

Cistanche tubulosa Extract Protects Cast Immobilization-Induced Declines of Muscle Strength and Muscle Mass

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ABSTRACT

Aim: One of the effects of *Cistanche* herb is described in the Chinese Pharmacopoeia as for lack of strength in muscles and bones. However, concrete evidence of this herb for muscle strength has been hardly reported. The present study aimed to clarify the effects of 30% ethanolic extract of *Cistanche tubulosa* fleshy stem on muscle strength and muscle mass in a wide range of doses.

Methods: Male ddY mice (10-weeks-old) were used. Muscle atrophy was induced via bilateral hindlimb immobilization by placing them in casts for 15 days. Thirty percent ethanol extract of *C. tubulosa* fleshy stem was orally administered from Day 2 to Day 15. Muscle strength was evaluated using an inclined wire mesh test. Wet weights of triceps surae and tibialis anterior and body weight were measured. Primary cultured skeletal myocytes were treated with acteoside or echinacoside, which are main constituents in *C. tubulosa*. Fiber-like shaped myocytes were quantified by immunocytochemistry.

Results: Although hanging ability of wired mesh significantly decreased in cast-attached hindlimbs atrophied mice, oral treatment of *C. tubulosa* fleshy stem extract dose-dependently protected the grasping ability. At 100 mg/kg, extract remarkably protected. Lower doses of 1 and 10 mg/kg extract indicated partial protection. For protection of muscle mass, only 100 mg/kg extract was effective. Acteoside treatment increased fiber length in fiber-like shaped mature myocytes in primary cultured myocytes, but echinacoside did not.

Conclusion: *C. tubulosa* fleshy stem extract has anti-muscle weakening effect and anti-muscle loss effect in disuse-induced muscle atrophy. Acteoside has been shown to potentially activate muscle cells.

1 | Introduction

Cistanche herb is defined in the Japanese Pharmacopoeia as fleshy stems of *Cistanche salsa* G.Beck, *C. deserticola* Y.C.Ma, or *C. tubulosa* Wight. In Chinese Pharmacopoeia, it is defined as fleshy stems of *C. deserticola* Y.C.Ma, or *C. tubulosa* Wight. *Cistanche* herb is famous for anti-aging [1, 2]. In the Chinese Pharmacopoeia, one of the effects of *Cistanche* herb is described as for lack of strength in muscles and bones.

Our previous screening approach identified that *Cistanche* herb extract stimulated primary cultured muscle cells (unpublished data). Therefore, we performed an animal study and a clinical study to evaluate the effects of *Cistanche tubulosa* fleshy stem extract on muscle strength. In the animal study, administration of 30% ethanolic extract of *C. tubulosa* fleshy stem at 100 mg/kg improved walking ability in a cast-immobilized skeletal muscle atrophy mouse model [3]. In the placebo-controlled, randomized, double-blind study, 26 subjects with pre-symptomatic or

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mild locomotive syndrome completed all tests [4]. Intake of 30% ethanolic extract of *C. tubulosa* fleshy stem at 1800mg/human for 12 weeks increased step width (two-step test) and gait speed (5-m walking test) in patients over 60 years old compared with those in a placebo control. No obvious adverse events were observed following the intake of 30% ethanolic extract of *C. tubulosa* fleshy stem [4].

Acteoside (synonym: verbascoside) is recognized as one of the major active constituents of this plant. In our previous study of the chronic phase in spinal cord injured mice, acteoside treatment significantly improved motor function and muscle weights in mice with spinal cord injuries [5]. In Bufo gastrocnemius study, acteoside pre-treatment significantly increased muscle contractility, indicating an anti-muscle fatigue effect of this compound [6].

In our previous study of cast-attached mice, although 100mg/kg of the extract improved walking a ladder without slipping, 50mg/kg of the extract showed no significant effect [3]. However, muscle strength was not evaluated. In addition, atrophied muscle masses were not improved by both doses of the extract [3]. At the study, effective dose for walking performance, 100mg/kg of 30% ethanolic extract of *C. tubulosa* fleshy stem contained 3.36 mg/kg/day of acteoside [3]. We suppose that *C. tubulosa* fleshy stem extract containing a higher amount of acteoside would be more effective. Therefore, the present study aimed to clarify the efficacy of *C. tubulosa* fleshy stem extract containing high content of acteoside for muscle strength and muscle mass.

