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Comparative Profiling of Bioactive Metabolites and Enzymatic Activities in Extracts from Stem-Base Waste and Fruiting Bodies of *Pleurotus eryngii*

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Abstract

This study aimed to compare the macronutrient composition, the antioxidant activity, and the enzymatic content of several enzymes in the stem base (SB-Pe) (non-commercial portion) of the cultivated *Pleurotus eryngii* mushroom with those of the commercial portion of its fruiting body (CP-Pe). Results showed that the stem waste contained significantly higher levels of carbohydrates and glucans compared to the fruiting bodies. Conversely, CP-Pe exhibited higher protein and fat content. The extract obtained from CP-Pe showed higher total phenolic and flavonoid content, as well as stronger scavenging activity towards DPPH and hydroxyl radicals, compared to the stem base extract. HPLC-MS/MS analysis identified sixteen analytes belonging to three compound classes, namely phenolic acids, sterols, and flavonoid aglycones and glycosides, widely recognized for their antioxidant, anti-inflammatory, and cytoprotective properties. Enzymatic assays also demonstrated higher activities of proteases, cellulases, amylases, hemicellulases, laccases, peroxidases, and lipases in CP-Pe than SB-Pe. In addition, the ethanolic extract of SB-Pe showed antifungal activity against four *Fusarium oxysporum* formae speciales plant pathogens. In conclusion, this study highlights the bioactive potential of stem-base by-products for integrated plant defense, along with their potential use as a source of enzymes with relevance for multiple industrial and biotechnological applications.

Keywords: *Pleurotus eryngii*; stem base; enzymes; glucans; polyphenols; bioactivity; valorization; antifungal activity

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1. Introduction

In the last three decades, mushroom production has increased more than 30-fold. According to the Food and Agriculture Organization of the United Nations (FAO), the global production of mushrooms and truffles has increased substantially (the world production of mushrooms and truffles exceeded 50 million tons for the first time in 2024), highlighting the growing economic importance of mushroom cultivation worldwide [1]. FAOSTAT data

provide a useful statistical framework for assessing the expansion of this sector and the increasing availability of mushroom-derived residual biomass. According to the Global Button Mushrooms Report, global production is projected to exceed 20 million tons by 2026 [2], leading to the simultaneous generation of massive amounts of by-products and waste—approximately 60 million tons each year. This rapid expansion raises significant environmental concerns and increases disposal and management costs for the industry. The growing scale of mushroom production further emphasizes the need to develop sustainable management strategies capable of transforming cultivation and processing residues into valuable secondary raw materials, thereby reducing environmental impacts while generating additional economic opportunities. This waste mainly consists of the residual biomass generated after harvesting the fruiting bodies, known as Spent Mushroom Substrate (SMS), as well as other two additional types of by-products: misshapen mushrooms that do not meet commercial standards, and stems or stipe. The latter correspond to the basal portion of the fruiting body, responsible for the nutrient transport from the growth substrate accounting for up to approximately 20% of the total fruiting-body mass [2]. These by-products have a high nutritional value and can be valorized in several applications [3]. The valorization of mushroom-processing waste is a highly relevant topic, as it integrates sustainability, circular economy principles, and agri-food innovation. Spent mushroom substrate (SMS) has demonstrated great potential in a wide range of applications, including reuse as a cultivation substrate, agronomic use as a fertilizer and soil amendment, incorporation into animal feed, bioenergy production via biochar, bioremediation processes, biomaterial synthesis, and extraction of industrially relevant enzymes [4–7]. In contrast, misshapen mushrooms generally retain the same nutritional profile as the commercial product; therefore, despite their morphological characteristics, they can be efficiently used after minimal processing such as slicing and crushing [8], contributing further to waste reduction strategies. Among mushroom by-products, stems represent a disposal problem but also a promising source of valuable compounds that can be exploited for their nutritional properties. Despite being typically discarded during processing, they present low caloric value and are rich in dietary fiber, polysaccharides such as β-glucans, micronutrients, phenolic compounds, and other molecules with high antioxidant properties. These properties make them ideal candidates for innovative applications in the food industry as functional ingredients [3–9], or in animal feed supplementation [10,11]. In this regard, Bermúdez-Gómez et al. [12] demonstrated that stem-derived flours, co-products from *Agaricus bisporus* and *Pleurotus ostreatus*, may have several food-related applications, such as potential for use as emulsifiers, sweeteners, or fortifiers in the development of functional food. Despite these promising findings, research on mushroom stems remains limited compared to the other major by-products as SMS, particularly regarding their enzymatic potential. In particular, there is a lack of systematic studies investigating stems as a source of enzymes with industrial and biotechnological relevance, representing a clear gap in current knowledge. In this context the recovery of bioactive compounds and enzymes from mushroom stem residues could support the development of integrated biorefinery approaches in which multiple valuable products are obtained from the same residual biomass. Such strategies are particularly relevant for improving the overall sustainability and economic efficiency of mushroom cultivation. Among edible mushrooms consumed worldwide, species of the genus *Pleurotus*, commonly known as oyster mushrooms, account for approximately 25% of the global cultivated mushroom market. In particular, *Pleurotus eryngii* (also known as king oyster mushroom) is widely appreciated for its excellent taste, flavor, and nutritional properties as well as for a broad spectrum of biological activities including antitumor, antioxidative, immunomodulatory, hepatoprotective, anti-inflammatory, antiviral, hypoglycemic, hypolipidemic, hypocholesterolemic, and fibrinolytic effects [13–17]. The most representative compounds recovered

from Pleurotus eryngii are polyphenols and related compounds, polysaccarides and glicoproteins, steroids and terpenoids, and enzymes [3]. The recovery of these compounds from mushroom by-products involves different extraction strategies depending on the target molecules. The recovery of phenolic and related compounds from mushroom tissue and their waste utilizes both traditional methods and innovative green technologies designed to maximize yield while minimizing environmental impact. Conventional solid–liquid extraction, primarily employing aqueous ethanol or methanol, is widely used due to its simplicity, low cost, and suitability for recovering compounds across a range of polarities. However, its efficiency is heavily dependent on solvent composition, temperature, and extraction time; furthermore, it generally lacks selectivity, which can lead to the co-extraction of interfering molecules. To overcome these limitations, alternative protocols have been developed that combine solvent extraction with ultrasound-assisted extraction (UAE) [18] or microwave-assisted extraction (MAE) [19] to disrupt fungal cell walls and facilitate the release of phenolic compounds. Recently, significant interest has also shifted toward deep eutectic solvents (DES). Operating as eco-friendly alternatives to classic organic solvents, DES enable the highly selective solubilization of fungal phenolic compounds [20].

Polysaccharides and glycoproteins from mushroom such as *Pleurotus eryngii* by-products are mainly recovered through hot-water extraction, alkaline or acidic treatments, and ultrasound-assisted extraction, depending on the target compounds and fungal species. Ethanol precipitation is frequently employed to recover polysaccharide fractions, followed by purification procedures such as dialysis, ultrafiltration, and chromatographic separation [21–24]. As summarized by Antunes al. [3], sterols and other lipophilic compounds require organic solvent-based procedures. In contrast, hydrolytic and lignocellulolytic enzymes are typically recovered using water or appropriate buffer solutions, followed by agitation, filtration, and centrifugation. Overall, these approaches highlight the importance of selecting extraction conditions according to the physicochemical properties of the target compounds to optimize the valorization of mushroom residues. Given this background, the present study investigates the macronutrient composition, the antioxidant activity, and the enzymatic profile of the stem base, non-commercial portion, of the cultivated *Pleurotus eryngii* mushroom, comparing it with the commercial fruiting body fraction. Furthermore, a targeted HPLC–MS/MS approach was employed to investigate the distribution of selected low-molecular-weight bioactive compounds in the two mushroom fractions, providing additional information on their chemical composition and potential functional properties. In addition, the antifungal activity of stem extracts against the plant pathogens *Fusarium oxysporum* formae speciales was evaluated with a view toward potential applications in integrated plant disease management.

2. Results and Discussion

2.1. Yield Performance and Biomass Partitioning of Pleurotus eryngii

After approximately 35 days of cultivation, each 1000 mL bottle produced for both the two cultivation batches an average of 320 g of fresh fruiting bodies of *Pleurotus eryngii* (Figure 1a). The harvested biomass was subsequently separated into commercial and non-commercial portions to evaluate both production performance and the generation of residual biomass potentially exploitable as a source of bioactive compounds. The commercial portion (CP-Pe) consisting of pileus and stipe (Figure 1b), accounted for 278 g of fresh biomass (86.9% of the total yield), whereas the non-commercial basal portion of the stipe (BS-Pe) represented 42 g (13.1% of the total yield) (Figure 1c). These results indicate a high proportion of marketable biomass and a relatively low production of waste. The biological efficiency (BE) reached 91.4%, whereas the commercial biological efficiency was 79.4%. These values are consistent with those reported for *P. eryngii* cultivated on lignocellulosic

substrates enriched with agro-industrial residues [25]. BE values above 80% are generally considered indicative of efficient substrate conversion and productive mycelial colonization. Similarly, Philippoussis et al. [26] reported that supplementation of agricultural residues significantly enhances the productivity of *Pleurotus* species by improving nutrient availability, enzymatic accessibility and water retention capacity. The high biological efficiency observed in the present study suggests that the substrate formulation, composed of wheat straw, corn cobs, cardoon wood chips, spelt husks, and sugar beet pulp, provided suitable physicochemical and nutritional conditions for fungal growth and fructification. The addition of sugar beet pulp may have contributed to enhanced carbohydrate availability, while the heterogeneous lignocellulosic matrix likely improved aeration and moisture retention. Moreover, the relatively low proportion of non-commercial biomass indicates a balanced morphological development of the basidiomes, characterized by limited basal thickening. From an applied perspective, these findings are particularly relevant in the context of sustainable mushroom production systems aimed at maximizing the yield of edible biomass while minimizing processing waste. At the same time, the presence of a defined non-commercial fraction (SB-Pe) supports the concept of mushroom cultivation as a dual-output bioprocess, in which both edible biomass and residual streams can be valorized. Indeed, mushroom-derived residues are increasingly recognized as valuable secondary raw materials rich in phenolic compounds, polysaccharides, and other bioactive metabolites with potential applications in agriculture, food preservation, and pharmaceutical formulations [3,7,27]. Recent studies further highlight mushroom by-products as promising substrates for circular bioeconomy strategies and enzyme recovery platforms, reinforcing their role within integrated biorefinery approaches [6].



