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Abstract

The effects of *Magnolia denudata* flower extract on the proliferation and differentiation of Hanwoo (Korean native cattle) satellite cells were examined to assess its suitability as a natural bioactive material for cultured meat production. The extract was prepared via ethanol extraction and freeze-drying, and HPLC used to quantify the contents of two major active compounds, magnolol and honokiol. Hanwoo satellite cells were isolated and cultured under proliferation and differentiation conditions with varying concentrations of (0, 0.5, 5, and 50) μg/mL of the extract. During the proliferation phase, no significant differences were observed in cell viability, cell count, or PAX7–positive nuclei across treatment groups, indicating that the extract did not affect cell proliferation. Likewise, the mRNA expression levels of *PAX7*, *MAPK1*, and *AKT1* showed no significant differences, whereas *MYOD1* expression was significantly decreased, suggesting a shift away from myogenic commitment rather than active progression toward differentiation. In contrast, during the differentiation phase, treatment with the extract significantly increased the mRNA and protein expression levels of myogenic markers MYOG and MYH1. The effect on myogenic differentiation was most pronounced at the highest concentration (T3, 50 μg/mL). These results suggest that *Magnolia denudata* flower extract promotes the differentiation of Hanwoo muscle satellite cells without affecting their proliferation, supporting its application in cultured meat production as a functional additive.

Keyword: Hanwoo muscle satellite cell, *Magnolia denudata* flower, Proliferation, Differentiation, Additive

Introduction

The current annual meat consumption worldwide amounts to approximately 360 million tons, and by 2050 is projected to increase by more than 70 % [1]. As global concerns grow regarding the sustainability of food and meat production, the development of alternative meat sources has emerged as a potential solution. Cultured meat, which involves cultivating stem cells or progenitor cells *in vitro* to form muscle tissue, is gaining attention as a sustainable alternative that can overcome the limitations of conventional livestock farming [2, 3]. However, cultured meat still faces several challenges that include high production costs, ethical issues surrounding the use of animal-derived components, and technical limitations, such as muscle cell proliferation and myofiber formation [4, 5].

To address these challenges, various functional materials with pharmacological activities, such as antioxidants and antimicrobial effects, have been actively developed, with natural products receiving particular attention as promising resources [6, 7]. Some natural products have been reported to possess bioactivities that promote muscle cell proliferation and myofiber formation, which are key processes in cultured meat production, by modulating critical signaling pathways, such as PI3K/Akt/mTOR, Wnt/β–catenin, and p38 MAPK [8]. The PI3K/Akt/mTOR pathway promotes protein synthesis and cell proliferation, the Wnt/β–catenin pathway regulates satellite cell self-renewal and differentiation, and the p38 MAPK pathway controls myofiber formation by regulating the expression of muscle-specific transcription factors [9-11]. For example, black ginseng extract has been reported to promote the differentiation of muscle progenitor cells through activation of the Akt pathway [12], while catechins and creatine, which are individual natural compounds, have shown beneficial effects on satellite cell proliferation and myofiber formation by activating both Akt and p38 MAPK pathways [13, 14].

Among these, *Magnolia denudata*, a deciduous shrub native to East Asia, has traditionally been used as an ornamental and medicinal plant [15]. *Magnolia denudata* is rich in polyphenols and flavonoids, and exhibits various pharmacological effects that include antioxidant, antimicrobial, anti-inflammatory, and sedative activities [16, 17]. The main bioactive compounds in *Magnolia denudata*,

magnolol and honokiol, are known to have strong antioxidant activities, and protect cells by reducing intracellular reactive oxygen species (ROS) [18, 19]. These compounds have also demonstrated anticancer and anti-obesity effects at the cellular level, particularly in cancer and adipocyte cells [20, 21], and are reported to influence key signaling pathways, such as PI3K/Akt/mTOR and MAPK, suggesting their potential role in regulating cell growth [22, 23].

Magnolia denudata flower has been traditionally used as an edible material, such as in tea, and has been reported to exhibit antioxidant-dominant functional properties that may contribute to the regulation of intracellular oxidative stress [24]. In addition, the flower has been reported to contain a relatively lower total phenolic content compared with the bark [25], suggesting a lower likelihood of excessive cellular stimulation associated with high phenolic levels.

Therefore, this study aimed to evaluate the effects of *Magnolia denudata* flower extract on the proliferation and myogenic differentiation of muscle satellite cells, thereby highlighting its potential as a functional natural material for cultured meat applications.

2. Materials and Methods

2.1. Preparation of freeze-dried *Magnolia denudata* flower extract

Magnolia denudata flowers used in this study were collected from ten trees planted for landscaping on the campus of Chungbuk National University in April 2024. Fully bloomed flowers were used. The collected samples were washed immediately after harvesting and dried at room temperature in a well-ventilated area. The dried flowers were extracted by adding 80% ethanol and incubating in a shaking water bath (Biofree, Korea) at 37°C for one week. Insoluble residues were removed to obtain a clarified extract, which was subsequently concentrated at 45°C, using a rotary vacuum evaporator (EYELA, Japan). This extraction process was repeated twice. The concentrated extract was subsequently freeze-dried at –80°C for 12 hours using a freeze dryer (Bondiro, Korea), and the

resulting powder, which was stored at −80 °C until experimental use [26]. All experiments were conducted using a single batch of freeze-dried *Magnolia denudata* flower extract to minimize batch-to-batch variability.

