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<https://ejournal.upi.edu/index.php/penjas/article/view/56199>DOI: <https://doi.org/10.17509/jpjo.v8i1.56199>**The Effect of Moringa leaf extract supplementation on IL-6 protein expression in endurance-training-induced inflammation of Wistar rat muscles****Afianti Sulastri^{1*}, Natasya Natasya¹, Muhammad Naufal Abdurahman², Pipit Pitriani³, Hamidie Ronald Daniel Ray⁴**¹Department of Nursing, Faculty of Sport and Health Education, Universitas Pendidikan Indonesia, Indonesia²Department of Sport Education, School of Postgraduate Program, Universitas Pendidikan Indonesia, Indonesia³Sport Coaching Study Program, Faculty of Sport and Health Education, Universitas Pendidikan Indonesia, Indonesia⁴Sport Science Study Program, Faculty of Sport and Health Education, Universitas Pendidikan Indonesia, Indonesia**Article Info***Article History :**Received March 2023**Revised March 2023**Accepted March 2023**Available online April 2023**Keywords :**anti-inflammatory, endurance training, IL-6, moringa oleifera***Abstract**

Overtraining can cause muscle inflammation. Untreated inflammation might cause permanent damage. Traumas can create dysfunction physically and functionally. One study demonstrated that moringa contained a high content of micro- and macronutrients, such as protein, energy, and vitamins, and had several therapeutic effects. This study aimed to evaluate the effects of moringa in inflammation reduction, due to endurance training (eTR), on skeletal muscle. The sample consisted of 12 adults male Wistar rats separated into three groups, including two exercise groups and one non-exercise group. One of the exercise groups was given moringa leaf extract (eTMO) at a dose of 0.02 mg/kg/day of body weight, which was suspended in 0.5% Na-CMC, taken orally for 28 days. The exercise rat groups were trained on a treadmill for 30 minutes every day for 28 days. Western blotting (WB) was utilized for the detection of IL-6 protein expression. These findings demonstrated that the treatment using *M. oleifera* leaf extract combined with endurance exercise reduced the expression of the protein IL-6 subunit in the gastrocnemius (Gas) muscles. This data revealed that the combination of *Moringa oleifera* and exercise could potentially lower IL-6 levels in skeletal muscle.

INTRODUCTION

To reach the goals, athletes must do exercises so that they can do the best performance during competitions. However, if the training program is not designed well, it can have harmful effects on the athletes. A continuous or excessive training can cause inflammatory disorders in the muscles. If not properly treated, the inflammation might worsen and result in persistent damage. Not only physiologically, trauma can also functionally cause dysfunction in the body. Meanwhile, fitness and physical conditions are valuable assets for athletes and have a significant impact on their careers. Overtraining results in a mismatch between physical training and recovery time. Excessive physical training can have a negative impact on homeostasis in the body, ultimately affecting the organ functioning systems (AlBasher et al., 2020a). During an intense physical activity, the high metabolic rate, shortage of oxygen, and rise of lactic acid will trigger the generation of free radicals that are integrated into the reactive oxygen species (ROS) (Khajehlandi et al., 2021).

Moringa oleifera is a tropical plant that produces bioactive chemicals with antioxidant and anti-inflammatory properties (Ma et al., 2020). We hypothesized that the *Moringa oleifera* (MO) leaves have bioactive chemicals that work as antioxidants and anti-inflammatory agents by lowering the levels of IL-2, IL-6, and TNF-, which are important pathways in the process of inflammation. The doses of *Moringa oleifera* were chosen based on the number of calories in a meal (3.37–3.44 kcal/g). The rise in antioxidant enzymes and the downregulation of IL-2, IL-6, and TNF- may explain how MO reduces inflammation by turning on the Nrf2/Keap1 complex and turning off NF- κ B (Sugiharto et al., 2022). Sailaja et al., (2022) state that moringin (MIC-1), one of the active ingredients in MO, affects inflammation and immunity-related genes in myoblasts and skeletal muscle tissue. Using *in vivo* models, the anti-inflammatory mechanisms of MIC-1 had also been explored. Sailaja previously demonstrated that daily oral supplementation with MIC-1 (80 mg/kg bw) for three days protected mice from LPS-induced systemic inflammation.