Figure 1. Morphological characteristics and biomass partitioning of *Pleurotus eryngii* fruiting bodies. (**a**) Representative mature fruiting bodies at harvest stage (full cap opening); (**b**) commercial portion (CP-Pe) after trimming; (**c**) non-commercial portion of stem base (SB-Pe) removed prior to market processing.

2.2. Proximate Composition and Glucan Content

A proximate analysis of fresh commercial fruiting bodies (CP-Pe) and stem base (SB-Pe) of *P. eryngii* is presented in Table 1. The CP-Pe samples showed that *P. eryngii* is characterized by a high moisture content of water (89.14 ± 0.68), carbohydrates (6.82 ± 0.33), minerals (0.71 ± 0.03), proteins (2.66 ± 0.13), and a low fat content (0.67 ± 0.02). These data are consistent with those reported by Manzi et al. [28], although in this study a slightly lower carbohydrate content was observed compared to their findings (9.6% of fresh weight) which may be attributed to differences in cultivation conditions, substrate composition, or harvest stage.

When comparing CP-Pe with SB-Pe, the commercial portion showed approximately 20.4% higher protein content and 63.4% higher lipid content. Conversely, carbohydrate content was approximately 24% lower in CP-Pe than in SB-Pe, while no significant differences were observed in both ash and moisture content. These findings are in agreement with Mau et al. [29], who reported that stems were characterized by a higher carbohydrate

content but a lower amount of proteins and fats, compared to the remaining portion of fruiting bodies, reflecting differences in tissue specialization and metabolic function.

Table 1. Proximate analysis of commercial portion (CP-Pe) and stem base (SB-Pe) of *Pleurotus eryngii*.

	Moisture	Proteins	Fat	Ash	Carbohydrates	Energy [#]
CP-Pe	89.14 ± 0.68	2.66 ± 0.13 *	0.67 ± 0.02 *	0.71 ± 0.03	6.82 ± 0.33 *	43.95 ± 2.17 *
SB-Pe	87.58 ± 0.95	2.21 ± 0.08	0.41 ± 0.01	0.75 ± 0.04	8.91 ± 0.41	48.17 ± 2.42

Values (g/100 fresh weight) represent the mean ± SD of two independent samples analyzed in triplicate ($n = 6$). [#] kCal/100 g fresh weight. Asterisks indicate a statistically significant difference between CP-Pe and SB-Pe (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

With regard to glucan content, the results reported in Table 2 show that stems exhibit significantly higher total glucan values (43.92 ± 1.13 g/100 g dry weight) compared to CP-Pe samples (35.21 ± 0.67 g/100 g dry weight). In both fractions, β-glucans account for more than 90% of total glucan. These findings indicate a higher β-glucan content in the stem base than in the commercial portion, in agreement with previous studies reporting tissue-dependent distribution of fungal polysaccharides [29–32]. Such differences may be related to the structural role of glucans in maintaining cell wall integrity and mechanical resistance, particularly in basal tissues exposed to substrate contact and nutrient transport functions. Glucans are widely distributed in edible mushrooms and exhibit differences both in their total content and in their distribution between the stem and the cap, which may be influenced by their structural and functional role [31–33]. Many of the biological activities of *P. eryngii*, including immunostimulating, antitumor, hepatoprotective, antioxidant, hypolipidemic, and hypoglycemic effects, have been largely attributed to its polysaccharide fraction, in particular, glucans [16,22,23,34,35].

Table 2. Glucans content in powdered commercial portion (CP-Pe) and stem base (SB-Pe) of *Pleurotus eryngii*.

	Total Glucans	α-Glucans	β-Glucans
CP-Pe	35.21 ± 0.67 (100%) *	3.14 ± 0.11 (8.92) *	32.07 ± 0.73 (91.08%) *
SB-Pe	43.92 ± 1.13 (100%)	4.23 ± 0.15 (9.63%)	39.69 ± 0.88 (90.37%)

Total and α-glucans were determined using an assay kit (Megazyme Cat. No. K-YBGL), while β-glucans were determined by difference. Values are expressed as g/100 g dry weight and represent the mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

2.3. Extraction Yield, and Total Phenolic and Flavonoid Content

The yields of ethanolic extracts obtained from CP-Pe and SB-Pe of *P. eryngii* are reported in Table 3. Both fractions showed extraction yields of approximately 40%, with no significant differences between the two mushroom portions. These values are comparable to those of Radzki et al. [36] in a study conducted on ethanolic extracts obtained from six cultivated edible mushrooms, including *P. eryngii*.

As shown in Table 3, the total phenolic content (TPC) of CP-Pe reached 3.85 ± 0.18 mg GAE/g of dry weight, corresponding to 9.56 ± 0.32 mg GAE/g of dry extract. These values were approximately 1.4-fold higher than those measured in SB-Pe (2.81 ± 0.12 mg GAE/g of dry weight or 7.08 ± 0.19 mg GAE/g of dry extract). These findings are consistent with previous studies reporting lower phenolic content in the hydroalcoholic stem-derived extracts compared to those obtained from the cup and upper portion of the fruiting body [13,36–39]. However, comparisons of TPC values across studies require careful consideration of extraction solvents, sample preparation procedures, extraction conditions, and reporting units. Radzki et al. [36] investigated ethanolic extracts of

freeze-dried stems and caps from six cultivated mushroom species, including *P. eryngii*, reporting phenolic and flavonoid contents on both dry-material and dry-extract bases. Their findings therefore provide a particularly relevant methodological reference for the present study, although differences in the anatomical fractions examined and extraction conditions should be considered. Li and Shah [40] reported TPC values ranging from 0.314 to 1.090 mg GAE/g dry weight in *P. eryngii* subjected to different thermal treatments, indicating that sample processing can substantially influence the measured phenolic content. Similarly, Si-Larbi et al. [41] investigated aqueous and 80% ethanolic extracts of *P. eryngii*, reporting a TPC of 20.65 ± 0.27 mg GAE/g dry extract for the aqueous fraction. Although this value exceeds those obtained in the present investigation, differences in solvent composition and extraction conditions preclude a direct quantitative comparison. Taken together, these findings highlight the importance of comparing TPC values obtained using similar extraction procedures and expressed in equivalent units.

Table 3. Extraction yield, total phenolic content (TPC) and total flavonoids content (TFC) of commercial portion (CP-Pe) and stem base (SB-Pe) of *Pleurotus eryngii*.

	Extraction Yield	**TPC**		**TFC**	
	(%)	**(mg GAE/g DW)**	**(mg GAE/g DE)**	**(mg QE/g DW)**	**(mg QE/g DE)**
CP-Pe	40.35 ± 0.43	3.85 ± 0.18 *	9.56 ± 0.32 *	0.57 ± 0.04 *	1.42 ± 0.11 *
SB-Pe	39.12 ± 0.82	2.81 ± 0.12	7.08 ± 0.19	0.31 ± 0.02	0.77 ± 0.06

TPC and TFC are expressed as mg/g of sample dry weight (DW) or mg/g of ethanolic extract dry weight (DE). GAE: gallic acid equivalents; QE: quercetin equivalents. Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

The interpretation of these results must also consider the inherent limitations of the Folin–Ciocalteu (FC) assay. Rather than selectively quantifying phenolic molecules, the FC reagent measures the overall reducing capacity of the sample [42]. Consequently, reducing non-phenolic compounds, including certain sugars, amino acids, organic acids, and vitamins, may contribute to the measured absorbance, potentially leading to an overestimation of the actual phenolic content. Therefore, TPC values should be interpreted as gallic acid equivalents of the Folin–Ciocalteu-reactive compounds rather than as direct measurements of the absolute concentrations of individual phenolic metabolites.

The CP-Pe extract showed a total flavonoid content of 0.57 ± 0.04 mg QE/g dry weight and 1.42 ± 0.11 mg QE/g dry extract, whereas the SB-Pe extract contained 0.31 ± 0.02 mg QE/g dry weight and 0.77 ± 0.06 mg QE/g dry extract, corresponding approximately to 54% of CP-Pe value. These findings differ from those reported by Radzky et al. [36], who observed a more homogeneous distribution of flavonoids among the different anatomical portions of *P. eryngii* fruiting bodies. Differences in sample preparation, extraction conditions, and the response of individual flavonoid structures to the aluminum chloride colorimetric assay may contribute to discrepancies among studies. Moreover, TFC values expressed as quercetin equivalents should be interpreted cautiously because the assay response depends on the chemical structure of the flavonoids present in the extract [43].

For both TPC and TFC, values calculated per gram of dry extract were substantially higher than those expressed per gram of dry weight, reflecting the concentration effect achieved during ethanolic extraction. Several studies have demonstrated that *Pleurotus* species contain a wide variety of phenolic and flavonoid compounds [38–44].