2.1.2 Magnolol and Honokiol analysis by High-Performance Liquid Chromatography (HPLC)

Magnolol and honokiol (HAWN, China) were individually dissolved in DMSO at a concentration of 10 mM. For calibration, the standards were diluted to appropriate concentrations. Ten milligrams of freeze-dried *Magnolia denudata* flower extract were dissolved in 1 mL of 70% methanol to obtain a sample concentration of 10 mg/mL.

Chromatographic separation was performed on a C18 column (250 mm × 4.6 mm, 5 μm) with UV detection. The run time was 45 min (flow rate 1.0 mL/min; injection 10 μL), with detection at 290 nm and the column maintained at 30°C. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was MeOH/ACN (50:50, v/v) containing 0.1% acetic acid. The gradient was: 0 min 60:40 (A:B), 10 min 40:60, 20 min 30:70, 30 min 10:90, and 35 min 60:40. Magnolol and honokiol were identified based on their specific retention times and quantified using peak area measurements.

2.2.1. Isolation of Hanwoo Satellite Cells

Semimembranosus muscle samples were collected from a 34-month-old castrated Hanwoo steer (Animal ID: 002 1317 3849 6) at a commercial slaughterhouse (Hanlaeng Farm Story, Eumseong, Republic of Korea) and immediately transported to the laboratory under chilled conditions. Satellite cells used in this study were derived from this single animal.

After non-muscle tissues were removed, the muscle was cut into small pieces and treated with collagenase type II. The digested tissue was subjected to sequential low- and high-speed centrifugation (70 × g and 800 × g) to enrich mononuclear cells. The suspension was filtered in sequence using 100 μm and 40 μm strainers, followed by erythrocyte removal using ACK

(Ammonium-Chloride-Potassium) lysis buffer.

Single cells were subsequently isolated by fluorescence-activated cell sorting (FACS Aria II, BD Biosciences). Satellite cells were identified and collected based on a $CD31^{-}CD45^{-}CD29^{+}CD56^{+}$ immunophenotype using antibodies against the established satellite cell markers CD29 and CD56 [27].

2.2.2. Cell Culture

Hanwoo satellite cells at passage 2 were cultured under proliferation conditions with Ham's F-10 medium (Gibco, USA) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin–amphotericin B mixture, and 0.1% DMSO. Cells were seeded at 3,000 cells/cm² and allowed to grow for 4 days under standard culture conditions (37°C, 5% CO_2). During the proliferation phase, *Magnolia denudata* flower extract was administered at the following concentrations: 0 μg/mL (control), 0.5 μg/mL (T1), 5 μg/mL (T2), and 50 μg/mL (T3). The treatment concentrations were selected based on previous studies of Magnolia-derived extracts, including reports on the biological activities of magnolol and honokiol [28, 29].

For differentiation, cells grown under proliferation conditions were switched to differentiation culture once they reached approximately 80–90% confluence. At this stage, the medium was replaced with DMEM containing 2% fetal bovine serum and 1% penicillin–streptomycin–amphotericin B. Differentiation was carried out for 3 days, and the same concentrations of *Magnolia denudata* flower extract were continuously applied throughout the differentiation period.

2.2.3. Proliferation Assay of Hanwoo Satellite Cells

Cell growth of Hanwoo satellite cells was assessed by measuring metabolic activity using an MTS colorimetric assay (CellTiter 96® AQueous One Solution, Promega, USA). Cells were seeded in 96-well plates and maintained under experimental conditions for 4 days. Following incubation, the medium was exchanged for fresh medium containing the MTS reagent at a volume ratio of 100:20.

The plates were incubated under standard culture conditions for 2 h, after which the formazan signal was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, USA).

2.2.4. Cell Count and Viability Assessment

At the designated time points, cells were collected for quantification of cell number and viability. Culture supernatants were aspirated, and the monolayers were gently rinsed with 1× PBS. Cells attached to the culture surface were enzymatically dissociated using 0.05% trypsin–EDTA and subsequently pelleted by centrifugation at 352 × g for 5 min.

After removal of the supernatant, the resulting cell pellets were resuspended and mixed with trypan blue solution for viability assessment. Cell counts were obtained with an automated cell counting system (Countess, Invitrogen, USA). In parallel, cellular morphology was examined using an EVOS FL Auto 5000 fluorescence microscope (Thermo Fisher Scientific, USA).

2.2.5. Immunofluorescence Staining and Image Analysis

Immunofluorescence analysis was used to examine myogenic marker expression in cells treated with or without *Magnolia denudata* flower extract. After medium removal, cells were rinsed with PBS, fixed with 2% paraformaldehyde (37°C, 45 min), permeabilized with 0.1% Triton X-100 (20 min), and blocked with 2% bovine serum albumin (30 min). Cells were incubated with a primary antibody against PAX7 at 4°C overnight, followed by Alexa Fluor® 488–conjugated secondary antibodies, and nuclei were stained with Hoechst 33342. For myotube visualization, a monoclonal anti-myosin antibody was used. Fluorescence images were obtained from five fields per well using an EVOS FL Auto 5000 microscope and analyzed with ImageJ to quantify total nuclei, myotube area, and fusion index [30, 31].

2.2.6. Quantitative analysis of gene expression by RT-qPCR

RNA samples were prepared from Hanwoo satellite cells using a commercial extraction kit (iNtRON Biotechnology, Korea) and converted to cDNA with a reverse transcription kit (Thermo Fisher Scientific, USA). Gene expression of *PAX7*, *MYOD1*, *MYOG*, and *MYH1* was analyzed by quantitative PCR using a SYBR Green master mix (ELPIS-BIOTECH, Korea), with β-actin serving as the reference gene. Reactions were run in a 20 μL volume, and relative expression levels were calculated using the 2^−ΔΔCT method [32]. Primer information is provided in Table 1.

