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CHARACTERIZATION OF AN AQUEOUS EXTRACT OBTAINED FROM *Pleurotus ostreatus* PROTEIN CONCENTRATE BY-PRODUCT

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ABSTRACT

Recently, the antioxidant activity of the *Pleurotus* genus has been associated with the content of polysaccharides, particularly in crude extracts, since they have the capacity to trap free radicals and inhibit lipid peroxidation. On the other hand, during the processing of protein concentrates obtained from these fungi, by-products rich in carbohydrates and proteins are produced. The present study focused on the determination of the antioxidant activity and the electrophoretic characterization of proteins present in the by-products obtained from the aqueous extract of protein concentrates of two parental strains (PCM and POS) and a hybrid strain (PCM × POS) of *Pleurotus ostreatus*. The hybrid strain had a higher total protein content (25.29 %) compared to the parental strains PCM (23.93 %) and POS (22.6 %). The electrophoretic profile of the aqueous extracts of *Pleurotus ostreatus* revealed protein bands with molecular weights between 14 and 99 kDa. In relation to the antioxidant capacity, strains POS and PCM showed higher mean inhibitory concentration (ED50), with values of 11.84 and 20.57 mg mL^{-1}, respectively, while the hybrid strain PCM × POS had an ED50 of 4.67 mg mL^{-1}. The results suggest that the hybrid strain extract with the lowest mean inhibitory concentration (ED50) has potential as an antioxidant and biological material for the production of metabolites for other biological functions.

Keywords: antioxidant, fungal hybrid.

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INTRODUCTION

Oxidative stress damages important biomolecules due to an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system (Pisoschi *et al*., 2021). High concentrations of ROS or an unbalanced defense system can lead to protein oxidation, lipid peroxidation, and nucleic acid destruction, and in turn, have an impact on cellular functions, causing health problems such as cancer, inflammation, and cardiovascular diseases. Therefore, there is currently great interest in the search for biomolecules with antioxidant activity (Zhang *et al*., 2020).

Macrofungal fruiting bodies are rich in dietary proteins and contain significant amounts of carbohydrates, fats, vitamins, fiber, and minerals (Mwangi *et al*., 2022). Characteristic fungal compounds, known as mycochemicals, include polysaccharides (β-glucans), peptides, proteins, lectins, phenolic compounds, polyketides, glycoproteins, terpenoids, and enzymes, which are generally isolated from fruiting bodies. Changes in substrate composition or culture conditions can increase the concentration and type of fungal compounds (Krishnamoorthi *et al*., 2022).

The genus *Pleurotus* is considered a functional food due to its organoleptic characteristics and high nutritional and medicinal properties (Raman *et al*., 2021). In addition, it is highly valued for its simple cultivation procedures, which are adaptable to a variety of substrates, including agroforestry residues, and can be used to produce food, enzymes, and nutraceuticals. This genus is widely used in the production of by-products, recognized for their therapeutic properties, such as antihypercholesterolemic, antihypertensive, antidiabetic, antiobesity, hepatoprotective, antiaging, antimicrobial, and antioxidant activities (Lesa *et al*., 2022).

Phenolic compounds are key contributors to the antioxidant activity of macromycetes; however, this activity has also been linked to the presence of polysaccharides, particularly in crude extracts (Carrasco-González *et al*., 2017), due to their ability to trap free radicals and inhibit lipid peroxidation (Barbosa *et al*., 2020). Industrial production of fungal by-products, such as broken fruiting bodies or stipes, accounts for approximately 20 % of total fruiting body weight and has a high recycling potential (Prandi *et al*., 2023). Moreover, when obtaining protein concentrates, highly enriched by-products are generated, especially in carbohydrates and proteins (Meganaharshini *et al*., 2023). Therefore, the objective of this research was to determine the antioxidant activity and to characterize electrophoretically the proteins of the by-products obtained from the aqueous extract of protein concentrates of *Pleurotus ostreatus* fruiting bodies.

MATERIALS AND METHODS

Fungal material

The study used three *Pleurotus* strains: PCM (collection strain), POS (commercial strain), and PCM × POS (hybrid generated from PCM and POS). The fungal strains belonged to the strain bank of the Cell Culture Laboratory of the Interdisciplinary Professional Unit of Biotechnology of the National Polytechnic Institute (IPN) in Mexico City, Mexico.

