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Effect of the combination of *Lithospermum erythrorhizon* and *Lonicera japonica* on dexamethasone-induced muscle atrophy in mice

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Abstract
Skeletal muscle atrophy occurs in several pathological conditions. Among other reasons, high-dose or long-term administration of glucocorticoids increases circulating glucocorticoid levels and causes muscle atrophy. The purpose of this study was to investigate whether *Lithospermum erythrorhizon* and *Lonicera japonica* complex extract (LELJ) has a beneficial effect on dexamethasone (Dexa)-induced muscle atrophy. In Dexa-induced myotube atrophy, treatment with LELJ increased myotube diameter, decreased the expression of muscle atrophy markers, and increased the expression of myosin heavy chain (MHC) isoforms. Supplementation with LELJ improved muscle function and performance in mice with Dexa-induced muscle atrophy as demonstrated by grip strength and running tests. Additionally, it increased skeletal muscle mass, size, and expression of MHC isoforms and protein synthesis-related markers. Furthermore, it reduced the upregulated protein levels of skeletal muscle atrophy markers in Dexa-treated mice. Supplementation with LELJ reversed Dexa-induced translocation of the glucocorticoid receptor and forkhead box O3 from the cytosol to the nucleus in skeletal muscles. LELJ also ameliorated age-related muscle loss by extending lifespan and increasing locomotor capacity in *Caenorhabditis elegans*. We identified loganin and lithospermic acid as bioactive compounds of LELJ and found that treatment with these agents increased myotube diameter, MHC isoform, and puromycin protein levels, and decreased atrophy markers in Dexa-treated myotubes. The current findings underscore how LELJ can prevent Dexa-induced skeletal muscle atrophy, attributing the effects to loganin and lithospermic acid.

Keywords *Lithospermum erythrorhizon*, *Lonicera japonica*, Muscle atrophy, Loganin, Lithospermic acid, Muscle protein synthesis

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Introduction

Glucocorticoids, released by the adrenal cortex as endocrine hormones, are recognized for their anti-inflammatory properties. However, sustained elevated levels are known to be involved in muscle atrophy, and the mechanisms that induce muscle wasting have been extensively studied [1]. Glucocorticoids have a direct effect on target gene transcription, primarily through the nuclear pathway; both natural glucocorticoids, such as corticosterone and cortisol, and synthetic glucocorticoids, such as dexamethasone (Dexa) and prednisolone, exert their effects through the glucocorticoid receptor (GR) [2]. Glucocorticoids bind to the cytosolic GR, migrate to the nucleus, and act either by interfering with the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway or by transcriptional stimulation of atrogenes through forkhead box protein O (FoxO)s [3]. This, in turn, blocks the signaling pathway regulating protein synthesis and leads to the upregulation of protein degradation and the ubiquitin-proteasome pathway in skeletal muscle [4].

Recent studies have focused on the safety and effectiveness of several traditional medicinal plants. Dried roots of *Lithospermum erythrorhizon Siebold et Zuccarini* (LE) have been used in traditional medicine for centuries in Korea, China, and Japan for the treatment of sore throats and burns, and are used as a traditional Korean distilled liquor, Jindo Hongju [5]. LE mainly contains various phytochemicals such as lithospermic acid (LA) and naphthoquinone compounds such as shikonin derivatives, which have been reported to have anti-inflammatory, antiviral, antibacterial, blood sugar-lowering, and anti-obesity effects [6–10].

Lonicera japonica Thunberg (LJ) has also been used as a medicinal plant in China, Japan, and Korea for curative purposes, such as treating exogenous wind fever, epidemic fever, ulcers, boils, and certain infectious diseases [11]. LJ exerts anti-inflammatory, antibacterial, antitumor, and neuroprotective effects [12–15], and contains various chemical components, such as loganin, sweroside, rutin, luteoloside, and lonicerin [16].

Because these medicinal plants consist of complex mixtures of major compounds and concomitant substances, the use of a combination of multiple medicinal plants may exhibit potential therapeutic effects, even at low concentrations, through synergistic interactions. In this study, we hypothesized that LE and LJ complex extract (LELJ) has a beneficial effect on Dexa-induced muscle atrophy. Therefore, we aimed to validate the synergistic potential of LELJ in mitigating Dexa-induced muscle atrophy in C2C12 cells. Additionally, we assessed the ability of LELJ in ameliorating Dexa-induced muscle atrophy both in vivo and in vitro, elucidated the underlying mechanism, and identified the bioactive compounds.

Methods/experimental

Sample preparation

Dried LE was purchased from Salim Global (Yongin, Republic of Korea). LE was extracted with 50% ethanol for two consecutive cycles, each lasting 8 and 6h, respectively. The ethanol extract was filtered and concentrated below 60 °C, and 20% dextrin was added. Sterilization was performed at 90 °C for 30 min. Finally, freeze-drying was performed to obtain the LE extract, which was stored at -20 ℃ until use. Dried LJ was purchased from H Food (Pocheon, Republic of Korea). LJ was extracted twice with 50% ethanol at 70 °C for 6h. The ethanol extract was filtered and concentrated below 60 °C, and 20% dextrin was added. Sterilization was performed at 90 °C for 30 min. Finally, freeze-drying was performed to obtain the LJ extract, which was stored at -20 °C until use. LELJ was prepared by mixing the LE and LJ extracts at a ratio of 1:1.

Cell culture

The culture of C2C12 myoblast cells (CRL-1772; ATCC, Manassas, VA, USA) was performed at 37 °C in a 5% CO_2 humidified atmosphere using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce myogenic differentiation, C2C12 cells were cultured in DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. To determine the fusion index, LELJ was added to the differentiation medium for 2 days. To examine efficacy against Dexa-induced muscle atrophy, samples containing LE, LJ, LELJ, loganin, or LA were treated with 50 μM Dexa for 24h on the fourth day of differentiation.

Myotube immunofluorescence and quantitative analysis

The myotubes were fixed in 10% formalin for 30 min, permeabilized with 0.05% saponin, and blocked with 1% BSA in phosphate-buffered saline (PBS). Next, overnight incubation at 4 °C was performed with a total myosin heavy chain (MHC) antibody. Following PBS rinsing, the myotubes were incubated for 30 min with Alexa Fluor 488-conjugated anti-mouse IgG (1:500 dilution, Cell Signaling Technology) and for 1 min with DAPI (1:10,000 dilution). Images were acquired using an Olympus IX71 microscope, and analysis was carried out using Olympus DP controller 3.1.1 software.

