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The modulatory effects of *Lonicera caerulea* L. extract and omega-3 on sarcopenia via regulating PI3K and FOXO signaling pathways in rats

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ABSTRACT

Introduction: Sarcopenia is an inflammatory disease caused by a disruption of muscle homeostasis. *Lonicera caerulea* L. (Haskap berry) (HB) extract and omega-3 (ω–3) possess antioxidant and anti-inflammatory activities. This study assesses the potential ameliorating effects of HB extract and ω–3 supplementation on dexamethasone (DEXA)-induced sarcopenia.

Methods: Rats were divided into five groups; the negative control group was injected with saline (i.p.); groups 2, 3, 4, and 5 were injected with DEXA (2 mg/kg/d, i.p.); groups 3 and 4 also received 400 mg/kg/d and 100 mg/kg/d of HB extract and ω–3, respectively, while group 5 received both treatments daily for 21 days. The ameliorative effects of treatments were investigated by measuring lysosomal proteolytic enzyme cathepsin activity, phosphatidylinositol 3 kinase (PI3K) activity, nuclear factor kappa beta (NF-κB) level, and heme oxygenase-1 (HO-1) activity. The gene expression levels of muscle ring-finger protein (MuRF) and forkhead box O (FOXO) transcription factor were also evaluated. Biochemical and histological examinations were done on muscle tissues.

Results: DEXA caused a significant elevation ($P<0.05$) in NF-κβ level and cathepsin activity in muscle tissue. Also, it significantly upregulated the muscle atrophy markers. Meanwhile, PI3K and HO-1 activities were significantly reduced. HB extract and ω–3 administration significantly ($P\leq0.05$) reversed these effects and downregulated the mRNA expression levels of MuRF and FOXO. Also, the histopathological examination confirmed these results.

Conclusion: The current study proved the ameliorative effects of HB extract and ω–3 on sarcopenia parameters. Therefore, they might be potential therapeutic agents for myopathy/sarcopenia.

Implication for health policy/practice/research/medical education:
Lonicera caerulea (haskap berry) extract and omega-3 reversed Dexamethasone-induced myopathies. Hence, they might be recommended to ameliorate sarcopenia by modulating muscle atrophy-related genes and oxidative stress.

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Introduction

Sarcopenia is a progressive loss of muscle mass, strength, and function syndrome (1). The pathophysiology of sarcopenia is multifactorial and classified as primary sarcopenia or age-related skeletal muscle loss and secondary sarcopenia caused by malnutrition, inflammation, sedentary lifestyle, endocrine imbalances, obesity, diabetes, cancer, and long-term use of glucocorticoids. Moreover, sarcopenia or muscle atrophy pathogenesis includes the imbalance of protein degradation and protein synthesis resulting in skeletal muscle mass loss. PI3K-Akt signaling pathway is one of the pathways that regulate muscle mass, regulating myogenesis (2,3). The loss of muscle mass is associated with the imbalance between ubiquitin-proteasome and lysosomal pathways (4). The imbalance between anabolism and catabolism leads to muscle atrophy. Muscle ring-finger protein (MuRF) and muscular atrophy F-box or Atrogin-1 are specific markers

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of muscle atrophy (5). In addition, MuRF and Atrogin-1 are regulated by the forkhead box O (FOXO) signaling pathway; activated FOXO can lead to muscle protein degradation by inducing the expression of MuRF and Atrogin-1 (6,7). Furthermore, FOXO is a key regulator of skeletal muscle mass through the ubiquitin/proteasome and autophagy/lysosome systems (4,8).

Oxidative stress and inflammation are crucial features of sarcopenia, thus highlighting the main targets for therapeutic interventions. Recent studies have revealed that reactive oxygen species (ROS) is involved in the development and progression of muscle atrophy by increasing the expression of muscle atrophy markers and activating the pro-inflammatory signaling pathways (2,9). Furthermore, glucocorticoids such as dexamethasone (DEXA) are widely used as anti-inflammatory agents for several diseases. One of the common side effects of long-term use of glucocorticoids is muscle atrophy (9,10). DEXA increases oxidative stress by activating the ubiquitin-proteasome system causing proteolysis and myopathy (11). Myostatin activation by DEXA further activates the expression of muscle degradation factors (MuRF and Atrogin-1), resulting in muscle atrophy (12).