Preparation of aqueous extracts

The *Pleurotus* strains were grown on pasteurized wheat straw substrate. After harvesting, the fruiting bodies were dried at 40 °C and ground using a homogenizer (Osterizer, Mexico) at 9000 rpm. The resulting flour was sieved through a number 50 mesh (Montinox, Mexico). The flour was then defatted with hexane at a 1:5 (w/v) ratio under continuous agitation for 8 h at 4 °C. The hexane was removed by decanting, and the defatted flour was placed in an extraction cabinet for 24 h. Finally, the flour was again sieved through a number 80 mesh (Montinox, Mexico) and stored at 4 °C. To obtain the aqueous extract of *Pleurotus*, the isoelectric precipitation method described by Cruz-Solorio *et al*. (2018) was used (Figure 1).

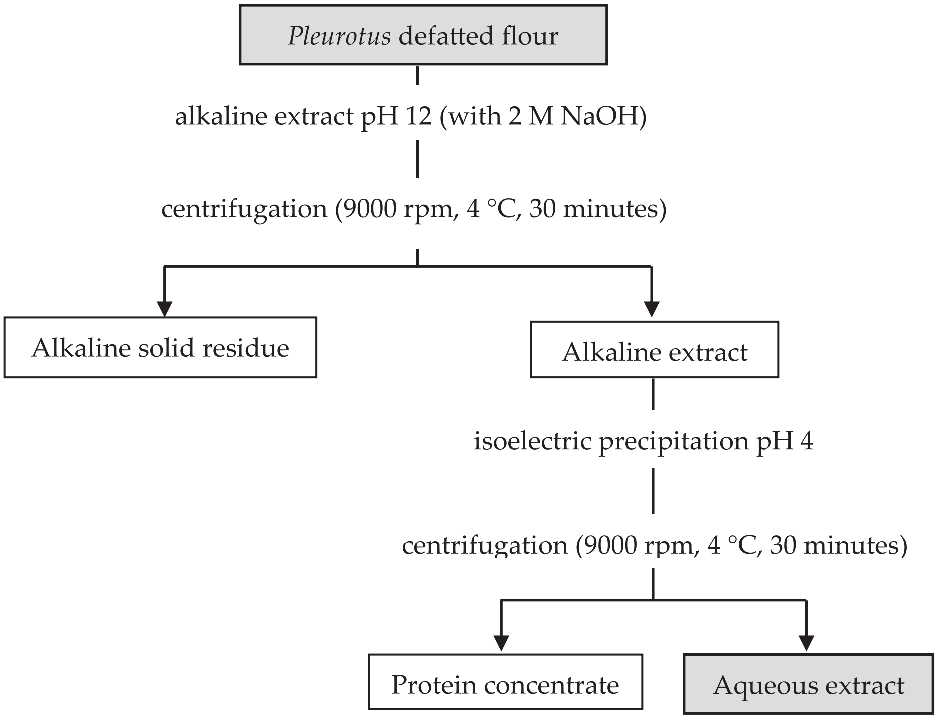

Figure 1. Schematic diagram for the preparation of *Pleurotus ostreatus* protein concentrate.

The aqueous extracts were spray dried using a Mini Spray Dryer B-290 (Buchi, Switzerland) under the following conditions: inlet temperature (Te) of 110 °C, outlet temperature (Ts) of 50 °C, and air flow (Fa) of 10 %. The powders obtained were stored at room temperature for later use.

Determination of total protein content

Crude protein content was determined using the micro-Kjeldahl method (AOAC, 1984). A total of 0.02 g of dry, defatted sample was weighed, and 0.25 g of a catalytic mixture of copper sulfate and potassium sulfate was added. The samples were placed in Micro-Kjeldahl flasks with 2 mL of concentrated sulfuric acid and subjected to the digestion process. The digested samples were processed in a K-350 distillation unit (Buchi, Switzerland), and the distillate was titrated with 0.1 N HCl. The protein content of the *P. ostreatus* aqueous extracts was calculated by multiplying the percentage of protein nitrogen obtained by a factor of 4.38.

Determination of soluble protein content

Soluble protein content was quantified using the Bradford method (Bradford, 1976) with some modifications. From each sample, 100 μL were taken, and 1500 μL of Bradford's reagent was added. The mixture was stirred gently and allowed to stand for 5 min. Finally, the absorbance of the samples was measured at 595 nm using a spectrophotometer (Thermo Genesys 10UV, USA). For analysis, a standard curve was prepared with bovine serum albumin (BSA).

