Ameliorative Effects of *Moringa oleifera* Leaf-based diet on Malnutrition- induced Skeletal Muscle Degeneration in Rats

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A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF ILORIN, ILORIN, NIGERIA.
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DECLARATION

I, Muinat Olayinka LAMBE (Matric number - 04/55EH103) declare that this entire thesis titled "Ameliorative effects of *Moringa oleifera* leaf-based diet on malnutrition induced skeletal muscle degeneration in rats" is the result of the study carried out by me under the supervision of Professor C. O. Bewaji in the Department of Biochemistry, University of Ilorin, Nigeria. I hereby confirm that the thesis presents the result of my research findings and that its contents are entirely my ideas otherwise stated with appropriate references. This thesis has not been submitted to any other University neither is it before any other university for the award of a Doctor of Philosophy (Ph. D.) in Biochemistry.

Lambe, M. O.

(September, 2019)

CERTIFICATION

I certify that this work was carried out by Lambe, Muinat Olayinka in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

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APPROVAL

This thesis has been read and approved as having met the requirement of the Department of Biochemistry, Faculty of Life Sciences and Postgraduate School, University of Ilorin, Ilorin, Nigeria, for the award of Philosophy (Ph.D.) degree in Biochemistry.

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DEDICATION

This thesis is dedicated to Mr. Kamaldeen O. JIMOH (my support, soul-mate and husband),

My precious gifts; Maryam (Taiye), Aisha (Kehinde), Abdullah and Zainab KAMALDEEN.

My dear Mum,

and

My first friends and siblings; Dr (Mrs) Kudirat, Dr (Mrs) Medinat, Dr Idris, Jummai, Mubaraq (LAMBEs).

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To ALLAH is the glory...

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ABSTRACT

Skeletal muscle degeneration (SMD) is a physiological condition characterized by decrease in muscle mass due to reduced protein synthesis and/or degradation. About 1 in 300 people is seen with a SDM. Chemotherapies generally used in the treatment of diseases like cancer and tuberculosis although expensive and inaccessible also contribute to SMD. Hence, the need to explore cheaper, safer and readily available options in *Moringa oleifera* leaf-based diet (MoLD). The objectives of the study were to: (i) identify the secondary metabolites in *M. oleifera* leaf; (ii) determine the proximate composition of MoLD; (iii) confirm the induction of skeletal muscle degeneration; (iv) evaluate the effects of MoLD on markers of muscle degeneration; and (v) evaluate the toxicity of MoLD in Wistar rats.

Skeletal muscle degeneration was induced by maintaining adult rats on 4% low protein iso-caloric diets (LPID) for four weeks. The control animals were fed on soy-based meal only while the SMD-induced rats were maintained on soy-based diet supplemented with methyl jasmonate (100 µg/kg) + vitamin E (20 mg/kg) + carnitine (3 mg/kg) (Soy-based), 12.5% MoLD and 25% MoLD. The feeding was done for four weeks after which serum and hind limb skeletal muscle homogenates were prepared for biochemical analyses. Data was analyzed using one-way Analysis of Variance and Tukey's *posthoc* test at $p < 0.05$.

The findings of this study were that:

i. *M. oleifera* leaf contained terpenoids (2.91mg/g), tannins (9.2mg/g), flavonoids (12.5mg/g), total phenols (42.34mg/g), alkaloids (30.86µg/g) and saponins (55.22µg/g);
ii. the crude protein (13.77%) and carbohydrate (62.89%) contents of 25% MoLD was higher than those of 12.5% MoLD but was similar to the control;
iii. MoLD significantly ($p < 0.05$) increased the activities of serum creatinine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine transaminase (ALT) and reduced Ca^{2+}- ATPase activity when compared with the control;
iv. activities of serum CK, LDH, AST and ALT were significantly ($p < 0.05$) reduced by the 12.5% and 25% MoLD whereas these enzymes in animals fed soy-based were not comparable with the control;
v. activity of cytosolic and mitochondrial Ca^{2+}- ATPase was significantly increased by all the treatments in the order: Soy-based > 25% MoLD > 12.5% MoLD;

vi. the 25% MoLD produced significantly ($p < 0.05$) higher activities of superoxide dismutase, glutathione-S-transferase, glutathione (reduced) and malondialdehyde when compared with other treatments;

vii. mitochondrial permeability and reactive oxygen species was significantly reduced while membrane potential was increased in all the treatment groups with the 25% MoLD producing the most profound change; and

viii. activities of serum AST, ALT and alkaline phosphatase (ALP) as well as albumin, urea and creatinine concentrations in the 12.5% and 25% MoLD fed animals were not significantly different from those of the control whereas those of Soy-base**d** were higher than the control values.

The study concluded that 25% MoLD ameliorated muscle degeneration in rats by positively modulating calcium homeostasis, oxidative stress, energy depletion and is also safe for consumption. The 25% *M. oleifera* leaf-based diet may be explored as an alternative therapeutic option for managing muscle degeneration.

CHAPTER ONE

1.0 INTRODUCTION

Muscle degeneration also known as “muscle atrophy” can be defined as a decrease in mass of the muscle, which could be a partial or complete wasting away. It occurs by changes in the basal balance between protein synthesis and protein degradation. In an atrophying tissue, there is a down-regulation of protein synthesis pathways, and activation of protein degradation (Sandri, 2008). A strict balance is maintained in the cell by Ca^{2+} (a very important molecule).

Muscle atrophies results into muscle weakness, since its ability to exert force is related to mass. Muscle atrophy results from a co-morbidity of several common diseases including cancer (Stefanie *et al.*, 2015), congestive heart failure, chronic obstructive pulmonary disease (COPD), infection (Bababunmi, 2002), acquired immunodeficiency syndrome (AIDS) (Baro and Deubel, 2006), renal failure, and severe burns; patients who have “cachexia” in these disease settings have poor prognosis. Moreover, starvation as well as disuse of muscle eventually leads to muscle atrophy.

Most of these diseases negatively affect calcium homeostasis, ATPase activity and redox state of cells causing oxidative stress (Bababunmi, 2002). There is large trans-membrane electrochemical gradient of calcium ions driving its entry into cells, and yet it is very important for cells to maintain low concentrations of calcium ions for proper cell signaling. This necessitatesthe cells to employ ion pumps to remove excess calcium ions (Carafoli, 1991). An example of such pumps is Calcium ATPase which is a form of P-ATPase that transfer calcium after muscle contraction. The enzyme exits in two forms namely, plasma membrane calcium ATPase (PMCA) and sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) (Carafoli, 1991). They are located on various membrane types and serve to

translocate calcium ions across these membranes against very steep concentration gradients (Bababunmi *et al*., 1994; Lee and East, 2001). PMCA is a transport protein in plasma membrane of cells were it serves to remove calcium ion from the cell. It is vital for regulating the amount of Calcium ion within cells (Jensen *et al.,* 2004). SERCA resides in the sarcoplasmic reticulum (SR) within muscle cells where it transfers Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation. Inhibition of Ca-ATPase (in the case of infection) prevents the pumping of calcium ion in the muscle cell that would otherwise be used for maintaining overall health of the cell, thus resulting in a wasting and degeneration of muscle cell tissue (Bababunmi, 2002).

Malnutrition is a medical condition resulting from improper or insufficient diets (Nikolaos, 2011). World Health Organisation (WHO) 2016, defined malnutrition as deficiencies, excesses or imbalances in a person's intake of energy and/or nutrients. Its definition covers 2 broad groups of conditions: (1) 'undernutrition'- stunting, wasting, underweight and micronutrient deficiencies or insufficiencies and (2) overweight, obesity and diet-related non-communicable diseases (such as heart disease, stroke, diabetes and cancer) (World Health Organization (WHO), 2016). However the condition is often used to mean just undernutrition from either inadequate calories or inadequate specific dietary components for whatever reason (Nikolaos, 2011). The term "severe malnutrition" is often used to refer specifically to protein-energy malnutrition (PEM) which is often associated with micronutrient deficiency (Christopher *et al.,* 2008).

Studies have led to the conclusion that reduced Ca-ATPase activity in protein-energy malnutrition may be linked with the generation of reactive oxygen species (Bababunmi, 2002; Riccardo *et al.,* 2014). Moreover, damage to skeletal muscle by different stresses has been found to occur by three common pathways or reactions vis; Loss of energy supply to the

cell, loss of intracellular calcium homeostasis, and over-activity of oxidizing free radical reactions (McArdle *et al.,* 2002; Bababunmi, 2002; Riccardo *et al.,* 2014).

The search for nutritionally therapeutic food materials has utmostly increased over the years as the use of orthodox medicines increasingly became unaffordable. More so, is the awearness of the fact that most modern drugs are of natural plant origin. In Asia and Africa, moringa has been used for centuries for its healing and nutritional properties. *Moringa oleifera* is an angiosperm belonging to the family Moringaceae (Dalziel, 1956). It is a fast growing, drought resistant tree native to the southern foot hills of the Himalayas in north western India. However it is now cultivated in all regions of the world. Several biological properties ascribed to various parts of *M. oleifera* tree have been reviewed in the past (Anwar *et al.,* 2007) part of this includes its use as an antioxidant (Verma *et al.,*2009) the leaves as a source of both macro- and micronutrients, β-carotene, protein, vitamin C, calcium, and potassium (Siddhuraju and Becker, 2003) amongst others. It has also been used in the treatment of malnutrition in infants and nursing mothers.

Due to availability and wide use, this research was designed to evaluate the mechanism of *M. oleifera* leaf in diet in ameliorating skeletal muscle degeneration *vis-a-vis* a patented formulation comprising of methyl jasmonate, vitamin E (an antioxidant) and carnitine in rat (*Rattus rattus*). This formulation was been used by Bababunmi, (2002) to ameliorate skeletal muscle degeneration caused by malnutrition and other muscle degenerating diseases such as HIV/AIDS (Baro and Deubel, 2006)), cancer (Ravasco *et al.,* 2003), infections (Bababunmi, 2002) and tuberculosis (Schaible and Kaufmann 2007).