Surface sensing of translation (SUnSET) assay

SUnSET is a non-radioactive technique that measures protein synthesis in mammalian cells [17]. After four days of differentiation, C2C12 cells were exposed to LELJ, loganin, or LA, with or without Dexa, for 24h. Puromycin (10 μg/ml or 1 μM) was applied for 1h before harvesting,

and its detection was performed by western blot analysis using a puromycin antibody.

Western blot analysis

Proteins from cells and skeletal muscle tissues were extracted using radioimmunoprecipitation assay buffer. Nuclear proteins were extracted from the quadriceps using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Total proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Bio-Rad Laboratories, CA, USA). Blocking of the membrane for 1h occurred using Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 (Junsei Chemical Co., Ltd., Tokyo, Japan), followed by overnight incubation at 4 ℃ with primary antibodies. Subsequently, the membrane was washed and incubated for 1h with a horseradish peroxidase-conjugated secondary antibody. Immunodetection was performed using enhanced chemiluminescence detection reagent (Bio-Rad).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from skeletal muscle tissues using the Qiagen RNeasy Mini Kit (QIAGEN Inc., Hilden, Germany). Subsequently, cDNA synthesis was conducted from the acquired total RNA using the ReverTra Ace® quantitative reverse transcription polymerase chain reaction (qPCR RT) master kit (Toyobo Co., Ltd., Osaka, Japan). SYBR Green real-time PCR Master Mix (Toyobo Co., Ltd.) and the ViiA7 PCR system (Applied Biosystems, Foster City, CA, USA) were utilized for quantitative PCR. The mRNA levels were normalized to 18s.

Animals

Three-week-old male C57BL/6 mice were housed in a controlled environment at a constant temperature (21–25 ℃) and humidity (50–60%), following a 12-h light/dark cycle, with unrestricted access to food and water. After 1 week of adaptation, the mice were divided into four groups (n=7/group). The Control (Con) and Dexa-treated groups received the AIN-93 M diet (Dyets, PA, USA), and the LELJ0.1 and LELJ0.2 groups received the AIN-93 M diet with 0.1% and 0.2% LELJ, respectively. After 4 weeks, the Con or Dexa, LELJ0.1, and LELJ0.2-treated groups were intraperitoneally injected with equal quantities of sterile distilled water or Dexa (D4902, Sigma) at 15 mg/kg/day over a 38-day period, respectively, while the experimental diet was concurrently supplied. Body weight was measured weekly. Upon completion of the experiment, all the mice were anesthetized using 2% isoflurane and euthanized. The skeletal muscle tissue was extracted and measured. All animal experiments were performed following the protocols outlined by the Institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI-IACUC, KFRI-M−21064).

Measurement of muscle mass and performance

To measure body composition, an InAlyzer (Medikors Inc., Seongnam, Korea) and its dual-energy X-ray absorptiometry function were used to determine lean body mass (%) in mice. Using a grip strength test machine (model GS3, Bioseb, Vitrolles, France), grip strength was measured five times and the results were normalized against body weight. All mice were acclimated to running on a treadmill (Ugo Basile, Gemonio, Italy) for 2 days at a speed of 10 m/min for 10 min. After adaptation, the running distance and time were measured. Starting at 10 m/min for 10 min, the mice experienced speed increments of 2 m/min every 3 min to ensure that the maximum speed did not exceed 20 m/min. The endpoint was set when the mouse contacted the shock grid for 10s.

Histological analysis

For cross-sectional area (CSA) measurements, gastrocnemius muscles were embedded in Tissue-Tek OCT compound (Sakura Finetech, Tokyo, Japan), frozen using liquid nitrogen on cryo-molds, and then stored at -80 °C. Using a cryo-microtome (CM1850, Leica Microsystems, Wetzlar, Germany) at -20 ℃, 7 μm sections were sliced from frozen tissue blocks and transferred onto slides pre-cooled to -20 °C. Detailed experimental conditions were established based on a previous study [18]. Images of the stained tissues were acquired using an Axio Imager Z2 microscope (Carl Zeiss, Jena, Germany).

Caenorhabditis elegans (*C. Elegans*) mobility and lifespan test

C. elegans (N2 wild-type strain) were maintained at 20 °C. To observe worm mobility, the body bend number was counted in liquid M9 buffer. The count involved tallying instances of reciprocating bending motions at the center of the body. Body bending was observed for 10s using an Olympus SZX7 Zoom Stereo Microscope (Olympus Corporation, Tokyo, Japan). Immobile worms were considered unable to swim. Lifespan analysis was conducted at 20 °C using solid nematode growth medium plates. Live animal assessments were performed at 1–2 day intervals until death, determined by the absence of a response to gentle probing with a platinum wire on the head or tail of the worm.

High performance liquid chromatography (HPLC) analysis

Loganin and LA content in LELJ extract was determined using an Agilent 1200 series HPLC system (Agilent Technologies, CA, USA) with C18 analytical columns (250×4.6 mm I.D. 5 μm, YMC, Kyoto, Japan). The absorbances of loganin and LA were measured at 254 nm and 312 nm, respectively, using a diode array detector (Agilent

Technologies). For loganin, solvents A, which comprised 0.1% formic acid in H_2O, and B (0.1% formic acid in acetonitrile) were used as the mobile phase. The gradient program began with an isocratic A/B ratio of 85:15 for the first 5 min, transitioned to an A/B ratio of 82:18 by 10 min, and was maintained at this ratio from 10 to 15 min. Following this phase, the gradient was shifted to an A/B ratio of 80:20 from 15 to 25 min, then transitioned to an A/B ratio of 30:70 by 35 min, and maintained at this ratio from 35 to 40 min. Subsequently, the gradient was shifted back to an A/B ratio of 85:15 from 40 to 45 min, and remained steady until 50 min. For LA, solvents A, which comprised 0.1% formic acid in H_2O, and B (acetonitrile) were used as the mobile phase. The gradient program began with an isocratic A/B ratio of 80:20 for the first 5 min, and then transitioned to an A/B ratio of 75:25, which was maintained from 5 to 30 min. Following this phase, the gradient was shifted to an A/B ratio of 80:20 from 30 to 35 min and concluded with an isocratic A/B ratio of 80:20 from 35 to 40 min. In both instances, the column temperature was maintained at 30 °C, and the flow rate and injection volume were set at 1 ml/min and 10 µl, respectively.