Under certain conditions, physical activity can be a stressor that causes muscle damage or injury caused by local inflammation. It causes the muscles to break down and grow back around the connective tissue

(Theret et al., 2021). In order to restore homeostasis, the inflammatory response must go through a number of carefully controlled stages, and cytokines are one of the most important parts that coordinate and boost different parts of inflammation. These molecules, such as IL-1b, TNF-alpha, and IL-6, or IL-6, IL-10, IL-4, and IL-5, can either make inflammation worse or stop it. Suzdaltseva et al. (2022) say that among these molecules, IL-6 and tNF- deserve special attention because they cause and stop inflammation, respectively. The antioxidant defense system primarily protects against cellular damage and lipid peroxidation (AlBasher et al., 2020b). IL-6 is an important cytokine that acts mainly on the differentiation of CD4+ T-cells, stimulating the Th17 pathway while inhibiting regulatory-T-cells (T-reg) differentiation. Th17 lymphocytes produce cytokines such as TNF, IL-1, IL-17, IL-21, and IL-22, which stimulate inflammation and the fibrotic reaction of the tissue. IL-6, on the other hand, inhibits the production of T-reg cells through transforming growth factor (TGF). In the right conditions, IL-6 is an autoinflammatory mediator, reducing the capacity of the body to distinguish self from not self and promoting fibrosis and the inflammatory reaction. It has been demonstrated and targeted in many autoimmune diseases (Pandolfi et al., 2020). This study aimed to assess the effect of *Moringa* supplementation on inflammatory conditions in an overtrained Wistar rat muscle injury. MO is a source of antioxidants, which when combined with bioactive chemicals, antioxidant, and anti-inflammatory properties, has an effect in reducing inflammation. Abdel-Daim et al., (2020) say that the antioxidant defense system is the main defense against cellular damage and lipid peroxidation. Presently, research on the association between *Moringa oleifera* consumption and muscle soreness is limited. For that reason, this research is required to add to the information around the use of organic substances for athlete endurance, particularly in overcoming exercise-related injuries.

METHODS

This study applied a true experimental design with a random posttest-only control group. PT. Biofarma provided a sample of 12 Wistar rats, aged 8 weeks, to be employed in this investigation (Bandung). They were placed with their backs to the light in an air-conditioned room with a 12-hour light/12-hour dark

cycle. The rats were given a meal of commercial standard rodent pellets and ad libitum water. All animals were treated humanely according to The National Academy of Science's Guide for the Care and Use of Laboratory Animals, 8th Edition (2011). Animals were randomly divided into three groups: 1) the non-exercise group with placebo (Na-CMC 0.5%) ($n = 4$) as the sedentary control group; 2) the exercise group with placebo ($n = 4$) named eT group; and 3) the exercise group treated with 0.02 mg/kg/day of MO suspension in CMC-Na 0.5%, named the eTMO group. All animals administered treatment orally an hour before the training program started for 28 days. On the harvest muscle day, the rats were anesthetized using 5% inhaled isoflurane until one minute after the breathing stopped. Harvest time was done immediately after the last training sessions in each group. The IL-6 protein expression was determined through western blotting method which was run based on our laboratory methods previously (Hamidie et al., 2021) at Central Laboratorium of Padjadjaran University.

Endurance training protocol

All rats were acclimated to the environment for two weeks and continued with habituation on the treadmill for two weeks. The treadmill was programmed for moderate-intensity, 30-minute-per-day workouts during the training sessions. Each exercise was divided into three 10-minute sets separated by five-minute intervals of rest. Training in endurance was undertaken five days a week for a total of 28 days. All training sessions were performed during the light cycle between 8 to 12 AM.

Western blotting analyses

The rats received anesthesia by administering 50 mg of pentobarbital for every 100 mg of body weight following the end of the treatment period. Thereafter, the gastrocnemius muscle was promptly removed and separated to examine protein expression. The tissue was cleansed from other tissues, then washed again and stored in cold NaCl solution before being promptly immersed in liquid nitrogen. Generally, 40 mg gastrocnemius muscle was homogenized in Buffer A made from 250 mM sucrose, 10 mM NaCl, 3 mM MgCl₂, 1 mmol/l dithiothreitol (DTT), and placed on 500 μ l of ice-cold for 30 seconds. After that, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 μ l protease inhibitor cocktail were added on Buffer A. Centrifuged was used at 500 g at 4°C in order to get supernatant on the

homogenized procedure. This condition was referred to as a full fraction. Furthermore, the remaining pellet was resuspended in 500 μ l of ice-cold buffer B containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 50 mM MgCl₂, 10% glycerol, 1% Triton X-100, and 1 mM PMSF. After that, 2 μ l tissue protease inhibitor cocktail was added on that buffer. For ten minutes, the buffer was incubated under cold conditions, with mixing occurring every minute. The resuspended pellet obtained from this process was spun at 3100 g at 4°C for 5–6 minutes in a centrifuge (Hamidie et al., 2021).