2.2.7. Western blot

Differentiated Hanwoo satellite cells were collected for protein analysis. After washing with cold Tris-buffered saline, cells were lysed in RIPA buffer, and protein levels were measured using a Bradford assay. Equal amounts of protein were resolved by SDS–PAGE using TGX precast gels (Bio-Rad) and transferred to PVDF membranes.

Membranes were treated with blocking buffer (Bio-Rad) and then incubated with primary antibodies against MYOG, MYH1, and β-actin at 4°C overnight. Following washing, HRP-linked secondary antibodies were applied at room temperature. Protein signals were detected using an enhanced chemiluminescence substrate (Bio-Rad) and analyzed with an ImageQuant 800 imaging system (Cytiva, USA).

10. Statistical Analysis

Data were analyzed using SPSS software, version 28.0 (SPSS Inc., USA). Differences among groups were evaluated by one-way ANOVA, followed by Duncan's multiple range test. Statistical significance was set at $p < 0.05$.

Results & Discussion

Quantitative analysis of magnolol and honokiol in *Magnolia denudata* flower extract

Table 2 presents the results of HPLC analysis for the major bioactive components of the *Magnolia denudata* flower extract, magnolol and honokiol. The concentrations of magnolol and honokiol were determined to be (6.209 and 5.479) mg/g, respectively, which are comparable to the findings of a previous study by Cristea [25]. Magnolol and honokiol have been reported to exhibit neuroprotective effects by preventing mitochondrial dysfunction and cellular damage, and also to enhance glucose uptake in skeletal muscle cells by activating the PI3K–dependent Akt signaling pathway [33, 34]. Therefore, based on their known antioxidant, metabolic regulatory, and cell survival-promoting properties, this study aimed to investigate the effects of magnolol and honokiol on the proliferation and differentiation of Hanwoo muscle satellite cells.

Effects of *Magnolia denudata* flower extract on cell viability, cell number, survival of Hanwoo satellite cells

Figure 1 shows the effect of *Magnolia denudata* flower extract on the proliferation of Hanwoo satellite cells as assessed using the MTS assay. No statistically significant differences in cell viability were observed among groups. Similarly, as Figure 2 shows, there were no significant differences in cell number or survival rate between the treated groups and the control. *Magnolia denudata* flower extract does not exert cytotoxic effects on satellite cell proliferation. These findings are also in line with a previous study by Kwon [28], which reported that *Magnolia denudata* seed extract did not significantly affect cell viability in C2C12 myoblasts under non-oxidative stress conditions.

Immunofluorescence staining and nuclei count of PAX7$^+$ Hanwoo satellite cells treated with *Magnolia denudata* flower extract

Figure 3 displays the results of PAX7 immunofluorescence staining and nuclei counting, conducted to evaluate the effect of *Magnolia denudata* flower extract on the maintenance of satellite cell identity. The number of nuclei showed a pattern similar to the total cell count, and no statistically significant differences were observed between the control and treatment groups. These results indicate that the extract does not alter PAX7 expression under the experimental conditions.

Changes in the expression of proliferation markers and signaling-related genes in Hanwoo satellite cells treated with *Magnolia denudata* flower extract

Figure 4 presents the mRNA expression levels of proliferation-related markers and signaling-related genes in Hanwoo satellite cells to assess the effects of *Magnolia denudata* flower extract on cell proliferation. The mRNA expression level of *PAX7* shows no significant differences between treatment groups, which is consistent with the results of PAX7$^+$ nuclei counts. In contrast, *MYOD1* expression significantly decreases with increasing concentrations of the *Magnolia denudata* flower extract ($p < 0.05$).

PAX7 is a transcription factor that plays a crucial role in maintaining the stemness and self-renewal capacity of satellite cells, and can remain expressed, even after activation [35, 36]. On the other hand, the downregulation of *MYOD1* expression has been associated with delayed or altered progression toward myogenic differentiation [37, 38]. In this study, the observed pattern of maintained *PAX7* expression alongside reduced *MYOD1* expression may reflect a modulation of myogenic commitment rather than a direct promotion of differentiation. Although previous studies have reported that specific signaling contexts, such as non-canonical Wnt activation or p38 MAPK inhibition, are associated with reserve cell formation [39-41], the present findings do not provide direct evidence for such mechanisms. Therefore, the observed expression pattern should be interpreted as suggestive of altered myogenic regulation.

In addition, the mRNA expression levels of the signaling-related genes *MAPK1* and *AKT1* do not

show significant differences, compared to the control group. MAPK1 and AKT1 are well-established components of the MAPK/ERK and PI3K/AKT signaling pathways, respectively, which are known to regulate muscle cell proliferation, differentiation, and survival [42-46]. However, in this study, the absence of significant changes in MAPK1 and AKT1 mRNA expression suggests that *Magnolia denudata* flower extract did not affect transcriptional regulation of major growth- and survival-related pathways under proliferative conditions.

Evaluation of myotube area and fusion index in Hanwoo satellite cells treated with *Magnolia denudata* flower extract

Figure 5 presents the immunofluorescence images stained with MYH1 and the corresponding quantification of myotube area and fusion index to assess the effects of *Magnolia denudata* flower extract on the differentiation of Hanwoo satellite cells. Compared to the control, treatment groups supplemented with the extract show enhanced formation of multinucleated myotubes, indicating promoted differentiation of the satellite cells. Quantitative analysis further confirms that both the myotube area and fusion index significantly increase with higher concentrations of the *Magnolia denudata* flower extract ($p < 0.05$). Muscle cell differentiation was enhanced by *Magnolia denudata* flower extract, with the greatest effect at T3 (50 μg/mL).