2 | Methods

2.1 | Animals

All animal experiments and protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama and were approved by the Committee for Animal Experiments of the Sugitani Campus of the University of Toyama (approval number: A2025 INM-5). Male closed-colony ddY mice (10-weeks-old; Japan SLC, Shizuoka, Japan) were used in this study. All mice were allowed ad libitum access to water and food and kept in a controlled environment ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 12-h light/dark cycle starting at 07:00). All efforts were made to minimize the number of experimental animals used.

2.2 | Induction of Hindlimb Muscle Atrophy by Cast-Attachment

To induce muscle atrophy, we immobilized the hind limbs, as previously described [7]. All animals were anesthetized via intraperitoneal injection of a mixture containing 0.75 mg/kg medetomidine (ZENOAQ, Fukushima, Japan), 4.0 mg/kg midazolam (Sandoz, Tokyo, Japan), and 5.0 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan).

Next, hair was removed from the thigh to the ankle. Ethylene propylene diene monomer (EPDM) rubber foam tape (Cemedine,

Tokyo, Japan) was then cut to approximately 3 cm and loosely wrapped to extend the ankle joint. Dental cement (UNIFAST III; GC International AG, Lucerne, Switzerland) was applied to the ankle edge of the foam for tightening. The foam's exterior was covered with a soft vinyl chloride insulating cap (TIC-22; Nichifu, Osaka, Japan) using double-sided tape. This cap was secured by wrapping it with strong adhesive tape (Asahipen, Osaka, Japan), aluminum foil tape (Nichiban, Tokyo, Japan), and vinyl tape (Nitto, Osaka, Japan). Finally, a 1-cm foam tape was attached to the back of the knee, fixed in an extended position with cloth surgical tape (Nitoms, Tokyo, Japan), and covered with vinyl tape. After the operation, the same amount of atipamezole hydrochloride (ZENOAQ) as an anti-anesthetic was intraperitoneally administered. To prevent anesthesia-induced body temperature drop, mice were kept warm on a hot plate at 40°C until arousal. For preparation of non-cast mice, anesthesia, hair shaving, and anti-anesthetic injection were carried out.

2.3 | Test Drug

Aqueous ethanol extract of *C. tubulosa* fleshy stem (Kanka extract-P25, Oryza Oil & Fat Chemical, Ichinomiya, Japan) was used. The extract contains 12.0% acteoside and 40.0% echinacoside. Contents of other compounds were trace levels. The extract was dissolved in water for administration to mice.

2.4 | Behavioral Assessments

Inclined wire mesh test is a method to evaluate muscle strength, which was established by us. A metal wire mesh (32 cm wide, 20 cm height, 1.3 cm square grid) was placed 40 cm above the floor. The wire mesh is leaning against an angle-adjustable stand. After habituating a mouse on the gently sloping wire mesh, the mouse was placed on a wire mesh stand positioned at a 90° angle (Figure 1B). The time spent grasping the mesh until falling to the floor was measured within a maximum of 1 min. The mesh angle increased in increments of 10° – 180° , and the time spent grasping was similarly measured. One test was conducted per degree of angle change.

2.5 | Tissue Isolation

ddY mice were anesthetized via isoflurane inhalation. Following cardiac perfusion with ice-cold saline, the skin was incised, and the tibialis anterior and triceps surae were isolated to weigh.

2.6 | Primary Skeletal Muscle Cell Culture and Immunocytochemistry

Male ddY mice at postnatal day 5 (Japan SLC) were decapitated under deep isoflurane anesthesia (FUJIFILM Wako Pure Chemical, Osaka Japan). For one experiment, 26 hindlimbs were used to make primary cultured myocytes. The same experiments were conducted twice. After cutting hindlimbs, the skin was peeled off. The skeletal muscle isolated from the whole hind limb was minced and dissociated in HBSS containing 480 U/mL collagenase (FUJIFILM Wako Pure Chemical) and 2.5 mM

