Applied Food Research 5 (2025) 101342

Contents lists available at ScienceDirect

Applied Food Research

journal homepage: www.elsevier.com/locate/afres

Boletus edulis as a healthy and prized edible mushroom: analysis of bioactive compounds and *in vitro* functional properties

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ARTICLE INFO

Keywords:
Edible fungi
Oxidative stress
Diabetes
Inflammation
Cholesterol
Functional food

ABSTRACT

Boletus edulis is a widely consumed edible mushroom, known for its high nutritional value and bioactive compounds of interest. This study aimed to explore its potential bioactive properties by evaluating the inhibitory effects of *B. edulis* extract on key digestive enzymes and its antioxidant capacity through *in vitro* assays. For this purpose, an hydroethanolic extract of *B. edulis* was evaluated phytochemically and through *in vitro* bioassays for antioxidant activity and enzyme inhibition. The extract contained 226 ± 3 mg of ergosterol/100 g, and phenolic compounds such as epigallocatechin-3-gallate (501 ± 18 mg/100 g) and caffeoyl tryptophan (15 ± 1 mg/100 g), consistent with fungal species of functional interest. Cytotoxicity effects of the extract were assessed in HepG2 cells, and results showed no significant effect on the loss of cell viability. Furthermore, it showed antioxidant capacity, including superoxide radical scavenging, nitric oxide and intracellular ROS production as well as a moderate inhibition of metabolic enzymes such as α-glucosidase (IC_{50} = 13 ± 1 mg/mL) and lipase (IC_{50} = 25 ± 11 mg/mL), and the pro-inflammatory enzyme 5-lipoxygenase, with an approximate IC_{50} value of 2.5 mg/mL. These results evidence a multi-targeted bioactive profile that could offer cumulative health benefits with regular consumption. However, further *in vivo* and clinical studies are necessary to confirm its potential as functional food.

1. Introduction

The current era, marked by the constant quest for improved health and wellness, emphasizes its role in nutrition (Witkamp, 2021). Therefore, the research for novel functional foods has become increasingly interesting due to their ability to offer additional benefits for health besides the basic nutrition (Essa et al., 2023). Among these, bioactive compounds of natural origin have demonstrated protective effects against various diseases (Vignesh et al., 2024). In addition, the increasing interest in plant-based diets, such as veganism, has encouraged the search for foods that cover basic nutritional requirements and provide additional health benefits (Łuszczki et al., 2023).

Edible mushrooms, in particular basidiomycetes, have become key elements of the modern diet (Dimopoulou et al., 2022). One of the most consumed and popular worldwide for its nutritional value and exceptional flavour, is the *Boletus edulis*, commonly known as porcini, which belongs to the Boletaceae family (Heleno et al., 2015). *B. edulis* typically grows in temperate forests across Europe, North America, and Asia, establishing symbiotic relationships with pine, oak, and other broadleaf trees. Environmental factors such as climate, soil composition, and altitude in its natural habitat can influence its phytochemical composition and bioactive potential (Sharma et al., 2015). This mushroom species are rich sources of proteins and carbohydrates (with 21.9 g and 59.2 g/100 g, respectively), with a low-fat content approximately 2.6 g/100 g and with a ash content of 6.4 g/100 g (Jaworska et al., 2015). In addition, this type of mushroom is rich in minerals, showing high contents of phosphorus, magnesium, sodium, calcium, among others (Quero et al., 2024). Therefore, the consumption of mushrooms such as *B. edulis*

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https://doi.org/10.1016/j.afres.2025.101342
Received 11 June 2025; Received in revised form 27 August 2025; Accepted 10 September 2025
Available online 11 September 2025

is highly recommended, especially in vegan diets where the supply of proteins, vitamins and minerals can be more difficult to obtain.

Several studies have identified *B. edulis* as a valuable source of polysaccharides, polyphenols, flavonoids, terpenoids, and unsaturated fatty acids. It is especially notable for its high content of ergosterol, a precursor of vitamin D_2, along with bioactive components such as β-glucans, triterpenoids, and antioxidants. These compounds of *B. edulis* not only contribute nutritionally but also therapeutically due to their immunomodulatory, antitumor, antioxidant, antimicrobial, antiviral, hepatoprotective, antiparasitic, anti-diabetic and anti-hypercholesterolemic properties (Chang & Wasser, 2012; Quero et al., 2024; Tan et al., 2022).

Despite its widespread popularity, the mechanisms underlying the health benefits of *B. edulis* remain incompletely understood. Its anti-carcinogenic properties and antioxidant activity have been well documented in several studies (Palacios et al., 2011; Lemieszek et al., 2013; Wang et al., 2014; Özyürek et al., 2014; Jaworska et al., 2015; Lemieszek et al., 2017; Morel et al., 2018; Quero et al., 2024). More recently, *B. edulis* extracts were shown to modulate the gut microbiota *in vitro* from individuals with type 2 diabetes and to reduce blood glucose levels *in vivo*, suggesting their potential in alleviating diabetes-associated dysbiosis (Zanfirescu et al., 2023). Additionally, polysaccharides from *B. edulis* demonstrated antidiabetic and hepatoprotective effects in diabetic rats by reducing blood glucose, improving liver function markers, and modulating oxidative stress and inflammation (Xiao et al., 2019). The extracts, rich in sugars, phenolic compounds, and minerals, have also exhibited anticancer and anti-inflammatory effects by modulating redox balance and inducing cell death in cancer models (Quero et al., 2024).

Although preliminary studies have demonstrated various bioactive properties of *B. edulis,* further research is still needed to characterize its bioactive compounds and elucidate their possible mechanisms of action (Monserrat-Mesquida et al., 2020). This is of particular interest in research since oxidative stress is a key factor in the development and progression of many chronic conditions, including cardiovascular diseases, diabetes, and metabolic syndrome, which affect millions of people worldwide and represent a major public health challenge. Given the global increase in diseases associated with oxidative stress, diabetes and metabolic syndrome, it is essential to further study natural foods such as *B. edulis* that may offer complementary or preventive alternatives.

Given this background and to extend the bioactive characterization of *B. edulis,* it is interesting to evaluate the possible inhibitory activity of the extract against key digestive enzymes, such as α-glucosidase, α-amylase and pancreatic lipase, all of which are fundamental in the enzymatic breakdown and subsequent absorption of carbohydrates and lipids. Inhibition of these enzymes represents a relevant therapeutic approach in the control of postprandial glycemia and fat absorption (Birari & Bhutani, 2007; Tundis et al., 2010; Kumar et al., 2011). Some studies have also determined the antidiabetic properties of mushrooms in general. According to some studies, homopolysaccharides such as β-glucans present in mushrooms may affect insulin metabolism by regulating insulin secretion through hormonal signaling mechanisms (Friedman, 2016). However, determining other mechanisms of action is of interest because diabetes, especially type 2 diabetes, is a multifactorial disease that is not solely reduced to a deficit in insulin production. One of the alternative approaches that has not been evaluated in *B. edulis* is the inhibition of glycosidases, such as α-glucosidase and α-amylase. By inhibiting these enzymes, enzymatic digestion and subsequent absorption of carbohydrates is delayed, contributing to a more controlled postprandial rise in blood glucose levels (Kaur et al., 2021).