1.1 Statement of Problem

A wide range of diseases (HIV/AIDS, infection, tuberculosis, malnutrition etc.) results in muscle degeneration and in most cases; the degeneration plays a key role in their prognosis

and progression. A number of these diseases are acute, chronic and some (e. g HIV/AIDS) are terminal. Hence, the incidence of muscle degeneration has been of great concern. Muscle degeneration plays in the prognosis of diseases; for instance, disuse muscle atrophy is a common clinical problem and there are no effective therapies to prevent the loss of muscle mass and tension resulting from it. Moreover, there are no good pharmaceutical options to enhance the recovery of muscle mass following atrophy-inducing events. Currently, only resistance exercise can be used to promote recovery of mass/strength following disuse atrophy. But in contrast, many patients are unable or unwilling to exercise at intensitysufficient to promote muscle growth (Bodine, 2013).

Tuberculosis and HIV/AIDS are chronic and mostly terminal diseases. Patients are weakened by the progression of the disease and by chemotherapy which usually leads to the production of free radicals. In some cases, the drugs used in the treatment of these diseases also contribute to muscle wasting because they produce free radicals; hence antioxidants are usually prescribed as part of the treatment regimen. It is usually the muscle wasting accompanying treatment that hastens the death of patients suffering from these diseases.

Skeletal muscle degeneration observed during the progression of these diseases is due to calcium homeostasis dysfunction; the major protein in skeletal muscle is the calcium pumping ATPase (Ca^{2+}-ATPase).

1.2 Justification of Study

Biological properties ascribed to different parts of *M. oleifera* have been reviewed in the past (Anwar *et al.,* 2007). These include its use as an antioxidant, anticarcinogenic, antiulcer, antibacterial, and antifungal. Phytochemical analyses have shown that its leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids as well as antioxidants (Amaglo *et al*., 2010). Studies on proximate and phytonutrient

analysis of the leaf by Bamishaiye *et al.* (2011) also showed that it has high percentage of carbohydrate and protein and compared favourably with other high protein/ carbohydrates food crops. It is however a potential leaf source of food that is suitable for fortification of foods and its use as a nutritional supplement is highly promising. Dietary consumption of its part is therefore promoted as a strategy of personal health preservation and self-medication in various diseases (Siddhuraju and Becker, 2003).

In view of the aforementioned problems associated with muscle degenerative diseases, it would be of great advantage to a feed-based therapy, that counteract the negative effects of skeletal muscle degenerative diseases without adversely affecting calcium homeostasis or the redox state of the muscle cells.

Hence the ultimate goal of this study was to develop an effective therapeutic nutritional supplement to prevent, limit or even reverse skeletal muscle atrophy resulting from different pathologic conditions with the hope of extending the lives of the patients by reducing/slowing down the rate of skeletal muscle wasting.

Although *M. oleifera* has been used effectively in cases of malnutrition, this study evaluates its mechanism of ameliorating skeletal muscle degeneration caused by malnutrition. This might be a useful tool in formulating drugs for the management and treatment of skeletal muscle degeneration resulting from different diseases of global concern.

1.3 Aim of Study

The aim of this study was to evaluate the possible mechanism of action of *Moringa oleifera* leaf and methyl jasmonate in ameliorating skeletal muscle degeneration caused by protein energy malnutrition and muscle disuse.

1.4 Specific Objectives

The specific objectives of the study were to:

1. Formulation and proximate composition analysis of control diet and MoLD
2. Induction of muscle atrophy using protein-enegy malnutrition (PEM) model (low-protein iso-caloric diets) and preliminary evaluation of formulated diets (soy -based + methyl jasmonate + Vit. E + carnitine and *M. oleifera* leaf-based) for treatment of muscle atrophy
3. Evaluation of biochemical markers relevant to muscle degeneration (Creatine kinase, lactate dehydrogenase, aspartate Transaminase (AST) and alanine transaminase (ALT)
4. Evaluation of oxidative stress markers associated with atrophy inducing events (Lipid peroxidation / malondialdehyde (MDA),Glutathione peroxidase, Glutathione reductase, Superoxide dismutase (SOD), Catalase)
5. Determination of Ca^{2+}-ATPase activity in the skeletal muscle
6. Evaluating the functionality and permeability of the mitochondrial membrane of the skeletal muscle (as a marker of energy depletion) by assessing the mitochondrial Membrane potential, ROS production and membrane swelling
7. Toxicological evaluation of the *M. oleifera* leaf formulated diet, through the liver function indices (AST, ALT, ALP) and kidney function tests (urea, creatine, serum and muscle electrolytes)
8. Phytochemical screening of the leaf

1.5 Experimental Design

The experiment was done in three phases described a follows:

1. **Preliminary phase (phase one):** objectives fulfilled in this phase where;

i. Feed formulation

ii. Proximate analysis of formulate feeds

iii. Test run of muscle induction by the formulated low protein (4%) iso-caloric feeds

iv. Phytochemical screening of the formulated feeds and *M. oleifera* leaf

2. Phase two

Evaluation of the effects of feeds on the biomarkers of muscle degeneration using the following parameter: calcium homeostasis, oxidatives tress, Energy depletion

3. Phase three: involves the toxicological evaluation of formulated feeds

In each set of the experiment, eight randomly picked animals were positive control (they were fed 25% protein all through). The remaining animals were fed iso-caloric low protein diet containing 4% low protein iso-caloric diets for four weeks for malnutrition to manifest and muscle degeneration induced. Anthropometric parameters were obtained from all the animals including the control weekly. After the first four weeks three animals were randomly selected from each group and sacrificed for analysis. The remaining animals were subdivided into treatment groups and treated for four weeks after which organ homogenates were used for analysis. The test feed and formulations were administered orally

1.6 Significance of Study

This study provides the basic mechanism through which *M. oleifera* leaf-baed diet corrects skeletal muscle degeneration, as this might be a useful tool in the treatment or management of muscle degenerative diseases or conditions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Skeltal Muscle

The muscle is a soft tissue derived from the mesodermal layer of embryonic germ cells in a process known as myogenesis (Mackenzie, 2015). Muscle action can be classified as being either voluntary or involuntary. Cardiac and smooth muscles contracts without conscious thought and are termed involuntary, whereas the skeletal muscles contract upon command (Mackenzie, 2015). Skeletal muscle is a plastic organ that is maintained by multiple pathways regulating cell and protein turnover (Bonald and Sandri, 2013).

Adult skeletal fibers are post-mitotic cells that possess an extraordinary capacity to adapt their size in response to the level of load and utilization. Such plasticity resides in their unique morphological and biological properties. Skeletal myofibers are the largest animal cells, thus, they cannot be supported by only one nucleus. Each nucleus has a limited synthetic capacity (Cavalier-Smith, 1978) therefore; muscle fibers rely on syncytia of hundreds or thousands of nuclei, where each nucleus regulates gene expression within the surrounding domain of its sarcoplasm (Hall and Ralston, 1989).

The smallest contractile unit of skeletal muscle is the muscle fiber also called myofiber. The myofiber is a long cylindrical cell that contains many nuclei, mitochondria, and sarcomeres (Boron and Boulpaep, 2009). Each muscle fiber is surrounded by a thin layer of connective tissue called the endomysium. Approximately 20–80 of these muscle fibers are grouped together in a parallel arrangement called a muscle fascicle or fiber bundle that is encapsulated by a perimysium, which is thicker than the epimysium enclosing each of the bundled muscle fibers. A distinct muscle is formed by enveloping a large number of muscle fascicles in a thick collagenous external sheath extending from the tendons called the epimysium (Boron

and Boulpaep, 2009). Within the muscle cells (myofiber) are bundles of protein filaments called myofibrils. A myofibril is a basic rod-like unit of a muscle cell (McCracken, 1999). It is a complex strand of several kinds of protein filaments organized together in repeated units called sacomeres which is responsible for the striated appearance of the skeletal muscle and cardiac muscles. Each filament in the sarcomere is composed of the actin and myosin fragments (thin and thin filaments respectively).

2.1.1 Types of Muscle Fibre

Each muscle fiber is classified by its histologic appearance, rapidity of contraction, and ability to resist fatigue (Boron and Boulpaep, 2009). Hence, skeletal muscles can be divided into fast and slow twitch fibers.

The Slow-twitch also known as type I fibers is generally thinner, invested by a denser capillary network, and appears red owing to the presence of a large amount of the oxygen-binding protein myoglobin. These type I fibers are resistant to fatigue, relying on oxidative metabolism for energy, and thus exhibit high mitochondrial numbers and oxidative enzyme content, and low glycogen levels and glycolytic enzyme activity. In contrast, fast-twitch or type II fibers differ among themselves with regard to fatigability. For instance, Type IIa fibers share some features with slow-twitch fibers in that they are fatigue-resistant, rely on oxidative metabolism, and contain myoglobin (and thus are red) (Armstrong, 1996; Boron and Boulpaep, 2009). Although, type IIa muscle fibers contain abundant glycogen and more mitochondria in contrast to the type I slow-twitch cells (Armstrong, 1996; Boron and Boulpaep, 2009). These distinctive features ensure adequate ATP generation to compensate for the accelerated rate of ATP hydrolysis in these fast-twitch fibers. The other type of fast-twitch fibers (type IIb) rely on the energy stored in glycogen and phosphocreatine because they contain fewer mitochondria, have low myoglobin (and thus are white muscle) and

oxidative enzyme content, and are invested by a less dense capillary network (Armstrong, 1996; Boron and Boulpaep, 2009). As a consequence of these deficiencies, type IIb muscle fibers are more easily fatigable. A third sub type of the type ii fibre is the type IIx. The three types vary in there contractile (Larson *et al*., 1991*)* speed and force generation (McCloud, 2011). Fast twitch fibers contract quickly and powerfully but fatigue very rapidly, sustaining only short anaerobic bursts of activity before muscle contraction becomes painful. They contribute most to muscle strength and have greater potential for increase in mass. Type IIb is anaerobic, glycolytic, "white" muscle that is least dense in mitochondria and myoglobin. In small animals (e.g., rodents) this is the major fast muscle type, explaining the pale color of their flesh. However, in most skeletal muscles, individual fascicles are composed of two or more of these fiber types, although one fiber type usually predominates in a given muscle.

