MORFAI JOURNAL Multidisciplinary Output Research For Actual and International Issue
INDEX COPERNICUS INTERNATIONAL
Multidiciplinary Output Research For Actual and International Issue (MORFAI Journal) ISSN (e): 2808-6635
Volumes 6 No. 5 (2026)

PROTECTIVE EFFECTS *OF HIBISCUS SABDARIFFA* LINN EXTRACT. PROTECTIVE EFFECTS OF HIBISCUS SABDARIFFA LINN. EXTRACT ON OXIDATIVE STRESS AND COLLAGEN DEPOSITION IN D-GALACTOSE-INDUCED SKELETAL MUSCLE AGING IN RATS

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Received : 01 June 2026 Accepted : 19 June 2026
Revised : 05 June 2026 Published : 08 July 2026

Abstract

Background: The aging process in skeletal muscle is often triggered by chronic oxidative stress that impacts extracellular matrix remodeling in the form of fibrosis and decreased motor function. *Hibiscus sabdariffa* Linn. (HSL) extract is known to be rich in polyphenol and anthocyanin compounds that have the potential as antioxidant agents. **Objective:** This study aims to analyze the potential of HSL intervention in regulating gastrocnemius muscle aging in an old rat model accelerated with D-galactose, reviewed from molecular, histopathological and functional aspects. **Methods:** A total of 18 rats were divided into three groups (n=6): Young Normal Group (NM), D-galactose aging acceleration Group (Ap), and D-galactose + HSL Group (ApH). Aging induction was carried out using D-galactose at a dose of 200 mg/KgBW, daily for 8 weeks orally via tube. HSL supplementation was mixed in drinking water at a dose of 400 mg/KgBW daily for 8 weeks. The parameters tested included serum *Malondialdehyde (MDA) levels, MMP-9 levels in muscle tissue, limb muscle grip strength, and the percentage of collagen deposition in histopathology preparations.* **Results:** MDA levels and MMP-9 enzyme activity in the Ap group were higher than the NM group ($P < 0.001$). The percentage of *gastrocnemius muscle tissue collagen* in the Ap group was lower than the NM group ($P < 0.05$). No differences were found in muscle grip strength between the Ap group and the NM group. MDA and MMP-9 levels in the ApH group given HSL supplementation were not significantly different from the NM group ($P > 0.05$). The percentage of *gastrocnemius muscle tissue collagen* in the ApH group was also not significantly different from the NM group ($P > 0.05$). **Conclusion:** Supplementation of HSL extract in the accelerated aging group was able to suppress MDA levels, reduce MMP-9 levels, and maintain the percentage of gastrocnemius muscle tissue collagen. *Hibiscus sabdariffa* Linn. extract proven to be effective in inhibiting the aging process of gastrocnemius muscle through the mechanism of lipid peroxidation chain cleavage and prevention of fibrotic remodeling, which contributes to the maintenance of the mechanical functional capacity of the muscle.

Keywords: *Hibiscus sabdariffa* Linn., D-galactose, Malondialdehyde, MMP-9, Collagen Percentage, muscle grip strength,

INTRODUCTION

The Global Burden of Disease 2021 (GBD 2021) data confirms that the demographic transition of an aging population is a global public health challenge. [1]A key manifestation of this challenge is the increasing burden of *disability-adjusted life years* (DALYs), which is associated with the increasing prevalence of degenerative diseases due to the aging process.[2] One of the organ systems affected is the musculoskeletal system, which is directly linked to disability in older adults. [3]One of the conditions of age-related musculoskeletal decline is sarcopenia.[4] Therefore, research to explore mechanisms that can inhibit aging-related muscle degeneration is very important. The muscle aging process is influenced by various factors such as decreased physical activity, hormonal disorders, oxidative stress, and chronic inflammation. [5]As the body ages, the capacity of endogenous antioxidant systems such as *Superoxide Dismutase* (SOD), *Catalase* (CAT), and *Glutathione Peroxidase* (GSH-px) decreases, leading to an increase in the accumulation of *Malondialdehyde* (MDA) and *Reactive Oxygen Species* (ROS) within cells. [6]Excess ROS triggers oxidative stress, which is a condition of imbalance between free radical production and the body's ability to neutralize them. [7]In the musculoskeletal system, the degeneration