Magnolol and honokiol, the key bioactive components of the *Magnolia denudata* flower, have been reported to inhibit muscle atrophy and to promote myogenic differentiation in previous studies [47, 48]. Moreover, magnolol has been suggested to modulate muscle growth–related pathways, including myostatin signaling, in other experimental models [29, 49]. However, these mechanisms were not directly evaluated in the present study, and thus their possible contribution to the observed phenotypic outcomes remains to be clarified.

Expression of myogenic marker genes and muscle-specific proteins in Hanwoo satellite cells treated with *Magnolia denudata* flower extract

Figure 6 shows the mRNA and protein expression levels of myogenic differentiation markers to

evaluate the effects of *Magnolia denudata* flower extract on the differentiation of Hanwoo satellite cells. *MYOG and MYH1*, well-known markers of muscle cell differentiation, are significantly upregulated in extract-treated groups, compared to the control ($p < 0.05$). Their expression levels tend to increase in a concentration-dependent manner, with the highest levels observable in the T3 (50 μg/mL) treatment group.

Similarly, protein expression analysis reveals that the relative levels of MYOG and MYH1 proteins also increases progressively with higher concentrations of the extract, with the T3 group showing significantly higher expression than the control ($p < 0.05$). During the myogenic differentiation process, the expression of myogenic regulatory factors (MRFs), including MyoD, Myf5, MyoG, and MRF4, increases in parallel with the upregulation of muscle-specific contractile proteins, such as myosin heavy chain (MHC) [50, 51].

The significant upregulation of MYOG and MYH1 by *Magnolia denudata* flower extract suggests its role in promoting muscle cell differentiation. These findings are consistent with previous studies that demonstrate that *Magnolia denudata*–derived compounds, such as magnolol and honokiol, protect muscle mass in atrophy models by maintaining MHC expression and preventing muscle degradation [52, 53].

Conclusion

In this study, *Magnolia denudata* flower extract did not significantly affect proliferation-related indicators in Hanwoo satellite cells; however, the lowest concentration (0.5 μg/mL) tended to support cell viability and the maintenance of $PAX7^+$ cells. In contrast, higher extract concentrations markedly promoted myogenic differentiation, as demonstrated by increased MYOG and MYH1 expression and enhanced myotube formation. These findings suggest that the differentiation-enhancing effects of the *Magnolia denudata* flower extract may be partially attributed to its major bioactive constituents, such as magnolol and honokiol, which have been reported to activate myogenic signaling pathways in previous studies.

Overall, the extract demonstrates potential as a food-grade natural material capable of supporting myogenic differentiation, highlighting its prospective value as a functional additive for future cultured meat applications. To further elucidate the mechanisms underlying these effects, additional studies using purified compounds will be required to determine the direct contribution of magnolol, honokiol, and other constituents, as well as to clarify the underlying signaling mechanisms regulating myogenic progression.

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460 **Table 1. Details of primers used in qPCR.**

Target	Orientation	Sequence (5´–3´)
PAX7	forward	TCCCTGAATGGACATCACCT
	reverse	TGGGTGTCCACTGCTACTAA
MYOD1	forward	CATCCGCTATATCGAAGGCC
	reverse	CTGTAGTCCATCATGCCGTC
MYOG	forward	ACAAACCATGCACATCTCCT
	reverse	TGGCAGCTTTACAAACAACA
MYH1	forward	GCTCCTTACCTCCGAAAGTC
	reverse	ATGGGGAAGACTTGATCCTC
β-actin	forward	AAATGCTTCTAGGCGGACTG
	reverse	TAAATCCTGAGTCAAGCGCC
MAPK1	forward	CAGACGTACTGCCAGAGAAC
	reverse	TTGCGTCTTCAAGAGCTTGT
AKT1	forward	ATCATGCAGCACCGATTCTT
	reverse	GGTGGCGTAATGGTGATCAT

461

462 **Table 2. The contents of Magnolol and Honokiol in the *Magnolia denudata* flower**
463 **extract were determined using HPLC.**

Traits (mg/g)	***Magnolia denudate* flower extract**
Magnolol	6.209± 0.02
Honokiol	5.479± 0.03