Determination of total carbohydrates content

The total sugar content was measured by the modified phenol-sulfur technique (Dubois *et al*., 1956). From each sample, 200 μL were mixed with 200 μL of 5 % phenol, followed by the addition of 1000 μL of concentrated sulfuric acid. The mixture was allowed to stand for 10 min. Subsequently, distilled water was added until a total volume of 2.5 mL was reached. The samples were shaken and incubated at 30 °C for 30 min. Finally, the absorbance of the samples was measured at 490 nm using a spectrophotometer (Thermo Genesys 10UV, USA). For analysis, a sucrose standard curve was prepared.

Determination of reducing sugar content

A stock solution was prepared from each of the extracts, with a concentration of 2 g L^{-1}. Then, 100 μL of the stock solution were mixed with 900 μL of distilled water. From this mixture, 250 μL were taken and combined with 250 μL of the 3,5-dinitrosalicylic acid (DNS) reagent in an Eppendorf tube. The samples were heated for 15 min in a boiling bath at 80–90 °C, and then cooled on ice for 5 min. Subsequently, distilled water was added until a final volume of 3 mL was reached. The absorbance of the samples was measured at 540 nm using a spectrophotometer (Thermo Genesys 10UV, USA). For analysis, a fructose standard curve was used as described by Miller (1972).

Electrophoretic profile

The determination of the molecular weights of the proteins present in the aqueous extracts was carried out by the method of Laemmli (1970) using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. A 12.5 % separating gel (pH 8.8) and a 5 % stacking gel (pH 6.8) were used. The run buffer contained 0.025 M Tris, 0.1 % SDS, 0.192 M glycine, and had a pH of 8.3. A 15 μL sample of 2 % (w/v) was prepared and mixed in a 1:1 ratio with Laemmli sample buffer (Bio-RAD, USA).

The assay was performed with a Mini PROTEAN-3 electrophoresis chamber (Bio-RAD, USA). The proteins were stained with Coomassie brilliant blue R-250. Electrophoresis was carried out at 200 V and 60 mA for 45 min. Subsequently, the gel was stained with 0.125 % Coomassie brilliant blue R-250 and decolorized with a solution of 25 % methanol and 10 % acetic acid until the background was transparent. The molecular weight of the protein bands was estimated using a standard Mark 12 protein marker with a weight range of 2.5 to 200 kDa (Invitrogen, USA).

Infrared spectroscopy analysis

Aqueous extracts of *P. ostreatus* were examined by Fourier transform infrared (FTIR) spectroscopy in a range from 400 to 4000 cm^{-1}, with a resolution of 8 cm^{-1} and a total of 12 scans, using a 16F polycarbonate (PC) FTIR spectrometer (Perkin-Elmer, Germany). Interpretation of the results was performed using Spekwin32 software.

Antioxidant activity

The ability of the aqueous extracts to scavenge free radicals was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Blois, 1958). Solutions with a concentration of 0.016 g mL^{-1} of the aqueous fractions were prepared as a starting point; subsequently, 500 µL of each sample were placed in test tubes, and 2500 µL of the DPPH reagent were added. The mixtures were shaken for 30 min, and the absorbance was measured in a spectrophotometer at 517 nm. Ascorbic acid was used as a positive control, and the percentage of free radicals was calculated from a standard curve prepared with different concentrations of ascorbic acid.

Statistical analysis

The results were analyzed by two-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 22. Differences between means were evaluated using Duncan's multiple comparison test ($p < 0.05$). Data presented in tables and figures are expressed as the average of three replicates ± standard deviation (SD).

RESULTS AND DISCUSSION

Total protein, soluble protein, total carbohydrates, and reducing sugar content

The hybrid strain presented 25.29 % total protein, while the parental strains were 23.94 % for PCM and 22.6 % for POS (Table 1). The protein content the aqueous extracts was higher than that reported for solid alkaline residues obtained from *Vicia faba* L., which presented 6.9 % total protein (Vioque *et al*., 2012).

Regarding soluble protein content (Table 1), values ranged from 1.24 to 1.25 g L^{-1}. The proteins present in the aqueous extract did not precipitate at pH 4. Cruz-Solorio *et al*. (2014) indicated that defatted meals of *Pleurotus* spp. exhibited values between 0.2 and 0.24 g L^{-1}, while for defatted meals of *P. ostreatus,* it remained between 0.035 and 0.51 g L^{-1} (González *et al*., 2021).