Hence, this article aims to evaluate the general bioactivity of *B. edulis* to demonstrate that in addition to its nutritional interest, it is also of interest for improving health. To do this, its phytochemical characterization and the evaluation of its antioxidant, anti-inflammatory, anti-lipidemic and anti-diabetic activity were performed to know the possible action mechanisms.

2. Material and methods

2.1. Experimental design

This study was designed to evaluate the phytochemical profile and multiple bioactivities of *B. edulis* extract. The workflow included: (1) collection, identification, and lyophilization of *B. edulis* fruiting bodies; (2) preparation of a crude hydroethanolic extract (80 % ethanol); (3) phytochemical characterization through spectrophotometric assays and HPLC analysis; (4) evaluation of antioxidant capacity using both chemical and cellular assays; (5) assessment of anti-inflammatory and enzymatic inhibitory activities, including lipoxygenase, α-glucosidase, α-amylase, and pancreatic lipase inhibition assays; and (6) statistical analysis of all results. All experiments were performed in triplicate to ensure reproducibility and reliability.

2.2. Boletus edulis collection and preservation

The mushroom species used in this study is wild *Boletus edulis*, which was collected in an Albarracín pine-oak forest (Teruel, Spain) during the year 2023. This region is characterized by a Mediterranean climate with marked seasonal variations, moderate altitude, and calcareous soils, which can influence the biosynthesis and accumulation of bioactive compounds in fungi. Given the widespread occurrence and distinctive characteristics of *B. edulis* in the collection area, specimens were identified based on macroscopic morphological characteristics by experienced mycologists familiar with the local mycobiota. The fruiting bodies were of the similar size according to a previous procedure; the mushrooms were lyophilised and kept at room temperature.

2.3. Bioactive compounds extraction from Boletus edulis

The extraction of *B. edulis* was carried out by solid-liquid extraction (SLE). For this purpose, an amount of 5 g of the material was exposed to 100 mL of an 80 % (v/v) ethanol solution and processed via ultrasonic extraction for a duration of 40 min at ambient conditions using an ultrasonic bath (AISI304 stainless steel chamber, 240 × 140 × 100 mm; frequency 40 kHz; ultrasound power 120 W; heating power 100 W; chamber volume ~3.3 L; power supply 220 V). Following the extraction, the suspension was clarified by removing particulate matter through filtration with standard-grade filter paper. Once filtered, the extract was brought to dryness by rotary evaporator at 37 °C and then lyophilised to remove possible humidity. The extracts were kept frozen at −20 °C until the subsequent analysis.

2.4. Phytochemical characterisation by chromatographic techniques

2.4.1. Target analysis of ergosterol

Ergosterol quantification was performed preparing the extracts at a concentration of 20 mg/mL in methanol and filtration was performed using a nylon membrane with a 0.22 μm pore diameter. The quantification was conducted using high-performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector system following a procedure reported by previously published authors (Barreira et al., 2014). Knauer HPLC system coupled to a Smartline 2500 UV detector (Knauer, Berlin, Germany) was used. Chromatographic separation was performed using a mobile phase of acetonitrile:methanol (70:30, v/v), pumped isocratically at 1 mL/min. The wavelength selected for the detection was 285 nm, and data were processed with Clarity 2.4 Software (DataApex, Pod Ohradska, Czech Republic). The amount of ergosterol (mg/100 g of extract) was calculated by a calibration curve generated using a commercially standard of ergosterol (E6510; CAS number: 57–87–4) adquired from Sigma-Aldrich (St. Louis, MO, USA).

2.4.2. Untargeted analysis of phenolic compounds

To this end, the extracts were redissolved in a solution of ethanol at

80 % (v/v) until 10 mg/mL and re-filtered with a 0.22 μm filter. This analysis was performed via high performance liquid chromatography equipped with a diode detector and mass spectrometer (HPLC-DAD-ESI-MS/MS), using the analytical parameters outlined by Bessada et al. (2016). An Orbitrap Exploris 120 (Thermo Scientific, Hemel Hempstead, UK) mass spectrometer, fitted with a negative-mode electrospray ionization (ESI) source, was connected to the LC system. Instrumental parameters included a source voltage of 2.5 kV, the sheath gas flow was adjusted to 50 arbitrary units (a.u.), with the auxiliary and sweep gas flows at 10 and at 1 a.u., respectively. The capillary and vaporizer temperatures were maintained at 325 °C and 350 °C, respectively. Fragmentation of precursor ions was carried out via high-energy collisional dissociation (HCD) at a fixed normalized collision energy of 30 %. Mass spectral data were collected over an *m/z* range of 100 to 1500. Instrument operation and data collection were conducted using Xcalibur software v4.6 (Thermo Fisher Scientific). Structural elucidation was based on precise mass determination, diagnostic fragmentation patterns, and previously published data. Quantification was performed using external calibration curves of gallic acid ($y = 23548x - 18{,}776$) for epigallocatechin-3-gallate and caffeic acid ($y = 50042x - 27{,}910$) for caffeoyl tryptophan. Commercial standards (gallic acid: G7384; CAS number: 149–91–7 and caffeic acid: C0625; CAS number: 331–39–5) used for calibration were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.5. Cell cultures and cell viability assay

HepG2 cells, derived from human hepatocellular carcinoma, were used to evaluate the possible cytotoxicity of *B. edulis*. Cell cultures obtained from ATCC were propagated in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) enriched with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Sigma-Aldrich). The cells were grown at 37 °C under a humidified atmosphere containing 5 % CO_2. The cytotoxic activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983) with some modifications. For this purpose, 20,000 cells were seeded into individual wells of a 96-well plate and incubated for 24 h to promote cell attachment. After this period, the culture medium was replaced with DMEM containing 10 % FBS. Extracts at different concentrations (between 0.03 and 1 mg/mL) were added to the test wells, whereas control wells contained only culture medium, without any extract. Treatments were applied for different times particularly 24, 48 and 72 h. After treatment, 100 μL of MTT stock solution (0.4 mg/mL in DMEM 10 % FBS) was added to each well. Subsequently, incubation continued for 3 h at 37 °C under 5 % CO_2, with the plates kept protected from light. Once formazan crystals formed, the MTT reagent was removed from the wells, and dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals, resulting in a purple-colored solution. Absorbance was then recorded at 550 nm using a Synergy H1 hybrid multimode reader (Biotek Instruments, Bad Friedrichshall, Germany). Then, the cell viability in this assay was measured according to the Eq. (1).

$$\textbf{\textit{Cell viability}}\ (\%) = \left(\frac{\textbf{\textit{Abs sample}}}{\textbf{\textit{Abs control}}}\right) \times 100 \quad (1)$$

