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Different Cultivation Protocols Could Influence the Anti-Atrophic Activity of *Achillea millefolium* and *Silybum marianum* against Sarcopenia: An *In Vitro* Study

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Abstract

Sarcopenia, the age-related loss of skeletal muscle mass and function, is a growing global health burden and currently no pharmacological treatment is approved for it. Nutraceutical and phytochemical strategies are one of many candidate approaches for this disease. This study evaluated the anti-atrophic potential of *Achillea millefolium* and *Silybum marianum* extracts, obtained from plants cultivated under different combinations of culture conditions, namely, plant growth-promoting rhizobacteria (PGPR) inoculation, fertilization, and water availability, using a murine C2C12 myotube-based model of skeletal muscle atrophy. Differentiated myotubes were exposed to two distinct atrophic stimuli: dexamethasone (5 µM), modelling glucocorticoid-induced atrophy, and a combination of TNF-α (20 ng/mL) and IFN-γ (10 ng/mL), modelling inflammation-driven atrophy. Protective effects of each extract (100 µg/mL) were assessed through myotube diameter, intracellular reactive oxygen species (ROS) levels, LysoTracker staining of the lysosomal compartment, and RT-qPCR quantification of atrophy- and autophagy-related transcripts (*Fbxo32*, *Trim63*, *Bnip3*, *Gabarap*, *Ctsl*). None of the extracts altered myotube diameter under basal conditions. Both atrophic stimuli significantly reduced myotube diameter, and co-treatment with several extracts counteracted this reduction; extracts that best preserved myotube diameter also attenuated ROS accumulation. In contrast, the selected extracts had only limited effects on *Fbxo32* and *Trim63* induction and produced variable changes in lysosomal and autophagy-related gene expression, indicating that their protective activity was not consistently accompanied by changes in these transcriptional programs and may instead be related to reduced oxidative stress. Overall, these findings show that selected *S. marianum* and *A. millefolium* extracts can protect C2C12 myotubes against glucocorticoid- and inflammation-induced atrophy, and that the cultivation conditions of the source plants can meaningfully influence this protective activity, providing a basis for further phytochemical and mechanistic studies aimed at developing nutraceutical strategies against sarcopenia.

Introduction

Sarcopenia and the molecular basis of muscle atrophy

Around the world, the rapid growth of the elderly population is placing increasing pressure on healthcare and economic systems. Aging is a well-established risk factor for the development of multiple chronic diseases, including cardiovascular disease, stroke, cancer, osteoarthritis, dementia and sarcopenia, whose diagnosis and treatment impose a considerable burden on national healthcare budgets (Atella et al. 2019). Sarcopenia is an age-related skeletal muscle disorder characterized by a progressive decline in muscle strength, muscle mass, and physical performance. It represents a major clinical concern in the ageing population, as it is associated with frailty, falls, disability, hospitalization, loss of independence, reduced quality of life, and increased mortality. Its prevalence was estimated at around 50 million individuals in 2010, a figure expected to rise to more than 200 million by 2050 (Kalan et al. 2025). Nevertheless, sarcopenia is still frequently under-recognized, partly because of the heterogeneity of diagnostic approaches and the limited use of standardized screening procedures in routine clinical practice (Kalan et al. 2025). Moreover, ageing is accompanied by a chronic, low-grade systemic inflammatory state, often referred to as “inflammaging”, characterized by persistently elevated circulating levels of pro-inflammatory cytokines such as TNF-α and IL-6; this condition has been implicated in the onset and progression of sarcopenia, as it promotes muscle protein breakdown and impairs muscle regeneration (Franceschi et al. 2018; Dalle, Rossmeislova, and Koppo 2017).

Skeletal muscle accounts for approximately 40% of total body weight and represents one of the most dynamic, heterogeneous, and plastic tissues in the human body. It is organized into numerous bundles of myofibers, striated multinucleated cells with diameters ranging from 10 to 100 micrometers and lengths extending up to several centimeters (McCuller, Jessu, and Callahan 2023). Far from being a static structure, muscle tissue continuously remodels itself in response to nutritional, mechanical, hormonal and inflammatory stimuli, shifting between states of hypertrophy and atrophy. An increase in protein and organelle content leading to cell volume expansion defines hypertrophy, whereas net protein loss and enhanced organelle clearance resulting in tissue reduction characterize atrophy (Sartori, Romanello, and Sandri 2021). This equilibrium is sustained by the coordinated balance between anabolic and catabolic signaling pathways. Its disruption represents a common downstream feature of multiple pathological conditions, including aging, chronic organ failure, and malignancy (Renzini et al. 2021). At the molecular level, skeletal muscle protein homeostasis is largely regulated by the insulin/IGF-1–PI3K–Akt–mTOR axis, which promotes protein synthesis while concomitantly suppressing proteolytic pathways. Inhibition of this signaling cascade reduces Akt-mediated repression of catabolic programs, thereby facilitating activation of the two major protein degradation systems: the ubiquitin-proteasome system (UPS) and the

autophagy-lysosome pathway (ALP) (Sandri 2013; Sartori, Romanello, and Sandri 2021). Specifically, reduced Akt activity decreases FoxO phosphorylation, thereby promoting the nuclear accumulation of FoxO1, FoxO3, and FoxO4 and the activation of a transcriptional program comprising multiple atrophy-related genes, collectively referred to as “atrogenes” (Sartori, Romanello, and Sandri 2021). Within the UPS, the muscle-specific E3 ubiquitin ligases Atrogin-1/MAFbx (encoded by *FBXO32*) and MuRF1 (encoded by *TRIM63*) are major effectors of muscle protein degradation and are consistently induced across diverse atrophic conditions (Sartori, Romanello, and Sandri 2021). In parallel, FoxO signaling also promotes the ALP by inducing genes involved in autophagosome formation, mitophagy, and lysosomal proteolysis, including LC3, GABARAP family members, BNIP3, and cathepsin L (CTSL) (Mammucari et al. 2007). This pathway is essential for the removal of damaged organelles and dysfunctional proteins; however, excessive or dysregulated activation can contribute to muscle fiber loss and functional impairment (Bechet et al. 2005; Sandri 2013; Sartori, Romanello, and Sandri 2021).