In addition, no specific cure is available to protect and ameliorate sarcopenia. Nutritional supplementation is being investigated for future therapeutic intervention with minimal side effects and ease of compliance (13). Considering the therapeutic effectiveness of medicinal plants, they may be feasible alternatives for treating sarcopenia (10). Haskap berry (HB) (*Lonicera caerulea* L.), also known as sweet berry, honeysuckle blue, honeyberry, or honeysuckle, is native to Siberia, Japan, China & North America (14). Several studies have been carried out to characterize the phytoconstituents of HB and its impact on human health (15). It has been used in traditional medicine since ancient times to manage inflammation, fever, digestive, ophthalmological, and cardiovascular conditions (16,17). HB is rich in anthocyanins, such as cyanidin-3-O-β-glucoside (C3G), and other polyphenols with potent anti-inflammatory and antioxidant activities (18). Compared to other berries that are more widely consumed, including blackberries and strawberries, HB exhibits antioxidant activity that is three to five times higher. Furthermore, out of all the fruits, it has the highest level of ascorbic acid. In addition, in a previous study, HB extract enhanced muscular performance by lowering inflammation linked to mouse skeletal muscle tissue fatigue, increasing energy storage, reducing the buildup of toxic metabolites, modulating apoptosis, and stimulating cell proliferation (19).

Polyunsaturated fatty acids (PUFAs) such as omega-3 (ω−3) and Omega-6 (ω−6) fatty acids are widely used as beneficial food supplements, particularly for cardiovascular and central nervous system health (20). The ω−3 PUFAs such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and α-linolenic acid (ALA) regulate various cellular functions, such as signaling, gene expression, and decreasing the activity of ubiquitin-proteasome system (21,22). Thereby, this medicinal plant and omega-3 may be used as potential natural therapies for sarcopenia. Herein, this study aims to investigate the protective effect of HB extract and ω−3 supplementation against sarcopenia in rats and to clarify their possible underlying mechanisms of action. This study proposes that HB and ω−3 rich foods can be used as functional foods to alleviate DEXA-induced muscle loss.

Materials and Methods

Chemicals

DEXA ampules were obtained from Amriya Pharmaceutical Industries, Egypt. All chemicals used for HB extract preparation were purchased from Sigma-Aldrich Co, St. Louis, MO, USA.

Plant materials and extract preparation

Raw HB (*L. caerulea* L.) fruits of blueberry variety (Herbarium number 04719) were obtained from LaHave Natural Farms, Blockhouse, Nova Scotia, Canada. HB extract was prepared as described previously (23) with some modifications. Raw HB fruits were finely ground with a blender and mixed with deionized water containing 1% citric acid at 1:10 (w/v). After incubating at 60 °C for 3 hours in a water bath, the mixture was filtered using No. 2 filter paper and concentrated with a rotary vacuum evaporator at 60 °C. The HB extract was stored at -20 °C until further use.

Animals

Adult male Sprague-Dawley rats (weighing 200 ± 20 g) were obtained from the Faculty of Medicine, Ain Shams University. All experiments were conducted following the ethical guidelines for the care and use of experimental animals. The animal experiment was performed at the Faculty of Medicine, Ain Shams Research Institute-Animal Facility. Rats were kept individually in stainless cages in constant conditions at room temperature of 25 ± 2 °C with 12/12 hours light/dark cycles; they were allowed to access commercial rodent chow diet (EL-Nasr Chemical Company, Cairo, Egypt) and tap water *ad libitum*.

Animal grouping and experimental design

The rats were acclimatized for 1 week before the start of the experiment. The rats were randomly divided into five groups (n=6 per group) and treated once daily for 21 days. Group 1 (Normal control group) received normal saline intraperitoneally (The same as DEXA). Group 2 (Sarcopenic group) received DEXA (2 mg/kg/d) via intraperitoneal injection (24). Group 3 (HB group) received DEXA (2 mg/kg/d) via intraperitoneal injection and Haskap berry extract (HB) (400 mg/kg/d, orally (25). Group 4 (ω−3 group) received DEXA (2 mg/kg) daily via intraperitoneal injection and Omega-3 (ω−3) (100 mg/

kg/d, orally (22). Group 5 (Mix group) received DEXA (2 mg/kg) via intraperitoneal injection and HB extract + ω-3 orally in the same doses as groups 3 and 4.