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process is characterized by changes in the structure and decreased function of cells and the extracellular matrix that make up muscle tissue. One of the changes that occurs is fibrosis in the muscle, which is an excessive accumulation of collagen fibers, especially types I and III, in the extracellular matrix (ECM) surrounding muscle fibers.[8] Fibrosis causes decreased muscle elasticity and impaired transmission of contractile force, ultimately reducing strength and movement efficiency. [8]Microscopically, fibrosis appears as a disproportionate thickening of the ECM structure, often accompanied by an increase in the number of myofibroblasts. These myofibroblasts are the result of fibroblast differentiation under the influence of *TGF-β1 Transforming Growth Factor-Beta 1* (TGF-β1), released during inflammation, and play an active role in collagen production. [8]Under normal conditions, collagen plays a role in maintaining the elasticity of muscle fibers, but with aging, unbalanced collagen synthesis can actually cause problems.[7] High collagen production is not balanced by proper regulation of its structure and quantity, resulting in excessive collagen accumulation in the ECM. This accumulation causes muscle fibrosis, which is the transformation of contractile muscle tissue into rigid, inelastic connective tissue. [7]Aging directly affects the balance between collagen synthesis and degradation in muscle tissue. Under normal conditions, collagen homeostasis is maintained through the activity of synthesis by fibroblasts and degradation by *Matrix Metalloproteinases* (MMPs). With aging, the expression of MMPs, especially MMP-9, actually increases. This enzyme plays a role in the degradation of collagen and other ECM proteins, and its excessive activation accelerates the breakdown of collagen that has already been formed. This causes the collagen that has already accumulated in aging muscles to become increasingly unstable and susceptible to damage.[9] Current research is developing the potential of natural ingredients as safe and effective anti-aging agents.[10] *Hibiscus Sabdariffa Linn. (HSL)* Ethanol Extract contains flavonoids and quercetin, the main bioactive components of which act as anti-inflammatories that can inhibit aging processes such as hypertension, hyperlipidemia, cancer, and other inflammatory diseases of the liver and kidneys.[11] To date, there has been no research on the effects of HSL ethanol extract on degenerative changes in muscle tissue in experimental animal models of accelerated aging. Aging research presents its own challenges. In vivo studies using mice are time-consuming and expensive. Therefore, an accelerated aging model that can mimic natural aging is needed. One method for creating an accelerated aging model is through D-galactose induction.[12] Administration of D-Galactose causes an increase in ROS which then triggers oxidative stress, inflammation, mitochondrial dysfunction, and apoptosis which can accelerate the aging process.[12 13 13]

METHODS

This study is an in vivo experimental study using *Sprague Dawley rats* aged 14 weeks with a body weight of 200-300 grams . The number of experimental animals in this study uses the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines, namely 18 rats. Exclusion criteria: Rats were sick, injured, or died during the study. Rats were randomly divided into three groups, namely: (1) Young normal group (NM) which was terminated at the age of 17 weeks. (2) Aging acceleration group (Ap). Aging acceleration was carried out by D-Galactose induction which was given daily for 8 weeks orally through a tube at a dose of 200 mg/KgBW. (3) Aging acceleration group (ApH). In this group, along with oral D-galactose administration, Hibiscus Sabdariffa Linn supplementation was added at a dose of 400 mg/KgBW daily for 8 weeks orally by mixing it with drinking water.
The parameters measured in this study include:

