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Crocin Mitigates Age Related Sarcopenia in Rats By Increased Mitochondrial Activity and Up-Regulation of Nrf2/HO-1 Pathway

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ABSTRACT

Background: Sarcopenia has always been associated with aging and the elderly. It is thought that crocin has anti-inflammatory and antioxidant qualities.

Objective: We looked at Crocin impact on age related sarcopenia and its potential underlying molecular processes.

Material and Methods: Thirty adult male rats were split into three groups: aged, control, and aged+crocin. In addition to muscle TNF-α, muscle IL-6, serum MIF, serum IGF-1, serum CK and muscle MDA assessment, rats were also assessed regarding EMG recordings, muscle citrate synthase, muscle GSH, and muscle Nrf-2, HO-1, and myogenin gene expression. The muscle was also evaluated histopathologically.

Results: While the aged group's muscle TNF-α, muscle IL-6, serum MIF, serum CK, and muscle MDA were dramatically higher than the control, the aged group's measured mean frequency, mean power, muscle citrate synthase, serum IGF-1, muscle GSH, muscle Nrf-2, HO-1, and myogenin gene expression were significantly lower. Crocin significantly reduced age-related alterations.

Conclusion: Through anti-oxidant, anti-inflammatory, and enhanced mitochondrial activity methods, as well as through up-regulating the expression of the Nrf-2, HO-1, and myogenin genes, crocin improved age-related sarcopenia.

Keywords: Crocin, Nrf-2, HO-1, Ageing, Sarcopenia, CK, IGF-1, Myogenin.

INTRODUCTION

Sarcopenia is the natural aging-related loss of muscle mass, contractility, innervation, strength, and quality (fiber type composition) along with decreasing vascularity [(1)].

Previously associated with aging and the elderly, sarcopenia is now known to start earlier in life due to a variety of reasons that go beyond age. It is believed that between 10 and 27 percent of those over 60 have sarcopenia, which emphasizes the significance of early intervention techniques. Under some circumstances, younger adults may also be impacted. People usually lose 20% of their cross-sectional area (CSA) and 30% of their muscle mass between the ages of 20 and 80 [(2)].

The apoptotic activity of myofibrils, a decrease in α-motor neurons, hormonal imbalance (a decrease in anabolic hormones), an increase in proinflammatory cytokines, biological changes, altered mitochondrial function, elevated oxidative stress, and other factors like energy deficiency can all contribute to sarcopenia, which has a complicated pathophysiology. Oxidative stress (OS) is one of the main indicators of sarcopenia that have been postulated. Because excessive mitochondrial radical generation raises reactive oxygen species (ROS) levels in muscle cells, OS may be the main cause of sarcopenia [(3)].

With the recent global aging trend, the prevalence rate of sarcopenia is rising quickly, with an annual increase of almost 0.8% in the population over 50.

Because it causes metabolic disturbances, increases the chance of impairments, and lowers life quality, it is considered a serious disorder in geriatric medicine [(4)].

More effective therapy and preventative measures may be developed as a result of the correlation between blood biomarkers, lifestyle variables, and the advancement of sarcopenia in older persons [(5)]. Furthermore, chronic inflammation, which is typified by disrupted acute phase proteins and systemic cytokines, is connected to both muscle mass loss and aging-related "inflammaging" [(6)]. In aging, systemic markers of inflammation may be employed as clinical indicators of skeletal muscle mass and strength [(7)].

The pathogenesis of sarcopenia has been attributed to hormone imbalance, chronic inflammation, oxidative stress, hereditary factors, and inadequate nutritional status [(8)]. The well-known antioxidant-related protein Nrf-2/HO-1 is crucial for controlling anti-cell-programmed cell death and inflammation as well as reducing oxidative stress [(9)]. Muscle complaints can be classified as originating largely from either the muscle or the nerve using neurophysiologic testing by EMG, which involves needle electrode examination of spontaneous muscle electrical activity. The distribution, intensity, and course of myopathic alterations are all shown by EMG [(10)].

Crocus sativus L. (C. sativus L.) has drawn attention as a possible source of pharmacologically

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bioactive chemicals (safranal and crocin), which are thought to have anticancer, anti-inflammatory, and antioxidant qualities [11]. They do, in fact, have the ability to scavenge free radicals and alter the creation of antioxidant enzymes, which may reduce the formation of ROS. Therefore, C. sativus L.'s abundance of these physiologically active metabolites offers various clinically demonstrated health benefits, including protection against oxidizing agents [8].