Statistical analysis

Data are expressed as mean ± SD for in vitro studies and mean ± SEM for in vivo studies. Statistical analyses were performed using GraphPad Prism, version 8.3.1 software (CA, USA). Kaplan–Meier survival analysis and unpaired t-tests were used to assess differences between two groups. One-way analysis of variance was used to compare multiple groups, followed by the Dunnett's test for multiple comparisons.

Results

The 1:1 mixture of LE and LJ showed a synergistic effect on the downregulation of atrogenes in C2C12 cells

To investigate the individual effects of LE and LJ on Dexa-induced myotube atrophy, differentiated C2C12 cells were treated with either LE or LJ at equal concentrations. Treatment with LE or LJ significantly suppressed the increase in the mRNA levels of muscle ring-finger protein 1 (MuRF1) and ubiquitin E3-ligase muscle-specific F-box protein (MAFbx/Atrogin-1), which are markers of Dexa-induced muscle atrophy (Fig. 1). To investigate the synergistic effect of LELJ, Dexa-treated C2C12 myotubes were treated with 2 µg/ml of a 1:1 mixture of LE and LJ. This treatment significantly reduced the mRNA levels of MuRF1 and Atrogin-1 compared to the results obtained with individual LE and LJ treatments. Colby's formula [19] was used to verify the synergistic effects of the complex. Based on this formula, a 97.1% and 92.1% inhibitory effect of MuRF1 and Atrogin-1, respectively, was required for the synergistic effect of LELJ. As shown in Fig. 1, treatment with LELJ resulted in a much higher inhibitory effect of 136.3% for MuRF1 and a similar inhibitory effect of 90.1% for Atrogin-1. Therefore, we suggest that LELJ exhibits a synergistic effect in a 1:1 ratio.

LELJ improved myotube atrophy and myogenesis in C2C12 cells

We investigated the effects of LELJ on Dexa-induced myotube atrophy in C2C12 cells. Immunofluorescence staining using a total MHC antibody revealed a notable decrease in myotube diameter following Dexa treatment compared with the control. Conversely, the myotube diameter significantly increased with LELJ treatment (Fig. 2A).

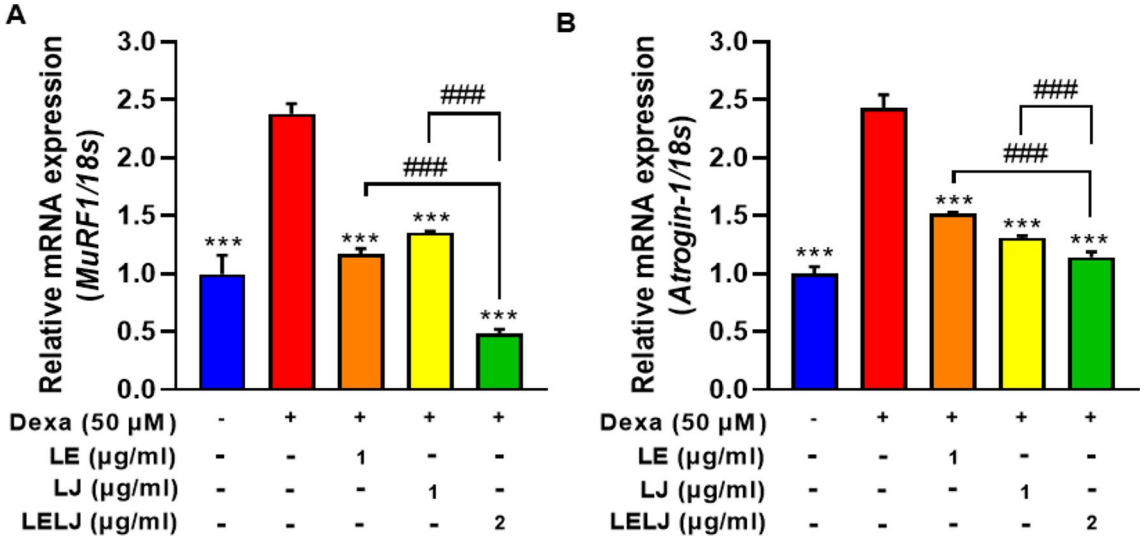


Fig. 1 Synergistic effect of LELJ on Dexa-induced muscle atrophy in C2C12 cells. **A** and **B** The expression levels of MuRF1 and Atrogin-1 quantified by qRT-PCR in Dexa-treated myotubes. Results are expressed as mean ± SD. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated myotubes. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the LELJ-treated myotubes