Overall, the present results indicate a greater abundance of compounds contributing to the TPC and TFC colorimetric responses in CP-Pe than in SB-Pe under identical extraction and analytical conditions.

2.4. Detection of Small Organic Molecules Putatively Responsible for Antioxidant Activity via HPLC-MS/MS Characterization

HPLC-MS/MS analysis was performed to identify a series of small organic molecules putatively responsible for antioxidant activity of both CP-Pe and SB-Pe extracts. Analytes were extracted via room temperature infusion with a methanol/water (70:30 *v*/*v*) mixture (Section 3.6) and analyzed by HPLC-MS/MS in Selected Reaction Monitoring (SRM) mode using a semi-quantitative approach, without authentic reference standards. Identification of analytes was performed by evaluating the m/z values of the precursor ions together with their fragmentation patterns, monitoring at least one quantifier and one qualifier transition for each analyte to ensure selectivity. Due to the absence of reference standards, identifications have to be regarded as putative.

Target compounds were selected among those frequently reported in the literature for the genus *Pleurotus* [45]. In particular, attention was focused on sixteen analytes (Table 4) belonging to three compound classes, namely phenolic acids, sterols, and flavonoid aglycones and glycosides, widely recognized for their antioxidant, anti-inflammatory, and cytoprotective properties [46]. Therefore, their occurrence in the extracts may contribute to rationalize the antioxidant activity observed herein (Section 2.5).

Table 4. Target compounds investigated in SRM mode, grouped by compound class, with the corresponding precursor ions and m/z values.

Compound Class	Bioactive Compound	ID	Precursor Ion	m/z	f2	f3
Phenolic acids	*trans*-ferulic acid	**1**	$[M\text{-}H]^-$	193.051	149.0	134.1
	p-coumaric acid	**2**	$[M\text{-}H]^-$	163.04	119.0	93.1
	gallic acid	**3**	$[M\text{-}H]^-$	169.014	125.2	107.0
	o-coumaric acid	**4**	$[M\text{-}H]^-$	163.039	119.0	89.1
	caffeic acid	**5**	$[M\text{-}H]^-$	179.035	135.4	107.4
Sterols	Campesterol	**6**	$[M+H\text{-}H_2O]^+$	383.367	951.0	161.1
	β-sitosterol	**7**	$[M+H]^+$	415.393	135.1	161.1
	Brassicasterol	**8**	$[M+H\text{-}H_2O]^+$	381.352	297.0	159.0
	Ergosterol	**9**	$[M+H\text{-}H_2O]^+$	379.335	694.0	295.2
Flavonoid aglycones and glycosides	Malvidin	**10**	$[M]^+$	331.081	316.0	287.0
	Hesperetin-7-*O*-rutinoside	**11**	$[M\text{-}H]^-$	609.182	286.0	301.0
	Isorhamnetin-3-*O*-rutinoside	**12**	$[M\text{-}H]^-$	623.162	300.0	315.0
	Luteolin-*O*-rutinoside	**13**	$[M\text{-}H]^-$	593.151	285.1	151.0
	Myricetin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one)	**14**	$[M\text{-}H]^-$	317.029	137.0	151.0
	Quercetin-3-*O*-rhamnoside	**15**	$[M\text{-}H]^-$	447.093	151.0	179.0
	Luteolin	**16**	$[M\text{-}H]^-$	285.041	151.0	133.1

Results are reported in Figure 2a–c, as peak areas of each analyte in CP-Pe and SB-Pe extracts, with areas normalized to internal standard. Since quantification was performed without authentic calibration standards, and ionization efficiencies differ markedly among compound classes and between polarities, the data in Figure 2 allow a reliable comparison of each analyte between the two matrices, but not a direct comparison of abundances among different analytes [47].

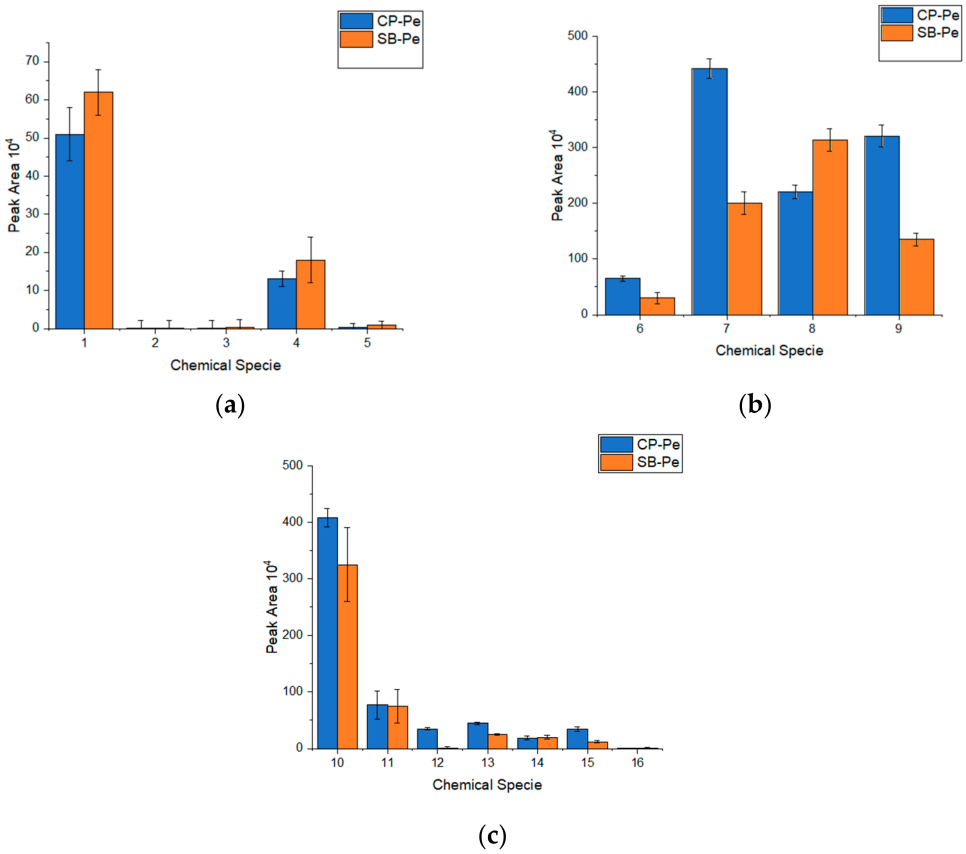


Figure 2. Peak areas of 16 different small target molecules, including phenolic acids ((**a**), 1–5), phytosterols ((**b**), 6–9) and simple or complex polyphenols ((**c**), 10–16). Statistical validation via ANOVA with Bonferroni correction revealed significant differences: p value < 0.05 for species 6–9 and 12, 13, 15, p value < 0.1 (trend) for species 1 and 10.

In addition to the preliminary screening performed via standardless SRM investigation, we successfully confirmed several key metabolites through absolute quantification using calibration curves constructed with authentic commercial standards (Table 5). A comparative analysis demonstrated a strong agreement between both analytical approaches, thereby validating the semi-quantitative screening reliability. Ultimately, the results obtained from the authentic standards consistently reinforce our initial findings, confirming both structural identity and concentration trends within acceptable analytical variance.

Table 5. Target compounds quantified through calibration curves and interpolation using authentic standard molecules.

Bioactive Compound	CP-Pe (mg/kg)	SP-Pe (mg/kg)	Standard
1	6.0 ± 0.6	7.2 ± 0.5	Authentic
2	<LOD	<LOD	Authentic
3	<LOD	<LOD	Authentic
4	1.0 ± 0.2	1.1 ± 0.4	Authentic
5	0.2 ± 0.1	0.2 ± 0.2	Authentic
6	6.3 ± 0.5	3.6 ± 0.5	Authentic
10	12.4 ± 0.5	9.6 ± 2.7	Authentic
13	1.5 ± 0.1	1.3 ± 0.1	Authentic
15	1.1 ± 0.1	0.6 ± 0.1	Authentic
16	<LOD	<LOD	Authentic

Among phenolic acids (Figure 2a), *trans*-ferulic acid (1) and *o*-coumaric acid (4) were the only species detected at appreciable levels, both slightly more abundant in SB-Pe than in CP-Pe, whereas *p*-coumaric (2), gallic (3) and caffeic (5) acids were found only at trace levels in both extracts. Sterols (Figure 2b) provided the clearest differentiation between the two matrices. Ergosterol (9), that is the main membrane sterol of fungi and the precursor of vitamin D_2, particularly abundant in *Pleurotus* species [48], was about 2.4-fold more abundant in the fruiting body than in the stem base. A similar trend was observed for *β*-sitosterol (7) and campesterol (6), whereas brassicasterol (8) was the only sterol more abundant in SB-Pe (about 1.4-fold). The occurrence of plant-type sterols, which are not typical products of fungal sterol biosynthesis, may reflect uptake from the lignocellulosic cultivation substrate, this being a hypothesis that deserves further investigation.

Flavonoid aglycones and glycosides (Figure 2c) were found to be more abundant in CP-Pe, with malvidin (10) showing the most intense signal of this group in both CP-Pe and SB-Pe extracts. Isorhamnetin-3-*O*-rutinoside (12) showed the largest difference in abundance between CP-Pe and SB-Pe, followed by quercetin-3-*O*-rhamnoside (15) and luteolin-*O*-rutinoside (13), while hesperetin-7-*O*-rutinoside (11) and myricetin (14) showed comparable levels in the two matrices, and luteolin (16) was detected only at trace levels. Representative chromatogram peaks related to the analyzed molecules are shown inMaterials and Methods, Section 3.6.

