

Article

Tuber borchii Extracts Buffer Galactose-Induced Skeletal Muscle Sarcopenia in C2C12 Myotubes

Vincenzo Aiello ^{1,2} , Leonardo Lupacchini ² , Manuel Belli ^{1,2} , Mario Cristina ³ , Luigi Sansone ^{1,2} , Gabriele Di Marco ⁴ , Angelo Gismondi ⁴ , Alessandro Pennesi ⁵, Maria Rosa Ciriolo ^{2,4}, Serena Castelli ^{1,2,†}  and Sara Baldelli ^{1,2,*,†} 

¹ Department for the Promotion of Human Science and Quality of Life, San Raffaele Open University, Via di Val Cannuta, 247, 00166 Rome, Italy; vincenzo.aiello@uniroma5.it (V.A.); manuel.belli@uniroma5.it (M.B.); luigi.sansone@uniroma5.it (L.S.); serena.castelli@uniroma5.it (S.C.)

² IRCCS San Raffaele Roma, 00166 Rome, Italy; leonardo.lupacchini@sanraffaele.it (L.L.); ciriolo@bio.uniroma2.it (M.R.C.)

³ San Raffaele Sulmona, Viale dell'Agricoltura, 67039 Sulmona, Italy; mario.cristina@sanraffaele.it

⁴ Department of Biology, University of Rome "Tor Vergata", 00133 Rome, Italy; gabriele.di.marco@uniroma2.it (G.D.M.); gismondi@scienze.uniroma2.it (A.G.)

⁵ Department of Biomolecular Sciences, University of Urbino, 61029 Urbino, Italy; a.pennesi@campus.uniurb.it

* Correspondence: sara.baldelli@uniroma5.it

† These authors contributed equally to this work.

Abstract

Background/Objectives: Sarcopenia involves a gradual decline in skeletal muscle mass that may occur during aging or in association with chronic pathological conditions. It markedly reduces muscle strength and mobility, thereby impairing quality of life. Because sarcopenia's severity directly correlates with frailty, it represents an important predictor of prognosis and disease risk. Current preventive and therapeutic strategies rely mainly on physical activity, which is not feasible for all patients. This study investigated the biological effects of two independently prepared *Tuber borchii* (*T. borchii*) extracts in an in vitro model of sarcopenic stress. **Methods:** The activity of *T. borchii* extracts was investigated in a cell-based model of sarcopenia, following previous observations that these preparations influence proliferation-related pathways, including ERK1/2 phosphorylation. Specifically, differentiated myotubes were exposed to D-galactose to reproduce atrophy-associated cellular changes, and the impact of *T. borchii* extracts on protein synthesis, turnover, and cell morphology was assessed. **Results:** *T. borchii* extracts enhanced protein synthesis and turnover in myotubes. Furthermore, the treatment significantly reduced the expression of key galactose-induced sarcopenia and atrophy markers, such as MuRF1. Morphological analysis confirmed this protective effect, showing that treated myotubes maintained greater thickness and exhibited a larger cross-sectional area despite exposure to the sarcopenic stimulus. **Conclusions:** These results indicate that *T. borchii* extracts can attenuate selected cellular alterations associated with muscle aging. Future identification of the most active components may support their development as nutraceutical supplements.

Keywords: myotubes senescence; skeletal muscle; *Tuber borchii*; atrophy; D-galactose; MuRF1; Atrogin-1



Received: 18 June 2026

Revised: 16 July 2026

Accepted: 21 July 2026

Published: 24 July 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Sarcopenia represents one of the most significant challenges arising in association with aging and metabolic impairment and involving a gradual decline in muscle mass,

force-generating capacity, and tissue quality. This condition not only compromises physical function but also contributes to a greater incidence of falls, fractures, disability, and adverse outcomes [1]. At the cellular level, sarcopenic muscle exhibits defective regulation of protein turnover together with persistent redox imbalance and age-associated inflammatory signaling [2–4]. Excessive production of reactive oxygen species (ROS) has been shown to accompany skeletal muscle sarcopenia, with increased oxidative damage to lipids, DNA, and proteins [5]. Inflammation also plays a crucial role: circulating cytokines signaling pathways controlling both anabolic processes and proteolytic activity in muscle [6]. Furthermore, one of the pillars of muscle loss in sarcopenia is the activation of the ubiquitin–proteasome system (UPS), with the upregulation of the muscle-specific E3 ligases Atrogin-1 and MuRF1, thereby enhancing the proteasomal removal of contractile components and contributing to myotube wasting [7,8]. In parallel, silencing of the mammalian target of rapamycin (mTOR) signaling pathway is often observed, resulting in a drastic reduction in overall protein synthesis [9,10]. In this scenario, scientific research is focused on identifying nutraceuticals capable of counteracting these processes. Truffles, hypogaeal fungi belonging to the *Tuber* genus, have emerged as promising sources of bioactive secondary metabolites, including phenols, flavonoids, terpenoids, and antioxidants such as glutathione [11]. Experimental evidence supports the potential health-promoting properties of these secondary metabolites, particularly their antioxidant, anticancer, and anti-inflammatory activities [12–14]. Through their ability to neutralize reactive species and limit oxidative cellular injury, these compounds may have therapeutic relevance, including a potential supportive role in the management of sarcopenia [15]. However, although effective, such interventions may not be applicable to all patients or may need to be complemented by a deeper understanding of the molecular mechanisms.

In this context, the identification of naturally occurring bioactive molecules represents a promising strategy to directly counteract or bypass the biochemical pathways of sarcopenia, offering targeted therapeutic solutions that complement traditional protocols.

Expanding upon prior findings that established the capacity of *Tuber borchii* (*T. borchii*) Vittad extracts to modulate key proliferative signaling factors—such as *p*-ERK1/2—this study investigated their therapeutic impact within an in vitro model of sarcopenia [12]. In fact, while other fungal extracts, such as *Cordyceps sinensis* or generic *Tuber* species, have previously been studied for their metabolic and antioxidant protective effects on skeletal muscle, *T. borchii* possesses a unique bioactive phytocomplex whose specific potential against sarcopenia remains completely unexplored [16,17]. The present evaluate the efficacy of *T. borchii* extracts in a D-galactose-induced sarcopenia model of C2C12 murine myotubes. The use of D-galactose effectively mimics the accelerated aging and oxidative stress typical of the clinical condition. Indeed, D-galactose exposure was selected as an in vitro strategy to induce an accelerated muscle aging phenotype in differentiated C2C12 myotubes. This model is commonly used to reproduce selected cellular alterations associated with aging, including oxidative stress, senescence-associated changes, impaired protein homeostasis, and atrophy-like morphological alterations. Specifically, the research focuses on the possibility that these extracts can counteract morphological atrophy, modulate the balance between synthesis (*p*70S6K/*puromycin* pathway) and degradation (Atrogin-1/MuRF1/ubiquitination), and attenuate the oxidative and inflammatory burden that characterizes skeletal muscle tissue degeneration.

2. Materials and Methods

2.1. Truffle Collection and Molecular Identification

Ascomata of *Tuber borchii* (Ascomycota) naturally growing in selected areas of the Marche region were collected in January 2025. The maturation stage was determined

according to the criteria established by Zeppa et al. (2004) [18]. Only ascomata classified as maturation grade 1, corresponding to 6–30% mature spores, were included in the analyses. Following harvesting, truffle samples were carefully cleaned to eliminate residual soil and individually coded. DNA was extracted from fresh gleba tissue according to the method reported by Paolocci et al. [19]. DNA quantity and quality were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Molecular authentication was performed by amplifying a 397-bp sequence specific to *T. borchii*. Genomic DNA (1 µL) was added to a 25-µL reaction mixture containing 1× TaKaRa Taq™ Buffer, 0.4 µM TboI primer (5'-TGTATGGGATGCCCTATCGGACT-3'), 0.4 µM TboII primer (5'-CTATTACCACGGTCAACTTC-3'), 200 µM dNTPs, and 1 U TaKaRa Taq DNA Polymerase. Reactions were run in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using 5 min at 95 °C, 30 amplification cycles comprising 20 s at 95 °C, 15 s at 55 °C, and 30 s at 72 °C, followed by 7 min at 72 °C. Amplicons were resolved in 1.4% agarose containing Midori Green Advance and visualized under UV illumination.

2.2. Preparation of the Ethanolic Extract

Two *T. borchii* fruiting bodies (identified as Extract 1 and Extract 2) were processed for ethanolic extraction in order to isolate bioactive compounds. The extraction procedure was adapted from the protocol described by Saltarelli et al. [20], with slight modifications optimized for truffle tissue. Briefly, internal gleba fragments (1–5 mm) were aseptically collected from each ascoma. Samples were weighed to determine fresh mass and subsequently dried at 60 °C for 5 h. The resulting dry weights were 1.73 g for Extract 1 and 1.72 g for Extract 2. Dried tissues were finely powdered in liquid nitrogen using a mortar and pestle and transferred into sterile 50-mL tubes. Metabolites were extracted overnight at 4 °C under gentle agitation with 20 mL of 80% (*v/v*) ethanol. Following extraction, samples were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatants were collected. The residual pellets were subjected to two additional extraction steps using 15 mL of 80% ethanol for 1 h under identical conditions. Supernatants obtained from the three sequential extractions were pooled and evaporated to dryness. At the end of the extraction procedure, the final yields were 0.028 g for Extract 1 and 0.098 g for Extract 2. Dried extracts were resuspended in 1 mL DMSO and stored at 4 °C until use. In all subsequent experiments, *T. borchii* extracts were administered and reported as µg/mL concentrations.

2.3. Cell Line and Treatments

The C2C12 murine myoblast cell line was purchased from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, supplemented with 10% fetal bovine serum, 10 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were grown at 37 °C in an atmosphere of 5% CO₂ and, for all experiments, were plated at a density of 1×10^5 cells/mL. Myogenic differentiation was triggered 24 h after plating by exchanging the growth medium for high-glucose DMEM supplemented with 2% horse serum, 2 mM L-glutamine, 10 U/mL penicillin–streptomycin, and 1 mM sodium pyruvate [21]. On day 1, 20 mg/mL galactose was added to this differentiation medium. On day 5, C2C12 myotubes were treated with two different *T. borchii* truffle extracts at a concentration of 100 µg/mL at 37 °C in differentiation medium for 48 h. An equal amount of DMSO was added to untreated cells. Brightfield images of C2C12 myotubes were acquired using a Nikon Eclipse Ts2 inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with a KoPa Lab digital camera using a 10× objective. For each biological replicate, at least three randomly selected non-overlapping fields were acquired while avoiding the edges of the wells. The measurement of myotube diameter was performed by the ImageJ software (version 1.53t,

National Institutes of Health, Bethesda, MD, USA) as previously described [22]. To ensure complete objectivity and eliminate potential operator bias, all quantifications were carried out using standardized, automated thresholding parameters uniformly applied across all experimental groups.

MG-132 was used at a final concentration of 10 μ M. Puromycin was used at a final concentration of 2 μ g/mL. C2C12 myotubes were treated with the compound and incubated for 16 h and 2 h, respectively, in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.4. Preparation of *T. borchii* Sample for HPLC-DAD and Spectrophotometric Analysis

The sample was dried to completion and resuspended in an equivalent volume of methanol/water (50:50, *v/v*) for 24 h under agitation, in the dark, at 4 °C. Following centrifugation at 10,000 $\times$ *g* for 10 min at 4 °C, the supernatant was collected for biochemical analyses. Specifically, targeted chromatographic characterization (HPLC-DAD) was performed to evaluate specific phenolic and flavonoid compounds, whereas spectrophotometric analysis was used to determine total phenolic and flavonoid content. All assays were carried out in triplicate.