464

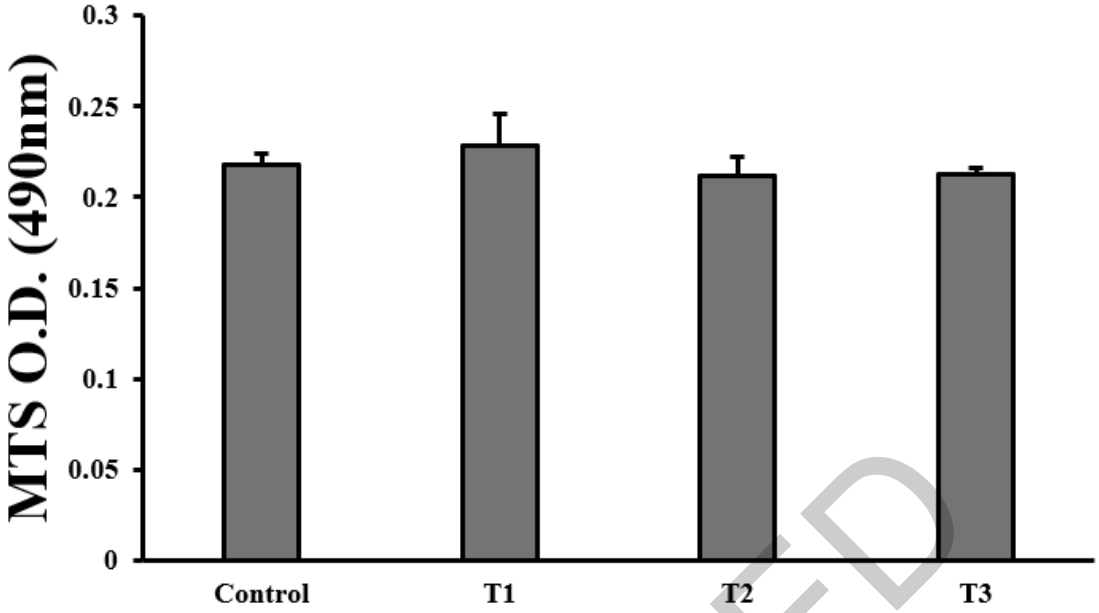

Figure 1. MTS values of Hanwoo myosatellite cells according to the concentration (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extract during proliferation culture for 4 days. Mean ± standard deviation.

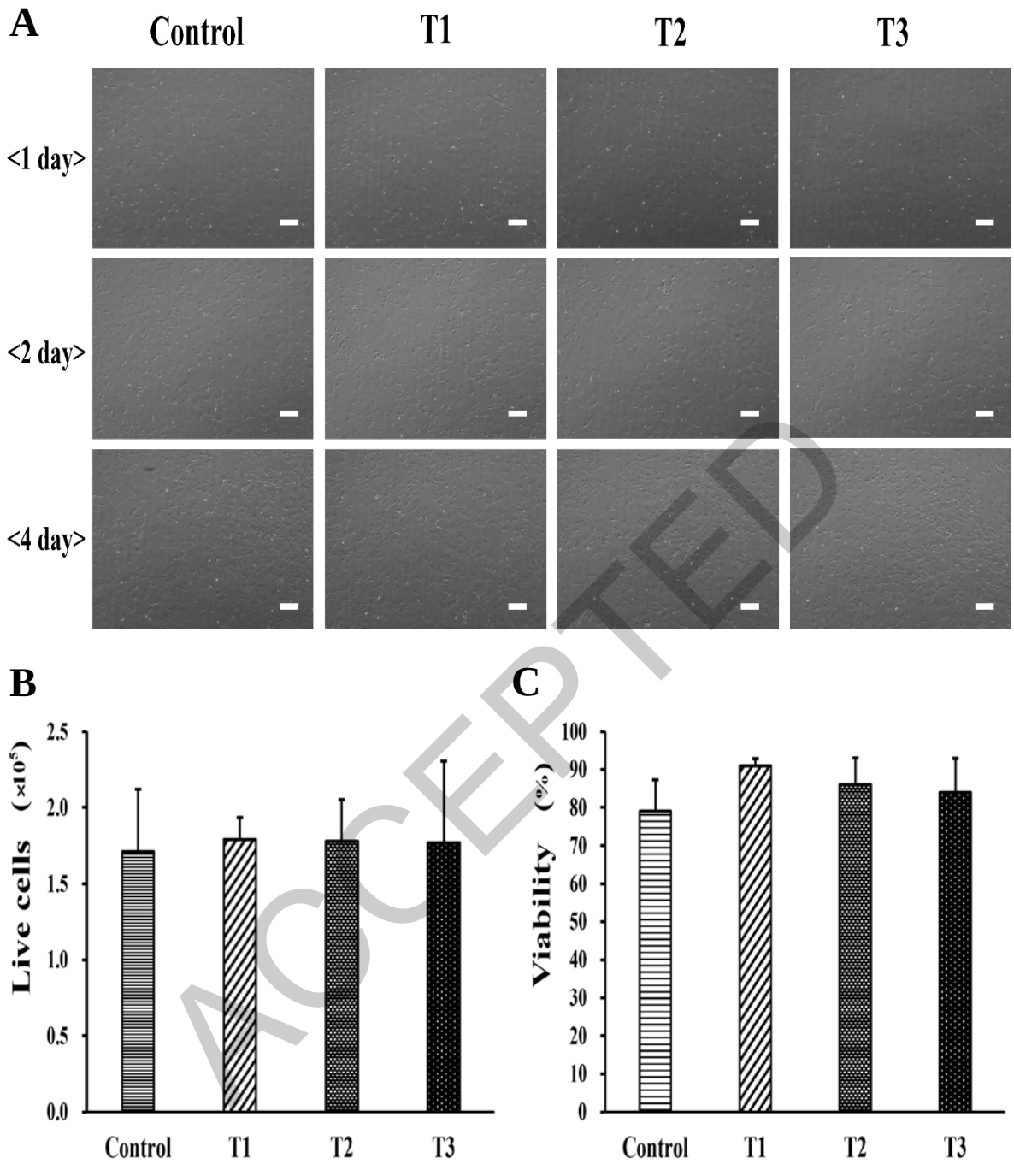

Figure 2. (A) Microscopic images (magnification: 400×) of Hanwoo myosatellite cell, (B) Cell count, and (C) Viability, according to the concentration of (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extract during proliferation culture for 4 days. Scale bar = 100 μm. Mean ± standard deviation.