Phenolic compounds commonly found in mushroom alcoholic extracts, particularly in *Pleurotus* species, are known to contribute substantially to their antioxidant properties [44,49–51]. Gasecka et al. [18] identified several phenolic acids and flavonoids in the methanolic extract of *P. eryngii* including 4-hydroxybenzoic, ferulic, *p*-coumaric, protocatechuic, *trans*-cinnamic and vanillic acids and naringenin, partially in line with the present results, particularly with regard to ferulic acid. The high variability in the qualitative and quantitative composition reported in different studies may depend on species, cultivation methods, environmental conditions, maturation stage, and genetic differences among strains [52]. Furthermore, the detection of flavonoid glycosides and sterols, together with polysaccharides such as β-glucans, helps to explain the nutraceutical and antioxidant value of these extracts, which are easily obtainable from natural sources, including agro-industrial by-products.

Overall, the fruiting body was richer in sterols, particularly ergosterol, and in glycosylated flavonoids, while the stem base showed comparable or slightly higher levels of hydroxycinnamic acids. Notably, SB-Pe, an agro-industrial by-product usually discarded, retained a substantial fraction of the bioactive compounds detected in the edible portion, supporting its potential valorization as a low-cost source of antioxidant molecules.

2.5. Antioxidant Activity

The antioxidant activity of the extracts was evaluated against DPPH and hydroxyl radicals (Figure 3). Results were expressed as IC_{50} values, representing the extract concentration required to scavenge 50% of radicals. The DPPH assay is one of the most commonly used methods to evaluate the antioxidant activity of mushroom extracts, whereas the hydroxyl radical (OH•) is considered among the most reactive oxygen species (ROS) capable of inducing severe oxidative damage to biomolecules. Thus, the scavenging activity against hydroxyl radical represents an important indicator of the protective effect against oxidative stress.

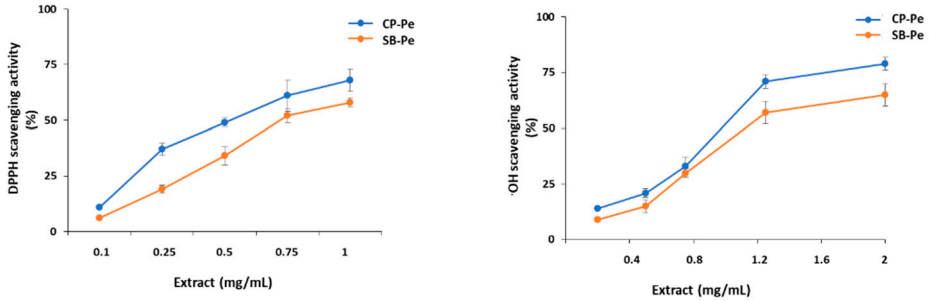


Figure 3. Scavenging activities of the ethanolic extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical (·OH). Data refer to the mean ± SD of two independent samples analyzed in triplicate ($n = 6$).

The extract obtained from CP-Pe exhibited stronger radical scavenging activity than SB-Pe. In particular, in the DPPH assay, CP-Pe showed a lower IC_{50} value (0.57 ± 0.04 mg/mL) compared to SB-Pe extract (0.75 ± 0.06 mg/mL), indicating higher antioxidant capacity. In both samples, DPPH scavenging activity of extracts increased in a dose-dependent manner (Figure 2). Specifically, CP-Pe exhibited inhibition values ranging from 11.2 to 68.7%, whereas SB-Pe ranged from 6.4 to 58.9%.

Lin et al. [44] reported IC_{50} values ranging from 1.08 to 1.3 mg dried extract/mL for ethanolic extracts obtained from *P. eryngii* harvested at different developmental stages. Similarly to the DPPH assay, in the hydroxyl radical scavenging assay, the extract obtained from the commercial portion of the mushroom exhibited higher scavenging activity (0.91 ± 0.04 mg/mL) compared to those from the stem base (1.22 ± 0.05 mg/mL). Results, also showed that the scavenging effect also increased in a concentration-dependent manner, ranging from 13.9 to 79.0% for CP-Pe and from 9.1 to 65.3% for SB-Pe.

The higher antioxidant activity observed in CP-Pe is consistent with its greater total phenolic and flavonoid contents, supporting the well-established relationship between phenolic compounds and radical scavenging activity in mushroom extracts [18]. These results further suggest that the scavenging capability towards the radicals varies according to the anatomical portion of the fruiting body from which the extracts are obtained.

The present findings are in agreement with those of several studies reporting lower DPPH scavenging activity in mushroom stems compared to caps or whole fruiting bodies [53,54]. Nevertheless, it should be noted that some authors have reported no significant difference among different mushroom tissues, suggesting that the antioxidant activity may also be influenced by many other factors, such as species, cultivation methods, substrate composition, extraction protocols, and harvest stage [23].

2.6. Protein Content and Total Proteolytic Activity

Protein content and enzymatic activity in the commercial portion (CP-Pe) and in the stem base (SB-Pe) of *P. eryngii* were measured in extracts prepared in sodium acetate buffer, pH 5.5. As reported in Table 6, the extract obtained from the commercial portion exhibited significantly higher protein content (40.04 ± 1.19 mg/g DW) and total proteolytic activity (1993.33 ± 44.87 U/g DW) than the stem base extract (30.75 ± 1.23 mg/g DW and 1027.75 ± 39.56 U/g DW, respectively). The amount and type of proteolytic enzymes produced by fungi are strongly influenced by both environmental conditions and the nature of the protein substrate available for growth [55]. In our recent study [7], performed on the same *P. eryngii* strain cultivated on the same substrate formulation used in the present work, the analysis of the proteolytic enzymes secreted by fungi into the growth medium increased during mycelial development and remained relatively stable throughout fruiting body formation suggesting additional physiological roles of fungal protases beyond

nutrient acquisition, possibly related to enzymatic regulation and protein turnover during morphogenesis. As already reported, proteolytic enzymes play an essential role in nitrogen mobilization and recycling in fungi. In addition, they may contribute to cellular protein homeostasis through the regulation of other enzymatic systems, including oxidative enzymes such as laccases and peroxidases [56,57].

Table 6. Protein concentration and total proteolytic activity.

	Proteins (mg prot/g DW)	Total Proteolytic Activity (U/g DW)	(U/mg prot)
CP-Pe	40.04 ± 1.19 *	1993.33 ± 44.87 *	49.78 ± 2.07 *
SB-Pe	30.75 ± 1.23	1027.75 ± 39.56	33.42 ± 1.39

The protein concentration was determined by the Bradford assay and expressed as mg proteins/g of sample dry weight (DW). Proteolytic activity was measured using azocasein and expressed as U/g DW or U/mg prot, where U corresponds to the amount of enzyme producing 0.001 absorbance units per min at 440 nm under the assay conditions. Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

The higher proteolytic activity observed in CP-Pe may therefore reflect the greater metabolic activity associated with actively differentiated fruiting tissues.

2.7. Carbohydrate-Hydrolizing Enzymes

Figures 4 and 5 show the cellulolytic, amylolytic and hemicellulolytic activities detected in the enzymatic extracts obtained from the commercial portion (CP-Pe) and stem base (SB-Pe) of *P. eryngii*. For all enzymes analyzed, significantly higher activity values ($p < 0.05$) were measured in CP-Pe compared to SB-Pe.

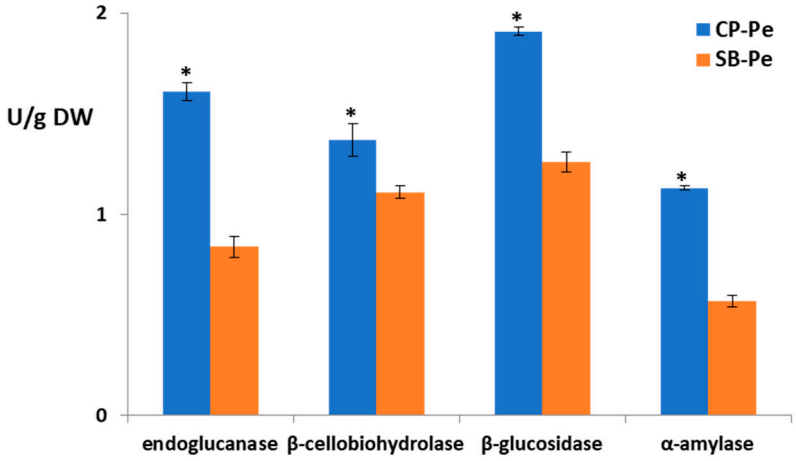


Figure 4. Cellulolytic and amylolytic activity of the enzymatic extract. The enzymatic activities were evaluated spectrophotometrically as described in Section 3 (Materials and Methods). Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

Among cellulolytic and amylolytic enzymes (Figure 4) the highest activity was observed for β-glucosidase reaching 1.91 ± 0.02 U/g DW in CP-Pe and 1.26 ± 0.04 U/g DW in SB-Pe.

Conversely, α-amylase showed the lowest activity values, corresponding to 1.13 ± 0.01 and 0.57 ± 0.03 U/g DW in CP-Pe and SB-Pe, respectively.