1. **Serum MDA Level Test**
   malondialdehyde (MDA) levels in serum using the modified *Thiobarbituric Acid Reactive Substances (TBARS) method by Buege and Aust (1978). The preparation of a standard curve (0 to 1.2 nmol) was carried out through gradual dilution of 6000 nmol MDA stock to a final concentration of 0.015 nmol in the third tube, then distributed into six series of tubes with a volume variation of 0-80* µL) and topped up with H_2O to 2000 µL. In sample processing, 200 µL of serum was diluted with 1800 µL of H_2O and then vortexed . Each standard and sample tube was then added with 1 ml of 20% TCA and 2 ml of 0.67% TBA, vortexed *,* then heated together at 100 ° C for 10 minutes. After reaching room temperature and vortexing *again* , all tubes were covered with parafilm and centrifuged at 3000 rpm for 10 minutes. The absorbance value of the supernatant formed was measured using a Spectrophotometer at a wavelength of 530 nm with a 1 cm cuvette, then the MDA concentration of the sample was determined based on the linear regression equation of the standard curve.[13]
2. **MMP-9 Level Test in Gastrocnemius Muscle Tissue Using ELISA**
   Matrix Metalloproteinase-9 (MMP)-9 levels in mouse gastrocnemius muscle tissue were determined using the Mouse MMP-9 ELISA Kit (*CLG4B ELISA Kit* , FineTest) according to the manufacturer's protocol. Gastrocnemius muscle pieces (50-100 mg) were washed and homogenized in cold PBS, then centrifuged at 5,000 × g for 5 minutes

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to obtain the supernatant. 100 µL of standard or sample solution was added to each well, the plate was covered, and static incubation was carried out for 90 minutes at 37°C. Washing: The plate was washed twice without soaking. 100 µL of standard or sample solution was added . µL of biotin-antibody working solution, the plate was covered, and static incubation was performed for 60 minutes at 37°C. Washing: The plate was washed three times and soaked for 1 minute each time. 100 µL of *HRP-Streptavidin Conjugate* (SABC) working solution was added, the plate was covered, and static incubation was performed for 30 minutes at 37°C. Washing: The plate was washed five times and soaked for 1 minute each time. 90 µL of TMB substrate solution was added, then the plate was covered, and static incubation was performed for 10-20 minutes at 37°C. (Accurate visualization control of TMB is required.). 50 µL of stop solution was added and absorbance was read at 450 nm to determine MMP-9 levels based on a standard curve.

3. **Collagen Percentage Test Using Masson Trichrome Staining**

The preparation of histological preparations begins with gradual dehydration of post-fixation muscle tissue (10% NBF) using graded ethanol (70% to absolute), followed by clearing *in* xylol 2-3 times, each 30 minutes). The tissue is then infiltrated in liquid paraffin at a temperature of ±60 ° C 2-3 times, each 1-2 hours), then (*embedding*) in a mold, then cut into 4-6 µm thick using a microtome. Before staining, the preparation is deparaffinized on *a hot plate* (± 60 ° C, 15-30 minutes), immersed in two series of xylol (each 5 minutes), and rehydrated through graded alcohol (100% to 70%) and distilled water. For *Masson's Trichrome* staining , the tissue was re-fixed in Bouin's solution (56 ° C, 1 hour), then sequentially stained using *Weigert's Iron Hematoxylin* (10 minutes) for the cell nucleus, *Biebrich Scarlet–Acid Fuchsin* (10-15 minutes) for the cytoplasm, *Phosphomolybdic/Phosphotungstic Acid* (10 minutes) for differentiation, and *Aniline Blue* (5-10 minutes) to stain collagen. Then, the preparation was fixed with 1% acetic acid (2 minutes), rinsed with distilled water, and covered with a coverslip. The percentage of collagen in the muscle tissue was analyzed and documented using an *inverted fluorescence microscope* .[14] For data analysis, the percentage of collagen in the muscle fiber area of each sample was calculated using high-quality image files (TIFF/PNG), then the Plugins > Color Deconvolution feature was run by selecting the Masson Trichrome vector to separate the color components into three new windows. Next, the Color_2 window (collagen component) was selected, then the segmentation threshold was set via the Image > Adjust > Threshold menu until the colored area accurately only covers the collagen tissue. Finally, open the Analyze > Set Measurements settings, ensure *the Area Fraction* and *Limit to Threshold options* are checked, then click Analyze > Measure to get the collagen percentage value in the % Area column.[15]

4. **Muscle Grip Strength Test**

Grip strength measurements of both hind legs were performed simultaneously. The net pull rod was held with the other hand and placed on the measuring frame at a 30° angle, tilted backward from the operator. Only the front legs were placed on the closer end of the net. The rat and the net pull rod were adjusted simultaneously and slowly until the rat could place its hind legs on the horizontal bar of the frame. The rat's body was ensured to remain upright with both legs attached to the rod. This technique was avoided if only one leg was attached. A slight force was applied and the hind legs were observed to ensure the rat was firmly gripping the rod. The rat was pulled horizontally from the measuring device until its hind legs were released from the rod. The net pull rod was moved at the same speed and direction as the hand holding the rat. The reading on the measuring device was recorded. The reading was reset to zero before being repeated. Three consecutive trials were conducted and the average value was taken. [16]Muscle grip strength tests were performed three times: before the intervention (D-Gal and HSL), in the fourth week, and in the eighth week before termination.