2.5. HPLC-DAD

The biochemical composition of the extracts was assessed by high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD). Analyses were performed using a Shimadzu chromatographic platform (Kyoto, Japan) comprising an SPD-M20A diode-array detector, a CBM-20A system controller, an LC-20AD solvent delivery module, and an autosampler. Chromatographic separation and compound analysis were conducted according to the protocol previously reported by D'Agostino et al. [23]. Chromatographic analyses were carried out with the column compartment set at 40 °C using a Luna 3u C18(2) analytical column (150 $\times$ 4.60 mm, 3 μ m particle size; Phenomenex, Torrance, CA, USA). For each run, 20 μ L of the sample was injected. The eluent system consisted of water containing 1% formic acid (*v/v*) as solvent A and methanol as solvent B, with a constant flow rate of 0.95 mL/min. The gradient started with 15% solvent B, which was maintained for 20 min. The proportion of methanol was then progressively increased to 35% over the next 20 min and subsequently raised to 90% by 55 min. The system was returned to the initial composition of 15% B at 70 min to allow column re-equilibration. Chromatographic data were collected and processed using the LAB-SOLUTION software (version 5.97, Shimadzu Corporation, Kyoto, Japan). Signals were monitored at 280 nm to detect phenolic constituents, including gallic acid, 3-hydroxytyrosol, vanillic acid, rosmarinic acid, 4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, salicylic acid, 1,1-dimethylallyl caffeate, caffeic acid phenethyl ester, and 5,7-dimethoxycoumarin, as well as flavonoids such as resveratrol, quercetin-3-glucoside, myricetin, quercetin, genistein, kaempferol, chrysin, and epicatechin. Compound identification and quantification were achieved by matching retention times, UV spectral profiles, and chromatographic peak areas with those of authentic analytical standards obtained from Sigma-Aldrich (Milan, Italy). Concentrations were reported as nanograms of standard equivalents per milligram of sample.

2.6. Determination of Total Phenolic Content

Total phenolic content was determined using the Folin–Ciocalteu spectrophotometric method [24]. For the assay, 50 μ L of each sample was combined with 250 μ L of Folin–Ciocalteu reagent diluted 1:10 (*v/v*) and 200 μ L of 0.7 M sodium carbonate. After incubation for 2 h at room temperature in the absence of light, absorbance was recorded at 760 nm with a Sunrise microplate reader (Tecan, Männedorf, Switzerland). Each sample was analyzed in six wells, using 200 μ L per well. Total phenolics were quantified against a gallic acid

calibration standard and reported as nanograms of gallic acid equivalents per milligram of sample (ng GAE/mg).

2.7. Determination of Total Flavonoid Content

For flavonoid determination, 100 µL of extract was mixed with 20 µL of 10% aluminium chloride (AlCl₃), 20 µL of 1 M potassium acetate (CH₃CO₂K), 300 µL of methanol, and 560 µL of ddH₂O [25]. The mixture was then incubated for 30 min in the dark at room temperature, and absorbance was measured spectrophotometrically at 415 nm using a microplate reader (Sunrise, Tecan), with six replicate wells per sample (200 µL per well). Total flavonoid content was quantified using a calibration curve prepared with increasing concentrations of quercetin, and results were expressed as ng quercetin equivalents (QE) per mg of sample weight (ng QE/mg).

2.8. Quantitative Real-Time PCR

Total RNA was isolated from cells lysed with TRIzol G reagent (PanReac AppliChem, Darmstadt, Germany; Cat. No. A4051) following the manufacturer's protocol. One microgram of purified RNA was reverse-transcribed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was subsequently carried out with iTaq Universal SYBR® Green Supermix (Bio-Rad) on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). The oligonucleotide sequences employed for gene expression analysis are reported below:

- Atrogin-1 (FBXO32): forward: 5'-GCGACCTTCCCCAACGCCTG-3'; reverse: 5'-GGCGACCGGGACAAGAGTGG-3'
- IL-6, forward: 5'-CTCTGCAAGAGACTTCCATCCA-3'; reverse: 5'-GACAGGTCTGTTGGGAGTGG-3'
- MuRF-1 (TRIM63): forward: 5'-AGGGGCTACCTTCCTCTCAAGTG-3'; reverse: 5'-TCTTCCCCAGCTGGCAGCCC-3'
- TGF-β, forward: 5'-TGACGTCACCTGGAGTTGTACGG-3'; reverse: 5'-GGTTCATGTCATGGATGGTGC-3'
- β-Actin, forward: 5'-CACACCCGCCACCAGTTTCGC-3'; reverse: 5'-TTGCACATGCCGGAGCCGTT-3'

The relative mRNA levels were determined by using the 2^{-ΔΔCt} method. Fold changes were calculated relative to the control, and normalization was performed using β-actin as the internal standard.

2.9. Western Blot Analysis

Cell pellets were disrupted in lysis solution containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% IGEPAL CA-630, and protease inhibitors. Protein content was measured by the Lowry procedure [24]. Equal amounts of protein lysate (20 µg per sample) were separated by SDS-PAGE using 8.5%, 10%, or 12% polyacrylamide gels, depending on the molecular weight of the target proteins. After separation, proteins were transferred to nitrocellulose and incubated with the antibodies listed in Table 1. Primary antibodies were diluted 1:1000, followed by the appropriate HRP-linked secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). Immunoreactive bands were visualized using ChemiGlow chemiluminescent reagent and acquired with a FluorChem imaging platform (Alpha Innotech Corporation–Analytica De Mori, Milan, Italy). Quantity One 4.6.8 was employed for band quantification, with β-actin used for normalization. To ensure complete objectivity and eliminate potential operator bias, all quantifications were carried out using standardized, automated thresholding parameters uniformly applied across all experimental groups.

Table 1. Antibodies used.

Antibody	Code	Brand
β -actin	4970S	Cell Signaling
p-mTOR Ser2448	2971	Cell Signaling
Myosin heavy chain (MHC)	MF20	DSHB
MuRF-1	4305	Cell Signaling
p-P70S6K Thr389	9205S	Cell Signaling
Puromycin	3RH11	Kerafast
Ubiquitinated Protein	3933	Cell Signaling

2.10. Staining with Senescence-Associated β -Galactosidase (SA- β -Gal)

C2C12 myoblasts were seeded on day 0. Twenty-four hours later, the cells were exposed to Extracts 1 and 2 or to their respective vehicle controls. Following 48 h of treatment, cellular senescence was assessed by measuring senescence-associated β -galactosidase activity using the SA- β -gal staining kit (Cell Signaling Technology, Inc., Danvers, MA, USA), in accordance with the manufacturer's protocol. Images were acquired from randomly selected non-overlapping fields while avoiding the edges of the wells until at least 100 cells per condition had been analyzed across three independent biological experiments. ImageJ was used to determine the proportion of stained cells by dividing the number of SA- β -gal-positive cells by the total cell count. To ensure complete objectivity and eliminate potential operator bias, all quantifications were carried out using standardized, automated thresholding parameters uniformly applied across all experimental groups.

2.11. Cell Proliferation Assay

Cell proliferation was measured using an MTS assay kit “Cell Titer 961 Aqueous One Solution Cell Proliferation assay” (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.12. Measurement of Intracellular ROS Production

ROS production was measured using DHE. C2C12 myoblasts were cultured and treated (as indicated above) on coverslips. After treatment, cells were incubated with 50 μ M DHE for 30 min at 37 °C. Cells were washed with PBS and fixed with 4% paraformaldehyde [26]. Coverslips were mounted with Mowiol (polyvinyl alcohol-based mounting medium) and allowed to cure overnight at room temperature. Cell images were digitized with a Nikon DS-Qi2 camera attached to a Nikon Eclipse Ti2 epifluorescence microscope (Nikon Europe B.V., Amstelveen, The Netherlands). All images were acquired under identical conditions to ensure comparability across experiments, and raw fluorescence images were used for analysis without further preprocessing. Fluorescence intensity was quantified using a custom CellProfiler pipeline (Broad Institute, Cambridge, MA, USA). Briefly, cells were identified as primary objects based on DHE fluorescence signal and used to measure the mean fluorescence intensity per cell. Fluorescence images were acquired from randomly selected non-overlapping fields while avoiding the edges of the coverslips until at least 100 cells per condition had been analyzed across three independent biological experiments.

2.13. ELISA Kit TNF- α

TNF- α level was measured by using a Mouse TNF- α ELISA Kit (Antibodies, Cambridge, UK, A1604) according to the manufacturer's instructions.

2.14. Transmission Electron Microscopy

Myotube samples were collected and fixed overnight in 2.5% glutaraldehyde solution (0.1 M phosphate buffer, pH 7.2), followed by six washes in phosphate buffer. Post-

fixation was carried out in 2% osmium tetroxide (0.1 M phosphate buffer) for 2 h at room temperature. Samples were subsequently processed following a standard EPON embedding protocol [27] and polymerized overnight at 65 °C. Ultrathin sections (70 nm) were obtained using a Leica Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany) and collected on copper grids. Sections were then stained with Uranylless and lead hydroxide and examined by transmission electron microscopy using a JEOL TEM-1400 Plus instrument (JEOL, Milan, Italy).

2.15. Statistical Analysis

Results are reported as mean $\pm$ SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism version 7.05 for Windows. Statistical significance was set at $p < 0.05$. Unless otherwise specified, all experiments were performed using independent biological replicates ($n \geq 3$), and the value of n refers to independent biological replicates.

3. Results

3.1. *T. borchii* Extracts Protect C2C12 Myotubes from D-Galactose-Induced Sarcopenia Without Impacting Viability

The analysis of the polyphenolic profile of two *T. borchii* samples (Extracts 1 and 2) conducted using spectrophotometric assays and HPLC-DAD allowed the identification and quantification of the main bioactive compounds present in the extracts. Due to the high consistency and reproducibility between the two profiles, the quantities reported below represent the aggregated mean value $\pm$ standard deviation (SD). The quantities reported below represent the mean value obtained from the comparative analysis of the two samples. Specifically, the total phenol content was estimated at 332.68 ± 2.75 ng/mg, while the total flavonoid fraction was 283.02 ± 17.79 ng/mg. Among the identified molecules, gallic acid was the predominant component, with a concentration of 84.32 ± 3.21 ng/mg. A significant presence of caffeic acid derivatives was also detected, particularly caffeic acid 1,1-dimethyl allyl ester (55 ± 7.07 ng/mg) and free caffeic acid (10.25 ± 1.06 ng/mg). Other significant constituents of the phytochemical profile include 3-hydroxytyrosol (33.5 ± 1.41 ng/mg), vanillic acid (22.25 ± 1.06 ng/mg), and 4-hydroxybenzoic acid (21 ± 1.41 ng/mg). Based on these results and with the aim of testing the bioactive potential of *T. borchii* extracts in counteracting muscle atrophy, we initially evaluated the impact of the two extracts on the viability of C2C12 murine myoblasts using the MTS assay. Cells were exposed to increasing concentrations of both extracts (100, 150, and 200 μ g/mL) for 24 h to evaluate whether they were cytotoxic and identify the optimal dosage range for subsequent experiments (Figure 1A,B). Treatment with the extracts showed no significant changes in optical density measured by the MTS assay kit, compared to control cells at any of the concentrations used, indicating that the extracts were not cytotoxic. Conversely, Ext2 shows a slight increase in cell proliferation. Based on these results and considering the results previously published [12], we selected the dose of 100 μ g/mL of Extracts 1 and 2 for subsequent experiments.

