Original Research

Effect of *Ferula hermonis* root extract on rat skeletal muscle adaptation to exercise

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Abstract

Ferula hermonis Boiss. is an aphrodisiac plant that grows in the Mediterranean region. It has been reported that treatment with acetonic extract from the root of this plant acutely increases serum testosterone in the rat. This study investigated the effects of *F. hermonis* extract alone or combined with exercise on rat skeletal muscle fibers. Adult male rats were divided into four groups: control-sedentary (CS) that had no treatment or exercise; ferula-sedentary (FS) that was orally treated with ferula extract at a dose of 60 mg/kg/rat every other day over a period of 20 d; control-exercised (CE) that was trained by swimming for 40 min every other day; and ferula-exercised (FE) that received ferula and performed exercise. At the end of experiments, the fiber diameter and number of muscle nuclei of tibialis anterior were measured by using immunofluorescent techniques and software analyses. The FE group showed significant increases in muscle weight, fiber size and nuclear number compared with the other groups. However, no significant changes in the aforementioned parameters were found among the CS, FS and CE groups. Ferula treatment and exercise were additive to each other. In conclusion, short-term exercise combined with administration of *F. hermonis* extract was more effective in enhancing the growth of skeletal muscle fibers than exercise alone.

Keywords: ferula, hypertrophy, phytoestrogen, testosterone

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Introduction

Ferula hermonis (Boiss.) is an aphrodisiac plant that belongs to the Apiaceae family and grows abundantly in the Middle East.[1,2] Its name belongs to the biblical mount Hermon in Lebanon where it was first discovered.[2] For centuries, the root extract of this plant has been used as a treatment for sexual impotence. Currently, the root extract is commercially available and is widely used among Middle Eastern populations to improve sexual performance and increase energy.[3]

Several phytochemical and pharmaceutical studies were conducted on the root of this plant to identify its active ingredients and their respective mechanism of action.[3–5] The main ingredients are four aromatic esters of sesquiterpene alcohol known as ferutinin (ferutinol *p*-hydroxybenzoate), teferdin (ferutinol benzoate), teferin (ferutinol vanillate) and epoxybenz (epoxyferutinol benzoate).[4] Among these compounds, ferutinin is a potent phytoestrogen and considered the primary active ingredient.[5,6] In addition to these esters, several naturally occurring vitamins and minerals were found in the root of *F. hermonis*.[1]

Zanoli *et al*.[3] reported that a single oral administration of acetonic extract from *F. hermonis* can significantly boost serum testosterone concentrations in adult male rats. They showed that ingestion of ferula extract at a dose of 60 mg/kg can increase the level of testosterone in rat serum by three-fold within 45 min. Testosterone is the primary male sex hormone that is responsible for both androgenic and anabolic activities in the body.[7] In recent years, there has been a great interest in determining the therapeutic value of using testosterone to treat sarcopenia, the age-related loss of muscle mass and function.[8,9] The interest in testosterone and its other synthetic derivatives stems from published literature showing their ability to increase skeletal muscle mass and strength.[10–12] Moreover, it has been reported that testosterone supplementation is associated with a dose-dependent increase in fiber size and nuclear number of skeletal muscle.[8]

The aim of this study was to investigate the effect of acetonic extract from the root of *F. hermonis* on skeletal muscle mass and fiber size. The study tested the hypothesis that administration of *F. hermonis* extract combined with short-term exercise is more effective in improving muscle growth than exercise alone. Some of the preliminary findings of this study were presented as a conference abstract.[13]

Materials and methods

Experimental model

Adult male albino rats of Sprague–Dawley strain, weighing about 300 g, were produced and raised in the Animal House Unit at Jordan University of Science and Technology (JUST) for this study. All animal care procedures and treatments were conducted with the approval of the JUST Committee on Animal Care, and in accordance with the guidelines of the National Institutes of Health on the use and care of laboratory animals (USA).

The rats were divided randomly into four groups: control-sedentary (CS) that had no treatment or exercise; ferula-sedentary (FS) that was orally treated with ferula extract; control-exercised (CE) that was trained by swimming; and ferula-exercised (FE) that received combined treatment and exercise. Each group contained four male rats. The animals were kept under a controlled temperature of 21 ± 1°C with a schedule of 12 h light and 12 h dark (lights on 06:00–18:00 h). Food and water were available *ad libitum*. The rats were given a two-week acclimatization period before starting the treatment.