Glucocorticoids and pro-inflammatory cytokines represent two of the best-characterized upstream drivers of the FoxO-mediated atrogene program and are widely used experimentally to model distinct forms of muscle wasting. Although synthetic glucocorticoids such as dexamethasone (DEXA) are primarily employed for their anti-inflammatory and immunosuppressive properties, prolonged or high-dose exposure is a well-established inducer of atrogene-associated skeletal muscle atrophy (Hwangbo et al. 2023). Mechanistically, glucocorticoid receptor activation impairs PI3K/Akt signaling, thereby favoring FoxO3 nuclear accumulation, with subsequent transcription of Atrogin-1 and MuRF1, leading to a muscle-wasting phenotype (Baehr, Furlow, and Bodine 2011; Hah et al. 2023; Hwangbo et al. 2023). A second major pathway contributing to muscle wasting is driven by chronic inflammation. Among pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) plays a prominent role by activating the IKK–IκB–NF-κB signaling axis. TNF receptor engagement promotes IKK-dependent degradation of IκB, thereby enabling NF-κB nuclear translocation and the transcriptional induction of catabolic genes, including *TRIM63*/*MuRF1*. In parallel, TNF-α can impair IRS1-dependent insulin/IGF-1 signaling, further shifting muscle protein homeostasis toward a catabolic state (Sartori, Romanello, and Sandri 2021). Although TNF-α alone is able to promote skeletal muscle atrophy, its combination with IFN-γ provides a more complex model of inflammation-driven muscle wasting by simultaneously engaging NF-κB- and JAK/STAT-dependent signaling. Previous studies in C2C12 myotubes have demonstrated a cooperative action of TNF-α and IFN-γ in promoting muscle catabolism, including activation of Atrogin-1 and MuRF1 and induction of the STAT3/NF-κB–iNOS axis (Pijet et al. 2013; Ma et al. 2017).

Beyond hormonal and cytokine-mediated signaling, oxidative stress represents another major contributor to skeletal muscle wasting. Excessive accumulation of reactive oxygen species (ROS) can damage cellular macromolecules, compromise mitochondrial function, and activate redox-sensitive signaling pathways, including NF-κB and FoxO3, thereby reinforcing catabolic programs involved in muscle protein degradation (Agrawal et al. 2023; Lee et al. 2025). In addition, oxidative stress can impair myogenic signaling and regenerative capacity, further contributing to the progressive loss of muscle mass and function (Lee et al. 2025). The close interplay among oxidative stress, inflammation, and disrupted protein homeostasis has therefore stimulated increasing interest in compounds with combined antioxidant and anti-inflammatory properties as potential countermeasures against muscle atrophy (Lee et al. 2025) (Figure 1).

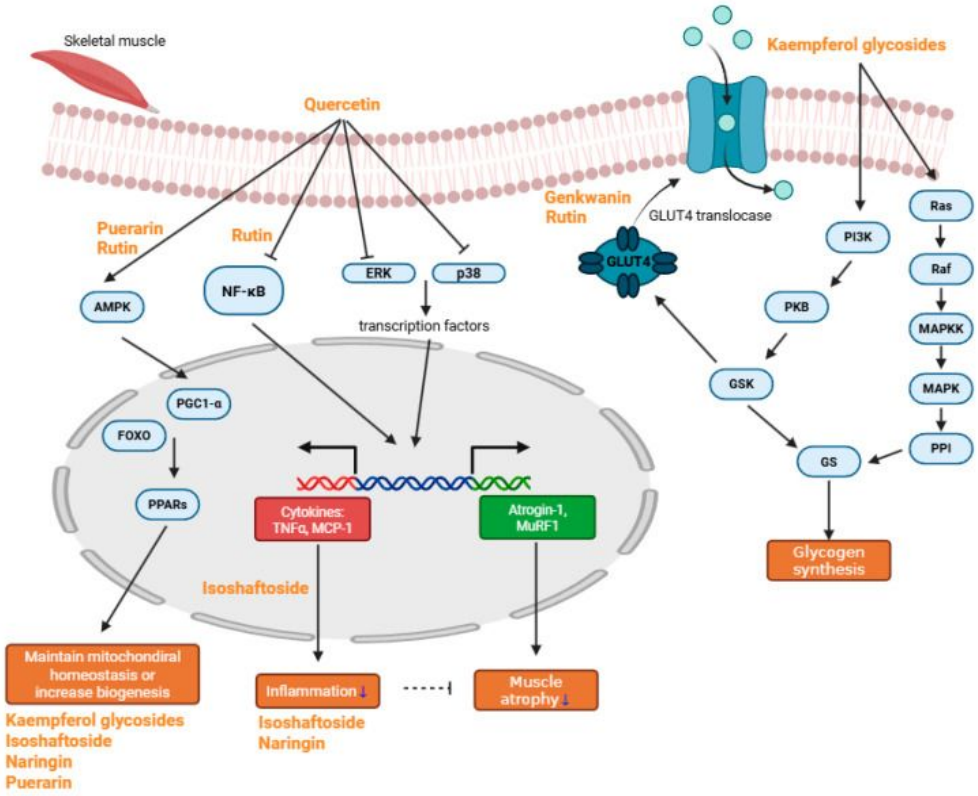


Figure 1. Signaling pathways involved in muscle atrophy.