Therefore, it has been difficult to identify novel therapeutic strategies to slow down age-myopathy. Crocin's protective properties were assessed in this study using aged rats. We also looked at the Nrf2/HO-1 pathway and its potential underlying molecular processes.

MATERIALS AND METHODS

Animals:

Thirty mature male Wistar albino rats (10 in each group) weighing between 240 and 290 g were used in this study. Rats had unrestricted access to food and water throughout the study. Exposing them to the normal cycles of light and dark, humidity, and room temperature. Rats were conditioned for two weeks prior to the study's start.

Rats were divided into three groups:

Control group: Adult rats (12 weeks of age) administered 1 ml normal saline i.p. for 12 weeks.

Aged group: Aged rats (age 18–22 months) administered 1 ml normal saline i.p. for 12 weeks [12].

Crocin-treated Aged group (Aged+Crocin): Rats that were 18–22 months old were given 100 mg/kg of crocin intraperitoneally [13]. At the required concentrations, crocin was generated in 0.5% normal saline. A 99.9% pure powdered crocin was purchased from the Tokyo Chemical Industry in Toshima, Tokyo, Japan (C1527). Rats were anesthetized for electromyography (EMG) recording at the conclusion of the 12-week experiment.

Measurements of electromyography:

The Biopack MP100 acquisition system, Inc., Goleta, California, United States, was used to conduct EMG recordings in vivo in order to identify electrical anomalies in muscle. To assess EMG activity, two thin concentric needle electrodes were placed into the gastrocnemius muscle of rats that had been anesthetized with thiopental sodium (50 mg/kg, i.p., as needed) [14].

One unshielded electrode (ground electrode) was inserted into the rat tail, and two shielded electrodes (LEAD110S/EL503 or EL508S) were employed for the signal inputs via a percutaneous puncture in the gastrocnemius muscle. After the electrodes were inserted, the EMG activity was regularly checked for three to four minutes. Rats that had fully recovered from anesthesia were immediately returned to their enclosures after the test. Using integrated EMG characteristics that included the mean power, frequency, and duration of electrical activity, a qualitative assessment of myotonic-like activity was obtained. Rats were given 100 mg/ml of ketamine to induce heart puncture anesthesia at the conclusion of the 12-week trial. Reduce to 50 mg/ml. Fill A sterile multidose vial was filled with 1 milliliter of ketamine (100 mg/ml) that was drawn into a sterile syringe. 1 milliliter of pyrogen-free sterile water or sterile 0.9% NaCl was added to this.

Collection of blood samples:

A heart puncture was used to draw five milliliters of blood. Each rat's blood was placed in a plain, sterile tube and allowed to coagulate at room temperature for about half an hour. Samples were centrifuged for 15 minutes. The samples were stored for biochemical analysis at -80 °C.

Biochemical analysis:

My BioSource (San Diego, CA, USA) ELISA kits were used to measure the levels of TNF-α and IL-6 in muscle homogenate. Muscle homogenate citrate synthase activity was assayed by ELISA kits (CS Activity Assay (Catalog No. E-BC-K178-M, Elabscience, Shizishan Ave, Hongshan, China). by Minneapolis, Minnesota, USA-based R&D Systems, Inc. ELISA kits, serum levels of insulin-like growth factor 1 (IGF-1) and the inhibitor of macrophage migration (MIF) were measured. Serum levels of creatine kinase (CK) were measured using ELISA kits from Abbexa Ltd. (Cambridge, UK) (Cat. No. abx157210). Muscle levels of GSH and MDA were spectrophotometrically determined using commercial kits supplied by Bio diagnostic, Egypt.

Quantitative RT-PCR (qRT-PCR):

In order to extract RNA and perform the Nrf2, HO-1, and myogenin gene assays, a single sample of fresh skeletal muscle tissue was taken from each rat and placed in a falcon tube and kept at -80 °C. A 7500 real-time PCR machine (Applied Biosystems, CA, United States) was used to identify Nrf2, HO-1, and myogenin. Using a direct-zol RNA miniprep kit (Cat. No. R2051; Zymo Research, USA), RNA was extracted from muscle cells. Next, the QuantiTect Reverse Transcription Kit (205311; Qiagen, Applied Biosystems, USA) was used to synthesize complementary deoxyribonucleic acid (DNA), and finally, the second PCR step (the real-time PCR step) was performed.

