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Moringa oleifera Improves Skeletal Muscle Metabolism and Running Performance in Mice

Siobhan M. Eze
Georgia State University

ACCEPTANCE

This dissertation, *MORINGA OLEIFERA* IMPROVES SKELETAL MUSCLE METABOLISM AND RUNNING PERFORMANCE IN MICE, by SIOBHAN M. EZE, was prepared under the direction of the candidate's Dissertation Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree, Doctor of Philosophy, in the College of Education, Georgia State University.

The Dissertation Advisory Committee and the student's Department Chairperson, as representatives of the faculty, certify that this dissertation has met all standards of excellence and scholarship as determined by the faculty. The Dean of the College of Education concurs.

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MORINGA OLEIFERA IMPROVES SKELETAL MUSCLE METABOLISM AND RUNNING PERFORMANCE IN MICE

by

SIOBHAN M. EZE

Under the Direction of Jeffrey S. Otis, Ph.D.

ABSTRACT

Background: Recent estimates suggest that 7% of Americans use plant-derived nutritional supplements to treat a variety of complications and/or to improve athletic performance and skeletal muscle health. Unfortunately, these supplements are largely unregulated and understudied. For example, *Moringa oleifera* (*M. oleifera*) is a subtropical plant and is routinely used to treat inflammation, diabetes, obesity, cancer and HIV. However, the mechanism of action of *M. oleifera* has not been fully elucidated, thus the purpose of this study is to evaluate the role of *M. oleifera* as a novel ergogenic aid to improve exercise performance by driving peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-dependent signaling pathways implicated in mitochondrial biogenesis and oxidative metabolism in skeletal muscle tissue. **Methods:** Adult male C57BL/6 mice were treated with 1.0 g of *M. oleifera* (N = 20) per day or vehicle control (N = 20) for a total of 5 weeks. Following 3 weeks of supplementation, half of each group (RUN) was given access to running wheels every night for 2 weeks (Remaining half = SED), distances ran were recorded daily. After treatment protocols were complete, the gastrocnemius muscles were excised and assayed for known markers of mitochondrial biogenesis, angiogenesis, endurance capacity, and capillary density using immunohistochemistry and RT-PCR. **Results:** Our results showed a significant increase in average distance run in the *M. oleifera* + SED and *M. oleifera* + RUN groups. This physiological trend was consistent with the molecular profile of key metabolic markers, i.e., there was an increase in levels of PGC-1α, PPARγ, SDHB, SUCLG1, VEGF, PGAM-2, PGK1, and MYLPF in the *M. oleifera* treated groups compared to vehicle + SED. Moreover, *M. oleifera* also increased CSA and decreased markers of protein degradation. **Conclusions:** This data suggests that *M. oleifera* has the potential to be an ergogenic aid via enhancing energy

metabolism in adult skeletal muscle by increasing the expression of key metabolic markers, including those involved in glycolysis, oxidative phosphorylation, mitochondrial biogenesis and angiogenesis.

INDEX WORDS: ergogenic aid, PGC-1α, *Moringa oleifera*, mitochondrial biogenesis.

MORINGA OLEIFERA IMPROVES SKELETAL MUSCLE METABOLISM AND RUNNING PERFORMANCE IN MICE

by

SIOBHAN M. EZE

A Dissertation

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2020

DEDICATION

I dedicate this doctoral dissertation to my loving and supportive family. First, I dedicate this to my husband & best friend, Chukwuma, I thank you for all your unwavering support and guidance. I appreciate your tough love, dedication, patience, and wisdom during this journey. Second, I dedicate this to my parents for their unfaltering support and patience throughout my entire life. I would not be the person I am today if it weren't for both of you. Third, I dedicate this to my siblings, nephews and future niece, and forever best friends, Sarah, Thomas, Lizzie, Carter, Grant, Baby Girl D, Mackenzie, and Lorcan. I will forever be grateful for your love, support, guidance, and laughs! Thank you all for pushing me to pursue my dreams and aspirations!

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ABBREVIATIONS

AMPK	5’ adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
BMR	Basal metabolic rate
CAIN/CABIN-1	Calcineurin inhibitory protein
CaMK	Calcium-calmodulin-dependent protein kinase
CsA	Cyclosporine A
DRP1	Dynamin-related protein 1
DSHEA	Dietary Supplement Health & Education Act
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases
ERRα	Estrogen-related receptor alpha
FDA	U.S. Food & Drug Administration
FIS1	Fission 1
GLUT	Glucose transporter
GTPase	Guanosine triphosphate enzyme
HDAC	Histone deacetylases
HIF	Hypoxia-inducible factor
IOC	International Olympic Committee
ISSN	International Society of Sports Nutrient
MAPK	Mitogen-activated protein kinases
MEF2	Myocyte enhancer factor
MFF	Mitochondrial fission factor
MFN	Mitofusions
MGM1/OPA1	Dynamin-like 120 kDa protein (mitofusion protein)
MHC	Myosin heavy chain
MICs	*Moringa* isothiocyanates
M. oleifera	*Moringa oleifera*
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
NRF	Nuclear respiratory factors
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator-1
PINK1	PTEN-induced putative kinase protein 1
PPARγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative real-time polymerase-chain reaction
ROS	Reactive oxygen species
SIRT1	Silent mating-type information regulation 2 homolog 1
TFAM	Mitochondrial transcription factor A
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor
VWR	Volunteer wheel running
WADA	World Anti-Doping Agency
WHO	World Health Organization

CHAPTER ONE

MORINGA OLEIFERA AS A POTENTIAL ERGOGENIC AID IN SKELETAL MUSCLE

Introduction

The benefits of physical activity are numerous, including increased skeletal muscle metabolism and preventing loss of mass, decreasing adipose tissue mass, improved cognition and preventing chronic diseases, such as coronary artery disease, stroke, diabetes and cancer [1-3]. However, because some individuals may not be able to participate in physical activity for various reasons, it is critical that alternative solutions to increase skeletal muscle metabolism and decrease adipose tissue mass are developed, such as ergogenic aids. An ergogenic aid is something that improves the performance capacity of exercise or enhances training adaptations [4-6]. They may also enhance energy production, provide individuals with a competitive advantage, prepare an individual to exercise, improve exercise efficiency, recovery time, or assist in injury prevention during intense training [4, 5]. An ergogenic aid may involve a training technique, mechanical device, nutritional ingredient, pharmacological method, or physiological technique [4, 5]. Due to the competitive nature of sports, performance enhancing substances or ergogenic aids have become more popular [7].

More recently, there has been more focus on the contribution of nutrition and dietary supplements to optimize training and athletic performance [17-21]. Research shows that roughly 50% of the general population [18] and about half of all athlete's report using supplements [19]. However, ergogenic aids that are classified as supplements are not required by law to be scientifically evaluated, which means the contents of these products and the claims on the label have not been evaluated by the U.S. Food and Drug Administration, and thus, may not have any

scientific basis [18]. A nutritional supplement is only defined as an ergogenic aid if peer-reviewed studies demonstrate that the supplement can significantly enhance metabolism or exercise performance after weeks to months of ingestion [17].

One common category of nutritional supplements are herbals and botanicals, which have become the most popular ergogenic aids on the market, in part due to the perception over the past few years that natural means healthy [22, 33, 34]. Plants contain nutrients and phytochemicals, thus providing essential and secondary metabolites, which are at the forefront of research due to their potential biological properties [35]. Approximately 7% of the U.S. population uses herbal supplements to enhance muscle growth, burn fat, improve endurance, or increase strength performance [34, 35]. Clinical outcomes have varied in previous studies due to factors such as the type of plant, soil type, geographic location and the method of extraction used [34]. Moreover, most studies only highlight the efficacy of the herb and do not mention probable risks or negative side effects to athletes [34]. Although research findings support the beneficial medicinal effects of specific herbs for specific health problems, research investigating the ergogenic effects of herbal supplements is limited [35].

Therefore, in the present study, we aim to investigate the role of *Moringa oleifera* (*M. oleifera*) in mouse skeletal muscle, a popular tropical and sub-tropical plant used to treat hundreds of common diseases and ailments, and importantly, is also believed to be a potential ergogenic aid. Specifically, there is significant evidence in literature that *M. oleifera* modulates metabolism and exerts anti-inflammation and -oxidant properties; however, there is not enough evidence on the underling mechanism of action of *M. oleifera* on skeletal muscle and its ergogenic aid effects. The present study is also designed to evaluate if *M. oleifera* attenuates oxidative stress, improves metabolism, and promotes endurance capacity by increasing

mitochondrial biogenesis and fiber-type switching in mouse skeletal muscle. We **hypothesize** that *M. oleifera* will increase endurance capacity in adult mouse skeletal muscle via key metabolic signaling pathways. We will rigorously test this hypothesis using three specific aims: 1) Aim 1: We will evaluate endurance capacity and fiber-type switching following *M. oleifera* supplementation and delineate the specific underlying mitochondrial signaling pathways; 2) Aim 2: We will examine the effects of *M. oleifera* on glucose metabolism in adult skeletal muscle and 3) Aim 3: We will investigate the effects of *M. oleifera* on angiogenesis and microcirculation in adult skeletal muscle. **Collectively, these specific aims will examine whether *M. oleifera* is an effective ergogenic aid and evaluate its use in the regulation of metabolism and circulation in skeletal muscle.**

Skeletal Muscle & Performance

Skeletal muscle is the most abundant tissue in the body of healthy individuals and is essential for motion and support [2]. In the adult human body, it comprises about 40-50% of the total body mass, with more than 600 individual muscles [1]. For this reason, skeletal muscle is the key determinant of basal metabolic rate (BMR), energy metabolism [2] and the major consumer of glucose, and is responsible for about 80% of insulin-mediated glucose uptake and fatty acid metabolism [2]. Furthermore, it can increase energy expenditure up to 30-fold during intense exercise by stimulating insulin-independent glucose uptake and increasing higher fatty acid uptake and oxidation [2]. Muscle tissue also plays a vital role and expends a significant amount of energy in maintaining muscle mass through protein synthesis, repair, and regeneration [2].

The loss of muscle mass can lead to physical and metabolic dysfunction [3]. Furthermore, a sedentary lifestyle or inactivity can lead to an increased risk of chronic diseases, such as coronary artery disease, stroke, diabetes, and cancer [8]. There is sufficient research demonstrating that physical activity provides the cardiovascular, neuroendocrine, respiratory, and musculoskeletal systems positive benefits [8]. Physical activity can reduce adipose tissue mass (obesity), improve glucose metabolism, and decrease the risk of metabolic syndrome [8]. Aerobic training (endurance) is associated with improvements in metabolic regulation, but current research suggests it may also promote skeletal muscle hypertrophy [9]. Skeletal muscle plays an important role in glucose homeostasis because it has the ability to take up glucose and store it, which aids in controlling blood glucose levels [10]. Glucose metabolism and skeletal muscle hypertrophy (protein synthesis) are crucial to maintaining a healthy weight and living a better quality of life. Physical activity is known to promote molecular changes in skeletal muscle including fiber-type switch, increasing mitochondrial biogenesis, angiogenesis, and oxidative capacity [11, 12].

Molecular Pathways Associated with Mitochondrial Biogenesis

Skeletal muscle fibers contain mitochondria which play a role in muscle contraction and energy production by generating ATP [13] through a process known as oxidative phosphorylation [13]. Mitochondrial biogenesis involves the growth or proliferation of pre-existing mitochondria, a process dependent on the coordination of nuclear and mitochondrial-encoded gene expression [13]. In response to stimuli, like endurance exercise, mitochondrial content increases because of the increase in capacity for sustained work [14]. Mitochondrial biogenesis is a complex process because mitochondria are comprised of proteins derived from

the nuclear and mitochondrial genomes [14]. For four decades, scientists have known that when muscle is exercised over long periods of time, mitochondrial content increases, which improves the tissue's capacity for oxygen consumption and ATP provision [14]. Mitochondrial biogenesis has many applied health benefits including improved muscular endurance and reduced fatiguability during normal daily activities [14].

The major steps involved in mitochondrial biogenesis include stimuli-induced activation of kinase/phosphatase signaling, such as what occurs with endurance exercise [14]. The kinase/phosphatase activation then activates transcription factors, mRNA stability proteins, and/or translation factors [14]. This leads to the translation of precursor proteins, followed by the import of proteins into mitochondrial compartments, leading to two different pathways [14]. First, the import of proteins can cause the assembly of multi-subunit complexes, which results in the increase in muscle aerobic capacity [14]. Second, the import of proteins can lead to mtDNA transcription and copy number, causing mtDNA-encoded proteins to assemble multi-subunit complexes, which results in the increase in muscle aerobic capacity [14].

In 1967, the first direct evidence of exercise training-induced mitochondrial biogenesis in skeletal muscle following treadmill running exercise was reported [13, 15]. More recently, studies have shown that mitochondrial dysfunction in skeletal muscle has been associated with the development of metabolic diseases [11]. Mitochondrial dysfunction can lead to a decrease in exercise performance and tolerance [2, 11, 16]. However, exercise can be the best treatment of many of these metabolic diseases [11]. Therefore, understanding the mechanisms of mitochondrial function in skeletal muscle in response to disease, training, injury, diet or other conditions is critically important.

The molecular mechanisms associated with skeletal muscle changes in response to aerobic exercise are well established [12]. Exercise can regulate a vast array of biological functions in the body [17], including cellular processes within skeletal muscle like fatty acid metabolism, and glucose and protein metabolism via the induction of a range of signal transduction pathways [17]. Most studies of exercise signaling have focused on the transient changes in phosphorylation of kinases, specifically AMP-activated protein kinase (AMPK), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), and mitogen-activated protein kinases p38 MAPK and ERK1/2 [12] [17]. Research has also established that acute exercise can activate signaling pathways, which alters the activity of coactivators and transcription factors, along with expression of genes that play a role in the regulation of angiogenesis, mitochondrial biogenesis, carbohydrate and lipid metabolism, and proteolysis [12]. Exercise causes acute metabolic and mechanical demands in the activated muscles, which requires the regulation of diverse signaling pathways that form a complex signaling network [17]. This network produces cellular homeostatic adaptations [17]. For example AMPK, plays an important role in whole body metabolic homeostasis and is a target for the effects of the antidiabetic drug, metformin [17]. However, research has yet to discover the full effects of AMPK's biological targets and narrow down the specific phosphorylation sites [17].

Other key molecules include peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1), a family member of transcriptional coactivators (PGC-1α, PGC-1β, and PRC), which have been identified as key regulators of mitochondrial function [11, 12]. In response to exercise, PGC-1α was the first transcriptional coactivator identified by its ability to interact with peroxisome proliferator-activated receptor gamma (PPARγ) transcription factor [11] [18]. More importantly, PGC-1α is an important regulator of exercise-induced adaptations

in skeletal muscle and has been shown to be induced in skeletal muscle in response to exercise [11] [19, 20]. Mitochondrial transcription factor A (TFAM) and PGC-1α are thought to be master regulators of mitochondrial biogenesis and adaptation of skeletal muscle in response to aerobic exercise [12, 13]. PGC-1α functions in the nucleus by controlling the expression of nuclear respiratory factors 1 and 2 (Nrf-1 and Nrf-2), which are responsible for controlling the expression of TFAM [13].

PGC-1α has a primary role in promoting mitochondrial biogenesis (Figure 1) [13]. An increase in PGC-1α expression can lead to expression of mitochondrial enzymes in the electron transport chain (ATP synthetase, cytochrome c oxidase subunits) [13]. One study has shown that overexpression of PGC-1α leads to upregulation of 151 genes [13], which are known to encode mitochondrial proteins involved in various metabolic functions [13]. Furthermore, endurance exercise induces mitochondrial biogenesis by promoting PGC-1α expression through transcriptional control and activity through post-translational modification [13]. Expression of PGC-1α mRNA, specifically over-expression or induction in skeletal muscle, have mimicked many effects of exercise on mitochondrial biogenesis [13]. In PGC-1α knockout mice, normal exercise training induced fiber-type transformation, but mitochondrial biogenesis and angiogenesis was significantly attenuated in the skeletal muscle [13]. These data suggest that PGC-1α is necessary for exercise-induced mitochondrial biogenesis in skeletal muscle.

Mitochondrial biogenesis in skeletal muscle can be promoted and controlled through the activation of PGC-1α and consequent translocation to the nucleus [13]. It has been shown that silent mating-type information regulation 2 homolog 1 (SIRT1) deacetylates PGC-1α, and SIRT1 activation is associated with increases in PGC-1α target gene expression [13]. It is suggested that SIRT1 activates PGC-1α through deacetylation, but this activity depends on prior

phosphorylation of PGC-1α by AMPK, which integrates both contractile and metabolic cues in exercise-induced skeletal muscle adaptation [13].

Mitochondrial Dynamics and Maintenance

In skeletal muscle, mitochondria form a network with the subsarcolemmal and intermyofibrillar population [13]. The intermyofibrillar mitochondria provide immediate high energy for contractile activities due to their proximity to the contractile apparatus [13]. Fusion is the joining of nearby individual mitochondria and fission is the separation of nearby mitochondria [13, 21]. These two processes are important in controlling mitochondrial structure and function [13, 21]. In healthy, metabolically active cells, there can be a large interconnected network of mitochondria, and dysfunctional mitochondria are separated from the network and removed through a process known as autophagy [13, 21].

Mitofusions 1 and 2 (MFN1 and MFN2) are proteins involved in the control of fusion of the mitochondrial outer membrane [13, 21]. A protein involved in the control of fusion of the mitochondrial inner membrane is MGM1/OPA1 [13]. Fission of the mitochondrial outer membrane occurs when a GTPase, dynamin-related protein 1 (DRP1), works with Fission1 (FIS1) or mitochondrial fission factor (MFF) [13, 21]. Fission and fusion of mitochondrial cells is crucial in maintaining healthy mitochondrial populations and cell function [13, 21]. Research suggests that exercise regulates both mitochondrial fusion and fission [13, 21], however it has been reported that excessive mitochondrial fission was linked to mitochondrial degradation [21]. It remains unclear how exercise precisely regulates mitochondrial fission and fusion and what the relationship of the pre-existing quality of the mitochondrial network region is to the response to exercise regimen [13].

Mitophagy is a category of autophagy and it is a highly selective process that can promote the elimination of dysfunctional or unnecessary mitochondria [21]. Damage to the mitochondrial network can be caused by toxins, free radicals, or other chemical agents [13]. Damaged mitochondria cannot fuse with healthy mitochondria and need to be removed for skeletal muscle to properly function, specifically contractile and metabolic functions [13]. Mitophagy is dependent on specific signals present on the damaged or dysfunctional region of the mitochondrial network (figure 2) [13, 21]. The most studied pathway of mitophagy involves PTEN-induced putative kinase protein 1 (PINK1) and PARKIN (component of a multiprotein E3 ubiquitin ligase complex) [13, 21]. These proteins relay the signals associated with damage to the induction of mitophagy [13]. As the mitochondrial inner membrane becomes depolarized, PINK1 stabilizes and recruits PARKIN from the cytosol onto the mitochondrial outer membrane [13, 21]. This process leads to polyubiquitination of the mitochondrial outer membrane proteins and mitophagy [13, 21].

Molecular Pathways Associated with Endurance Capacity

Skeletal muscle fibers are multinucleated cells and contain sarcomeres, which are the structural contractile unit [13, 22]. The phenotype and gene expression of skeletal muscle fibers are categorized based on their specific contractile functions of speed (fast or slow), which is, in turn, determined by their contractile protein isoform, and the density of mitochondria (high or low) [13, 22]. Human skeletal muscle is comprised of two general types of fibers, type I (slow-twitch) fibers and type II (fast-twitch) fibers [13, 22].

Type I fibers are sometimes termed slow oxidative fibers and have the slowest contractile speed and a high density of mitochondria, which supports endurance capacity [13]. They contain

the myosin heavy chain protein isoform that has a slow contractile speed and uses less ATP per unit of work because of its lower myosin ATPase activity [13]. This allows the fibers to be more metabolically efficient, while the high mitochondrial density allows an oxidative supply of ATP for contractile work [13]. Furthermore, muscle fiber adaptations to endurance exercise may be restricted to intrinsic ranges within a given fiber type that limit mechanical adaptations [13].