5. **Statistical Analysis**

Statistical analysis was performed using Graphpad Prism 8.0. The normality of data distribution was tested using the Shapiro-Wilk test. Homogeneity of variance was tested using the Levene test. Data with normal distribution and homogeneous variance were then tested using one-way ANOVA or two-way ANOVA. Data that were not normally distributed and had heterogeneous variance were tested using the non-parametric Kruskal-Wallis test. All results are presented as mean ± standard deviation or median (minimum - maximum). A p-value <0.05 is considered statistically significant.

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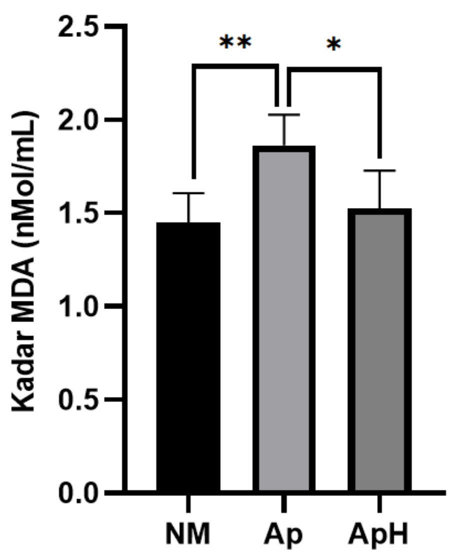
RESULTS

Malondialdehyde level measurement Serum MDA was measured after termination at week 8 as an indicator of oxidative stress and cellular lipid peroxidation levels. According to the research data (Table 1, Graph 1), serum MDA levels in the Ap group (1.86 ± 0.164 nMol/mL) were significantly higher than those in the NM group (1.45 ± 0.16 nMol/mL). ; p = 0.0023. These results prove that the accumulation of D-galactose produces excessive [17]*Reactive Oxygen Species (ROS), thus causing lipid peroxidation and MDA production, a typical pathological condition commonly found in animal models of sarcopenia.* These free radicals that are not compensated by the body's defense system will attack unsaturated fatty acids in the muscle cell membrane (sarcolemma), initiating a chain of lipid peroxidation, and releasing chronic pro-inflammatory cytokines that accelerate muscle fiber atrophy while triggering the accumulation of interstitial fibrotic collagen matrix.[18]

Figure 1. Serum MDA levels



Information:
*p < 0.05
**p < 0.01
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

Table 1. Results of serum MDA level measurements

	Group		
	NM nMol/mL	**Ap** nMol/mL	**ApH** nMol/mL
MDA levels	1.45 ± 0.16	1.86 ± 0.164	1.53 ± 0.20

Data are displayed as Mean ± SD

NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleratio In contrast, serum MDA levels in the ApH group (1.53 ± 0.20 nMol/mL) were not significantly different from the NM group; p = 0.7380. This demonstrates the ability of HSL to suppress lipid peroxidation end products and demonstrates the presence of antioxidant protective activity. The bioactive compounds polyphenols and anthocyanins in HSL likely stabilize radical compounds before they can damage cell membranes. [19]These results strengthen the hypothesis that the protective effect of HSL in maintaining tissue integrity and inhibiting the aging process is through inhibition of oxidative stress.[20]

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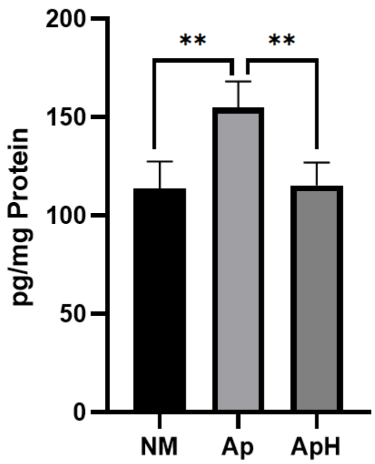
Figure 2. MMP-9 Levels in Muscle Tissue