2.2 Muscle Degeneration

Atrophy is defined as a decrease in the size of a tissue or organ resulting from cellular shrinkage; the decrease in cell size is caused by the loss of organelles, cytoplasm and proteins (Paolo and Marco, 2013). Muscles serve as a source of amino acids that can be used for energy production by various organs (such as the heart, liver and brain) during catabolic periods (physiological conditions), such as in cancer, sepsis, burn injury, heart failure and AIDS. Excessive protein degradation in skeletal muscle, and the resulting muscle loss (cachexia), ultimately aggravates diseases, and increases morbidity and mortality because it is highly detrimental for the economy of the body and can lead to death. Moreover, excessive loss of muscle mass is a poor prognostic indicator and can impair the efficacy of many different therapeutic treatments (Paolo and Marco, 2013). The maintenance of healthy muscles is however crucial for preventing metabolic disorders, maintaining healthy aging and providing energy to vital organs during stress conditions.

Inactivity and starvation in mammals lead to atrophy of skeletal muscle, a decrease in muscle mass that may be accompanied by a smaller number and size of the muscle cells as well as lower protein content (Fuster *et al.,* 2007). Muscle atrophy may also result from the natural aging process or from disease.

Muscle atrophy occurs in numerous pathologies such as cancer, sepsis, uremia, and diabetes (Hasselgren and Fischer, 1997; Jagoe and Goldberg, 2001). However, muscle atrophy can also occur in the absence of disease during prolonged periods of reduced muscle activity (Booth, 1982). Based on their causes, three primary categories of skeletal muscle loss exist. These include starvation, sarcopenia, and cachexia. Different types of conditions producing atrophy imply different types of molecular triggers and signaling pathways for muscle wasting (Robert and Susan, 2004). Starvation is a pure protein-energy deficiency, thus forcing a reduction in both fat and fat-free mass. The key physiological sign of starvation is that it is reversed solely by the replenishment of nutrients (American Society of Parenteral and Enteral Nutrition, 2002). Observed age-related decline in muscle mass has been termed sarcopenia. Sarcopenia was coined from the Greek ‘‘sarx,’’ or ‘‘flesh,’’ and ‘‘penia,’’ or ‘‘loss.’’ The term was first introduced by Irwin Rosenberg in 1988 (Rosenberg, 1989), and the first Sarcopenia Workshop held by the National Institute on Aging in 1994. Severe wasting of both fat and fat-free mass is termed cachexia. Cachexia is from the Greek ‘‘kak’’ or ‘‘cac,’’ meaning ‘‘bad,’’ and ‘‘hexis,’’ or ‘‘condition.’’ Cachexia is widely recognized as severe wasting accompanying disease states such as cancer or immunodeficiency disease, but does not have a widely accepted definition. Although there is no widely accepted definition, cachexia is best viewed as the cytokine-associated wasting of protein and energy stores due to the effects of disease (Thomas, 2002). Persons with cachexia lose roughly equal amounts of fat and fat-free mass, while maintaining extracellular water and intracellular potassium. The loss of fat-free mass is mainly from the skeletal muscle (David, 2007).

All skeletal muscle loss is seen as resulting from an inadequate intake of nutrients. Under this paradigm, hypercaloric feeding would be expected to result in repair of the nutritional deficit and weight gain. Conversely, in most studies of hypercaloric feeding, it has been difficult to produce a significant gain in body weight. This suggests that factors other than pure starvation are operational, since a response to refeeding is the hallmark of starvation. Skeletal muscle loss due to sarcopenia and especially cachexia has however been remarkably resistant to nutritional therapy (David, 2007).

Epidemiological data demonstrates that sarcopenia is the most frequent cause while cachexia is the next most common cause of loss of muscle mass, occurring in a number of disease states. In the extreme situations, starvation can lead to cachexia but is usually not as common, at least in developed countries where access to food is not a factor. Although starvation, cachexia, and sarcopenia can be defined as distinct clinical syndromes, the understanding of the process is complicated by a certain degree of overlap. Starvation, from whatever cause, will ultimately lead to a loss of muscle mass and strength indistinguishable from that produced by cachexia or sarcopenia. In cachexia, proinflammatory cytokines have a direct effect on muscle mass. This leads to a loss of muscle mass indistinguishable from sarcopenia. On the other hand, sarcopenia alone has not been shown to lead to a decrease in appetite or to loss of fat mass similar to that associated with cachexia. It is not clear however, whether aging itself in some persons, in the absence of any defined inflammatory disease, is associated with elevated proinflammatory cytokines that lead to sarcopenia (David, 2007).

2.2.1 Mechanisms of Muscle Atrophy

Like any other tissue, muscle mass depends on protein turnover and cell turnover (Sartorelli and Fulco, 2004). The main players of muscle regeneration are satellite cells, which are a population of undifferentiated myogenic cells located between the basal lamina and the

plasma membrane of muscle fibers. Although these cells play a major role in postnatal muscle growth, recent studies have challenged the idea that they are required for muscle mass maintenance and hypertrophy in adulthood (Amthor *et al*., 2009; Blaauw *et al*., 2009; McCarthy *et al*., 2011; Raffaello *et al*., 2010; Sartori *et al*., 2009).

The pathways controlling muscle atrophy in physiological and pathological conditions are mainly the ubiquitin-proteasome machinery and the autophagy -lysosome machinery; these two most important cell proteolytic systems control protein turnover in muscle. Evidence indicates that these two processes play a pivotal role in regulating overall muscle homeostasis (Bonaldo and Sandr, 2013). Thus, different types of muscle atrophy share a common transcriptional program that is activated in many systemic diseases (Lecker *et al*, 2004). Generally, the proteolytic pathways involved in skeletal muscleprotein breakdown in eukaryotic cells includes; lysosomal (involves cathepsins B, H, L, etc.); ATP-dependent, that require or not ubiquitin (comprises at least two large cytosolic proteases, UCDEN and proteasome), Cytosolic Ca^{2+}-dependent (involves Ca^{2+}-dependent proteasescalpains I and II); and ATP-independent (it is not known which proteases are involved in this degradative system) (Kettelhut *et al*., 1994).

Despite recent dramatic progress, the relative contribution of these pathways to the accelerated proteolysis occurring in normal and pathological states is still largely unknown (Kettelhut *et al*., 1994). It is also becoming evident that interactions among known proteolytic pathways (ubiquitin- proteasome, lysosomal, and calpain) are involved in muscle proteolysis during atrophy (Robert *et al*., 2004).

2.2.1.1 The Lysosomal Pathway

This pathway is also referred to as the autophagy-lysosome system. Autophagy is a self-eating process through which cells degrade their own components, recycles amino acids and

other building blocks that can eventually be re-used (Kroemer *et al.,* 2010). Macroautophagy (hereby referred to as autophagy) is the best characterized form of autophagy, and consists a genetically-programmed, evolutionarily-conserved catabolic process that degrades damaged or unnecessary cellular proteins and organelles through their sequestration into a double-membrane structure known as the autophagosome (Kroemer *et al.,* 2010). Autophagosomes then fuse with lysosomes to form auto lysosomes, wherein the enveloped content is degraded (Figure 1).

Autophagy was first described many years ago; however, its involvement in muscle protein breakdown during atrophy was not recognized for a long time. Early evidence showed that lysosomal degradation contributes to protein breakdown in denervated muscle (Furuno *et al*., 1990). A lysosomal protease, Cathepsin l, was also shown to be up-regulated during muscle atrophy (Deval *et al*., 2001). The development of molecular and imaging tools to follow autophagosome formation has greatly improved the characterization of autophagy in normal and atrophying muscles. In fact, analysis of different organs revealed that skeletal muscle is one of the tissues with the highest rates of vesicle formation during fasting (Klionsky *et al*., 2008). However, fasting glycolytic muscles display a higher content of autophagosomes than slow β-oxidative muscles (Mizushima *et al*., 2004).