Type II fibers have poor aerobic endurance capacity compared to type I fibers [13]. In humans, faster contracting muscle fibers have two common classifications, fast oxidative (type IIa) and fast oxidative-glycolytic (type IIx) [13, 22]. Rodents contain a third type of fast contracting fibers, which is classified as fast glycolytic (type IIb) [13]. Each of the three types of type II fibers has its own myosin heavy chain (MHC) gene [13, 22]. Furthermore, mitochondrial density is high in type I and IIa and is decreased in type IIx and IIb fibers (type IIb fibers contain the least number of mitochondria) [13, 22]. A comparison of skeletal muscle fiber types is outlined in Table 1. After long periods of inactivity or injury, type I (slow) fibers can switch to type II (fast) fibers. Fiber-type switching refers to the MHC protein and gene isoforms within a single fiber "switching" or changing due to physical activity, physical inactivity, electrical stimulations, or gene modifications [13]. Increased endurance activity can induce a fiber-type switching to a more oxidative fast phenotype, for example in humans, type IIx may switch to type IIa with endurance training [13, 22]. Moreover, research suggests that endurance exercise increases fiber oxidative capacity without changing the MHC composition, further suggesting that the metabolic and contractile differences may be controlled by distinct signaling pathways [13]. Endurance exercise also promotes other phenotypic changes including improved insulin sensitivity and metabolic flexibility [22-25].

Several studies have demonstrated that endurance exercise promotes a fiber type switch from a glycolytic to an oxidative phenotype within the fast-twitch fiber types (e.g., MHC type IIx to IIa) [22-24, 26, 27]. Both MHC1 and IIa isoforms coexist in human skeletal muscle fibers following endurance training, and professional athletes have increased type I fibers, but exercise-induced fiber type switching to type I fibers remains elusive [22-24]. Advancements in molecular genetics have led to research in exercise-induced fiber type transformation [22, 23, 27]. Some studies have shown that muscle contractions can activate the calcium-calmodulin dependent serine/threonine protein phosphatase, calcineurin [22, 28]. Activation of calcineurin is thought to promote the expression of slow-twitch muscle genes through dephosphorylation and activation of the nuclear factor of activated T-cells (NFAT) [22, 28]. Furthermore, inhibition of the calcineurin-NFAT pathway using cyclosporine A (CsA), FK506, calcineurin inhibitory protein (CAIN/CABIN-1), or peptide VIVIT lead to a reduction in the percentage of slow-twitch fibers and/or blocked slow-twitch muscle promoter activity and gene expression in rodents [22, 28-30]. Transgenic mice overexpressing a constitutively active form of calcineurin in skeletal muscle had an increased number of slow-twitch fibers, as well as elevated expression of slow-twitch troponin I, myoglobin, glucose transporter 4 (GLUT4), pyruvate dehydrogenase kinase 4 (PDK4), mitochondrial enzymes, and PGC-1α [22, 31, 32]. Moreover, carbohydrate oxidation was decreased, and lipid oxidation increased in skeletal muscle of these mice [22, 32, 33].

In adult skeletal muscle, CaMK has also been shown to have a role in exercise-induced genetic reprogramming of fiber types [22, 34-36]. The interaction between calcineurin and CaMKIV has been shown to stimulate the activities of transcriptional factors, myocyte enhancer factor 2 (MEF2) and NFAT [22, 34-36]. It is thought that CaMK activates MEF2 proteins via class II histone deacetylases, such as HDAC4, HDAC5, and HDAC9 [22]. These histones

interact and inhibit MEF2, which results in the repression of target genes [22, 37]. Deletion of HDAC5 and HDAC9 genes demonstrated an increase in type I and IIa fibers in soleus and plantaris muscles, whereas muscle-specific inducible expression of an HDAC that cannot be phosphorylated prevented running-induced fiber type switching [22, 34-37]. Data from research studies suggest that CaMK activates MEF2 through depression of HDACs in fiber type transformation [22, 37, 38].

As described previously, PGC-1α plays a vital role in endurance exercise-induced muscle adaptation [22, 39, 40]. Some studies suggest that overexpressing PGC-1α results in an increased percentage of slow-twitch fibers, and improved exercise capacity in mice [22, 39, 40]. Total knockout, or muscle-specific knockout of the PGC-1α gene (*Ppargc1a*), results in a reduction of the oxidative phenotype in skeletal muscle [22, 41-43]. Deletion of targets upstream of PGC-1α, like p38 MAPK, led to normal fiber type transformation (type IIx to IIa), but attenuated mitochondrial biogenesis and angiogenesis [22, 44, 45]. More recent research revealed that PGC-1α may influence the maintenance of slow-twitch, type I fibers, but it is not required for exercise-induce fiber type switching [22].

Vascular Improvements in Skeletal Muscle

The metabolic demands of skeletal muscle increase during aerobic endurance exercise [13]. Exercise training is associated with a decreased risk of cardiovascular diseases [46]. In response to regular physical exercise, the structure and function of the skeletal muscle fibers adapt (mitochondrial biogenesis, fiber-type switching), and cardiovascular changes lead to additional positive effects [47]. Some of the cardiovascular adaptations to endurance exercise include functional changes to the heart and blood vessels [47], more specifically the radius of

large vessels increases and there is an increase of muscle capillarity through angiogenesis [22]. An increase in blood flow to the skeletal muscle during exercise is required to provide an additional supply of oxygen and nutrients [22]. More recently, research suggests that the smallest diameter vessels, capillaries, may also undergo microvascular remodeling in response to continuous training stimulus [47]. Angiogenesis is an adaptive process that causes an expansion of the capillary network from pre-existing capillaries [22]. This process improves gas and nutrient exchange in peripheral tissues, which is thought to contribute to improved physical performance [22]. Angiogenesis promoted by exercise is thought to be mediated by a combination of growth factors, hypoxia, and shear and mechanical stresses [22].

Angiogenesis in skeletal muscle is a tightly regulated process at many different levels [46]. When microcirculation cannot meet the metabolic demands imposed on the muscle by cellular and physical influences, these influences exert an upstream effect on the capillary network to proliferate, which affects the up-stream input [46]. Muscle fibers and endothelial cells can sense the stress from cellular or metabolic demands, which leads to the activation of appropriate gene expression [46]. The process of angiogenesis ceases once metabolic homeostasis is reestablished [46]. However, this process is multifactorial, and the molecular mechanisms are only beginning to be elucidated [46].

The molecular AMPK/PGC-1α/Vascular endothelial growth factor (VEGF) axis is thought to play a pivotal role in angiogenesis in skeletal muscle [47]. Research has demonstrated that after exercise, endothelial cells and/or the muscle fibers respond to AMPK, sirtuins, and PPARs [47]. Data suggests that the presence of VEGF within the muscle fiber is necessary for endurance training responses in angiogenesis and oxidative capacity [13]. Furthermore, an increase in VEGF expression has been shown to improve running speed and endurance [13].

Providing adequate oxygen to the working muscles and mitochondria depends on the surrounding capillaries [48]. Low levels of VEGF in skeletal muscle have been associated with diminished capillary density [48]. It has also been suggested that VEGF plays a role in the regulation of vasodilation in several vascular beds through a nitric-oxide dependent mechanism [48]. Studies have demonstrated an increase in VEGF expression in response to exercise [48]. However, little attention has been given to the requirement of VEGF signaling for maintaining the microvascular network in skeletal muscle [48].

A single bout of exercise has been shown to induce mRNA expression of multiple angiogenic growth factors and related receptors in skeletal muscle, including VEGF (*Vegfa)* [22]. Muscle fiber expression and secretion of VEGF has been shown to promote angiogenesis through its paracrine effects [22]. However, the signaling cascade within muscle fibers that decodes muscle contractile activity signals in regulating VEGF activity has yet to be elucidated [22]. It is thought that PGC-1α coactivates the orphan nuclear receptor estrogen-related receptor-α (ERRα) [22]. This hypothesis was tested using a whole body knock out of the PGC-1α gene, which resulted in a reduced VEGF protein expression and blunted response to acute and chronic exercise training [22]. The functional role of PGC-1α in exercise-induced VEGF expression and angiogenesis may be dependent on p38 MAPK signaling upstream and ERRα downstream [22].

Nutritional Interventions & Performance

Ergogenic Aids

An ergogenic aid can be a training technique, mechanical device, nutritional ingredient, pharmacological method, or physiological technique that can improve exercise performance

capacity or enhance training adaptations [4-6]. Ergogenic aids may enhance energy production, provide individuals with a competitive advantage, prepare an individual to exercise, improve exercise efficiency, improve recovery time, or assist in injury prevention during intense training [4, 5]. Due to the intense competitive nature of sports, the use of performance-enhancing substances (PESs) has become increasingly popular [7]. Sporting federations have created regulations and developed testing programs to detect substances and discourage doping in athletics [49]. The World Anti-Doping Agency (WADA) was created in 1999 to address doping across sporting disciplines and regions [7, 49]. Detection of banned substances was originally done by assaying biological fluids for illicit substances or their metabolites [7, 49]. However, these methods were limited to detecting only the substances or their metabolites, but were not able to detect synthetic versions of naturally occurring endogenous hormones, like growth hormone [49].

Currently, indirect detection methods are used, which test for the biological effects of the substance rather than the substance itself [7, 49]. There are limitations and advantages to both detection methods, which means new approaches to anti-doping are needed [49]. A more recent method to detection is using performance as a marker for doping [49]. Most people who dope with substances or methods, do so with the intention to enhance performance [7, 49]. Therefore, raw performance data, profiles, or derived metrics could serve as indirect markers of doping [49]. However, using performance markers for doping detection is most reliable in sports like track and field, weight lifting, and swimming because the sport settings are relatively standardized, the outcome is a discrete, objective measurement of distance covered, mass lifted, or time achieved [49]. It may be more difficult to use performance markers as a detection of

doping in other sports, like soccer or basketball, due to less standardized competition settings and the physical capacity of the athlete may not be the primary determinant of performance [49].

In 2004, WADA released the first comprehensive list of prohibited substances [7, 49]. The list breaks down violations into substances and methods. Substances are classified as anabolic agents; peptide hormones, growth factors, and related substances; β_2 adrenergic receptor agonists; hormone and metabolic modulators; and diuretics and masking agents [7, 49]. Substances that have not been approved for human therapeutic use are prohibited, even if they are not listed [7, 49]. The list of banned methods includes manipulation of blood and blood components; chemical and physical manipulation, and gene doping [7, 49]. If a substance or method is not listed, it may be considered prohibited if it meets two of the following criteria: (a) it has the potential to enhance sport performance, (b) it represents a health risk to the athletes, or (c) it violates the spirit of sport [7, 49]. When an individual decides to use an ergogenic aid, whether it be a dietary supplement, method, or other substance, they need to consider the issue of efficacy, safety, and legality associated with the product before use [6].

More recently, there has been more focus on the contribution of nutrition and dietary supplements to optimize training and athletic performance [4-7, 49]. Dietary supplements are used by a variety of individuals, but individuals who participate in athletics and physical activity represent the largest portion of the population purchasing these products [6]. Most data reports that about 50% of the general population reported using supplements, while about 75% of college athletes, 100% of body builders [4], and about half of all athletes reported using supplements [6]. There is a wide variation between different sports and between athletes of differing performance levels, ages, and cultural backgrounds who have reported supplement use [6]. In 2013, the Nutrition Business Journal report estimated that the global supplements market

stood at $104 billion, while the sports supplements broke the $10 billion retail value sales mark in 2014 [6].

New ergogenic aids appear on the market almost daily and most are classified as a dietary supplement [4]. Aids that are classified as supplements means the contents of the product and the claims on the label have not been evaluated by the U.S. Food and Drug Administration (FDA), and thus, may not have any scientific basis [4]. Dietary supplements can be defined and categorized based on their function, action, or ingredients [50]. Some categories include sports foods (gels, bars, drinks, protein powders), vitamins and minerals, herbals and botanicals, or an ergogenic supplement [50]. Furthermore, there are specific categories for weight loss, improving libido, gluten-free, lactose-free, allergen-free, and functional foods [50]. Functional foods, herbs and botanicals, and super food categories are difficult to study and define due to the multiple, complex compounds and the heterogenous content of biologically-active ingredients [50]. The FDA has defined dietary supplements as, "a product intended for ingestion that contains a 'dietary ingredient' intended to add further nutritional value to (supplement) the diet" [50]. A "dietary ingredient" can be a vitamin, mineral, herb or other botanical, amino acid, metabolite, constituents, or extracts [50].

Nutritional Supplements as Ergogenic Aids

It is suggested that a nutritional supplement can be defined as an ergogenic aid if peer-reviewed studies demonstrate the supplement to significantly enhance exercise performance after hours to months of ingestion [5]. This may include, but is not limited to an increase in maximal strength, running speed, and/or work during a given exercise task [5]. A supplement may also be classified as ergogenic if it can acutely enhance the ability of an athlete to perform an exercise

task or enhances recovery from a single bout of exercise [5]. The International Society of Sports Nutrition (ISSN) has defined the ergogenic value of supplements more broadly [5]. The view of the ISSN is that a supplement is ergogenic if most human studies support the ingredient as being effective in promoting further increases in muscle hypertrophy or performance with exercise training, but a supplement cannot be classified as ergogenic if data is only supported by cell culture or rodent studies [5].

Complementary and alternative medicine is defined as the use of medical products and practices that are not part of the standard medical care [51]. Plants are widely used around the world in the management and treatment of a wide range of ailments and thus, are considered complementary and alternative medicine [51-58]. The World Health Organization (WHO) estimates that up to 80% of people rely primarily on traditional remedies, such as herbs, for medicinal purposes [59]. The medicinal value of certain plants is due to the presence of phytochemicals, polyphenols, and their elemental composition [54, 59].

Polyphenol compounds in plants have been associated with disease prevention or control due to the antioxidant properties of their constituents [59]. Plants are also rich in other phytochemicals, which are sources of nutrition and medicine for humans [60]. Furthermore, herbal and botanical remedies have attracted interest in the research community because of their rich source of potential chemical entities for the development of new effective drugs for neglected diseases [61]. A group of phytochemicals in plants are antioxidants and include chemical compounds such as polyphenols, vitamins, and fatty acids [60]. These chemical compounds have been shown to react with unstable oxidants, such as free radical species, like reactive oxygen (ROS) and nitrogen species and stabilize them [60]. In humans, an increase in

oxidants can lead to oxidative stress causing degenerative diseases like cancer, neurological disorders, Alzheimer's, cardiovascular disease, and pulmonary diseases [60].

Dietary supplements have become one of the most popular ergogenic aids on the market. One category of dietary supplements are herbals and botanicals. The use of herbal supplements has increased over the past few years due in part to the perception that natural means healthy [50, 62]. Plants provide us with essential nutrients and contain naturally occurring substances, known as phytochemicals [63]. Herbal supplements may be derived or extracted from seeds, gums, roots, leaves, bark, berries, or flowers of the plant [62, 63]. These supplements contain phytochemicals like carotenoids and polyphenols, including phenolic acids, flavonoids, saponins, alkaloids, glycosides, and lignans, which are thought to have nutritive or medicinal value [62, 63]. For example, capsaicin, found in cayenne, red, and chili peppers, have medicinal properties that are comparable to caffeine [63]. Caffeine and capsaicin are stimulants, which may induce sympathetic activation of the central nervous system, increasing catecholamine secretion and enhancing lipid oxidation, thus decreasing the use of glycogen [63].

Throughout history, herbals and botanicals have been used as medicine, with the earliest evidence of human use for healing dating back to the Neanderthal era [63]. Currently, some modern medicines may be classified as herbals [63]. In Germany, herbals are regulated as medicine, but in the United States, most herbals are regulated by the Dietary Supplement Health and Education Act (DSHEA), more like food than drugs [63]. Due to the pharmacological effects of herbals, health professionals are emphasizing the need for regulations in order to standardize herbal therapy [62, 63].

A number of herbal and botanical supplements have not been proven safe and effective under current FDA standards and others have been excluded from FDA requirements because

they present a source of drug production [62]. Research has shown that plants provide essential metabolites (carbohydrates, lipids, nucleic acids) and secondary metabolites (terpenoids, alkaloids, phenolic compounds) [62]. Secondary metabolites have been at the forefront of research due to their potential biological properties like anti-allergic, anti-atherogenic, anti-inflammatory, hepato-protective, antimicrobial, antiviral, antibacterial, anticarcinogenic, antithrombotic, cardioprotective, and vasodilatory effects [62]. The biological properties of herbs are mediated by a variety of characterisitcs and compounds, such as antioxidant and redox properties [62]. The antioxidant characteristics and redox properties play an important role in oxidative damage stabilization by free radical neutralization, oxygen scavenging, or decomposition of peroxides [62]. This information has led to studies focusing on the role of herbal supplements in reducing exercise-induced oxidative stress in athletes [62]. The reduction of oxidative stress may enhance muscle recovery and energy maintenance during intensive exercises [62].

As stated earlier, approximately 7% of the U.S. population takes herbal or botanical supplements [62, 63] and about 17% of female collegiate athletes reported using herbal or botanical supplements [63]. Most herbal supplements used in sports or exercise are used to enhance muscle growth and burn fat [62] and are also used by athletes and non-athletes to improve endurance and strength performance [62, 63]. However, clinical outcomes have varied in previous studies due to factors like the type of the plant, the geographic location from which the plant was gathered, and the method of extraction used [62]. Moreover, most studies highlight the efficacy of the herb and do not mention probable risks or negative side effects in athletes [62]. Research supports the beneficial medicinal effects of specific herbs for specific health problems, but research investigating the ergogenic effects of herbal supplements remains limited

[63]. The most common perception of herbal supplements is that they are healthy, but some natural substances pose a serious health threat, even though they are marketed to improve health and physical performance [50, 62, 64]. For example, some natural substances may interact with cardiac medications, increase the risk of bleeding when taken with blood-thinning drugs, and can cause an overdose if not taken correctly [50].

Athletes need to be aware that some plants may contain doping substances, as well as some products based on herbal extracts may be contaminated by agents prohibited by sports [62, 63]. For example, ma huang, an herbal ephedrine, is a stimulant that may be found in plants and is banned by the International Olympic Committee (IOC) [4]. Individuals, specifically athletes, who are interested in the performance enhancing benefits of herbal supplements are urged to try the supplement in training or simulated competition before implementation in competition [64]. The effects of most herbal or botanical supplements in sports and exercise remains inconclusive [62-64] and more studies investigating the mechanism of action and/or off target effects need to be done.

Herbal Medicine as Ergogenic Aids

The use of plants and herbal supplements has increased significantly over the past decade [62]. Research has demonstrated that herbs have specific beneficial medicinal effects, but research investigating the ergogenic effects of herbal supplements is limited [63]. Herbal supplements like capsaicin, ginseng, ginkgo biloba, caffeine, and guarana have been used to study their ergogenic effects.

Ginseng is one of the more popular herbal supplement and is one of the most studied herb with regards to physical performance [62, 63]. This plant has been shown to have anti-

inflammatory and antioxidant properties, as well as stimulate brain function, anabolic and immuno-stimulant, and improve endurance performance [62, 63]. Other potential ergogenic effects of ginseng include favorable metabolic, hematologic, and cardiovascular improvements [63]. Capsaicin, guarana, and caffeine are similar, in that they are stimulants of the central nervous system, which increases catecholamine secretions and enhances lipid oxidation [63]. Ginkgo biloba is thought to exert its mode of action when its active ingredients, the flavonoids and terpenoids, work together [62, 63]. Ginkgo stimulates the release of endothelium-derived relaxing factor, nitric oxide, which is thought to enhance muscle tissue blood flow through improved microcirculation, ultimately improving aerobic endurance by enhancing muscle tissue oxidation [63].

There are a variety of herbal supplements that are marketed as ergogenic aids, but there is a shortage of well-controlled research evaluating the efficacy of herbal supplements on exercise and performance [63]. Most of the knowledge surrounding the efficacy of herbal supplements being used as an ergogenic aid is subjective, and research suffers from methodological problems [63]. These problems include poor research design and use of a variety of substances where the purity and content of the plant is suspect [63]. Future research efforts need to focus on experimental design, product purity, standardized dosages, subject compliance, and statistical power [63]. Due to the attention and popularity of herbal supplements, it is imperative to have quality quantitative evidence to provide to the population.

Moringa oleifera

Plant Background

Moringa oleifera (*M. oleifera*) is a tropical and subtropical plant species belonging to the Moringaceae family (Papaverales Order) (Figure 3) [54, 65-67]. This plant is commonly referred to as Moringa, drumstick tree, horseradish and ben oil tree [68, 69]. *M. oleifera* is widely cultivated around the world, especially in sub-Himalayan regions of Northwest India [52, 53, 58, 59, 67, 70]. However, it can grow under a variety of conditions in tropical and subtropical areas throughout Africa, Saudi Arabia, Southeast Asia, the Caribbean Islands, and South America, that may be both humid and hot dry lands and can even survive in less fertile soils [59-61, 65-67, 71, 72]. It is a fast-growing and is widely cultivated for its young seed pods and leaves (Figure 4), which are used as vegetables and traditional herbal medicine. The seed of the plant can also be processed and used for water purification [68, 69, 73].