Extract preparation and analysis

F. hermonis roots were obtained from a local market in Jordan and further identified by specialized botanists in The Department of Biological Sciences at Yarmouk University, Jordan. The roots were ground into powder and the acetonic extract was prepared using a Soxhlet apparatus.[14] The extract was then filtered and the solvent from the filtrate was removed by distillation. The remaining solid extract was preserved in a refrigerator until treatment.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) was applied in order to reveal the main active ingredients of the extract. A sample of 100 mg of the solid root extract was dissolved in 4 mL methanol and centrifuged at 20,000 rpm. The supernatant was then separated and injected into a LC-MS-MS analyzer (API3200Qtrap; AB Sciex, Concord, ON, Canada) with electrospray ionization-positive ion mode. A reverse-phase chromatography of the water/acetonitrile gradient program was performed for 12 min run on a symmetry C18 column (5 μm, 2.1×50 mm; Waters, Milford, MA, USA) to elute the extract at a flow rate of 350 μL/min. Chromatographic data were processed using Analyst software (Version 1.5; AB Life Technologies Corporation, Foster City, CA, USA).

Animal treatments

Just before treatment, the extract was solubilized in Tween-80 (10%) and distilled water (90%) to facilitate its oral administration to the animals in the ferula-treated groups at a dose of 60 mg/kg body weight. This dose was chosen based on a previous study that reported a significant increase in serum testosterone concentration within 45 min after treatment with a similar dose of ferula extract.[3] The dose was given every other day using animal feeding intubation needles (Popper & Sons, New Hyde Park, NY, USA). Each animal received 10 doses over a period of 20 d. Animals in the control groups received the same volume of vehicle media only.

Testosterone assay

In order to confirm the efficacy of our ferula extract to increase serum testosterone, 10 male rats were obtained and divided equally into two groups. One group received 60 mg/kg/rat ferula extract and the other group received vehicle. Forty-five minutes later, the rats were sacrificed by an overdose of diethyl ether and trunk blood was collected into centrifuge tubes. Serum was prepared by centrifugation at 3000 rpm for 30 min, and then stored frozen (−20°C) until the testosterone assay. The testosterone concentration was determined in duplicate experiments using the Testosterone Enzyme Immunoassay test kit (BioCheck Inc, Foster City, CA, USA). The minimum detectable concentration of this assay was estimated to be 0.05 ng/mL and cross-reactivity with other corticosteroids was minimal (<0.05%). The same procedure was also performed at the end of the experiments to measure the serum levels of testosterone in all treatment groups.

Swimming exercise

Rats in the exercised groups were trained to swim in a rectangular water tank (1.5 × 1.0 m) with a water depth of 40 cm and a temperature of approximately 34–36°C. Swimming was selected as the mode of exercise because it is considered to be physically less traumatic for the animals and does not cause foot injures. The swimming protocol was conducted in two phases: adaptation and training. The adaptation phase started in the last five days of the acclimatization period, before the treatments began. The rats were exposed for 10-min swimming on the first day of adaptation. The swimming period was extended by 10 min every day until the rats were swimming for a total of 40 min. On the final day of the adaptation phase, the animals were allowed to rest. The training phase consisted of swimming for 40 min on the same day of ferula treatment, 45 min after receiving the oral dose. The rats were trained 10 sessions over a period of 20 d. All swimming sessions were supervised to avoid floating and/or clinging of the rats.

Animal sacrifice and muscle weights

Animals were euthanized 24 h after the last treatment and trunk blood was collected from each animal for the testosterone assay later on. The left tibialis anterior (TA), gastrocnemius and soleus muscles were dissected out, trimmed free of fat and weighed. The results were then compared among the four groups.

Tissue preparation and sectioning

Muscle samples were excised from the superficial portion of the TA muscle of each rat. The samples were cut parallel to the orientation of the muscle fibers. Each sample was then coated with optimal cutting temperature compound

(Bio-Optica, Milano, Italy) and immediately frozen in 2-methylbutane cooled via liquid nitrogen. Samples were then stored at −40°C until sectioning.