Nutraceutical potential of *Achillea millefolium* and *Silybum marianum*

To date, no pharmacological treatment has been specifically approved for the management of sarcopenia, and current recommendations remain largely confined to nutritional supplementation and resistance exercise (Xue et al. 2026). In this context, nutraceutical and phytochemical interventions have gained increasing attention as potential preventive or adjunctive strategies, either alone or in combination with pharmacological and exercise-based approaches, particularly given the multifactorial nature of muscle wasting (Renzini et al. 2021). As previously discussed, sarcopenia is

closely associated with a state of chronic low-grade inflammation and oxidative stress, both of which actively contribute to the activation of proteolytic pathways and the progressive decline of skeletal muscle mass and function (Lee et al. 2025). Natural compounds exhibiting antioxidant and anti-inflammatory properties therefore represent attractive candidates in this setting. Among these, *Achillea millefolium* and *Silybum marianum* have attracted interest due to their traditional medicinal use and their rich phytochemical profiles, which include several bioactive compounds with potential antioxidant and anti-inflammatory activities (Villalva et al. 2022). In particular, extracts of *A. millefolium* obtained using different solvent systems have been reported to contain chlorogenic acid, dicaffeoylquinic acid isomers, luteolin, apigenin, and their glucosides, with antioxidant and antimicrobial activities that vary according to the extraction procedure employed (Ivanović et al. 2022). *S. marianum* is a medicinal plant widely studied for the biological properties of its major flavonolignan complex, silymarin. Beyond its well-established hepatoprotective activity, increasing evidence suggests that silymarin may exert beneficial effects in metabolic and oxidative stress-related conditions (Surai 2015). In experimental models of diet-induced obesity, silymarin supplementation has been associated with preservation of skeletal muscle mass and function, together with improvements in muscle performance (Kim et al. 2022). However, its potential protective effects in different models of muscle wasting, and the underlying molecular mechanisms, remain incompletely defined. Importantly, the biological activity of plant-derived extracts may be influenced by the cultivation conditions under which the plants are grown, as these can affect the production and accumulation of secondary metabolites. Plant growth-promoting rhizobacteria (PGPR), for instance, can enhance nutrient acquisition and modulate plant physiological responses, potentially influencing secondary metabolite biosynthesis and antioxidant properties. Similarly, fertilization regimes and water availability can affect the accumulation of bioactive compounds in medicinal plants (Ahemad and Kibret 2014).

In this context, a recent study on *S. marianum*, *A. millefolium*, and *Trifolium pratense* demonstrated that different combinations of PGPR inoculation, fertilization, and water availability significantly influenced the total antioxidant capacity of the resulting phytoextracts (Trabalzini et al. 2025). These findings suggest that modifying cultivation conditions may represent a promising strategy to enhance the biological activity of plant-derived extracts, potentially through changes in their phytochemical composition. Nevertheless, despite their reported antioxidant and anti-inflammatory properties, the anti-atrophic potential of *A. millefolium* and *S. marianum* extracts remains insufficiently investigated, particularly regarding the influence of different agronomic conditions on their protective activity against skeletal muscle atrophy.

Aim of the study

The aim of this thesis is to evaluate the protective effect of *Achillea millefolium* and *Silybum marianum* extracts obtained under different cultivation conditions against skeletal muscle atrophy using the murine C2C12 myotube cell line. Differentiated myotubes were exposed to two distinct atrophic stimuli, DEXA and TNF-α/IFN-γ, in the presence or absence of each extract. Protective effects were assessed by evaluating myotube morphology, intracellular oxidative stress, and the expression of molecular markers associated with the ubiquitin–proteasome and autophagy–lysosome pathways, including *Fbxo32*, *Trim63*, *Bnip3*, *Gabarap* and *Ctsl*. For each plant species, extracts obtained under the different cultivation conditions were compared with the corresponding original extract. By comparing extracts across both atrophic models, this study aims to determine whether their effects on skeletal muscle cells are reproducible, stimulus-dependent, and influenced by the cultivation conditions from which the extracts were derived. Overall, this work aims to lay the foundation for the development of novel nutraceutical strategies to counteract sarcopenia and, more broadly, age-related skeletal muscle atrophy.

Materials and methods

Reagents

Standardized dried extracts of *Achillea millefolium* and *Silybum marianum* were provided by Prof. Guglielmo Sorci (University of Perugia, Italy). Extracts were obtained from plants grown under standard cultivation conditions, hereafter referred to as original extracts (A-OG and C-OG, respectively), or from plants cultivated under different combinations of three agronomic factors, namely plant growth-promoting rhizobacteria (PGPR) inoculation, fertilization with K_2O and P_2O_5, and water availability. Two irrigation conditions were applied: full irrigation at 100% of field capacity and water stress at 40% of field capacity. Water stress was maintained for five weeks, before and during the early flowering stage (Trabalzini et al. 2025). Each extract was assigned a three-digit binary code indicating the cultivation conditions in the following order: PGPR inoculation (first digit), fertilization (second digit), and water availability (third digit). For PGPR inoculation and fertilization, 0 indicates the absence of treatment and 1 indicates its presence. For water availability, 0 indicates water stress (40% of field capacity), whereas 1 indicates full irrigation (100% of field capacity). Extracts derived from *A. millefolium* were coded A000–A111, while those derived from *S. marianum* were coded C000–C111 (Table 1). The same batch of each extract was used throughout the experiments. Dried extracts were dissolved in water to obtain stock solutions of 10 mg/mL and subsequently diluted to a final concentration of 100 µg/mL for the cellular assays.

Digit position	Agronomic factor	0	1
1st	PGPR	Absent	Present
2nd	Fertilizers (K_2O, P_2O_5)	Absent	Present
3rd	Water availability	Water stress (40% of field capacity)	Full irrigation (100% of field capacity)

Table 1. Three-digit coding of the cultivation conditions of the plant extracts.