The total carbohydrate content ranged (Table 1) between 27.26 and 37.11 µg µL^{-1}, which partially agrees with that reported by Santhosharajan and Sarawathy (2014)

Table 1. Contents of protein and carbohydrates in Pleurotus ostreatus aqueous extracts.

	PCM	POS	PCM × POS
Total protein (%)	23.94 ± 0.52^{a}	22.60 ± 0.44^{a}	25.29 ± 1.02^{a}
Soluble protein (g L^{-1})	1.24 ± 0.01^{a}	1.24 ± 0.01^{a}	1.25 ± 0.01^{a}
Total carbohydrates ($\mu g\ \mu L^{-1}$)	37.11 ± 0.01^{b}	34.48 ± 0.01^{a}	27.26 ± 0.02^{a}
Reducing sugar content ($\mu g\ \mu L^{-1}$)	3.72 ± 0.00^{a}	7.44 ± 0.01^{c}	5.81 ± 0.00^{b}

Values are expressed as mean ± standard deviation (n = 3). Means with different letters within a row differ significantly ($p < 0.05$).

for *Pleurotus* polysaccharide extracts (15–30 μg μL^{-1}). In contrast, the concentration of reducing sugars was lower in aqueous extracts than that reported by the same authors (18–20 μg μL^{-1}). On the other hand, Shukla and Jaitly (2011) reported a range of reducing sugars between 0.26 and 0.35 μg μL^{-1} lower than that of total soluble sugars (130.5 to 192.8 μg μL^{-1}) for species of the *Pleurotus* genus. Beltrán-Delgado *et al.* (2021) reported that, in edible fungi, carbohydrates are present in greater proportion, while reducing sugars constitute only a minor fraction of their total content.

The alkali extraction method contributes to a higher release of polysaccharides, attributable to the possibility of disrupting the existing ester and hydrogen bonding, which increases the rate of polysaccharide extraction (Janardhanan *et al.*, 2024). Furthermore, it has been observed that carbohydrate composition depends on the strain, the substrate used in its cultivation, and its conditions (Yadav and Negi, 2021).

Electrophoretic profile

The electrophoretic profile of the aqueous extracts of *P. ostreatus* (Figure 2) revealed the presence of protein bands with molecular weights between 14 and 99 kDa, with six representative bands standing out in each extract, with greater intensity in the range of 20 to 30 kDa. The bands detected in the aqueous extracts could correspond to different proteins previously reported for the genus *Pleurotus*. Liao *et al*. (2020) found polypeptides with molecular weights of 27, 30, 55, 88, and 115 kDa, with antioxidant activity in *P. geesteranus*. Likewise, Jin *et al*. (2022) reported five representative proteins in the protein extract of *P. geesteranus*, obtained by isoelectric precipitation, with molecular weights >15, 27~31, 46~50, 15~16, and 42~43 kDa, highlighting their physicochemical and functional properties.

Bands with molecular weights in the range of 17 to 94 kDa correspond to proteins from *Pleurotus eryngii*. These proteins are an alternative resource with applications in the food industry due to their functional properties and antioxidant activity (Wu *et al*., 2023). Likewise, a ribotoxin-like protein has been purified from *Pleurotus ostreatus* fruiting bodies, with a molecular weight of 15 kDa (Landi *et al*., 2020). The results obtained in the present investigation are consistent with molecular weights previously reported for this genus.