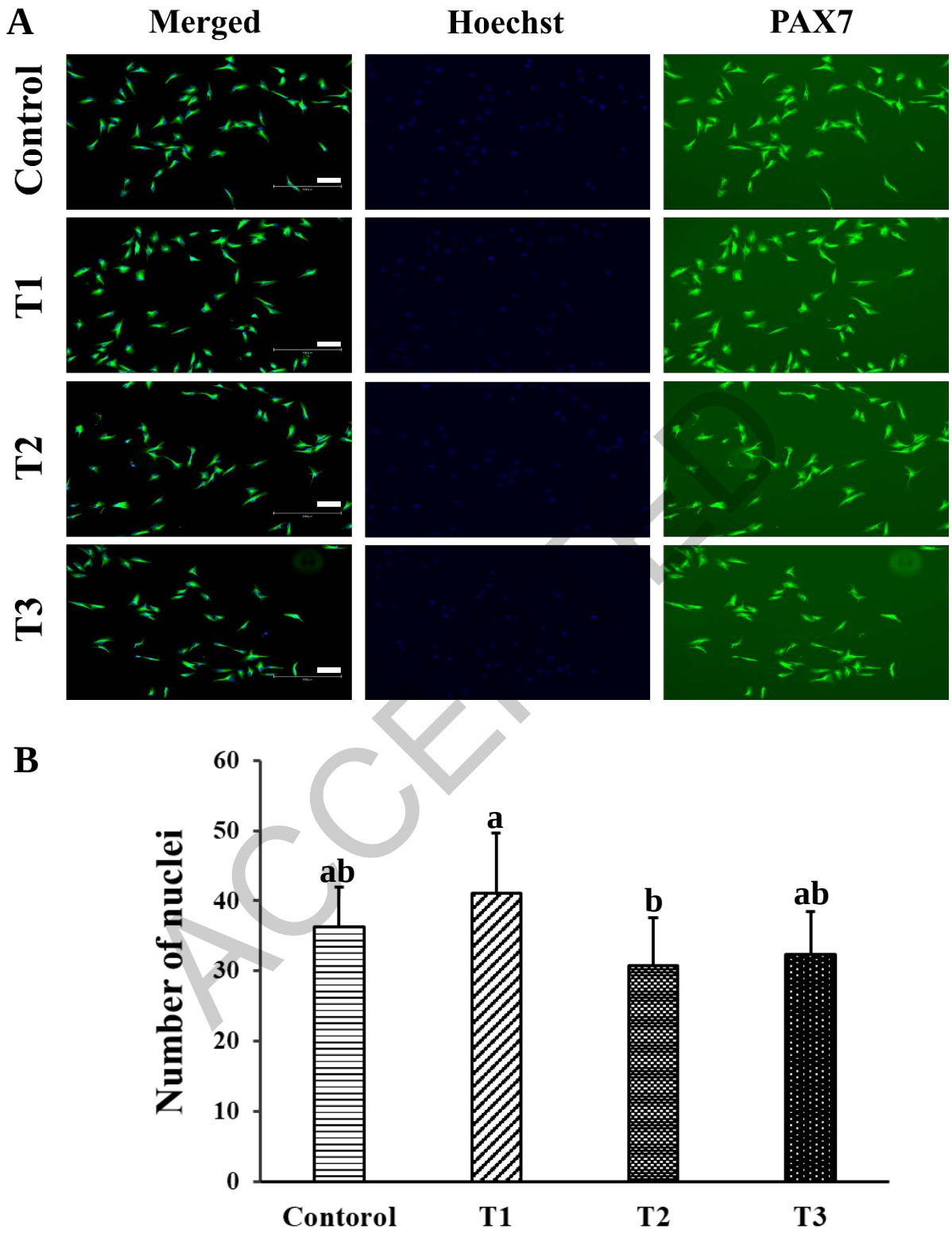

Figure 3. Representative image (magnification: ×100) of the immunofluorescence staining PAX7 (green) and nuclei counterstained with DAPI (blue) in Hanwoo myosatellite cells (A) and (B) number of nuclei by concentration of (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extract during proliferation culture for 4 days. Scale bar = 100 μm. a–b Different letters above the bars indicate significant differences based on the mean ± standard deviation ($p < 0.05$).