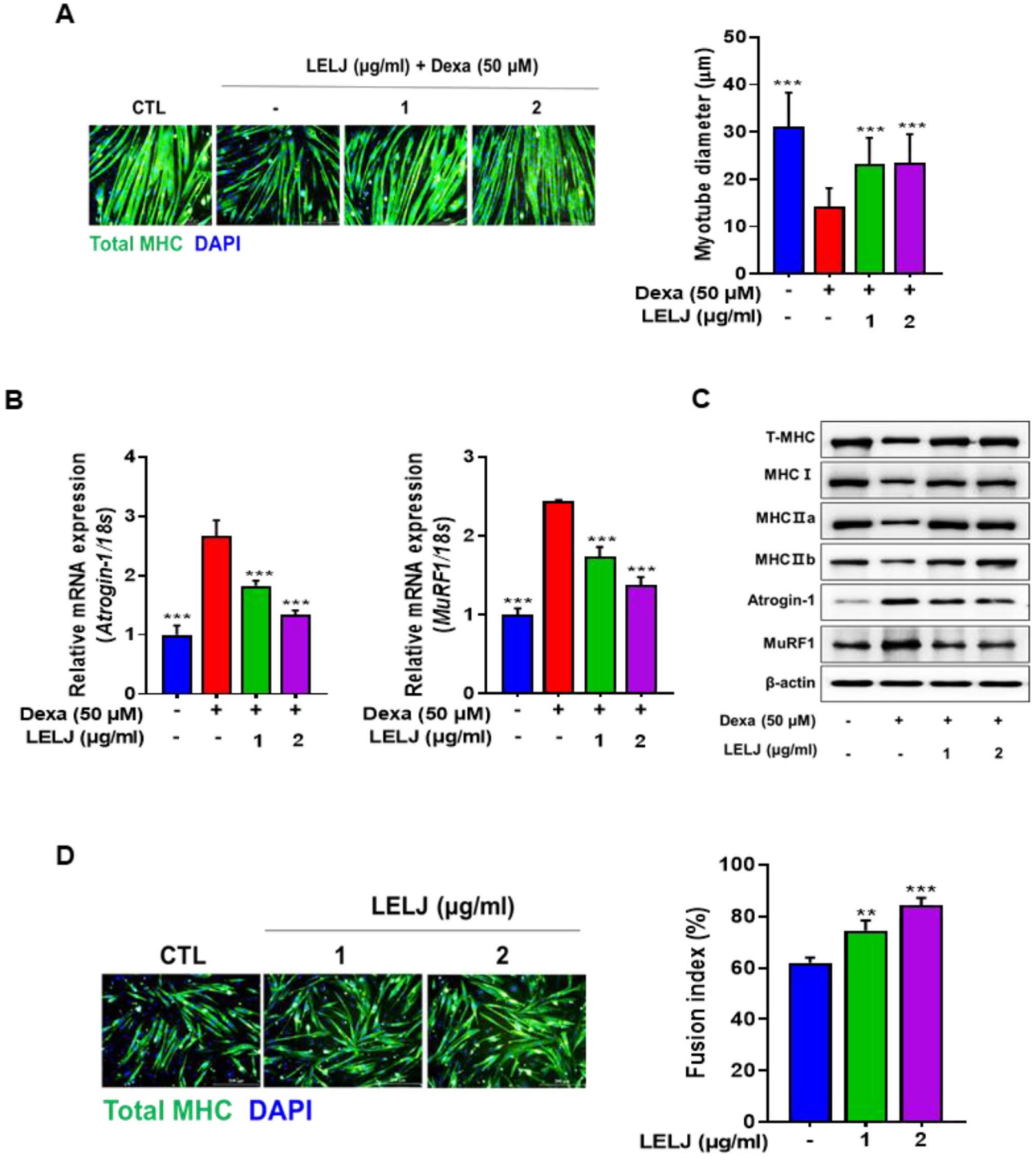


Fig. 2 Effect of LELJ on Dexa-induced muscle atrophy in C2C12 cells. **A** C2C12 cells were fixed and stained with total MHC antibody (scale bar, 200 μm). Myotube diameter was calculated as the average diameter of MHC-positive multinucleated myotubes. **B** The expression levels of Atrogin-1 and MuRF1 quantified by qRT-PCR in Dexa-treated myotubes. The protein levels of **C** total MHC, MHC1, 2 A, 2B, Atrogin-1, MuRF1, and β-actin measured by western blotting in Dexa-treated C2C12 cells. **D** C2C12 cells were fixed and stained with total MHC antibody (scale bar, 200 μm). Fusion index was calculated as the average number of nuclei in MHC positive multinucleated cells above total nucleus. Results are expressed as mean ± SD. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated myotubes

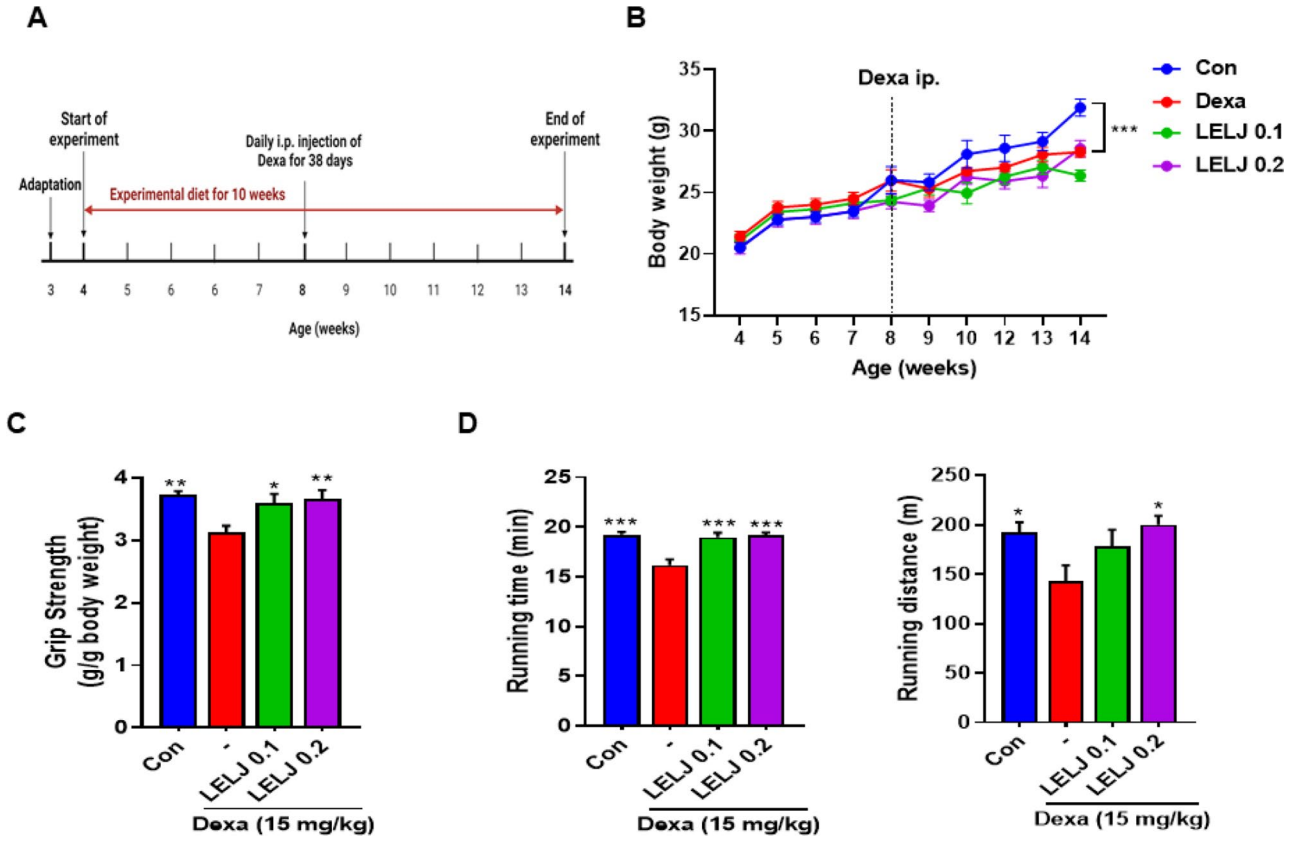


Fig. 3 Effect of LELJ on grip strength, running time and distance in mice. **A** Experimental design. **B** Weekly body weight. **C** Muscle function was measured on grip strength (g/g BW). **D** Total running time to exhaustion (min) and running distance (m). Results are expressed as mean ± SEM. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated group

Furthermore, we observed elevated levels of Atrogin-1 and MuRF1 mRNA and protein in Dexa-treated C2C12 cells, which were reversed by LELJ treatment (Fig. 2B and C). Protein levels of MHC isoforms decreased after Dexa treatment in C2C12 myotubes. However, LELJ treatment increased these protein levels in C2C12 cells (Fig. 2C). In addition, to investigate the myogenic capacity of LELJ, we treated cells with LELJ in differentiation medium for 2 days; LELJ treatment significantly increased the fusion index (Fig. 2D).