M. oleifera can grow to heights of up to 40 feet, with a trunk diameter of 1.5 feet. The bark is a whitish-grey color and is surrounded by thick cork. The flowers contain yellowish-white petals and are fragrant and asexual (Figure 4). Flowering begins within the first four to six months after planting. However, in cooler climates, flowering only occurs once a year between April and June, and in more constant higher temperatures, flowering can occur twice or all year round. The fruit of *M. oleifera* looks like giant bean-pods about 20-45 cm long (Figure 4) and contains dark brown, globular seeds with a diameter around 1 cm (Figure 4). The seeds are dispersed by wind and water and the plant is trimmed and cut down annually to about 3-6 ft to increase its leaf biomass. This is done so the pods and leaves remain within arm's reach [68, 69, 73-75].

There are 13 known varieties of *Moringa* species that exist, but *M. oleifera* is the most widely known and used [52, 54, 65]. Different parts of *M. oleifera* may have nutritional, prophylactic and therapeutic properties [58]. Most of the plant parts, including the root, bark, gum, leaf, fruit (pods), flowers, seed, and seed oil of *M. oleifera* are consumed and added to the daily diet in a number of different cultures [59, 72] or used as herbal therapies for various ailments [51, 65, 72]. *M. oleifera* is an easily accessible and inexpensive plant and may have complementary therapeutic affects [72]. For this reason, it is commonly used as a medicinal herb to treat common diseases and ailments, such as inflammation-related disorders, diabetes, obesity, cancer, HIV, blood pressure and epilepsy [52-58]. However, there is still a need to conduct more extensive research to validate its traditional therapeutic effects cited above.

Phytoconstituents of Moringa oleifera

M. oleifera plant contains a variety of phytochemicals, such as zeatin, quercetin, beta-sitosterol, kaempferol, and caffeoylquinic acid, terpenoids, alkaloids, tannins, steroidal aglycones and reducing sugars, as well as other bioactive compounds like glucosinolates and isothiocyanates [54, 66], which exert multiple pharmacological activities cited earlier, including anti-inflammatory, anti-bacterial, anti-oxidant, anti-cancer, hepatoprotective, and neuroprotective activities [52, 65, 66]. Chlorogenic acid in *M. oleifera* has been shown to lower blood glucose [66]. Phytochemicals or phytoconstituents also provide color, act as anti-feedants, or protect macromolecules [76] and when consumed by humans, phytoconstituents exert bioactive properties, including antioxidant, anti-inflammatory, and vasoactive [76]. Studies have shown improvements in disease outcomes and tissue function, such as in endothelial function, blood pressure, and circulating lipoproteins when plants rich in these phytoconstituents are consumed

[76]. A hundred grams (100 g) of dry *M. oleifera* leaf powder contains 9 times the vitamin A of carrots, 15 times the potassium of bananas, 17 times the calcium of milk, 12 times the vitamin C of oranges, and 25 times the iron of spinach [66]. For this reason, *M. oleifera* has been used to supplement malnourished populations and mitigate a variety of disorders, such as diabetes (hyperglycemia), HIV, cancers and other diseases and conditions [52, 61, 66, 67, 71, 77].

Because of the high number of *M. oleifera* phytochemicals, only a few will be briefly reviewed here. Zeatin is a plant hormone and a member of the cytokinin growth hormone family [78]. It has been reported to have anti-aging effects on human skin fibroblasts [78]. Quercetin is a polyphenolic flavonoid with potential chemo-preventive activity [78]. It has also been shown to produce antiproliferative effects resulting from the modulation of epidermal growth factor receptor (EGFR) or estrogen-receptor mediated pathways, but the exact mechanism of action has not been elucidated [78]. Furthermore, research has demonstrated that quercetin also produces anti-inflammatory and anti-allergy effects mediated through the inhibition of lipoxygenase and cyclooxygenase pathways [78]. Beta-sitosterol is a phytosterol, which when oxidized acquire anti-carcinogenic and anti-atherogenic properties [78]. However, their exact mechanism of action is not fully understood [78]. Kaempferol is a natural flavonoid that has been shown to act as an antioxidant by reducing oxidative stress and is being investigated as a possible cancer treatment [78]. Research also suggests that it may be an antibacterial agent [78]. Caffeoylquinic acid is an alkyl caffeate ester that may have a role in the inhibition of the NFκB pathway, as an anti-neoplastic agent, and an antioxidant [78].

The other key bioactive compounds in *M. oleifera* are glucosinolates and isothiocyanates [79]. Glucosinolates are plant secondary metabolites that are known for their fungicidal, bactericidal, nematocidal, and allelopathic properties [80], whereas isothiocyanates

are derived from glucosinolates [76]. Sulforaphane is an isothiocyanate from broccoli and has been shown to induce expression of mammalian cyto-protective proteins through the Keap1-Nrf2-ARE pathway [79]. Furthermore, sulforaphane has been shown to inhibit enzymes responsible for the activation of carcinogens and induces detoxification enzymes [80]

Extraction Methods

Various types of solvents have been used to extract a set of desired bioactive compounds for research and therapy and the most common include aqueous, ethanolic and methanolic [66, 71]. Furthermore, the extraction method, along with the part of the plant used, will affect the phytochemical content. Table 2 lists the constituents based on the part of the plant and solvent used for extraction [66]. The pharmacological activity of *M. oleifera* is listed in Table 3.

To extract specific phytoconstituents from the leaves using polar and non-polar methods, the leaves must first be dried and hand-milled [54]. The dried leaves are then extracted by successive maceration for 48 h with hexane at room temperature followed by filtration [54]. The residue is then extracted again, but this time with absolute ethanol followed by filtration [54]. The final residue is discarded, and the filtrates concentrated under vacuum to eliminate the solvent [54]. As a result, this method of extraction yields two different extracts: a dark green greasy hexane extract and a dark green semisolid ethanol extract [54]. A UHPLC analysis of the ethanol crude extract is used to identify a characteristic flavonoid and a mass spectroscopy negative ion mode is applied to identify the presence of fatty acids in the non-polar extract [54]. The UHPLC analysis of the ethanol extract contains Kaempferol-3-glucoside [54]. This extract is used for pain and inflammation treatment and research [54].

An increasing amount of experimental research and epidemiological evidence suggests that *M. oleifera* has anti-diabetic and antioxidant effects against the harmful damages of oxidative stress and diabetic complications [81] [82]. Several studies have also shown that *M. oleifera* has beneficial effects on carbohydrate metabolism via several mechanism, including preventing and restoring the integrity and function of pancreatic β-cells; increasing insulin action; improving glucose uptake and utilization [81] [82]. During adipogenesis, it has been shown that *M. oleifera* improves adipocyte functionality and upregulates the expression of uncoupling protein (UCP1), sirtuin 1(SIRT1), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is involved in thermogenesis by modulating lipid metabolism [81]. Furthermore, it is thought that mitochondrial proton leak (UCP activity) might play a role in the pathophysiology of diabetic complications and cardiovascular diseases [81]. Collectively, these data suggest that *M. oleifera* may exerts its effects intracellularly by modulating several metabolic pathways within the mitochondria [81]. However, most of the data have been limited to the investigation of *M. oleifera* role in the liver, kidney, or heart tissue using methanolic extraction methods.

Moringa oleifera & Exercise Performance

While the overall underlying mechanism of action of *M. oleifera* in most disorders is not fully understood, research conducted thus far has demonstrated its overall biological effects and its ability to regulate the function and expression of different proteins in the body. For example, it has been shown that *M. oleifera* improves adipocyte functionality and upregulates the expression of SIRT1 and PGC-1α involved in thermogenesis and modulation of lipid metabolism [81]. Based on these and other findings, we hypothesize that *M. oleifera* may improve exercise

performance by upregulating expression of PGC-1α in skeletal muscle tissue, which may subsequently induce or facilitate mitochondrial biogenesis and endurance capacity. Furthermore, we speculate that supplementation with *M. oleifera* could increase SIRT1 deacetylase activity in skeletal muscle, which would then promote mitochondrial biogenesis.

Specific Aims and Hypotheses

With roughly 50% of the general population and athletes reportedly using supplements, there is an urgent need to fully understand the effects and associated side effects of these substances. Although there is significant evidence in literature that shows that *M. oleifera* modulates metabolism and exerts anti-inflammation and -oxidant properties, there is not enough data on the specific underlying mechanism of action of *M. oleifera* on skeletal muscle and its ergogenic aid effects. The present study will investigate *M. oleifera*'s ergogenic effects on skeletal muscles, namely whether there is improvement in performance, change in metabolism, microcirculation and induction of mitochondrial biogenesis or switch in fiber-type.

The exact *M. oleifera* phytochemical that is responsible for inducing its ergogenic effects are not known. We do know that *M. oleifera* leaves contain several phytoconstituents, such as quercetin, zeatin, and methionine, as well as biological compounds like isothiocyanates. The leaves also contain an abundance of secondary metabolites, principally polyphenols and four unique *M. oleifera* isothiocyanates (MICs) [57]. MICs have strong biological activity and are thought to be the principal therapeutically active constituents found in *M. oleifera* [57] and perhaps maybe the phytochemical responsible for *M. oleifera*'s ergogenic effects. We **hypothesize** that *M. oleifera* will increase endurance capacity in adult skeletal muscle via key metabolic signaling pathways. We will rigorously test this hypothesis using three **specific aims:**

1. **Specific Aim 1:** We will evaluate endurance capacity and fiber-type switching following *M. oleifera* supplementation and delineate the specific underlying mitochondrial signaling pathways,
2. **Specific Aim 2:** We will examine the effects of *M. oleifera* on glucose metabolism in adult skeletal muscle,
3. **Specific Aim 3:** We will investigate the histological effects of *M. oleifera* by examining changes in skeletal muscle angiogenesis and microcirculation of adult animals.

Collectively, these specific aims will rigorously examine whether *M. oleifera* is an effective ergogenic aid and evaluate its use in the regulation of metabolism and circulation in skeletal muscle.

Materials & Methods

Overview of Experimental Design

Adult, sexually mature male C57BL/6 mice will be divided into four treatment groups: 1) Negative control group ($N = 10$), 2) *M. oleifera* supplemented group ($N = 10$), 3) Negative control + volunteer wheel running (VWR) ($N = 10$), or 4) *M. oleifera* + VWR ($N = 10$). We will use C57BL/6 mice because they are the most widely used inbred strain and they are commonly used for general purposes. Male mice will be used due to the lack of estrous cycle and thus, the absence of hormones that could potentially alter results. Skeletal muscle tissue from the gastrocnemius will be dissected into three equal pieces for analysis, including histological and quantitative real-time PCR to evaluate the effect of *M. oleifera* on protein and gene expressions associated with skeletal muscle metabolism, including signaling factors implicated in

mitochondrial biogenesis, endurance capacity, mitophagy (fission/fusion) and angiogenesis. Muscle capillarity will be assessed in histological sections.

Animals & Supplementation of M. oleifera

Sexually mature male (6-8 wks) C57BL/6 mice will be individually housed in a specific pathogen-free animal facility with a 12-12h light-dark cycle. Each mouse will be fed at 6:30 am every day to ensure the light/dark cycle of the room is kept constant. They will be fed their *M. oleifera* supplemental pellet first to ensure they eat the whole pellet, then 5.000g of mouse chow. What remains of the 5.000g will be taken out and subtracted from the feed the next day when the mouse receives the next feeding. All the mice will be able to drink water throughout the day and night without restriction, and the total volume drank will be measured. The ingredients of the *M. oleifera* pellet will be as follows: 1) 2.0 g bleached & enriched cassava flour, peanut butter (a pinch for flavor), 3.32 mL DI water, and 0.070 g *M. oleifera* leaf powder. All animal experiments will be approved by the IACUC of Georgia State University and Appalachian State University and will conform to the National Institutes of Health (NIH) guidelines. Following the 3-week supplementation of *M. oleifera,* half of the mice from each group will participate in VWR for an additional 2-weeks to examine if *M. oleifera* increases endurance capacity [83]. During the VWR testing, all groups will continue respective treatments. The animals will be killed by CO_2 asphyxiation, and their gastrocnemius muscles dissected and either snap frozen or fixed immediately. Figure 5 demonstrates the timeline of treatment with *M. oleifera* or vehicle control, VWR, and harvesting of skeletal muscle tissues.

Volunteer Wheel Running

Following 3-weeks of *M. oleifera* or vehicle control supplementation, half of the mice from each treatment group will have access to a running wheel every night for 2-weeks. The mice will be separated into single cages that have a running wheel. The distance of each mice will be tracked nightly and the average distances of each treatment group will be calculated and compared using a student's t-test.

Quantitative RT-PCR

Total RNA will be extracted from the gastrocnemius muscle via homogenization using a RNeasy purification kit and treated with DNase, which will then be used for the reverse transcription protocol. Reverse transcription is the process of converting RNA to cDNA using a reverse transcriptase enzyme and dNTPs. Total RNA will be used to prepare cDNA to ensure a global cDNA is produced that is representative of all the RNA transcripts in the sample using random primers and the Superscript III first strand synthesis kit. Relative levels of mRNA will be determined by real-time quantitative PCR using an Applied Biosystems cycler and the TaqMan Universal PCR master mix. Taqman primers for 18s, PPARγ, PGC-1α, MYLRF, and VEGFa will be used. Table 3 lists the gene name, primer name, and forward and reverse gene sequences used to design the primers of interest. After amplification, data will be normalized to 18S levels and analyzed by delta Ct method. Triplicates will be included per group, and experiments will be repeated at least twice.

Immunohistomchemical Staining Procedure for Capillary Density

An indirect immunoperoxidase staining technique will be used to stain for collagen type IV. This method is based on antibodies directed against collagen type IV followed by development with secondary enzyme-labelled antibody and substrate. Collagen type IV is the only collagen present in the basement membrane, is only present in capillaries, and can be used to measure the capillary density. Muscles will be histologically processed to evaluate the degree of capillarity. Briefly, tissues will be harvested and embedded in OCT and immediately frozen in isopentane cooled in liquid nitrogen. The muscles will be cut transversely in serial sections at 10μm beginning at the mid-belly of the muscle. After tissues are sectioned using a cryostat, the sections will be fixed in acetone for 10 min. After fixing, the sections will be washed for 1 min in TBS buffer and then washed in PBS buffer for 5 min. The sections will be incubated in 10% w/v normal swine serum in PBS for 10 min and then incubated for 30 min with rabbit-anti-collagen IV antibody diluted in 10% human AB serum in PBS (1:7500) and then washed in PBS for 10 min. After the slides are wiped gently, they will be incubated for 30 min with peroxidase-labelled swine anti-rabbit IgG diluted in 10% v/v human AB serum in PBS (1:50). After washing for 5 min with PBS and 5 min with distilled water, the slides will be incubated with carbazole buffer for 10 min and washed for 10 min with distilled water. The sections will be mounted with Permount.

The stained slides will be viewed under a Leica light microscope at the magnification of 90x, photographed, and copied. An area with at least 100 fibers in each sample will be selected and used for the capillary counting. Transversely cut capillaries in the area will be counted. If a capillary is sectioned longitudinally, it will be counted as one each time it crossed a junction

between three or more fibers. Capillaries per fiber will be calculated as the number of capillaries in the area divided by the number of fibers in the area.

Statistics

Data will be presented as means ± SE. All experiments will be performed with independent tissue isolates from at least three different animals for each treatment condition. All measures or reactions will be performed in triplicate. A two-way ANOVA will be used to determine significant differences using SPSS. Significance will be accepted at $p < 0.05$.

Conclusion

This study aims to investigate the role of *Moringa oleifera* as a potential ergogenic aid. *M. oleifera* has shown to be a promising medicinal therapeutic in combating a variety of ailments, conditions, and diseases. Specifically, there is significant evidence that *M. oleifera* modulates metabolism and exerts anti-inflammation and -oxidant properties. There is currently no substantial evidence demonstrating the underlying mechanism of action of *M. oleifera* on skeletal muscle and its ergogenic aid effects. The current study is designed to evaluate the role of *M. oleifera* on skeletal muscle, specifically evaluating the effects on oxidative stress, metabolism, and endurance. We **hypothesize** that *M. oleifera* will increase endurance capacity in adult skeletal muscle via key metabolic signaling pathways. Furthermore, we aim to delineate the specific underlying mitochondrial signaling pathways associated with endurance capacity and fiber-type switching, as well as investigate the role of *M. oleifera* on glucose metabolism, angiogenesis, and microcirculation in adult skeletal muscle. **These specific aims will examine whether** *M. oleifera* **is an effective ergogenic aid, evaluate its use in the regulation of**

metabolism and circulation, and discern signaling pathways in skeletal muscle it may affect.

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CHAPTER 2

MORINGA OLEIFERA IMPROVES SKELETAL MUSCLE METABOLISM AND RUNNING PERFORMANCE IN MICE

Introduction

Moringa oleifera (*M. oleifera*) is a tropical and subtropical plant that contains a variety of phytochemicals and bioactive compounds harvested for its medicinal qualities, including anti-inflammatory, antibacterial, antioxidant, anti-cancer, hepatoprotective, and neuroprotective activities [1-3]. For example, *M. oleifera* has been used to treat inflammation in rats and it inhibits the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) in macrophage cell culture, key factors that drive the inflammatory response [4-6]. Further, pre-treatment with *M. oleifera* leaf extract prevented lipid peroxidation in the liver by effectively restoring available pools of glutathione (GSH) [6, 7]. While the mechanism of action of *M. oleifera* in most disorders is not fully understood, most research has demonstrated a significant impact on substrate metabolism.

Several studies have shown that *M. oleifera* has beneficial effects on carbohydrate metabolism, such as restoring the integrity and functionality of pancreatic β-cells, increasing insulin action, and improving glucose uptake and utilization [8, 9]. *M. oleifera* improves adipocyte functionality during adipogenesis and upregulates key signaling factors implicated in lipid metabolism, including uncoupling protein-1 (UCP1), sirtuin 1(SIRT1), and peroxisome proliferator receptor gamma coactivator 1 alpha (PGC-1α) [8]. Interestingly, PGC-1α is an important regulator of exercise-induced adaptations in skeletal muscle [10]. Further, mitochondrial transcription factor A (TFAM) and PGC-1α are thought to be master regulators of mitochondrial biogenesis in skeletal muscle following aerobic exercise [11, 12]. Within

mitochondria, PGC-1α can also increase content of enzymes associated with the Krebs Cycle, fatty acid oxidation, oxidative phosphorylation, and electron transport chain [13]. Collectively, these data suggest that *M. oleifera* may exert its effects intracellularly by modulating several metabolic pathways within the mitochondria, or by increasing total mitochondrial density [8]. While the majority of *M. oleifera* research quantifies adaptations in liver, kidney, or heart tissues, *M. oleifera* may exert similar effects in skeletal muscle.

Approximately 7% of the U.S. population takes herbal or botanical supplements to improve endurance and strength performance [14, 15]. For example, ginseng, a Chinese herb used for the past two centuries, stimulates brain function, enhances protein synthesis, and improves endurance performance in humans [14, 15]. Quercetin, a bioflavonoid isolate of *M. oleifera,* increased skeletal muscle PGC-1α expression, attenuated insulin resistance, minimized fat mass accumulation, and increased energy expenditure in rodents and humans [16]. Further, rats supplemented with *M. oleifera* had a delayed onset of fatigue and swam for longer periods of time during a forced swim test [17], suggesting that *M. oleifera* could be an effective ergogenic aid.

Increased endurance-like activity is also associated with increases in vascular endothelial growth factor (VEGF), which plays a vital role in stimulating angiogenesis and increasing capillary density in skeletal muscle [11]. Interestingly, *M. oleifera* supplementation upregulated the angiogenic marker, VEGF, in wounded tissue from diabetic rats [18]. Further, angiogenesis was stimulated in fertilized whit leghorn chicken eggs that received *M. oleifera* supplementation [19]. Together, these data suggest that *M. oleifera* supplementation stimulates vascular remodeling and may improve blood flow. However, the role *M. oleifera* supplementation might play in vascular remodeling in skeletal muscle and performance remains unknown.

Endurance-like activity may also promote skeletal muscle hypertrophy [20, 21]. Published research examining the effects of *M. oleifera* and the maintenance of skeletal muscle mass is limited. However, *M. oleifera* supplementation has been successfully used to improve skeletal muscle mass in atrophied muscles in an experimental model of malnutrition [22]. Furthermore, skeletal muscle atrophy was blunted in obese rats that received quercetin extract [23]. Importantly, skeletal muscles from these obese rats reported lower levels of atrogin-1, an E3 ligase associated with skeletal muscle atrophy. Taken together, these data suggest that *M. oleifera* may also play a role in altering markers of skeletal muscle protein balance, specifically by decreasing atrophic markers and protein catabolism.