Cell Culture and treatments

C2C12 myoblasts were grown at low density in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL antimycotic. To induce differentiation, cells were allowed to reach confluence, and the growth medium was replaced with differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum (GE Healthcare Bio-Sciences) and the same antibiotic/antimycotic supplementation described above. Following differentiation into myotubes, muscle atrophy was induced using two distinct experimental models. To model glucocorticoid-induced atrophy (Klein and Vaughan 2026), myotubes were treated for 48 h with 5 µM DEXA, used as a positive control for atrophy induction, in the presence or absence of phytoextracts. To model inflammatory-type, chronic atrophy (Pijet et al. 2013), myotubes were

treated for 48 h with a combination of 20 ng/mL TNF-α and 10 ng/mL IFN-γ, used as a positive control of atrophy, in the presence or absence of phytoextracts. All treatments were performed in triplicate in 24-well culture plates. Experimental groups included untreated controls (NT), atrophy-only controls (DEXA or TNF-α/IFN-γ), and each atrophy inducer combined with the respective phytoextract. For every experiment, at least 10 myotube diameters were measured for each field, five different fields for each replicate, and three technical replicates for each used treatment. Myotube diameters were measured with JMicroVision software.

RNA extraction, reverse transcription, and RT-qPCR

Total RNA from myotubes was extracted by Nucleozol (Macherey Nagel). The RNA was retro-transcribed with High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) and real-time PCR was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad) using the following TaqMan probes (Thermo Fisher Scientific): *Bnip3* (Mm01275600_g1), *Gabarap* (Mm00490678_m1), *Ctsl* (Mm00515597_m1), *Fbxo32* (Mm00499523_m1), *Trim63* (Mm01185221_m1) and *Gusb* (Mm01197698_m1).

Reactive Oxygen Species (ROS) production

To measure cellular ROS production, C2C12 myotubes were stained with CellROX® Deep Red Reagent for 30 min at 37 °C and washed with PBS. Images were acquired using the EVOS™ XL Core imaging System with a 40× objective, and the mean fluorescence signal intensity was measured using ImageJ. For every experiment assessing cellular oxidative stress, at least three myotubes in each field, five different fields for each replicate, three technical replicates for each treatment were measured.

LysoTracker staining

To assess the lysosomal compartment, C2C12 myoblasts were differentiated and treated on glass coverslips in 24-well culture plates. At the end of the treatments, cells were incubated with 50 nM LysoTracker Red DND-99 for 45 min at 37 °C in 5% CO_2. Following incubation, the excess of fluorophore was removed by washing with PBS, and coverslips were transferred to a µ-Dish 35 mm, high (Ibidi GmbH) for live-cell imaging, performed using a Leica TCS SP8 confocal laser scanning microscope with a 63× immersion objective.

Statistical Analysis

Data are presented as the mean ± SEM. Outliers were identified using the interquartile range (IQR) method, defined as values below Q1 − 1.5×IQR or above Q3 + 1.5×IQR, and were excluded from the analysis. Differences among groups were evaluated by one-way ANOVA, unless otherwise stated. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Effects of *Silybum marianum* and *Achillea millefolium* extracts on C2C12 myotube diameter

Before assessing their anti-atrophic activity, *Silybum marianum* and *Achillea millefolium* extracts obtained from plants grown under different cultivation conditions were tested on differentiated C2C12 myotubes under basal conditions. None of the extracts significantly altered myotube diameter compared with untreated controls (Figure 2A and 2D), excluding an intrinsic atrophic or hypertrophic effect of the extracts *per se*. Their protective activity was subsequently investigated using DEXA or the combination of TNF-α and IFN-γ to induce atrophy in C2C12 myotubes. Both treatments reduced myotube diameter compared with untreated cells, confirming the establishment of an atrophic phenotype. Co-treatment with the plant-derived extracts counteracted the reduction in myotube diameter to different extents, depending on the plant species, the atrophic stimulus, and the specific growth condition tested. Following TNF-α/IFN-γ exposure, C-OG and C101 significantly preserved myotube diameter among *S. marianum* extracts, while A010, A011, A100, A101 and A110 produced a significant protective effect among *A. millefolium* extracts (Figure 2B and 2E). A comparable protective response was observed in the DEXA model: C-OG, C100 and C111 significantly attenuated the loss of myotube diameter compared with DEXA alone, restoring values close to those observed in untreated cells, while among *A. millefolium* extracts, A011, A100, A101 and A110 significantly counteracted DEXA-induced atrophy (Figure 2C and 2F). These findings show that several extracts were able to attenuate the reduction in myotube diameter, although the protective response varied among the tested preparations and between the two atrophic stimuli.