Serial cross-sections of 10 μm thickness were cut at −20°C using a cryostat. Every two serial sections were picked up on a Superfrost Plus microscopic slide (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). The reason for collecting two sections on each slide was to increase the possibility of choosing better fields for imaging. Serial slides bearing sections were numbered and stored at −20°C.

Antibodies and nuclear labeling agent

The primary antibody used for immunostaining was antilaminin (Sigma Chemical Co, St Louis, MO, USA). Antilaminin is a rabbit polyclonal antibody that was developed against the glycoprotein laminin of mouse origin and was used to detect the basal laminae of skeletal muscle fibers at a dilution of 1:200. Tetramethyl rhodamine secondary antibody (Sigma Chemical Co) was used to label antilaminin in red when viewed with an epifluorescent microscope. The secondary antibody was prepared in phosphate-buffered saline (PBS) at a dilution of 1:400. Hoechst 33258 (Bisbenzimide; Sigma Chemical Co) was applied at a dilution of 1:1,500,000 in PBS to label the DNA in all nuclei blue under epifluorescent microscopy.

Immunocytochemical protocol

Slides were removed from the −20°C freezer and air-dried for 15 min. Sections were then treated for 30 min with 200 μL of blocking solution, which consisted of 5 mmol/L ethylenediaminetetraacetic acid (EDTA) in PBS (0.02 mol/L sodium phosphate buffer, 0.15 mol/L sodium chloride, pH 7.2), 5% horse serum and 1% bovine serum albumin. After that, blocking solution was drained from each slide and antilaminin primary antibody diluted in blocking solution was added to each slide (150 μL) and incubated overnight at 4°C. Slides were then washed three times in fresh PBS solution for five minutes per wash. The secondary antibody was then applied over the sections on each slide for 40 min at room temperature, followed by two five-minute washes in fresh PBS. Hoechst 33258 was applied over the sections for five minutes, followed by two additional five-minute washes in PBS. All slides were then fixed with 4% formaldehyde in PBS for three minutes, and washed twice for five minutes each wash in PBS. Finally, slides were mounted with cover slips using an aqueous mounting medium (Santa Cruz Biotechnology Inc) and left for 20 min to harden before being examined under epifluorescent microscopy.

Image analysis and data collection

Four different fields of view were captured from each immunofluorescent slide using a fluorescent microscope equipped with a digital camera. Two epifluorescent images, each viewed through a different wavelength filter, were acquired from each field. Images were subsequently transferred to a computer, and the two images of each field of view were superimposed using Adobe Photoshop program (Adobe System Inc, San Jose, CA, USA). The resultant immunofluorescent images showed all nuclei in blue and basal laminae in red (Figure 1).

The ellipse minor axis (shorter diameter of an ellipse structure) was used to assess fiber size. Minor axes of 250 contiguous fiber cross-sections were measured from each animal. The basal laminae images were used to measure the ellipse minor axis of individual fibers by using Image J program (developed by US National Institutes of Health, Bethesda, MD, USA and available on the Internet, Version 1.43u).

The number of all muscle nuclei beneath the basal lamina (myonuclei and satellite cell nuclei) was counted for each of the 250 fibers. After that, the number of nuclei per unit length of fiber was calculated for each rat using the formula demonstrated by Castillo de Maruenda and Franzini-Armstrong.[15] This formula is: $N = A/(L_n + M)$, where N is the number of cells per unit length of fiber, A is the mean number of nuclei per fiber cross-section, L_n is the average length of the nucleus and M is the thickness of tissue section.

Statistics

Samples were blocked into four groups. Levene's test for the equality of variance was first applied. After determining the homogeneity of variance, data were evaluated by one- and two-way analysis of variance (ANOVA) at 5% level of significance. If a significant difference ($P < 0.05$) was detected, Scheffe's *post hoc* analysis test was performed to examine statistical differences among groups.