While published research on *M. oleifera* may suggest it could be an effective ergogenic aid, a preliminary analysis on the basic adaptations to skeletal muscle structure, physiology, and performance, as well as potential alterations to metabolism is required. As such, we have designed unique experiments to evaluate the extent that *M. oleifera* supplementation affects energy metabolism and voluntary wheel running performance in mice. We hypothesized that *M. oleifera* would stimulate mitochondrial biogenesis by increasing gene expression of PGC-1α and PPARγ. We also hypothesized that increased mitochondrial content would increase succinate dehydrogenase (SHDB) and succinate-CoA ligase GDP/ADP-forming subunit alpha (SUCLG1), important enzymes in the oxidative phosphorylation pathway. Lastly, we hypothesize that *M. oleifera* would promote vascular health through increased expression of vascular endothelial growth factor (VEGF), a marker of angiogenesis, and endothelial nitric oxide (eNOS), a marker of vasodilation. Because compounds in *M. oleifera* have been shown to positively regulate protein balance [1, 5, 6, 8, 9, 17, 24, 25], we hypothesized that *M. oleifera* would increase fiber cross-sectional area (CSA) and alter expression levels of key factors implicated in protein

turnover such as atrogin-1 and myostatin. Together, if our hypotheses are supported, we will provide the initial data quantifying the impact of *M. oleifera* on muscle physiology in untrained subjects and establish a framework for future work to potentially identify the herb as an effective ergogenic aid.

Materials & Methods

Animals & Experimental Groups

Male C57BL/6 mice (~6-8 wks of age) were randomly assigned to one of the following four experimental groups (N = 10/group): (1) Vehicle control + Sedentary (Vehicle + SED), (2) *M. oleifera*-supplemented + Sedentary (*M. oleifera* + SED), (3) Vehicle + volunteer wheel running (Vehicle + RUN), or (4) *M. oleifera* + RUN. Effect size, power, and required sample size to attain a power of 0.8 at an alpha level of 0.05 have been calculated and suggested 10 animals per experimental group was required. Animals were housed in groups in a pathogen-free animal facility under a 12-12h light-dark cycle. Mice were fed normal chow *ad libitum* at 8:00 am every day, allowed free access to drinking water, and provided with supplemental pellets (vehicle or *M. oleifera*) at 5pm every evening. These supplemental pellets were tracked the following morning to assess complete consumption. Animals were weighed daily as a surrogate marker of total dietary intake. All experiments were approved by the Institutional

animal Care and Use Committee of Georgia State University and conformed to the National Institutes of Health (NIH) guidelines.

Moringa Oleifera Formulation and Treatment Plan

The ingredients of the *M. oleifera* pellet were as follows: 1) 2.0 g bleached & enriched cassava flour (Whole Foods, Atlanta, GA), organic peanut butter (a pinch for flavor) (Whole Foods, Atlanta, GA), 3.32 mL distilled water (Deer Park), and 0.10 g *M. oleifera* leaf powder (NC A & T University, Greensboro, NC), as previously described [26]. Pellets were formed manually, and all pellets were made the same size, containing approximately 0.10g of *M. oleifera* powder. Mice in the control-supplementation groups were fed pellets of similar size and contained the same ingredients, but that did not contain *M. oleifera*. Regardless of supplementation group, each cage of mice received one pellet per mouse (i.e., 5 pellets/cage) a day. The amounts of supplemental pellets consumed were tracked daily.

Mice received experimental pellets for a total of 5 weeks (See Figure 6). After 3-weeks of supplementation, half of the mice from each group were singly housed in a cage that allowed free access to food and water. Their cages were provided with a voluntary running wheel (RUN) for the final 2-weeks of the study protocol. All groups continued their experimental diets during the volunteer wheel running experiments. At the end of the experimental protocol, mice were euthanized by CO_2 asphyxiation followed by thoracotomy. Gastrocnemius, soleus, and plantaris muscles from one limb and the whole triceps surae group from the other limb were dissected and either snap frozen in liquid nitrogen or fixed in OCT compound immersed in melting isopentane for sectioning and subsequent histological analyses.

Volunteer Wheel Running

To assess the influence of *M. oleifera* on voluntary running activity, subgroups of vehicle supplemented, or *M. oleifera*-fed mice were maintained on their respective diet and provided free access to a running wheel (Super pet comfort wheel, 5.5" diameter, PetSmart, Inc., Phoenix, AZ). This wheel was equipped with a cycle monitor (Velo 8 computer, CatEye Co. LTD, Osaka, Japan) and was used to calculate total distance run over a 12-hour period. To accomplish this, animals were placed in these modified cages at 7pm every evening and cycle monitors were checked at 7am the next morning. Mice were returned to group housing during the day and reintroduced into the running cages on this daily schedule for the next 14 days.

Quantitative RT-PCR

Total RNA was extracted from the gastrocnemius muscle using a RNeasy Mini Kit per manufacturer's instructions (Qiagen, Germantown, MD). Total RNA was then used to prepare cDNA using random primers and the iScript cDNA Synthesis kit (Biorad, Hercules, CA). Relative levels of mRNA were determined by real-time quantitative PCR using an Applied Biosystems cycler (Applied Biosystems, Foster City, CA) and the iQ SYBR Green Supermix (Biorad, Hercules, CA). Primers for beta-actin, PPARγ, PGC-1α, MYLPF, VEGFa, PGAM-1, PGK-1, SDHB, SUCLG1, AKT, atrogin-1, and myostatin (Integrated DNA Technologies, Coralville, IA) were used (Table 5). Briefly, cDNA samples were incubated at 95°C for 15 min, followed by 40 cycles of denaturation, annealing, and extensions at 95°C, 60°C, and 72°C, respectively. Fluorescence was recorded at the end of each annealing and extension step. All reactions were performed in duplicate and the starting quantity of the gene of interest was

normalized to beta-actin rRNA for each sample. The delta-delta Ct method was used to analyze alterations in gene expression and values are expressed as total mRNA expression.

Muscle Histology (Fiber Cross-Sectional Area)

Triceps surae (TS) muscles were removed, embedded in OCT, and immediately frozen in isopentane cooled in liquid nitrogen. Muscles were cut in 10 μm serial sections maintained at -20°C using a cryostat (Leica CM1520, Buffalo Grove, IL). Sections were then processed for hematoxylin and eosin staining, dehydrated, mounted, and visualized at 20X with a Leica microscope. Cross-sectional areas of approximately 100 fibers per muscle were calculated using ImageJ software (NIH, Bethesda, MD).

Endothelial Nitric Oxide Synthase Immunoassay

eNOS protein content was examined in gastrocnemius skeletal muscle homogenates using a commercially available ELISA-based assay kit according to manufacturer's instructions (Abcam, Cambridge, MA). Briefly, frozen gastrocnemius muscles were washed, minced, and homogenized in ice-cold PBS. Homogenates were centrifuged for 20 min at 18,000g and supernatants were used for quantification of total eNOS protein content.

Statistical Analysis

An *a priori* power analysis was conducted using G*Power3.1.9.7 [27] to test the difference between four independent group means using an ANOVA test, a medium effect size ($d = 0.35$), and an alpha level of 0.05. Results indicated that a total sample of 40 mice with 4 equal sized groups of $N = 10$ were required to achieve a power of 0.80. Gene of interest (GOI)

mRNA expression were analyzed using a two-way ANOVA with factors of treatment (Vehicle and *M. oleifera*) and exercise (SED and RUN). Tukey correction factors were used to account for multiple pairwise comparisons. All data were analyzed and graphed using commercially available software (GraphPad Prism 8, San Diego, CA). Data are presented as mean + standard error. $P < 0.05$ was used for statistical significance.

Results

M. oleifera Improves Voluntary Wheel Running Performance

Over 14 days, the average daily voluntary wheel running distance was greater in *M. oleifera* mice compared to vehicle treated mice (4.21 + 0.16 vs. 3.63 +0.24 km, respectively; $p = 0.04$) (Figure 7B). Body weights and growth curves were similar between groups suggesting that 5 weeks of supplementation, regardless of experimental diet, did not body size or weight (Figure 7C).

M. oleifera Promotes Mitochondrial Biogenesis & Oxidative Capacity

We next examined the effects of *M. oleifera* and voluntary running activity on mitochondrial biogenesis. There was not a statistically significant interaction between the effects of voluntary running activity and *M. oleifera* supplementation on PGC-1α expression. *M. oleifera* + SED appeared to increase PGC-1α expression compared to vehicle + SED (3.22 + 0.87 vs. 1.05 + 0.68 AU, respectively; $p = 0.68$) (Figure 8A). Voluntary running (vehicle + RUN) increased the expression of PGC-1α compared to vehicle + SED (9.99 + 5.17 vs. 1.05 + 0.68 AU, respectively; $p = 0.11$).

PPARγ, a key regulator of mitochondrial biogenesis, was increased in the *M. oleifera* + SED mice compared to the vehicle + SED mice (7.15 + 2.18 vs. 1.81 + 0.92 AU, respectively; $p = 0.03$) (Figure 8B). PPARγ mRNA expression was also increased in the vehicle + RUN group compared to the vehicle + SED group (14.41 + 6.81 vs. 1.81 + 0.92 AU, respectively; $p = 0.01$). Expression of PPARγ was also increased in the *M. oleifera* + RUN group compared to the vehicle + SED group (8.53 + 2.62 vs. 1.81 + 0.92 AU, respectively; $p = 0.02$).

SDHB, an enzyme associated with Complex II of the Electron Transport Chain, appeared to increase in the *M. oleifera* + SED group compared to vehicle + SED (3.05 + 1.20 vs. 1.14 + 0.25 AU, respectively; $p = 0.36$) (Figure 8C). SDHB was also increased in the vehicle + RUN group (4.67 + 2.01 vs. 1.14 + 0.25 AU, respectively; $p = 0.09$). Gene levels of SUCLG1, an enzyme that regulates the formation of succinate and ATP from succinyl CoA and ADP or GDP, were unchanged regardless of group (Figure 8D).

M. oleifera Improves Vascular Health

Functional adaptations that improve running performance may also include angiogenesis and increased ability to deliver blood to working musculature while simultaneously removing metabolic byproducts. As such, we next investigated the effect of *M. oleifera* and exercise on markers of general vascular health by quantifying endothelial nitric oxide (eNOS) protein content and vascular endothelial growth factor (VEGF) gene levels. Protein content of eNOS was not altered in the *M. oleifera* + SED group compared to vehicle + SED group (3,793 + 410.6 vs. 4,569 + 379.4 pg/mL, respectively; $p = 0.68$) (Figure 9A). *M. oleifera* + RUN mice did not have an affect on protein content compared to vehicle + SED group (3,149 + 451.9 vs. 4,569 + 379.4 pg/mL, respectively; $p = 0.19$). Furthermore, eNOS protein content remained unaffected in the

vehicle + RUN group compared to the vehicle + SED group (3,605 + 797.0 vs. 4,569 + 379.4 pg/mL, respectively; $p = 0.70$).

There was not a statistically significant interaction between the effects of voluntary running activity and *M. oleifera* supplementation on VEGF (Figure 9B). Results indicate that VEGF appears to be increased in the *M. oleifera* + SED group compared to the vehicle + SED group (3.61 + 0.77 vs. 1.87 + 0.94 AU, respectively; $p = 0.21$). *M. oleifera* + RUN also increased VEGF expression compared to the vehicle + SED group (3.91 + 1.41 vs. 1.87 + 0.94 AU, respectively; $p = 0.38$). VEGF was increased in the vehicle + RUN group compared to vehicle + SED group (6.33 + 2.55 vs. 1.87 + 0.94 AU, respectively; $p = 0.22$).

Markers of Glycolysis and Fast-Twitch Fibers are Increased by M. oleifera

Because *M. oleifera* improved voluntary wheel running distance, we next analyzed the impact *M. oleifera* had on key components of glycolysis. PGAM-2, an enzyme that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis appears to be increased in the *M. oleifera* + SED group compared to vehicle + sed group (4.39 + 1.02 vs. 2.10 + 1.09 AU, respectively; $p = 0.07$) (Figure 10A). PGAM-2 was increased in the *M. oleifera* + RUN group compared to the vehicle + SED group (3.51 + 0.77 vs. 2.10 + 1.09 AU, respectively; $p = 0.16$). Vehicle + RUN mice also had an increase in PGAM-2 compared to the vehicle + SED group (6.58 + 2.76 vs. 2.10 + 1.09 AU, respectively; $p = 0.10$) (Figure 10A).

PGK-1, which produces ATP and 3-phosphoglycerate was increased in the *M. oleifera* + SED group compared to vehicle + SED group (3.32 + 0.59 vs. 1.35 + 0.45 AU, respectively; $p = 0.04$) (Figure 10B). *M. oleifera* + RUN mice had an increase in PGK-1 compared to the vehicle + SED mice (3.45 + 0.59 vs. 1.35 + 0.45 AU, respectively; $p = 0.04$). PGK-1 expression was also

increased in the vehicle + RUN group compared to the vehicle + SED group (5.83 + 2.64 vs. 1.35 + 0.45 AU, respectively; $p = 0.04$).

To examine the fiber-type profile in the gastrocnemius muscle in mice, we measured the mRNA expression of myosin regulatory light chain 2, skeletal muscle isoform (MYLPF). Results indicate that MYLPF is increased in the *M. oleifera* + SED group compared to the vehicle + SED group (5.70 + 0.88 vs. 1.67 + 0.77 AU, respectively; $p < 0.01$) (Figure 10C). *M. oleifera* + RUN mice had an increase in MYLPF expression compared to vehicle + SED mice (7.50 + 2.63 vs. 1.67 + 0.77 AU, respectively; $p < 0.01$). MYLPF expression was increased in the vehicle + RUN group compared to the vehicle + SED group (5.73 + 1.96 vs. 1.67 + 0.77 AU, respectively; $p = 0.02$).

Cross-Sectional Area is Augmented by M. oleifera

Because *M. oleifera* increased voluntary running activity and altered metabolism, we next analyzed its potential role on triceps surae muscle fiber cross-sectional area (CSA). Results indicate that CSA is greater in the *M. oleifera* + SED (253.2 + 15.04 μm^2, $p < 0.01$) and *M. oleifera* + RUN (278.0 + 18.24 μm^2, $p < 0.01$) groups compared to both the vehicle + RUN (174.9 + 22.11 μm^2) and the vehicle + SED (172.2 + 6.82 μm^2) groups (Figure 11A). Representative histological images are shown in Figure 11B.

M. oleifera Decreases Markers of Atrophy

We showed that *M. oleifera* was associated with larger fiber areas, therefore; we next analyzed key signaling components associated with protein synthesis and degradation. Protein kinase B (AKT) is a serine/threonine-specific protein kinase shown to play a vital role in many

cellular processes including regulation of skeletal muscle mass [28, 29]. Results indicate that AKT is increased in the vehicle + RUN group compared to the vehicle + SED group (5.40 + 1.14 vs. 2.33 + 1.13 AU, respectively; $p = 0.01$). There appears to be little to no change in AKT expression in the *M. oleifera* + SED (1.81 + 0.38 vs. 2.33 + 1.13 AU, respectively; $p = 0.94$) and *M. oleifera* + RUN (2.18 + 0.63 vs. 2.33 + 1.13 AU, respectively; $p = 0.84$) groups compared to the vehicle + SED group (Figure 12A).

In contrast, gene levels of atrogin-1, an E3 ligase associated with proteasomal degradation, had no statistically significant interaction between the effects of voluntary running activity and *M. oleifera* supplementation. Results indicate that atrogin-1 appears to be decreased in the *M. oleifera* + SED (0.83 + 0.18 vs. 1.21 + 0.33 AU, respectively; $p = 0.48$) and *M. oleifera* + RUN (0.61 + 0.10 vs. 1.21 + 0.33 AU, respectively; $p = 0.53$) groups compared to the vehicle + SED group (Figure 12B).

Furthermore, gene levels of myostatin, an inhibitor of muscle cell growth and differentiation, had a statistically significant interaction between the effects of voluntary running activity and *M. oleifera* supplementation. Results indicate that myostatin expression appears to be unaffected in the *M. oleifera* + SED group compared to the vehicle + SED group (1.44 + 0.47 vs. 3.31 + 2.07, respectively; $p = 0.99$). Expression of myostatin in the *M. oleifera* + RUN group appears to be unchanged compared to the vehicle + SED group (1.63 + 0.37 vs. 3.31 + 2.07 AU, respectively; $p = 0.98$). Myostatin expression appears to decrease in the vehicle + RUN group compared to the vehicle + SED group (0.50 + 0.32 vs. 3.31 + 2.07 AU, respectively; $p = 0.11$). Myostatin was also significantly less in the vehicle + RUN group compared to *M. oleifera* + RUN group (0.50 + 0.32 vs. 1.63 + 0.37 AU, respectively; $p = 0.02$) (Figure 12C).

DISCUSSION

The main findings of this study suggest that *M. oleifera* may be a potential ergogenic aid and associated with: 1) increased endurance capacity by stimulating oxidative metabolism and mitochondrial biogenesis; 2) improved glycolytic metabolism; 3) enhanced vascular health; and 4) decreased markers of protein degradation and increased fiber cross-sectional area. To our knowledge, this is the first study that explored the impact of *M. oleifera* supplementation on skeletal muscle physiology, metabolism, and performance.

M. oleifera Increases Oxidative Capacity

Our data showed that after 5 weeks of *M. oleifera* supplementation several genes associated with oxidative phosphorylation (PGC-1α, PPARγ, SDHB, SUCLG1) and glycolysis (PGAM-2, PGK1) were increased. Together, these improvements in energy metabolism likely influenced voluntary running distance during the final two weeks of supplementation. In concert with our data, rats supplemented with *M. oleifera* for 4 weeks swam significantly longer compared to the vehicle group suggesting that *M. oleifera* may improve fatigue resistance [17].

Because supplemented mice ran longer distances, we next examined the impact of *M. oleifera* on markers of mitochondrial biogenesis. Mitochondrial biogenesis involves the expansion of pre-existing mitochondria, a process dependent on the coordination of nuclear and mitochondrial-encoded gene expression [11, 30]. In response to exercise, PPARγ can be stimulated through the induction of PGC-1α, which when activated interacts with nuclear respiratory factors (NRFs) to promote mitochondrial biogenesis [31-34]. In concert with previous work, our data showed a significant increase in PGC-1α mRNA expression in mice with free access to a running wheel compared to sedentary controls. Interestingly, PGC-1α levels also

increased in mice supplemented with *M. oleifera,* suggesting that supplementation alone may be enough to drive mitochondrial biogenesis in the absence of increased physical activity. However, more research is required to truly define *M. oleifera* as a potential exercise mimetic.

In addition to expanding the pool of mitochondria, increased voluntary activity also increases the content of oxidative enzymes in mitochondria [35, 36]. Accordingly, we measured gene expression levels of two proteins associated with oxidative phosphorylation, succinate dehydrogenase B (SDHB) and succinate CoA ligase 1 (SUCLG). SDHB is an enzyme complex involved in the Krebs cycle and electron transport chain that oxidizes succinate to fumarate [37, 38]. In agreement with our results, exercise was shown to attenuate SDHB expression in mouse muscle after disuse [39]. Furthermore, exercise also increased citrate synthase, another enzyme active in the Krebs cycle [39]. As expected, we showed a significant increase in SDHB in the RUN group. Surprisingly, *M. oleifera* also increased SDHB mRNA expression in the absence of increased physical activity suggesting similar improvements to oxidative metabolism. Increases in citric acid cycle intermediates, like SUCLG and SDHB, have been associated with higher peak VO_2 during acute exercise and faster marathon times [40-42]. Together, these genes are critical in maintaining oxidative metabolism during prolonged exercise [40-42]. As expected, our data showed that SUCLG1 mRNA was increased in the RUN and *M. oleifera* groups compared to the vehicle + SED group. This data suggests that *M. oleifera* may induce the same outcomes as running, possibly promoting oxidative metabolism.

M. oleifera Promotes VEGF-Induced Oxidative Capacity

With increased physical activity, vascular endothelial growth factor (VEGF) plays a central role in stimulating angiogenesis and increasing capillary density in skeletal muscle [11].