Autophagy is primarily considered to be a non-selective degradation pathway, but the significance of more of its selective forms is becoming increasingly evident withseveral cargo-specific autophagic processes been identified in mammals and yeasts; each of these has been named based on the nature of the target. Examples include pexophagy for the disposal of peroxisomes, ERphagy for endoplasmic reticulum degradation, ribophagy for the elimination of ribosomes, aggrephagy for the removal of aggregates (Reggiori *et al.,* 2012),

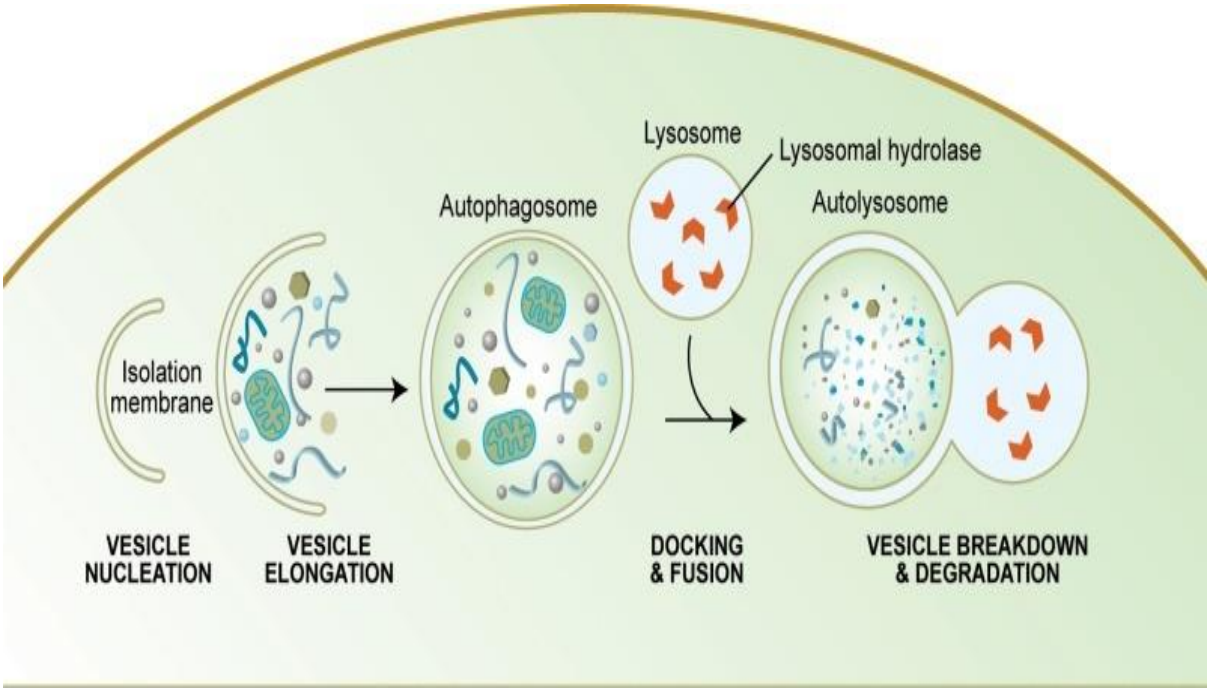


Figure 1: **Schematic Diagram of the Steps Involved in Autophagy**

Source: Melèndez and Levine (2009)

and mitochondria via mitophagy (Bonaldoand Sandri, 2013). The process regulates mitochondrial number to match metabolic demands and also serves as a quality control mechanism for the removal of damaged mitochondria (Ding and Yin, 2012). Mitophagy is preceded by mitochondrial fission, which divides mitochondria into pieces of manageable size for engulfment and mediates the segregation of damaged material for subsequent disposal (Twig *et al.*, 2008a). The loss of the mitochondrial membrane potential ($\Delta\psi_m$) is a major trigger of mitophagy (Twig *et al.*, 2008a). Recent evidence also suggests that opening of the mitochondrial permeability transition pore (mPTP) may be required for the selective removal of damaged mitochondria by inducing $\Delta\psi_m$ dissipation (Carreira *et al.*, 2010). In addition to the relevance of autophagy to mitochondrial quality control, several lines of evidence also suggest that mitochondria can substantially influence the autophagic process. Indeed, mitochondria have been linked to virtually every step of autophagy through the localization of numerous autophagic regulators on mitochondrial membranes, the integration of these organelles in several signaling networks, and also their ability to modulate these pathways (Rambold and Lippincott-Schwartz, 2011). Hence, the crosstalk between mitochondria and autophagy places these two elements in a unique position, where defects in one or the other system can increase the risk for various disease conditions. Impairment of basal mitophagy is however deleterious to muscle homeostasis, and leads to the accumulation of damaged and dysfunctional mitochondria (Grumati *et al*., 2010). Besides, mitophagy and other forms of selective autophagy probably play important roles in the maintenance of skeletal muscle homeostasis. For instance, a proper rate of nucleophagy seems essential for nuclear remodeling of muscle fibers, as indicated by nuclear envelopathies, a group of genetic disorders characterized by increased nuclear fragility and giant autophagosomes that contain nuclear components (Park *et al*., 2009). However, in skeletal muscle, the regulation of autophagy presents some interesting peculiarities.

In atrophying muscle, the mitochondrial network is dramatically remodeled following fasting or denervation, and autophagy via Bnip3 contributes to mitochondrial remodeling (Romanello *et al*., 2010; Romanello and Sandri, 2010). Expression of the fission machinery is enough to cause muscle wasting in mice, whereas inhibition of mitochondrial fission prevents muscle loss during denervation, indicating that disruption of the mitochondrial network is a crucial amplificatory loop of the muscle atrophy program (Romanello *et al*., 2010; Romanello and Sandri, 2010).

Autophagy has been shown to be involved in various biological functions such as starvation adaptation, turnover of unfolded proteins and damaged organelles, cell metabolism, development, immunity, and cell death. Emerging studies showed that autophagy also plays roles in the pathology of different human diseases including degenerative diseases, aging, and cancer (Kroemer and White, 2010). It is activated as an adaptive catabolic process in response to different forms of metabolic stress, including nutrient deprivation, growth factor depletion, and hypoxia. This bulk form of degradation generates free amino and fatty acids that can be recycled in a cell-autonomous fashion or delivered systemically to distant sites within the organism. Presumably, the amino acids generated are used for the *de novo* synthesis of proteins that are essential for stress adaptation (Levine and Kroemer, 2008). These amino acids can be further processed and together with the fatty acids, used by the tricarboxylic acid cycle (TCA) to maintain cellular ATP production. The importance of autophagy in fueling the TCA cycle is supported by studies showing that certain phenotypes of autophagy-deficient cells can be reversed by supplying them with a TCA substrate such as pyruvate (or its membrane-permeable derivative methylpyruvate) (Lum *et al.,* 2005). It can also restore ATP production, the generation of engulfment signals, and effective corpse removal in autophagy-deficient cells during embryonic development (Qu *et al.,* 2007).

A critical physiological role of autophagy seems to be the mobilization of intracellular energy resources to meet cellular and organismal demands for metabolic substrates. The requirement for this function of autophagy is not limited to settings of nutrient starvation. Because growth factors are often required for nutrient uptake, loss of growth factor signaling can result in reduced intracellular metabolite concentrations and activation of autophagy-dependent survival mechanisms (Lum *et al.,* 2005). Thus, the role of autophagy in maintaining macromolecular synthesis and ATP production (Levine and Kroemer, 2008).

Moreover, in certain conditions, especially when cells suddenly have high metabolic needs, autophagy may be needed in a cell-autonomous fashion to generate sufficient intracellular metabolic substrates to maintain cellular energy homeostasis. This hypothesis may explain why there are high levels of autophagy in the mouse heart and diaphragm immediately following birth (Kuma *et al.,* 2004).

2.2.1.2 Ubiquitin-Proteasome System

A decrease in muscle mass is associated with an increased conjugation of ubiquitin to muscle proteins (Figure 2), increased proteasomal ATP-dependent activity, increased protein breakdown that can be efficiently blocked by proteasome inhibitors; and an up regulation of transcripts encoding ubiquitin, some ubiquitin-conjugating enzymes (E2), a few ubiquitin-protein ligases (E3) and several proteasome subunits (Lecker *et al*., 2006). Human genome encodes more than 650 ubiquitin ligases (Lee and Goldberg, 2011), which are involved in the precise regulation of different cellular processes, and dramatic progress has been made recently in elucidating the roles of different E3s in regulating metabolism, transcription, cell cycle, oncogenesis and muscle size.

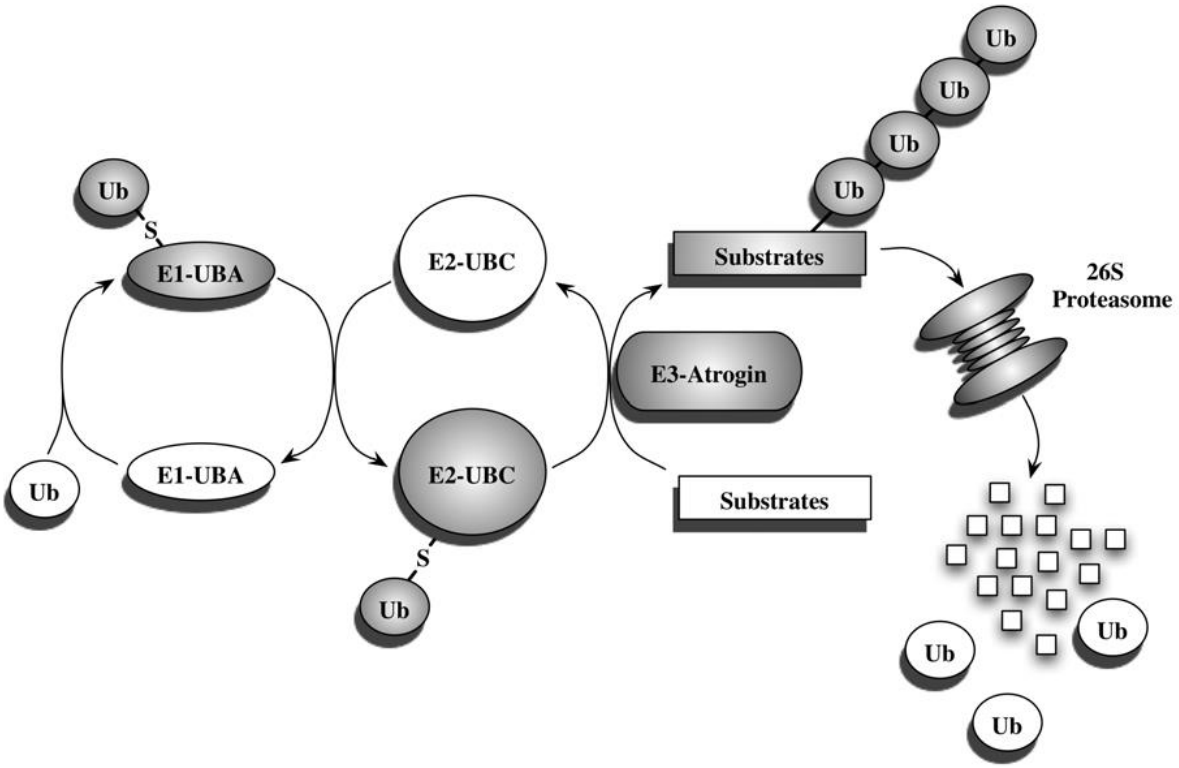


Figure 2: **The Ubiquitin Proteasome Pathway**

Source: Reid, 2005.