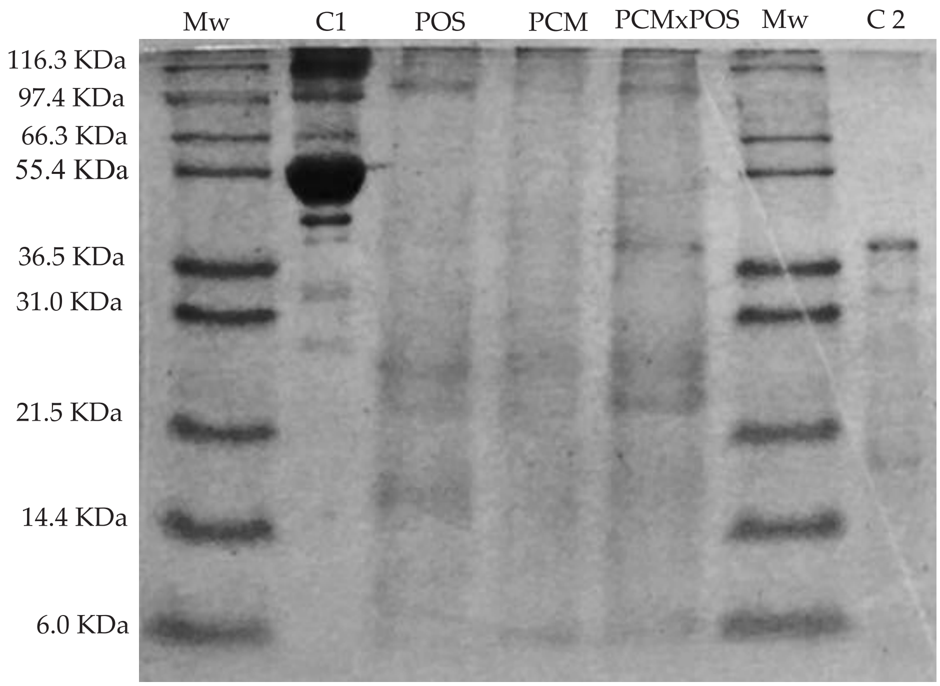

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) pattern of *Pleurotus ostreatus* aqueous extracts. Mw: standard marker Invitrogen; C1: albumin; C2: pepsine.

Infrared spectroscopy analysis

A similar pattern was observed for all fungal extracts (Figure 3). All three spectra showed a C-H bond around 2932 cm^{-1}, carboxyl groups in absorption peaks near 1642

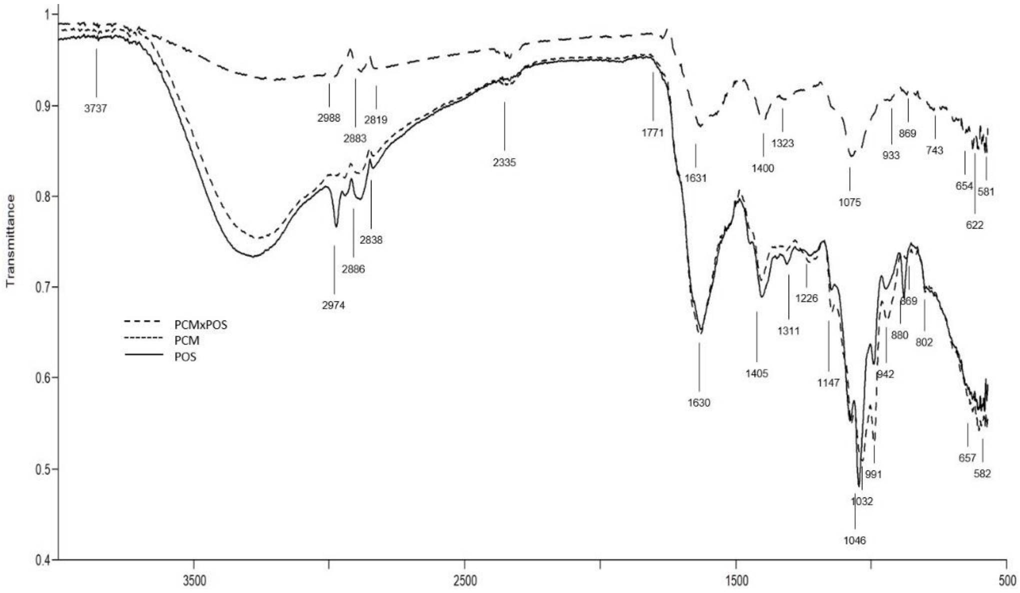

Figure 3. Infrared spectrum of *Pleurotus ostreatus* aqueous extracts.

cm^{-1}, and α-configuration in sugar units at 847 cm^{-1} (Wang *et al*., 2020). Peaks at 1000–1200 cm^{-1} were attributed to C-O glycosidic bonds, while pyranose rings were found at 500–1000 cm^{-1} (Ji *et al*., 2019).

According to Jin *et al*. (2022), the absorption peaks in the 1650–1660, 1610–1642, 1660–1680, 1680–1700, and 1642–1650 cm^{-1} regions corresponded to secondary structures of proteins α-helix, β-strand, β-turns, β-antiparallel, and random coil, respectively. On the other hand, peaks at 1375, 1312, 1100, 1065, 1038, and 890 cm^{-1} were associated with the presence of β-D-glucans (Bikmurzin *et al*., 2022). Based on these results, the present study confirms the presence of polysaccharides and proteins in the aqueous extracts of the fungal strains by infrared spectroscopy.