Furthermore, an increase in VEGF has been associated with both improved running speed and time to fatigue during endurance events [11]. In response to an 8-wk treadmill running program, VEGF-null skeletal myofibers of mice had diminished exercise capacity and decreases in markers of oxidative capacity (citrate synthase and beta-hydroxyacyl CoA dehydrogenase) [43]. Moreover, maximal running speed was unchanged in the VEGF-null mice, but increased in the control mice [43]. Also, VEGF-null mice exhibited a 30% reduction in the time to exhaustion compared with untrained, VEGF-positive mice [43]. Most importantly, the VEGF-null mice did not improve endurance capacity, but the VEGF-positive mice increased the time to reach exhaustion by 99% [43]

Here, mice supplemented with *M. oleifera* had greater VEGF expression compared to vehicle control, which could explain, in part, why *M. oleifera*-supplemented mice voluntarily ran farther distances and had increased markers of oxidative phosphorylation. Multiple groups have reported that the formation of new capillaries within the muscle depends on dynamic cell to cell interactions [44-47]. Deletion of the VEGF gene from skeletal myofibers was shown to not affect capillarity in skeletal muscle [43, 44]. In VEGF-null gastrocnemius muscles fatigue onset was faster, there was no reduction in capillarity nor evidence of muscle contractile or metabolic dysfunction, which suggests a VEGF-dependent change in vascular reactivity and perfusion of the capillaries [44]. More importantly, the results suggest that VEGF may play a critical role in regulating oxygen delivery to the muscles [44]. These findings are in concert with our data. VEGF was increased with running, but more importantly *M. oleifera* was also able to induce VEGF expression.

Skeletal muscle microcirculation is an important regulator of health and disease [48]. It also plays a critical role in exercise because it matches perfusion with metabolic demands [48].

A reduction in exercise-induced perfusion could be due to limited nitric oxide (NO) secondary to a decrease in VEGF levels in skeletal muscle [44]. Ideally then, VEGF-induced eNOS would increase NO release from endothelial cells [44, 49]. Although there were no changes in eNOS activity in the current work, running or *M. oleifera* supplementation may still increase vasodilation or blood flow through the release of prostaglandins, endothelium-derived hyperpolarizing factor, or AMPK [44, 50-52].

Glycolytic Activity was Increased by M. oleifera-Supplementation

Not only were oxidative related genes increased in the skeletal muscle of mice treated with *M. oleifera*, but enzymes associated with glycolysis were also induced, such as phosphoglycerate kinase-1 (PGK1) and phosphoglycerate mutase (PGAM2). During the first few minutes of exercise, these enzymes are important for the generation of ATP. Upregulation of genes involved in muscle glucose uptake and muscle glycolysis, including PGK1, suggests a compensatory response to maintain energy homeostasis and could lead to an increase in carbohydrate oxidation [53]. Healthy skeletal muscle has flexibility to switch between fatty acid and carbohydrate metabolism depending on substrate availability and exercise intensity [53, 54]. Furthermore, athletes and sports nutritionists routinely attempt to improve sports performance by manipulating fuel metabolism in concert with exercise training [54]. Our data shows that *M. oleifera* has the potential to promote markers of glycolysis (PGK1 and PGAM2), as well as markers of oxidative metabolism (SUCLG1 and SDHB), which may suggest an increase in metabolic flexibility.

Alternatively, changes in both oxidative and glycolytic markers may be because endurance exercise is more dependent on oxidative type muscle fibers (Type I and IIa) [55].

Furthermore, the recruitment of glycolytic muscle fibers (Type IIb and IIx) during endurance training may cause a "metabolic challenge" that leads to the induction of transient changes in gene expression [55]. These transient changes could contribute to alteration in muscle phenotype that could result in a more oxidative muscle [55]. When comparing our data to other studies, several variables may influence the gene induction response to exercise [55]. For example, we did not measure our gene response after one bout of exercise (acute), rather than analyzing changes following two weeks of voluntary wheel running activity. We also did not monitor the time of food intake (pre- or post-exercise). Feeding is an important consideration because fasting and feeding have both been shown to alter metabolic gene expression [55, 56]. For example, during short-term fasting, substrate utilization in skeletal muscle switches from carbohydrate to fat in order to conserve glucose [56]. After 20-h of fasting, transcription of pyruvate dehydrogenase was increased nine-fold compared to control, but was enhanced following refeeding [56].

We analyzed whole gastrocnemius muscles for gene levels of fast skeletal myosin light chain (MYLPF). Running and *M. oleifera* increased the expression of MYLPF, suggesting an increase in fast fiber phenotype. Different types of exercise have been shown to induce differential expression of myosin protein isoforms in skeletal muscle [57, 58]. Most studies have focused on the composition of myosin heavy chain (MHC) because of its role in myosin ATPase activity and in the velocity of muscle fiber shortening [58, 59]. It is not well understood how exercise of different intensity and duration may affect the changes in the relative content of MLC isoforms and their distribution in different muscles [58]. However, it has been suggested that myosin light chains are necessary for full force development and shortening velocity [60, 61]. In support of our data, 5-weeks of endurance training has been shown to increase the content of

MYLPF in fast twitch muscles [58]. Furthermore, resistance training lead to an increase in MYLPF in the soleus muscle suggesting a transition toward a faster phenotype [58]. Based on these findings, our data may suggest that an increase of MYLPF could be aiding in force development and shortening velocity. However, due to the lack of specificity in isoform analysis, it is difficult to conclude the exact affect an increase in MLC-fast isoform may be having in the gastrocnemius muscle. Further studies will need to be performed to determine the effects *M. oleifera* may have on fiber-type phenotype.

Protein Degradation is Decreased by M. oleifera-Supplementation

While aerobic training and increased endurance-like activity are clearly associated with improvements in metabolism and refinement to substrate utilization, emerging research suggests these types of activities may promote skeletal muscle hypertrophy [20, 21]. To assess this, we analyzed key factors implicated in the regulation of muscle mass. Atrogin-1 is an E3 ligase involved in the ubiquitin proteasome pathway (UPP), which is responsible for most of the intracellular protein degradation in skeletal muscle [20]. Further, myostatin is also involved in the UPP, but is also a potent inhibitor of muscle growth due to its role in satellite regulation, muscle protein synthesis, and augmenting muscle protein breakdown [20]. Interestingly, both factors were decreased with *M. oleifera* supplementation. Together, this suggests that *M. oleifera* may increase net protein accretion by possibly decreasing the content of common catabolic factors. Similarly, chronic exercise training decreases atrogin-1 expression, which has preventative effects against muscle atrophy [62-64]. Both acute and repeated bouts of aerobic exercise have been shown to reduce myostatin mRNA expression, leading to increased muscle protein synthesis, myofiber and whole muscle hypertrophy, increased protein turnover, and

myocellular remodeling [65, 66]. In concert with these findings, we showed decreased atrogin-1 and myostatin mRNA expression, as well as an increase in fiber CSA, suggesting that *M. oleifera* is associated with promoting hypertrophy and attenuating muscle atrophy.

In summary, this is the first study to investigate the effects of *M. oleifera* on energy metabolism, vascular health, protein synthesis/degradation, and fiber-type profiles in mouse skeletal muscle. This is evidenced by the facts that *M. oleifera* increased both glycolytic and oxidative metabolic genes, as well as increased markers of mitochondrial biogenesis and the MLC fast-type fiber phenotype gene, MYLPF. Further, *M. oleifera* decreased the expression of genes associated with protein degradation and promoted an increase in fiber CSA. Although further studies are required, our data suggest that *M. oleifera* supplementation may confer benefits to basal muscle metabolism, increase the performance or duration of voluntary activity, and, therefore, be considered as a possible ergogenic aid.

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APPENDICES

Appendix A: Chapter 1 Tables and Figures

Table 1. **Skeletal Muscle Fiber Types.** A comparison of slow-twitch (type I) and fast-twitch (type II) skeletal muscle fibers.

	Type I	Type IIa	Type IIx	Type IIb
Contraction time	Slow	Moderately Fast	Fast	Very Fast
Size of motor neuron	Small	Medium	Large	Very large
Resistance to fatigue	High	Fairly High	Intermediate	Low
Activity Used for	Aerobic	Long-term anaerobic	Short-term anaerobic	Short-term anaerobic
Power Produced	Low	Medium	High	Very High
Mitochondrial density	High	High	Medium	Low
Capillary density	High	Intermediate	Low	Low
Oxidative Capacity	High	High	Intermediate	Low
Glycolytic Capacity	Low	High	High	High
Myosin Heavy Chain (human genes)	*MYH7*	*MYH2*	*MYH1*	*MYH4*

Table 2. Extract Methods & Phytoconstituents of *M. oleifera* [69]. Various types of solvents have been used to extract a set of desired bioactive compounds for research and therapy, the most common include aqueous, ethanolic, and methanolic. The type of phytochemical constituents extract from *M. oleifera* varies based on the part of the plant used.

Plant Part	Extract	Phytoconstituents	Potential Therapeutic & Ergogenic Effect
Leaves	Aqueous & alcoholic	Niazirin and Niazirinin – nitrile glycosides,4-[(4'-O-acetylalpha-L-rhamnosyloxy) benzyl isothiocyanate, Niaziminin A, Niaziminin B, three mustard oil glycosides, niaziminin, a thiocarbamate,4-alpha-1-rhamnopyranosloxy)-benzylglucosinolate, quercetin-3-O-glucoside and quercetin-3-O-(6"-Malonyl-glucoside), Niazimicin. Pyrrole alkaloid (pyrrolemarumine 400-O-a-L-rhamnopyranoside) and 40-hydroxypheylethenamide (marumoside A and B) 4 alpha and gamma-tocopherol.2	Hypotensive effect
Seeds	Aqueous & Hydro-alcoholic	Methionine, cysteine, 4-(alpha-L-rhamnopyranosyloxy) benzylglucosinolate, Moringine, benzylglucosinolate, niazimicin niazirin	Metabolism & Angiogenesis
Pods	Hydro-alcoholic	Isothiocyanate, nitrites, thiocarbamates, O-(1heptenyloxy) propyl undecanoate, O-ethyl-4(alpha-L-rhamnosyloxy) benzyl carbamate, methyl-p-hydroxybenzoate, beta-sitosterol.	Effects Nrf2 Activity
Bark	Alcoholic	4-(alpha-L-rhamnopyranosyloxy) benzyl glucosinolate.	Unknown
Flowers	Hydro-alcoholic	D-glucose, quercetin, isoquercetin, kaempferol, kaempferitin and ascorbic acid, protein, D-mannose.	Metabolism, Anti-inflammatory, Antioxidant
Root	Alcoholic	Moringine, moringinine, spirachin, 1,3-dibenzyl urea, alpha-phellandrene, p-cymene, Deoxy-niazimicine, 4-(alpha-L-rhamnopyranosyloxy) benzylglucosinolate.	Unknown
Stem	Aqueous and Hydro-alcoholic	4-hydroxyl mullein, vanillin, octacosonoic acid, beta-sitosterone and beta-sitosterol.	Anti-inflammatory, Antioxidant, Anti-hyperlipidemia

Table 3. P**harmacological Activities of *M. oleifera*** [69]. A representation of the pharmacological and medicinal uses of *M. oleifera* comparing different parts of the plant and extraction methods.

Part of Plant	Extract	*In-vitro/In-vivo*	Activity	Standard Use
Leaves & Roots	Aqueous and alcoholic extracts	*In-vitro*	Anti-oxidant & radical scavenging activity	-
Leaves	Methanolic extract	*In-vivo*	Antiepileptic activity & anti-convulsant activity	Pentylenetetrazole
Leaves	Aqueous extract	*In-vivo*	Anti-diabetic activity	Streptozotocin
Leaves	Ethanolic extract	*In-vivo*	Cardiovascular activity/anti-hypertensive	-
Roots	Aqueous extract	*In-vivo*	Anti-fertility activity	-
Bark	Aqueous and alcoholic extracts	*In-vitro*	Anti-urolithiatic activity	Ethylene glycol
Seed Kernel	Alcoholic extract	Human Study	Anti-asthmatic activity	-
Leaves & Seeds	Ethanolic Extract	*In-vivo*	Hepatoprotective activity	-
Leaves & Seeds	Ethanolic Extracts	*In-vivo*	Anti-cancer activity	-
Root, Bark, leaves, flowers, seeds, stalks	Methanolic & aqueous	*In-vitro*	Anti-inflammatory activity	Carrageen
Leaves	Macerated & infused aqueous extract, & ethanolic extract	*In-vitro*	Anthelminitic activity	Piperazine citrate
Leaves	Aqueous extract	*In-vitro*	CNS activity	Penicillin

Table 4. Gene Sequences. The gene name, primer name, forward & reverse sequence, and the signaling pathway the GOI is involved in.

Gene Name	Primer Name	Forward Sequence	Reverse Sequence	Signaling Pathway
Peroxisome proliferator-activated receptor gamma	PPARγ	AGTACTGTCGGTTTCGAAG	GGGAAGGACTTTATGTATGAG	Mitochondrial Biogenesis; Angiogenesis
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1α	TCCTCTTCAAGATCCTGTTAC	CACATACAAGGGAGAATTGC	Mitochondrial Biogenesis; Angiogenesis
Myosin light chain, phosphorylatable, fast skeletal muscle	MYLPF	CATCAACCTTCACTGTCTTCC	ATGTTCTTGATCTCCTCCTG	Endurance Capacity (Fiber type "switching")
Vascular Endothelial Growth Factor	VEGFa	TAGAGTACATCTTCAAGCCG	TCTTTCTTTGGTCTGCATTC	Angiogenesis
Kelch-like ECH-associated protein-1	KEAP1	GGCTAATTGAGTTCGCCTACAC	GCAGTGTGACAGGTTGAAGAA	Mitochondrial Biogenesis
Nuclear factor (erythroid-derived 2)-like 2	NRF-2	AGAGCACTCTGTGGAGCTTCC	AGGCTGTACTGTATCCCCAGAA	Mitochondrial Biogenesis

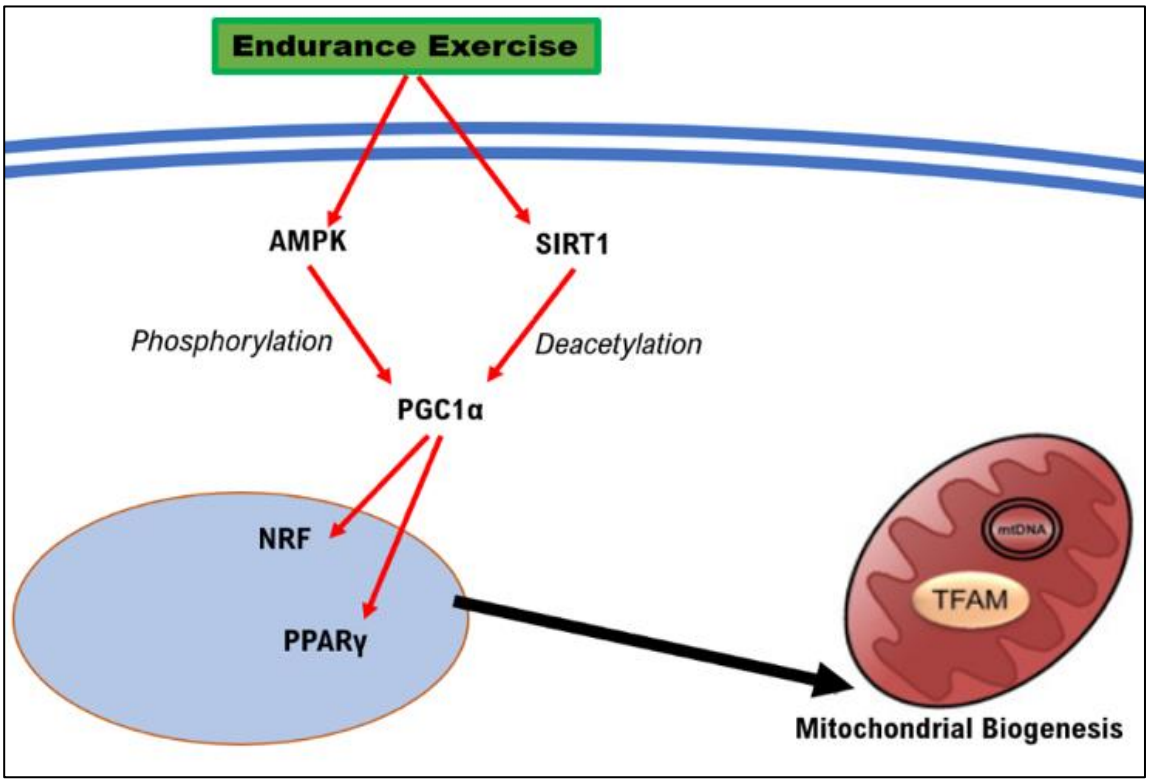

Figure 1. **Molecular Signaling Pathway of Endurance Exercise-Induced Mitochondrial Biogenesis**. AMP-activated protein kinase (AMPK), Sirtuin (SIRT1), Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC-1α), Nuclear Respiratory Factor (NRF), Peroxisome Proliferator-Activated Receptor Gamma (PPARγ), Transcription Factor A Mitochondrial (TFAM)

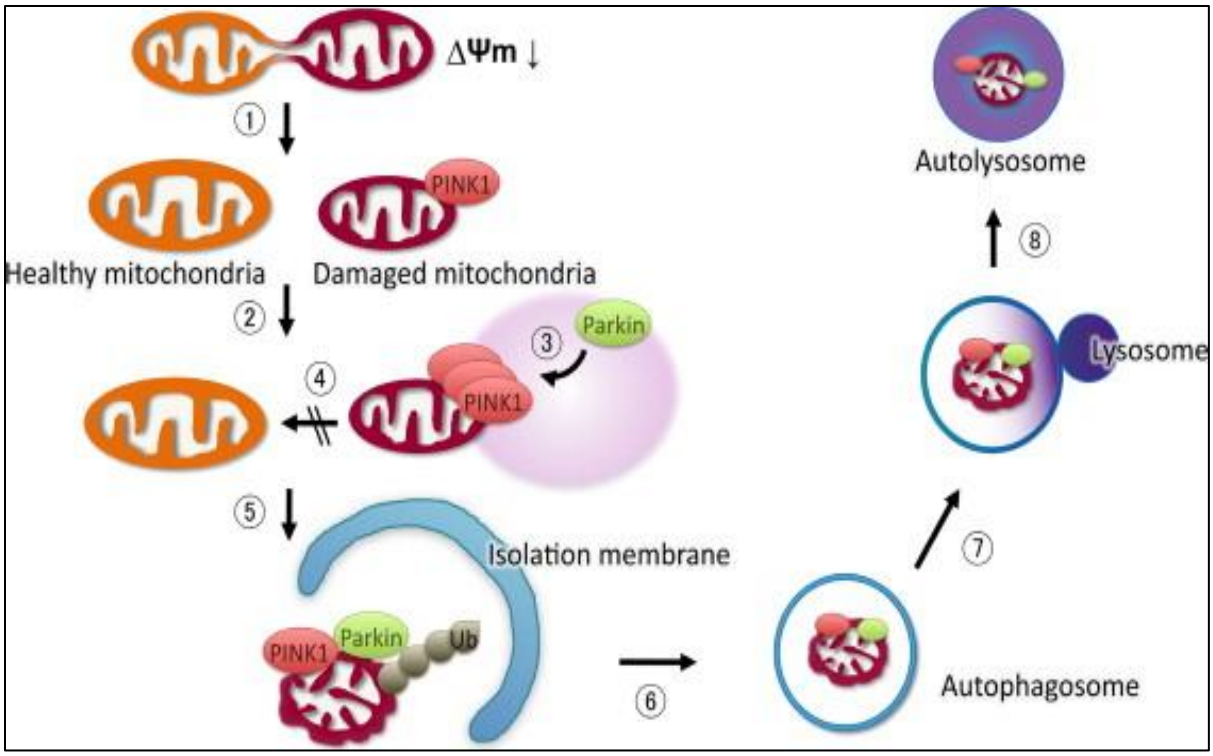

Figure 2. **Mitophagy PINK1-PARKIN pathway of mitochondrial cells [1].** Fission and fusion of mitochondrial cells is crucial in maintaining healthy mitochondrial populations and cell function. The most studied pathway of mitophagy involves PTEN-induced putative kinase protein 1 (PINK1) and PARKIN (component of a multiprotein E3 ubiquitin ligase complex). These proteins relay the signals associated with damage to the induction of mitophagy. As the mitochondrial inner membrane becomes depolarized, PINK1 stabilizes and recruits PARKIN from the cytosol onto the mitochondrial outer membrane. This process leads to polyubiquitination of the mitochondrial outer membrane proteins and mitophagy.

Figure 3. **Plant of *M. oleifera.*** Moringaceae is a tropical and subtropical plant species cultivated around the world, especially in sub-Himalayan regions of Northwest India. It can grow to heights of up to 40 feet, with a trunk diameter of 1.5 feet.