2.6. Evaluation of antiradical properties by in vitro bioassays

2.6.1. Superoxide radical scavenging capacity

The xanthine/xanthine oxidase (X/XO) system was employed to generate superoxide radicals following the protocol described by Les et al. (2015). In each well, 240 μL of NBT solution—composed of 90 μM xanthine, 22.8 μM NBT (pH 7.0, phosphate buffer), and 16 mM Na_2CO_3—was combined with 30 μL of extract at various concentrations (between 0.03 and 1 mg/mL). The reaction commenced upon addition of 30 μL xanthine oxidase (168 U/L). The activity of the *B. edulis* extract was determined by its capacity to prevent the NBT reduction to the blue formazan, indicating superoxide radical scavenging, with absorbance recorded at 560 nm and calculated by Eq. (2). Quercetin (Fluorochem, Barcelona, Spain) served as the assay's positive control. All assays were carried out three times.

$$\textbf{\textit{Inhibition}}\ (\%) = \left[\frac{(\textbf{\textit{Abs control}} - \textbf{\textit{Abs sample}})}{\textbf{\textit{Abs control}}}\right] \times 100 \quad (2)$$

2.6.2. Nitric oxide-scavenging activity

Nitric oxide scavenging activity was assessed following the protocol described by Sreejayan and Rao (1997) by preparing different concentrations of *B. edulis* extract (between 0.12 and 1 mg/mL) in 0.1 M phosphate buffer (pH 7.4), which were then incubated with sodium nitroprusside dihydrate (Sigma-Aldrich) in 96-well plates. The mixtures were subjected to light exposure at room temperature for 1 h. After this incubation, Griess reagent (Sigma-Aldrich) was added to each well, followed by a 10-minute incubation period in darkness to allow for colorimetric reaction development. Absorbance measurements were performed at 560 nm to quantify nitric oxide neutralization. Quercetin served as the assay's positive control. All assays were carried out three times. The inhibition values were calculated using Eq. (2).

2.6.3. Assessment of the possible production of intracellular reactive oxygen species (ROS)

The evaluation of the possible production of intracellular reactive oxygen species (ROS) was carried out following the protocol of Lebel et al. (1992) with minor modifications. For this purpose, HepG2 cells were initially plated in 96-well microplates and allowed to attach over a 24-hour period, as detailed in Section 2.4. Following this, the medium was replaced with a freshly prepared solution containing phosphate-buffered saline (PBS), glucose, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Barcelona, Spain). This solution was protected from light and incubated with the cells at 37 °C for 30 min in a humidified environment supplemented with 5 % CO_2. Following incubation, the wells were double-washed with fresh glucose-PBS to eliminate excess DCFH-DA and different concentrations of *B. edulis* (between 0.12 and 1 mg/mL) in PBS, along with 500 μM hydrogen peroxide were added to the plate. Finally, measurements of fluorescence were carried out with a wavelength of excitation of 480 nm and an emission of 520 nm. Kinetic fluorescence readings were recorded over a 90-minute period, with measurements taken at ten time points. Results were reported as the intracellular percentage of ROS production relative to non-treated control. Tests were conducted in triplicate.

2.7. Determination of enzymes inhibition with relevant biological activities

In this section, anti-diabetic activity was evaluated by assessing the reduction in the function of digestive enzymes catalyzing carbohydrate hydrolysis like α-glucosidase and α-amylase. Subsequently, the activity against obesity and lipid metabolism was evaluated by inhibition of pancreatic lipase. And finally, the anti-inflammatory capacity was assessed by inhibition of the 5-LOX pathway related to leukotriene synthesis.

2.7.1. Inhibitory effects on carbohydrate-metabolising enzymes: α-glucosidase and α-amylase

The inhibitory effect of α-glucosidase of *B. edulis* extract was evaluated using the enzyme derived from *Saccharomyces cerevisiae* (Sigma-Aldrich, Barcelona, Spain) utilizing a 96-well microplate setup following the protocol described by Kazeem et al. (2013) with slight modifications. Each well was treated with 50 μL of the test sample at different concentrations (between 0.2 and 25 mg/mL), followed by the addition of 100 μL of α-glucosidase solution at a concentration of 1 U/mL, and 50 μL of 4-nitrophenyl α-d-glucopyranoside (pNPG) at a final concentration of

3 mM (Sigma-Aldrich, Barcelona, Spain). All reagents were prepared using a phosphate buffer composed of 12.5 mM disodium hydrogen phosphate (Na_2HPO_4) and 3.3 mM monosodium dihydrogen phosphate (NaH_2PO_4), adjusted to pH 6.9. Buffer (50 μL) was administered to control wells as a substitute for the sample, while blank wells were set up by substituting the enzyme with 100 μL of buffer to account for background absorbance. Absorbance at 405 nm was determined after a 10-minute incubation period at 37 °C to assess enzyme inhibition following Eq. (2). Acarbose served as the positive control (reference inhibitor) in this assay. All assays were carried out three times.

Inhibition of α-amylase activity was evaluated following the protocol described by Kazeem et al. (2013) with slight modifications by combining equal volumes (100 μL each) of *B. edulis* extract at different concentrations (between 0.2 and 25 mg/mL), and centrifuged porcine pancreatic α-amylase solution (0.5 mg/mL) in 2 mL Eppendorf tubes, followed by incubation at 37 °C for 5 min prior to further analysis. All substances were prepared in a phosphate buffer containing 20 mM Na_2HPO_4 and 6 mM NaCl at pH 6.9. Then, 1 % starch solution (Sigma-Aldrich) was introduced into each tube, and the reaction mixtures were subsequently incubated for 10 min under identical temperature conditions. To stop the enzymatic reaction and develop colour, 200 μL of solution 1 % of 3,5-dinitrosalicylic acid (DNS) (Sigma-Aldrich, Barcelona, Spain) and 50 μL of sodium hydroxide solution (1 M) were added and the mixture was incubated at 100 °C for 5 min. After the reaction concluded and a color change was observed, samples were immediately cooled in an ice bath. In control wells, 100 μL of buffer was added instead of the sample, while blanks were prepared by substituting the enzyme with an equal volume of buffer to account for background absorbance. Subsequently, each solution (200 μL) was loaded into a 96-well microplate, and the absorbance was recorded at 540 nm using a microplate spectrophotometer to evaluate inhibitory activity according to the specified Eq. (2). Gallic acid was employed as the positive control in this assay. All assays were carried out three times.