Next, we established an in vitro model of sarcopenia/atrophy using differentiated C2C12 murine myoblasts treated with 20 mg/mL D-galactose. The experimental protocol involved the differentiation of myoblasts into mature myotubes over a period of 7 days with a differentiation medium supplemented with or without 20 mg/mL D-galactose to induce a senescent and atrophic phenotype. Finally, on day 5, myotubes were treated with 100 μ g/mL *T. borchii* extracts (Figure 2A). On day 7, myotubes were subjected to morphological analysis by phase-contrast microscopy, which revealed that D-galactose treatment produced significant structural alterations compared to control cells. Quantifi-

cation of morphological parameters confirmed a marked reduction in myotube diameter and area induced by D-galactose, demonstrating the validity of the experimental system. The addition of Extracts 1 and 2 effectively counteracted these morphological changes (Figure 2B–D). Both treatments preserved the structural integrity of the myotubes, showing a statistically significant recovery of both myotube diameter and area (Figure 2C,D). These results suggest that both extracts are able to significantly antagonize the atrophic stress induced by D-galactose, promoting the maintenance of the muscle phenotype and preventing the structural degradation of the myotubes.

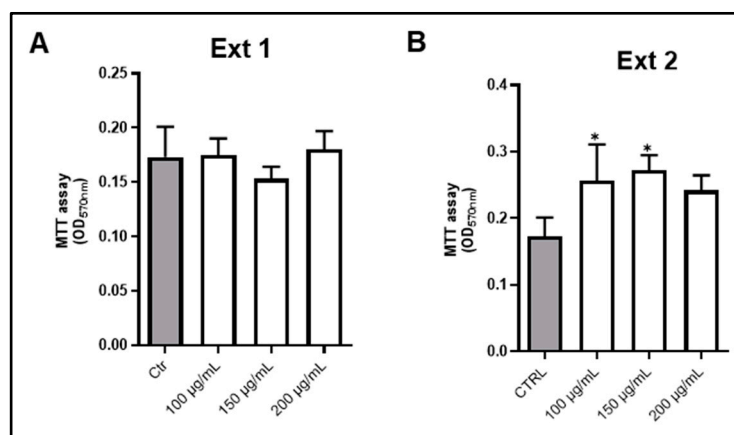


Figure 1. Analysis of cell viability of C2C12 myoblasts treated with *T. borchii* extracts. (A,B) Cell proliferation was evaluated by MTS assay 24 h after treatment with increasing concentrations (100, 150, and 200 µg/mL) of Extracts 1 and 2 of *T. borchii*. Data are expressed as means $\pm$ SD; $n = 3$ independent biological experiments; * $p < 0.05$.

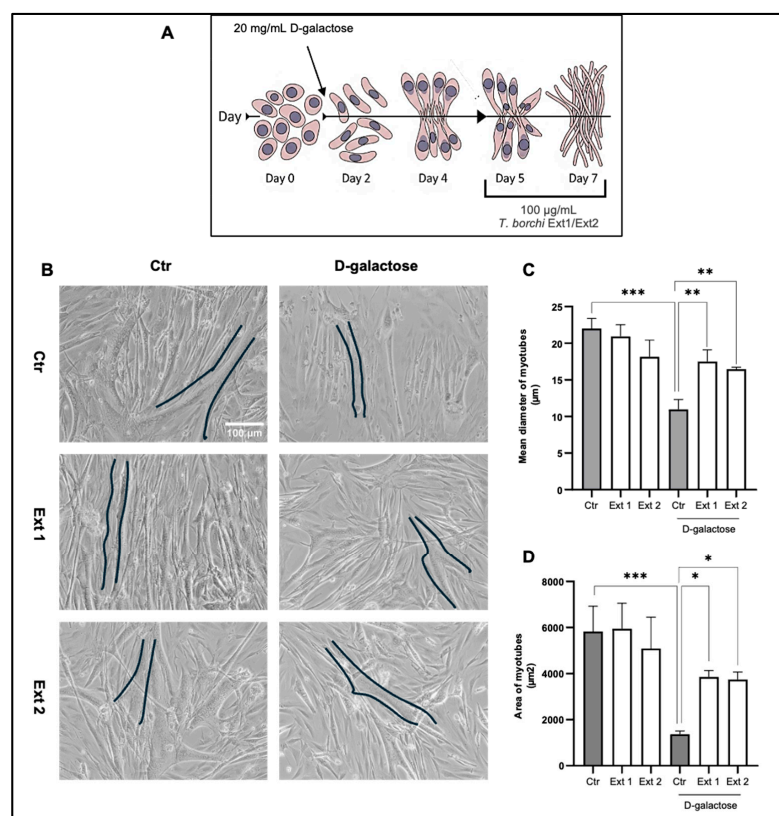


Figure 2. *T. borchii* restores the phenotypic characteristics of myotubes in a D-galactose-induced C2C12 model of sarcopenia. (A) Timeline diagram of the experimental protocol used to induce a

senescent and atrophic phenotype in differentiated C2C12 myotubes by treatment with D-galactose (20 mg/mL), followed by treatment with 100 µg/mL *T. borchii* Extracts 1 and 2 (Ext 1 and Ext2) for 48 h. **(B)** Brightfield images (10×) of C2C12 myotubes to assess differentiation, myotube diameter and area in control cells (Ctr), with or without D-galactose in combination with Extract 1 or 2. Scale bar: 100 µm. Morphometric analysis of C2C12 myotubes, expressed as diameter **(C)** and area **(D)**, in control cells (Ctr), D-galactose-treated cells, and cells treated with D-galactose in combination with Extract 1 or 2. Quantification was performed on three independent biological replicates. For each replicate, at least three randomly selected non-overlapping fields were analyzed. Data are expressed as means ± SD; $n = 3$ independent biological experiments; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, as indicated by brackets. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

3.2. *T. borchii* Extracts Are Associated with Preserved Ultrastructural Features in D-Galactose-Treated C2C12 Myotubes

TEM analysis was performed as a qualitative and descriptive assessment of representative ultrastructural features. At the ultrastructural level, myotubes treated with D-galactose appeared as elongated, multinucleated syncytia delimited by a continuous sarcolemma. Nuclei were typically centrally located and either clustered or aligned along the longitudinal axis of the cell (Figure 3A,B); they often showed an altered chromatin profile (Figure 3A). Numerous mitochondria appeared vacuolated and with a reduced density of cristae (Figure 3B–D). TEM evaluation showed a large number of concentric membranous structures containing dense material and dense bodies, as well as swollen and degenerating mitochondria (Figure 3A,B). Cytoplasmic vacuolization and concentric multilamellar bodies and membranous fragments, consistent with autophagic–lysosomal elements, were also detectable (Figure 3A), confirming the loss of integrity of the cell population. Elements of the vesicular trafficking system were also frequently found in the sarcoplasm. Uncoated vesicles of variable size were dispersed throughout the cytoplasm (Figure 3A). Coated vesicles, recognizable by the characteristic electron-dense, bristle-like coat surrounding their limiting membrane, were also present, frequently in continuity with, or in close proximity to, the plasma membrane (Figure 3D). The presence of both vesicle populations, together with the above-mentioned signs of organelle swelling and membrane disruption, may reflect altered metabolic activity. Overall, the ultrastructural features observed by TEM further validate the reliability of the sarcopenic model, confirming that the induced alterations closely recapitulate the morphological hallmarks of sarcopenia [28].

As observed in the D-galactose-treated control group, myotubes also treated with Extract 1 appeared as elongated, multinucleated syncytia bounded by a continuous, well-preserved sarcolemma. Nuclei occupied a predominantly central position and displayed a mainly euchromatic profile with one or more prominent nucleoli (Figure 4A). TEM micrographs rarely showed alterations of the chromatin profile. Unlike the control group, TEM analysis revealed an increased number of normal mitochondria with well-defined cristae induced by *T. borchii* Extract 1 (Figure 4A, inset). Nevertheless, vacuolated mitochondria, as well as mitochondria enclosed within concentric membranous structures, were also observed (Figure 4B). In addition, concentric multilamellar bodies and membranous fragments, consistent with autophagic–lysosomal vesicles, were present (Figure 4B).

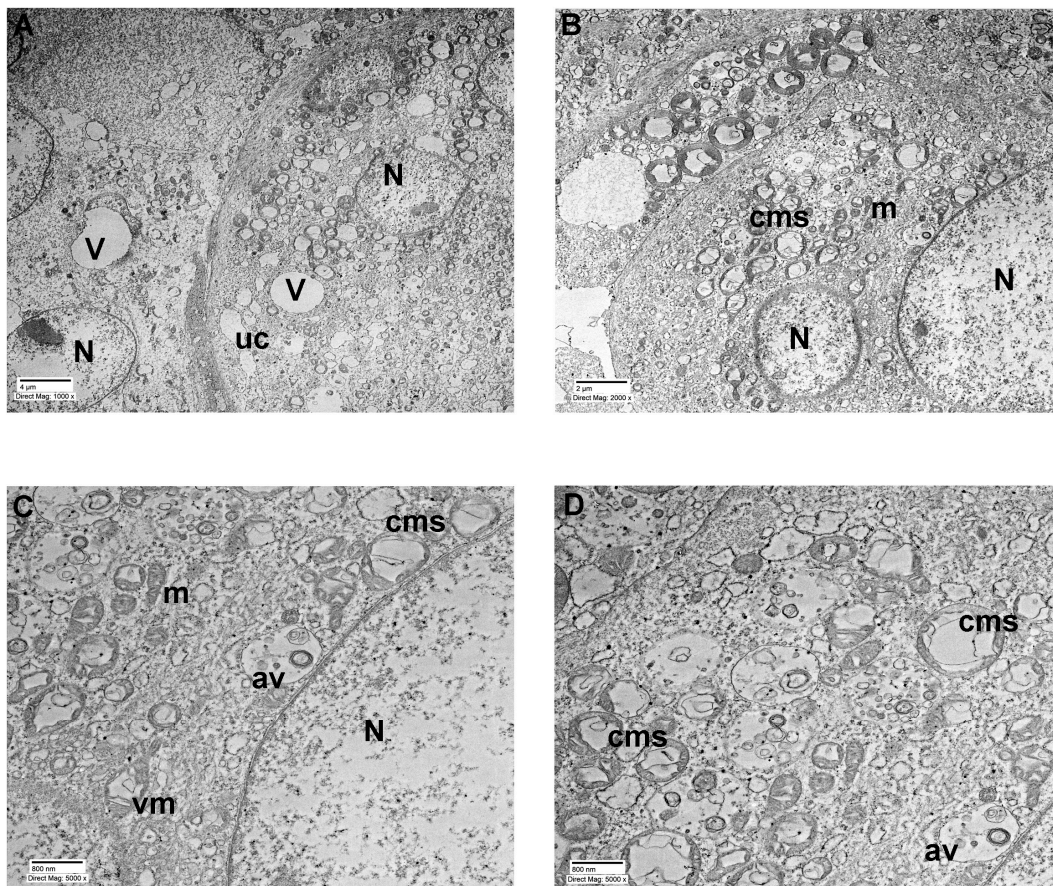


Figure 3. Ultrastructural evaluation of C2C12 myotubes treated with D-galactose. Representative TEM micrographs illustrating qualitative ultrastructural features. (A) Multinucleated syncytium with centrally located, ovoid nuclei (N) showing an altered chromatin profile; cytoplasmic vacuolization (V), concentric multilamellar bodies (cms), and uncoated vesicles (uv) were observed. TEM bar: 4 μ m. (B) TEM micrograph showing numerous concentric membranous structures containing dense material and dense bodies, together with degenerating mitochondria (m). TEM bar: 2 μ m. (C,D) Higher-magnification TEM micrographs showing vacuolated mitochondria (vm), autophagic vesicles (av), and cms. TEM bars: 800 nm.