Tissue sampling

At the end of the treatment protocol, rats were anesthetized with 1% pentobarbital sodium (50 mg/kg), intraperitoneally. The gastrocnemius muscle was then dissected and rinsed with saline. The first segment of the skeletal muscle was preserved in 10% formalin for histological examinations. Another segment of the skeletal muscle was stored at −80 ℃ for biochemical and molecular assays. Furthermore, gastrocnemius muscle tissue homogenate was prepared by homogenization in normal saline. The supernatant was separated by centrifugation and used for further analysis.

Assessment of lysosomal proteolytic enzyme cathepsin activity

Lysosomal proteolytic enzyme cathepsin activity was assayed in muscle tissue samples using rat Cathepsin K (CTSK) ELISA kit (MyBioSource – catalog number. MBS731525).

Assessment of phosphatidylinositol 3 kinase (PI3K) levels

PI3K activities were determined in muscle tissue samples using an ELISA kit (MyBioSource – catalog number: MBS260381).

Assessment of nuclear factor kappa B (NF-κB) levels

NF-κB levels were assayed in muscle tissue samples using an ELISA kit (Cloud-Clone Corp. – catalog number: SEB824Mu)

Assessment of heme oxygenase 1 (HO-1) activity

HO-1 activity was determined in muscle tissues using an ELISA kit (BioVision-catalog number. E4525).

Quantitative gene expression of MuRF and FOXO

Relative gene expression of MuRF and FOXO were evaluated by quantitative real-time PCR using the following primers (Table 1). The total RNA isolation kit was provided by Thermo Fisher Scientific Inc., Germany (GeneJET Kit, #K0732). The relative levels of mRNA expression for the target genes were determined using GAPDH as the reference/housekeeping gene. Relative gene expression levels were statistically analyzed using the $2^{-\Delta\Delta Ct}$ method (4).

Histopathological examinations

Gastrocnemius muscle samples were fixed with a 10% formalin solution and embedded in paraffin wax, then the samples were stained with hematoxylin and eosin (H&E) and examined under an Olympus Provis microscope at 50X magnification.

Statistical analysis

The statistical analysis was done using the Statistical Package for Social Science (SPSS) software program, version 21.0. Data were expressed as mean ± standard deviation (SD). Statistical differences between groups were performed using a one-way analysis of variance (ANOVA) at $P \leq 0.05$ significance level (26).

Results

Results presented in Figure 1A showed a significant elevation ($P \leq 0.05$) in lysosomal proteolytic enzyme cathepsin in the DEXA group compared with the healthy control group. Meanwhile, HB extract and omega-3 administration significantly decreased muscle cathepsin activity compared to the DEXA group. Moreover, results presented in Figures 1 (B & C) revealed that DEXA caused a significant decrease in PI3K activity and a significant elevation in NF-κB level in muscle tissue compared to the healthy control group ($P \leq 0.05$). Haskap extract and omega-3 administration caused a significant elevation in PI3K activity and a significant decrease in NF-κB level as compared to the DEXA group. The results of the Figure 1D demonstrated that DEXA caused a significant decrease ($P \leq 0.05$) in HO-1 activity as compared to the healthy control group. Compared to the DEXA control group, muscle activity of HO-1 was significantly increased ($P \leq 0.05$) in the HB extract and ω−3 administration groups thus reversing oxidative injury of muscle. Furthermore, results presented in Figures 1 (E & F) revealed that the muscle atrophy regulator MuRF and FOXO mRNA expression was significantly upregulated ($P \leq 0.05$) in the DEXA control group revealing muscle damage as compared to the healthy control group. Whereas HB extract and ω−3 administration, significantly ($P \leq 0.05$) downregulated MuRF and FOXO mRNA expression thus effectively counteracting the DEXA-induced upregulation.

Table 1. Genes primers sequences

Gene	Primers	
GAPDH	Forward sequence	5′ GTCCATGCCATCACTGCCACTC 3′
	Reverse sequence	5′ CGCCTGCTTCACCACCTTCTTG 3′
MuRF	Forward sequence	5′ GTTTGACGCCCTCTACGCCATC 3′
	Reverse sequence	5′ CTTGATCCTCCTCCTCCTCCTTC 3′
FOXO3	Forward sequence	5′ CCTTCCGTAAGCAAGCCGTGTAC 3′
	Reverse sequence	5′ CGCCTGCTTCACCACCTTCTTG 3′