LELJ improved muscle function and performance in Dexa-induced muscle atrophy

Next, we investigated the effects of LELJ on muscle atrophy in mice. Figure 3A shows the experimental design. First, we measured the body weight of mice and observed a significant decrease following intraperitoneal injection of Dexa in the final week of the experiment. Interestingly, no significant changes in body weight were observed in the LELJ groups compared to the Dexa-treated group (Fig. 3B). Grip strength and treadmill performance were assessed to examine muscle function and performance. The Dexa-treated group exhibited a decrease in grip strength normalized by body weight, as well as reduced running time and distance to exhaustion compared with the Con group. However, LELJ significantly increased grip strength, running time, and distance to exhaustion (Fig. 3C and D).

LELJ increased skeletal muscle mass in Dexa-induced muscle atrophy

Dual-energy X-ray absorptiometry analysis revealed a significant increase in lean body mass in the LELJ groups compared to that in the Dexa-treated group (Fig. 4A and B). Additionally, muscle weight was reduced in the Dexa-treated group compared with the Con group, while the LELJ groups exhibited increased skeletal muscle weight, especially the quadriceps (Fig. 4C). We also measured the CSA of skeletal muscle fibers and observed that the Dexa-treated group had decreased CSA compared with Con mice. However, the LELJ groups demonstrated a muscle fiber size shift mirroring that of the Con group (Fig. 4D and E). Furthermore, we assessed the protein levels of MHC isoforms in the quadriceps and noted decreased protein levels of total MHC, MHC1, 2 A, and 2B in the Dexa-treated group. However, the LELJ groups demonstrated increased protein expression of MHC

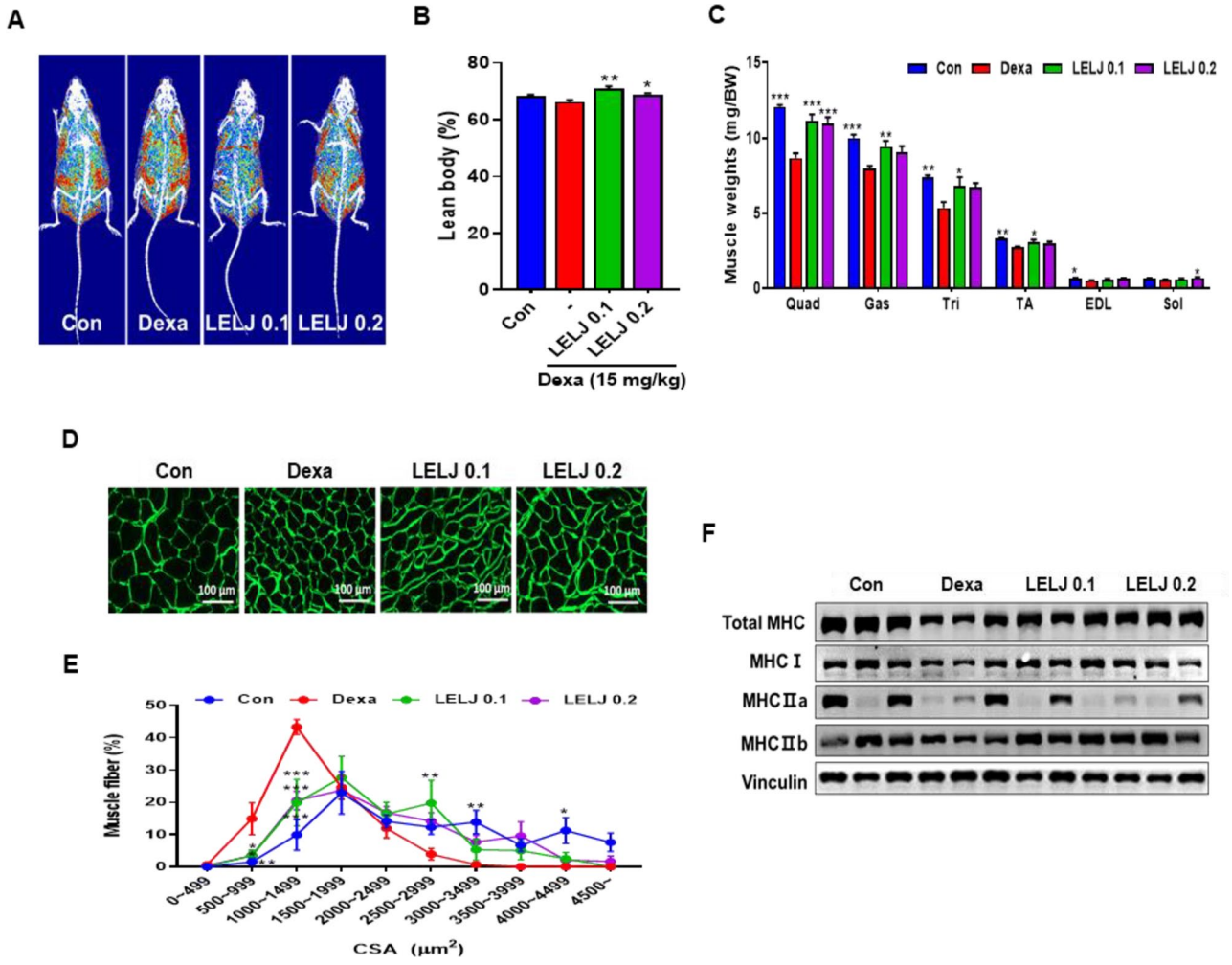


Fig. 4 Effect of LELJ on skeletal muscle mass in mice with Dexa-induced muscle atrophy. **A** and **B** Body composition images and bar chart showing lean body mass (%). **C** The weights of skeletal muscle from the experimental mice (mg/g BW). **D** and **E** Representative image of skeletal muscle sections stained with antibody to laminin. Images were analyzed by ImageJ, and CSA were measured. **F** The expression of total MHC, 1, 2 A, and 2B in quadriceps as determined by western blotting. Results are expressed as mean ± SEM. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated group

isoforms, whereas the protein expression of MHC2A did not change (Fig. 4F).