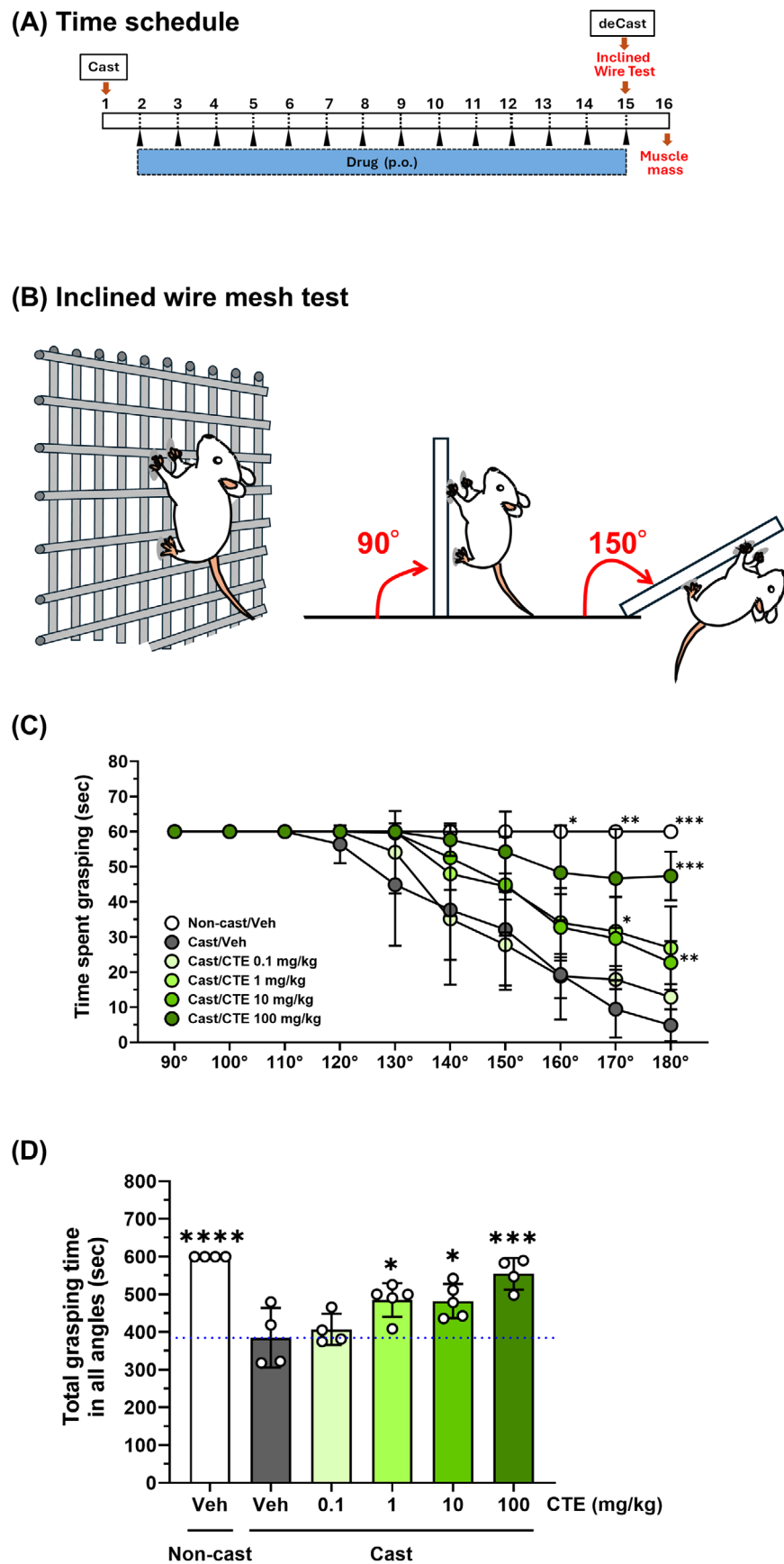


FIGURE 1 | Legend on next page.

FIGURE 1 | Effect of *C. tubulosa* fleshy stem extract on motor function evaluated by inclined wire mesh test. (A) Experimental timeline. Oral administration of vehicle solution (Veh, water) and *C. tubulosa* fleshy stem extract (0.1, 1, 10, 1, and 100 mg/kg) to cast-attached mice were continued for 14 days. Veh-treated non-cast-attached mice were used as control. (B) Inclined wire mesh test was performed from 90° to 180° angles with increment every 10°. (C) Time spent grasping mesh was plotted in each angle. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus cast-attached vehicle treated group; repeated measures two-way ANOVA, post hoc Bonferroni test; $n = 4-5$ mice. (D) Summed time spent grasping mesh was shown. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ versus cast-attached vehicle treated group; one-way ANOVA, post hoc Bonferroni test; $n = 4-5$ mice. CTE: *C. tubulosa* fleshy stem extract. Data are presented as mean $\pm$ SD.