The following primers were used for the Nrf-2 gene:
(1) Forward primer: 5- GGTTGCCCACATTCCCAAATC-3
(2) Reverse primer: 5- CAAGTGACTGAAACGTAGCCG-3

The following primers were used for the HO-1 gene:
(1) Forward primer: 5-AGGTGCACATCCGTGCAGAG-3
(2) Reverse primer: 5-CTTCCAGGGCCGTATAGATATGGTA-3

The following primers were used for the myogenin gene:

(1) Forward primer: GAGCCCCACTTCTATGACGG
(2) Reverse primer: GTTGAGCAGGGTGCTTCTCT

The forward and reverse primers for actin were 5′-GAC GGC CAG GTC ATC ACT AT-3′ and 5′-CTT CTG CAT CCT GTC AGC AA -3′, which served as an endogenous control. Ten microliters of SYBR Green (2× QuantiTect PCR Master Mix), three microliters of cDNA, one microliter of forward primer, one microliter of reverse primer, and five milliliters of RNase-free water were used in each PCR reaction, which was carried out in a final volume of 20 microliters. Denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 31 s were the next 55 cycles. The data were processed using the Applied Biosystems 7500 software version 2.0.1. Gene expression was measured relative to one another using the comparative Ct technique. The plot of amplification and melting curve of the Nrf2, HO-1 and myogenin genes.

Histopathological methods

For H&E, the chosen muscle tissues were preserved for 24 hours in 10% formaldehyde. Following the standard procedure, the samples were embedded in paraffin, divided into sections that were stained with H&E.

Ethical approval:

The Guide for the Care and Use of Laboratory Animals was followed in the execution of all experimental procedures and methodology. The experimental procedure was approved by the Menoufia University Faculty of Medicine Ethics Committee with approval code 3/2025PHYS18.

Statistical analysis

Following data collection and analysis, they were found to satisfy the parametric assumptions based on the results of the Shapiro-Wilk test. As a result, one-way ANOVA and post hoc Bonferroni's tests were applied to the data. The data were displayed using the mean ± SD. Significance was considered to exist when the p value was 0.05 or less. The data were analyzed using Graph-Pad Prism software (version 9.3.1, San Diego, CA, USA).

RESULTS

The measured aged group's muscle TNF-α, muscle IL-6, serum MIF, serum CK and muscle MDA were dramatically elevated than the control, but their mean frequency, mean power, muscle citrate synthase, serum IGF-1, muscle GSH, muscle Nrf-2, HO-1, and myogenin gene expression were significantly lower.

When compared to the Aged group, the Aged+Crocin group's measured mean frequency, mean power, muscle citrate synthase, serum IGF-1, muscle GSH, muscle Nrf2, HO-1, and myogenin gene expression were dramatically elevated; however, the Aged group's measured muscle TNF-α, muscle IL-6, serum MIF, serum CK and muscle MDA were significantly lower (Table 1).

Table (1): The measured mean frequency, mean power, muscle TNF-α, muscle IL-6, muscle citrate synthase, serum MIF, serum IGF-1, muscle MDA, muscle GSH, serum CK and muscle Nrf2, HO-1 and myogenin genes expression in all studied groups.

	Control group	Aged group	Aged+Crocin group
Mean frequency (mv/sec)	521.9±9.18	321.19±7.2 *	420.8±6.33 *#
Mean power (mv)	9.22±0.32	2.99±0.21 *	5.86±0.20 *#
Muscle TNF-α (ng/ml)	18.9±1.19	39.6±0.9 *	31.2±1.47 *#
Muscle IL-6 (ng/ml)	60.3±3.1	99.8±2.3 *	61.9±3.4 *#
Muscle Citrate Synthase (U/L)	16.9±0.35	7.6±0.22 *	11.9±0.99 *#
MIF (ng/ml)	18.9±1.09	39.6±1.18 *	31.8±1.12 *#
IGF-1 (ng/ml)	150.8±3.1	92.3±3.12 *	123.87±3.89 *#
Muscle MDA (nmol/ gm Tissue)	6.33 ±0.9	28.2± 1.93*	16.8± 0.9*#
Muscle GSH (U/gm Tissue)	4.9±0.03	1.02±0.09*	2.91±0.11*#
Serum CK (ng/ml)	11.2±0.33	32.08±1.09*	20.9±1.11*#
Muscle Nrf-2 gene expression	1	0.33±0.06*	0.71±0.11*#
Muscle HO-1 gene expression	1	0.41±0.03*	0.75±0.06*#
Muscle Myogenin gene expression	1	0.31±0.02*	0.61±0.02*#

Data are presented as mean + standard deviation, * Significant compared with control, # Significant compared with Aged Group.