LELJ promoted muscle protein synthesis via the mammalian target of rapamycin (mTOR) pathway and inhibited GR translation

To investigate the effect of LELJ on muscle protein synthesis, a SUnSET assay using puromycin was performed in C2C12 cells. LELJ treatment increased the rate of protein synthesis, which was reduced by Dexa treatment (Fig. 5A). Additionally, we assessed the phosphorylation of key proteins involved in muscle protein synthesis, including p70 S6 kinase (S6K) and eIF4E-binding protein 1 (4EBP1), and the phosphorylation levels of mTOR and Akt, which are upstream regulators. Dexa treatment decreased the phosphorylation of mTOR, Akt, S6K, and 4EBP1 compared with the control in C2C12 cells. However, LELJ treatment increased their phosphorylation (Fig. 5B). It has been reported that Dexa triggers the nuclear translocation of FoxO through dephosphorylation, and subsequently controls muscle atrophy [20]. As shown in Fig. 5B, phosphorylation of the FoxO3 protein was decreased in Dexa-treated cells compared with that in Con cells; however, phospho-FoxO3 protein expression was upregulated in LELJ-treated cells compared to Dexa-treated cells (Fig. 5B).

The effect of LELJ on muscle protein synthesis was assessed by western blotting in mice muscle tissue. The Dexa-treated group showed upregulated protein levels of Atrogin-1, MuRF1, and myostatin compared with the Con group, but the LELJ groups showed decreased levels of these proteins (Fig. 5C). The activation of the mTOR pathway by LELJ was also observed in the skeletal muscles of mice (Fig. 5D). Furthermore, to examine whether

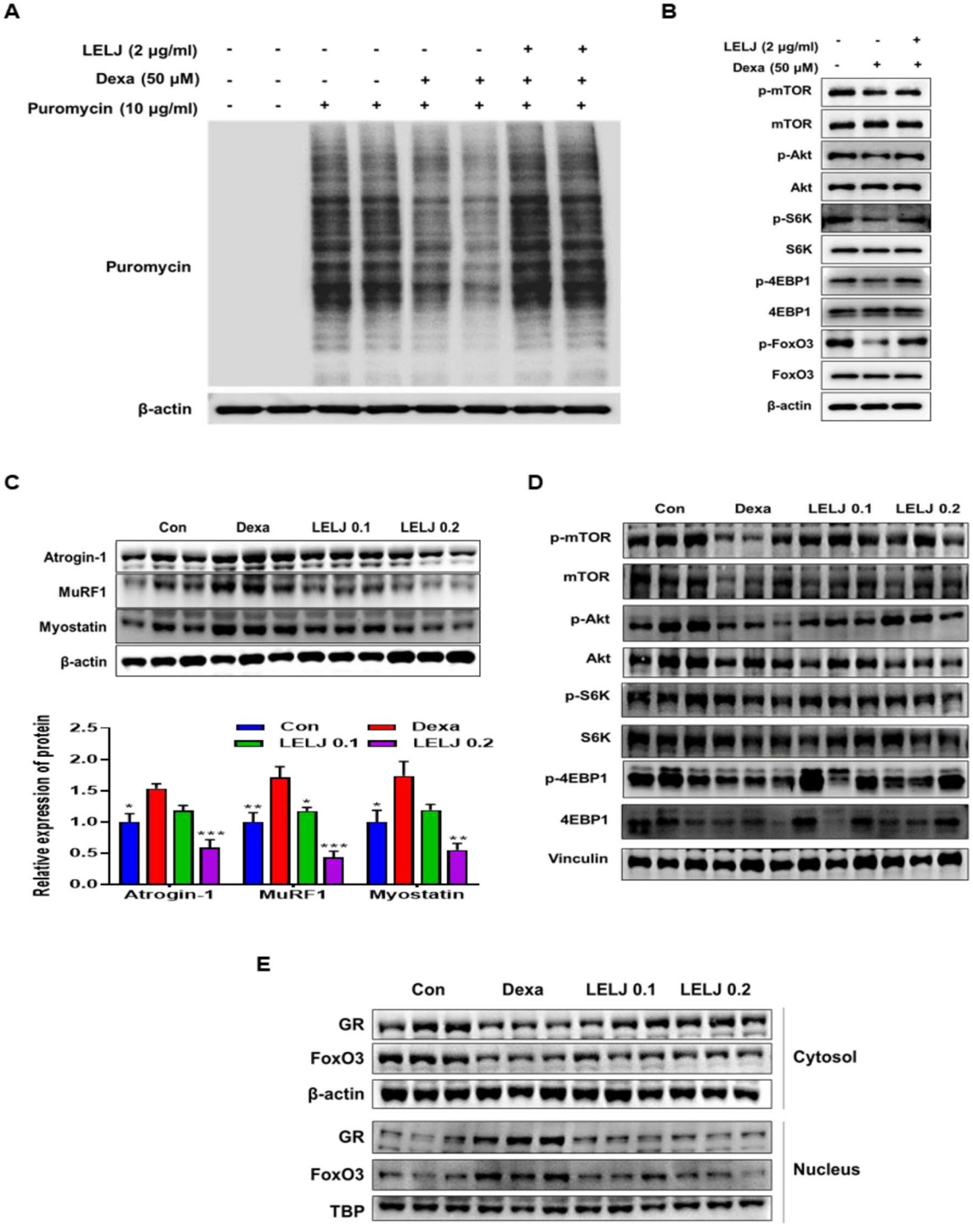


Fig. 5 Effect of LELJ on muscle protein synthesis and muscle atrophy in vivo and in vitro. **A** and **B** The protein levels of puromycin, p-mTOR, mTOR, p-Akt, Akt, p-S6K, S6K, p-4EBP1, 4EBP1, p-FoxO3, FoxO3, and β-actin measured by western blotting in Dexa-treated C2C12 cells. **C** and **D** The expression of Atrogin-1, MuRF1, Myostatin, β-actin, p-mTOR, mTOR, p-Akt, Akt, p-S6K, S6K, p-4EBP1, 4EBP1, and Vinculin in quadriceps as determined by western blotting. **E** The protein levels of cytosol and nucleus GR, FoxO3, β-actin, and TBP measured by western blotting in quadriceps. The band density was analyzed by ImageJ software. Results are expressed as mean ± SEM. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated group