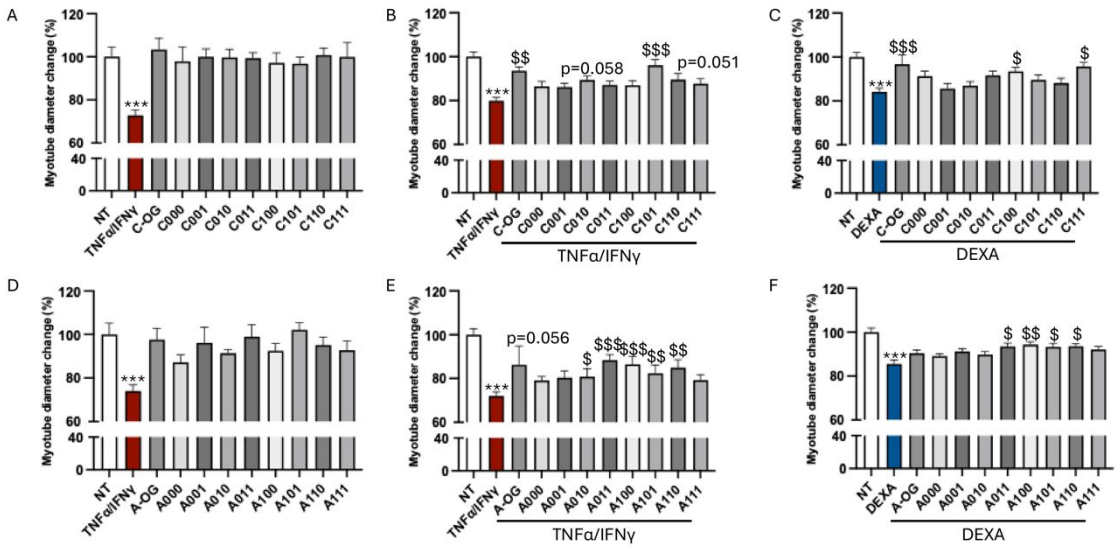


Figure 2. Effects of *Silybum marianum* and *Achillea millefolium* extracts on C2C12 myotube diameter in basal and atrophic conditions. Differentiated C2C12 myotubes were treated for 48 h with *S. marianum* (A–C) and *A. millefolium* (D–F) extracts (100 µg/mL), obtained from plants grown under different cultivation conditions, either alone (A, D) or in combination with TNF-α/IFN-γ (20 ng/mL and 10 ng/mL, respectively) (B, E) or DEXA (5 µM) (C, F). In panels A and D, TNF-α/IFN-γ-treated myotubes are shown as the atrophic reference. Myotube diameter was measured as an index of muscle atrophy and compared with untreated (NT) and atrophic controls. Data are presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Data were analyzed by one-way ANOVA. ***P < 0.001 vs NT; $$$P < 0.001, $$P < 0.01, and $P < 0.05 vs the corresponding atrophic stimulus.

Phytoextracts reduce oxidative stress in C2C12 myotubes exposed to atrophic stimuli

Since oxidative stress represents an important component of skeletal muscle atrophy (Agrawal et al. 2023), we first verified whether the atrophic stimuli used in this study increased intracellular ROS levels. ROS were measured in live myotubes using the fluorogenic probe CellROX Deep Red, and the mean fluorescence intensity was quantified from fluorescence images. Both TNF-α/IFN-γ and DEXA significantly increased CellROX fluorescence compared with untreated myotubes, confirming that both atrophic models were associated with oxidative stress (Figure 3). We then assessed whether the phytoextracts were also able to preserve cellular redox homeostasis. In TNF-α/IFN-γ-treated myotubes, most *S. marianum* extracts attenuated ROS accumulation, with several conditions almost completely abolishing the increase in intracellular ROS production. In particular, C-OG, C010, C011, C101, C110 and C111 significantly reduced ROS levels, with C101 and C110 showing the most evident effect. A similar response was observed for *A. millefolium*, although in this case the effect was restricted to a smaller number of conditions, A011, A100, A110 and A111, all of which significantly reduced ROS accumulation (Figure 3A–B). Under DEXA exposure, the antioxidant response was more selective. Among the *S. marianum* preparations, C001, C101 and C110 significantly attenuated DEXA-induced ROS accumulation. Likewise, several *A. millefolium* extracts, including A-OG, A000, A010, A011, A110 and A111, significantly reduced intracellular ROS production compared with DEXA-treated myotubes (Figure 3A and 3C).

Only the extracts able to reduce ROS levels induced by the atrophic stimuli were used for further analyses alongside their corresponding original preparations.

