze-dried extract are presented as mean ± standard deviation. tr: traces, below the quantitation limit. nd: not detected. More than one compound per peak indicates coelution into the same chromatographic peak.

Table 4. Identification (chromatographic and spectroscopic characteristics) and quantification of compounds in *L. tigrinus* cultivated on sawdust (Lt1_S) extract.

Peak [a]	Rt (min) [b]	[M−H]$^-$ (*m*/*z*)	MS^2 Fragments (*m*/*z*)	Tentative Identification	Quantification (mg/g Extract dw) [c]
1a	4.34	239 (adduct)	193, 175, 161, 131, 119, 89	ni	10.84 ± 0.19
1b		218	146, 116, 88	Piperic acid	
1c		117	99, 73	Succinic acid	
1d		133	115, 71	Malic acid	
1e		115	71	Fumaric acid	
1f		173	155, 129, 111, 85	Aconitic acid	
2a	4.76	312	265, 134, 107	Vinylguaiacol derivative	6.79 ± 0.37
2b		341	179	Caffeic acid-*O*-hexoside	
2c		325	nd	Coumaric acid-*O*-hexoside	
2d		181	163	Tyrosine	
3a	7.34	225	181, 166, 123	Syringaldehyde derivative	0.60 ± 0.12
3b		181	166, 137, 123	Syringaldehyde	
4	11.26	443	293, 249, 207, 193, 161,134	1,3-*O*-Diferuloylglycerol	0.29 ± 0.01
5	13.05	151	135, 108	Vanillin	25.29 ± 0.80
6	14.35	401	357	Dihydroxybenzoic-malonyl-hexoside	3.16 ± 0.32

[a] Peaks numbered according to the chromatogram shown in Figure 2. [b] Retention time on C_{18} column. [c] Quantitative data expressed in mg/g of the freeze-dried extract are presented as mean ± standard deviation. More than one row per peak indicates coelution. tr: traces, below the quantitation limit. nd: not detected.

Table 5. Identification (chromatographic and spectroscopic characteristics) and quantification of compounds in *L. tigrinus* cultivated on hazelnut shell (Lt1_ HS) extract.

Peak [a]	Rt (min) [b]	$[M-H]^-$ (*m*/*z*)	MS^2 Fragments (*m*/*z*)	Tentative Identification	Quantification (mg/g Extract dw) [c]
1a	4.34	117	99, 73	Succinic acid	2.47 ± 0.01
1b		239 (adduct)	193, 175, 119, 101	ni	
2a	5.15	312 (adduct)	266, 134, 107	Vinylguaiacol derivative	2.25 ± 0.06
2b		341	179, 119	Caffeic acid-*O*-hexoside	
3	7.4	225	181, 166, 123	Syringaldehyde derivative	0.04 ± 0.00
4a	14.74	707	471, 353	Caffeoylquinic acid derivative	2.48 ± 0.01
4b		401	357	Dihydroxybenzoic-malonyl-hexoside	

[a] Peaks numbered according to the chromatogram shown in Figure 3. [b] Retention time on C_{18} column. [c] Quantitative data expressed as mg/g of the freeze-dried extract are presented as mean± standard deviation. FA: formic acid adduct. nd: not detected. ni: not identified. More than one row per peak indicates coelution.

In the HS extract, 17 peaks were present, and 22 bioactive compounds were tentatively identified. The ones with the highest concentration were syringic acid-O-hexoside (23.33 mg/g extract dw), tyrosol (15.47 mg/g extract dw), gallic acid (11.42 mg/g extract dw), and protocatechuic acid (9.74 mg/g extract dw). It should be noted that the majority of the bioactive compounds that were identified in HS extract are polyphenols (Table 3).

In the Lt1_S extract, a total of six peaks were present, and 17 bioactive compounds were identified. Vanillin was the major compound tentatively identified in the sample (25.29 mg/g extract dw). It should be noted that a total of 8 out of the 17 compounds were identified as phenolic compounds (Table 4). In addition, three organic acids, malic, fumaric, and succinic, were also identified.