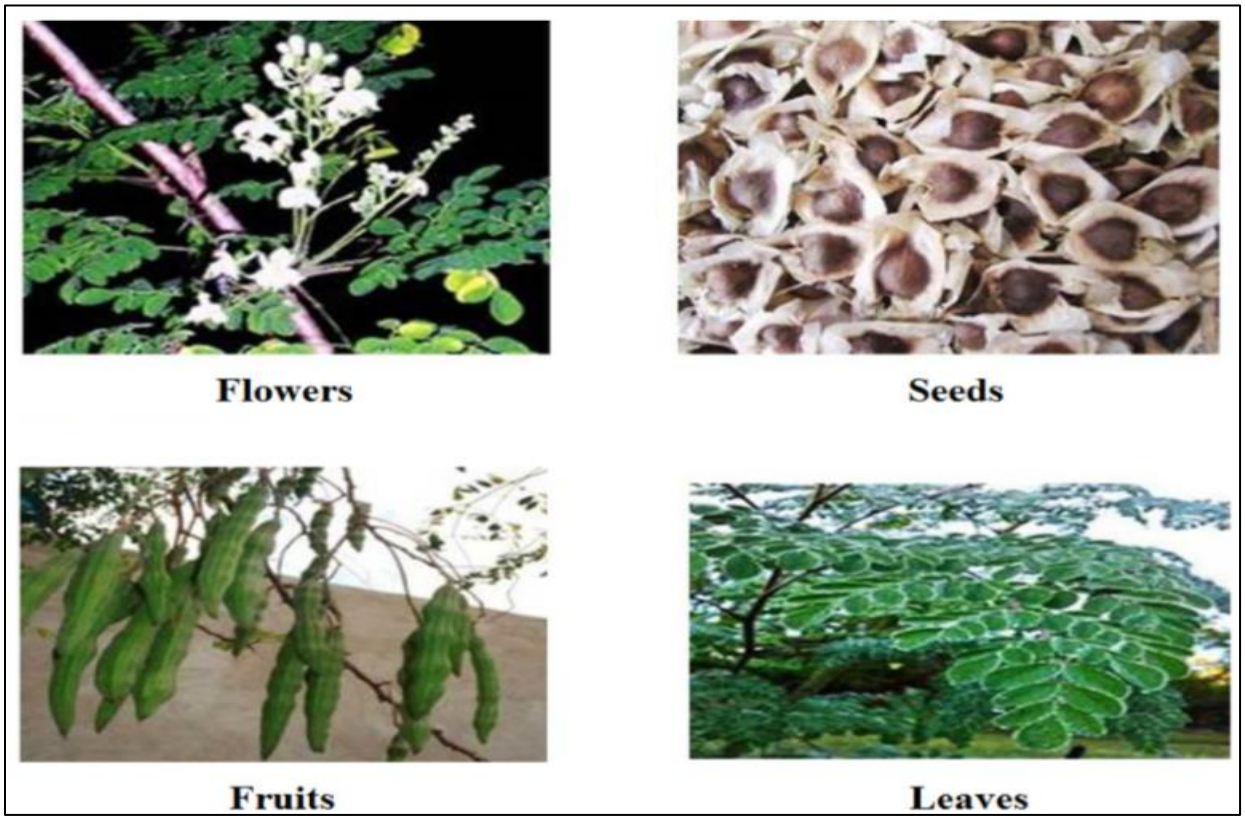

Figure 4. **Plant parts of *M. oleifera.*** The flowers of *M. oleifera* contain yellowish-white petals, are fragrant, and asexual. The fruit looks like giant bean-pods about 20-45 cm long. The seeds are found within the fruit and are dark brown with a diameter around 1 cm. The seeds are dispersed by wind and water. The plant is trimmed and cut down annually to about 3-6 ft to increase its leaf biomass.

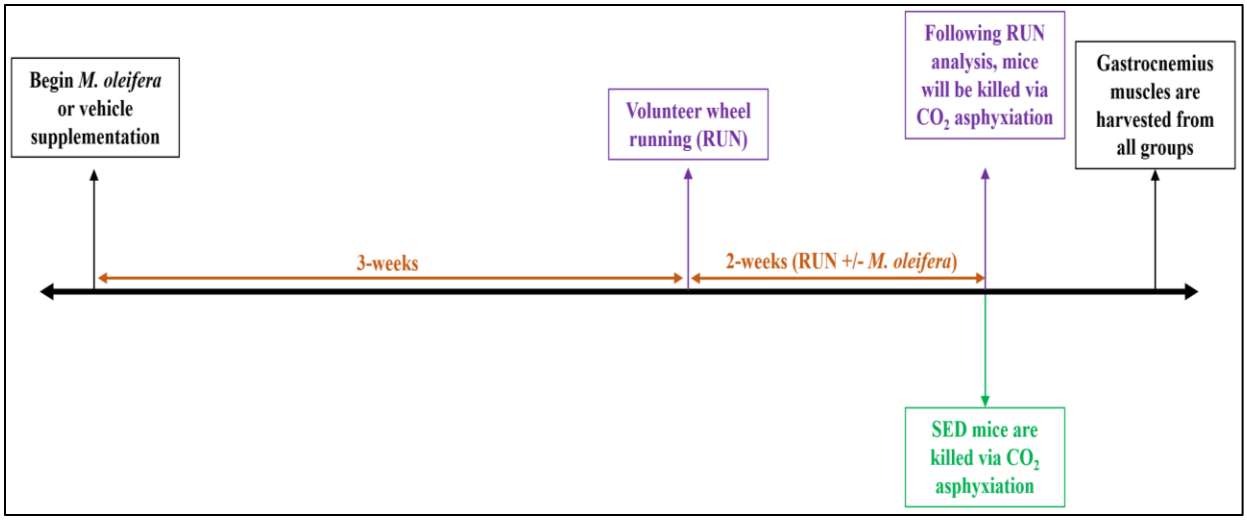

Figure 5. Proposed Experimental Design. This shows the proposed schematic of a 5-week *M. oleifera* supplementation and 2-week exercise protocol.

Appendix B: Chapter 2 Tables and Figures

Table 5. Gene Sequences. The gene name, primer name, forward & reverse sequence, and the signaling pathway the GOI is involved in.

Gene Name	Primer Name	Forward Sequence	Reverse Sequence	Signaling Pathway
Peroxisome proliferator-activated receptor gamma	PPARγ	AGTACTGTCGGTTTCGAAG	GGGAAGGACTTTATGTATGAG	Mitochondrial Biogenesis; Angiogenesis
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1α	ATGTGTCGCCTTCTTGCTCT	ATCTACTGCCTGGGGACCTT	Mitochondrial Biogenesis
Myosin light chain, phosphorylatable, fast skeletal muscle	MYLPF	CATCAACCTTCACTGTCTTCC	ATGTTCTTGATCTCCTCCTG	Endurance Capacity (Fiber-type "switching")
Vascular endothelial growth factor	VEGFa	TAGAGTACATCTTCAAGCCG	TCTTTCTTTGGTCTGCATTC	Angiogenesis
Phosphoglycerate mutase 1	PGAM-1	ACACCTCCATCAGCAAGGAC	GGGCTGCAATAAGCACTCTC	Glycolysis
Phosphoglycerate kinase 1	PGK1	GAAGGGAAGGGAAAAGATGC	TCAAAAATCCACCAGCCTTC	Glycolysis
Succinyl-CoA Ligase (GDP-forming) subunit alpha	SUCLG1	GGGATGACCGCAACAGTAGT	AGGTACCCTGTTTGCCTGTG	Krebs Cycle
Succinate dehydrogenase	SDHB	ACTGGTGGAACGGAGACAAG	GTTAAGCCAATGCTCGCTTC	Krebs Cycle
Protein kinase B	AKT	CCCTTCTACAACCAGGACCA	ATACACATCCTGCCACACGA	Protein Synthesis/Degradation
Atrogin-1	Atrogin-1	CTCTGCTGTGAGTGCCACAT	CAATGAGCCTGGGTACCACT	Protein Synthesis/Degradation
Myostatin	Myostatin	TGCAAAATTGGCTCAAACAG	GCAGTCAAGCCCAAAGTCTC	Protein Synthesis/Degradation

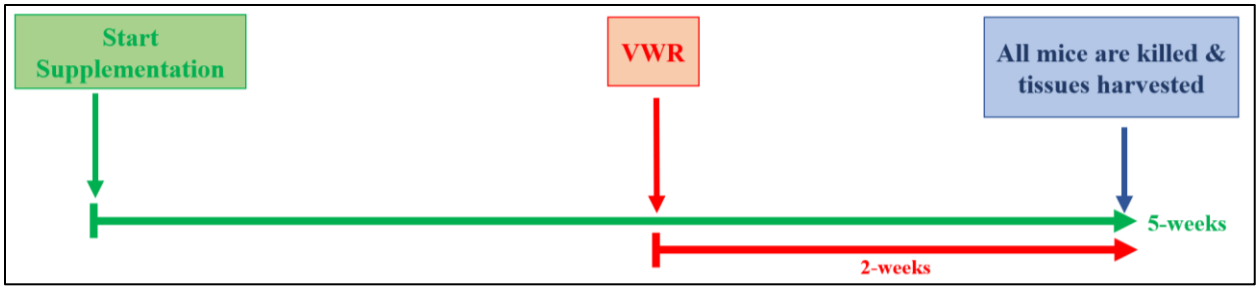

Figure 6. **Experimental Design.** This shows the schematic of a 5-week *M. oleifera* supplementation and 2-week exercise protocol.

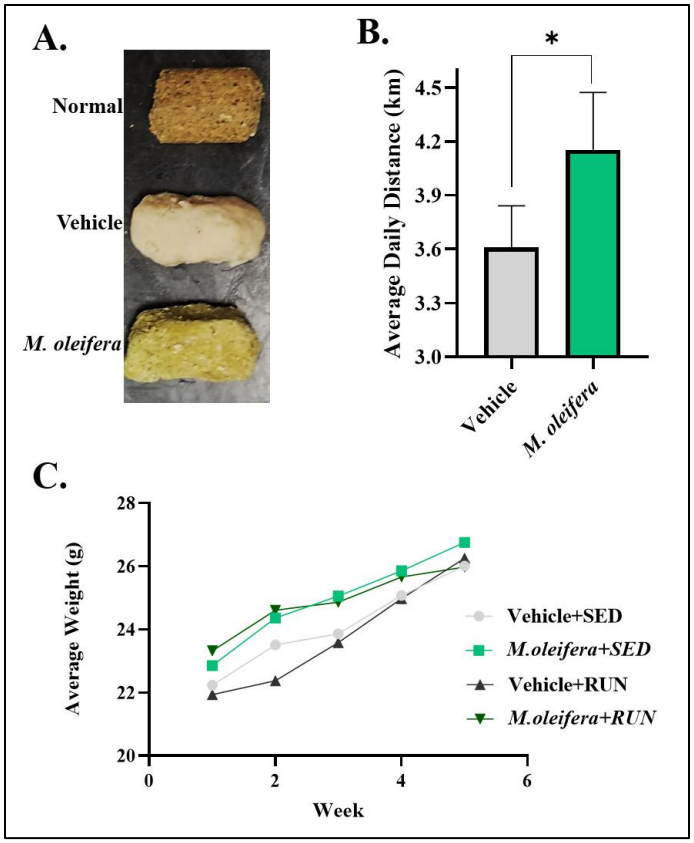

Figure 7. *M. oleifera* improves voluntary wheel running performance. (A) Representative images of normal mouse chow, vehicle pellets, and *M. oleifera* pellets. (B) Voluntary wheel running distance was significantly longer in *M. oleifera*-treated mice compared to vehicle-treated mice. Data are expressed as means + standard error. (C) Body weights and growth curves were similar between groups suggesting that 5 weeks of supplementation, regardless of experimental diet, did not affect caloric intake nor appetite. *$p < 0.05$ compared with vehicle.

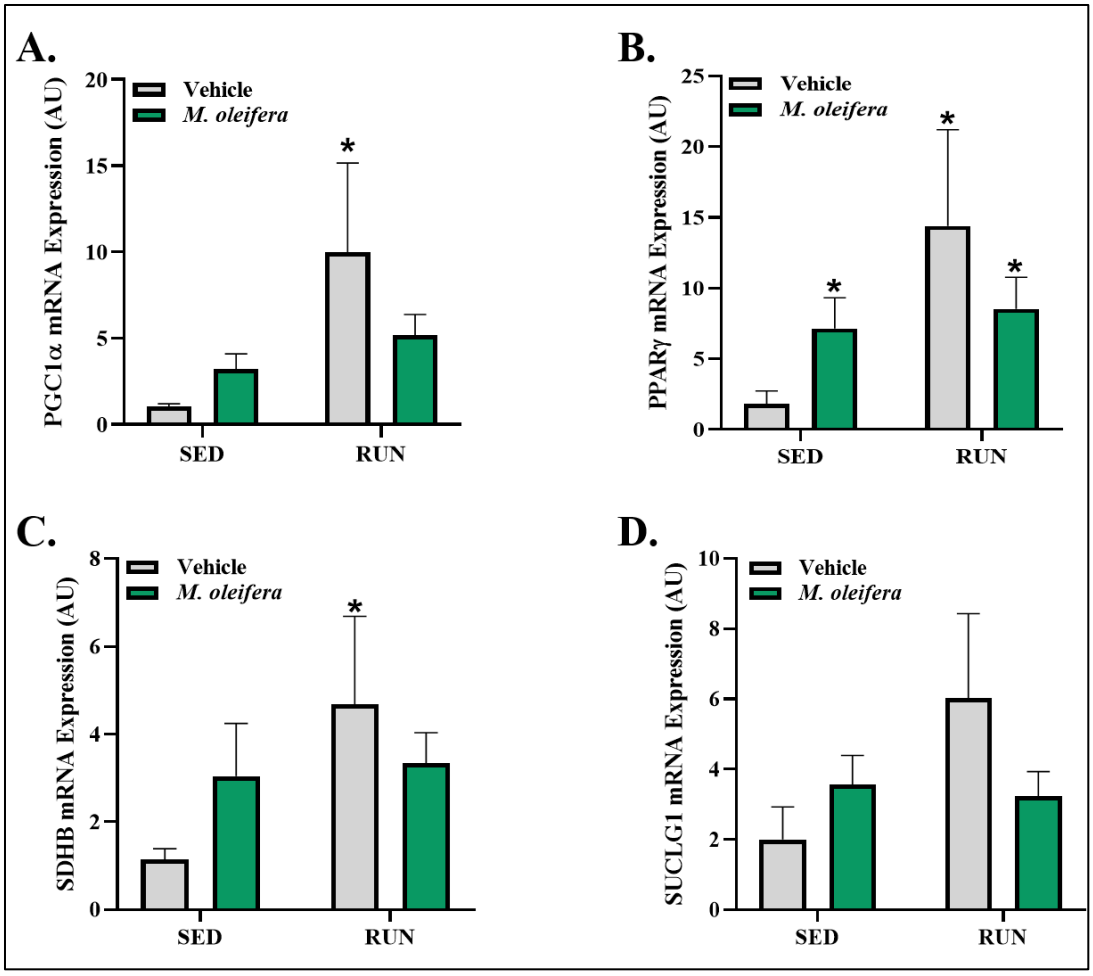

Figure 8. ***M. oleifera* promotes oxidative capacity.** (A) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) appeared to increase in the *M. oleifera*-supplemented group but was significantly increased in the running group. (B) Peroxisome proliferator-activated receptor gamma (PPARγ) was significantly increased in the *M. oleifera*-supplemented, running, and *M. oleifera* + RUN groups. (C) Succinate dehydrogenase-B (SDHB) mRNA expression appeared to be increased by *M. oleifera*-supplementation but was significantly greater in the RUN group. (D) Succinyl-CoA Ligase (GDP-forming) subunit alpha (SUCLG1) expression appeared to be increased by *M. oleifera* supplementation & running. *$p < 0.05$ compared with vehicle. Data are expressed as means + standard error.

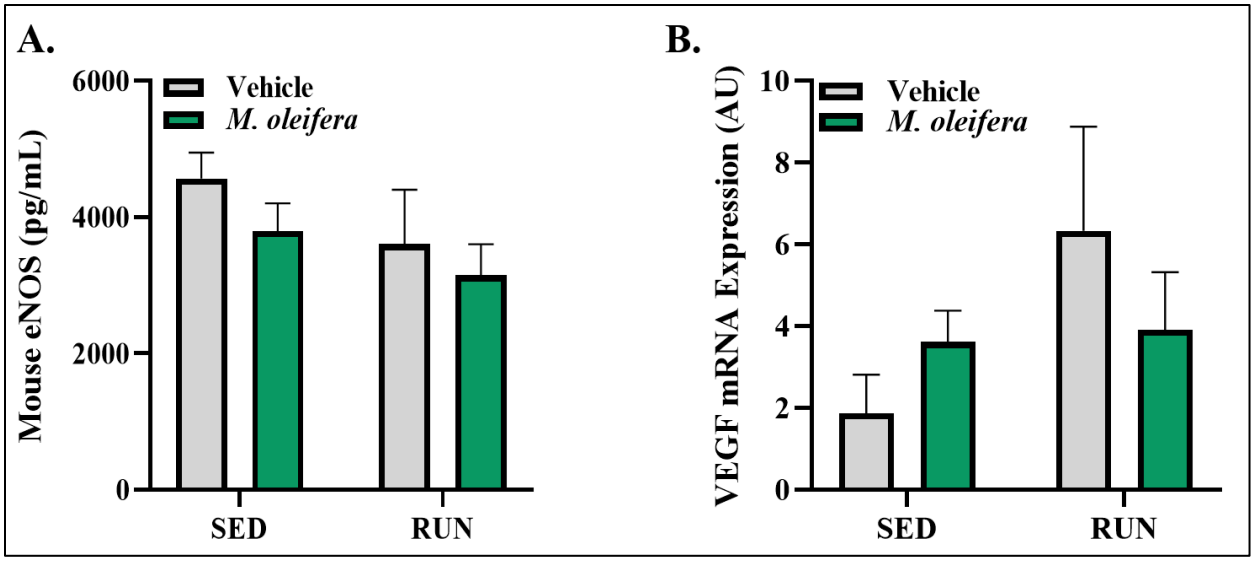

Figure 9. ***M. oleifera*** **Improves Vascular Health.** (A) Epithelial nitric oxide synthase (eNOS) was not affected by running nor by *M. oleifera*-supplementation. (B) Vascular endothelial growth factor (VEGF) was increased in *M. oleifera*-supplemented mice and in the running group. Data are expressed as means + standard error.

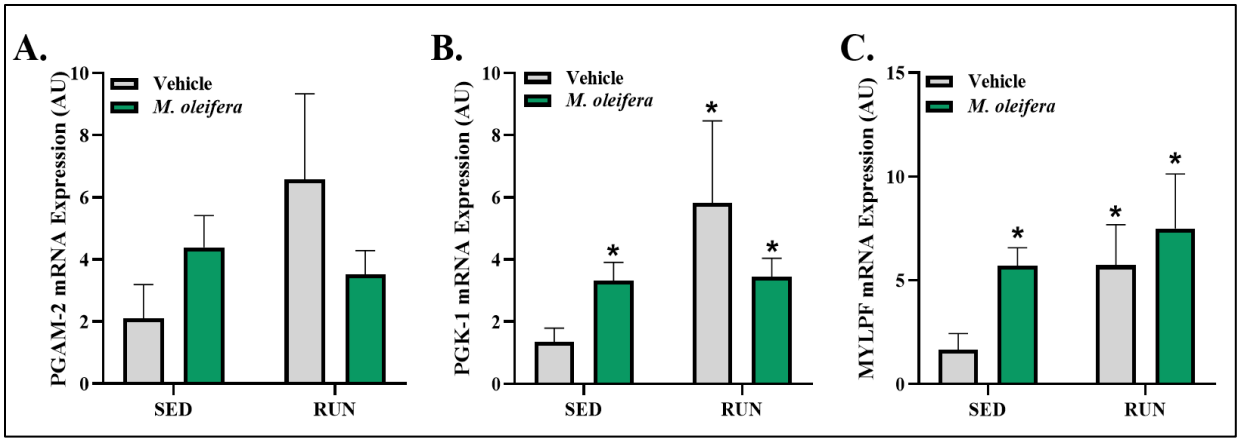

Figure 10. ***M. oleifera* Promotes Glycolytic Activity.** (A) Phosphoglycerate mutase (PGAM-2) appeared to be increased in the running and *M. oleifera*-supplemented groups. (B) Phosphoglycerate kinase-1 (PGK-1) was significantly increased in *M. oleifera*-supplemented mice and in the running group. (C) Myosin regulatory light chain 2, skeletal muscle isoform (MYLPF) was significantly increased in *M. oleifera*-supplemented mice and in the running group. *$p < 0.05$ compared with vehicle. Data are expressed as means + standard error.