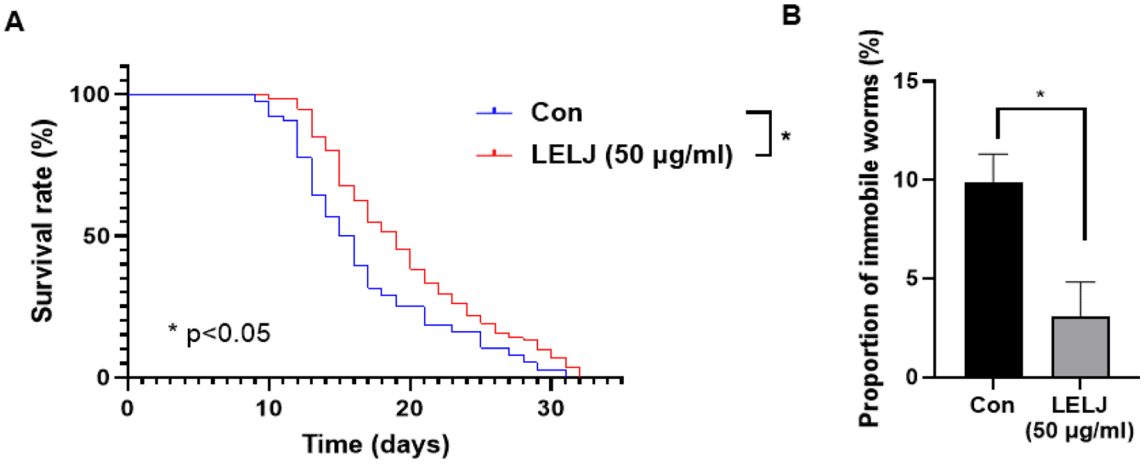


Fig. 6 Effect of LELJ on lifespan and muscle activity in *C. elegans*.**A** Survival rate. **B** Proportion of immobile worms. Results are expressed as mean ± SEM. Kaplan-Meier survival analysis and unpaired t-test were used to assess the differences between the two groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Con group

Dexa translocated the GR from the cytosol to the nucleus, the nucleus and cytosol were separated from the muscle tissues of mice. The translocation of GR protein to the nucleus was increased in the Dexa-treated group compared with that in the Con group and was decreased in the LELJ groups. In addition, Dexa injection increased the expression of FoxO3 protein in the nucleus, whereas the LELJ groups exhibited decreased FoxO3 protein expression compared to the Dexa-treated group (Fig. 5E).

LELJ extended lifespan and mitigated decline in muscle activity in C. Elegans

To evaluate the effect of LELJ on age-related loss of muscle activity, we used a *C. elegans* model and monitored lifespan and worm mobility. We found that LELJ extended the median lifespan of *C. elegans* by 16.7% compared with that of the Con group (Fig. 6A). To measure locomotor capacity, we recorded the number of body bends in *C. elegans* and found that the proportion of immobile worms in the Con group (9.9%) was significantly higher than that in the LELJ-treated group (3.1%) ($p < 0.05$) (Fig. 6B).

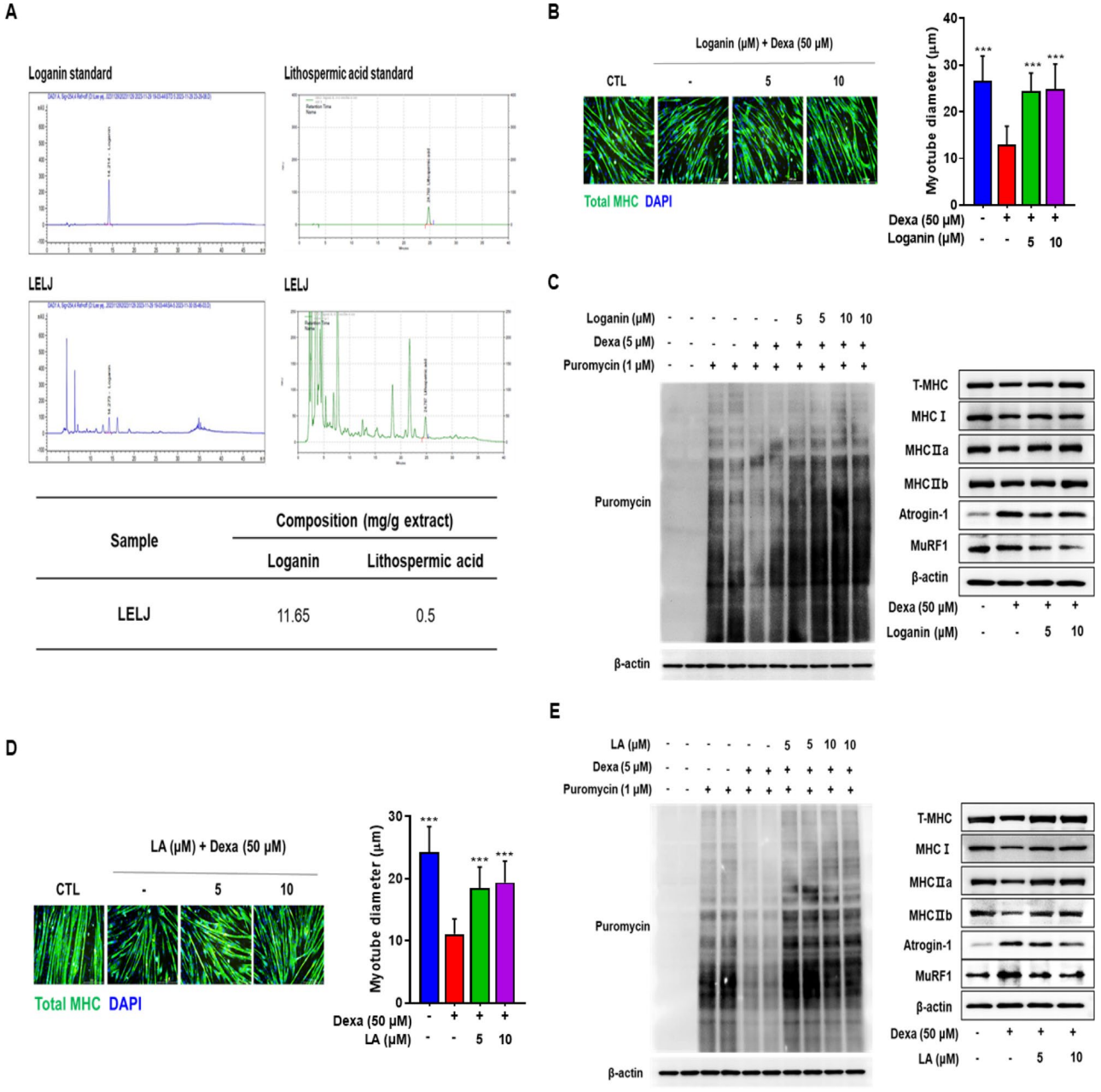
Loganin and LA, the bioactive compounds of LELJ, ameliorated Dexa-induced myotube atrophy in C2C12 cells