CaCl₂ for 1 h. The obtained myocytes were grown in Myocyte Medium (Promo Cell, Heidelberg, Germany) with poly D-lysine coating at 1.45 or 7.25×10^4 cells/cm² in 8-well chamber slides (Corning, Corning, NY, USA).

After drug treatment (acteoside and echinacoside), myocytes were fixed with 4% paraformaldehyde for 30 min and immunostained at 4°C for 24 h in 0.3% Triton X-PBS with mouse anti-myosin heavy chain (MHC) antibody (1:500, #05-716-I, Merck, Rahway, NJ, USA). Secondary antibodies, Alexa Fluor 488 Plus-conjugated goat anti-mouse IgG (1:1000, #A32723, Thermo Fisher Scientific, Waltham, MA, USA) were incubated for 2 h at room temperature in 0.3% Triton X-PBS. Counterstaining by 1 μ g/mL DAPI (Enzo Life Science, Farmingdale, NY, USA) was followed. Fluorescence images were captured using a fluorescence microscope (Cell observer, Carl Zeiss, Oberkochen, Germany). Ten to twelve photos per well at regular intervals were captured (well area: 0.69 cm²). To take photos without bias, selecting locations was blinded. Photos were captured by a 10 $\times$ objective lens. Numbers of total myocytes were automatically counted using MetaMorph version 7.8 (Molecular Devices, San Jose, CA, USA). Fiber-like myocytes were manually counted looking at all photos. Fiber lengths were automatically measured by MetaMorph version 7.8. Experiments were conducted twice, and data were combined.

2.7 | Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA) was used to perform one-way analysis of variance (ANOVA) and post hoc Bonferroni tests, and repeated measures two-way ANOVA and post hoc Bonferroni tests. The significance level was set at $p < 0.05$.

3 | Results

At one day after cast attachment, oral administration of *C. tubulosa* fleshy stem extract was started and continued for 14 days (Figure 1A). Doses of *C. tubulosa* fleshy stem extract were 0.1, 1, 10 and 100 mg/kg/day. After the last treatment of *C. tubulosa* fleshy stem extract, cast was removed. Inclined wire mesh test was performed at 4–5 h after de-casting. Non-cast control mice showed full scores in all angles of the wire mesh. In contrast, Cast-attached and vehicle solution (water)-treated mice began to fall at 120°, and as the angle of the wire mesh became steeper, mice began to fall in a shorter time (Figure 1C). *C. tubulosa* fleshy stem extract treatment dose-dependently prevented the falling from the wire mesh. As shown in Figure 1C, repeated

measures two-way ANOVA showed a significant effect of drug effect ($F(5, 20) = 12.13$, $p < 0.0001$), and angle $\times$ drug interaction ($F(13.41, 53.64) = 6.901$, $p < 0.0001$). By post hoc Bonferroni test, 1 mg/kg extract at 170°, 10 mg/kg extract at 180°, and 100 mg/kg at 180° were significantly improved compared with cast-attached vehicle treatment.

Summed values in all angles were shown in Figure 1D. *C. tubulosa* fleshy stem extract at 0.1 mg/kg showed no effect. The extract at 1 and 10 mg/kg significantly but partially extended holding time on the wire mesh. *C. tubulosa* fleshy stem extract at 100 mg/kg indicated a remarkable effect nearly to the non-cast group.