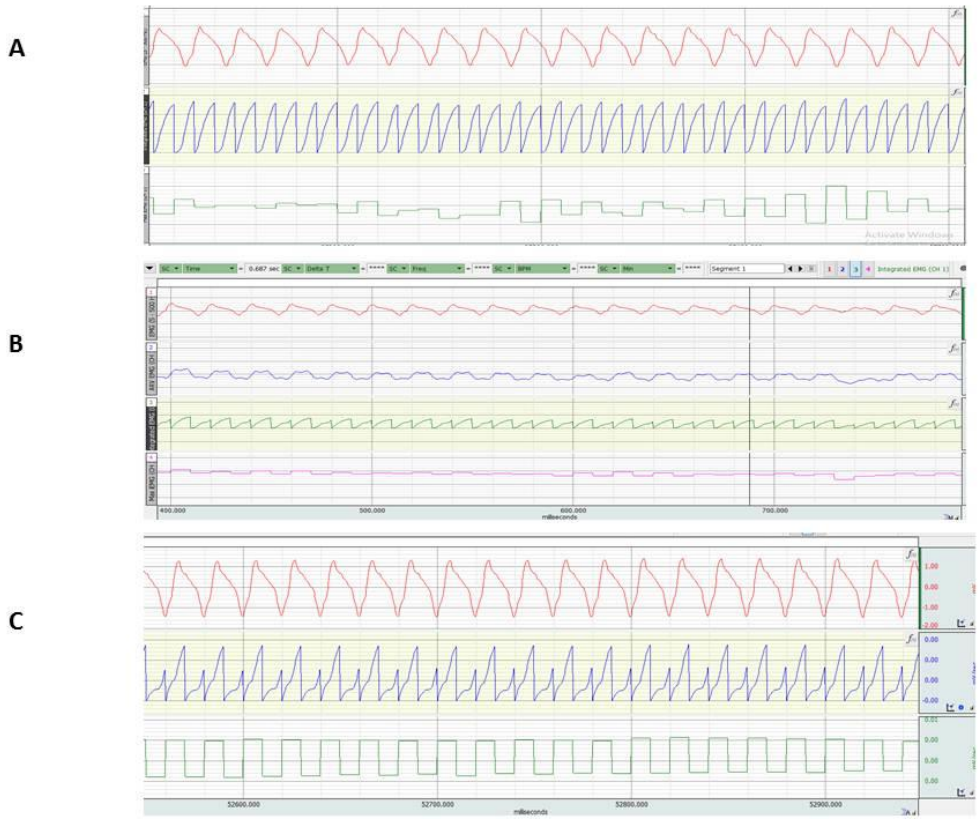

Fig. (1): EMG record in all studied groups A) Control, B) Aged and C) Aged+Crocin.

Histopathological evaluation:

Normal muscle was seen in the control group. The skeletal muscle's histological architecture was normal in the elderly group. Pathological alterations, including persistent inflammation, fibrous tissue, and fat accumulated between muscle fibers, were observed in the elderly group. When coupled with crocin, the elderly group's skeletal muscle improved with less fibrosis and pathological alterations (Fig. 2).