2.7.2. Effects on pancreatic lipase activity

The inhibitory activity of lipase was quantified using a 96-well microplate based on a previous protocol with some modifications (Spínola et al., 2017). Each well was done by combining 40 μL of *B. edulis* extracts at different concentrations (between 0.8 and 100 mg/mL), with 40 μL of centrifuged porcine pancreatic lipase type II with porcine origin (Sigma-Aldrich, Barcelona, Spain) prepared at 2.5 mg/mL and subsequently incubated for 15 min at room temperature. Subsequently, 20 μL of a 10 mM solution of *p*-nitrophenyl butyrate (NPB) (Sigma-Aldrich, Barcelona, Spain) was added to initiate the reaction. Absorbance at 405 nm was recorded after incubating the reaction mixtures at 37 °C for 15 min. All components, including the extract, were previously dissolved in phosphate buffer. The percentage of enzyme inhibition was determined in accordance with Eq. (2). Orlistat was used as the reference inhibitor in this assay and all assays were carried out three times.

2.7.3. 5-lipoxigenase (5-LOX) inhibition as anti-inflammatory mechanism

5-Lipoxygenase (5-LOX) inhibitory activity was evaluated by monitoring the enzymatic conversion of linoleic acid into 13-hydroperoxylinoleic acid, using a microplate-based assay adapted from a previously established protocol (Macedo et al., 2020). Different concentrations of *B. edulis* extract (between 0.15 and 2.5 mg/mL) were done in sodium phosphate buffer (100 mM) adjusted to pH 9.0. Each well contained phosphate buffer, sample extract, and 20 μL of soybean-derived 5-LOX (Glycine max; Sigma-Aldrich). Following a 5-minute preincubation at room temperature, 20 μL of linoleic acid solution (4.18 mM) were added. The oxidation process was tracked by measuring absorbance at 234 nm over a period of 3 min using a spectrophotometer. The inhibition percentage was obtained by calculating the slopes of the kinetic measurements and using Eq. (2). Quercetin (Fluorochem, Barcelona, Spain) was employed as a positive control under identical conditions. Each experiment was performed in triplicates.

2.8. Statistical analysis

Experimental procedures were independently performed on at least three separate occasions to ensure reproducibility. The presented data correspond to the mean values accompanied by the standard error of the mean (SEM). Statistical analyses were conducted utilizing GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, *CA*, USA). Outlier detection was performed using Grubbs' test. Data analysis included nonlinear regression modeling, as well as one-way analysis of variance (ANOVA), coupled with Dunnett's post hoc test for multiple comparisons, was used to assess statistical significance, which was accepted at $p < 0.05$ and 95 % confidence.

3. Results

3.1. Phytochemical characterisation by HPLC

B. edulis extract was analyzed by UHPLC-MS/MS and two peaks were tentatively identified (Fig. 1). Peak 1 showed retention time of 3.68 min, had [M-H]- at *m/z* 457, which showed a neutral loss of 288 Da that could be assigned to the elimination of dehydrated epigallocatechin molecule. The compound exhibited predominant fragment ions at *m/z* 125 and 169, which are indicative of gallic acid, as previously reported (Singh et al., 2016). Consequently, this substance was provisionally identified as epigallocatechin 3-gallate. Peak 2, detected at a retention time of 13.55 min, presented a deprotonated molecular ion at *m/z* 365 and a primary fragment ion at *m/z* 203. Considering this fragmentation profile alongside existing literature, the compound was tentatively characterized as caffeoyl tryptophan, which has been reported in *Coriandrum sativum* L., coffee beans and *Bituminaria bituminosa* (Baeza et al., 2016; Barros et al., 2012; Llorent-Martinez et al., 2015). Quantitative analysis revealed that the levels of these compounds were 501 ± 18 mg/100 g and 15 ± 1 mg/100 g, respectively.

On the other hand, HPLC-UV was used to carry out ergosterol quantification with a calibration line of the ergosterol standard acquired from Acrōs Organics (Fair Lawn, NJ, USA). Confirmation of the compound's identity was achieved by matching the retention time of the sample (14.550 min) with that of the corresponding commercial standard (14.533 min). The chromatogram is presented in Fig. 2. A total of 226 ± 3 mg of ergosterol per 100 g of extract was obtained.

3.2. Cell viability assay

Cell viability (%) of *B. edulis* extract was evaluated by MTT assay, treating HepG2 cells at three different times (24, 48 and 72 h). Subsequently, the cells were treated with varying concentrations of *B. edulis* (between 0.03 and 1 mg/mL). As shown in Fig. 3, cell viability decreased to values close to 70 % only at the highest concentration and longest exposure time. Additionally, no morphological alterations were observed under light microscopy, further supporting the absence of acute cytotoxic effects.

3.3. Evaluation of antiradical properties by in vitro bioassays

Different studies were performed to assess the antiradical properties of *B. edulis* extract. For this purpose, the following activities were evaluated: superoxide radical scavenging, nitric oxide scavenging and ROS generation in cells.

First, to evaluate superoxide radical scavenging activity of *B. edulis* extract, concentrations up to 1 mg/mL were tested, obtaining an inhibition (%) of X/XO of 40 ± 0.01 % (data not shown). In this case, quercetine was used such as positive control, obtaining a IC_{50} of 10.1 ± 0.1 μg/mL.