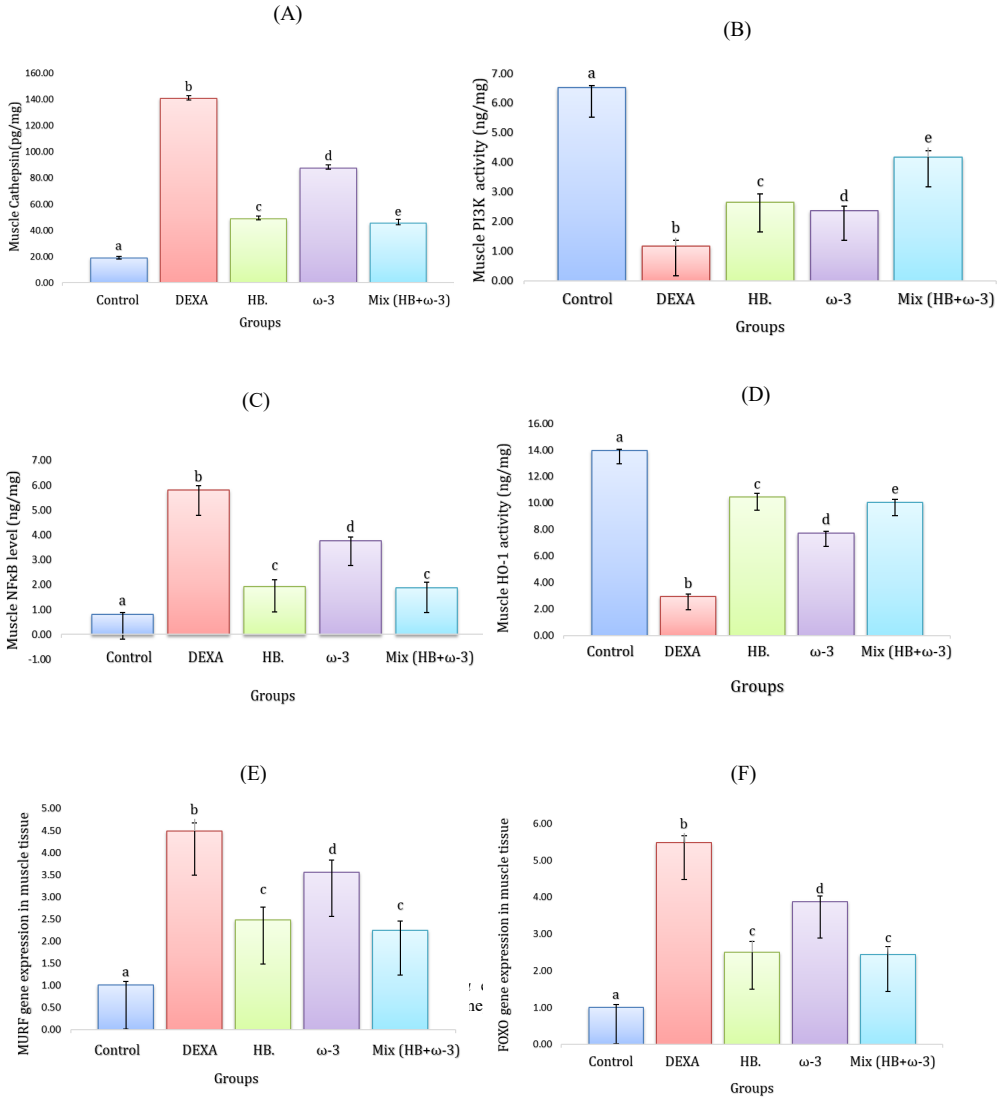

Figure 1. Ameliorative effect of *Lonicera caerulea* extract and omega-3 administrations on dexamethasone (DEXA)-induced muscle atrophy. HB; Haskap, ω-3; Omega-3 and MIX; Mixture. A: Cathepsin activity (pg/mg); B: Phosphatidylinositol 3 kinase (PI3K) activity (ng/mg); C: Nuclear factor kappa B (NF-κB) concentration (ng/mg); D: Heme oxygenase 1 (HO-1) activity in muscle tissue; E: Muscle ring-finger protein 1 (MuRF1) gene expression in muscle tissue; F: Forkhead box O (FOXO) gene expression. Data are expressed as mean ± SD. Different letters on columns (a, b, c, and d) indicate significant differences between groups at $P \leq 0.05$.