Six monomers of Ubiquitin (Ub) are activated by the E1 or ubiquitin-activating protein (E1-UBA), transferred to the E2 or ubiquitin-conjugating protein (E2-UBC), and attached to substrate proteins in cooperation with E3 proteins such as atrogin1/MAFbx (E3-atrogin); repetition of this process creates ubiquitin polymers that trigger substrate degradation by the 26S proteasome.

Different E2–E3 pairs degrade different proteins; the specificity of E3s for certain groups of proteins provides exquisite selectivity to this degradation. The amount of different E2 and E3 proteins varies between tissues types and under different physiological conditions, but it is still unknown which specific E2s and E3s normally operate in muscle. Among the known E3s, only a few of them are muscle-specific and are up regulated during muscle loss (Sacheck *et al.,* 2007). In the Ubiquitin-Proteasome Systems, E1 enzymes activate ubiquitin proteins after the cleavage of ATP. The ubiquitin is then moved from E1 to members of the E2 enzyme class. The final ubiquitylation reaction is catalyzed by members of the E3 enzyme class. E3 binds to E2 and the protein substrate, inducing the transfer of ubiquitin from E2 to the substrate. Once the substrate is polyubiquitylated, it is docked (joined) to the proteasome for degradation (Figure 2). Note that polyubiquitin chains can be removed by de-ubiquitylating enzymes [ubiquitin-specific processing proteases (USPs)]. ZNF216 is involved in the recognition and delivery to the proteasome of ubiquitylated proteins during muscle atrophy. Atrogin-1 regulates the half-life of the MyoD transcription factor and of eIF3f, which is crucial for protein synthesis. Fbxo40 regulates the half-life of insulin receptor substrate (IRS1), an essential factor for IGF1/insulin signaling, whereas MuRF1 regulates the half-life of several sarcomeric proteins. Note that ubiquitin ligases can have different cellular localizations and can shuttle into the nucleus (Bonaldo and Sandr, 2013).

The commonly up- or down regulated genes believed to regulate the loss of muscle components are called the atrophy-related genes or ‘atrogenes’ (Sacheck *et al*., 2007). Experimental findings revealed that muscle atrophy is an active process controlled by specific signaling pathways and transcriptional programs. Furthermore, the genes induced most strongly were found to encode two muscle-specific ubiquitin ligases, atrogin-1 (also known as MAFbx) and MuRF1 (Bodine *et al.,* 2001; Gomes *et al.,* 2001). Notably, the transcription

factor myogenin, an essential regulator of muscle development, is required for the maximal activation of these two genes, at least during denervation (Moresi *et al*., 2010).

Valuable information on the role of specific components of the ubiquitin-proteasome system in muscle was obtained by generating genetically modified animals. For instance, mice lacking atrogin-1 and MuRF1 are resistant to muscle atrophy induced by denervation (Bodine *et al*., 2001). Moreover, knockdown of atrogin-1 prevents muscle loss during fasting (Cong *et al*., 2011), whereas MuRF1 knockout mice (but not atrogin-1 knockout mice) are resistant to dexamethasone-induced muscle atrophy (Baehr *et al*., 2011). However, only a few muscle proteins have been identified as substrates for atrogin-1 so far, and they all seem to be involved in growth-related processes or survival pathways. Atrogin-1 promotes degradation of MyoD, a key muscle transcription factor, and of eIF3f, an important activator of protein synthesis (Csibi *et al*., 2010; Tintignac *et al*., 2005).

Muscle atrophy appears to be caused by the rise in oxidant activity since administration of exogenous antioxidants can blunt muscle loss in TNF-treated animals (Buck and Chojkier, 1996). Genes that code for ubiquitin, E-proteins, and proteasome subunits are upregulated in response to TNF or ROS exposure. Other examples include atrogin1/MAFbx and MuRF-1 expression which are positively regulated by FoxO. MuRF-1 is also sensitive to nuclear factor-κB (NF-κB), a transcription factor that is activated by inflammatory mediators, including TNF and ROS (Moylan *et al.*, 2008). These include genes that are strongly associated with muscle catabolism. E2 and E3 proteins determine specificity of the ubiquitin-proteasome pathway. They are rate-limiting elements of the pathway, determining the maximal rate of ubiquitin conjugation to substrates. Increased expression of E2 and E3 proteins can influence the composition of proteolyzed substrates and can accelerate the overall rate of proteasomal degradation, favoring net loss of muscle protein.

2.2.1.3 Mechanisms of Cachexia

Individuals with cachexia experience progressive severe loss of skeletal muscle with a relative preservation of visceral protein reserves. The loss of skeletal muscle mass is due to a combination of reduced protein synthesis and increased protein degradation. While reduced protein synthesis plays a role, protein degradation is the major cause of loss of skeletal muscle mass in cachexia (David, 2007). Lysosomal protease cathespins B probably play a role in early protein breakdown, as they are elevated in skeletal muscle biopsies from patients with lung cancer and minimal weight loss (Jagoe *et al.,* 2002). In more established cachexia, the ubiquitin-proteasome dependent proteolytic pathway is up regulated and this is the predominant pathway for protein degradation (Gordon *et al.,* 2005). The underlying mechanism(s) appears to involve the induction of muscle-specific ubiquitin ligases by catabolic hormones, such as the glucocorticoids, but also the inhibition of anabolic pathways as those controlled by insulin-like growth factor-1, phosphatidylinositol-3-kinase/ Akt, and mammalian target of rapamycin (Frost and Lang, 2005). Several cytokines, including tumor necrosis factor-alpha, IL-6, IL-1-beta and gamma interferon, reproduce symptoms of cachexia in animal models, although individually they have not been shown to produce full-blown cachexia syndrome (Murray *et al.,* 1997). Direct infusion of IL-6 into a mouse muscle decreased myofibrillar protein by 17% at 14 days, suggesting a direct effect on muscle (Haddad *et al.,* 2005).

2.2.2 The Role of Oxidative Stress and Calcium Ion in Muscle Degeneration

Prolonged periods of muscle inactivity lead to increased production of reactive oxygen species (ROS), which are important signaling molecules responsible for mediating downstream muscle dysfunction and atrophy. Early evidence indicating that enhanced oxidative stress plays a role in muscle loss with disuse was provided over 20 years ago by Kondo *et al*., (1991). This pioneer study demonstrated that the immobilization of rodent

muscle was associated with increased levels of oxidative stress, which could be partially rescued by vitamin E supplementation. Subsequent investigations have however provided the definitive mechanistic link between oxidative stress and acute muscle loss (Agten *et al.,* 2011; Min *et al.,* 2011). Evidence indicates that mitochondria are the primary source of ROS during chronic muscle inactivity (Powers *et al.,* 2012). Remarkably, administration of the mitochondria-targeted antioxidant SS-31 has recently been shown to attenuate ROS production and myofiber atrophy in hind-limb muscles (Min *et al.,* 2011) and the diaphragm (Powers *et al.,* 2011a) of mice subjected to cast immobilization and mechanical ventilation, respectively.

Experimental evidence also suggests that chronic muscle inactivity causes major disturbances in intracellular calcium homeostasis (Ingalls *et al.,* 1999; Weiss *et al.,* 2010), which might lead to altered mitochondrial calcium handling and increased oxidant production (Brookes *et al.,* 2004). Mitochondrial calcium overload can promote ROS generation through several mechanisms including activation of the citric acid cycle, which results in increased NADH formation, stimulation of nitric oxide synthase, and activation of ROS-generating enzymes such as α-ketoglutarate dehydrogenase (Peng and Jou, 2010). Alternatively, chronic muscle inactivity could induce the accumulation of fatty acid hydroperoxides in mitochondria, which may lead to enhanced ROS production by acting as uncouplers, interfering with complex I and III of the ETC, and inhibiting adenine nucleotide translocase (Bhattacharya *et al.*,2011).

Though mitochondria appear to be the primary source of oxidants during muscle inactivity, non-mitochondrial processes may also participate in the overall increase in ROS generation. For example, xanthine oxidase (Kondo *et al.,* 1993) and/or NAD(P)H oxidase located in the cytosolic fraction (Javesghani *et al.,* 2002) may contribute slightly to oxidative stress associated with chronic muscle disuse. Regardless of the mechanism(s) responsible for

excessive ROS generation during disuse atrophy, oxidative stress activates transcription factors that regulate the expression of mediators involved in catabolic pathways (Li *et al.*, 2003; Dodd *et al.,* 2010; McClung *et al.,* 2010). In addition, ROS can directly activate key intracellular proteolytic enzymes such as calpains and promote the initiation of the apoptotic cascade via the release of mitochondrial pro-apoptotic factors (Adhihetty *et al.*, 2005; Smuder *et al.,* 2010). Lastly, oxidative stress can modify contractile elements, thereby making them targets for proteolysis (Grune *et al.*, 2003).