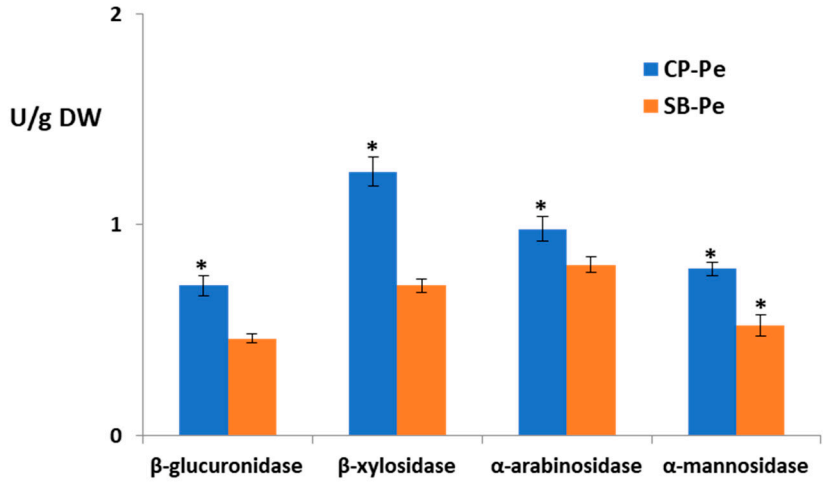


Figure 5. Hemicellulolytic activity of the enzymatic extract. The enzymatic activities were evaluated spectrophotometrically as described in Section 3 (Materials and Methods). Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

As shown in Figure 5, β-glucuronidase and α-mannosidase exhibited the lowest activity levels of h emicellulolytic enzymes in both extracts. In CP-Pe, these activities corresponded to 0.71 ± 0.05 and 0.79 ± 0.03 U/g DW, respectively, whereas in SB-Pe they reached 0.46 ± 0.02 and 0.52 ± 0.05 U/g DW, respectively. In CP-Pe the most active enzyme was β-xyloxidase (1.25 ± 0.07 U/g DW), whereas α-arabinosidase (0.81 ± 0.04 U/g DW) represented the predominant activity in SB-Pe (0.81 ± 0.04 U/g DW).

The predominance of β-glucosidase activity in both extracts highlights the central role of this enzyme in the final stages of cellulose degradation, where cellobiose is converted into glucose. Similarly, the relatively low α-amylase activity suggests that starch-degrading enzymes are less expressed in basidiomycetes grown on lignocellulosic substrates, in which starch is not a primary structural component.

Nevertheless, our previous study conducted on the same fungal strain and substrate formulation demonstrated that *P. eryngii*, in the early stages of mycelium development, preferentially utilized starch as the main carbon source, suggesting a dynamic modulation of carbohydrate-metabolizing enzymes during fungal growth and differentiation [7].

The predominance of β-xylosidase activity in CP-Pe and α-arabinosidase activity in SB-Pe may reflect differences in hemicellulose composition between the two tissue types, resulting in selective induction of specific enzymatic pathways. The higher enzymatic activities observed in CP-Pe may also be associated with the greater physiological and metabolic demand for carbohydrate mobilization in the upper portion of the fruiting body, where active growth, structural remodeling, and nutrient redistribution are more intense [58]. These findings further indicate that enzyme distribution within the fruiting body is not homogeneous.

Basidiomycetes, and, in particular, mushrooms belonging to the genus *Pleurotus*, are recognized as efficient producers of lignocellulose-degrading enzymes with wide industrial relevance. Different classes of enzymes derived from edible mushrooms are currently exploited in several industrial sectors including dairy production, food and feed processing, brewery, leather treatment, textile manufacturing, paper processing, agrochemicals, and pharmaceutical applications [59]. However, despite the increasing interest in mushroom-derived enzymes, the available literature is still mainly focused on enzymes recovered from spent mushroom substrate [3–6], whereas studies investigating mushroom stems as

a potential enzymatic source remain very limited, highlighting the novelty and potential biotechnological relevance of the present findings.

2.8. Laccase and Peroxidase Activities

The laccase and peroxidase activities detected in the enzymatic extracts are shown in Figure 6. For both enzymes, significantly higher activity values ($p < 0.05$) were observed in the extract obtained from CP-Pe compared to SB-Pe. In particular, laccase and peroxidase activities in CP-Pe reached 3.21 ± 0.21 and 2.12 ± 0.12 U/g DW, respectively, whereas SB-Pe samples showed values of 1.89 ± 0.13 and 1.33 ± 0.22 U/g DW for laccase and peroxidase, respectively. Laccase activity is widely distributed throughout mushroom fruiting bodies, particularly in white-rot fungi, although its distribution is not homogeneous. It is most heavily concentrated in the cap, and to a lesser extent in the stem, playing crucial roles in pigment formation and detoxification of phenol compounds produced during lignin degradation. In addition, it has been reported that laccase acts synergistically with peroxidases and other enzymes in lignin depolymerization [60]. Fungal laccases and peroxidases are ligninolytic enzymes naturally produced by mushrooms to mineralize lignin, which serves as a carbon source for their survival [6]. Due to their broad substrate specificity and oxidative capabilities, these enzymes are extensively exploited in several industrial and biotechnological applications, including bioremediation, biosensing, wastewater treatment, textile and wood processing, food technology, pharmaceutical production, and chemical sectors [61–65].

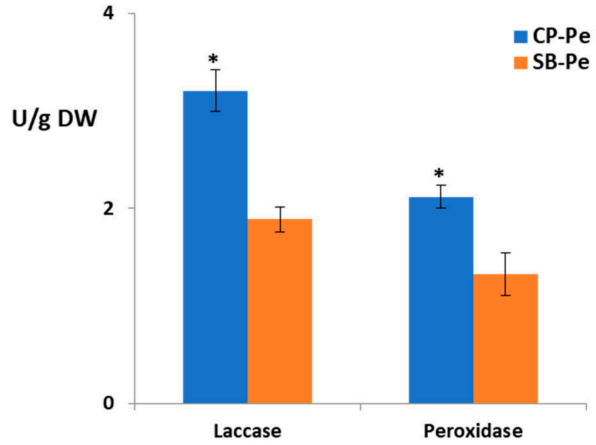


Figure 6. Laccase and peroxidase activities of the enzymatic extract obtained from commercial portion (CP-Pe) and stem base (SB-Pe) of *Pleurotus eryngii*. The enzymatic activities were detected spectrophotometrically using syringaldazine as substrate. Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

As widely reported, among the waste materials generated during mushroom production, the resulting SMS are commonly used as a source for recovering lignin-degrading enzymes [66,67]. However, the present results show that mushroom stem residues may also represent an eco-friendly, renewable, and enzymatically active source of laccases and peroxidases. These findings further support the potential valorization of mushroom-processing by-products within sustainable biotechnological and circular economy approaches.

2.9. Lipolytic Activity

Lipolytic activity of the extracts was evaluated spectrophotometrically using three different *p*-nitrophenyl-labeled fatty acids as substrates: butyrate (C4), decanoate (C10),

and palmitate (C16). As shown in Figure 7, both extracts from CP-Pe and SB-Pe exhibited higher activity toward medium- and long-chain substrates than the short-chain fatty acids. No statistically significant differences were observed between the activities measured against *p*-nitrophenyl palmitate (C16) and *p*-nitrophenyl decanoate C10). The comparison between the two mushroom fractions revealed that the extract obtained from CP-Pe displayed significantly higher lipolytic activity compared to SB-Pe ($p < 0.05$) for all the substrates tested. Fungal lipases are versatile enzymes widely employed in industrial and biotechnological applications in different sectors including leather, textile, cosmetics, biodiesel production, medicine and pharmaceutical, foods, fatty acid production, and the oleochemical industry [68–71]. Their broad substrate specificity, catalytic efficiency, and stability under different operational conditions make them particularly attractive for sustainable bioprocesses. However, unlike the extracellular lipases recovered from the SMS or produced through solid state fermentation [7,72,73], only limited information is currently available for lipases present in mushroom stem tissues.

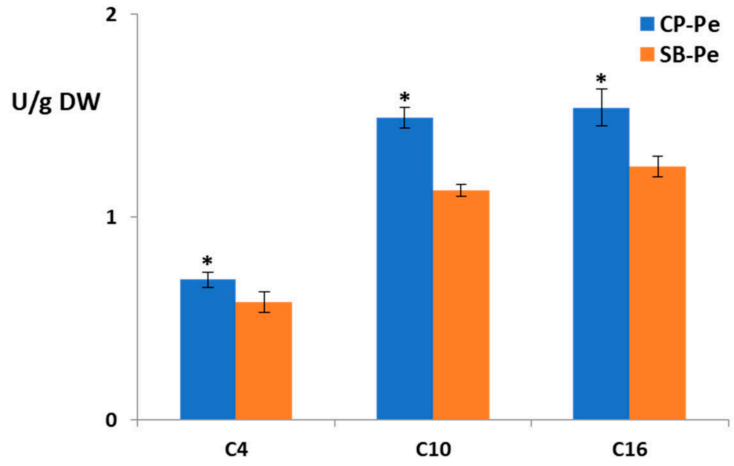


Figure 7. Lipolytic activity of the enzymatic extracts obtained from commercial portion (CP-Pe) and stem base (SB-Pe) of *Pleurotus eryngii*. Lipolytic activity was evaluated spectrophotometrically using *p*-nitrophenyl-labeled substrates: *p*-nitrophenyl-butyrate (C4), *p*-nitrophenyl-decanoate (C10), and *p*-nitrophenyl-palmitate (C16). Values are reported as mean ± SD of two independent samples analyzed in triplicate ($n = 6$). Asterisks indicate statistically significant differences (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

2.10. Antifungal Activity of the Basal Stem Extract

The hydroalcoholic extract obtained from the non-commercial basal stem portion (SB-Pe) of *Pleurotus eryngii* exhibited reproducible antifungal activity against all tested *Fusarium oxysporum* formae speciales, including *F. oxysporum f.* sp. *lycopersici, capsici, niveum*, and *melonis*. Visual evaluation of fungal colonies after 18 days of incubation demonstrated a clear reduction in radial mycelial expansion in treated plates compared with untreated controls (Figure 8). Control colonies showed dense and homogeneous growth with complete occupation of the Petri dish surface, whereas treated colonies exhibited reduced diameter, lower mycelial density, and altered colony morphology. No inhibitory effects were observed in the negative control plates treated with 80% ethanol, confirming that fungal growth inhibition was exclusively associated with compounds present in the mushroom extract.