In the Lt1_HS extract, only six bioactive compounds could be tentatively identified. It should be noted that five molecules are phenolic compounds, besides succinic acid, which is an organic acid (Table 5). The highest concentrations were found for succinic acid and vinylguaiacol derivative, which were equal to 2.47 ± 0.01 mg/g, and 2.25 ± 0.06 mg/g of extract dw, respectively.

A comparison was made among the three extracts to identify the molecules present in all samples (Table 6). The comparison reveals significant differences between the HS and Lt1_S samples. Indeed, the only shared molecules are caffeic acid-*O*-hexoside and vanillin, the latter being particularly important as it is a product of lignin degradation, as reported in the literature [44].

Interestingly, the only phenolic compounds that were present in HS and Lt1_HS hydroalcoholic extracts, at similar concentrations, but that were absent in Lt1_S, were caffeoylquinic acid and its derivative. Syringaldehyde and vinylguaiacol derivatives, dihydroxybenzoic-malonyl-hexoside, and succinic acid were present in both Lt1_HS and Lt1_S but were absent in HS extract, suggesting that these molecules are not dependent on the two different substrates.

Table 6. The polyphenols common to the three extracts HS, Lt1_S, and Lt1_HS.

Molecules Identified	HS (mg/g Extract dw) [d]	Lt1_S (mg/g Extract dw) [d]	Lt1_HS (mg/g Extract dw) [d]
Syringaldehyde derivative	np	0.60 ± 0.12	0.04 ± 0.00
Vinylguaiacol derivative	np	6.79 ± 0.37	2.25 ± 0.06
Caffeic acid-*O*-hexoside	9.74 ± 0.27	6.79 ± 0.37	2.25 ± 0.06
Caffeoylquinic acid derivative	2.34 ± 0.01	np	2.48 ± 0.01
Succinic acid	np	10.84 ± 0.19	2.46 ± 0.01
Vanillin	23.33 ± 0.09	25.29 ± 0.80	np
Dihydroxybenzoic-malonyl-hexoside	np	3.16 ± 0.32	2.48 ± 0.01

np: not present in the extract. [d] Quantitative data expressed as mg/g of the freeze-dried extract are presented as mean± standard deviation.

4. Discussion

L. tigrinus is an edible, white-rot fungus able to grow on various substrates, such as hazelnut shells, which are difficult to degrade due to their high lignin content [45–47]. *L. tigrinus* is particularly known for its bioactive compounds, such as polyphenols, tocopherol, flavonoids, and terpenes [48,49]. All these bioactive compounds present different biological properties, and their antioxidant activity is the most well known and deeply studied [50,51]. Research is focusing on investigating natural products rich in antioxidant compounds, specifically mushrooms, to develop novel, innovative, and health-promoting food products and contribute to new drug discovery efforts [52–55]. Therefore, thanks to their potential, mushrooms could find applications in various fields, including nutraceuticals and cosmeceuticals [56,57]. This novel approach aligns with the issue of the circular economy, which enhances the valorization of waste as a new resource.

The present work aimed to employ hazelnut shells, an agricultural waste difficult to recycle, as a growth substrate for *L. tigrinus*, to investigate how antioxidant molecules present in hazelnut shells could be employed or metabolized by the mushroom during sporophore growth. Therefore, we investigated the antioxidant activity expressed by the hydroalcoholic extract obtained from sporophores cultivated on hazelnut shells in comparison with the hydroalcoholic extract obtained from sporophores cultivated on sawdust. The choice to use hazelnut shells as a substrate for *L. tigrinus* cultivation is based on their high polyphenol content, proving that hazelnut shells are a natural source of antioxidants and could enhance fungal growth and enzyme production [58]. This is the first study addressing the growth of *L. tigrinus* on hazelnut shells.