Analyses utilizing WB were conducted as previously mentioned. In a summary, 12.5% SDS-PAGE gel was used to load equivalent protein levels of materials, including IL-6 and TNF-, before being transferred to a membrane made of a polyvinylidene fluoride (PVDF). Blocking buffer was used to prepare the membrane for overnight incubation with the antibodies, IL-6 (Cell Signaling Technologies, Danvers, MA, USA), TNF- (Calbiochem, San Diego, CA, USA), and GAPDH (Abcam, Cambridge, UK). Additionally, the membrane was washed three times for a total of 10 minutes after each washing. Amersham ECL western blotting detection reagent chemiluminescence (ECL) was employed to analyze the protein signal (GE Healthcare, Piscataway, NJ, USA). We used Image J from the NIH to quantify the intensity of the analysis (Maryland, USA).

Data Analysis

The data were presented as mean \pm standard deviation (SD) and analysed by Oneway analysis of variance (ANOVA) by GraphPad 8.0. The post hoc Holm-Sidak's multiple comparison test was used to evaluate the significance among sedentary control, endurance exercise (eTR), and endurance exercise with *M. oleifera* (eTMO) groups with a $p < 0.05$.

RESULT

The findings of the ANOVA analysis for the three groups are shown in Figure 1, where the calculated value of $F = 14.0$ with a sig p of 0.0017 ($p < 0.05$). The p value for the post hoc Bartlett test was 0.0149. ($p < 0.05$). These findings indicate that the administration of MO extracts reduced the level of IL-6 protein in skeletal muscle more effectively than in the control group. Figure 1 illustrates that the group that exercised and was

supplemented with MO extract had the lowest regulation of IL-6 protein expression. This might mean that the group who received eTMO showed the least level of inflammation. In comparison to the control group, the exercise group without supplements had better health. The fact that the expression of the IL-6 protein was lower in the exercise group than in the control group demonstrates the health benefits of the exercise program.

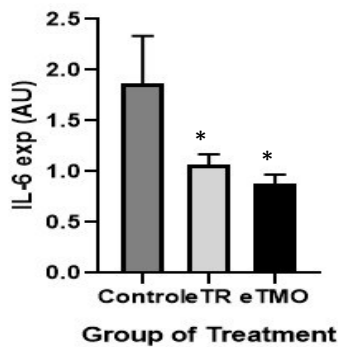


Fig 1. Comparison of protein expression of IL-6 in the three treatment groups

DISCUSSION

The reduction in IL-6 levels shown in the exercise group administering MO was an indication of a faster recovery from muscle injury compared to the other two groups. This is consistent with the findings of the research conducted by Toft et al., (2002), revealing that individuals who had suffered a muscle injury had significantly higher levels of IL-6 in their blood. Throughout the healing phase, IL-6 levels and inflammatory symptoms, such as pain and swelling, decreased. This suggests that a reduction in IL-6 levels may be an indication that injured muscles are recovering. In addition, Zaldivar et al., (2006) revealed that a significant decrease in IL-6 levels after exercise might represent how well the body handles exercise-induced stress. This is due to the fact that IL-6 can encourage the body to produce more growth hormone and insulin-like growth factor, which aid in muscle repair and the production of new muscle cells.

Meanwhile, the exercise group without moringa supplementation showed better conditions than the control group, indicating that the proper exercise regimen prevented cell damage compared to the sedentary control group. The control group, on the other hand, was in

worse shape compared to the exercise group which did not take moringa. This shows that the right exercise routine prevented cell damage. These results back up what we found in a previous study (Ray et al., 2020) about how a combination of Moringa and endurance exercise could improve mitochondrial biogenesis. In the past, the effects of exercise on inflammatory markers (neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and systemic-immune-inflammation index (SII)) and patient-reported outcomes in CCPs were studied in randomized controlled experiments. Inflammation markers were linked to patient outcomes. SII was considerably lower after exercise ($p = 0.036$). Exercise anti-inflammatory effects may underlie exercise-induced symptom improvements (Winker et al., 2022).

Previous research has demonstrated that the *Moringa oleifera* plant contains flavonoids, which are known to have an anti-inflammatory effect. Chemicals belonging to the phenolic group that can be found in high concentrations in nature are called flavonoids. Flavonoids are found in most seed plants, so it is reasonable to assume that they are also present in powdered plant extracts. In most cases, the solubility of flavonoids can be raised by using polar solvents such as methanol, acetone, water, or ethanol (Silva et al., 2022). In particular, flavonoid molecules can stop the body from making and releasing chemicals that cause inflammation when the body has an allergic reaction. Flavonoids are a group of anti-inflammatory chemicals that can work in a number of different ways. Flavonoids can stop enzymes like COX and lipoxygenase from doing what they are supposed to do. This stops the body from making the chemicals that are made when these things happen, like prostaglandins and leukotrienes. This is just one of the many ways in which flavonoids are able to exert their anti-inflammatory effects on the body (Zhou et al., 2016).