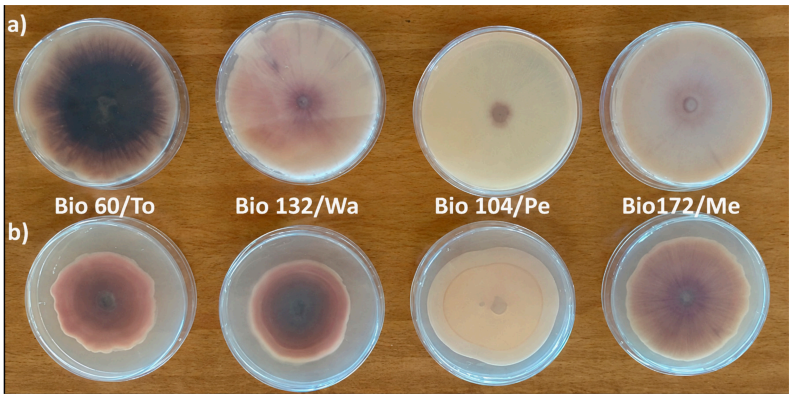


Figure 8. Antifungal activity of the SB-Pe extract against different *Fusarium oxysporum* formae speciales: *lycopersici* (Bio 60/To), *capsici* (Bio 104/Pe), *niveum* (Bio 132/Wa), and *melonis* (Bio 172/Me). Plates show mycelial growth at 18 days after inoculation. (**a**) Control plates without extract (upper row); (**b**) plates supplemented with the extract (lower row). For each plate, 0.6 mL of ethanolic extract corresponding to 13.8 mg dried extract, was applied.

The temporal analysis of radial mycelial growth showed in Figure 9 demonstrated that fungal inhibition became evident from the early incubation stages, starting from day 6, and remained detectable throughout the experimental period. After 18 days of inoculation, all untreated control colonies reached the maximum plate diameter (85 mm), whereas treated colonies consistently exhibited smaller diameters. Among the tested pathogen strains, the strongest inhibition was observed against *F. oxysporum f*. sp. *niveum* and *lycopersici*, whereas *capsici* and *melonis* appeared comparatively less sensitive to the extract treatment.

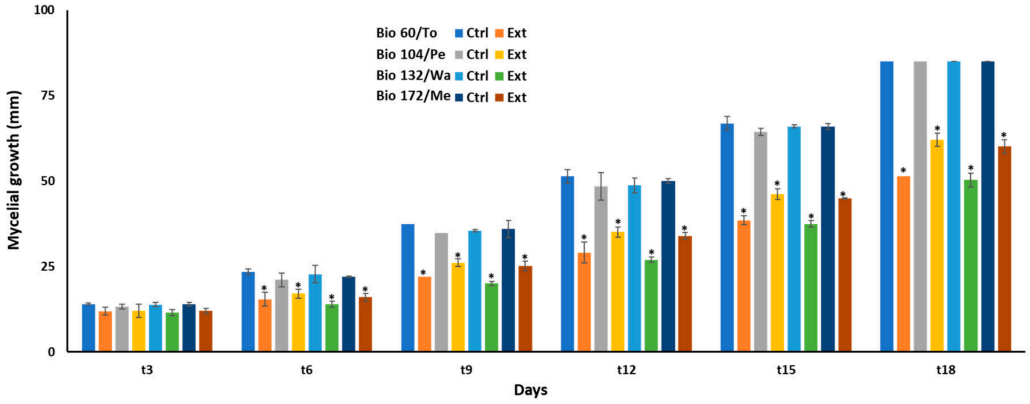


Figure 9. Time-course of radial mycelial growth (mm) of different *Fusarium oxysporum* formae speciales—*lycopersici* (Bio 60/To), *capsici* (Bio 104/Pe), *niveum* (Bio 132/Wa), and *melonis* (Bio 172/Me)—measured at regular intervals in the presence of the tested extract (Ext), compared with the untreated control (Ctrl). For each time point, the second, fourth, sixth, and eighth histogram bars correspond to the extract-treated samples, whereas the first, third, fifth, and seventh bars represent the respective untreated controls. Data are expressed as mean ± standard deviation (SD) of three replicates ($n = 3$). Asterisks indicate statistically significant differences between each of the four sample types treated with the pooled extracts and their corresponding untreated controls (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

The percentage reduction in mycelial growth confirmed these observations, with inhibition values ranging from approximately 30% to 45% depending on the fungal strain and incubation time. In all samples, the maximum antifungal effect was observed after 9 days of incubation and remained constant during the later stages of incubation (Figure 10). This trend may reflect a partial physiological adaptation of the fungal colonies to the bioactive

compounds present in the extract or a progressive decrease in the availability of active metabolites within the solid medium. Although the antifungal effect can be considered moderate, the activity observed is noteworthy considering that the extract was obtained from a non-commercial mushroom by-product without any purification or enrichment procedures. Similar findings have been reported for extracts derived from *Pleurotus* species, whose antimicrobial properties have frequently been associated with phenolic compounds, terpenoids, peptides, and low-molecular-weight secondary metabolites. In this study, each sample was treated with 0.6 mL of ethanolic extract, corresponding to 13.8 mg of dried extract, containing a total phenolic amount of 130 µg gallic acid equivalents (GAE) per plate. Phenolic compounds are known to interfere with fungal cell membrane integrity, mitochondrial activity, and oxidative metabolism, thereby reducing fungal growth and sporulation [74,75]. Several studies have demonstrated that mushroom-derived phenolics may exert antifungal activity through the induction of oxidative stress and disruption of membrane permeability [66]. In addition, extracts from *Pleurotus* spp. have been reported to inhibit phytopathogenic fungi including *Fusarium*, *Alternaria*, and *Botrytis* species [76]. The highest inhibition observed against *F. oxysporum f.* sp. *niveum* and *lycopersici* suggests possible differences in sensitivity among formae speciales, potentially related to variations in membrane composition, detoxification mechanisms, or growth rate.

Such strain-dependent variability has previously been described for natural antifungal compounds derived from fungi and plants [71,72]. Recent studies have further demonstrated that different *Fusarium* formae speciales may exhibit strain-dependent responses to natural antifungal compounds due to differences in cell wall composition, oxidative stress tolerance, and detoxification mechanisms [77]. Moreover, extracts obtained from mushroom by-products have been shown to possess antimicrobial and antifungal activities associated with phenolic compounds and other secondary metabolites capable of altering fungal membrane integrity and oxidative metabolism [78].

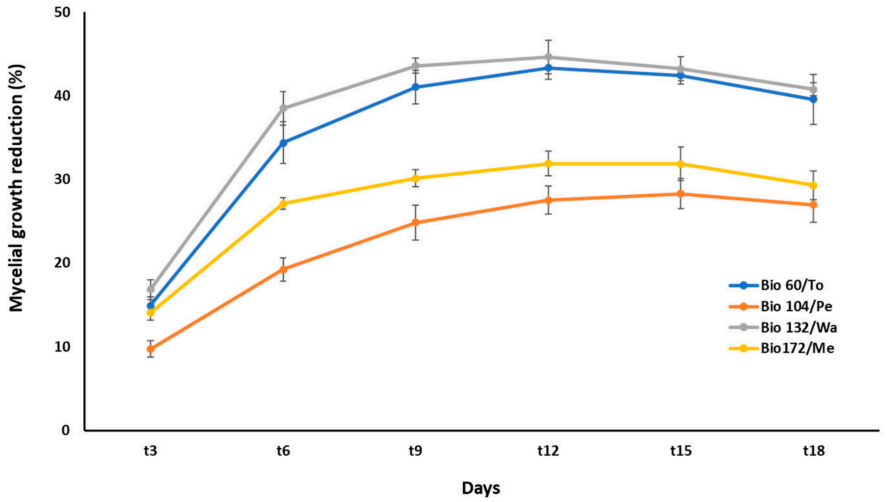


Figure 10. Percentage reduction in mycelial growth of different *Fusarium oxysporum* formae speciales: *lycopersici* (Bio 60/To), *capsici* (Bio 104/Pe), *niveum* (Bio 132/Wa), and *melonis* (Bio 172/Me), calculated according to [79]. Values were determined at different time points corresponding to days after inoculation, calculated by comparing extract-treated plates with untreated control plates. Data are expressed as mean ± standard deviation (SD) of three replicates ($n = 3$).

3. Materials and Methods

3.1. Fungal Material and Spawn Preparation

A strain of *Pleurotus eryngii* (Bio 175/Pe), preserved in the fungal collection of the Bioagritest Research Center (Bioagritest srl-Centro Interregionale di Diagnosi Vegetale, Pignola, Italy), was used in this study. The strain was originally isolated in the Basilicata region (Southern Italy) at approximately 800 m above sea level and identified based on morphological and molecular characteristics following standard taxonomic approaches for edible mushrooms.