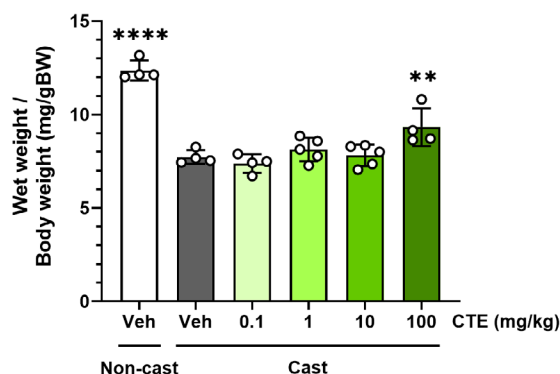
After completion of the behavioral test, triceps surae and tibialis anterior muscles were isolated to weigh. Muscle masses of triceps surae and tibialis anterior were significantly decreased in the cast-attached vehicle-treated group. *C. tubulosa* fleshy stem extract at 100 mg/kg significantly protected the loss of triceps surae (Figure 2A). Although the muscle mass of tibialis anterior tended to be protected by 100 mg/kg *C. tubulosa* fleshy stem extract, it was not a significant difference (Figure 2B). Lower doses of *C. tubulosa* fleshy stem extract (0.1, 1, and 10 mg/kg) showed no effects on those muscle mass losses. By cast attachment, body weights were significantly decreased, and any doses of *C. tubulosa* fleshy stem extract didn't affect the body weight loss (Figure 2C).

C. tubulosa fleshy stem extract used in the present study contains 12% acteoside and 40% echinacoside. Primary cultures of mouse hindlimb skeletal muscle cells were treated with acteoside or echinacoside for 4 days. Undifferentiated myocytes are round in shape, while differentiated myocytes exhibit a fiber-like shape. The percentage of fiber-like myocytes per total myocytes tended to elevate in acteoside-treated myocytes, but not in echinacoside-treated myocytes (Figure 3A,B).

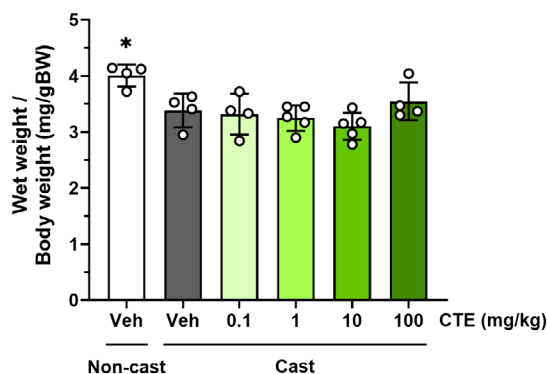
4 | Discussion

Although *Cistanche* herb including *C. tubulosa* is not used in Traditional Kampo Medicine, this herb has been widely used in China as medicines, teas, foods, and so on [8]. In the Chinese Pharmacopoeia, one of the effects of *Cistanche* herb is described as for lack of strength in muscles and bones. However, concrete evidence of this herb for muscle strength has been hardly reported. Only our group reported improvement of walking performance by 30% ethanol extract of *C. tubulosa* fleshy stem in cast-attached muscle atrophied mice [3] and humans with locomotive syndrome [4]. However, the muscle strength was not evaluated, and the extract didn't ameliorate muscle atrophy in