However, in comparing the treated groups with the positive control group (DEXA), a significant amelioration was observed in all parameters of the treated groups. HB and mix groups showed significant amelioration in MuRF, FOXO, and NF-κB as compared to the ω-3 group.

A visual examination of muscle tissue size showed that DEXA significantly reduced gastrocnemius muscle size as compared to those of the treated group as shown in Figure 2.

Microscopic examination of rat's muscle tissue showed that DEXA induced muscle injury. The administration of HB extract and omega-3 showed ameliorated the muscle damage caused by DEXA. Thus, the biochemical analysis results obtained were in line with the microscopic observations. Microscopic examination of the G1 group revealed normal skeletal muscle fibers that revealed multinucleated cells, and the nuclei were located peripherally within the cell (Figure 3A). Meanwhile, examination of the G2 group showed the atrophy of skeletal muscle fibers that were characterized by a reduction in myofiber diameter. The affected myofibers appeared rounded to angular with hyper-eosinophilic sarcoplasm, hyalinized, and fragmented myofibers. The examined muscle fibers also revealed increased numbers of plump reactive fibroblasts with prominent vesiculated nuclei, with separating and surrounding adjacent myofibers with collagen deposition (Figure 3B). Examination of the G3 group showed normal myofibers

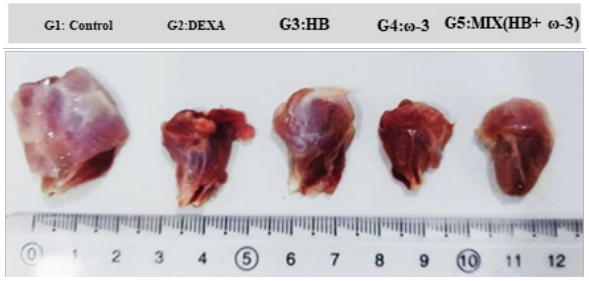

Figure 2. AEffect of *Lonicera caerulea* extract and omega-3 administrations on gastrocnemius muscle size. G1: Healthy control group (negative control group); G2: Dexamethasone (DEXA) injected group (Sarcopenic group); G3: Haskap berry (HB) extract group; G4: Omega-3 (ω-3) group; G5: MIX (HB+ ω-3) group.

in several examined sections. Some examined sections showed fewer hypertrophied myofibers with an increase in myofiber diameter (Figure 3C). In addition, congestion of interstitial blood vessels was observed in the G4 group with intact surrounding myofibers (Figure 3D). Normal myofibers were detected in almost all examined sections of the G5 group (Figure 3E).

Discussion

The present study objective was to examine the effect of *L. caerulea* (haskap berry) extract and Omega-3 supplementation on modulating sarcopenia induced by DEXA via regulating PI3K and FOXO signaling pathways.

Chronic or long-term consumption of glucocorticoids such as DEXA induce muscle atrophy via the activation of the ubiquitin-proteasome system (precisely, the expression of E3 ligases MuRF and atrogin-1) and autophagy of lysosomal system (such as lysosomal cathepsin). The PI3K/Akt pathway is an important signaling pathway for myogenesis and the maintenance of protein homeostasis. The inhibition of the PI3K-Akt signaling pathway activates the FOXO. Hence, the modulation of the PI3kt/FOXO pathway can be an effective therapeutic target to manage and prevent muscle atrophy (27).

Oxidative stress, inflammation, and apoptosis are involved in the pathogenesis of skeletal muscle damage. Medicinal plants are well-known for their antioxidant, anti-inflammatory, and anti-aging properties. Thus, they may exert a beneficial effect in protecting muscle integrity by affecting muscle regeneration and differentiation (28). Plant polyphenols have been widely studied for their diverse functions and rare side effects. Polyphenols have several biological functions including anti-aging, antioxidant, neuroprotective, hepatoprotective, cardioprotective, anti-cancer, and anti-diabetic. Several studies reported that the *Lonicera caerulea* berry or haskap berry is rich in polyphenols and bioactive components that provide antioxidant and anti-inflammatory effects (29).

Results of the present study showed that treatment with haskap berry extract and Omega-3 effectively ameliorated the DEXA-induced upregulation of FOXO and MuRF mRNA expression. DEXA induced muscle atrophy by inhibiting PI3KT/Akt phosphorylation, which stimulates FOXO transcription factors, activating ubiquitin ligase proteins, Atrogin-1, and MuRF (muscle atrophy signaling hallmarks). Haskap berry extract alone or in combination with Omega-3 effectively provided anti-sarcopenic effects by diminishing muscle protein degradation and inhibiting oxidative stress thus markedly increasing protein synthesis through the modulation of the PI3KT/FOXO pathway.