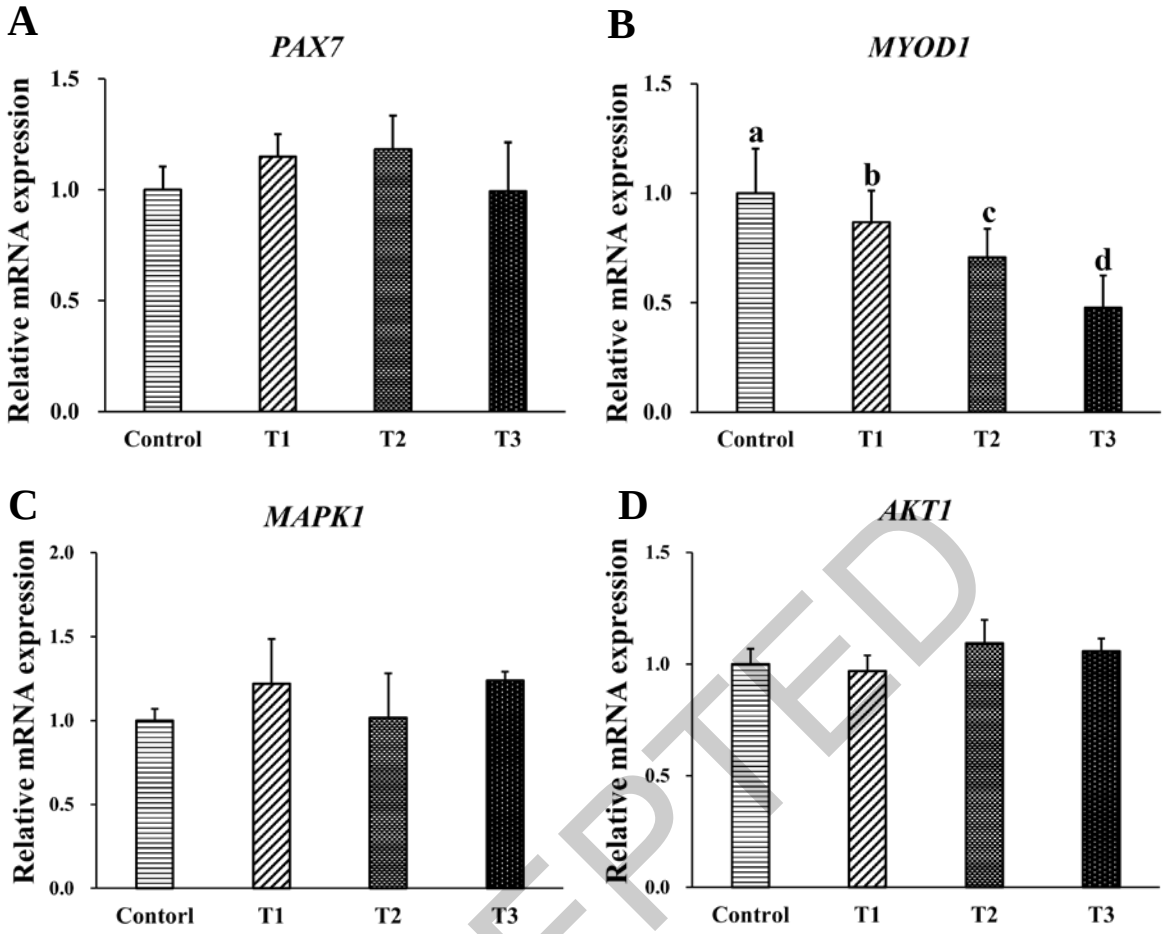

Figure 4. Relative expression of proliferation-related mRNA of (A) *PAX7*, (B) *MYOD1*, (C) *MAPK1*, and (D) *AKT1* by concentration of (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extract during proliferation culture for 4 days. a–d Different letters above the bars indicate significant differences based on the mean ± standard deviation ($p < 0.05$).