Results

Extract active ingredients

LC-MS-MS analysis revealed the presence of many aromatic esters of sesquiterpene alcohol in the extract. The main

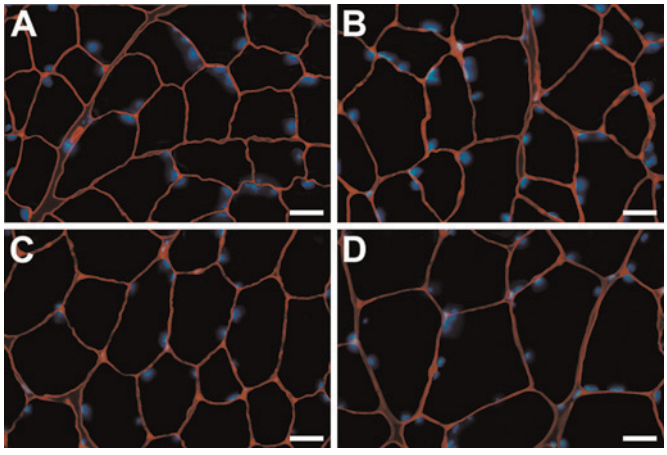

Figure 1 Immunocytochemical labeling of cross-sections from rat tibialis anterior muscle. Muscles were from four different groups: control-sedentary (A), ferula-sedentary (B), control-exercised (C) and ferula-exercised (D). In all images, laminin is colored in red and all nuclei in blue. Scale bars = 50 μm

Table 1 Major active sesquiterpenes identified from *Ferula hermonis* acetonic root extract

Ingredient	Molecular mass (Da)	Retention time (min)	Intensity (CPS)
Teferin	388	6.84	1.9×10^5
Teferdin	342	7.08	9.3×10^4
Ferutinin	358	7.18	2.4×10^5

CPS, count per second

active ingredients in the extract were ferutinin, teferdin and teferin. Their molecular weight, retention time and intensity are summarized in Table 1.

Testosterone serum concentration

Serum testosterone concentration was significantly ($P < 0.001$) increased within 45 min in rats treated with *F. hermonis* extract compared with vehicle-treated rats. Serum testosterone in *F. hermonis*-treated rats was acutely elevated to 5.12 ± 0.44 ng/mL while in control rats it was 2.42 ± 0.37 ng/mL (mean $\pm$ SD). The coefficient of variation was 8.6% in the ferula-treated group and 15.3% in control.

In addition, the serum levels of testosterone in all treatment groups were measured (Figure 2). Testosterone concentrations were elevated in both the FS and CE groups compared with the CS group, but not to significant levels. Only the FE group showed a significant increase ($P < 0.01$) in serum testosterone concentration compared with the CS group. The two-way ANOVA revealed a significant effect ($P < 0.01$) of exercise alone, and a significant effect ($P < 0.01$) of ferula treatment alone on serum testosterone concentration. However, there was no significant interaction between the two factors ($P = 0.45$).

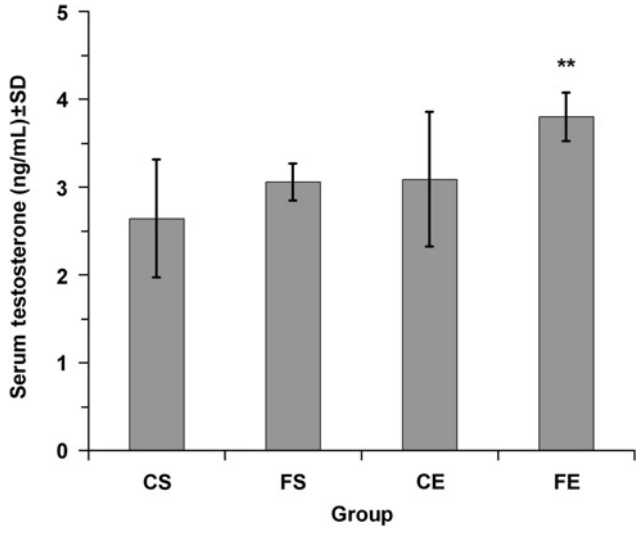

Figure 2 Influence of short-term oral administration of *Ferula hermonis* acetonic root extract (60 mg/kg body weight) on serum testosterone concentration within sedentary and exercised male rats. Values are representing the mean serum level of testosterone $\pm$ standard deviation (SD) for four different groups (CS, control-sedentary; FS, ferula-sedentary; CE, control-exercised; FE, ferula-exercised). Each group contained four male rats. $^{**}P < 0.01$, significantly differs from the CS group (analysis of variance)

Body and muscle weights

In addition to total body weight, TA, gastrocnemius and soleus muscle weights were measured at the end of the experiment and compared among the four groups (Table 2). There was no significant difference ($P = 0.29$) in body weight among the different groups. The weights of TA and gastrocnemius muscles were significantly ($P < 0.05$) increased in the FE group in comparison to the CS group. However, there was no significant difference ($P = 0.27$) in soleus muscle weight among the four groups.