(A) Triceps surae



(B) Tibialis anterior



(C) Body weight

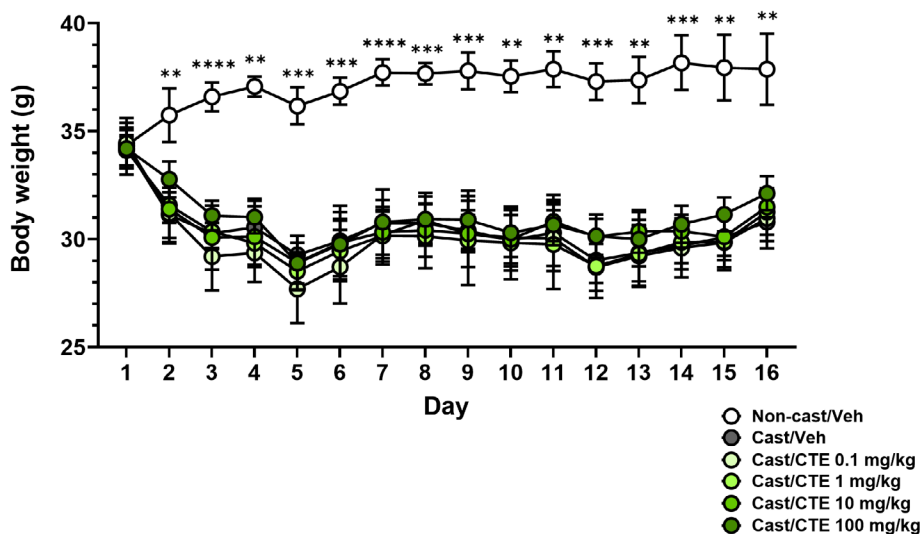


FIGURE 2 | Effect of *C. tubulosa* fleshy stem extract on muscle mass and body weight. Oral administration of vehicle solution (Veh, water) and *C. tubulosa* fleshy stem extract (0.1, 1, 10, 1, and 100 mg/kg) to cast-attached mice was continued for 14 days. Veh-treated non-cast-attached mice were used as control. The next day of de-casting, wet weights of triceps surae (A) and tibialis anterior (B) were measured. Each muscle weight was divided by initial body weight. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ versus cast-attached vehicle treated group; one-way ANOVA, post hoc Bonferroni test; $n = 4-5$ mice. (C) Body weights in entire experimental period. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus cast-attached vehicle treated group; repeated measures two-way ANOVA, post hoc Bonferroni test; $n = 4-5$ mice. CTE: *C. tubulosa* fleshy stem extract. Data are presented as mean $\pm$ SD.

the previous mouse study [3]. Efficacy of plant extracts depends on contents of active ingredients. Therefore, the present study used acetoside-rich *C. tubulosa* fleshy stem extract and evaluated muscle strength and muscle masses.

Hanging ability of the wired mesh significantly decreased in cast-attached hindlimbs of atrophied mice. Oral treatment of *C. tubulosa* fleshy stem extract dose-dependently protected the grasping ability. At 100 mg/kg, extract showed remarkable

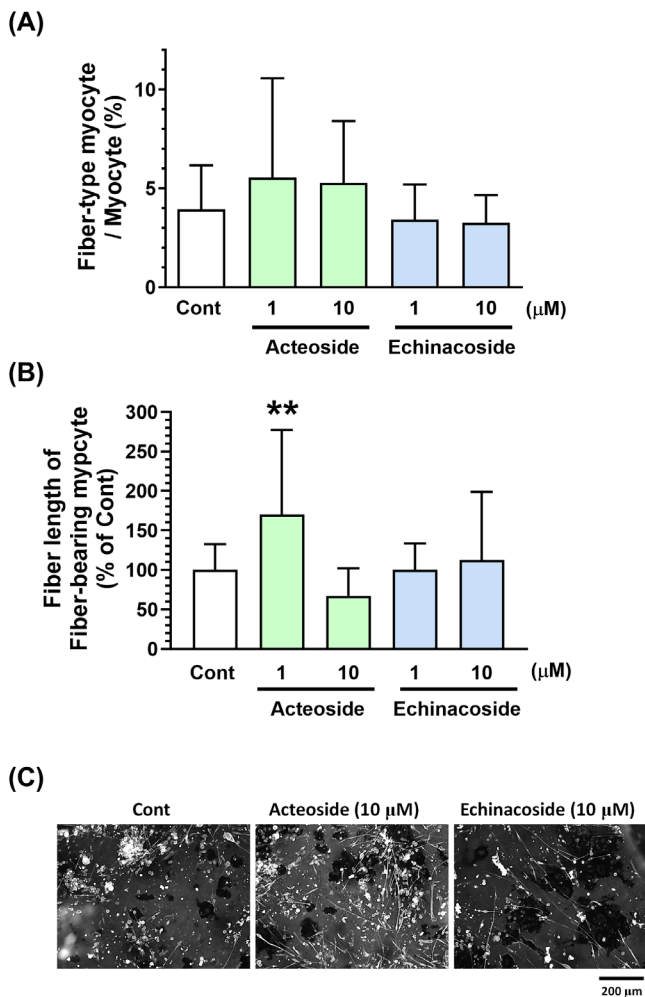


FIGURE 3 | Effects of acteoside and echinacoside on myotube differentiation in cultured skeletal myocytes. Primary cultured P5 myocytes were treated by vehicle solution (Cont, water), acteoside (1 or 10 μM), or echinacoside (1 or 10 μM). After 4 days, cells were immunostained with MHC. (A) Fiber-like shaped myocytes and any shaped myocytes were quantified (total myocytes). The percentage of fiber-like shaped myocytes to total myocytes was shown. One-way ANOVA, post hoc Bonferroni test; $n=18-22$ photos in two experiments. Data are presented as mean $\pm$ SD. (B) Fiber length in a fiber-bearing myocyte was shown. Since basal lengths varied in two experiments, data are shown as the percentage of control. ** $p < 0.01$ versus Cont, One-way ANOVA, post hoc Bonferroni test; $n=18-22$ photos in two experiments. Data are presented as mean $\pm$ SD. (C) Representative images of MHC-positive myocytes. Scale bar is 200 μm.

improvement (Figure 1C,D). Lower doses of 1 and 10 mg/kg extract indicated partial protection. For protection of muscle mass, only 100 mg/kg extract was effective (Figure 2A).