Atrogin-1 and MuRF or 'atrogens' are specific markers of skeletal muscle atrophy. Additionally, FOXO is a crucial

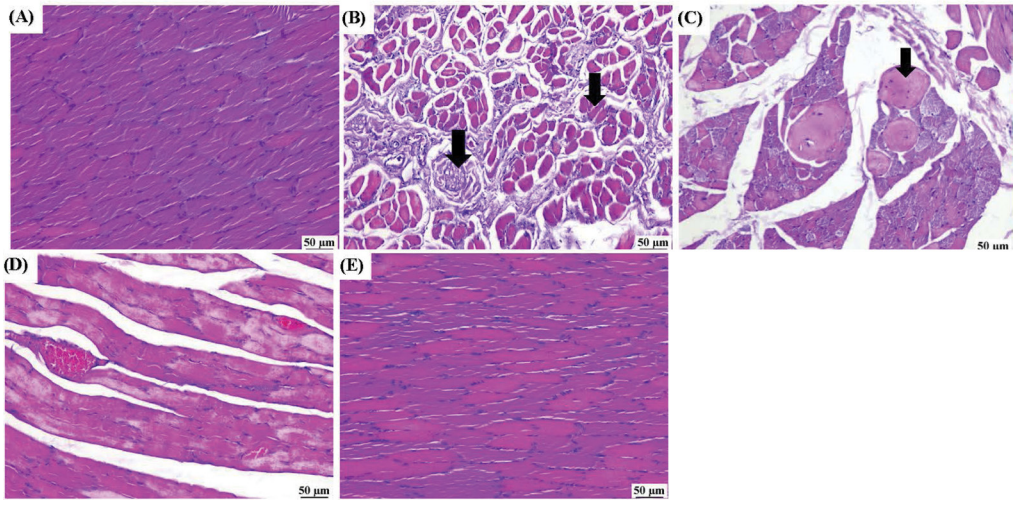

Figure 3. Microscopic examination of gastrocnemius muscle tissues (H&E). A: Healthy control group (negative control group; G1) showing normal skeletal muscle represented in a longitudinal section. B: Dexamethasone (DEXA) injected group (Sarcopenic group; G2) showing marked degeneration and atrophy of myofibers with perimysial deposits of collagen bundles. C: Haskap berry (HB) extract group (G3) showing fewer hypertrophied myofibers. D: Omega-3 (ω-3) group (G4) showing dilated endothelial-lined vessels filled with blood. E: Mixture (MIX). (HB+ ω-3) group (G5) showing dilated endothelial-lined vessels filled with blood and normal myofibers.

key player in skeletal muscle protein turnover. Moreover, protein degradation systems include the ubiquitin-proteasome system and autophagy-lysosome pathway with the participation of the FOXO transcription factor family. Thus, FOXO, being the most critical gene in muscle atrophy, independently controls the ubiquitin-proteasome system and autophagy-lysosome pathway by initiating the transcription of target genes (30).

Furthermore, omega-3 PUFAs, especially EPA and DHA are used for the prevention and treatment of several inflammatory disorders through inhibiting pro-inflammatory cytokine mediators. Also, omega-3 supplementation was reported to prevent obesity-induced muscle atrophy by up-regulation of AKT/mTOR pathway and down-regulation of atrogin-1 and MuRF through a negative regulation of FOXOs that is normally blocked by AKT activation (27).

In concordance with our results, muscle atrophy was suppressed by blocking the ubiquitin-proteasome pathway through suppressing FOXO. Meanwhile, FOXO activation leads to lysosomal degradation mediated by cathepsin L and atrogens (4). Another study regarding the possible mechanism of DEXA-induced sarcopenia suggested that it increased ROS production, stimulating muscle-specific E3 ubiquitin ligase, atrogin-1, MuRF, and lysosomal cathepsin L. The expression of the cathepsin family members is upregulated during various forms of skeletal muscle atrophy. Furthermore, increased ROS production results in mitochondrial dysfunction causing decreased expression of the respiratory chain and resting membrane potential. Also, it alters the expression of antioxidant genes and NF-κB activation (31).