Flavonoids have the ability to reduce both the number of activated complement molecules as well as the number of leukocytes. This results in a weakening of the inflammatory response that is normally produced by the body and alters the way that leukocytes adhere to the endothelium. Flavonoids contain a number of anti-inflammatory properties, including the ability to prevent the inflamed and proliferative phases of the inflammatory process, as well as the ability to prevent neutrophils and endothelial cells from releasing lysoso-

mal enzymes and arachidonic acid (Kang et al., 2019). Because of the inability of inflammatory cells to release arachidonic acid, there will be no arachidonic substrate for the cyclooxygenase (COX) or lipoxygenase (LOX) pathways. This will reduce the concentrations of prostaglandins, prostacyclins, endoperoxides, and thromboxane while increasing the concentrations of hydroperoxide acid, hydrosiecosatetraenoic acid, and thromboxane. Inhibition of the COX pathway hinders leukocyte aggregation and neutrophil degranulation, which directly reduce the quantity of arachidonic acid released by neutrophils and suppress histamine production. In normal circumstances, leukocytes can penetrate the endothelium wall without obstruction. Many endothelial mediators and complement components collaborate during an inflammatory response to facilitate leukocyte adhesion to the endothelial wall (Pandolfi et al., 2020).

Quercetin and kaempferol are the primary flavonoid components of Moringa leaf (Chen et al., 2021). The endurance training of Wistar rats caused inflammation in their muscle tissue, which in turn led to the activation of muscle macrophages (Kupffer cells). The stimulation of CD4+ T cells by macrophages leads to the production of cytokines. It has been demonstrated that quercetin and other flavonoids present in the leaves of Moringa oleifera can prevent injured muscle Kupffer cells from producing IL-6. These flavonoids can be found in the leaves. Quercetin and other flavonoids are able to neutralize and prevent the inflammatory process due to their capacity to either bind atoms to free radicals or act as scavengers. It gives them the potential to do both (Ridker & Rane, 2021; Sulastri et al., 2022). Quercetin is a flavonoid that occurs naturally and can be found in a wide range of fruits and vegetables. It has the potential to maintain healthy muscles and improve how well they function. Anyone who is interested in enhancing their muscle health and performance, whether they are athletes, fitness enthusiasts, or just regular people, should take into account the research on quercetin and its advantages for muscle function.

Quercetin is a natural and safe alternative to many performance-enhancing pharmaceuticals, which makes it an intriguing option for individuals who are seeking a natural approach to boost the health of their muscles and their performance. Quercetin has been proven to boost endurance, reduce inflammation and oxidative stress, and enhance mitochondrial biogenesis, all of which can lead to an increase in the amount of energy

produced by muscle cells. In addition, including foods high in quercetin in your diet is an easy and convenient way to potentially gain the benefits of doing so. In addition, quercetin pills are readily available, and taking them might be an easy and practical approach to make sure that we are consuming a suitable quantity of this flavonoid through the food that we eat. According to research by Kressler et al., (2011), the effect of quercetin on human endurance exercise capacity (VO₂max) and endurance exercise performance is statistically significant but between minor and small. Another study by Lira et al. (2020) demonstrated that supplementation with quercetin for eight weeks could enhance the performance of elite female volleyball players. Another study by Roldan-Ruiz et al. (2020) demonstrated that elite male sprint performance could be enhanced by 14 days of quercetin intake. Even while these results are encouraging, additional studies are required to figure out the optimal MO dose and how quercetin boosts an athlete endurance.

CONCLUSION

An important finding of this study is that well-planned physical activity can play a role in enhancing immunity, as demonstrated by a decrease in IL-6 protein expression. Exercise can encourage the body to produce more growth hormone and insulin-like growth factor, which aid in muscle repair and the production of new muscle cells. Anti-inflammatory flavonoids function in many ways. They inhibit COX and lipoxygenase, diminish activated complement molecules and leukocytes, and block the endothelial wall. They also block neutrophils and endothelial cells from producing lysosomal enzymes and arachidonic acid during the inflamed and proliferative phases of the inflammatory process. Moringa leaf contains mostly quercetin and kaempferol. Quercetin, a natural and safe alternative to performance-enhancing drugs, increases endurance, reduces inflammation and oxidative stress, and enhances mitochondrial biogenesis, which increases muscle cell energy production. Due to their ability to attach atoms to free radicals or act as scavengers, it is a simple and convenient technique to neutralize and avoid inflammation. Quercetin improves endurance exercise capacity (VO₂max) and performance. Further research is needed to determine the ideal MO dose and how quercetin improves an athlete endurance.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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