Although the main components of *C. tubulosa* have been recognized as acteoside and echinacoside, the content of these components can vary depending on the raw materials used. It was not clarified previously which compound was related to muscle strength. Therefore, we compared effects of acteoside and echinacoside on myocyte differentiation in primary cultured skeletal muscle cells. Acteoside treatment tended to increase the rate of fiber-bearing mature myocytes, but not

echinacoside (Figure 3A,C). The fiber length of mature myocytes was significantly enhanced by 1 μM acteoside treatment (Figure 3B). We supposed the acteoside-mediated myocyte differentiation effect might be related to upregulation of muscle mass. For concerning the relationship about myocyte differentiation in vitro and muscle strength in vivo, Cho et al. reported the Slit guidance ligand (SLIT) 3 protein-mediated phenomena [9]. Slit3-deficient mice exhibited decreased skeletal muscle mass, muscle strength, and physical activity. In addition, SLIT3 treatment to C2C12 myoblast cells induced myotube formation.

In our previous study, *C. tubulosa* fleshy stem extract containing 3.36% acteoside was effective for waking ability at 100 mg/kg, but not at 50 mg/kg in cast-attached mice [3]. In contrast, the present extract containing 12% acteoside showed remarkable effect on the muscle strength and muscle mass at 100 mg/kg, but just partial effects on the muscle at 1 and 10 mg/kg (Figures 1 and 2). We consider that the concentration of acteoside in the extract may serve as a criterion for predicting the extract's efficacy. However, we cannot yet state with certainty that acteoside-induced myocyte differentiation is only reason for muscle mass upregulation. Other group reported that paclitaxel-induced fatigue and muscle mitochondrial damage in BALB/c mice were inhibited by acteoside treatment per oral, and acteoside treatment to cachexia-like cell model, C26 tumor cell-conditioned medium-treated murine C2C12 cells, ameliorated mitophagy, ROS production, and abnormal expression changes of key factors for mitochondrial function and mitophagy (COXIV, CytoC, LC3B, p62, PINK1 and Parkin) [10]. They suggested in the report that a direct target protein of acteoside was proline hydroxylase 2. Same group recently reported that colon cancer inoculation-induced fatigue-associated behaviors and skeletal muscle abnormal morphology were ameliorated by acteoside oral treatment [11]. By metabolome and network analyses of plasma, several hub protein candidates such as CYP3A4 and BCL-2 were supposed interacting with acteoside [11]. Therefore, multiple molecules were proposed as target proteins for acteoside. We believe it is crucial to identify the acteoside signaling pathway that is genuinely involved in restoring muscle function. From this perspective, we are currently investigating the key acteoside signaling pathways involved in muscle function recovery.

5 | Conclusion

In summary, *C. tubulosa* fleshy stem extract has clearly showed anti-muscle weakening effect and anti-muscle loss effect in disuse-induced muscle atrophy. Acteoside potentially activates muscle cells. A remarkable effective dose of *C. tubulosa* fleshy stem extract was 100 mg/kg, in the case of containing 12 mg/kg of acteoside. In contrast, 0.1 mg/kg of *C. tubulosa* fleshy stem extract was completely ineffective for muscle strength. Middle doses of *C. tubulosa* fleshy stem extract (1 and 10 mg/kg) showed partial effects on muscle strength and no effects on muscle mass. In an aging society, preventing dysfunction and restoring function of the locomotive organs are necessary for long-term quality of life. *C. tubulosa* fleshy stem is a promising crude drug capable of preventing muscle wasting.

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Ethics Statement

All animal experiments and protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama and were approved by the Committee for Animal Experiments of the Sugitani Campus of the University of Toyama (approval number: A2025 INM-5).

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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