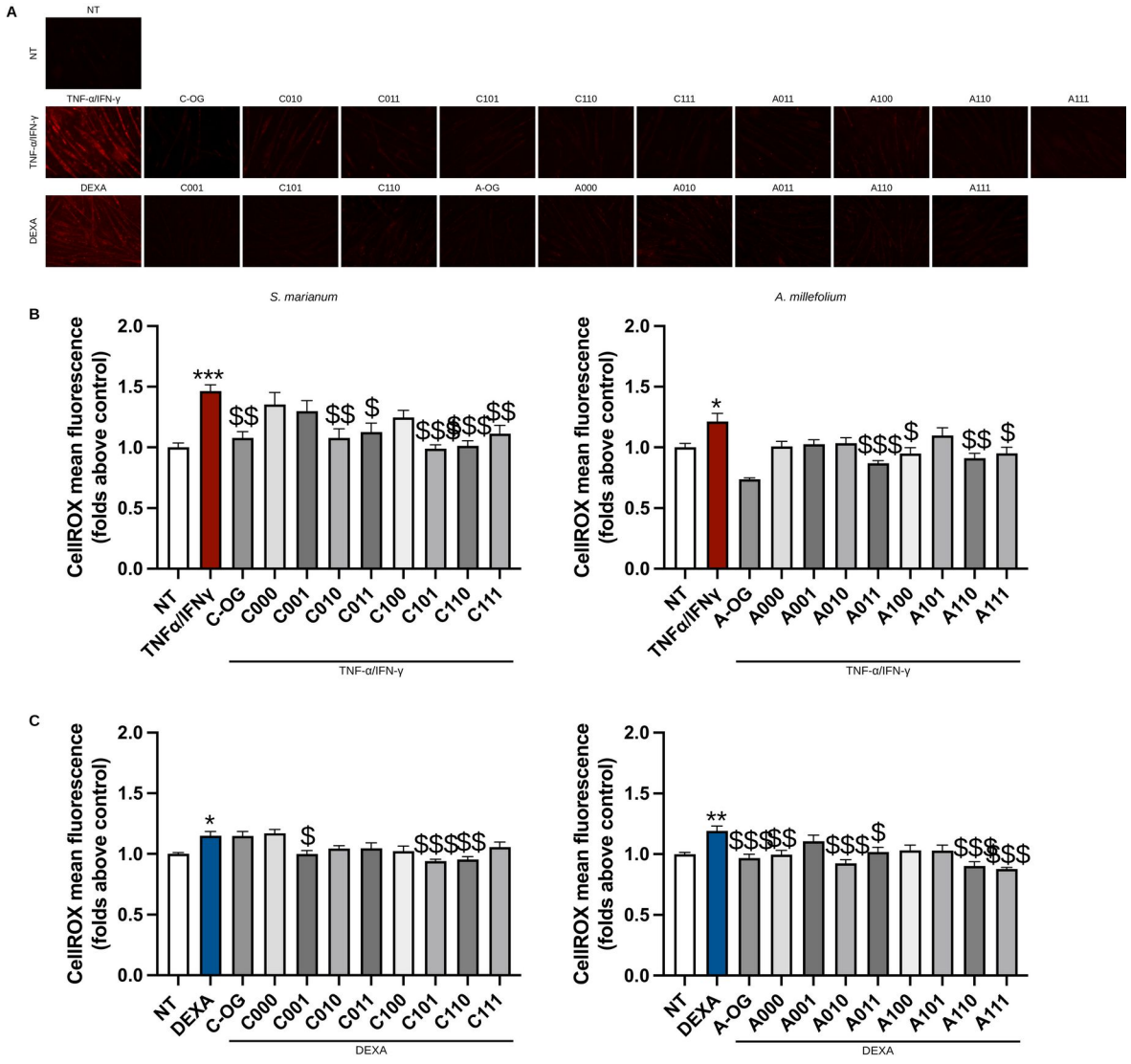


Figure 3. Intracellular ROS levels in C2C12 myotubes treated with *Silybum marianum* and *Achillea millefolium* extracts under atrophic conditions. Differentiated C2C12 myotubes were exposed for 48 h to TNF-α/IFN-γ (20 ng/mL and 10 ng/mL, respectively) (B) or DEXA (5 µM) (C), in the presence or absence of *S. marianum* and *A. millefolium* extracts (100 µg/mL). (A) Representative CellROX Deep Red images (40× objective) of untreated (NT) and atrophic myotubes and of myotubes co-treated with the extracts that significantly reduced ROS accumulation. (B, C) CellROX mean fluorescence intensity was quantified and normalized to the untreated control. Data are presented as mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA. ***P < 0.001, **P < 0.01, and *P < 0.05 vs NT; $$$P < 0.001, $$P < 0.01, and $P < 0.05 vs the corresponding atrophic stimulus (TNF-α/IFN-γ or DEXA).

Expression of atrophy-related genes following phytoextract co-treatment

To determine whether the protective phenotype was accompanied by modulation of the canonical atrophy program, we assessed the expression of *Fbxo32* (Atrogin-1) and *Trim63* (MuRF1), two muscle-specific E3 ubiquitin ligases that act as main regulators of proteasome-mediated proteolysis during skeletal muscle atrophy. Co-treatment with the selected phytoextracts generally tended to reduce *Fbxo32* expression compared with the corresponding atrophic stimuli, an effect that reached statistical significance only for A-OG under DEXA exposure. In contrast, *Trim63* expression was

not significantly modulated by any of the tested phytoextracts under TNF-α/IFN-γ stimulation, although a trend toward reduced expression was observed following DEXA co-treatment (Figure 4A–B). Overall, although the selected phytoextracts preserved myotube diameter and reduced intracellular ROS accumulation, these protective effects were not accompanied by a significant modulation in *Fbxo32* and *Trim63* expression. These findings suggest that additional cellular mechanisms may contribute to the anti-atrophic activity of the extracts.

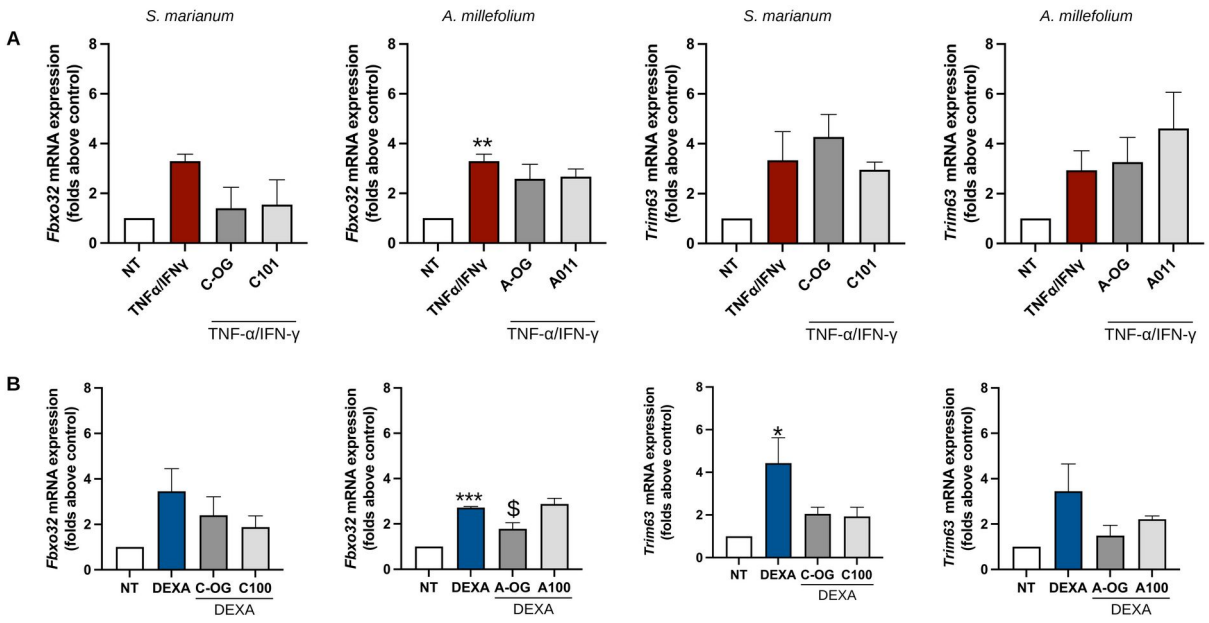


Figure 4. Effects of *Silybum marianum* and *Achillea millefolium* extracts on atrophy-related gene expression in C2C12 myotubes. Differentiated C2C12 myotubes were exposed for 24 h to TNF-α/IFN-γ (20 ng/mL and 10 ng/mL, respectively) (A) or DEXA (5 µM) (B), in the presence or absence of the selected *S. marianum* and *A. millefolium* extracts (100 µg/mL) or of the corresponding original extracts (C-OG, A-OG). mRNA expression levels of *Fbxo32* (Atrogin-1) and *Trim63* (MuRF1) were assessed by real-time PCR and normalized to *Gusb* as the housekeeping gene. Data are presented as mean ± standard error of the mean (SEM) from two to three independent experiments. Data were analyzed by one-way ANOVA. ***P < 0.001, **P < 0.01, and *P < 0.05 vs NT; $P < 0.05 vs DEXA.