The scavenging potential of *B. edulis* upon nitric oxide radical was

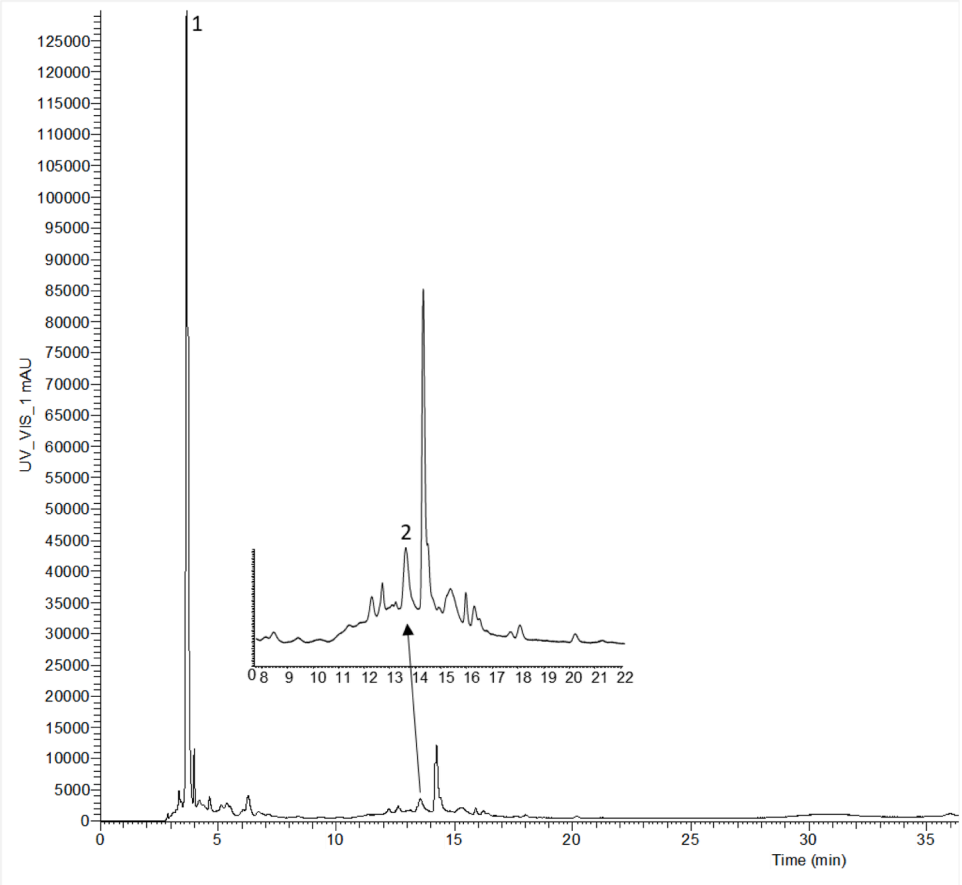

Fig. 1. Representative chromatogram of phenolic compounds in *Boletus edulis* extract recorded at 280 nm. **Peak 1**: Epigallocatechin-3-gallate**; Peak 2**: Tryptophan caffeoyl.

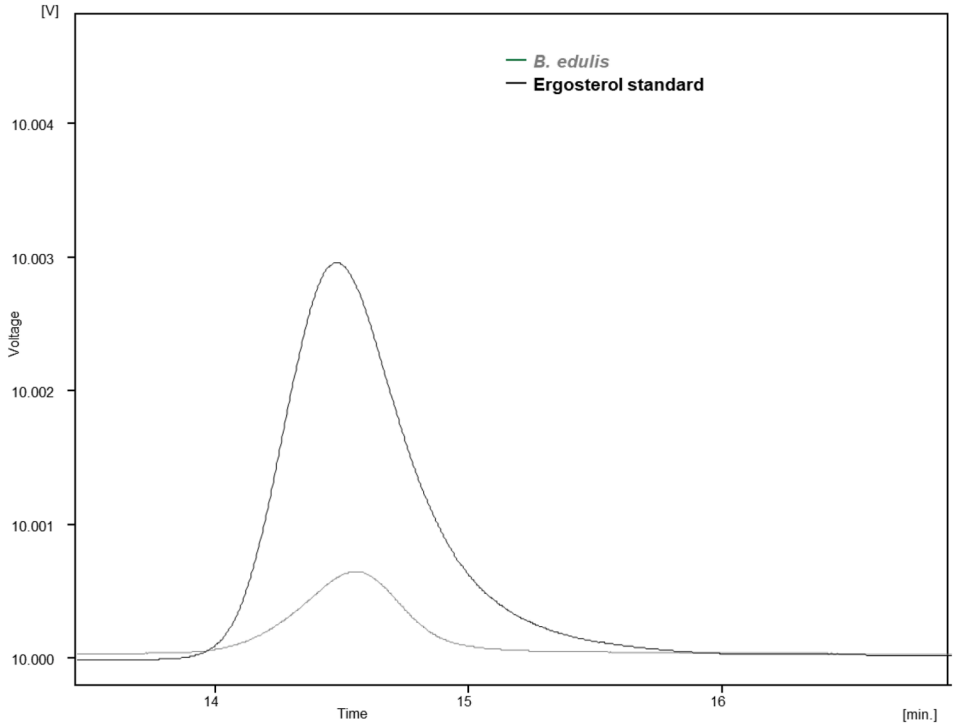

Fig. 2. Ergosterol chromatogram profile of *B. edulis* extract.

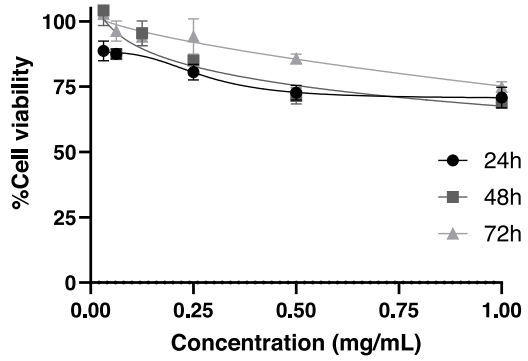

Fig. 3. HepG2 cell viability at 24 h, 48 h and 72 h after incubation with boletus extract. Results are presented as the mean ± standard error of the mean (SEM). Note: *p*-values ≤ 0.05 (*), 0.01 (**), and 0.001 (***), performing a minimum of 3 independent experiments.

assessed employing a cell-free assay. Concentrations between 0.12 and 1 mg/mL were tested. Such as shown in Fig. 4a, the extract exhibited a concentration-dependent activity, showing significant scavenging effects at 0.5 mg/mL and 1 mg/mL ($p < 0.05$) and $p < 0.01$, respectively), with a maximum nitric oxide radical scavenging of 20.18 %. In this case, quercetine was used such as positive control, obtaining a IC_{50} of 0.08 ± 0.01 mg/mL.

Finally, the impact of *B. edulis* extract in the reduction of ROS production was evaluated in HepG2 cells stressed with H_2O_2. Concentrations between 0.12 and 1 mg/mL were tested. The percentage of ROS production was normalized to the non-treated control at the beginning of the reaction. As shown in Fig. 4b, intracellular ROS production was evaluated for 90 min, during which ROS production in H_2O_2 stressed cells without treatment increased to 415.04 %. Some stress was also seen in the non-treated control showing values of 190.3 % at 90 min. Concentrations of 0.5 and 1 mg/mL managed to reduce the percentage of ROS production to 347.3 and 280.3 % respectively.

3.4. Determination of enzymes inhibition with relevant biological activities

The assessment of the inhibitory effects on various enzymes implicated in key biological activities, including anti-diabetic, antilipidemic, and anti-inflammatory functions, was conducted (Fig. 5).

First, the inhibitory effects on carbohydrate-metabolizing enzymes showed that *B. edulis* had activity against α-glucosidase (Fig. 5a) but no significant inhibition of α-amylase was detected (data not shown). To do this, concentrations between 0.2 and 25 mg/mL were performed in three different days, obtaining in the case of α-glucosidase a IC_{50} value from *B. edulis* extract of 13 ± 1 mg/mL. Besides, acarbose was performed such