Myotubes treated with D-galactose and Extract 2, similarly to the previous groups, appeared as elongated, multinucleated syncytia delimited by a continuous, well-preserved sarcolemma. Nuclei were typically centrally located, with an intact nuclear membrane, and showed a prevalent euchromatic profile with one or more prominent nucleoli (Figure 4C). Coated and uncoated vesicles were also detected in this group. Unlike the previous groups, numerous mitochondria displaying an intact double membrane and well-defined cristae were distributed throughout the sarcoplasm (Figure 4D). Profiles of rough endoplasmic reticulum, free ribosomes, and elements of the sarcoplasmic reticulum were also present. Overall, the organelles appeared well preserved, and altered chromatin profile and cytoplasmic vacuolization were reduced compared with the other groups, demonstrating a prominent protective effect of *T. borchii* extracts against the hallmark features of sarcopenia.

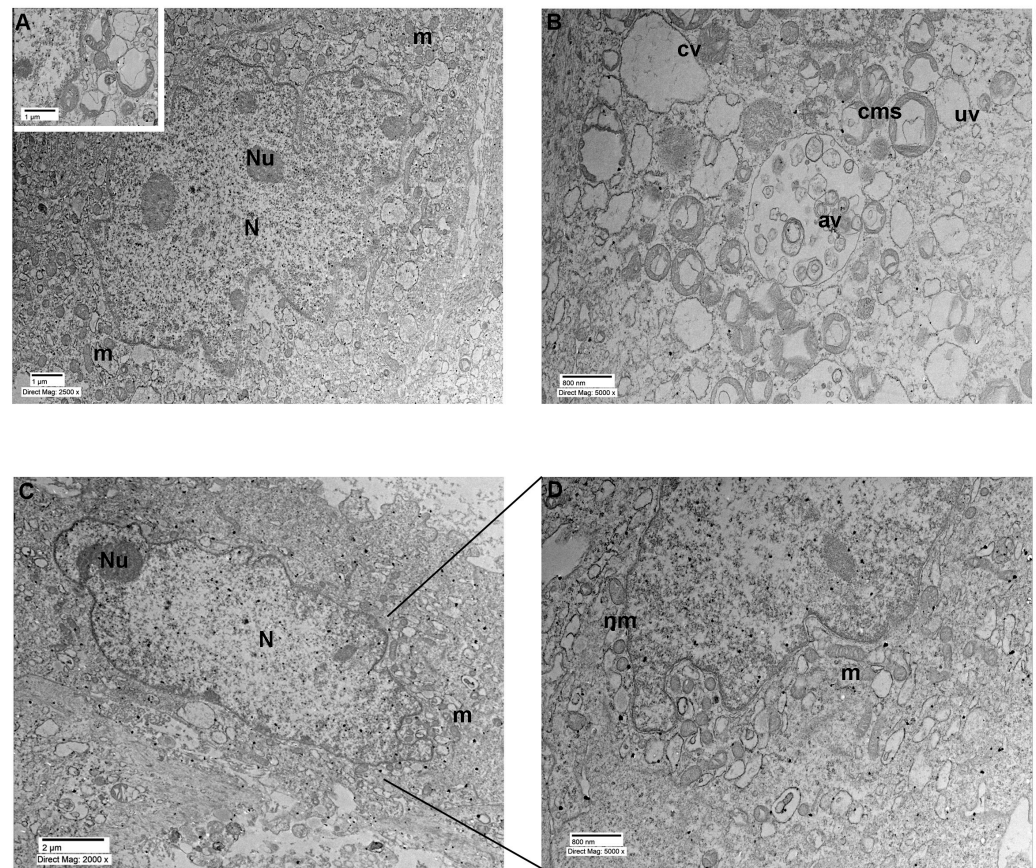


Figure 4. Ultrastructural evaluation of C2C12 myotubes treated with D-galactose and *T. borchii* extracts. Representative TEM micrographs illustrating qualitative ultrastructural features. (A) Ext 1 TEM micrograph of a myotube showing a well-preserved nucleus (N) with numerous nucleoli (Nu) and numerous mitochondria (m). TEM bar: 1 μ m. Inset in (A): detail of mitochondria (m) with visible cristae. TEM bar: 1 μ m. (B) Ext 1, higher magnification showing concentric membranous structures (cms), autophagic vesicles (av), uncoated (uv) and coated vesicles (cv). TEM bar: 800 nm. (C) Ext 2 micrograph showing a myotube section with a well-preserved nucleus and evident nucleoli; the cytoplasm appears rich in healthy mitochondria. TEM bar: 2 μ m. (D) Ext 2, higher magnification showing nuclear detail with an intact nuclear membrane (nm). Numerous mitochondria (m) with evident cristae are visible. TEM bar: 800 nm.

3.3. *T. borchii* Extracts Modulate the Ubiquitin–Proteasome System and Preserve MHC Integrity in D-Galactose-Induced Muscle Sarcopenia

To investigate the molecular mechanisms underlying the cytoprotective effect of *T. borchii* extracts observed at the morphological level, we analyzed the expression of key proteins involved in muscle catabolism and structural maintenance. As shown in Figure 5A,B, qPCR analysis revealed that D-galactose treatment resulted in an approximately 3-fold increase in the mRNA levels of Atrogin-1 (Fbxo32) and MuRF-1 (muscle RING-finger protein-1) compared to controls, whereas the combined treatment with truffle extracts reduced the levels of atrophic markers to values comparable to the control. These two muscle-specific E3 ubiquitin ligases play a key role in regulating muscle mass by promoting the degradation of contractile proteins through the ubiquitin–proteasome system [29]. Western blotting data (Figure 5C) and related densitometric analysis (Figure 5D) confirmed this trend at the protein level, demonstrating a reduction in MuRF-1 protein levels (Figure 5C,D), suggesting an association with attenuated activation of proteolytic pathways induced by the accelerated sarcopenia model. In parallel, we assessed the levels of the myosin heavy chain (MHC), the main structural protein of the sarcomere, essential

for contractile function [30]. Consistent with the activation of atrophy markers, D-galactose treatment caused a significant reduction in MHC levels, indicating a loss of muscle fiber integrity. Supplementation with *T. borchii* extracts was associated with a recovery of MHC levels (Figure 5C,E). Indeed, densitometric analysis showed a statistically significant recovery of MHC levels in response to both extracts, suggesting a possible positive effect on sarcomere stability (Figure 5C,E).

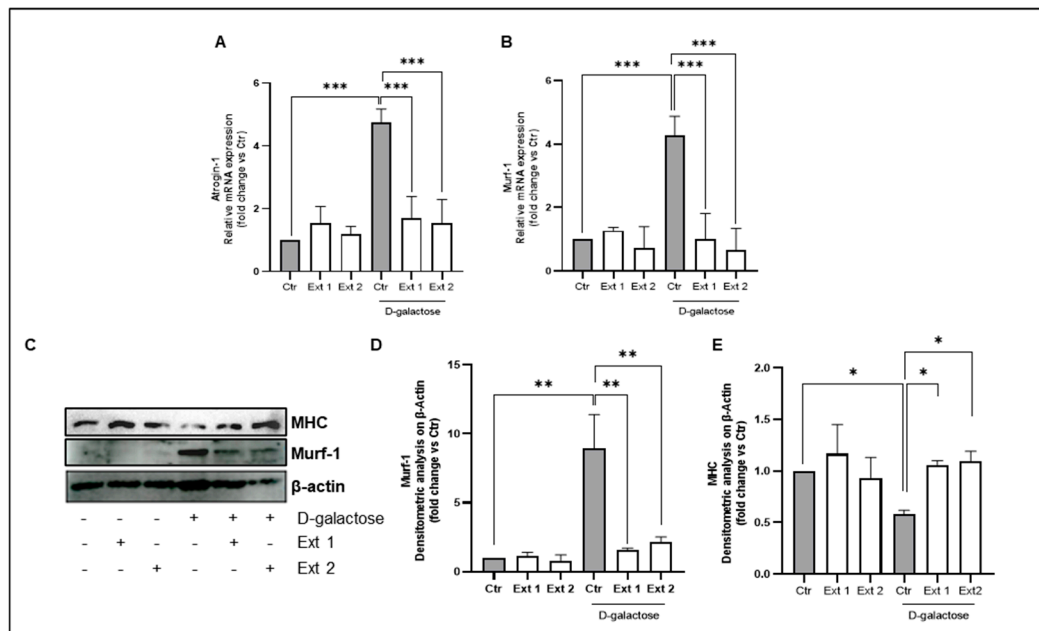


Figure 5. Effects of *T. borchii* extracts on the expression of muscle atrophy markers and MHC levels in sarcopenic myotubes. D-galactose (20 mg/mL) was added on day 1 of C2C12 differentiation and maintained throughout the experiment. On day 5, myotubes, with and without 20 mg/mL D-galactose, were treated with 100 µg/mL *T. borchii* extracts (Ext 1 and Ext2) for 48 h. (A,B) Relative mRNA levels of Atrogin-1 and MuRF-1 were analyzed by RT-qPCR. Data are expressed as means ± SD ($n = 3$ independent biological experiments; *** $p < 0.005$ as indicated by brackets). (C) Western blot analyses of MHC and MuRF-1. All the images presented are from one experiment representative of three independent biological experiments that showed similar results. β-actin was used as a loading control. (D,E) Density of immunoreactive bands was calculated using the software ImageJ, and data are shown as the ratio of MuRF-1/β-actin (D) and MHC/β-actin (E). Data are expressed as means ± SD from three independent biological replicates ($n = 3$, * $p < 0.05$, ** $p < 0.005$ as indicated by brackets). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test.

3.4. *T. borchii* Extracts Modulate Protein Homeostasis by Modulating p70S6K Signaling and the Ubiquitin–Proteasome System

Considering that sarcopenia/atrophy causes an imbalance in protein turnover, favoring protein degradation while reducing protein synthesis [31], we assessed the potential of *T. borchii* to modulate the pathways regulating protein synthesis and degradation. We initially analyzed the phosphorylation status of p70S6K (Thr389), a key downstream effector of the mTORC1 complex that promotes protein synthesis [32]. Treatment with D-galactose alone significantly reduced p70S6K phosphorylation relative to control cells, indicating marked inhibition of cellular anabolic signaling, as expected. Combined treatment with *T. borchii* extracts attenuated this trend: in particular, both Extracts 1 and 2 partially counteract the decrease in p70S6K phosphorylation levels, suggesting a positive modulation of the protein synthesis pathway (Figure 6A,B). Subsequently, to further explore this pathway, we measured the actual protein synthesis rate using the puromycin incorporation assay

(Figure 6C,D). Consistent with the p70S6K data, treatment with both Extracts 1 and 2 effectively mitigated the reduction induced by D-galactose, helping to sustain the protein synthesis rate at levels comparable to the control (Figure 6C,D). Finally, we assessed the potential modulation of protein degradation by analyzing the accumulation of polyubiquitinated proteins [33]. As shown in Figure 6E,F, both *T. borchii* extracts reduced the accumulation of ubiquitinated proteins induced by the sarcopenic stimulus, suggesting a favorable modulation of the proteolytic balance.