Effects of selected phytoextracts on the lysosomal compartment

Since changes in *Fbxo32* and *Trim63* expression only partially reflected the protective phenotype observed at the morphological and oxidative stress levels, we next investigated whether autophagy, another pathway crucially involved in muscle atrophy (Sandri 2013), might contribute to the response to the selected phytoextracts. To this aim, changes in the lysosomal compartment were assessed by LysoTracker staining in both atrophic models. Co-treatment with the selected phytoextracts attenuated TNF-α/IFN-γ-induced LysoTracker fluorescence, although the extent of the effect differed between the two plant species. Specifically, C101 produced a more pronounced reduction in fluorescence than its corresponding original extract. Under DEXA exposure, C100 showed a slight reduction in LysoTracker fluorescence compared with its corresponding original

extract, whereas A100 and A-OG displayed comparable fluorescence levels, indicating no additional protective effect of the selected condition over the original extract (Figure 5A–C). Overall, these results highlight a stimulus- and extract-dependent modulation of the lysosomal compartment: while *A. millefolium* displayed a protective profile in both atrophic models, the effect of *S. marianum* appeared to be restricted to the TNF-α/IFN-γ condition, indicating that the lysosomal response to the phytoextracts depends on both the nature of the atrophic stimulus and the plant-derived preparation.

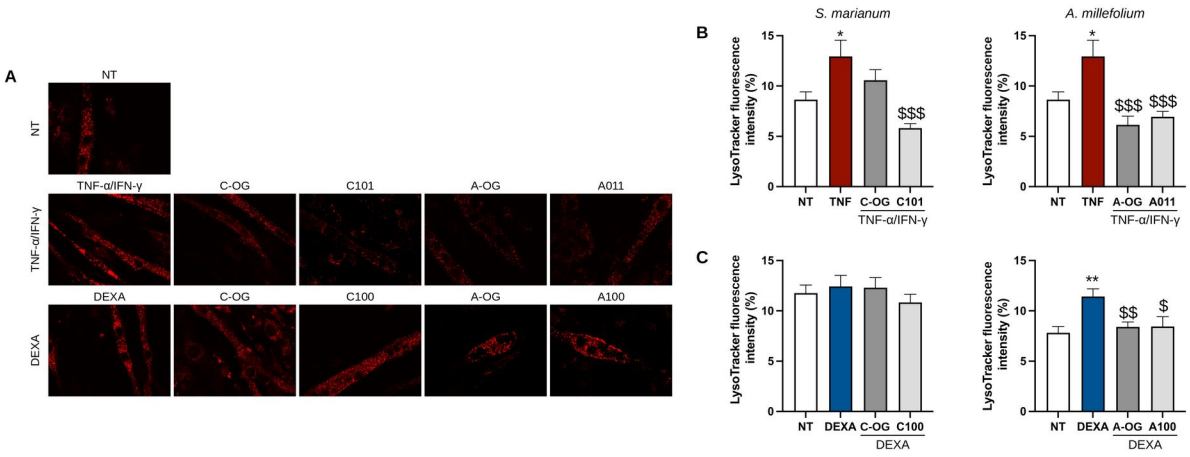


Figure 5. Modulation of the lysosomal compartment by selected phytoextracts under atrophic conditions. Differentiated C2C12 myotubes were treated for 48 h with TNF-α/IFN-γ (20 ng/mL and 10 ng/mL, respectively) or DEXA (5 µM), in the presence or absence of the selected *S. marianum* and *A. millefolium* extracts (100 µg/mL) or of the corresponding original extracts. (A) Representative live-cell confocal images of LysoTracker Red-stained myotubes (63× objective). (B, C) LysoTracker fluorescence intensity, quantified as an indirect readout of changes in acidic intracellular compartments, in TNF-α/IFN-γ-treated (B) and DEXA-treated (C) myotubes. Data are presented as mean ± SEM. Data were analyzed by one-way ANOVA. **P < 0.01 and *P < 0.05 vs NT; $$$P < 0.001, $$P < 0.01, and $P < 0.05 vs the corresponding atrophic stimulus.

Expression of lysosomal and autophagy-related genes

To further investigate the molecular changes associated with the lysosomal compartment, the expression of the lysosomal- and autophagy-related genes *Bnip3* (Irazoki, Zorzano, and Sebastian 2022), *Ctsl* (Deval et al. 2001), and *Gabarap* was evaluated in both atrophic models. Under TNF-α/IFN-γ treatment, *Bnip3* expression tended to decrease with both *S. marianum* and *A. millefolium* extracts. In contrast, *Ctsl* expression tended to further increase following phytoextract co-treatment in both C101 and A011, showing a more pronounced increase than their corresponding original extracts. *Gabarap* showed a divergent pattern between the two species: while both C-OG and C101 tended to reduce, A011 increased the expression levels compared with A-OG (Figure 6A). A different transcriptional response was observed following DEXA exposure. In this model, phytoextract generally resulted in a tendency toward reduced expression of *Bnip3*, *Ctsl*, and

Gabarap in both plant conditions. The only exception was observed for *Bnip3* where C-OG was associated with increased expression, whereas C100 showed a tendency toward reduction. Overall, both *S. marianum* and *A. millefolium* extracts tended to attenuate the expression of the investigated genes under DEXA-induced atrophy (Figure 6B).