The contents of loganin and LA, which are bioactive compounds of LELJ, were determined to be 11.65 mg/g and 0.5 mg/g, respectively (Fig. 7A). We investigated the effects of loganin and LA on Dexa-induced myotube atrophy in C2C12 cells. Treatment with loganin or LA significantly increased the Dexa-induced reduction in myotube diameter (Fig. 7B and D). We also observed an increase in puromycin and MHC isoform protein levels in C2C12 cells treated with loganin or LA compared to those treated with Dexa (Fig. 7C and E). Furthermore, loganin and LA treatment decreased Atrogin-1 and MuRF1 protein levels in C2C12 cells compared to those in Dexa-treated cells (Fig. 7C and E).

Discussion

In this study, we demonstrated the ability of LELJ to alleviate Dexa-induced muscle atrophy in C2C12 myotubes and skeletal muscles of mice. LELJ also ameliorated age-related muscle loss by extending lifespan and increasing locomotor capacity in *C. elegans*.

First, we observed the individual alleviating effects of LE and LJ extracts on Dexa-induced muscle atrophy in C2C12 cells. Additionally, based on studying different LE and LJ ratios, we determined that the 1:1 ratio was optimal for subsequent experiments (data not shown). We found that LELJ prevented Dexa-induced muscle atrophy in C2C12 cells and skeletal muscle of mice by increasing protein synthesis and improving protein degradation. Because glucocorticoids are catabolic in skeletal muscle, high-dose or long-term administration causes steroid myopathy [21], and treatment with Dexa in this study resulted in protein degradation in myotubes and skeletal muscle. It is also known that the catabolic effects of glucocorticoids on skeletal muscle are mediated through several cellular mechanisms that may contribute to the breakdown of muscle proteins. Several studies have indicated that glucocorticoids exert catabolic effects by inhibiting the phosphoinositide 3-kinase (PI3K)/Akt pathway, which mediates the anabolic action of insulin/IGF-1. PI3K/Akt pathway inhibition causes the suppression of mTOR to stimulate proteolysis and decrease protein synthesis, which directly affects muscle protein content [3]. In this study, LELJ increased Dexa-induced reduction in Akt and mTOR phosphorylation in C2C12 cells and skeletal muscle of mice, thus alleviating the negative effects of glucocorticoids on protein synthesis and muscle mass.



Sample	Composition (mg/g extract)	
	Loganin	Lithospermic acid
LELJ	11.65	0.5

Fig. 7 Effect of loganin and lithospermic acid on Dexa-induced muscle atrophy in C2C12 cells. **A** Quantitative HPLC analysis of loganin and lithospermic acid in LELJ. **B** and **D** C2C12 cells were fixed and stained with total MHC antibody (scale bar, 200 μm). Myotube diameter was calculated as the average diameter of MHC-positive multinucleated myotubes. **C** and **E** The protein levels of puromycin, total MHC, MHC1, 2 A, 2B, Atrogin-1, MuRF1, and β-actin measured by western blotting in Dexa-treated C2C12 cells. Results are expressed as mean ± SD. One-way ANOVA was used to compare more than two groups, followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Dexa-treated myotubes

Myostatin, a negative regulator of muscle growth, inhibits Akt phosphorylation in C2C12 cells [22]. Inhibition of the PI3K/Akt pathway activates FoxO, triggering the ubiquitin-proteasome pathway. In this study, the Dexa-treated group showed upregulated myostatin protein levels and reduced phosphorylation of FoxO3, leading to its nuclear translocation. However, LELJ downregulated the protein level of myostatin compared to the Dexa-treated group and reversed the Dexa-induced dephosphorylation and nuclear translocation of FoxO3. In addition, the expression of Atrogin-1 and MuRF1 was decreased. This indicates that LELJ inhibits the dephosphorylation and nuclear translocation of the FoxO transcription factor, which in turn suppresses the expression of Atrogin-1 and MuRF1, thereby alleviating muscle atrophy [23, 24].

Muscle function refers to the ability to exert the maximum force in the shortest possible time, whereas physical performance involves objective measurements of whole-body function related to mobility [25]. The administration of Dexa has been shown to worsen both. Conversely, LELJ administration had a positive effect on Dexa-induced exacerbations.

Aging is one of the many causes of sarcopenia [26]. Therefore, we investigated the effectiveness of addressing age-related muscle loss. In this study, the *C. elegans* model was utilized because of the time limitations associated with the use of a mouse model. After administering LELJ, we observed an extension in the lifespan and an increase in the locomotor capacity of *C. elegans*. It is expected that LELJ could alleviate sarcopenia caused by aging; however, further research is required.

We identified loganin and LA as the bioactive compounds in LELJ. Remarkably, our study revealed, for the first time, that treatment with loganin and LA increased myotube diameter, MHC isoforms, and puromycin protein levels, while decreasing atrophy markers in Dexa-induced C2C12 cells. Notably, shikonin, a major pigment component widely recognized as an indicator of LE, was not detected in this study (data not shown). Because shikonin is known to be unstable at high temperatures, above 80 ℃, which was the sterilization temperature, we used LA as a bioactive compound of LELJ instead of a shikonin derivative [27]. Additionally, loganin was utilized in this study because it is designated as a bioactive compound of LJ in the Korean Pharmacopoeia.

In conclusion, we report the effects of LELJ on Dexa-induced muscle atrophy. We demonstrated that LELJ improved protein degradation and increased protein synthesis. The present results highlight the potential of LELJ in preventing and treating Dexa-induced skeletal muscle atrophy and the contribution of loganin and LA to the therapeutic effects of LELJ.

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Author contributions
J.A. was responsible for study conception and design and wrote the manuscript. A.Y. wrote original draft manuscript and performed the experimental work as well as data analysis. H.L. and JI.K. performed experiments. JH. H. and C.H.J. contributed to discussion and analyzed the data.

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Data availability
The data presented in this study are available from the corresponding authors on reasonable request.

Declarations

Competing interests
The authors declare that they have no conflict of interest.

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