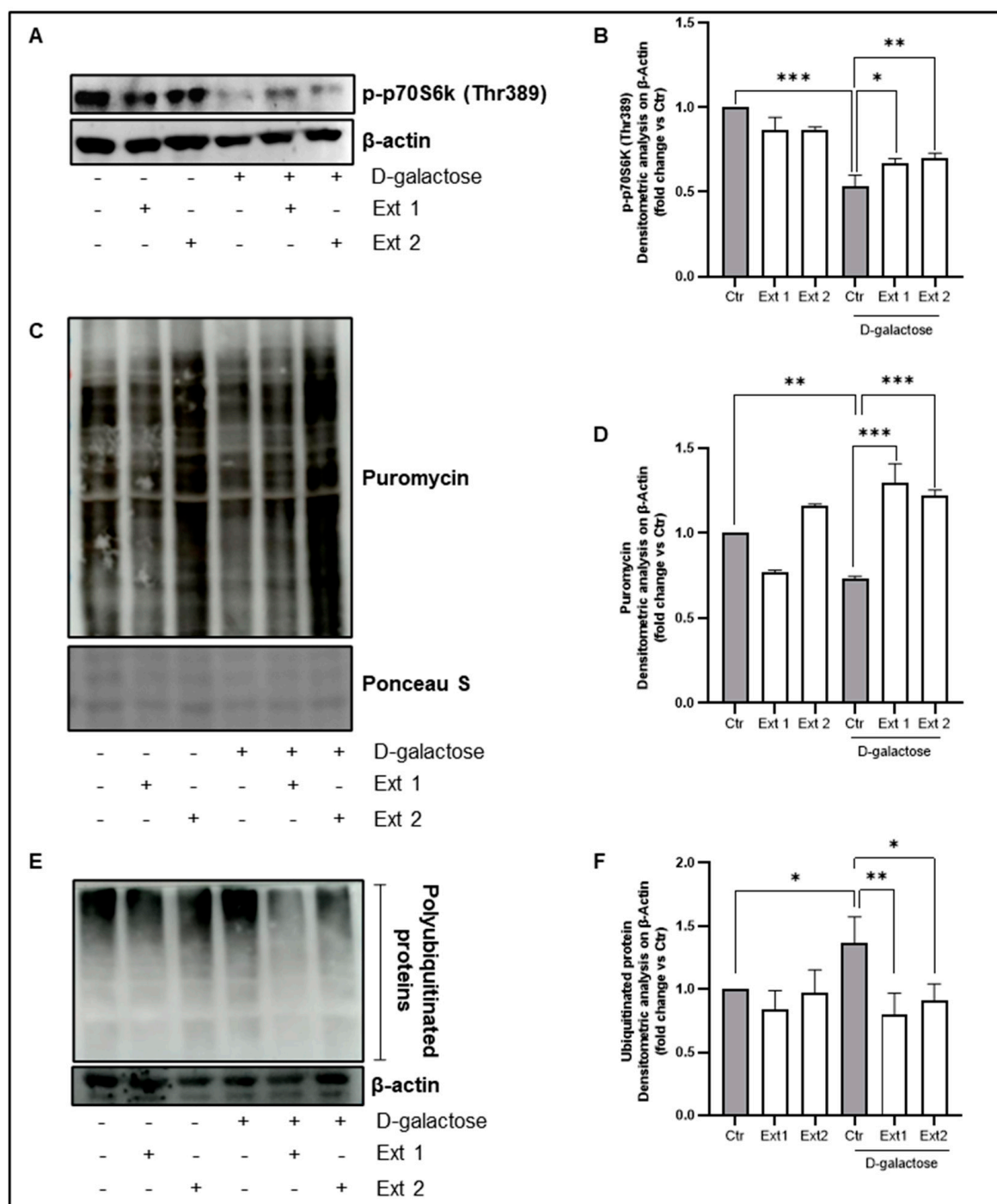


Figure 6. *T. borchii* extracts modulate p70S6K signaling and the ubiquitin–proteasome system in C2C12 myotubes. D-galactose (20 mg/mL) was added on day 1 of C2C12 differentiation and maintained throughout the experiment. On day 5, myotubes, with and without 20 mg/mL D-galactose, were

treated with 100 µg/mL *T. borchii* extracts (Ext 1 and Ext2) for 48 h. (A) Western blot analysis of p-p70S6k (Thr389). (B) The density of immunoreactive bands was calculated using the software ImageJ, and data are shown as the ratio of p-p70S6k (Thr389)/β-actin. (C) All experimental conditions of C2C12 myotubes were treated with 2 µg/mL puromycin 2 h before completing the experiment. Western blot analysis was used to evaluate puromycin incorporation levels. Ponceau S was used as a loading control. (D) The ImageJ software was used to quantify the density of immunoreactive bands, and data are shown as the ratio of Puromycin/Ponceau S. (E) In order to block proteasome activity and the accumulation of polyubiquitinated proteins, all experimental conditions of C2C12 myotubes were treated with the proteasome inhibitor MG-132 (10 µM) for 16 h. Western blot analysis of polyubiquitinated proteins. (F) The density of immunoreactive bands was calculated using the software ImageJ, and data are shown as the ratio of ubiquitinated proteins/β-actin. All the immunoblots reported are from one experiment representative of three independent biological replicates that gave similar results. β-actin was used as a loading control. Data are expressed as means ± SD; *n* = 3 independent biological experiments; * *p* < 0.05, ** *p* < 0.005, *** *p* < 0.0005 vs. control D-galactose, as indicated by brackets. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test.

3.5. *T. borchii* Extracts Attenuate Oxidative Stress and Prevent Cellular Senescence in Myotubes Treated with D-Galactose

Based on the results obtained by HPLC-DAD and considering that truffle extracts are known in the literature to possess antioxidant activity [13], we analyzed the potential antioxidant activity of *T. borchii* extracts. Firstly, we assessed intracellular ROS production using the fluorescent probe dihydroethidium (DHE). DHE, once oxidized by ROS, intercalates into DNA, emitting a bright red fluorescence. Myotubes treated with D-galactose alone showed a marked increase in signal intensity compared to the control, whereas co-treatment with *T. borchii* Extracts 1 and 2 dramatically reversed this profile (Figure 7A,B). Indeed, both extracts were able to reduce ROS production to levels comparable to those observed in control cells. The antioxidant capacity of the extracts on C2C12 myotubes was further confirmed by analyzing the accumulation of carbonyl groups in amino acid side chains, a well-established marker of irreversible oxidative damage induced by ROS [34]. As shown in Figure 7C,D, treatment with D-galactose induced a significant increase in the content of carbonylated proteins compared to control cells, and treatment with *T. borchii* extracts showed a marked protective effect. These results confirm that the cytoprotective efficacy of *T. borchii* is closely related to its strong antioxidant activity, capable of neutralizing excess ROS before they can trigger irreversible molecular damage and subsequent muscle catabolism.

In parallel with oxidative stress, inflammaging, characterized by chronic low-grade inflammation, is known to be a key factor in the pathogenesis of sarcopenia, acting as a potent stimulus for muscle catabolic pathways and protein degradation [35]. To determine whether treatment with *T. borchii* could mitigate this inflammatory state in the sarcopenia model, we initially assessed levels of the pro-inflammatory cytokine TNF-α by ELISA assay. As expected, D-galactose treatment induced a significant increase in TNF-α secretion compared to control cells, whereas co-treatment with both *T. borchii* Extracts 1 and 2 effectively counteracted these alterations (Figure 8A). Consistent with these data, qPCR analysis revealed that truffle extracts markedly reduced D-galactose-induced expression of IL-6 (Figure 8B) and TGF-β (Figure 8C), critical mediators of the systemic inflammatory response and tissue fibrosis processes associated with muscle loss [36]. These results demonstrate that *T. borchii* extracts exert a potent anti-inflammatory action, capable of suppressing the catabolic signals that drive the degradation of aged muscle tissue.