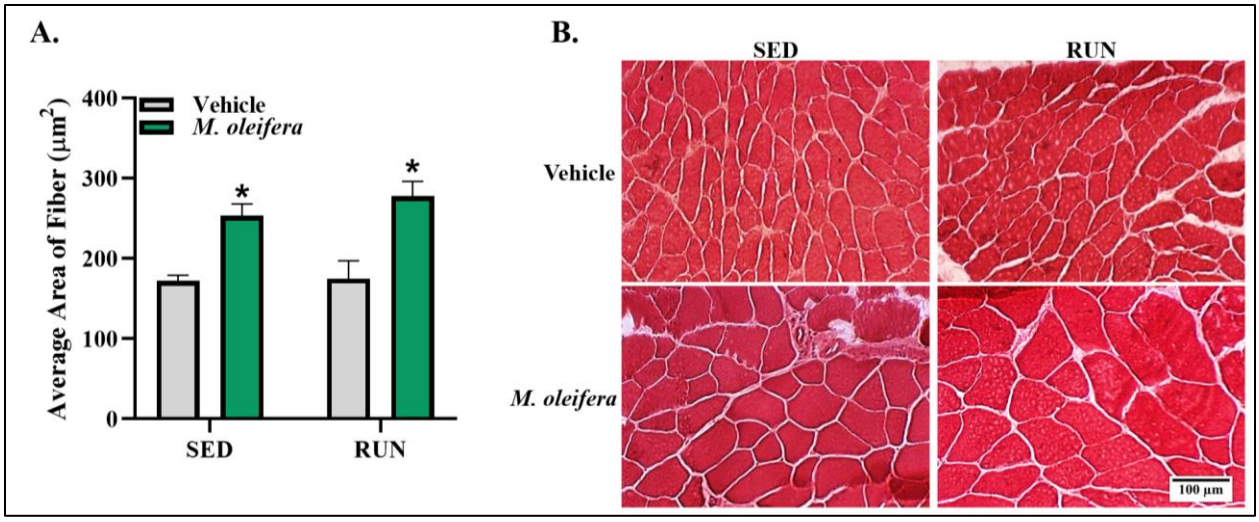

Figure 11. **Cross-Sectional Area is Augmented by *M. oleifera*-Supplementation.** (A) Average area of fiber diameter (μ^2) was significantly greater in the gastrocnemius muscles of mice supplemented with *M. oleifera*. (B) Representative histological images of hematoxylin and eosin stained gastrocnemius muscles (20x). Magnification bar is 100 μm. *$p < 0.05$ compared with vehicle. Data are expressed as means + standard error.

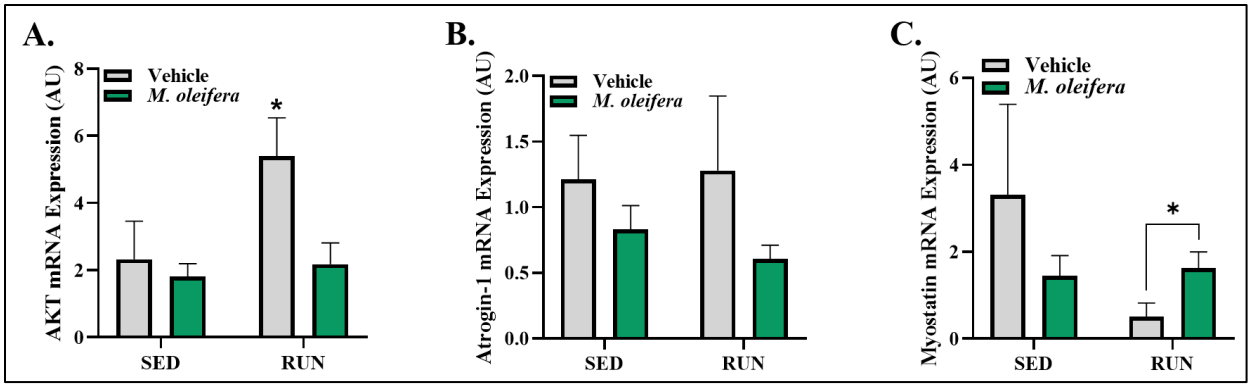

Figure 12. ***M. oleifera* Decreases Atrophic-Related Gene Expression.** (A) The mRNA expression of protein kinase B (AKT) was significantly greater in the RUN group but was not affected by *M. oleifera*-supplementation. (B) Atrogin-1 mRNA expression appeared to be decreased in mice supplemented with *M. oleifera*. (C) Myostatin mRNA expression appeared to be down regulated in mice supplemented with *M. oleifera* compared to vehicle, but it was significantly decreased in the vehicle + RUN group compared to the *M. oleifera* + RUN group. *$p < 0.05$ compared with vehicle. Data are expressed as means + standard error.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Mail: P. O. Box 3999 In Person: 58 Edgewood
Atlanta, Georgia 30302-3999 Suite 300
Phone: **404.413.3508**
Email: **iacuc@gsu.edu**
Web: **http://ursa.research.gsu.edu/iacuc/**

May 07, 2019

MEMORANDUM

To: Jeffrey Otis
Kinesiology & Health

FROM: Dr. Richard Plemper, Chair
Institutional Animal Care and Use Committee

RE: Protocol Number: A19043
Protocol Title: Moringa Oliefera and Skeletal Muscle Health
Animal Type and Quantity: *Mus musculus* (48)
Approval Period: 05/07/2019 - 03/22/2022

Your protocol referenced above was approved by the Georgia State University's Animal Care and Use Committee on May 07, 2019. This approval is for the following amendment to your previously approved protocol:

Add Desiree Wanders as Approved Personnel

This approval will remain in effect for the three-year proposal period.

Any additional changes to the current protocol must be approved before implementation. Submit any additional changes on the Amendment Form.

Any unexpected adverse effects of the experiments described in this protocol must be reported to the chair of the IACUC immediately.

Protocol approval does not guarantee animal housing space. Please contact Dean Blake to request animal housing space. Note: When ordering animals please refer to your full protocol number **A19043**.

Georgia State University has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is **D16-00527** (**A3914-01)**.

Richard K. Plemper, Ph.D.

Richard Plemper

Research Protocol for Animal Use (IACUC Application) (Version 1.3)

1.0 General Information

***Please enter the full title of your study:**

Moringa Oliefera and Skeletal Muscle Health

***Please enter a short title for your own personal reference.**

MO & Muscle
* This field allows you to enter an abbreviated version of the Study Title to quickly identify this study.

2.0 Add Department(s)

2.1 Your department is listed below; click "add" to add an additional department, or select the check box next to the department and select "remove" to remove it. PLEASE DO NOT LEAVE "GSU - Georgia State University" AS YOUR PRIMARY DEPARTMENT.:

Primary Dept?	Department Name
◉	**GSU** - Kinesiology & Health

3.0 Assign Study Personnel

3.1 *Please add a Principal Investigator for the study:

Otis, Jeffrey

3.2 If applicable, please select the Research Staff personnel:

A) Additional Investigators

B) Research Support Staff

Eze, Siobhan M
Graduate Student

3.3 *Please add a Study Contact:

Otis, Jeffrey

The Study Contact(s) will receive all important system notifications along with the Principal Investigator. (e.g. The project contact(s) are typically either the Study Coordinator or the Principal Investigator themselves).

3.4 If applicable, please select the Designated Department Approval(s):

Add the name of the individual authorized to approve and sign off on this protocol from your Department (e.g. the Department Chair or Dean). THIS DOES NOT APPLY TO IACUC		
3.5 If applicable, please select the Administrative Assistant(s):		
Administrative Assistant Note		

4.0

Georgia State University

Institutional Animal Care and Use Committee

The Institutional Animal Care and use Committee (IACUC) is by law responsible for ensuring that the use of animals at Georgia State University is performed according to the highest standards and in an ethical manner. This responsibility is shared with university faculty, staff, and students. The use of animals at the university is a privilege, not a right. Maintaining this privilege requires compliance with the following regulations, policies, and guidelines:

Animal Welfare Act Regulations
Public Health Service Policy on Humane Care and Use of Laboratory Animals
The Guide for the Care and Use of Laboratory Animals
U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training

The authority of the IACUC is derived from these laws and policies. The IACUC's role in institutional self-regulation ensures that animals are not subject to unnecessary pain and distress. Furthermore, by assuring compliance with these animal welfare laws and guidelines the IACUC also protects the investigator and the institution. The IACUC must review all aspects of the animal care and use program. The animal care and use program must include:

A properly constituted and functioning IACUC
Procedures for self-monitoring
An adequate veterinary care program
An occupational health and safety program
A training program for personnel
An environment, housing, and management program for animals
Appropriately maintained facilities for housing and support

Central to the IACUC's mandated functions are (1) reviewing and approving animal use protocols submitted by investigators and (2) semi-annual program reviews and facility inspections.
This form is intended to facilitate review of requests to use animals for specific research, instruction, or biological testing projects.

- **This completed form must be reviewed and approved by the IACUC before the project or course is initiated and before animals can be procured.**
- **After 3 years a new complete AUP must be submitted, approved and assigned a new number.**
- **The number of animals used must be declared annually and their documentation is the responsibility of the Principal Investigator.**

The Georgia State University IACUC Policies and Procedures Manual can be found on line at: GSU IACUC Policies and Procedures Manual This document contains information on Completing the Protocol Form, Protocol Review and Approval, Required Training, The Occupational Health Program, Additional Considerations Pertaining to Protocols, and more.

5.0

Basic Protocol Information

5.1 General Animal Information

Please select a category in which your protocol falls:

- (•) Research Protocol Only
- ( ) Breeding Protocol Only
- ( ) Research and Breeding Protocol

5.2 Provide details related to your grant:

Please indicate from which source your project is funded:

- [x] External (NIH, NSF, Other)
- [ ] Internal
- [ ] Not Funded

5.3 Sponsor Details:

Is your project sponsored by the NSF or NIH

- ( ) NSF
- ( ) NIH
- ( ) Both
- (•) Neither

5.4 Will any aspect of the study (course) or animal husbandry be conducted at another institution?

- (•) Yes
- ( ) No

Please provide institution name and collaborating PI's name.

We are collaborating with Dr. Nathan Mowa from Appalachian State University. Dr. Mowa will be receiving research funds that will be used, in large part, to purchase, house, feed, and test mice at GSU (under the direction of Jeffrey S. Otis). Dr. Mowa's lab will also formulate the research diet and ship to us (see sections 14.0 and 18.0 for clarity)

5.5 Will field investigations be employed?

- ( ) Yes
- (•) No

5.6 Is this a three year renewal?

- ( ) Yes
- (•) No

6.0 Sponsor Information

6.1 Please select your sponsor(s) for your project:

View Details	Sponsor Name	Sponsor Type	Contract Type:	Award Number
⊟	Departmental Funds	Personal	Unfunded Research Collaboration	

Sponsor Name:	Departmental Funds
Sponsor Type:	Personal
Contract Type:	Unfunded Research Collaboration
Award Number:	
Grant Title:	

7.0 Other Institution

7.1 Location of work on project:

Other than GSU, where will any aspect of the study (course) or animal husbandry be conducted? List specific location, i.e. building name and room number.

219 Rankin Science North, Appalachian State University, Boone, NC 28608

Please provide the PHS Assurance number of the institution where this work will occur:

D16-00581 (A4078-01)

Has this proposal been approved by the IACUC of that institution?

- ○ Yes
- ◉ No

8.0 Lay Project Summary and Overview

8.1 Objective(s) (What are you doing?)

Please provide a brief statement, in LAY TERMINOLOGY understandable by someone with a college education, with no acronyms or scientific jargon, outlining the objective of the procedures of this protocol. Begin with a broad statement concerning the overall problem (*e.g.*, "Pancreatic cancer kills ~50,000 Americans each year. We are addressing this problem by …."). Include a statement of your experimental hypothesis or objectives. Please do not submit your grant proposal abstract for this section. Define all abbreviations the first time they are used and explain medical terms. You will be asked to provide a scientific summary of the project in a later section.

Approximately 7% of the US population takes an herbal or botanical supplement, which has created a multi-billion dollar industry. Herbal supplements are largely unregulated, but have been taken to treat chronic illnesses or used as ergogenic aids to improve athletic performance.

Moringa Oleifera (MO) is a subtropical tree cultivated throughout Africa and Southeast Asia, and through the Caribbean Island into South America. Native cultures routinely use MO to treat a variety of complications, including inflammation, diabetes, obesity, cancer, and HIV. While MO has been used for centuries in these population to treat disease, recent research suggests that MO has powerful anti-inflammatory, anti-bacterial, and anti-oxidant properites.

Our lab became interested in MO because some of the published effects following chronic supplementation may directly impact skeletal muscle structure and function. Specifically, research has shown that MO

may improve mitochondrial health, capillarity, and glucose metabolism. In skeletal muscle, we would logically hypothesize that chronic MO supplementation my improve endurance capacity and exercise tolerance. Accordingly, this small, pilot study has been designed to test the role of chronic MO supplementation on: (1) signaling mechanisms implicated in controlling the number of mitochondria (mitochondrial biogenesis), capillary density (angiogenesis), and fiber type expression, and (2) voluntary wheel running as a surrogate marker of exercise endurance capacity.

8.2 Rationale and Significance (Why are you doing it?)

The IACUC is obliged to weigh the objectives of the study against potential animal welfare concerns. This protocol in its entirety is reviewed by the IACUC to assess animal welfare concerns. As a component of analyzing the potential animal welfare concerns as compared to potential benefits derived from the study (e.g. harm/benefit analysis), please provide a brief statement about how contributions from your proposed work might benefit human/animal wellbeing or the expansion of knowledge. This must be written in LAY TERMINOLOGY, understandable by someone with a college education, with no acronyms or scientific jargon. Please do not submit your grant proposal abstract for this section. Define all abbreviations the first time they are used and explain medical terms

Moringa Oliefera appears to play broad physiolgical roles following chronic supplementation, including anti-inflammatory, anti-oxidant, and pro-metabolic properties, and because of this, is commonly used in subtropical cultures to treat a wide range of chronic diseases. Interestingly, these systemic effects of *Mori nga Oliefera* supplementation have not been adequately evaluated in otherwise healthy models with a particular focus on skeletal muscle health.

Accordingly, we will supplement otherwise healthy, male, C57BL/6 mice with *Moringa oleifera* and analyze it's impact on skeletal muscle structure and signaling mechanisms (e.g., mitochondrial density, capillarity, fiber type, fiber area), as well as a surrogate measure of skeletal muscle performance (e.g., voluntary wheel running). Importantly, we and our collaborator (Dr. Nathan Mowa, Appalachian State University) have not found evidence of side effects of *Moringa oleifera* and, as such, **predict a very low harm/benefit ratio**. If our hypotheses are proven, the potential benefits of increased capillarity, mitochondrial biogenesis, fiber type switching to a more fatigue-resistance phenotype, and increased endurance capacity could provide the first data on *Moringa oleifera* use as an ergogenic aid to enhance training and athletic performance.

9.0

USDA Animal Use Category Classification

USDA Classifications and Examples of Pain Categories:
<u>Classification B:</u> Animals being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery, but not yet used for such purposes.
Examples:

- **Breeding colonies - Includes parents and offspring.**
- **Animals held under proper captive conditions or wild animals that are being observed.**

<u>Classification C:</u> Animals upon which teaching, research, experiments, or tests will be conducted involving no pain, distress, or use of pain-relieving drugs.
Examples:

- **Procedures performed correctly by trained personnel such as the administration of electrolytes /fluids, administration of oral medication, blood collection from a common peripheral vein per standard veterinary practice or catheterization of same, standard radiography, parenteral injections of non-irritating substances.**
- **Euthanasia performed in accordance with the recommendations of the most recent AVMA Panel on Euthanasia, utilizing procedures that produce rapid unconsciousness and subsequent humane death.**
- **Manual restraint that is no longer than would be required for a simple exam; short period of chair restraint for an adapted nonhuman primate.**

Classification D: **Animals upon which experiments, teaching, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs will be used.**
Examples:

- **Surgical procedures conducted by trained personnel in accordance with standard veterinary practice such as biopsies, gonadectomy, exposure of blood vessels, chronic catheter implantation, laparotomy or laparoscopy.**
- **Blood collection by more invasive routes such as intracardiac or periorbital collection from species without a true orbital sinus such as rats and guinea pigs.**
- **Administration of drugs, chemicals, toxins, or organisms that would be expected to produce pain or distress but which will be alleviated by analgesics.**

Classification E: **Animals upon which teaching, experiments, research, surgery, or tests will be conducted involving accompanying pain or distress or leading to illness to the animals and for which the use of appropriate anesthetic, analgesic, or tranquilizing drugs will adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests.**
Examples:

- **Procedures producing pain or distress unrelieved by analgesics such as toxicity studies, microbial virulence testing, radiation sickness, and research on stress, shock, or pain.**
- **Surgical and postsurgical sequella from invasion of body cavities, orthopedic procedures, dentistry or other hard or soft tissue damage that produces unrelieved pain or distress.**
- **Negative conditioning via electric shocks that would cause pain in humans.**
- **Chairing of nonhuman primates not conditioned to the procedure for the time period used.**

9.1 Selection of Pain Category
Please classify the project according to the level of perceived pain / stress / distress experienced by the animal(s). Animals must be claimed under the highest class involved at any point prior to euthanasia or release. Highest Pain Category within this protocol:(enter B, C, D, or E)

Pain category C

9.4 Will any adverse effects or overt signs of illness be expected?

- ○ Yes
- ◉ No

9.5 Monitoring of Animal Pain and Comfort Levels

Who will be responsible for monitoring the animals for pain and/or distress during the experimental procedure(s)?

In addition to the DAR, the PI (Jeffrey S. Otis) and his PhD student (Siobhan Eze) will be responsible for monitoring pain or stress during the experimental procedures.

How will the comfort level of the animals be determined? Include a description of what records would be kept if applicable.

Animals will be monitored by the PI or Siobhan Eze daily (M-F) for signs of potential distress due to Moringa Oliefera supplemenation. Although highly unlikely, potential signs of distress may include: increased vocalization, failure to groom, decreased appetite, and lethargy.

In the event that an animal needs to be euthanized or removed from the experiment, humane endpoint criteria must be identified by the researcher. I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the Humane Endpoint policy.

- (•) Yes
- ( ) No

If your species is not included in the Humane Endpoint policy, then please describe the humane endpoint criteria that you will use to determine whether an animal needs to be euthanized or removed from the experiment.

Describe the frequency of monitoring for these humane endpoints:

Animals will be monitored by Dr. Otis or Siobhan Eze daily during the work week (Monday-Friday).

Who will determine if euthanasia is required?

The PI will defer to the recommendations of the University Vet and DAR staff.

Will weight loss be used as a humane endpoint criterion?

- (•) Yes
- ( ) No

I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the Weight Loss Policy.

- (•) Yes
- ( ) No

Will rectal prolapse be an expected complication of this model?

- ( ) Yes
- (•) No

Will animal models with tumors be utilized?

- ( ) Yes
- (•) No

Will non tumor cells be administered to the animals?

- ( ) Yes
- (•) No

10.0 Justification for Animal Use and Species Selection

10.1 Animal characteristics

Species Name	Common Name	Sex	Age (at use)
Mus musculus	mouse	Male	8 weeks

Please click "Add a new row" button for each Strain.

To use Jax Database to search for the strain click here

Specific Strain Name	Stock No. (JAX Mice only)	Describe any adverse phenotype
C57BL/6	000664	

10.2 This species has been selected because (check all that apply)

- [x] Previous work in the biomedical literature validates the use of this species as an animal model for this disease or biological process.
- [x] This is the lowest sentient species that provides appropriate size, tissue or anatomy of the proposed work.
- [ ] There is a large body of existing data that would need to be repeated if another species was used instead.
- [ ] Available reagents or research tools necessary for this research are unique to this species.
- [ ] Characteristics of this species make it uniquely suited for the proposed research. Explain below:
- [ ] Other (Explain below):

10.3 Please justify why animals must be used instead of using methodology that does not require vertebrate animal use (e.g., cell culture, computer modeling).

Skeletal muscle cultures would be useful to identify discrete signaling pathways induced by *Moringa Oliefera.* However, the complex metabolism of Moringa Oliefera supplementation, it's metabolites, and the consequent physiological/biochemical/metabolic adaptations can only be analyzed in a whole animal model. Further, our lab is interested in skeletal muscle structural adapations and how these directly effect functional improvements.

As such, we have chosen the mouse model to identify the role of *Moringa Oliefera* supplementation on skeletal muscle health.

Upon the acquisition of new animals I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the Animal Acclimation Policy

- (•) Yes
- ( ) No

11.0 Justification for the number of animals that will be used

11.1 Over a period of three (3) years what is the total number of animals you would like to use?

48

11.2 Group sizes are expected to represent the minimum number of animals that are needed to achieve the scientific or instructional objectives. Please indicate all the methods used to determine these numbers.