Reactive oxygen species (ROS) are constantly generated within cells by several enzymatic reactions, including those catalyzed by cyclooxygenases, NAD(P)H oxidase, and xanthine oxidase; however, the bulk of ROS production (~ 90 %) occurs as a byproduct of mitochondrial oxidative phosphorylation (OXPHOS). In physiological conditions, 0.2 – 2.0 % of biatomic oxygen is incompletely reduced to superoxide anion ($O_2^{\bullet -}$) by electrons leaking at complex I and III of the electron transport chain (ETC) (Chance *et al*., 1979). However, the mitochondria have evolved a multileveled defense network comprising detoxifying enzymes and non-enzymatic antioxidants to deal with the ordeal such that, if the mitochondrial antioxidant defenses are fully functioning and electron leakage occurs within the physiological range, oxidative damage is almost completely prevented. In such circumstances, the small amounts of ROS generated function as second messenger molecules that modulate the expression of several genes involved in metabolic regulation and stress resistance (mitochondrial hormesis or mitohormesis) (Handy and Loscalzo, 2012).

Excessive ROS generation, defective oxidant scavenging, or both, have been implicated in the aging process and in the pathogenesis of several conditions, including acute muscle atrophy and sarcopenia (Powers *et al*., 2011; Handy and Loscalzo, 2012).

It is clear that skeletal muscle inactivity is associated with an increase in both cytosolic calcium levels and calpain activity (Kandarian and Stevenson, 2002). Although the mechanism responsible for the inactivity-mediated calcium overload is unknown, it is possible that intracellular production of ROS could play a key role in the disturbances of calcium homeostasis (Kandarian and Stevenson, 2002). A potential biochemical mechanism to link oxidative stress with calcium overload is that ROS-mediated formation of reactive aldehydes (i.e., 4-hydroxy-2, 3-trans-nonenal) can inhibit plasma membrane Ca^{2+}-ATPase activity (Siems *et al*., 2003). Therefore, the oxidative stress induced decrease in membrane Ca^{2+}-ATPase activity would impede Ca^{2+} removal from the cell thereby promoting intracellular Ca^{2+} accumulation. However, it is currently unknown as to whether this mechanism is the single explanation for inactivity mediated calcium overload in muscle.

2.2.3 Intracellular Calcium Homeostasis

Calcium is a universal carrier of information (Brini *et al.*, 2013).The calcium ion (Ca^{2+}) acts as a ubiquitous second messenger signal in the control of eukaryotic cell function (Carafoli, 1987; Miller, 1991; Strehler, 1996) and therefore changes in the intracellular Ca^{2+} concentration needs to be under tight spatial and temporal control. Ca^{2+} entering the cell from the extracellular space increases the total calcium concentration present in that cell. An unchecked Ca^{2+} entry overtime may lead to Ca^{2+} overload, eventually saturating the intracellular buffering and organellar storage systems (Carafoli, 1987; Miller, 1991; Strehler, 1996).

Calcium homeostasis refers to the regulation of calcium ion within the extracellular fluid (Brini *et al.*, 2013). In higher animals, most of the Ca^{2+} is immobilized within the bones and teeth in the form of hydroxyapatite (Ca_{10} $(PO_4)_6(OH_2)$) with only a negligible amount available within the extracellular and intracellular fluid (Carafoli, 1987) under controlled

conditions. However, Ca^{2+} concentration in the intracellular fluid is controlled by its movement in and out of the bone deposits though fixed at just about 3 mM, half of which are ionized (Carafoli, 1987).

Intracellular (i.e within the cytoplasm) calcium ion is maintained at a very lower concentration (about 10,000 time) than the extracellular concentration to maintain a steep electrochemical gradient (Carafoli, 1991) which is necessary for cellular wellbeing. To maintain a resting concentration of the Ca^{2+} in the cytoplasm which is normally maintained at around 100nM, about 20,000 – 100,000 folds lower than the extracellular concentration (Clapham, 2007; Demauríex, 2016), Ca^{2+} is actively pumped out of the cytosol to the extracellular space, endoplasmic reticulum (ER), and sometimes the mitochondria.

Calcium signaling occurs when the cell is stimulated to release calcium ions from the intracellular (cytosolic) pool, and/or when it enters into the cell through the plasma membrane ion channels (Clapham, 2007). To maintain the steep electrochemical gradient at low intracellular concentration, it is necessary for the cell to employ ion pumps to remove excess calcium ions (Carafoli, 1991).

Calcium adenosine triphosphatase (ATPase) is a form of P-ATPase that transfer calcium after muscle contraction. The enzyme exits in two forms namely, Plasma Membrane Calcium ATPase (PMCA) and Sarcoplasmic Endoplasmic Reticulum Calcium ATPase (SERCA) (Carafoli, 1991; Bewaji, 1993; Bababunmi *et al*., 1994; Bewaji and Dawson, 1995). They are located on various membrane types and serve to translocate calcium ions across these membranes against very steep concentration gradients (Bababunmi *et al.,* 1994). PMCA is a transport protein in plasma membrane of cells that serves to remove calcium ion from the cell. It is vital for regulating the amount of calcium ion within cells (Jensen *et al.,* 2004). SERCA resides in the sarcoplasmic reticulum (SR) within muscle cells where it transfers

Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation.

Many mechanisms have been proposed to explain muscle degeneration seen in muscle degenerative diseases. Among these, one of the most championed mechanisms entails the loss of calcium homeostasis as a result of calcium influx through ion channels in the membrane (Franco and Lansman, 1990). The increase in cytoplasmic calcium is known to then regulate ROS primarily through disrupting mitochondrial function (Nethery *et al*., 2000; Brookes and Darley-Usmar, 2004).

The two plasmalemmal systems primarily responsible for Ca^{2+} extrusion are the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/ Ca^{2+} exchanger (Carafoli, 1987; Miller, 1991; Strehler, 1996). The control of cytoplasmic Ca^{2+} concentrations is also mediated, in part, by the uptake or release of Ca^{2+} from the intracellular organelles (Bewaji and Bababunmi, 2008).

The sodium-calcium exchanger (often denoted as Na^{+}/Ca^{2+} exchanger, NCX, or exchange protein) is an antiportermembrane protein that removes calcium from cells using the energy that is stored in the electrochemical gradient of sodium (Na^{+}) by allowing Na^{+} to flow down its gradient across the plasma membrane in exchange for the counter transport of calcium ions (Ca^{2+}). The NCX removes a single calcium ion in exchange for the import of three sodium ions (Yu and Choi, 1997). The exchanger exists in many different cell types and animal species (Dipolo and Beaugé, 2006). It is considered one of the most important cellular mechanisms for removing Ca^{2+} (Dipolo and Beaugé, 2006) usually found in the plasma membranes, mitochondria and endoplasmic reticulum of excitable cells (Patterson *et al.*, 2007).

2.2.3.1 The Plasma Membrane Calcium ATPase

The plasma membrane calcium ATPase (PMCA) (Figure 3) was first isolated from erythrocyte membranes (Schatzmann, 1966) and was one of the first enzymes shown to be regulated by the calcium sensor protein calmodulin (Carafoli, 1987; Penniston, 1983). Subsequent work has determined that the PMCAs are generated from a gene family consisting of four distinct members, designated ATP2B1, ATP2B2, ATP2B3, and ATP2B4 in the human genome database (GDB) nomenclature. These four major isoforms encoded by these genes (PMCAs 1-4) are expressed in a tissue specific manner. Vis; PMCA isoforms 1 and 4 are considered housekeeping forms because of their almost ubiquitous expression. In rats and humans, PMCA isoform 1 has been detected in all tissues examined to date (Shull, 1988; Brandt *et al.,* 1992; Keeton *et al.,* 1993; Stauffer *et al.,* 1994). In humans, PMCA isoform 4 has also been found in all tissues examined (Stauffer *et al.,* 1994; Stauffer *et al.,* 1995; Carafoli and Stauffer, 1994) whereas in rats, this isoform is less abundant and appears to be absent in liver (Keeton and Shull, 1995). PMCA isoform 2 is expressed primarily in brain and heart, and PMCA isoform 3 is essentially confined to brain and skeletal muscle (Shull and Greeb, 1988; Brandt *et al.,* 1992; Keeton *et al.,* 1993; Stauffer *et al.,* 1993; Stauffer *et al.,* 1995; Greeb and Shull, 1989). Further isoform variability is achieved by mRNA splicing which occurs at two splice sites A and C (Strehler, 1991).The functional significance for alternative splicing at site A has not yet been elucidated although it has been hypothesized that differential regulation by phospholipids may be involved (Strehler, 1991 and Hilfiker *et al.,* 1994). Splicing at site A affects a region of the protein previously shown to be involved in lipid regulation of the pump (Zvaritch *et al.,* 1990; Filoteo *et al.,* 1992; Brodin *et al.,* 1992). In contrast to the sparse information on the significance of splice site A variants, splicing events at site C have been much better characterized with respect to their functional consequences. Splicing at site C has been shown to alter the PMCA's ability to

bind calmodulin and to affect the apparent Ca^{2+} affinity (i.e., the K1/2(Ca^{2+})) of the final protein product (Enyedi *et al.,* 1994; Verma *et al.,* 1996; Elwess *et al.,* 1997).

The PMCAs range in molecular weight from 125-140 kDa, and are members of the P-type class of ion motive ATPases (Pedersen and Carafoli, 1987; Carafoli, 1991).They contain ten predicted transmembrane domains, and the C and N-termini are located intra-cellularly (Strehler, 1991; Carafoli, 1991). The two major intracellular loops, located between transmembrane domains 2 and 3 and between transmembrane domains 4 and 5, comprise the transduction domain and the catalytic region of the PMCA, respectively. The extended C-terminal tail of the pump is the main regulatory region. It contains the calmodulin-binding domain, sites for phosphorylation by protein kinases A and C, and sequences for additional protein interactions that may determine the membrane localization, targeting, and cross-talk with other signaling molecules of the pump (Carafoli, 1994; Penniston and Enyedi, 1998; Kim *et al.,* 1998; Strehler *et al.,* 1998). The calmdulin binding sequence is thought to act as autoinhibitory domain, effectively keeping the pump in an inactive state in the absence of calcium-calmodulin (i.e. in the presence of low intracellular free Ca^{2+}) (Carafoli, 1994; Penniston and Enyedi, 1998; Kim *et al.,* 1998; Strehler *et al.,* 1998).