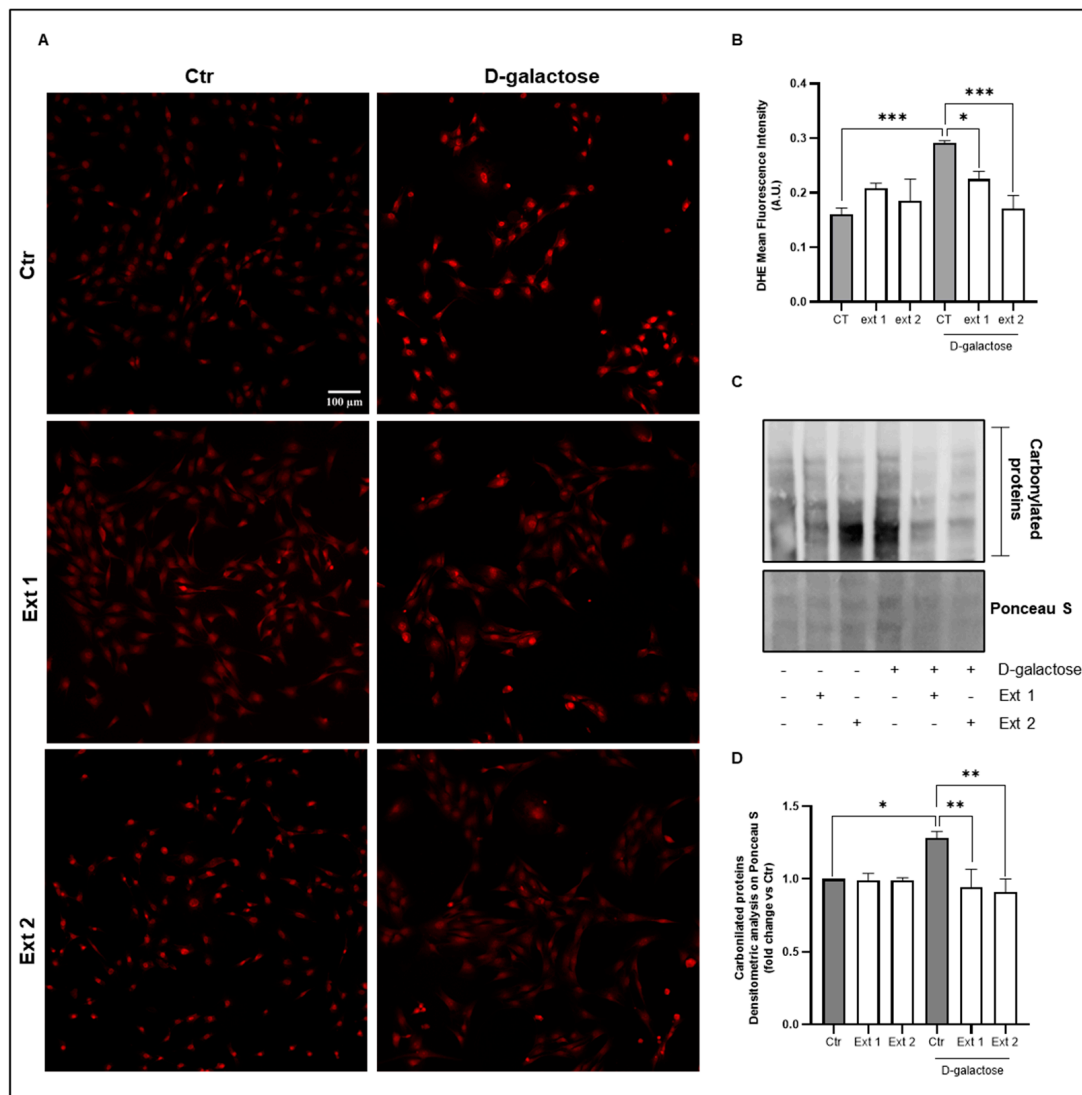


Figure 7. *T. borchii* extracts reduce oxidative protein damage in sarcopenic myotubes. D-galactose (20 mg/mL) was added on day 1 of C2C12 differentiation and maintained throughout the experiment. On day 5, myotubes, with and without 20 mg/mL D-galactose, were treated with 100 µg/mL *T. borchii* extracts (Ext 1 and Ext2) for 48 h. (A) ROS were quantified by cytofluorimetric analysis. Cells were treated with 2 µM DHE 30 min before the end of the experiment. The cells were analyzed by fluorescence microscopy. (B) Quantification was performed on three independent biological replicates. For each replicate, at least three randomly selected non-overlapping fields were analyzed. Data are expressed as mean $\pm$ SD; $n = 3$ independent biological experiments; * $p < 0.05$, *** $p < 0.005$ vs. control D-galactose, as indicated by brackets. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. (C) Western blot of carbonylated proteins. All the immunoblots reported are from one experiment representative of three independent biological experiments that gave similar results. Ponceau S was used as a loading control. (D) Density of Western blot bands was evaluated using the software ImageJ, and data are shown as the ratio of carbonylated proteins/Ponceau S. Data are expressed as means $\pm$ SD; $n = 3$ independent biological experiments; * $p < 0.05$, ** $p < 0.005$ vs. control D-galactose, as indicated by brackets. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test.

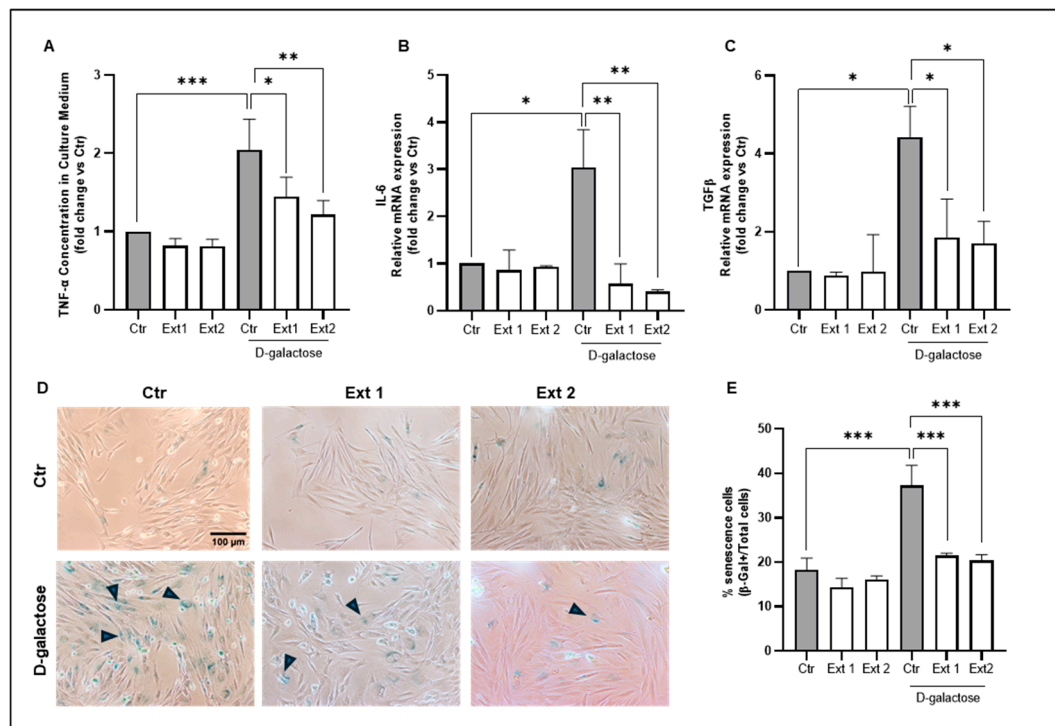


Figure 8. *T. borchii* extracts reduce inflammation and counteract the D-galactose-induced senescent phenotype in C2C12 myotubes. D-galactose (20 mg/mL) was added on day 1 of C2C12 differentiation and maintained throughout the experiment. On day 5, myotubes, with and without 20 mg/mL D-galactose, were treated with 100 µg/mL *T. borchii* extracts (Ext 1 and Ext2) for 48 h. (A) TNF-α concentration was determined by ELISA assay. Data are expressed as means ± SD; $n = 3$ independent biological experiments; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, as indicated by brackets. (B,C) Total RNA was isolated, and relative mRNA levels of IL-6 and TGF-β were analyzed by RT-qPCR. Data are expressed as means ± SD; $n = 3$ independent biological experiments; * $p < 0.05$, ** $p < 0.005$, as indicated by brackets. (D) Brightfield images (10×) to assess cellular senescence by histochemical staining for senescence-associated β-galactosidase (SA-β-Gal) in control C2C12 myoblasts, D-galactose-treated myoblasts, and myoblasts treated with D-galactose in combination with Extract 1 or Extract 2. Scale bar: 100 µm. (E) Quantification was performed on four independent biological replicates. For each replicate, at least three randomly selected non-overlapping fields were analyzed. Results of senescence-associated β-galactosidase are expressed as the percentage of SA-β-Gal-positive cells and reported as means ± SD; $n = 4$ independent biological experiments; *** $p < 0.0005$, as indicated by brackets. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test.

Definitive confirmation of the senescent state was obtained by histochemical staining for senescence-associated β-galactosidase (SA-β-gal). As shown in Figure 8D,E, while the percentage of positive cells was minimal in control cells, treatment with D-galactose caused a marked increase in senescence, with approximately 40% of cells displaying the characteristic blue cytoplasmic staining. The addition of Extracts 1 and 2 effectively counteracted the onset of the senescent phenotype (Figure 8D,E). In conclusion, these results demonstrate that *T. borchii* extracts not only preserve protein homeostasis and myotube morphology but also act upstream by preventing oxidative damage and blocking the transition to the senescent phenotype induced by D-galactose metabolic stress.

4. Discussion

Sarcopenia is a typical condition characterized by the loss of muscle tissue that occurs under physiological conditions, such as aging, as well as in pathological conditions leading

to a reduction in the body's lean mass. The health consequences of sarcopenia are broad, as it represents a key component of the individual frailty index and predicts the risk of disease development and hospitalization [37].

Given the major clinical and public health relevance of sarcopenia, numerous studies have focused on identifying preventive and therapeutic strategies capable of reducing its clinical consequences, including falls and frailty. Current strategies are mainly based on counteracting the pathways commonly activated during sarcopenia, such as the imbalance in protein turnover favoring protein degradation, oxidative stress, and the activation of inflammatory pathways. Many of these approaches include nutritional interventions and physical exercise programs aimed at restoring the lost muscle mass [30]. Additional approaches involve the use of dietary supplements and natural compounds, such as curcumin and green tea catechins, which have been shown to reduce the sarcopenic phenotype [38]. However, many of these supplements still present limitations related to bioavailability or to their inability to simultaneously target the multiple pathways involved in sarcopenia.

In this study, we lay the groundwork for exploring a novel nutraceutical perspective. Although the findings are limited to extracts obtained from only two *T. borchii* fruiting bodies, both extracts were able to attenuate the D-galactose-induced sarcopenic phenotype. This effect was observed at the phenotypic level, through the partial recovery of morphological alterations, and at the molecular level, through the reduction in sarcopenia-associated markers, inflammation, and oxidative stress. Moreover, treatment with the tested *T. borchii* extracts was associated with positive modulation of the mTOR/p70S6K pathway and with increased protein translation. Accordingly, qualitative TEM observations showed that representative myotubes treated with the tested extracts displayed mitochondria with comparatively preserved membranes and cristae, together with reduced cytoplasmic vacuolization in some examined fields. These findings suggest a preservation of mitochondrial integrity and a potential improvement in mitochondrial function, with a reduction in mitophagy, typically observed in sarcopenic models.

The attenuation of the D-galactose-induced phenotype observed with the two tested extracts suggests that they may warrant further investigation for their potential relevance in both preventive and intervention-oriented approaches to sarcopenia. This aspect may support the translational and clinical relevance of the truffle extracts, thereby providing a rationale for future studies to determine whether these effects can be reproduced in more representative preclinical models, even after the onset of the sarcopenic phenotype. Moreover, this evidence, together with the lack of detectable in vitro toxicity of the extracts, further supports their potential safety and applicability as promising candidates for the management of sarcopenia. On the other hand, it is important to clarify that the D-galactose-treated C2C12 system used in this study should be considered a reductionist in vitro model of accelerated muscle aging and sarcopenia-like atrophy, rather than a complete representation of clinical sarcopenia. D-galactose exposure reproduces selected cellular features relevant to age-associated muscle deterioration, including oxidative stress, senescence-associated alterations, impaired protein homeostasis, and myotube atrophy. However, clinical sarcopenia is a multifactorial condition involving systemic inflammation, endocrine changes, neuromuscular remodeling, altered physical activity, nutritional status, and tissue-level interactions that cannot be fully recapitulated in differentiated C2C12 myotubes.

HPLC-DAD analysis of the extracts highlighted the presence of compounds with well-known antioxidant and anti-inflammatory activity, including gallic acid [39], 3-hydroxytyrosol [40], vanillic acid [41], 4-hydroxybenzoic acid [42], and caffeic acid [43], further supporting the hypothesis that the beneficial effects of the *T. borchii* extracts may be at least partially mediated by their antioxidant and anti-inflammatory properties. Based on

these findings and considering that inflammation and oxidative stress represent key drivers of sarcopenia, we focused our study on investigating the potential effects of the tested extracts of *T. borchii* on these two processes. Interestingly, treatment with truffle extracts markedly reduced both oxidative stress and inflammation induced by the pro-sarcopenic stimulus. The reduction in oxidative stress and inflammation consequently contributes to the attenuation of cellular senescence, a hallmark of skeletal muscle aging and dysfunction. Indeed, chronic oxidative damage and persistent inflammatory signaling are known to promote the establishment of a senescent phenotype, characterized by impaired cellular homeostasis, reduced regenerative capacity, and the secretion of pro-inflammatory mediators [44,45]. From a speculative perspective, by counteracting these processes, treatment with *T. borchii* extracts may help preserve cellular functionality and limit the progression of the senescence-associated alterations typically observed in sarcopenic muscle. Although several phenolic compounds were identified in the two extracts, any potential contribution of these individual molecules to the observed biological effects remains hypothetical. For some of the compounds identified within the extracts, evidence already exists in the literature regarding their beneficial effects on sarcopenia and skeletal muscle homeostasis in general. In particular, gallic acid has been reported to enhance muscle function and improve muscle capacity through the induction of myogenesis [46], further supporting the potential contribution of this compound to the protective effects exerted by the *T. borchii* extracts observed in our study. Indeed, gallic acid has been shown to reduce exercise-induced muscle damage through its antioxidant activity, primarily exerted at the mitochondrial level. In particular, this compound was reported to improve mitochondrial function and ATP production, thereby contributing to the preservation of muscle integrity and functionality under stress conditions [47]. Similarly, caffeic acid has also been reported to alleviate muscle atrophy [48] and prevent sarcopenia [49], mainly through the modulation and reduction in inflammatory pathways. In particular, its anti-inflammatory activity appears to counteract the chronic inflammatory state associated with muscle wasting, thereby contributing to the maintenance of skeletal muscle homeostasis and functionality.