Fiber size and muscle nuclei

TA muscle fibers were significantly ($P < 0.05$) larger in the FE group compared with the other three groups. There was no significant difference ($P > 0.05$) in fiber size in the CE or FS groups in comparison to the CS group. The mean ellipse minor axis in the FE group was 12.6% larger than the CE group, and 25.7% larger than the CS group (Table 3).

The number of nuclei per millimeter of fiber length was significantly ($P < 0.05$) greater in the FE group compared with the other three groups. No significant increase ($P > 0.05$) in the number of nuclei per millimeter of fiber was found among the CE, FS and CS groups. There was on average ~21 more nuclei per mm in TA fibers from the FE group in comparison to the CE group (Table 2).

The two-way ANOVA revealed significant effects ($P < 0.01$) of exercise alone, and significant effects ($P < 0.01$) of ferula treatment alone on both fiber size and nuclear numbers. However, there was no significant interaction between the two factors for both fiber size ($P = 0.38$) and nuclear numbers ($P = 0.20$).

Discussion

The major finding from this study was the ability of ferula extract to potentiate the effect of exercise on skeletal muscle. Rats that exercised and received ferula extract showed an increase in TA mass, fiber size and number of nuclei, whereas exercise or ferula extract treatment individually caused no change in TA mass or morphology. The statistical results indicate clearly that ferula treatment and exercise are additive to each other and not synergistic. While the mechanism by which ferula extract is able to enhance the effect of exercise on skeletal muscle is not known, a likely scenario involves testosterone, given the ability of ferula extract treatment to significantly boost serum testosterone concentration.

Several herbs and herbal extracts have been used as anabolics through providing testosterone-like effects. These include *Tribulus terrestris*, *Muria puama* (potency wood), *Serenoa repens* (saw palmetto berries) and *Smilax officinalis*.[16] A number of hypothetical mechanisms have been suggested for how these plants exert their anabolic effects. They are believed to contain chemical structures such as sterols and steroidal saponins, which are claimed to undergo bioconversion into testosterone within the human body.[16] However, there is no scientific evidence to support a mechanism involving the bioconversion of these molecules into

Table 2 Influence of *Ferula hermonis* acetonic root extract and short-term exercise on body and wet muscle weights of adult male rat

Group	Body (g)	TA (g)	GC (g)	Sol (g)
CS	278 ± 11.4	0.44 ± 0.04[a]	1.20 ± 0.06[a]	0.102 ± 0.002
FS	309.7 ± 12.7	0.50 ± 0.02[ab]	1.28 ± 0.07[ab]	0.102 ± 0.002
CE	288.7 ± 14.5	0.49 ± 0.01[ab]	1.28 ± 0.04[ab]	0.112 ± 0.011
FE	290.7 ± 30.1	0.54 ± 0.03[b**]	1.44 ± 0.14[b*]	0.107 ± 0.007

Each value is representing the mean weight ± standard deviation for the body and three muscles (TA, tibialis anterior; GC, gastrocnemius; Sol, soleus) in four different groups (CS, control-sedentary; FS, ferula-sedentary; CE, control-exercised; FE, ferula-exercised). Different letters indicate significantly different values. $^{*}P < 0.05$, $^{**}P < 0.01$ (analysis of variance)

testosterone.[17] Another mechanism includes the presence of steroid-like compounds in these herbs that may act directly on androgenic receptors or mediate a feedback loop regulation.[18] A third possible mechanism is the presence of anticatabolic contents that operate by blocking cortisol receptors, although there is no evidence to support such a mechanism.