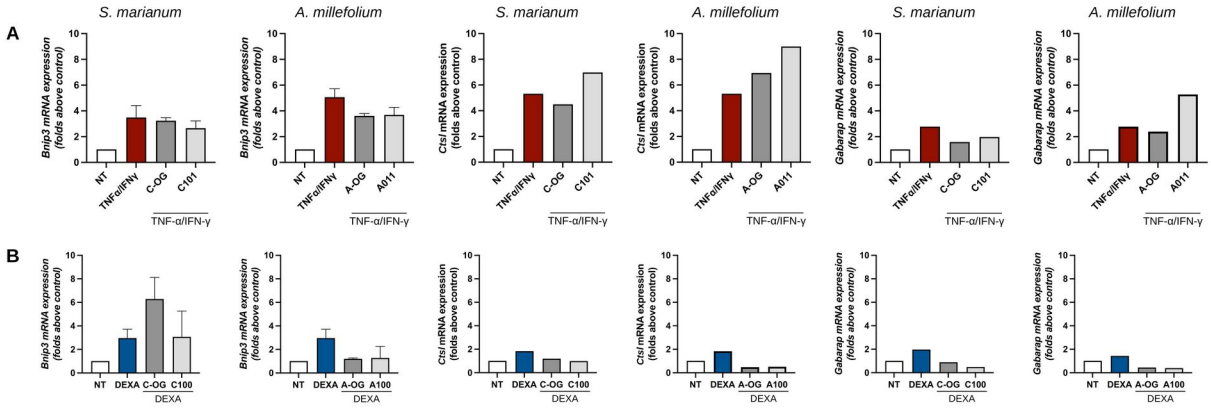


Figure 6. Expression of lysosomal and autophagy-related genes in C2C12 myotubes under atrophic conditions. Differentiated C2C12 myotubes were treated for 24 h with TNF-α/IFN-γ (20 ng/mL and 10 ng/mL, respectively) (A) or DEXA (5 µM) (B), in the presence or absence of the selected *Silybum marianum* and *Achillea millefolium* extracts (100 µg/mL) or of the corresponding original extracts. mRNA expression levels of *Bnip3*, *Ctsl*, and *Gabarap* were assessed by real-time PCR and normalized to *Gusb* as the housekeeping gene. *Bnip3* data are presented as mean ± SEM of two to three independent experiments; *Ctsl* and *Gabarap* data derive from a single experiment and are therefore shown without error bars and were not analyzed statistically.

Discussion

Sarcopenia is an age-related skeletal muscle disorder that highlights the need for additional strategies to preserve muscle mass and function. In this context, plant-derived extracts have attracted interest as potential sources of bioactive compounds involved in muscle homeostasis (Hwangbo et al. 2023; Salucci et al. 2024; Lee et al. 2025).

The present study investigated whether different cultivation conditions, including plant growth-promoting rhizobacteria (PGPR) inoculation, fertilization, and water availability, could influence the protective activity of *S. marianum* and *A. millefolium* extracts against skeletal muscle atrophy. These conditions may affect the accumulation and composition of plant bioactive compounds, potentially altering the biological activity of the resulting extracts (Trabalzini et al. 2025). Based on myotube diameter and intracellular ROS levels, different preparations were selected for the two atrophic models. Under DEXA exposure, A100 and C100, both obtained from plants grown with PGPR inoculation under water stress and without fertilization, were selected. Under TNF-α/IFN-γ exposure, the selected extracts were A011, obtained with fertilization and water supplementation but without PGPR inoculation, and C101, obtained with PGPR inoculation and water supplementation but without fertilization. These results indicate that the protective response varied according to the plant species, cultivation conditions, and atrophic stimulus.

At the molecular level, the selected extracts showed limited effects on the expression of the atrophy-related genes *Fbxo32* and *Trim63*, despite preserving myotube diameter. This indicates that their protective activity was not consistently accompanied by changes in the expression of these genes. Similarly, changes in LysoTracker fluorescence and in the expression of lysosomal- and autophagy-related genes differed between the two atrophic models. Together, these results suggest that additional cellular mechanisms may contribute to the protective effects of the extracts, potentially including the reduction of oxidative stress.

Interestingly, in a previous study by the group of Prof. Guglielmo Sorci (University of Perugia), selected phytoextracts were characterized by mass spectrometry and their antioxidant capacity was evaluated (Trabalzini et al. 2025). Among the *S. marianum* preparations, C101, obtained with PGPR inoculation and full irrigation, without fertilization, showed the highest total antioxidant capacity. This is consistent with the selection of C101 in the present study under TNF-α/IFN-γ exposure, where it preserved myotube diameter and reduced intracellular ROS accumulation. Its antioxidant properties may therefore contribute to the protective activity observed in this model.

This study has some limitations. Although mass spectrometry analysis was previously performed on selected preparations (Trabalzini et al. 2025), a comprehensive comparison of the phytochemical composition of the extracts selected in the present study and their corresponding original preparations was not available. In addition, the molecular analyses focused on a limited number of atrophy- and autophagy-related genes, without investigating other crucial signaling pathways

involved in atrophy or mitochondrial function, which may also contribute to the protective effects observed. Future studies should therefore compare the phytochemical composition of the selected extracts with that of their original preparations and investigate additional molecular mechanisms to better understand their anti-atrophic activity.

Overall, this study shows that selected *S. marianum* and *A. millefolium* extracts can protect C2C12 myotubes against glucocorticoid- and inflammation-induced atrophy. These findings highlight the importance of considering cultivation conditions when investigating the anti-atrophic activity of plant-derived extracts and provide a basis for further studies on their potential use in strategies aimed at counteracting skeletal muscle atrophy.

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