Information:
*p < 0.05
**p < 0.01
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

Table 2. Results of measuring MMP-9 levels in muscle tissue

	Group		
	NM pg/mg Protein	**Ap** pg/mg Protein	**ApH** pg/mg Protein
MMP-9 Enzyme Activity	113.5 ± 13.89	154.7 ± 13.39	115.3 ± 11.64

Information:
Data are displayed as Mean ± SD
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

Referring to research data (Table 2, Graph 2), the activity level of *the Matrix Metalloproteinase-9* (MMP-9) enzyme in *gastrocnemius muscle tissue* in the Ap group (154.7 ± 13.39 pg/mg Protein) was significantly higher than in the NM group (113.5 ± 13.89 pg/mg Protein) . These results indicate that D-galactose administration creates excessive ROS conditions and triggers oxidative stress. [21]As a result, there is increased local inflammation in aging muscle tissue. This inflammation, in addition to accelerating muscle protein degradation, also affects collagen-producing cells such as fibroblasts and myofibroblasts. Fibroblasts are activated by inflammatory signals, especially types I and III, in response to tissue damage. [22]In aging, unbalanced collagen synthesis actually causes problems. This accumulation causes muscle fibrosis, which is the replacement of contractile muscle tissue with stiff and inelastic connective tissue. [23]On the other hand, ROS also damages the structure of collagen that has been formed through oxidation and fragmentation processes. Oxidized collagen tends to lose its tensile strength and elasticity and is more susceptible to degradation by proteolytic enzymes such as MMPs. Therefore, despite increased collagen production in aging muscle, the quality and strength of the tissue decline due to a combination of oxidative stress and chronic inflammation.[24] In contrast, MMP-9 levels in the ApH group (115.3 ± 11.64 pg/mg Protein) was not significantly different compared to the NM group; p = 0.9707. This proves that HSL administration suppresses MMP-9 levels in the mouse model of accelerated aging. This therapeutic effect is thought to be influenced by the content of phytochemical compounds in *Hibiscus sabdariffa* Linn. extract, such as polyphenols and anthocyanins, which have high antioxidants to neutralize cellular free radicals. The structural impact of

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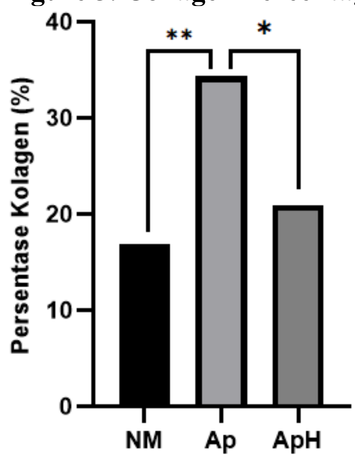
HSL extract in controlling MMP-9 activity affects the structural protection of *gastrocnemius muscle myocyte fibers* . Controlled MMP-9 activity prevents excessive proteolysis of the main components of the skeletal muscle extracellular matrix (ECM), especially type IV collagen in the basal lamina. The ECM layer not only optimizes the efficiency of lateral muscle contractile force transmission but also ensures the stability *of the satellite cell niche* or muscle stem cell micro niche required for cellular repair and regeneration processes.[25]

Figure 3. Collagen Percentage



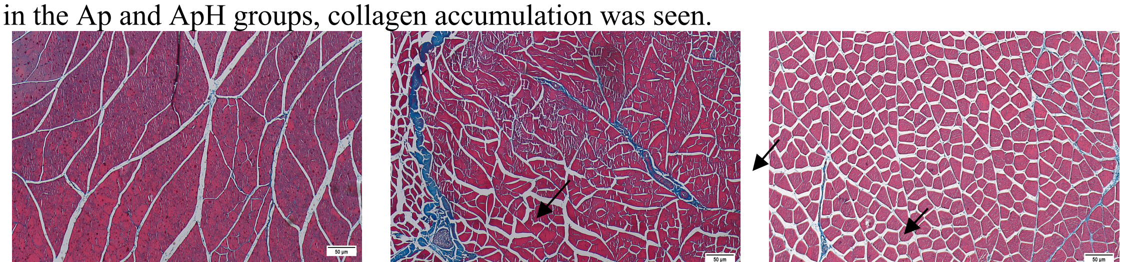
* $p < 0.05$
** $p < 0.01$
Data is displayed in Median
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