Among the compounds identified within the extracts, some have not yet been specifically investigated in the context of skeletal muscle biology and sarcopenia, such as hydroxytyrosol. This makes these molecules particularly interesting targets for future investigations aimed at further elucidating the mechanisms underlying the beneficial effects of *T. borchii* extracts. However, it should be noted that the chemical characterization performed in this study was limited to a targeted panel of phenolic and flavonoid compounds. Therefore, the extracts likely contain additional unidentified metabolites that may contribute, either individually or synergistically, to the biological effects observed in C2C12 myotubes. The active components responsible for these effects, therefore, remain to be identified. Future studies based on broader untargeted metabolomic profiling, extract fractionation, and validation experiments using purified compounds or enriched fractions will be required to define the molecular determinants of the observed bioactivity.

A potential advantage of using the tested extracts lies in their complex composition, characterized by a mixture of antioxidant and anti-inflammatory compounds. Indeed, our findings suggest that the extracts may simultaneously modulate multiple aspects of sarcopenia. In particular, treatment with *T. borchii* extracts is associated with an increase in protein synthesis over protein degradation, as indicated by the positive modulation of the p70S6K signaling pathway, which is associated with enhanced translational activity and anabolic processes. Consistently, puromycin incorporation assays showed a recovery in the rate of protein translation in treated cells. At the same time, proteasome-dependent protein degradation was attenuated, further supporting the ability of the extracts to favorably influence protein turnover balance and counteract the catabolic state typically associated

with sarcopenia. Moreover, the expression of the main inflammatory markers was down-regulated following truffle extracts treatment. Although both extracts produced broadly comparable effects, we identified small changes between Extracts 1 and 2. In particular, Extract 2 is able to increase cell proliferation more and is able to preserve mitochondrial ultrastructure by maintaining mitochondrial cristae compared to Extract 1.

The multifaceted phytochemical profile may allow the simultaneous modulation of multiple pathways involved in sarcopenia pathogenesis. Such a multi-target approach could lead to broader and more effective protective effects compared with single-compound interventions, thereby increasing the translational potential of these extracts for the prevention and treatment of sarcopenia. The complex phytochemical composition of the extracts may represent an advantage, as multiple identified and unidentified metabolites could act additively or synergistically to modulate different cellular processes involved in muscle wasting, including oxidative stress, inflammatory signaling, and proteostasis. A limitation of the present study is that the biological experiments were performed using two independently prepared extracts obtained from two individual *T. borchii* fruiting bodies. Although the two extracts were processed and tested separately, this sample size does not capture the broad biological and phytochemical variability that may characterize *T. borchii*, including variability related to genotype, maturation stage, environmental conditions, geographical origin, and host species. Therefore, future studies including a larger number of independently collected fruiting bodies and a broader phytochemical characterization will be required to establish the reproducibility, variability, and translational relevance of these effects.

5. Conclusions

In conclusion, these findings show that the two *T. borchii* extracts analyzed in this study exerted protective effects against several morphological and molecular features of D-galactose-induced sarcopenic stress in C2C12 myotubes. These data support the potential bioactivity of the tested extracts, but the limited number of fruiting bodies analyzed prevents broad generalization to *T. borchii* extracts as a whole. The results obtained with the two extracts examined in this study provide preliminary evidence that they may attenuate selected cellular alterations associated with D-galactose-induced sarcopenic stress. However, given that the extracts were derived from only two individual *T. borchii* fruiting bodies, these findings cannot be generalized to the species as a whole or interpreted as evidence of therapeutic efficacy. Rather, they provide a rationale for future studies using a larger number of independently collected specimens and more representative preclinical models to determine the reproducibility, safety, and potential translational relevance of the observed effects. In particular, the multifactorial activity of *T. borchii* extracts, encompassing antioxidant, anti-inflammatory, and pro-anabolic effects, makes them especially attractive candidates for future translational and clinical investigations. Overall, the present data should be considered as a promising basis for further preclinical investigation rather than as evidence of therapeutic applicability. Future studies using additional biological replicates, broader chemical characterization, primary muscle cells, and animal models of age-related muscle wasting will be required to confirm the reproducibility and biological relevance of these effects.

Author Contributions: Conceptualization, V.A., S.C., M.R.C., and S.B.; writing—original draft preparation, S.C., V.A., and S.B.; writing—review and editing, S.C., M.R.C., and S.B.; preparation of *T. borchii* extracts, A.P.; HPLC-DAD analysis, G.D.M. and A.G.; microscopy analysis, L.L., M.B., L.S., and M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Union—NextGenerationEU, Ministero dell’Università e della Ricerca, under the National Recovery and Resilience Plan (PNRR), Mission 4 (Education and Research), Component 2 (From Research to Business), Investment 1.1 (Notice Prin 2022, DD N. 104, 2 February 2022), from the title “Interactions of the white truffle *Tuber magnatum* with soil microbiome and plants” (proposal code: 2022K272X8—CUP J53D23010090006) and by the Italian Ministry of Health (Fondo Ricerca Corrente).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and analyzed during the current study are available upon request from the corresponding author.

Acknowledgments: The authors would like to thank Francesca Romana Pellegrini and Gaia Fattorini for their valuable advice and fruitful discussions.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

<i>T. borchii</i>	<i>Tuber borchii</i>
MuRF1	Muscle RING Finger 1
ROS	Reactive Oxygen Species
UPS	Ubiquitin–Proteasome System
Atrogin-1	FBXO32
mTOR	Mammalian Target of Rapamycin
p70S6K	Ribosomal Protein S6 Kinase β -1
HPLC-DAD	High-Performance Liquid Chromatography with Diode-Array Detection
MHC	Myosin Heavy Chain
mTORC1	Mechanistic Target of Rapamycin Complex 1
DHE	Dihydroethidium
IL-6	Interleukin-6
TGF- α	Transforming Growth Factor- β
SA- β -gal	Senescence-Associated β -galactosidase

References

1. Sayer, A.A.; Cooper, R.; Arai, H.; Cawthon, P.M.; Ntsama Essomba, M.-J.; Fielding, R.A.; Grounds, M.D.; Witham, M.D.; Cruz-Jentoft, A.J. Sarcopenia. *Nat. Rev. Dis. Prim.* **2024**, *10*, 68. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Ryall, J.G.; Schertzer, J.D.; Lynch, G.S. Cellular and Molecular Mechanisms Underlying Age-Related Skeletal Muscle Wasting and Weakness. *Biogerontology* **2008**, *9*, 213–228. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Meng, S.-J.; Yu, L.-J. Oxidative Stress, Molecular Inflammation and Sarcopenia. *Int. J. Mol. Sci.* **2010**, *11*, 1509–1526. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Park, S.S.; Kwon, E.-S.; Kwon, K.-S. Molecular Mechanisms and Therapeutic Interventions in Sarcopenia. *Osteoporos. Sarcopenia* **2017**, *3*, 117–122. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Vasilaki, A.; Mansouri, A.; Van Remmen, H.; van der Meulen, J.H.; Larkin, L.; Richardson, A.G.; McArdle, A.; Faulkner, J.A.; Jackson, M.J. Free Radical Generation by Skeletal Muscle of Adult and Old Mice: Effect of Contractile Activity. *Aging Cell* **2006**, *5*, 109–117. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Jo, E.; Lee, S.-R.; Park, B.-S.; Kim, J.-S. Potential Mechanisms Underlying the Role of Chronic Inflammation in Age-Related Muscle Wasting. *Aging Clin. Exp. Res.* **2012**, *24*, 412–422. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Clavel, S.; Coldefy, A.-S.; Kurkdjian, E.; Salles, J.; Margaritis, I.; Derjard, B. Atrophy-Related Ubiquitin Ligases, Atrogin-1 and MuRF1 Are Up-Regulated in Aged Rat *Tibialis Anterior* Muscle. *Mech. Ageing Dev.* **2006**, *127*, 794–801. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Gumucio, J.P.; Mendias, C.L. Atrogin-1, MuRF-1, and Sarcopenia. *Endocrine* **2013**, *43*, 12–21. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Sirago, G.; Picca, A.; Calvani, R.; Coelho-Júnior, H.J.; Marzetti, E. Mammalian Target of Rapamycin (mTOR) Signaling at the Crossroad of Muscle Fiber Fate in Sarcopenia. *Int. J. Mol. Sci.* **2022**, *23*, 13823. [\[CrossRef\]](#) [\[PubMed\]](#)