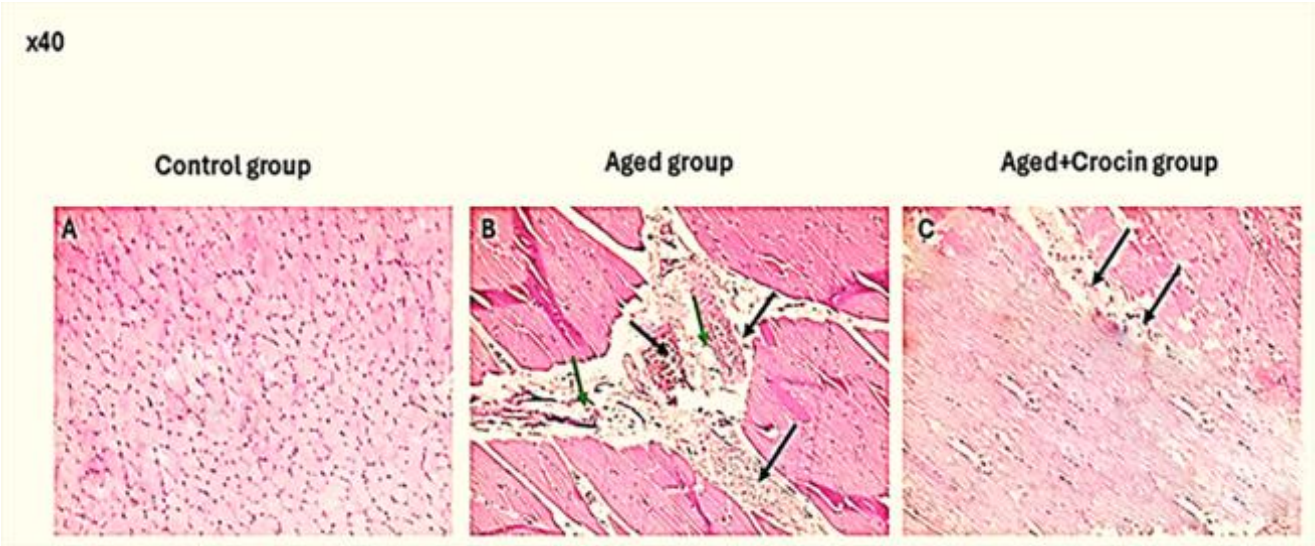

Fig. (2): H&E-stained sections (x40): (A) the control group showed a normal histological architecture of the skeletal muscle. (B) Aged group showed fibrous tissue (blue arrow) together with chronic inflammation (black arrows) and adipose tissue in between the muscle fibers (green arrows). (c) Aged+Corcin group showed improvement of the muscle tissue but there was still fibrosis found in between the muscle fibers (black arrows).

DISCUSSION

To the best of our knowledge, few researches have been done on the connections between crocin and sarcopenia in terms of antioxidant anti-inflammatory action. Most frequently, IL-6, and TNFα are used to examine the relationship between inflammation and muscle mass and strength. Sarcopenia is regarded as a handicap that increases the healthcare system's socioeconomic cost. As a result, an animal model was employed in this work to investigate a potential therapeutic strategy to postpone its effects. The mean power and frequency of the elderly non-treated group in this study were dramatically lower than those of the control. This concurred with **Gilmartin *et al.*** [15].

Skeletal muscle proteins undergo balanced, ongoing breakdown and resynthesis under normal circumstances. However, as people age, their skeletal muscle cells degrade and oxidative stress levels rise, upsetting this equilibrium. Thus, oxidative stress, inflammation, endocrine alterations, inactivity, and undernutrition are all implicated in the complex pathophysiology of sarcopenia [16].

Additionally, aging lowers functional and regenerative capacity as well as myogenic regulatory factors (Myogenin, MyoD, and Myf5), according to **Plate *et al.*** [17]. The skeletal muscles of embryos, fetuses, and adults only express myogenin in myogenic tissues. Skeletal muscle contraction power decreased as a result of myogenin depletion with aging. Crocin's anti-inflammatory and antioxidant properties are responsible for the elderly treated group's markedly increased muscle power and frequency on EMG recordings [18,12].

The elevation of myogenic factors in comparison to their baseline levels was previously maintained by Crocin therapy. Therefore, the low level of ROS generation in cells pretreated with crocin may be associated to these results by activating the PI3K/Akt/mTORC1 pathway [12].

Regarding inflammation, the elderly group's levels of muscle IL-6 and TNF-α were noticeably greater than those of the control group. This was consistent with a prior study [19] that found sarcopenia to be linked to chronic low-grade inflammation. Long-term exposure to IL-6 has been demonstrated to promote muscle atrophy by suppressing muscle anabolism and energy balance. IL-6 is another well-known myokine that is produced by skeletal muscle and improves muscular glucose metabolism [20].

Decreased muscle mass and power, as well as poorer physical performance, can result from elevated IL-6 levels. Additionally, it has been determined that serum IL-6 may be a biomarker for sarcopenia [21].

Our study's findings, which are consistent with earlier research[22], suggest that a crocin-based age control strategy may reduce inflammation.

When compared to controls, the older group showed significantly higher muscle MDA and significantly lower GSH levels in terms of oxidative stress. This concurred with Chuang and colleagues [8].

The crucial roles that chronic inflammation, OS, and mitochondrial dysfunction play in muscle atrophy have been highlighted by recent research. Apoptosis, a major process causing a substantial loss of muscle mass, is triggered when these factors interact and perhaps converge on several intracellular signaling pathways, upsetting the balance between protein production and breakdown [1].