During times of low intracellular calcium, intramolecular interactions of the C - terminal autoinhibitory tail with two sites located within the first and second cytosolic loop prevent high-affinity Ca^{2+} binding and transport (Strehler, 1996; Carafoli, 1994; Strehler *et al.,* 1998). The functioning of the PMCA is therefore, regulated by Ca^{2+}. Although the precise mechanism of Ca^{2+} transport is not yet known, site-directed mutagenesis studies have shown that conserved amino acid residues within transmembrane domains 4, 6 and 8 are essential for calcium transport by the PMCA (Guerini *et al.,* 1996; Adebayo *et al.,* 1995).

Due to the high affinity of PMCAs for Ca^{2+}, they have been long believed to play a major role only in maintaining the basal level of Ca^{2+} (Carafoli, 1991). Recent evidence, however, suggests a more active role for the PMCAs in returning Ca^{2+} to basal levels after cellular stimulation (Toescu and Petersen, 1995; Monteith and Roufogalis, 1995; Penniston and Enyedi, 1998; Tepikin *et al.,* 1992). A more dynamic involvement of the PMCA in shaping intracellular Ca^{2+} signals is also suggested by the multitude of isoforms and splice variants showing different regulatory and transport properties.

PMCA 1 has a molecular weight of 129,500 and 1,176 amino acid residues while PMCA 2 has a molecular weight of 132,600 and 1,198 amino acid residues. The two proteins exhibit 82% amino acid similarity to each other and are encoded by separate genes (Bewaji and Bababunmi, 2008).

2.2.3.2 Sarcoplasmic Recticulum Calcium ATPase

The major Ca^{2+} ion reservoir in the muscle cell is the sarcoplasmic reticulum (SR). The (SR) release of Ca^{2+}causes contraction while the subsequent active transport of Ca^{2+} back into the SR, through the activity of membrane-bound Ca^{2+}-ATPase, causes relaxation (Tada *et al.,* 1978).

The Ca^{2+}-pumping ATPase of the sarcoplasmic reticulum has a molecular weight of about 100,000 Daltons which accounts for up to 90 per cent of the total protein (Tada *et al.,* 1978). Cleavage of an intact 100,000 Dalton polypeptide of the enzyme by trypsin in situ, produces two fragments with molecular weights of 55,000 and 45,000 daltons without loss of Ca^{2+} transport function (Klip *et al.,* 1980). The 55,000-dalton fragment is subsequently cleaved into 30,000- and 20,000-dalton fragments. However, this latter cleavage is accompanied by loss of Ca^{2+} transport activity but no loss of ATPase activity.

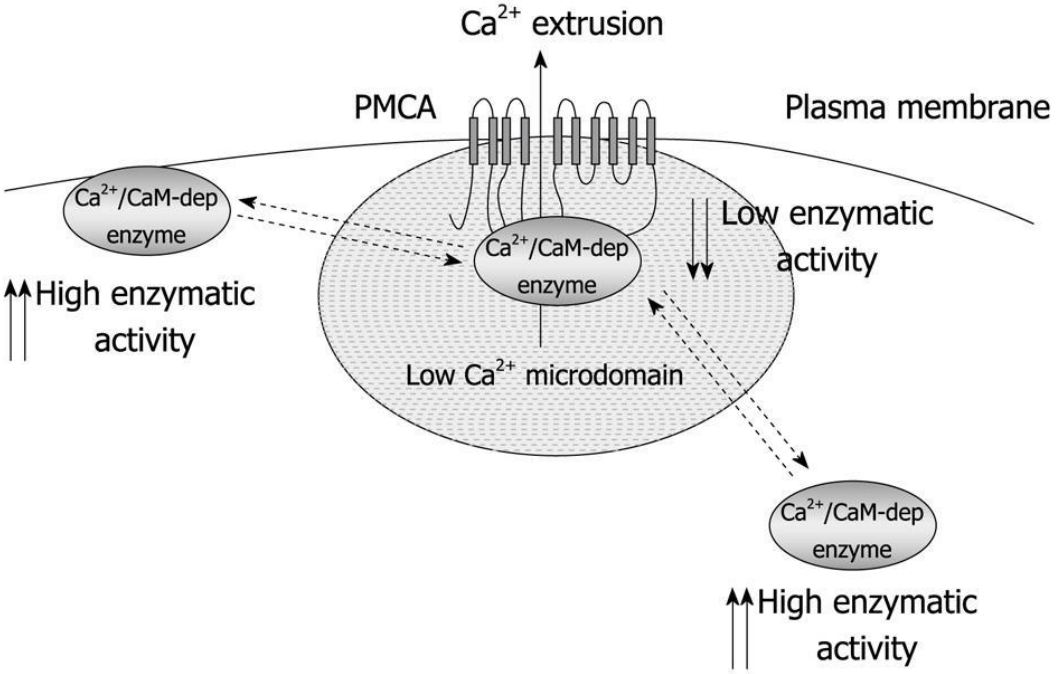


Figure 3: **Model Structure of the Plasma Membrane Calcium Adenosine Triphosphatase (PMCA) as a Regulator of Signal Transduction.**

PMCA pumps calcium ion out of the cell generating a microenvironment where the intracellular calcium concentration is very low. Interaction with the intracellular domains of the PMCA tethers, partner proteins to this low-calcium microenvironment, which results into downregulation of the enzymatic activity of calcium/calmodulin- dependent proteins (Ca^{2+}/CaM-dep).

Source: Holton *et al.*, (2010).

The major storage organelle in non-muscle cells are the endoplasmic reticulum or the recently discovered Ca^{2+} accumulating structure which has been named calciosome. The ATP-dependent pumps responsible for sequestering Ca^{2+} in these storage vesicles belong to the E1-E2 family of ATPases which are also referred to as P-type or aspartyl-phosphate ATPases. They cross-react with antibodies directed against the sarcoplasmic reticulum Ca^{2+}-ATPase (Papp *et al.*, 1992). They also form phosphorylated intermediates during their reaction cycle and are sensitive to vanadate, these properties are shared with other members of the E1-E2 family of enzymes (Figure 4) (such as Na^{+}, K^{+}-ATPase, the gastric H^{+}, K^{+}-ATPase and the plasma membrane Ca^{2+}-ATPase).

Three different mammalian SERCA genes have been reported: SERCA 1, SERCA 2 and SERCA 3 (Verboomen *et al.*, 1992). Alternative splicing of the SERCA 2 gene transcript gives rise to two distinct protein isoforms: SERCA 2a, which is expressed in slow skeletal muscle, heart and to a limited extent, in smooth muscle; SERCA 2b which is expressed in smooth muscle and non-muscle tissues. The last four amino acids of the expressed SERCA 2a ATPase are replaced by an extended tail of 49 amino acids in the SERCA 2b isoform. SERCA3 is expressed at high levels in platelets, lymphoid cells, and some endothelial cells (Bobe *et al*., 1994; Wuytack *et al*., 1994). Wu *et al*. (1995), by in situ hybridization, found that SERCA3 is expressed most abundantly in large and small intestine, thymus, and cerebellum and in several other tissues in lower abundance. High levels of expression were also detected in various lymphoid and endothelial cells. It was therefore concluded that, SERCA 3 plays a critical role in regulating physiological processes in cells in which Ca^{2+} signaling is important. SERCA3 has also been found to be present transiently together with SERCA 2a in early heart development (Anger *et al*., 1994).