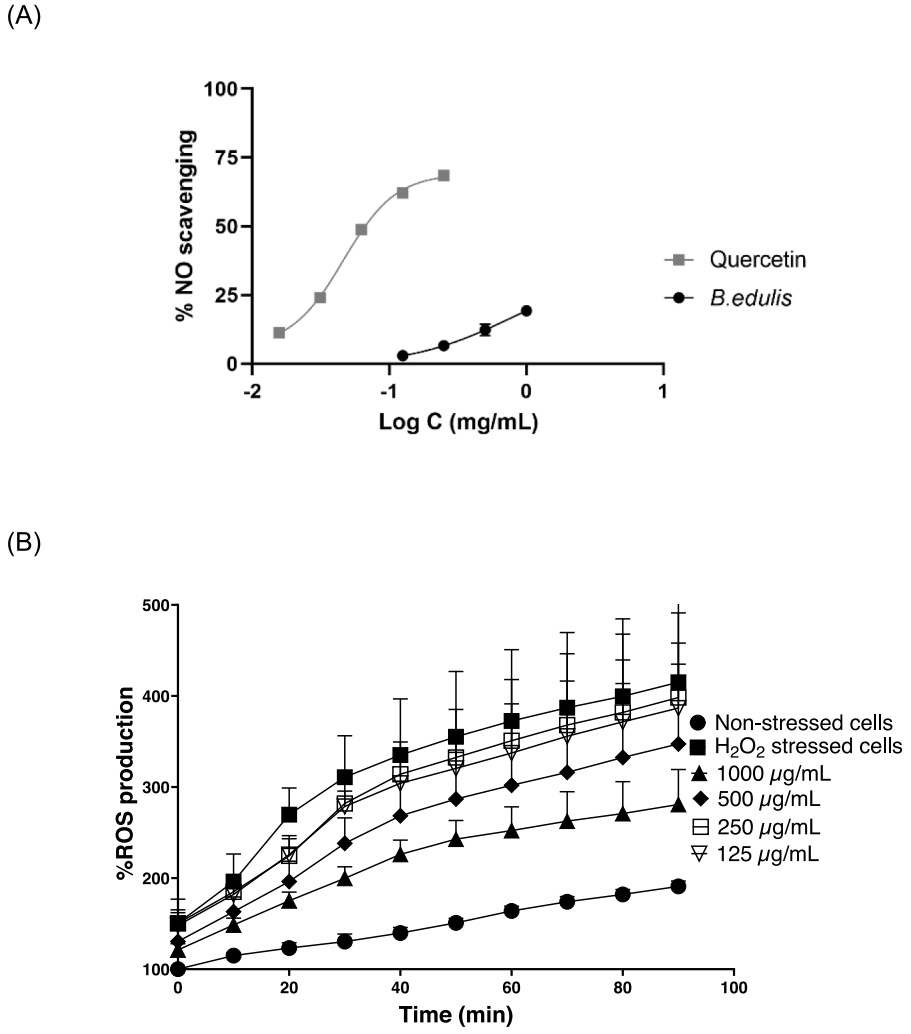

Fig. 4. Antiradical properties evaluated in *B. edulis* extract: (A) nitric oxide scavenging assessed in a cell-free system and (B) intracellular ROS levels in HepG2 cells exposed to hydrogen peroxide-induced oxidative stress, followed by treatment with *B. edulis* extract. The results are reported as the mean ± standard error of the mean (SEM), based on a minimum of three independent experiments, each carried out in triplicate.

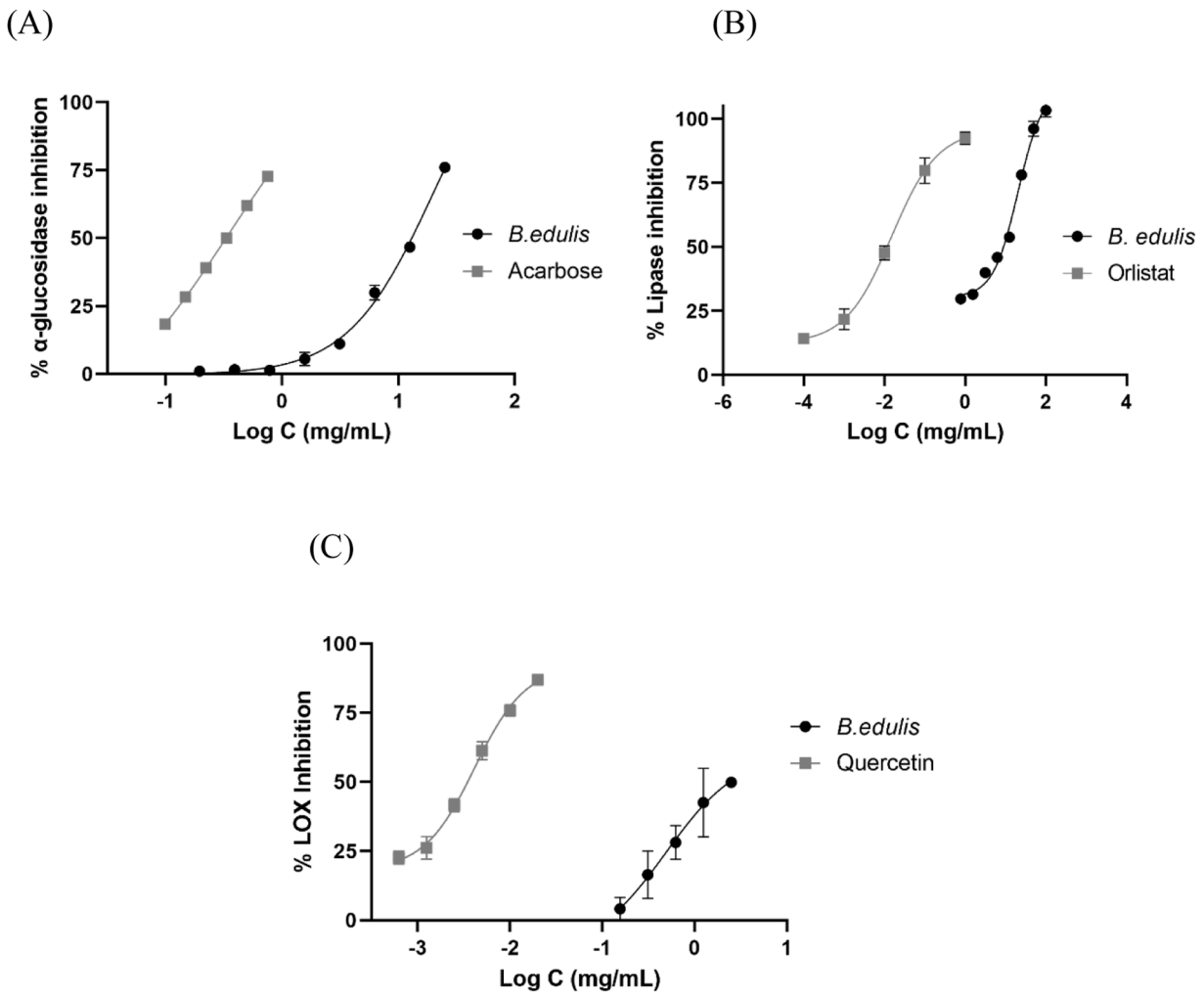

Fig. 5. Inhibition of different enzymes with relevant biological activities: (A) α-glucosidase, (B) lipase, and (C) LOX. Data are presented as mean ± SEM from at least three independent experiments conducted in triplicate. Inhibition of α-amylase was not detected.

as positive control, obtaining 0.34 ± 0.02 mg/mL.