Despite *L. tigrinus* cultivation on hazelnut shells showing a lower yield growth compared to sawdust, the biological efficiency obtained is comparable to that reported by Puliga et al. [32]. The first significant finding is that *L. tigrinus* can develop and grow on a substrate composed entirely of hazelnut shells, indicating its adaptability and the potential to utilize this resource. *L. tigrinus* has shown great potential as a candidate for cultivation on hazelnut shells, supported by the literature highlighting its hydrolytic action on lignocellulosic and recalcitrant substrate [59,60]. The growth of fungal species on hazelnut shells represents an ecological peculiarity of considerable interest because these agricultural wastes are typically considered challenging to degrade [29]. Indeed, hazelnut shells are characterized by a high content of lignin, a complex biopolymer difficult to degrade [28], but wood-decay fungi, in particular white-rot, act as powerful agents of decomposition, producing enzymes, such as laccases and peroxidases, that break down lignin [61,62]. Lignin degradation releases various nutrients such as sugars, cellulose, and organic compounds, especially in the presence of substrates rich in bioactive molecules such as hazelnut shells, creating a favorable environment for fungal growth [63]. The mycelium absorbs essential nutrients and bioactive compounds which include flavonoids, a big group of phenols [64,65]. As

a result of the high availability due to the breakdown of lignin, it could be assumed that the mushrooms are enriched in bioactive molecules such as polyphenols, increasing their antioxidant activity [66,67]. Another hypothesis suggests that the increased availability of these released molecules stimulates fungal metabolism, leading to the production of secondary metabolites [68]. Thus, the waste substrate is not only biodegraded but also produces high-value fungal biomass with potential antioxidant effects. Biological assays were performed to evaluate the anti-peroxidant and antioxidant properties [15] of the two extracts, Lt1_S and Lt1_HS.

The TBARS assay reveals a superior anti-peroxidant effect expressed by the Lt1_HS extract compared with Lt1_S. The difference in phenolic composition between Lt1_S and Lt1_HS may explain the variation in the anti-peroxidant activity, supporting the hypothesis that HS substrate enriches the sporophore with bioactive molecules [66,69]. Recent articles by Bouyahya et al. [70] and Deore et al. [71] indicate that various phenolic compounds are crucial contributors to anti-peroxidant effects, and the results align with our own. Specifically, from published results, caffeoyl derivatives, such as caffeoylquinic acids, present both in Lt1_HS and HS extracts, are known to inhibit lipid peroxidation, and the synergy among these molecules may enhance the anti-peroxidant activity [72–75].

The DCFH-DA assay used to assess intracellular ROS levels demonstrates that Lt1_HS significantly reduced ROS production both in basal and in induced oxidative stress conditions in HaCaT dermal cells, indicating a robust antioxidant capacity likely due to the synergistic activity of its phenolic components [76]. Notably, the DCFH-DA assay is a reliable test widely used to measure oxidative stress, and it has also been employed in numerous EFSA studies, further supporting its credibility in scientific research [77,78]. We hypothesize that the antioxidant-enriched Lt1_HS extract could not exhibit any potential risks of consumption at the usual recommended dose (1 g/day of dried mushroom). This consideration is based on two main points: (1) the in vitro MTT assay displayed that at the tested concentrations, the extracts exhibited only a decrease of 25% in cell viability, considered a low cytotoxicity percentage; (2) furthermore, the polyphenol content measured is considerably lower compared to the upper limit reported in a DRA (Dietary Risk Assessment). As reported by Liu et al. [79], the antioxidant effect of an extract is greatly enhanced by the synergistic interaction of its molecules, particularly polyphenols. This synergy amplifies the extract's overall antioxidant capacity, suggesting that complex mixtures of polyphenols and other bioactive compounds provide stronger, more comprehensive protection against oxidative stress than individual components.