Antioxidant activity

The free radical scavenging activity assay by DPPH is one of the most widely used methods to evaluate antioxidant capacity. The hybrid strain PCM × POS showed 93.97 % antioxidant activity (percentage of inhibition) at a concentration of 54.79 μg μL^{-1}, being the highest activity recorded compared to the parental strains PCM and POS, which presented 64.29 % at 44.63 μg μL^{-1} and 79.73 % at 44.63 μg μL^{-1}, respectively (Figure 4). Aqueous extracts of PCM × POS evidenced higher DPPH radical scavenging capacity compared to extracts of the parental *P. ostreatus* strains, which could be attributed to their high affinity for DPPH radicals.

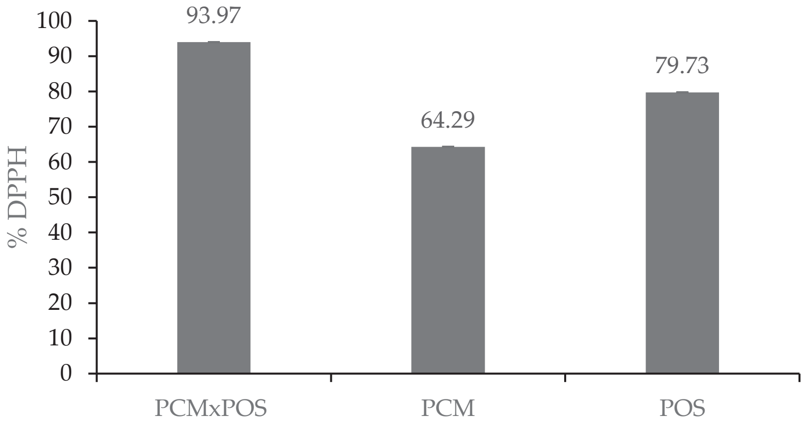

Figure 4. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (%) of *Pleurotus ostreatus* aqueous extracts.

On the other hand, IC50 is defined as the amount of an antioxidant-containing material required to scavenge 50 % of a radical. In addition, an antioxidant substance or extract is considered more effective in scavenging radicals when it has a lower IC50 value. Therefore, a lower IC50 indicates higher antioxidant activity (Effiong *et al*., 2024). The results showed that aqueous extracts of PCM × POS had an IC50 of 4.67 mg mL^{-1}, higher than aqueous extracts of PCM and POS (11.84 and 20.57 mg mL^{-1}, respectively). A previous study showed that *P. ostreatus* protein hydrolysates presented antioxidant activity between 16.55 and 31 %, with a minimum IC50 value of 5.24 mg mL^{-1} (Goswami *et al*., 2021). For their part, Beltrán-Delgado *et al*. (2021) reported the antioxidant activity of the aqueous extract of the fruiting body of *P. ostreatus*, with DPPH uptake percentages between 16 % and 26 %, and an IC50 of 1.72 mg mL^{-1}. These results suggest the presence of proteins, reducing sugars, carbohydrates, and flavonoids.

Phenolic compounds have an impact on the antioxidant activity of macromycetes. However, recent studies have associated this activity with the content of polysaccharides, especially in crude extracts (Carrasco-González *et al*., 2017), due to their ability to trap free radicals and inhibit lipid peroxidation (Barbosa *et al*., 2020). The antioxidant effectiveness of these polysaccharides is influenced by factors such as their molecular weight, glycosidic bond type, uronic acid content, water solubility, and monosaccharide composition (Wang *et al*., 2018). For example, a polysaccharide isolated from *P. djamor* showed DPPH free radical scavenging activity with percentages of 27.4 and 52.3 %, and IC50 of 3.83 mg mL^{-1} (Maity *et al*., 2021).

Likewise, fungal antioxidant agents, such as proteins and peptides, have been identified. Liao *et al*. (2020) reported a DPPH free radical inhibition of 91.2 % using protein hydrolysates from *P. geesteranus* when evaluating the antioxidant activity of this primary metabolite. On the other hand, Huang *et al*. (2023) found that proteins and uronic acid present in polysaccharides can enhance their ability to scavenge free radicals.

CONCLUSIONS

Aqueous extracts of *Pleurotus ostreatus* from parental and hybrid strains exhibit antioxidant activity. Soluble sugars, polysaccharides, and proteins were found in lower proportions, potentially contributing to their biological activity. In addition, the hybrid strain, which had the lowest average inhibitory concentration (IC50), represented a promising extract with antioxidant properties.

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