The fungus was cultured on acidified potato dextrose agar (PDA) supplemented with streptomycin sulfate (100 mg L^{-1}) at 25 °C. Subcultures were maintained on the same medium at 5 °C for up to five months. Spawn was prepared in 1000 mL glass bottles equipped with aerated caps filled with wheat straw (4.1%), corn cobs (4.1%), cardoon wood chips (4.1%), spelt husks (4.1%), sugar beet pulp (15%), and $CaCO_3$ (3.6%). Moisture content was adjusted to 65% using deionized water, and then sterilized at 121 °C for 60 min. After cooling to room temperature, bottles were inoculated with 1-cm-diameter agar plugs of actively growing *P. eryngii* mycelium. Two independent cultivation batches were prepared for the experiments.

3.2. Basidiome Production

The inoculated bottles were incubated at 24–26 °C under an 8 h light (50 lux)/16 h dark photoperiod until complete substrate colonization. After full colonization, the bottles were transferred to a cold chamber at 10 °C for 5 days to induce primordia formation. Subsequently, bottles were moved to the production chamber for fruiting body development. Fruiting bodies were harvested at commercial maturity corresponding to full cap opening. The harvested mushrooms were separated into a commercial portion (CP-Pe), comprising stem and cap, and non-commercial portion, represented by the stem base (SB-Pe). Both portions were weighed separately.

Biological efficiency (BE) was used to evaluate substrate conversion into fruiting bodies and was calculated as the ratio of the total fresh weight of harvested mushrooms to the initial dry weight of the substrate, expressed as a percentage (BE (%) = fresh weight of fruiting bodies/dry substrate weight × 100), according to Chang and Miles [80].

3.3. Proximate Composition and Glucan Content

Samples were analyzed for proximate composition including moisture, protein, fat, carbohydrates, and ash content, following the procedures of the Association of Official Analytical Chemists (AOAC) [81]. Moisture content was determined by measuring weight loss after drying at 105 °C to constant weight. Protein content was estimated using the macro-Kjeldahl method with a conversion factor of 4.38. Fat content was determined by extracting a known weight of powdered sample with petroleum ether using a Soxhlet apparatus, whereas ash content was measured after incineration in a muffle furnace at 550 °C. Total carbohydrates were calculated by difference. Finally, energy content was calculated using the following equation:

$$\text{Energy (kcal)} = 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g fat})$$

Glucan content (total, α- and β-glucans) was quantified in 100 mg of powdered sample using the Mushroom and Yeast β-Glucan Kit (K-YBGL, Megazyme, Bray, Ireland), according to the manufacturer's instructions. Total glucans were hydrolyzed and solubilized with 12 M H_2SO_4 and subsequently incubated with exo-1,3-β-glucanase and β-glucosidase. The glucose specifically released was quantified using a glucose oxidase/peroxidase (GOPOD) assay. α-Glucans were solubilized in 2 M KOH and quantified after enzymatic hydrolysis of

starch-like glucans to glucose using glucoamylase, followed by glucose quantification via GOPOD assay. β-Glucan content was calculated as the difference between glucose released after acidic hydrolysis of total glucans and that released following specific enzymatic hydrolysis of α-glucans. Results were expressed as percentage of dry mass [82].

3.4. Preparation of Ethanolic Extracts

For the analyses, two independent biological extracts obtained from two independent cultivation batches were prepared. One gram of powdered commercial portion (CP-Pe) of mushroom or stem base (SB-Pe) of *Pleurotus eryngii* was suspended in 20 mL of 80% (v/v) ethanol. Extractions were carried out for 12 h at 20 °C in conical flasks under continuous orbital agitation (125 rpm). The extracts were recovered by centrifugation at 12,000× g for 15 min at 10 °C. Supernatants were filtered through filter paper, aliquoted, and stored at −20 °C until analysis. To determine extraction yield (%), an aliquot of each extract was freeze-dried and weighed. Extraction yield was calculated according to the following equation:

$$\text{Extraction yield (\%)} = (\text{dry weight of extract})/(\text{dry weight of powdered sample}) \times 100$$

3.5. Total Phenolic and Flavonoid Content

Total phenolic content (TPC) of CP-Pe and SB-Pe extracts was determined spectrophotometrically using the Folin–Ciocalteu reagent [42], with minor modifications. Briefly, 0.5 mL of Folin–Ciocalteu reagent (diluted 1:10 with water) was added to 0.1 mL of extract. After 3 min, 0.4 mL of 7.5% sodium carbonate solution was added. The reaction mixtures were incubated for 45 min at room temperature in the dark, and absorbance was measured at 765 nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Total phenolic content was expressed as gallic acid equivalents (GAE), reported as mg GAE/g of sample dry weight (DW) or mg GAE/g of ethanolic extract dry weight (DE). Total flavonoid content (TFC) was determined using the aluminum chloride method, with quercetin as the reference compound [83]. Briefly, 0.3 mL of each extract was mixed with 0.9 mL of methanol, 0.06 mL of 10% aluminum chloride, 0.06 mL of 1 M potassium acetate, and 1.7 mL of distilled water. The reaction mixtures were incubated at room temperature for 30 min and absorbance was measured at 415 nm. Total flavonoid content was expressed as quercetin equivalents (QE), reported as mg GAE/g of sample dry weight (DW) or mg GAE/g of ethanolic extract dry weight (DE).

3.6. Extraction of the Small-Molecule Fraction and LC-MS/MS Conditions

Analytes were extracted from the matrix pellets following a modified protocol [84]. Briefly, pellets were gently mixed with 0.1 mL of an extraction solution composed of methanol and water (70:30 v/v). The mixture was shaken mechanically for 30 min at 25 °C and then centrifuged at 3000 rpm for 10 min. The resulting supernatant served as the basis for preparing the standard addition solutions.

LC-MS/MS analyses were performed using a TSQ Altis™ Plus triple quadrupole mass spectrometer (Thermo Scientific™, Waltham, MA, USA) coupled to a Vanquish ultra-high pressure liquid chromatography (UHPLC) system equipped with a heated electrospray ionization (HESI) source (Thermo Scientific™, USA). The chromatographic separation of 10 µL of injected sample was performed on a Hypersil Gold™ column (50 × 2.1 mm, 1.9 µm particle size) with eluents A: water (0.1% formic acid) and B: methanol (both LC-MS grade, Sigma-Aldrich, Milan, Italy). Temperatures were set at 10 °C for sampler, 25 °C for column, and rate flow was kept constant at 0.3 mL/min. Table 7 reports the LC program.

Table 7. Elution method for LC-MS/MS analysis.

Time (min)	Flow (mL/min)	%B
0.0	0.3	5.0
2.0	0.3	5.0
8.0	0.3	35.0
12.0	0.3	60.0
13.5	0.3	95.0
16.0	0.3	5.0

The total run time was 16 min (Figure 11). Compound detection was performed in selected reaction monitoring (SRM) mode using negative or positive ionization, depending on the target molecules [85,86].

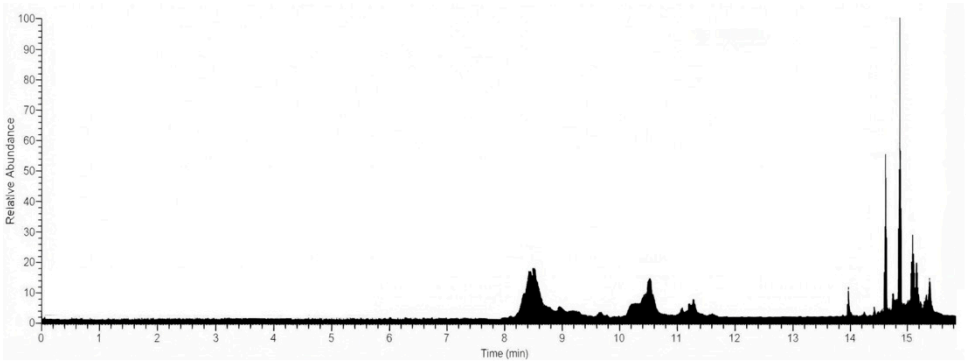


Figure 11. Chromatogram peaks related to the analyzed molecules searched using SRM table.

SRM peak areas were normalized to the area of 4-(2-amminoethyl)benzene-1,2-diol (dopamine), used as internal standard, and results were reported as relative peak areas. Compound identification was based on SRM transitions. Data acquisition and processing were carried out using TraceFinder 5.2 software (Thermo Scientific™, USA). Moreover, a quantitative confirmation of some metabolites was obtained via LC–MS/MS using compound-specific calibration curves built with authentic standards, processed through TraceFinder 5.2 software and using a validated Master Method input.

3.7. DPPH and Hydroxyl Radical Scavenging Activities

For the DPPH assay, 0.1 mL of sample extract at different concentrations was added to 0.7 mL of 0.2 mM 2,2-diphenyl-1-picrylhydraziyl (DPPH) in ethanol and incubated at room temperature for 30 min in the dark [87]. The absorbance was measured at 517 nm. Results were expressed as percentage of DPPH radical scavenging activity using the following equation: $(\%) = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

For the hydroxyl radical scavenging activity assay, 0.3 mL of extracts (prepared at different concentrations) were mixed with 0.3 mL of 6 mM ferrous sulfate solution and 0.3 mL of ethanolic salicylic acid solution (6 mM). The reaction was initiated by adding 0.3 mL of 7.5 mM H_2O_2. After incubation at 37° C for 60 min, the absorbance was read at 510 nm [88]. Results were expressed as percentage of hydroxyl (OH) radical scavenging activity using the following equation: $(\%) = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

For both radicals, antioxidant activity was expressed as IC_{50}, where IC_{50} values indicate the sample concentration required to inhibit (scavenge) 50% of radicals.