From the chemical analysis of the three extracts, it arises that the composition of the HS extract is in accordance with the literature [80]. Instead for the Lt1_S extract, although bibliographic research on polyphenols in *L. tigrinus* is limited, our findings support the conclusions obtained by Sanchez [15] and Karaman et al. [13]. Specifically, Sanchez identified several polyphenols in the sporophore extract of *L. tigrinus*, including *p*-coumaric acid, caffeic acid, and protocatechuic acid, which align with our findings. However, while Sanchez did not detect vanillin, vanillic acid was present. On the other hand, in the results of Karaman et al. [13], only protocatechuic acid. Interestingly, vanillic acid and caffeic and *p*-coumaroyl acid derivatives, which were absent in their findings, are clearly present in our Lt1_S extract. The comparison of the three extracts, HS, Lt1_S, and Lt1_HS, highlights a notable difference in their compositions, particularly concerning vanillin, which is present in the Lt1_S and HS extracts and absent in the Lt1_HS extract. The presence of vanillin in the Lt1_S extract aligns with the studies of Deore et al. [71], Zhu et al. [81], and Bogdan et al. [82], who indicate vanillin as a degradation product of lignin with various biological properties, including anti-microbial and antioxidant activities. It is known that the production of vanillin is heavily influenced by the activity

of specific ligninolytic enzymes, such as laccases and peroxidases, and the environmental conditions of the degradation process, including pH, oxygen availability, and substrate composition [83,84]. The absence of vanillin in the Lt1_HS extract leads us to hypothesize that lignin catabolism could be expressed by different enzymes, or that other metabolic pathways may be activated. This could lead to differences in fungal or microbial species involved, variations in the composition of the substrate, or enzymatic inefficiencies [85].

Instead, caffeoylquinic acid and its derivative are present in Lt1_HS and HS extracts. Literature shows that caffeoylquinic acid and its derivatives are metabolites produced by plants, such as *Ipomoea batatas*, *Coffea arabica*, *Corylus avellana*, and other plant species [75,86]. We hypothesize that the sporophore developed on hazelnut shells may have absorbed molecules directly from the substrate itself, enhancing its antioxidant bioactivity [87].

Although the results obtained for antioxidant activities are promising, it remains unclear which polyphenols, among those detected, or other molecules not yet detected, are involved in antioxidant activity. However, what is clear is that the use of a substrate, such as HS, increased the antioxidant activity. Chemical analyses indicate that the Lt1_HS extract contains a limited number of polyphenols. For this reason, a deep chemical characterization will be performed to investigate which other molecules, such as flavonoids, terpenes, or tocopherols, may contribute to the antioxidant effect [88–90].

5. Conclusions

In conclusion, utilizing hazelnut shells as a growth substrate for *L. tigrinus* presents a promising approach for enriching sporophores with bioactive compounds that offer health benefits, particularly in reducing oxidative stress. Hazelnut shells, agricultural waste rich in lignin and polyphenols, are used not only as a nutrient source for fungi but also as catalysts in stimulating the production of antioxidant molecules within the mushrooms.

Specifically, the sporophores of *L. tigrinus* cultivated on hazelnut shells enhanced anti-peroxidant and antioxidant activities, indicating a promising strategy for maximizing the bioactive potential of these agricultural residues. This kind of waste substrate enriches the mushrooms with compounds known to neutralize free radicals and protect against cellular oxidation, contributing to overall health maintenance and potentially lowering the risk of oxidative-stress-related diseases. By transforming hazelnut shells into a valuable substrate, this approach supports sustainable agriculture and provides a natural, eco-friendly source of antioxidants, showcasing the potential of mushroom-based biofortified products as functional foods with health-promoting properties. This study aimed to investigate if the lignin degradation process could enrich *L. tigrinus* sporophores. Additionally, an important focus will be on understanding the molecular pathways involved. In the near future, we are committed to addressing this significant question.

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