10. Joseph, G.A.; Wang, S.X.; Jacobs, C.E.; Zhou, W.; Kimble, G.C.; Tse, H.W.; Eash, J.K.; Shavlakadze, T.; Glass, D.J. Partial Inhibition of mTORC1 in Aged Rats Counteracts the Decline in Muscle Mass and Reverses Molecular Signaling Associated with Sarcopenia. *Mol. Cell. Biol.* **2019**, *39*, e00141–19. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Tejedor-Calvo, E.; Amara, K.; Reis, F.S.; Barros, L.; Martins, A.; Calhella, R.C.; Venturini, M.E.; Blanco, D.; Redondo, D.; Marco, P.; et al. Chemical Composition and Evaluation of Antioxidant, Antimicrobial and Antiproliferative Activities of *Tuber* and *Terfezia* Truffles. *Food Res. Int.* **2021**, *140*, 110071. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Carinci, E.; Castelli, S.; Vitiello, L.; Pennesi, A.; Amicucci, A.; Zambonelli, A.; Ciriolo, M.R.; Stocchi, V.; Baldelli, S. Antiproliferative and Pro-Apoptotic Effects of *Tuber Borchii* Extracts on Human Colorectal Cancer Cells via P53-Dependent Pathway Activation. *Metabolites* **2025**, *15*, 796. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Baldelli, S.; Aiello, G.; Bruno, A.D.; Castelli, S.; Lombardo, M.; Stocchi, V.; Tripodi, G. Bioactive Compounds and Antioxidant Potential of Truffles: A Comprehensive Review. *Antioxidants* **2025**, *14*, 1341. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Lee, H.; Nam, K.; Zahra, Z.; Farooqi, M.Q.U. Potentials of Truffles in Nutritional and Medicinal Applications: A Review. *Fungal Biol. Biotechnol.* **2020**, *7*, 9. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Moretti, A.; Tomaino, F.; Paoletta, M.; Liguori, S.; Migliaccio, S.; Rondanelli, M.; Di Iorio, A.; Pellegrino, R.; Donnarumma, D.; Di Nunzio, D.; et al. Physical Exercise for Primary Sarcopenia: An Expert Opinion. *Front. Rehabil. Sci.* **2025**, *6*, 1538336. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Jiang, X.; Chu, Q.; Li, L.; Qin, L.; Hao, J.; Kou, L.; Lin, F.; Wang, D. The Anti-Fatigue Activities of *Tuber Melanosporum* in a Mouse Model. *Exp. Ther. Med.* **2018**, *15*, 3066–3073. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Kumar, R.; Negi, P.S.; Singh, B.; Ilavazhagan, G.; Bhargava, K.; Sethy, N.K. Cordyceps Sinensis Promotes Exercise Endurance Capacity of Rats by Activating Skeletal Muscle Metabolic Regulators. *J. Ethnopharmacol.* **2011**, *136*, 260–266. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Zeppa, S.; Gioacchini, A.M.; Guidi, C.; Guescini, M.; Pierleoni, R.; Zambonelli, A.; Stocchi, V. Determination of Specific Volatile Organic Compounds Synthesised During *Tuber borchii* Fruit Body Development by Solid-Phase Microextraction and Gas Chromatography/Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 199–205. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Paolocci, F.; Rubini, A.; Granetti, B.; Arcioni, S. Rapid Molecular Approach for a Reliable Identification of *Tuber* Spp. Ectomycorrhizae. *FEMS Microbiol. Ecol.* **1999**, *28*, 23–30. [\[CrossRef\]](#)
20. Saltarelli, R.; Palma, F.; Gioacchini, A.M.; Calcabrini, C.; Mancini, U.; De Bellis, R.; Stocchi, V.; Potenza, L. Phytochemical Composition, Antioxidant and Antiproliferative Activities and Effects on Nuclear DNA of Ethanolic Extract from an Italian Mycelial Isolate of *Ganoderma lucidum*. *J. Ethnopharmacol.* **2019**, *231*, 464–473. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Baldelli, S.; Ciriolo, M.R. Altered S-Nitrosylation of P53 Is Responsible for Impaired Antioxidant Response in Skeletal Muscle During Aging. *Aging* **2016**, *8*, 3450–3467. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Castelli, S.; Desideri, E.; Laureti, L.; Felice, F.; De Cristofaro, A.; Scaricamazza, S.; Lazzarino, G.; Ciriolo, M.R.; Ciccarone, F. N-Acetylaspartate Promotes Glycolytic-to-Oxidative Fiber-Type Switch and Resistance to Atrophic Stimuli in Myotubes. *Cell Death Dis.* **2024**, *15*, 686. [\[CrossRef\]](#) [\[PubMed\]](#)
23. D'Agostino, A.; Di Marco, G.; Canini, A.; Gismondi, A. Gallic Acid as a Phytostimulant Enhancing Yield and Quality of *Mentha spicata* L. under Deficit- and Well-Watered Conditions. *Environ. Exp. Bot.* **2024**, *219*, 105656. [\[CrossRef\]](#)
24. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [\[CrossRef\]](#)
25. Di Marco, G.; D'Agostino, A.; Braglia, R.; Redi, E.L.; Iacobelli, S.; Gismondi, A.; Canini, A. Pollen Variability in *Quercus* L. Species and Relative Systematic Implications. *Plant Physiol. Biochem.* **2023**, *204*, 108079. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Castelli, S.; Ciccarone, F.; Taviani, D.; Ciriolo, M.R. ROS-Dependent HIF1 α Activation under Forced Lipid Catabolism Entails Glycolysis and Mitophagy as Mediators of Higher Proliferation Rate in Cervical Cancer Cells. *J. Exp. Clin. Cancer Res.* **2021**, *40*, 94. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Sansone, L.; de Iure, A.; Cristina, M.; Belli, M.; Vitiello, L.; Marcolongo, F.; Rosellini, A.; Macera, L.; Spezia, P.G.; Tomino, C.; et al. Nicotine in Combination with SARS-CoV-2 Affects Cells Viability, Inflammatory Response and Ultrastructural Integrity. *Int. J. Mol. Sci.* **2022**, *23*, 9488. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Malatesta, M.; Giagnacovo, M.; Renna, L.V.; Cardani, R.; Meola, G.; Pellicciari, C. Cultured Myoblasts from Patients Affected by Myotonic Dystrophy Type 2 Exhibit Senescence-Related Features: Ultrastructural Evidence. *Eur. J. Histochem.* **2011**, *55*, e26. [\[CrossRef\]](#) [\[PubMed\]](#)
29. de Souza, A.L.G.; Alves, A.L.R.; Martinez, C.G.; de Sousa, J.C.; Kurtenbach, E. Biomarkers of Skeletal Muscle Atrophy Based on Atrogenes Evaluation: A Systematic Review and Meta-Analysis Study. *Int. J. Mol. Sci.* **2025**, *26*, 3516. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Watchko, J.F.; Daoud, M.J.; Sieck, G.C. Myosin Heavy Chain Transitions During Development: Functional Implications for the Respiratory musculature. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **1998**, *119*, 459–470. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Castelli, S.; Aiello, G.; Aiello, V.; Massimino, E.; Pieri, M.; Amoah, I.; Lombardo, M.; Tripodi, G.; Baldelli, S. Nutritional Strategies and Aging: Current Evidence and Future Directions. *Molecules* **2026**, *31*, 756. [\[CrossRef\]](#) [\[PubMed\]](#)

32. Hornberger, T.A.; Sukhija, K.B.; Wang, X.-R.; Chien, S. mTOR Is the Rapamycin-Sensitive Kinase That Confers Mechanically-Induced Phosphorylation of the Hydrophobic Motif Site Thr(389) in P70^{S6k}. *FEBS Lett.* **2007**, *581*, 4562–4566. [[CrossRef](#)] [[PubMed](#)]
33. Pang, X.; Zhang, P.; Chen, X.; Liu, W. Ubiquitin-Proteasome Pathway in Skeletal Muscle Atrophy. *Front. Physiol.* **2023**, *14*, 1289537. [[CrossRef](#)] [[PubMed](#)]
34. Nyström, T. Role of Oxidative Carbonylation in Protein Quality Control and Senescence. *EMBO J.* **2005**, *24*, 1311–1317. [[CrossRef](#)] [[PubMed](#)]
35. Antuña, E.; Cachán-Vega, C.; Bermejo-Millo, J.C.; Potes, Y.; Caballero, B.; Vega-Naredo, I.; Coto-Montes, A.; Garcia-Gonzalez, C. Inflammaging: Implications in Sarcopenia. *Int. J. Mol. Sci.* **2022**, *23*, 15039. [[CrossRef](#)] [[PubMed](#)]
36. Zhang, X.; Li, H.; He, M.; Wang, J.; Wu, Y.; Li, Y. Immune System and Sarcopenia: Presented Relationship and Future Perspective. *Exp. Gerontol.* **2022**, *164*, 111823. [[CrossRef](#)] [[PubMed](#)]
37. Altherr, C.A.; Luo, X.; Ding, H.; Liu, Z.; Warden, S.J.; Moorthi, R.N.; Imel, E.A. Using an Electronic Frailty Index and Patient Reported Outcomes to Predict Sarcopenia Risk. *Sci. Rep.* **2025**, *15*, 35372. [[CrossRef](#)] [[PubMed](#)]
38. Najm, A.; Niculescu, A.-G.; Grumezescu, A.M.; Beuran, M. Emerging Therapeutic Strategies in Sarcopenia: An Updated Review on Pathogenesis and Treatment Advances. *Int. J. Mol. Sci.* **2024**, *25*, 4300. [[CrossRef](#)] [[PubMed](#)]
39. Hadidi, M.; Liñán-Atero, R.; Tarahi, M.; Christodoulou, M.C.; Aghababaei, F. The Potential Health Benefits of Gallic Acid: Therapeutic and Food Applications. *Antioxidants* **2024**, *13*, 1001. [[CrossRef](#)] [[PubMed](#)]
40. Moratilla-Rivera, I.; Pérez-Jiménez, J.; Ramos, S.; Portillo, M.P.; Martín, M.Á.; Mateos, R. Hydroxytyrosol Supplementation Improves Antioxidant and Anti-Inflammatory Status in Individuals with Overweight and Prediabetes: A Randomized, Double-Blind, Placebo-Controlled Parallel Trial. *Clin. Nutr.* **2025**, *52*, 17–26. [[CrossRef](#)] [[PubMed](#)]
41. Magiera, A.; Kołodziejczyk-Czepas, J.; Olszewska, M.A. Antioxidant and Anti-Inflammatory Effects of Vanillic Acid in Human Plasma, Human Neutrophils, and Non-Cellular Models In Vitro. *Molecules* **2025**, *30*, 467. [[CrossRef](#)] [[PubMed](#)]
42. Kou, Y.; Jing, Q.; Yan, X.; Chen, J.; Shen, Y.; Ma, Y.; Xiang, Y.; Li, X.; Liu, X.; Liu, Z.; et al. 4-Hydroxybenzoic Acid Restrains Nlrp3 Inflammasome Priming and Activation via Disrupting PU.1 DNA Binding Activity and Direct Antioxidation. *Chem.-Biol. Interact.* **2024**, *404*, 111262. [[CrossRef](#)] [[PubMed](#)]
43. Spagnol, C.M.; Assis, R.P.; Brunetti, I.L.; Isaac, V.L.B.; Salgado, H.R.N.; Corrêa, M.A. In Vitro Methods to Determine the Antioxidant Activity of Caffeic Acid. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2019**, *219*, 358–366. [[CrossRef](#)] [[PubMed](#)]
44. Falvino, A.; Gasperini, B.; Cariati, I.; Bonanni, R.; Chiavoghilefu, A.; Gasbarra, E.; Botta, A.; Tancredi, V.; Tarantino, U. Cellular Senescence: The Driving Force of Musculoskeletal Diseases. *Biomedicines* **2024**, *12*, 1948. [[CrossRef](#)] [[PubMed](#)]
45. He, Y.; Xie, W.; Li, H.; Jin, H.; Zhang, Y.; Li, Y. Cellular Senescence in Sarcopenia: Possible Mechanisms and Therapeutic Potential. *Front. Cell Dev. Biol.* **2021**, *9*, 793088. [[CrossRef](#)] [[PubMed](#)]
46. Lee, S.; Han, D.-Y.; Kim, K.K. Gallic Acid Improves Muscular Function Through Enhanced Myoblast Myogenesis in Mice. *Food Sci. Nutr.* **2025**, *13*, e70667. [[CrossRef](#)] [[PubMed](#)]
47. Yu, L.; Tian, D.; Su, Z.; Zhang, L.; Guo, S.; Zhu, W.; Fang, Y.; Wang, P.; Zhang, N. Gallic Acid Alleviates Exercise-Induced Muscle Damage by Inhibiting Mitochondrial Oxidative Stress and Ferroptosis. *J. Transl. Med.* **2025**, *23*, 30. [[CrossRef](#)] [[PubMed](#)]
48. He, J.; He, Z.; Wang, H.; Zhang, C.; Pei, T.; Yan, S.; Yan, Y.; Wang, F.; Chen, Y.; Yuan, N.; et al. Caffeic Acid Alleviates Skeletal Muscle Atrophy in 5/6 Nephrectomy Rats through the TLR4/MYD88/NF-κB Pathway. *Biomed. Pharmacother.* **2024**, *174*, 116556. [[CrossRef](#)] [[PubMed](#)]
49. Guo, Y.; Niu, K.; Okazaki, T.; Wu, H.; Yoshikawa, T.; Ohru, T.; Furukawa, K.; Ichinose, M.; Yanai, K.; Arai, H.; et al. Coffee Treatment Prevents the Progression of Sarcopenia in Aged Mice in Vivo and in Vitro. *Exp. Gerontol.* **2014**, *50*, 1–8. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.