3.8. Preparation of Enzymatic Extracts

For each of the two cultivation batches, powdered samples (1 g) were transferred into Falcon tubes and mixed with 15 mL of cold 50 mM sodium acetate buffer (pH 5.5), followed by vortexing for 2 min. The mixtures were then sonicated using an ultrasonic probe for 5 min on ice. Subsequently, the suspensions were transferred into beakers, and the extraction was continued for 2 h under cold homogenization. After centrifugation at 12,000× *g*, for 10 min at 4 °C the supernatants were filtered on Whatman N.3 filter paper. The resulting filtrates were assayed for enzymatic activities. Results were expressed as U/g of dry sample. All analysis were performed in triplicate.

3.9. Protein Content and Total Proteolytic Activity

Protein content was determined according to the Bradford method [89] and expressed as mg of proteins per g of dry sample. Total proteolytic activity was assessed spectrophotometrically using the using azocasein as substrate according to Petraglia et al. [90]. Briefly, 0.1 mL of extract was added to 0.4 mL of 1% (*w*/*v*) azocasein solution (Sigma-Aldrich, St. Louis, MO, USA) in 40 mM Tris-HCl buffer pH 7.0, then mixtures were incubated at 37 °C. The reaction was stopped after 45 min by adding 0.5 mL of 10% trichloroacetic acid (TCA) and centrifuged for 4 min at 12,000 rpm (Amicon MC-13 microcentrifuge; Amicon, Beverly, MA, USA). An equal volume of 4 N NaOH was added to the supernatants and the absorbance was measured at 440 nm. A blank sample, in which TCA was added before substrate addition, was included in each assay. Total proteolytic activity was expressed as U/g of dry sample, where U corresponds to the amount of enzyme producing 0.001 absorbance units per min at 440 nm under the assay conditions.

3.10. Cellulolytic and Amylolytic Activities

Endoglucanase activity was determined by measuring the amount of glucose released from carboxymethyl cellulose (Sigma,-Aldrich St. Louis, MO, USA) [7]. For measurement of the activity, 0.5 mL of extract was added to 0.5 mL of 2% (*w*/*v*) carboxymethyl cellulose solution prepared in 40 mM sodium acetate buffer (pH 5.5) and incubated (with shaking) for 30 min at 50 °C. After that, the mixture was centrifuged for 5 min at 12,000× *g* and the supernatant was used for the assay. Glucose estimation was performed by glucose oxidase (GOD) and peroxidase (POD) method [82]. Results were obtained from a calibration curve using glucose as standard and the activity was expressed as U/g, where one unit (U) is defined as the quantity of enzyme which releases 1 µmol of glucose in 1 min. β-glucosidase and β-cellobiohydrolase activities were determined by monitoring the release of *p*-nitrophenol from the synthetic substrates *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-cellobioside (Sigma-Aldrich, St. Louis, MO, USA). For the assay, 0.05 mL of 10 mM substrates prepared in 40 mM sodium acetate buffer pH 5.5 were mixed with the same volume of extract, then 0.1 mL of acetate buffer were added. After 20 min of incubation at 30 °C, the enzymatic reaction was stopped by adding 0.8 mL of 200 mM Na_2CO_3. The absorbance was measured at a wavelength of 405 nm (ε = 18.4 mM^{-1} cm^{-1}) and results were expressed as U/g, where one unit (U) is defined as the quantity of enzyme which releases 1 µmol of *p*-nitrophenol in 1 min.

Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) reagent as described by Miller [91]. In total, 20 µL of enzymatic extracts were added to 180 µL of 1% starch in distilled water and incubated at 50 °C for 60 min, followed by the addition of 200 µL of DNS to stop the reaction. The solution was boiled for 10 min, placed on ice for 5 min, and then 800 µL of water was added. The amount of reducing sugar was determined using glucose as standard by measuring absorbance at 575 nm. Amylase activity (1 U) was defined as the quantity of enzyme required for release of 1 µmol of glucose per min.

3.11. Hemicellulolytic Activity

Hemicellulolytic activities of secretomes were assayed spectrophotometrically by using the following synthetic 4-nitrophenyl-labeled substrates: 4-nitrophenyl β-D-xylopyranoside; 4-nitrophenyl β-D-glucuronide; 4-nitrophenyl α-L-arabinopyranoside and 4-nitrophenyl α-D-mannopyranoside (Sigma-Aldrich, St. Louis, MO, USA). The assays were performed under the same conditions described above.

3.12. Laccase and Peroxidase Activities

Laccase activity was measured spectrophotometrically by monitoring the oxidation of syringaldazine (Sigma-Aldrich, St. Louis, MO, USA) at 530 nm ($\varepsilon = 65\ mM^{-1}\ cm^{-1}$) [92]. The assay mixture consisted of 0.90 mL of 40 mM sodium acetate buffer (pH 5.0), 0.05 mL of enzyme extract, and 0.05 mL of 2 mM syringaldazine (prepared in ethanol).

For peroxidase activity, the reaction mixture was supplemented with H_2O_2 to a final concentration of 4 mM. For both enzymatic activities, assays were carried out at 40 °C, and the increase in absorbance at 530 nm was measured after 15 min. Enzyme activity was expressed as International Units (U/g), where one Unit corresponds to the oxidation of 1 µmol of syringaldazine per minute at 40 °C. The blank contained all components except the enzyme extract.

3.13. Lipolytic Activity

Lipolytic activity was determined spectrophotometrically using the following pNP-labeled substrates: *p*-nitrophenyl butyrate, *p*-nitrophenyl decanoate, and *p*-nitrophenyl palmitate (Sigma-Aldrich, St. Louis, MO, USA). The reaction mixture contained 0.05 mL of enzyme extract, 0.05 mL of 10 mM substrate dissolved in acetonitrile, and 0.9 mL of 20 mM phosphate buffer (pH 6.8). After incubation at 30 °C for 20 min, the absorbance of the released *p*-nitrophenol was measured at 405 nm. Results, calculated using a calibration curve generated with *p*-nitrophenol (2–100 nmol), were expressed as U/g, where one Unit (U) corresponds to the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute [7].

3.14. Antifungal Activity of BS-Pe Extract

The two ethanolic extracts obtained from the two independent cultivation batches of SB-Pe were pooled into a single extract, which was subsequently used to evaluate the antifungal activity against different *Fusarium oxysporum* formae speciales: lycopersici (Bio 60/To), capsici (Bio 104/Pe), niveum (Bio 132/Wa), and melonis (Bio 172/Me). These isolates were sourced from the fungal collection of the Bioagritest Research Center and were originally isolated from tomato, pepper, watermelon, and melon plants, respectively, cultivated in Southern Italy, all exhibiting typical symptoms of Fusarium wilt. Prior to assays, pathogens were cultured on potato dextrose agar (PDA) supplemented with streptomycin sulfate (100 mg L^{-1}) and incubated at 25 °C for 7 days. The extract was tested at a concentration of 23 mg/mL, corresponding to 13.8 mg per plate. The total phenolic content (TPC) was 213 µg/mL, equivalent to approximately 128 µg gallic acid equivalents (GAE) per plate.

For the antifungal assay, 600 µL of the extract was aseptically distributed onto the surface of PDA plates previously inoculated with a 9 mm diameter mycelial plug of *F. oxysporum* and allowed to dry under laminar airflow conditions. The plates were then incubated at 28 °C for 21 days. Control plates treated with 80% ethanol served as negative controls. Treatments were performed in triplicate. Colony diameter was measured every three days and the percentage reduction in mycelial growth (Mr) was calculated according to Nduagu et al. [79] as follows: $Mr = (Mc - Mt) / Mc \times 100$, where

Mr = percentage reduction in colony diameter
Mc = colony diameter in control plates

Mt = colony diameter in treated plates

4. Conclusions

The present study demonstrates that the non-commercial stem base fraction of *Pleurotus eryngii*, commonly discarded during mushroom processing, represents a valuable source of bioactive compounds and industrially relevant enzymes. Although the commercial portion exhibited higher phenolic content, antioxidant activity, and overall enzymatic activities, the stem base showed a remarkably high content of carbohydrates and β-glucans together with significant antifungal activity against several *Fusarium oxysporum* formae speciales.

Importantly, the exploratory HPLC-MS/MS profiling provided a partial, putative characterization of the extracts' small-molecule composition, offering insight into their distinct antioxidant profiles without establishing causal contributions of individual metabolites.

These findings highlight that mushroom-processing residues should no longer be considered merely as waste materials but rather as promising secondary raw materials suitable for valorization within circular bioeconomy strategies. In particular, the recovery of bioactive metabolites and hydrolytic or oxidative enzymes from stem residues may contribute to the development of sustainable biotechnological processes with potential applications in agriculture, food preservation, feed supplementation, bioremediation, and enzyme-based industrial sectors. The antifungal activity observed in the stem-base extract is especially relevant considering that the extract was obtained without purification or enrichment procedures, supporting the possibility of developing low-cost and eco-friendly formulations for integrated plant disease management. At the same time, the detection of cellulolytic, hemicellulolytic, proteolytic, lipolytic, laccase, and peroxidase activities further confirms the biotechnological potential of these by-products as renewable enzymatic sources. Overall, this work supports a more sustainable and integrated approach to mushroom production, in which both commercial fruiting bodies and residual biomass streams are fully exploited. The recovery and use of mushroom-processing waste may therefore reduce environmental impact, lower disposal costs, and generate added-value products, contributing to the transition toward zero-waste agro-industrial systems and sustainable resource management.

Future studies should focus on a deeper investigation of the specific metabolites responsible for the antifungal activity, optimization of extraction procedures, scale-up strategies for enzyme recovery, and the evaluation of the efficacy of stem-derived extracts under field conditions.

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