Table 3. Results of collagen percentage measurements

	Group		
	NM	**Ap**	**ApH**
Collagen Percentage	15.84 (15.93-24.82)	34.34 (30.21-40.58)	20.91 (20.47-22.78)

* $p < 0.05$
Data is displayed in Median (Minimum – Maximum)
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

Data analysis was continued on muscle fibers, in the form of calculating the average percentage of collagen in 5 microscopic cross-sectional fields of muscle fibers for each sample with *Masson Trichrome staining* . Morphologically, there were differences in muscle fibers in the NM group, the Ap group, and the ApH group (Figure 4). The morphology of collagen distribution (in blue) in muscle tissue in the NM group appeared even and there was no collagen accumulation. Meanwhile, in the Ap and ApH groups, collagen accumulation was seen.



(a) (b) (c)

Figure 4. Comparison of the results of *Masson Trichrome histological examination* of gastrocnemius muscle tissue.

(a)NM: Young Normal Group; (b) Ap: D-galactose acceleration model; (c) ApH: D-galactose+HSL acceleration (scale bar 100µm)

There were striking structural differences in collagen deposition between the three groups. In the NM group (Figure 1a), muscle fibers appeared neatly arranged, dense, and uniform, with a thin blue distribution of interstitial connective tissue (both endomysium and perimysium). This condition differed from the Ap group (Figure 1b). A clear morphological difference was seen in the form of a smaller muscle fiber diameter, which was exacerbated by massive collagen

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accumulation in the intercellular space. This accumulation of extracellular matrix or fibrosis is strong evidence of chronic oxidative stress due to D-galactose induction, which in turn disrupts the regulation of *MMPs* (MMP) enzymes and triggers pro-fibrotic pathways. [26]Microscopic changes in the aging-accelerated muscle were supported by the results of quantitative analysis of the percentage of collagen area in the Ap group (34.34 ; 30.21-40.58) which was significantly higher than the NM group (16.84; 15.93-24.82) with p = 0.0029 . Interestingly, there was no significant difference between the ApH group (20.91; 20.47-22.78) and the NM group; p = 0.0002. This proves that supplementation with *Hibiscus sabdariffa* Linn. ethanol extract is able to inhibit structural damage. Visually, muscle fiber density in the ApH group appeared to recover and approach its normal condition, followed by reduced collagen deposition when compared to the Ap group. This protective effect indicates that the bioactive compounds in HSL, especially anthocyanins and polyphenols as antioxidants, effectively dampen and reduce free radicals due to D-galactose toxicity. [27]The protective ability of HSL in reducing interstitial fibrosis is in line with several previous studies that reported that HSL polyphenol extract is able to repair cell organelle damage while suppressing extracellular matrix deposition.[28] As shown in Based on the results of the muscle strength test, in the first week before termination for the NM group and given D-gal treatment in the Ap group and oral administration of D-gal + HSL mixed with drinking water for the ApH group showed that before being given D-gal and D-gal + HSL treatment, the difference in muscle strength in the three groups was not too far (Table 3).