- [x] Statistical tools, such as power analysis, were employed to determine appropriate group sizes to ensure statistically valid outcomes. (Please retain print outs from calculations.)
- [x] Previous experience with this experimental paradigm indicates this is the minimum number of animals needed.
- [ ] Consultation with a biostatistician
- [x] This is a pilot study used to determine feasibility before proceeding with larger, more tightly controlled experiments
- [ ] This is an instructional activity. This is the minimum number of animals needed based on class size and optimal student to instructor ratios.
- [ ] Other (Explain):

11.3 Using the specifics of your experimental plan demonstrate how the numbers of animals required to achieve your scientific objectives for this project were calculated. Include details of numbers of animals per group, control groups, treatment groups, pilot studies, and potential experimental failure. Information MUST be provided in the form of a table (recommend attaching a file for multiple tables).

Justify the number of animals required for each procedure/experiment using power analysis, if possible. For help with power analyses see: http://www.gpower.hhu.de/

Effect size, power, and required sample size to attain power of 0.8 at an alpha level of 0.05 have been calculated and suggested 10 animals per experimental group will be required. With an additional 2 mice requested per experimental group (thus, n = 12/group), we anticipate that **48 mice** will be required to complete this pilot work. These mice will be randomly assigned to one of four groups:

(1) vehicle + locked wheel (control:sedentary)
(2) vehicle + unlocked wheel (control:exercise)
(3) MO + locked wheel (treatment:control)
(4) MO + unlocked wheel (treatment:exercise)

12.0

Animal Use Narrative

12.1 Please describe in narrative form all experimental or instructional procedures to be performed on the animals. Please note that it is not necessary to provide the details already provided elsewhere in the protocol (e.g. procedure descriptions, volumes of blood collected, dosages, routes of administration, use of aseptic procedures, etc.). However, it is important that one is able to ascertain what procedure or set of procedures is conducted on each group of animals. Include the time frames and intervals between procedures and describe the procedures in the order they will be performed.

Does your narrative include any of the following?

- **images**
- **tables**

○ Yes
◉ No

Experimental Design
This pilot study is designed to identify the role of *Moringa Oliefera* (MO) on skeletal muscle structure, signaling, and function in otherwise healthy mice. To accomplish this, male, C57BL/6 mice will be randomized in to one of four experimental groups:

(1) vehicle + locked wheel (control:sedentary)
(2) vehicle + unlocked wheel (control:exercise)
(3) MO + locked wheel (treatment:control)
(4) MO + unlocked wheel (treatment:exercise)

Moringa Oliefera (MO) supplementation
MO will be formulated into a pellet at Appalachian State University and shipped to the DAR at Georgia State University (see Substance Administration and Husbandry sections for details on diet formulation). This final formulation will be concentrated and provided to treatment animals in both their littermate cages, as well as their modified cages containing the voluntary running wheel. Experimental groups will be fed MO pellets for **3 weeks** prior to the introduction of the voluntary running wheels. Pellets will continue to be provided to the mice for the subsequent **2 weeks** as we collect voluntary running activity. Thus, animals will be on the experimental diets for a total of **5 weeks**.

Voluntary Wheel Running (VWR)
Subgroups of MO- or control-fed mice will be provided free access to a standard running wheel that is equipped with a cycle monitor (Velo 8 computer, CatEye Co. LTD, Osaka, Japan) to count revolutions. Standard chow, pellet, and water will be provided. For accuracy, mice need to be singly housed with the wheel between our data collection hours (5-pm through 9-am the following morning). We will have multiple cages and will collect voluntary wheel running data for **14 consecutive days, occuring during the final 2 weeks of the 5 weeks feeding protocol**. Although not expected, mice will be checked daily for injuries that may occur as a result of using the wheel. In the rare case that a mouse is injured, they will be removed from the study (euthanized with tissues collected as described below). Removal of injured mice is required because we must collect 14 days of injury-free voluntary wheel running activity. Mice will return to their host cage with their littermates between 9-am and 5-pm. We are aware that returning male mice back to their home cage may precipitate fighting. To reduce the chances of this occuring, the experimental plan requires all littermates to run and rest together (i.e., in run cages from 5-pm through 9-am, and re-grouped 9-am through 5-pm for 14 consecutive days). However, if fighting occurs, involved animals will be identified (us or DAR) and removed from the study (euthanized with tissues collected as described below) as potential injuries may impact our data.
Monitoring during VWR

Please note that animals assigned to VWR (sedentary or exercise) will be monitored everyday for 14 days by the PI or Siobhan Eze. Before animals have been assigned to groups 1-4 listed above, they will be monitored by the PI or Siobhan Eze during the work week (M-F).

Euthanasia & Tissue Collections

After the experimental time period has elapsed, mice will be transported to our lab (G19 Sports Arena). Mice will be euthanized in a CO_2 chamber and death confirmed following thoracotomy and diaphragm removal and collection. Several skeletal muscles (gastrocnemius, plantaris, soleus, extensor digitorum longus, tibialis anterior, diaphragm), as well as the heart, liver, and lungs will be frozen for future analyses. Importantly, tissue collections are **ONLY** performed in euthanized animals - collections will not be performed in anesthetized animals.

13.0 Procedures

13.1 Will any substance such as Complete Freund's Adjuvant or other adjuvants be injected which could cause chronic inflammation and /or pain?

- ○ Yes
- ◉ No

13.2 Will your study involve prolonged physical restraint (greater or equal to 30 mins)?

- ○ Yes
- ◉ No

13.3 Will you perform surgical procedures under this protocol on animals?

- ○ Yes
- ◉ No

13.4 Please select the non-surgical procedures that will be used under this protocol:

- ☐ Blood Collection (Axiliary Cutdown)
- ☐ Blood Collection (Cardiac)
- ☐ Blood Collection (Facial vein - Mice Only)
- ☐ Blood Collection (Retro-Orbital)
- ☐ Blood Collection (Saphenous)
- ☐ Blood Collection (Tail Nick)
- ☐ Genotyping or Blood Collection (Tail Snip)
- ☐ Blood Collection (Wing Vein)
- ☐ Retro-orbital sinus injection (Alternative to tail vein)
- ☐ Intramuscular (IM) Injection
- ☐ Subcutaneous (SC) Injection
- ☐ Intraperitoneal (IP) Injection
- ☐ Intradermal (ID) Injection
- ☐ Intravenous (IV) Injection Utilizing Lateral Tail Veins
- ☐ Oral Gavage
- ☐ Genotyping (Ear Punch)
- ☐ Other Non-surgical Procedure Not Listed

14.0 Method(s) of Euthanasia

14.1 Describe in detail all the methods of euthanasia (if any) you will use. If the method involves the use of pharmaceuticals, please specify agent, dose, and route of administration.

Please note that methods of euthanasia must be in accordance with the most current American Veterinary Medical Association Panel on Euthanasia. References must be cited to justify deviations from this document, or to justify use of cervical dislocation or decapitation without anesthesia.

- [x] Carbon dioxide
- [ ] Commercial euthanasia solution containing sodium pentobarbital (please use text box below to indicate dose and route of administration)
- [ ] Transcardial perfusion
- [ ] Cervical dislocation in an awake animal (requires scientific justification supported by literature references to be indicated in the text box below)
- [ ] Decapitation in an awake animal (requires scientific justification supported by literature references to be indicated in the text box below)
- [ ] Isoflurane anesthesia using a precision vaporizer
- [ ] Isoflurane anesthesia using a drop jar
- [ ] Other (please use the text box below to describe the procedure as well as the anesthetic agent, dose, and route of administration, as applicable)
- [ ] None (No Planned Euthanasia (e.g. Non-Human Primates))

Carbon dioxide inhalation will be our primary euthanization method confirmed by thoracotomy and removal/collection of the diaphragm.

14.2 How will death be confirmed?

- [x] With animals euthanized with CO2 or Isoflurane, a secondary physical method will be employed (e.g. cervical dislocation, bilateral thoracotomy, major vessel transection)
- [ ] Animals euthanized with a commercial euthanasia solution (sodium pentobarbital product), no secondary physical method is needed but the animal will be checked to assure there is has been a cessation of the heart beat as well as respirations.
- [ ] Other (please indicate in text box below)

14.3 How will remains be disposed?

- [x] Carcasses will be placed in a carcass bag, affixed with the completed DAR carcass bag label, and disposed of in the carcass freezer for ultimate disposal by the DAR staff
- [ ] Other (please indicate the method to be utilized in the text box below)

I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the IACUC Carbon Dioxide Euthanasia Policy

- (•) Yes
- ( ) No

15.0 Substance Administration

15.1 Please indicate below what type of substance(s) you will be using on your protocol:

Which type of Substances will you be using in this Protocol?

- [ ] Pharmaceutical Grade
- [ ] Non-pharmaceutical Grade (please select this if any of the substances administered BY INJECTION (as opposed to orally or topically) are of a chemical-grade)

- [x] Non-pharmaceutical Grade (please select this if any of the substances administered ORALLY or TOPICALLY are of a Chemical-grade)
- [ ] Biologicals (e.g. cells, infectious agents to include viral vectors)
- [ ] N/A

I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the Expired Materials Policy, Anesthetic/Analgesia Formulary Policy, Mixing or Diluting Anesthetic /Analgesic Agents Policy.

- (•) Yes
- ( ) No

15.3 Non-Pharmaceutical Substance Description
If anesthetics, analgesics, or any other substances are administered during the conduct of a procedure, please describe:

Please complete the information below:

Substance:

Cassava flour

Route:

Oral (into the mouth)

If other, please indicate route:

Dose:

10.1 g per pellet

Frequency:

daily

Substance:

Moringa leaf powder

Route:

Oral (into the mouth)

If other, please indicate route:

Dose:

60 mg per pellet

Frequency:

daily

Substance:

Peanut butter

Route:

Oral (into the mouth)

If other, please indicate route:

Dose:

2 g per pellet

Frequency:

daily

As applicable, I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the GSU IACUC Policy on the Use of Non-Pharmaceutical Grade Substances (Topical or Oral Use).

- (•) Yes
- ( ) No

If applicable, state how anesthetic depth will be assessed:

NA

16.0 Non-duplication of Research

16.1 Consideration of non-duplication of research

I assure that the proposed work is not unnecessarily duplicative.

- (•) Yes
- ( ) No

17.0 Personnel and Their Experience and Training

For each person listed below check which procedure(s) they will perform and describe their level of competency with the procedure(s). If personnel are not experienced, please list the name of the individual(s) who will be responsible for training on all procedures. Please note that DAR and/or the IACUC reserve the right to observe procedures being performed prior to protocol approval.

If training has not been completed click here to find out what requirements must be met in order to be considered approved personnel on this protocol. Click here if you are a first time user of the AALAS Learning Library. After the initial registration with the AALAS Learning Library, any future modules in the library can be accessed by visting the AALAS Learning Library website and selecting "AALAS Learning Library Training" after logging in.

All employees of Georgia State University who work with vertebrate animals must enroll in the Medical Monitoring Program for Vertebrate Animal Exposure. To enroll click here.

Name	Non-Surgical /Surgical Procedure (s)	Describe level of competency /ability with these procedures in this species	If training is needed who will provide	Have you enrolled in MMPVAE
Otis, Jeffrey	☐ Blood Collection (Axiliary Cutdown) ☐ Blood	Dr. Otis has been working with animal models for the past 20 years	NA	(•) Yes ( ) No

		Collection (Cardiac) ☐ Blood Collection (Facial Vein - Mice Only) ☐ Blood Collection (Retro-Orbital) ☐ Blood Collection (Saphenous) ☐ Blood Collection (Tail Nick) ☐ Genotyping or Blood Collection (Tail Snip) ☐ Blood Collection (Wing Vein) ☐ Retro-orbital sinus injection (Alternative to tail vein) ☐ Intramuscular (IM) Injection ☐ Subcutaneous (SC) Injection ☐ Intraperitoneal (IP) Injection ☐ Intradermal (ID) Injection ☐ Intravenous (IV) Injection Utilizing Lateral Tail Veins ☐ Oral Gavage ☐ Genotyping (Ear Punch) ☐ Other Non-surgical procedure Not Listed ☐ Gonadectomy - Castration Using Scrotal Method ☐ Gonadectomy - Castration Using Abdominal Method ☐ Intracerebral Microinjection ☐ Lipectomy ☐ Osmotic Minipump Implantation - Subcutaneous Infusions ☐ Osmotic	and routinely uses dietary and exercise interventions to induce skeletal muscle adaptations and uses CO2+thoracotomy as my primary euthanasia strategy. I published a paper using this exact VWR design (see: Chronic alcohol ingestion delays skeletal muscle regeneration following injury, Dekeyser et al., Reg Med Res, 2013)					

		Minipump Implantation - Infusion Into a Brain Cannula ☐ Osmotic Minipump Implantation - Infusion Into a Vessel ☐ Ovariectomy ☐ Perfusion ☐ Pinealectomy ☐ Silastic Capsule Implantation ☐ Vessel Cannulation ☑ Euthanasia Method - Carbon Dioxide ☐ Commercial euthanasia solution containing sodium pentobarbital ☐ Transcardial perfusion ☐ Cervical dislocation in an awake animal ☐ Decapitation in an awake animal ☐ Isoflurane anesthesia using a precision vaporizer ☐ Isoflurane anesthesia using a drop jar ☐ Stereotaxic Surgery ☐ Other Surgical Procedures Not Listed				
	Eze, Siobhan M	☐ Blood Collection (Axiliary Cutdown) ☐ Blood Collection (Cardiac) ☐ Blood Collection (Facial Vein - Mice Only) ☐ Blood Collection (Retro-Orbital)	Siobhan is my PhD student (3rd year) and spent 4-5 years working with laboratory mice at Emory University. She is fully proficient with small animal handling. While she is new to the skeletal muscle biology		◉ Yes ○ No	

		☐ Blood Collection (Saphenous) ☐ Blood Collection (Tail Nick) ☐ Genotyping or Blood Collection (Tail Snip) ☐ Blood Collection (Wing Vein) ☐ Retro-orbital sinus injection (Alternative to tail vein) ☐ Intramuscular (IM) Injection ☐ Subcutaneous (SC) Injection ☐ Intraperitoneal (IP) Injection ☐ Intradermal (ID) Injection ☐ Intravenous (IV) Injection Utilizing Lateral Tail Veins ☐ Oral Gavage ☐ Genotyping (Ear Punch) ☐ Other Non-surgical procedure Not Listed ☐ Gonadectomy - Castration Using Scrotal Method ☐ Gonadectomy - Castration Using Abdominal Method ☐ Intracerebral Microinjection ☐ Lipectomy ☐ Osmotic Minipump Implantation - Subcutaneous Infusions ☐ Osmotic Minipump Implantation - Infusion Into a Brain Cannula ☐ Osmotic Minipump Implantation - Infusion Into a Vessel	field, she will be fully trained by the PI on VWR and animal monitoring, and euthanasia.				

- [ ] Ovariectomy
- [ ] Perfusion
- [ ] Pinealectomy
- [ ] Silastic Capsule Implantation
- [ ] Vessel Cannulation
- [x] Euthanasia Method - Carbon Dioxide
- [ ] Commercial euthanasia solution containing sodium pentobarbital
- [ ] Transcardial perfusion
- [ ] Cervical dislocation in an awake animal
- [ ] Decapitation in an awake animal
- [ ] Isoflurane anesthesia using a precision vaporizer
- [ ] Isoflurane anesthesia using a drop jar
- [ ] Stereotaxic Surgery
- [ ] Other Surgical Procedures Not Listed

18.0

Hazard Use

All Hazardous Materials used must be identified.
For more information on the Biosafety program please click: GSU Biosafety Program
For more information on the Radiation Safety program please click: GSU Radiation Safety
For more information on GSU Lab Safety and Hazard Communication please click: https://ursa.research.gsu.edu/laboratory-ennvironmental-safety/
For more information on GSU Chemical Safety please click: https://ursa.research.gsu.edu/chemical-safety/

18.1 Are you working with infectious agents and/or biologically-derived toxins?

- ○ Yes
- ◉ No

18.2 Does the project use any recombinant DNA (i.e. cloning/expression systems, viral vectors, etc.) material in animals?

-

Yes
(•) No

18.3 Will any carcinogenic or toxic compounds be used in animals?

( ) Yes
(•) No

If your chemical or substance is not listed in Chematix, please upload the SDS (Safety Data Sheet) here.

Version	Title	Category	Expiration Date	Document Outcome	Checked Out	View Document
No Document(s) have been attached to this form.						

18.4 Will any radioactive compounds be administered to the animals or will the study otherwise involve a radiation device (e.g. an x-irradiator for total body irradiation, an x-ray machine, etc.)?

( ) Yes
(•) No

18.5 Will Controlled Substances be used?

( ) Yes
(•) No

19.0 Animal Housing and Husbandry

19.1 Do the animals require housing other than standard caging/bedding or tanks/water?

(•) Yes (List housing required and explain below):
( ) No

Explain:

To accomodate the voluntary running wheels, we will need to modify standard rat cages. The larger rat cages are required to fit the height of our running wheels. These cages will still contain standard bedding, food (experimental or control), and water.

19.2 Will animals be housed singly?

For more information see the Social Housing Policy

(•) Yes (List reason single housing is required and explain why below):
( ) No

Explain:

Mice will need to be singly housed between 5PM-9AM to accurately measure their voluntary wheel running activity. These modified cages will contain food and water. Animals will be returned to their home cages with their littermates between 9AM-5PM.

In addition, mice may have to be singly housed, in addition to the times they are in the running wheel cages, if they start to fight when they are returned to the group housing scenario.

Non-Human Primate Housing and Social Housing of Social Species criteria options can be found at: http://grants.nih.gov

19.3 Do the animals require special care?

- ○ Yes (List special care and explain below):
- ◉ No

19.4 Do the animals require a diet other than the standard diet for this species used at GSU?

- ◉ Yes (List diet, diet manufacturer and explain below):
- ○ No

Explain:

**Mice in the experimental diet groups that receive Moringa Oliefera (MO) are still fed the standard chow in the hopper. Two pellets are provided per mouse and are placed in the cage (pre and post pellet weights are calculated daily to get an average consumption/cage/mouse).

Moringa Oliefera pellet formulation
All formulation is completed under a sterile hood in the laboratory of Dr. Nathan Mowa, Appalachian State University. A single Moringa pellet is formed using sterile water, and contains:
10.1 g of cassava flour
60 mg of Moringa leaf powder (sourced from a manufacturer in Research Triangle Park, NC)
2 g peanut butter (to enhance flavor)

*Control pellets add 60 mg of cassava flour in place of MO.

Pellets are air-dried under the hood over night and refrigerated until use. Our feeding protocol is 5 total weeks. We will receive 1 shipment, store accordingly, and be solely responsible for providing the special diet (special diet cages will be clearly labelled for the DAR staff). All experimental diets will be well-labelled, dated to ensure freshness and not expired.

19.5 Will food and/or fluid restriction(s) be necessary?

- ○ Yes
- ◉ No

19.6 Will animals remain outside the IACUC approved Animal Housing Area for more than 12 hours?

IACUC approved Animal Housing Areas

- ○ Yes (List building and room number and explain below):
- ◉ No

19.7 Will animals undergo experimental manipulations outside the IACUC approved Animal Housing Area?

- ◉ Yes
- ○ No

If you selected Yes, please justify the move to this location and indicate the building name and room number.

Animals will be euthanized and tissues removed in our lab (G18 Sports Arena). We collect multiple tissues and process them immediately for gene or protein analyses (flash freezing in liquid nitrogen or store in cold-saline), while at the same time process portions of these tissues for immunohistochemistry and histology (melting isopentane). We can certainly consider doing this all in the DAR facility, but for convenience of sample processing and storage, bringing animals back to G18 on our schedule seems more efficient.

If Yes, complete the following: See GSU IACUC Animal Transportation Policy

- [x] I agree to adhere to the GSU IACUC Animal Transportation Policy.
- [ ] I request a deviation from the GSU IACUC Animal Transportation Policy.

19.8 Can animals be provided environmental enrichment?

- (•) Yes (by selecting yes the default enrichment will be provided unless otherwise specified):
- ( ) No (Explain reason for lack of enrichment here):

19.10 Does this protocol involve the use of any rodent species?

- (•) Yes
- ( ) No

I will ensure that all relevant lab personnel have read and will adhere to the standards specified in the Capture of Escaped Rodents policy

- (•) Yes

20.0 PHS Policy

20.1 The PHS Policy on Humane Care and Use of Laboratory Animals and Animal Welfare Regulations define the responsibilities of the IACUC regarding review and approval of animal activities.
Changes that may be handled administratively without IACUC-approved policies, consultations, or notifications include:

1. **correction of typographical errors**
2. **correction of grammar**
3. **contact information updates**

- (•) I AGREE to allow the IACUC Office to make the above regulatory approved changes to my IACUC protocol
- ( ) I DO NOT AGREE to allow the IACUC Office to make the above regulatory approved changes to my IACUC protocol