Evaluating the effects on lipase activity to determine the anti-lipidemic activity concentrations between 0.8 and 100 mg/mL were studied with pancreatic lipase. Such as is shown in Fig. 5b, the IC_{50} of *B. edulis* was 25 ± 11 mg/mL and of Orlistat like reference inhibitor was 11 ± 1 μg/mL.

Finally, to evaluate the inhibition of 5-LOX as anti-inflammatory mechanism, concentrations between 0.16 and 2.5 mg/mL were determined. In this case, as shown in Fig. 5c, the highest concentration tested reached 49.9 % inhibition of this enzyme ($p < 0.01$). In this case, quercetine was used such as positive control, obtaining 4 ± 1 μg/mL.

4. Discussion

Edible mushrooms are increasingly recognised as valuable matrices of phytochemicals and compounds with functional properties. However, many species, including *B. edulis*, remain poorly characterized beyond their basic nutritional content (Zhang et al., 2016). This species of mushroom is one of the most prized in gastronomy, is consumed primarily for its flavour and nutritional profile. However, its application as a functional food or natural ingredient remains largely unexplored. Accordingly, the present investigation was designed to assess the *in vitro* biological effects of *B. edulis* extract, focusing on its potential to modulate oxidative stress, carbohydrate and lipid metabolism, as well as inflammatory pathways. These four processes are intimately linked to common diet-associated chronic diseases, including metabolic syndrome, obesity, and type 2 diabetes. The results provide new insights into the functional value of *B. edulis*, revealing moderate but significant effects in assays, showing multi-target action and highlighting its potential as a multifunctional food ingredient with applications in health-oriented food design (Kozarski et al., 2023).

Regarding the safety of the extract, in all tested conditions in this study, viability remained above the commonly accepted cytotoxicity threshold of 70 %, indicating low cytotoxic potential of the extract in HepG2 cells. Besides, the cells retained their characteristic morphology and adherence throughout the incubation period, which is consistent with the cell viability results and suggests that the extracts did not cause visible cell damage under the assay conditions. These results were meaningful, since HepG2 cells, derived from human hepatocarcinoma, are widely used as a representative model of liver metabolism and allow a first approximation on the safety of bioactive compounds in physiological contexts. Similarly, Quero et al. (2024) did not observe cytotoxicity in differentiated Caco-2 cells with hydroethanolic extracts of *B. edulis*. These results reinforce the potential of *B. edulis* extract for use in functional foods or nutraceuticals. In contrast, another study reported antitumor activity in tumour cell lines, although extracts from different fungal species and cell types were used (Kosanić et al., 2016). This also coincides with the results obtained in other types of mushrooms such as *Ganoderma lucidum* which has shown cytotoxic effects on hematological malignant cell lines, with IC_{50} values between 26.8 and 78.4 ppm (Dulay et al., 2022). Therefore, despite the results obtained in *B. edulis* in this and other studies, it is recognised that additional studies are needed,

both in more specific cellular models and *in vivo* studies, to confirm its safety profile under real consumption conditions.

The results obtained show that the hydroethanolic extract of *B. edulis* has a moderate antioxidant activity, with relevant effects in both chemical and cellular assays. The capacity to neutralise the superoxide radical and nitric oxide, although limited in comparison with reference antioxidants, suggests the presence of compounds with antiradical potential, possibly associated with its phenolic content. In line with this, the dose-dependent reduction of ROS in HepG2 cells subjected to oxidative stress by H_2O_2 indicates a possible cellular protective effect, although not completely preventive. These results agree with previous works that have described antioxidant properties in extracts of *B. edulis* and other edible fungi (Nascimento et al., 2020; Quero et al., 2024; Souilem et al., 2017). Overall, the antioxidant capacity of mushrooms is predominantly attributed to their content of bioactive compounds, including phenolic compounds, tocopherols, flavonoids, vitamins, and carotenoids (Reis et al., 2011) and in this study, phenolic compounds such as epigallocatechin-3-gallate (Wang et al., 2025), and caffeoyl tryptophan (Clifford, 2004), were quantified, both in significant concentrations (501 ± 18 mg/100 g and 15 ± 1 mg/100 g respectively).

Specifically, epigallocatechin-3-gallate is a widely studied polyphenol that exhibits potent antioxidant properties (He et al., 2018), as well as a recognized ability to inhibit key enzymes involved in carbohydrate digestion, such as α-glucosidase and α-amylase. Its mechanism of action is based on competitive binding to the active sites of these enzymes, which slows down the release and absorption of glucose (Dai et al., 2020).

As for caffeoyltryptophan, it is a particularly interesting compound with potential antioxidant activity (Ramalakshmi et al., 2008; Babova et al., 2016). However, its biological activities in fungi have been scarcely explored. To the best of our knowledge, some caffeic acid derivatives have been identified in some mushroom species (Lia et al., 2013), although they have not been the subject of detailed studies. This highlights the need for future research aimed at clarifying their role in the bioactivities observed in *B. edulis*.

In addition, significant concentrations of ergosterol were quantified in the present work, namely 226 ± 3 mg ergosterol /100 g extract. This amount is consistent with the levels reported in other species of edible mushrooms (Román-Hidalgo et al., 2021). It is the predominant sterol in fungal species and acts as a precursor of vitamin D2, attracting attention due to its numerous potential health-promoting activities. Besides its antioxidant activity, as it can help neutralise free radicals, contributing to the prevention of cell damage, its main function is its hypocholesterolemic effect due to its competition for absorption in the intestine because of its cholesterol-like structure, thus decreasing its incorporation into the bloodstream (Das & Kumar 2021).

It is important to highlight that *B. edulis* samples analyzed in this study were collected from a pine-oak forest in Albarracín (Teruel, Spain), an area characterized by a Mediterranean climate with hot, dry summers and cold, wet winters, and acidic, well-drained soils favorable for mushroom growth. These environmental conditions, including climate, soil composition, altitude, and local flora, can influence the metabolic pathways and phytochemical profile of the mushrooms. Therefore, the phytochemical properties and bioactive compound concentrations observed in these samples may reflect specific adaptations to the local habitat, which could differ from those found in specimens collected in other geographic regions (Fogarasi et al., 2018; Luo et al., 2024). Therefore, natural variability is inherent to wild fungi and should be considered when extrapolating or comparing results to those obtained from other regions or harvest periods.

In line with the importance of inhibiting key digestive enzymes for the control of postprandial glycemia, this study evaluated the inhibitory activity of *B. edulis* extract on α-glucosidase, α-amylase and pancreatic lipase. The obtained results provide new evidence on the potential of this species as a natural modulator of carbohydrate and lipid metabolism, expanding the knowledge on its previously described bioactive properties.