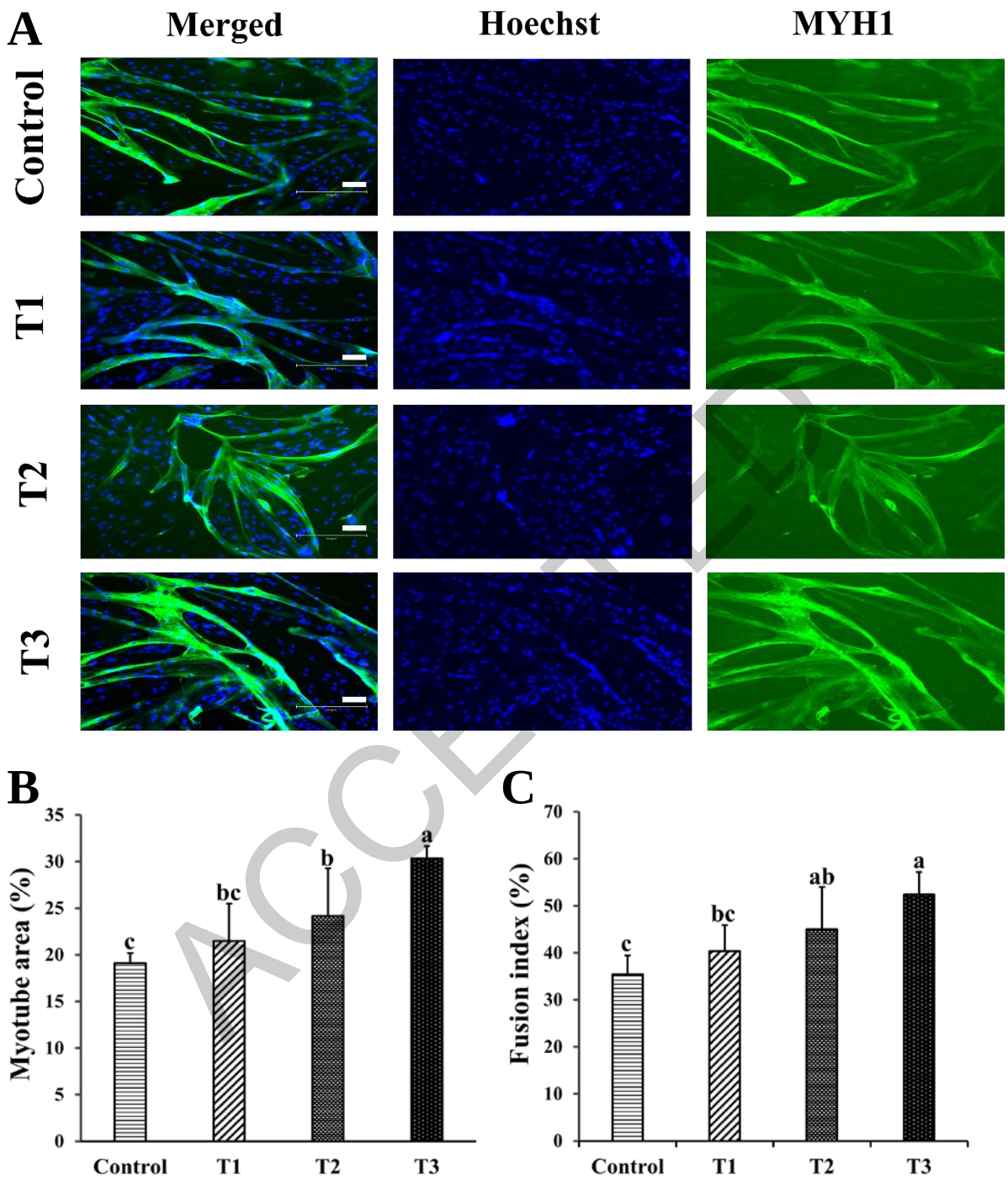

Figure 5. (A) Representative images (magnification: 100×) of the immunofluorescence staining MYH1 (green) and nuclei counterstained with DAPI (blue) in Hanwoo myosatellite cells, (B) Myotube area, and (C) Fusion index, by concentration of (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extract after 3 days of differentiation culture. Scale bar = 100 μm. a–c Different letters above the bars indicate significant differences based on the mean ± standard deviation ($p < 0.05$).

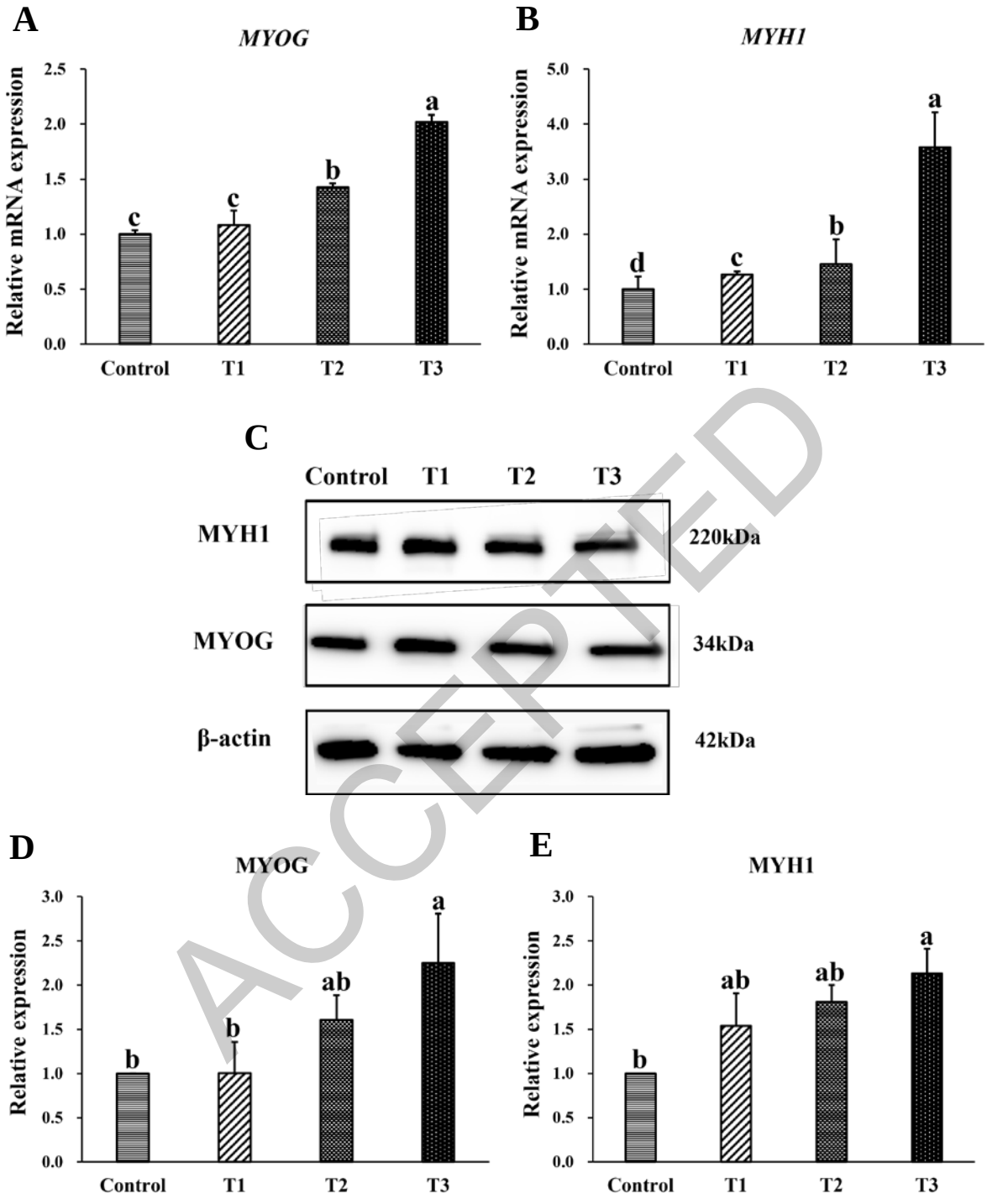

Figure 6. **Relative expression of differentiation-related mRNA (A) *MYOG*, and (B) *MYH1*. (C) Representative western blot images of MYOG, MYH1, and β–actin, and protein expression levels of (D) MYOG, and (E) MYH1, by concentration of (0, 0.5, 5, and 50) μg/mL (Control, T1, T2, and T3, respectively) of *Magnolia denudata* flower extracts after 3 days of differentiation culture.** a–d Different letters above the bars indicate significant differences based on the mean ± standard deviation ($p < 0.05$).