Figure 5. Muscle Grip Strength

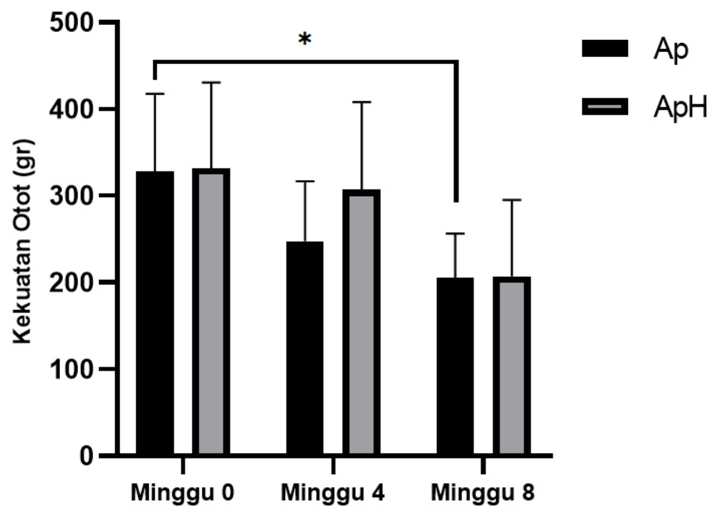

Table 4. Muscle Grip Strength

Measurement Time	Group		
	NM grams	**Ap** grams	**ApH** grams
Week 0 (Before Treatment)	317.78 ± 100.7	328.33 ± 89.21	331.94 ± 98.50
Week 4	-	247.61 ± 69.42	307.39 ± 100.76
Week 8 (Before Termination)	-	205.08 ± 51.38	207.39 ± 87.70

*$p < 0.05$
Data are displayed as Mean ± SD
NM: Young Normal Group; Ap: D-galactose acceleration model; ApH: D-galactose+HSL acceleration

The results of the statistical test using *Two-Way Repeated Measures* (RM) ANOVA showed that there was no significant interaction between the time factor and the treatment group (p = 0.6674). There was also no difference between the groups in the mean muscle strength. There was a decrease in muscle strength in all 3 groups with increasing age, however, a significant decrease only occurred in the Ap group, namely between Week 0 and Week 8 (p = 0.0097). This indicates the role of accelerated aging by D-galactose in causing a decline in musculoskeletal function that resembles sarcopenia. Although in Week 4 the ApH group showed a higher average muscle strength compared to the Ap group, in general there was no significant difference (p = 0.4097). Thus, it can be concluded that the administration of *Hibiscus sabdariffa* Linn. extract at this dose or study duration has not been able to provide a statistically significant protective effect in preventing

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the rate of decline in functional muscle strength due to D-galactose-induced accelerated aging.

Molecularly, the chronic accumulation of free radicals due to D-galactose induces abnormalities and dysfunction of the mitochondrial complex in skeletal muscle, which directly triggers a decrease in ATP production and disrupts the aerobic metabolism of muscle cells. [29]This prolonged energy deficit and oxidative stress damages myocyte integrity, triggers muscle fiber atrophy, and triggers pathological extracellular matrix remodeling through thick and massive collagen accumulation (fibrosis) in the endomysium and perimysium areas, as visualized in *Masson's Trichrome staining* of the Ap group (Figure 1b).[30] In contrast, in the group receiving *Hibiscus sabdariffa* Linn. (ApH) extract intervention, the rate of decline in muscle mechanical functional performance could be better restrained. This protective effect was seen in the 4th week observation, which showed that the grip strength value of the ApH group was relatively the same as week 0. The ability of HSL to maintain contractile function and delay the onset of muscle weakness is related to differences in serum MDA levels and proves that the many exogenous antioxidant compounds in HSL such as polyphenols and anthocyanins work effectively to cut the lipid peroxidation chain in the sarcolemma and protect the muscle macromolecular growth signaling pathway from overtraining stress and oxidative aging stress. [20]By attenuating intracellular oxidative stress by HSL, interstitial connective tissue homeostasis is restored, which is microscopically indicated by the return of gastrocnemius muscle fiber density and a massive reduction in pathological collagen deposition (Figure 1c).

CONCLUSION

This study shows that supplementation with ethanol extract *of Hibiscus sabdariffa* Linn. (HSL) has therapeutic potential in inhibiting the degeneration process of gastrocnemius muscle in a mouse model of accelerated aging at the molecular, histopathological, and functional levels. At the molecular level, the activity of HSL bioactive compounds has been shown to be effective in reducing oxidative stress by cutting the lipid peroxidation chain in the sarcolemma, which is indicated by a decrease in *Malondialdehyde* (MDA) levels. This decrease in intracellular oxidative stress has positive implications for the morphological defense of tissue structures, as demonstrated by the activity of the MMP-9 enzyme and the accumulation of pathological collagen (fibrosis). Although there was no evidence of a difference in grip strength between groups, HSL supplementation has been shown to have the potential to inhibit the decline in grip strength.

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