In this study, *B. edulis* extract exhibited a moderate inhibitory effect on α-glucosidase, with an IC_{50} value of 13 ± 1 mg/mL, although less potent than acarbose, the standard pharmacological inhibitor. No relevant inhibition was observed against α-amylase, suggesting selective enzymatic activity. Several studies have reported that extracts from medicinal and edible mushrooms can inhibit digestive enzymes relevant to carbohydrate metabolism, supporting their potential as natural antidiabetic agents. For instance, hexane extracts of *Cystoderma rutilus* and *Pleurotus ostreatus* demonstrated stronger α-amylase and α-glucosidase inhibition than acarbose itself (Deveci et al., 2021). Likewise, a comparative study with seven edible mushroom species commercially available in Japan found that the methanolic extract of *B. edulis* had the highest α-glucosidase inhibitory activity, with an IC_{50} of 1.27 mg/mL (Bhattacharjya et al., 2020). These differences highlight the influence of extraction methods, solvent polarity, and geographical or environmental factors on the bioactive profile and potency of mushroom-derived compounds. Although the inhibitory activity observed in the present study was moderate compared to some of these reports, it remains relevant, particularly when considering the use of 80 % ethanol, a food-grade, efficient solvents, reflects realistic extraction conditions for potential nutraceutical development. Therefore, the obtained results contribute to the expanding evidence base supporting *B. edulis* as a functional food candidate for the dietary management of postprandial glycemia and metabolic disorders.

Edible and medicinal mushrooms of different species have been shown to reduce triglyceride levels in the blood (Anusiya et al., 2021). The results of the present work showed that pancreatic lipase inhibition was modest, with an IC_{50} value of 25 ± 11 mg/mL, and significantly higher (less potent) than that of orlistat. However, the observed inhibitory activity suggests that the extract may have some potential to influence lipid absorption and metabolism.

Finally, mushrooms are a source of bioactive constituents such as triterpenoids and polysaccharides, which have been associated with notable anti-inflammatory activity (Muszyńska et al., 2018). In the present work it was shown that 2.5 mg/mL was the approximate IC_{50} value. This reflects a moderate level of inhibition, implying the presence of bioactive constituents with anti-inflammatory properties capable of modulating the LOX pathway, a key route in the biosynthesis of pro-inflammatory leukotrienes. The inhibition of LOX is of particular relevance, as it may play a role in mitigating inflammatory responses linked to the pathophysiology of various chronic disorders. This effect may be synergistically enhanced by the previously mentioned mild NO scavenging activity, given the dual role of nitric oxide in inflammation, as its excessive production is associated with various inflammatory disorders (Kim & Lee, 2025).

In conclusion, the results obtained with 1 mg/mL of *B. edulis* extract show a balanced bioactive profile. At this concentration, acceptable levels of cell viability (70 %) were maintained, while notable inhibition of xanthine oxidase (40 %), 5-lipoxygenase (35 %), and lipase (30 %) was observed, as well as a reduction in the percentage of ROS production (280 %). In contrast, nitric oxide inhibition was moderate (20 %), α-glucosidase inhibition was lower (5 %), and no inhibitory activity was detected against α-amylase. These findings, when compared across the different tests performed, suggest that the extract has a more relevant profile in terms of antioxidant and anti-inflammatory activity, with a secondary contribution to the modulation of carbohydrate digestive enzymes. Consequently, these results support its potential in regulating oxidative stress and lipid metabolism, rather than in directly controlling postprandial blood glucose levels.

Overall, the results confirm that *B. edulis*, in addition to its nutritional value, presents several biologically relevant activities *in vitro*, which may act synergistically to offer health benefits. It is important to note that the concentrations used in these *in vitro* assays are often higher than those achievable through normal dietary intake. Therefore, while *B. edulis* extracts show promising multi-target bioactivities, these

findings should be interpreted cautiously regarding physiological relevance. Nevertheless, regular consumption may provide cumulative or synergistic effects contributing to health promotion. These findings justify future research on its bioactive components, bioavailability and *in vivo* efficacy, highlighting its potential for applications in functional foods and preventive nutrition.

5. Conclusions

This study underscores the relevance of *B. edulis* not only as a nutritionally rich traditional food, but also as a matrix of molecules with functional and therapeutic applications. The hydroethanolic extract analysed presented a considerable concentration of ergosterol and relevant phenolic compounds, such as epigallocatechin-3-gallate and tryptophan caffeoyl, which agrees with what has been observed in other fungal species of nutraceutical interest. In terms of function, the extract exhibited moderate antioxidant effects, demonstrated by its capacity to scavenge superoxide radicals and nitric oxide, as well as to decrease intracellular reactive oxygen species (ROS) production. Furthermore, moderate inhibitory activity against enzymes involved in carbohydrate and lipid digestion, specifically α-glucosidase and pancreatic lipase, as well as of the pro-inflammatory enzyme 5-lipoxygenase was observed, suggesting a multi-targeted bioactive profile, capable of intervening in different key physiological processes.

Preliminary assays in the HepG2 cell line indicate that the *B. edulis* extract does not produce significant cytotoxic effects under the evaluated conditions, suggesting a favorable safety profile. Nevertheless, as these findings are limited to a single *in vitro* model, additional investigations across different cell lines and *in vivo* systems are necessary to comprehensively establish its safety and toxicity profile. Overall, these results support the potential of *B. edulis* as a functional ingredient within the preventive nutrition and health promotion approach, thanks to its broad and moderate bioactive profile, and justify further studies to explore its bioavailability, mechanisms of action and practical applications in the design of functional foods or nutraceutical supplements.

CRediT authorship contribution statement

Gema Casado-Hidalgo: Writing – original draft, Investigation, Formal analysis. **Pilar Cebollada:** Investigation, Formal analysis. **Javier Cano-Lou:** Investigation. **Rossana V.C. Cardoso:** Investigation. **Lillian Barros:** Investigation. **Maria Jesús Rodrígez-Yoldi:** Conceptualization. **Víctor López:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Government of Aragon for funding the Phyto-Pharm group (ref. B44_23R) and Universidad San Jorge for supporting Proyecto Interno 2425013 through the Internal Research Projects Call for 2024-2025 academic year, as well as for providing the postdoctoral grant for Gema Casado-Hidalgo.

The authors express their gratitude to the Foundation for Science and Technology (Portugal) for providing funds FCT/MCTES (PIDDAC) allocated to CIMO, under project numbers UIDB/00690/2020 (DOI: 10.54499/UIDB/00690/2020) and UIDP/00690/2020 (DOI: 10.54499/UIDP/00690/2020); and SusTEC, LA/P/0007/2020 (DOI: 10.54499/LA/P/0007/2020). Appreciation is also extended to the Agenda VIIA-FOOD - Platform for Valorization, Industrialization and Commercial Innovation for Agrifood (contract no. C644929456–00000040) supporting R. Cardoso's contract. This is funded by PRR (www.recuperarportugal.gov.pt) and financed by the European Union throughNext Generation EU. The authors thank Rubén Escribano and Fernando (CITA, Zaragoza) for providing the mushrooms and Eduardo Aranda-Cañada for helping with some bioassays.

Data availability

Data will be made available on request.

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