The NF-κB family regulates many genes involved in several cellular processes, such as cell differentiation, proliferation, and apoptosis. Also, it is the main regulator of inflammatory cytokines-mediated muscle atrophy. Moreover, it was demonstrated that the increased NF-κB signaling upregulates inflammatory cytokine release and increases protein degradation by the ubiquitin-proteasome system with upregulation of MuRF and atrogin-1 expression (32), which is similar to our results. In addition, omega-3 PUFA supplements inhibit oxidative stress, inflammation, and apoptosis. Proposed mechanisms of action include the production of pro-resolving mediators, promoting anti-inflammatory activity (such as resolvins, protectins, and maresins), and inhibition of NF-κB activation thus, inhibiting muscle protein degradation via regulating MuRF expression (33). Similarly, EPA and DHA supplementation was reported to be involved in the recovery of muscle homeostasis. Omega-3 supplementation downregulated the markers of muscle atrophy the atrogin-1 and MuRF ligases involved in the ubiquitin-proteasome pathway through the activation of NF-κB (22). In addition, some studies have reported that cathepsin inhibition limits NF-κB activation (34); this observation interprets our results where DEXA reduced HO-1 level which inhibited NF-κB activation, while treatment inhibited the NF-κB activity and increased HO-1 levels (32).

A study reported that the anti-sarcopenic effect of haskap berry extract was due to its bioactive components, mainly anthocyanins such as cyanidin-3-O-glucoside, flavanols, and phenolic acids (25). Results of this study showed that the haskap berry extract ameliorated muscle atrophy by downregulating the expression of MuRF and atrogin-1 by upregulating protein synthesis-related genes. Also, HB extract increased the mRNA expression levels of antioxidant genes such as HO-1 which interpret our results.

The pharmacological effect of *L. caerulea* extract is due to the synergistic activity of all phenolic compounds present in it, besides vitamin C. Anthocyanins are the predominant compounds found in *L. caerulea* fruits; it also contains several phenolic acids (chlorogenic, neo-chlorogenic acids, ferulic acid and caffeic acids), flavonoids (quercetin), apigenin, catechin, and epicatechin. Furthermore, iridoids (a group of monoterpenoids) such as loganic acid are the main bioactive components in Haskap berry extract (19). Several studies have reported that the antioxidant effect of haskap berries is much higher (three to five times higher) than that of blueberries, blackberries, bearberries, chokeberries, blueberries, lingonberries, and raspberries (35).

It was reported that the administration of haskap berry extracts reduced exercise fatigue-associated inflammation, and oxidative stress by modulating apoptosis, enhancing mitochondrial biosynthesis, and stimulating cell proliferation, thus enhancing muscle performance (29). Moreover, the biochemical data of the present study are consistent with the histological findings and similar to several studies that reported the damaging effect of DEXA on muscle fibers. Lastly, the results of the present study highlighted the role of omega-3 and haskap in ameliorating sarcopenia by suppressing the ubiquitin-proteasome system activated by DEXA. Moreover, the best modulation was observed by administering a mixture of (ω-3 & HB) in some parameters while the haskap group showed better amelioration than other treated groups. Thus, administering haskap extract alone or with omega-3 might be beneficial as an anti-sarcopenic candidate therapy to prevent muscle atrophy.

Conclusion

In this study, DEXA-induced muscle loss due to the activation of ubiquitin-proteasome and lysosomal pathways. HB extract and ω−3 possessed a protective effect against DEXA-induced muscle atrophy in rats, possibly through blocking the FOXO/MuRf pathway associated with catabolic protein degradation. So, HB and ω−3 supplementation may be considered a promising

therapeutic agent for DEXA-induced muscle atrophy. Future studies are needed to investigate the therapeutic effects of HB and ω−3 supplementation in human, as well as on the other types of muscle atrophy.

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Authors' contribution

Conceptualization: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
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Formal analysis: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
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Methodology: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Resources: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Software: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Supervision: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Validation: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Visualization: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Writing–original draft: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany
Writing–review & editing: Radwa Wahid Mohamed and Nourhan Gamal El-Rahmany

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The study was examined and approved by the research ethics committee, Ain Shams University, Faculty of Medicine Ain Shams Research Institute- Animal Facility (MASRI- Animal Facility) with ethical code: SCI1432404001 assigned on April 30, 2024.

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