Serum creatinine and creatine kinase levels may increase due to sarcopenia. It can be used to differentiate sarcopenia from other degenerative diseases of the muscles [23]. Crocin aged group showed decreased skeletal oxidative stress markers when compared to adults. This goes hand by hand with previous study [24].

According to **Bua *et al.*** [25] crocin decreased MMP-1 production and IL-1β expression, possibly by inhibiting the NF-κB pathway. Our findings are supported by the possibility that crocin reduces tissue oxidative degradation.

Since mitochondrial citrate synthase is a unique indicator of the mitochondrial matrix and the pace-making enzyme in the Krebs cycle, muscle citrate synthetase was considerably lower in the old group of rats than in the adult group. The presence of undamaged mitochondria is indicated by the activity and quantity of citrate synthase [26].

A biomarker for mitochondrial malfunction that may affect the skeletal muscle system's energy requirements is citrate synthetase. Therefore, some of the main characteristics of age-related sarcopenia may be caused by oxidative stress in conjunction with anomalies seen in the mitochondrial DNA or electron transport chain functioning [27].

In this investigation, compared to the aged group working with a prior study, crocin consumption dramatically reduced mitochondrial dysfunction [28].

One well-known modulator of muscle development and regeneration is IGF1, also known as somatomedin C [29]. Phosphatidylinositol-3-kinase (PI3K) is activated by IGF-1 signaling, which causes muscle hypertrophy by promoting protein synthesis pathways and inhibiting muscle atrophy pathways. It also causes human myotube hypertrophy by hastening the recruitment of reserve cells. This was consistent with the study **Goldspink** [29], which showed that the elderly group's IGF1 levels were dramatically lower than those of the control. Furthermore, human skeletal muscle contains IGF-1.

MIF is a pro-inflammatory cytokine that has an impact on glucose homeostasis and muscle damage. This outcome was consistent with the current study's findings. The elderly rats' MIF significantly increased in comparison to the control group. One essential organ for using glucose is the skeletal muscle. The MIF levels may reflect the metabolic state of sarcopenia [30].

Crocin's anti-inflammatory and antioxidant properties significantly reduced the MIF level.

Compared to adult control animals, we discovered that aged rats exhibited greater serum levels of CK. **Prajapati *et al.*** [31] found that the loss of muscle fibers caused an increase in CK. Numerous variables, including as aging-related ROS-induced damage and a drop in antioxidant enzyme levels, contribute to mitochondrial malfunction and damage. Thankfully, crocin helped to improve this [32].

Age-related downregulation of Nrf-2/HO-1 gene mRNA levels was observed. All things considered, this study showed that the crocin-aged treated group exhibited antioxidant qualities due to an upregulation of Nrf-2 and HO-1 gene mRNA levels. The findings of **Yaribeygi *et al.*** [33] who found a significant increase in H_2O_2-induced senescent cells with aging that was counteracted by the antioxidant qualities of crocin, were in agreement with this. By encouraging the transcription of many cytodefensive factors, such as heme oxygenase-1 (HO-1), Nrf-2 effectively prevents tissue damage [34].

The pathogenesis of sarcopenia includes hormone imbalance, chronic inflammation, oxidative stress, hereditary factors, and inadequate nutritional status [8].

Numerous studies have demonstrated that frequent consumption of herbal bioactive ingredients enhanced quality of life, inflammation, and oxidative stress. Consumption of herbal components such as curcumin and crocin has been linked in animal studies to increased muscle mass, resistance to exhaustion, improved muscle force and strength production, and suppression of muscle atrophy. These preclinical investigations have shown a number of molecular pathways, including the antioxidant-related Nrf-2/HO-1 genes. A stress-responsive protein, HO-1 reacts to several forms of oxidative stress that are created chemically or biologically [35].

The expression of Nrf-2/HO-1 genes is clearly impacted by C. sativus L. stigmas extracts, which have recently been demonstrated to be the most effective antioxidant in lowering oxidative stress in several cell lines. This is regarded as a useful instrument for evaluating the possible cytoprotective and oxidative stress impacts of substances on muscle metabolism [32].

Overall, this research shows that medicinal plants like crocin can effectively treat sarcopenia without causing any negative side effects. This is due to their molecular, antioxidant, mitigating, mitochondrial, and anti-inflammatory mechanisms of action, which improve the physiological function of the muscular system.

CONCLUSION

Through its anti-oxidant, anti-inflammatory, and mitochondrial dysfunction-reduction properties, as well as its up-regulation of the Nrf-2 and HO-1 pathways and muscle myogenin, crocin helped to alleviate age-related sarcopenia.

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