The two main components of ferula root that are speculated to elevate testosterone concentration are ferutinin and teferdin. These are phytoestrogen compounds that have the ability to bind to the estrogen receptors.[6,19] Zanoli *et al*.[20] reported that a single administration of ferutinin or teferdin was able to increase serum testosterone concentrations in male rats within a short period of time. The exact mechanism of how these phytoestrogen components act is still unclear. However, a recent study reported that phytoestrogens can stimulate the synthesis of testosterone in male goats by increasing the secretion of triiodothyronine (T_3).[21] This is a reasonable possibility given the well-established ability of T_3 to stimulate the steroidogenic activity of Leydig cells.[22–24] Moreover, it has been reported that T_3 deficiency was associated with a reduction in serum testosterone concentration, due to suppression of Leydig cell function.[25] Another possible mechanism is that those phytoestrogens, either directly or indirectly, can acutely stimulate the release of gonadotropins from the pituitary gland which, in turn, leads to testosterone secretion. In fact, Colman-Saizarbitoria *et al*.[26] reported the ability of ferutinin to stimulate the synthesis of nitric oxide within the median eminence of rat hypothalamus. Nitric oxide is a key intercellular messenger that is involved in the activation of the hypothalamus–pituitary–gonadal axis.[27]

The type of solvent used in the extraction procedure is considered a critical factor in this study. Different effects of *F. hermonis* on male rat sexual behavior were reported based on the type of extraction solvent used. While methanolic extract improved the sexual behavior in the male rat by increasing the number of mounts, the aqueous extract repressed this behavior in the same animal model by increasing the intromission latency.[28] In addition, administering an aqueous extract of ferula for six weeks resulted in a reduction in body weight gain and in the weight of testes in mice.[29] These weight reductions suggest an antiandrogenic action for the aqueous extract from *F. hermonis*. On the other hand, Zanoli *et al*.[3] reported an acute elevation in serum testosterone concentration in the rat following oral administration of the acetonic extract of ferula.

No potential side-effects on body organs are expected from the ferula extract treatment, since the dose given was 176 times less than LD_{50} (10.6 g/kg body weight).[30] An increase in liver weight was only reported in rats treated with a daily dose of 500 mg/kg for a period of 28 d.[30] Also, it must be pointed out that we avoided the repeated daily administration of the extract, since continuous treatment with *F. hermonis* acetonic extract may lead to potentially negative effects. Zanoli *et al*.[3] showed that repeated daily ingestion of *F. hermonis* acetonic root extract for 10 d resulted in a significant reduction of testosterone serum level in the male rat. This could be due to continuous exposure of estrogen receptors, on a daily basis, to the phytoestrogen compounds found in the extract.[31] In this study, testosterone concentrations were normal in FS rats. Thus, it is anticipated that no potential negative effects on fertility may occur in those animals due to extract treatment.

An increase in the total number of muscle nuclei, which include both myonuclei and satellite cell nuclei, was found in TA fibers of the FE group when compared with the other three groups. Satellite cells (SCs) are mononuclear myogenic stem cells that reside between the basal lamina and plasmalemma of the muscle fiber.[32] They are considered the main source of new myonuclei in mature skeletal muscle fibers.[33] The accretion in SCs and myonuclei is well substantiated in hypertrophied muscle fibers.[12,34] The accretion of myonuclei during hypertrophy is thought to be required to meet the greater demand for protein synthesis in muscle fibers.

In conclusion, this study demonstrated, for the first time, that short-term exercise combined with intermittent administration of *F. hermonis* acetonic root extract is more effective in enhancing the growth of skeletal muscle fibers when compared with exercise alone. The anticipated mechanism of action of the extract is via boosting serum testosterone concentration. This finding supports the idea that

Table 3 Influence of *Ferula hermonis* acetonic root extract and short-term exercise on the morphology of skeletal muscle fibers within tibialis anterior of adult male rat

Group	Fiber diameter (μm)	Number of nuclei per fiber cross-section	Length of nuclei (μm)	Number of nuclei per mm of fiber
CS	74.59 ± 1.80	2.3 ± 0.21	20.54 ± 3.5	74.2 ± 6.8
FS	81.52 ± 3.82	2.5 ± 0.15	20.19 ± 2.8	83.9 ± 5.0
CE	83.26 ± 2.56	2.5 ± 0.10	20.43 ± 3.6	82.2 ± 3.3
FE	93.76 ± 4.56*	3.1 ± 0.25*	20.11 ± 3.3	101.9 ± 8.4*

Values are representing the mean ± standard deviation for four different groups (CS, control-sedentary; FS, ferula-sedentary; CE, control-exercised; FE, ferula-exercised). $^{*}P < 0.05$, significantly differs from all other groups (analysis of variance)

F. hermonis root extract may be appropriate for inclusion in short-term rehabilitative programs. However, further research is needed to determine the effects of long-term treatment with *F. hermonis* extract on skeletal muscle.

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