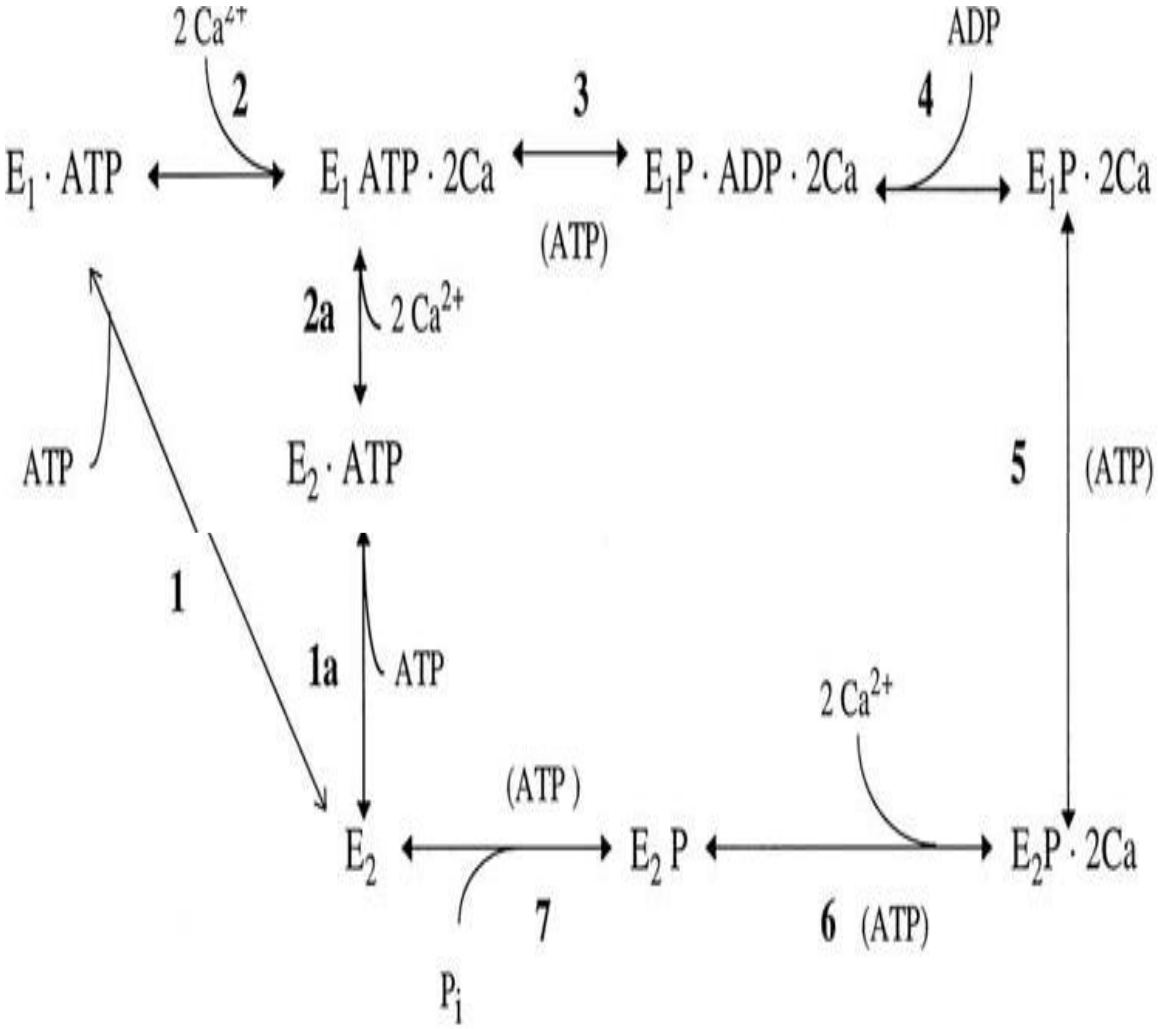


Figure 4: The Catalytic Cycle of Cardiac Sarcoplasmic Reticulum Ca^{2+}-ATPase.

The enzyme exists in two major functional states, E_1 an E_2, whose equilibrium is shifted towards E_1 in the presence of either ATP or Ca^{2+}. ATP, in the presence of Mg^{2+}, binds to E_1 with high affinity and accelerates the convertion of E to E_1 and the conformational change involved in the binding of Ca^{2+}, which also binds to E1 with high affinity (step 2) (Champeil *et al.,* 1986). ATP may also bind to E_2 with reduced affinity (step 1a) and accelerate the conversion of E_2 to E_1, followed by high affinity binding of Ca^{2+} to the latter (Wakabayash and Shigekawa, 1990) (step 2a). After hydrolysis of the ATP and occlusion of Ca^{2+} (step 3), AP is released on the cytoplasmic side of the SR membrane (step 4), and Ca^{2+} is vectorially transferred across the membrane (step 5) and released on its luminal side (step 6). Finally, E_2P decomposition and release of inorganic phosphate on the cytoplasmic side of the membrane return the enzyme to the ligand-free E_2 state (step 7). However, ATP may bind with reduced affinity to enzyme already at step 3, 5, and 7 of the cycle and produce forward acceleratiom of the transitions (Cable and Briggs, 1988; Champeil *et al.,* 1986)

Source: Bewaji and Bababunmi, 2008.

Example of Ca^{2+}-ATPase activity Modulator is phospholamban, while examples of inhibitor includes thapsigargin (Papp *et al.*, 1991), which does not inhibit the plasma membrane Ca^{2+} pumps (PMCA) or other ion-motive ATPases (Papp *et al.,* 1991; Bewaji and Dawson, 1995). Two other inhibitors have been reported to elevate Ca^{2+} in many cells by causing Ca^{2+} to be released from intracellular stores. These are 2,5-di (tert-butyl)-1,4-hydroquinone (DBHQ) (Robinson and Burgoyne, 1991) and cyclopiazonic acid (CPA) (Demaurex *et al.,* 1992). It is also well established that cyclopiazonic acid (CPA) is a specific inhibitor of sarcoplasmic Ca^{2+}-ATPase in skeletal muscle (Demaurex *et al.,* 1992; Bewaji, 1996). Thus, the fact that the effect of methyl jasmonate on the activation curve was opposite to that of CPA suggests that methyl jasmonate is a stimulator of sarcoplasmic Ca^{2+}-ATPase (Bewaji and Bababunmi, 2008).

2.2.4 Oxidative Stress

Oxidative stress can simply be defined as a disturbance of the pro-oxidant/antioxidant balance of the body in favour of the former (Bababunmi and Bewaji, 2002). Free radical is defined as any species that contains one or more unpaired electrons e.g superoxide radical (O_2•), hydroxyl radical (OH•), nitric oxide (NO•). These disturbances can occur as a result of exposure to excess free radicals in the environment or excess endogenous production of free radicals in some disease states (Bababunmi and Bewaji, 2002)..

Research evidences indicated that muscle wasting observed in diseases such as cancer, coronary heart disease, tuberculosis, malaria, malnutrition and AIDS can be prevented or delayed to some extent by dietary intervention (Byers, 1993; Ames *et al.,* 1993; Garcia de la Asuncion *et al.,* 1998; Davis *et al.,* 1999; Davis *et al.,* 2000; Bababunmi, 2002). Dietary intake of nutrient substances known as ‘antioxidant nutrients’ has been recommended. These

include the vitamins C and E, carotenoids (particularly β-carotene) and trace element selenium (Reznick and Cross, 2000).

The idea that antioxidants play a role in slowing down the progression of muscle degenerative diseases) is novel (Bababunmi and Bewaji, 2002). There is considerable experimental evidence that free radicals are involved in the aetiology of disease, either as causative agents or as participants in disease processes that have been initiated by some other means. Evidence has also been presented to show that antioxidant nutrients modulate free radical events in mammalian systems that lead to disease (Rice-Evans and Bruckdorfer, 1992; Rice-Evans and Diplock, 1993).

Various antioxidant defences have evolved to counteract the free radicals which are being constantly produced *in vivo*. One of such is Superoxide dismutase, which is an enzyme that removes O_2•, and converts it to hydrogen peroxide (H_2O_2) usually removed by enzymes such as catalase and glutathione peroxidase (Halliwell and Gutteridge, 1989; Sies, 1991). The human body also has a complex system of metal ion and storage proteins that ensures the mop up of essential metals (such as iron and copper ions which are promoters of free radical damage by accelerating lipid peroxidation, causing formation of OH• radicals) disallowing them from being in a free state. Examples of such scavengers include reduced glutathione, α-tocopherol and ascorbate (usually obtained from diets). However, some of these free radicals manage to escape and causes damage to DNA molecules (Ames *et al.,* 1993).

2.2.4.1 Markers of Oxidative Stress

Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS in the microenvironment; and molecules of the antioxidant system that change in response to increased redox stress. DNA, lipids, proteins and carbohydrates can be modified by excessive ROS *in vivo*. Of these modifications, some are known to have direct

effects on function of the molecule (e.g. inhibit enzyme function), but others merely reflect the degree of oxidative stress in the local environment. The functional significance or causal role of the oxidative modification on cell, organ and system function is recognized as a key determinant of the validity of the marker (Dalle-Donne, 2005).

Superoxide dismutase (SOD) (EC 1.15.1.1), is an enzyme that alternately catalyzes the adding or removing an electron (dismutation or partitioning) from the superoxide molecules it encounters, converting them into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxides are produced as by-products of oxygen metabolism and, if not regulated, because many types of cellular damage (Hayyan *et al.,* 2016). Hydrogen peroxide is also damaging and is degraded by other enzymes such as catalase.

Catalase (CAT) is a ubiquitously expressed protein mainly localized in peroxisomes and it is able to reduce H_2O_2 efficiently (Nordgren and Fransen, 2014). CAT, together with GPx, has the function of inactivating peroxides also by H^+ ion donors to facilitate the reduction of organic hydroperoxides. It is present at higher levels in peroxisomes and vesicles attached to the plasma membrane and are still found in significant levels, mainly in the liver and erythrocytes (Vávrová *et al*., 2013).

Malondialdehyde (MDA) is generated *in vivo* via peroxidation of polyunsaturated fatty acids (a major product of lipid peroxidation a highly reactive dialdehyde, formed from the reaction of the lipid hydroperoxide (LOOH) with alkoxyl radical (LO•) (Taniguchi, 2011). MDA interacts with proteins and is itself potentially atherogenic. MDA's reaction with lysine residues generates lysine–lysine cross-links (Uchida, 2000) which have been identified in apolipoprotein B (apoB) fractions of oxidized low density lipoprotein (OxLDL). MDA is typically quantified from plasma samples with the most popular method being a colorimetric assay based on the reaction between MDA and thiobarbituric acid (TBA).

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other molecules, such as reactive oxygen species to neutralize them, or to protein cysteines to maintain their reduced forms. While donating an electron, glutathione itself becomes reactive and readily reacts with another reactive glutathione to form glutathione disulphide (GSSG) (Kaplowitz, 1981).

Generally, interactions between GSH and other molecules with higher relative electrophilicity deplete GSH levels within the cell (Bannai and tateishi, 1986; Bannai, 1984). GSH can be regenerated from GSSG by the enzyme glutathione reductase (GSR) (Couto *et al.,* 2013): NADPH reduces FAD present in GSR to produce a transient FADH-anion. This anion then quickly breaks a disulfide bond (Cys58 – Cys63) and leads to Cys63's nucleophilically attacking the nearest sulfide unit in the GSSG molecule (promoted by His467), which creates a mixed disulfide bond (GS-Cys58) and a GS-anion. His467 of GSR then protonates the GS-anion to form the first GSH. Next, Cys63 nucleophilically attacks the sulfide of Cys58, releasing a GS-anion, which, in turn, picks up a solvent proton and is released from the enzyme, thereby creating the second GSH. So, for every GSSG and NADPH, two reduced GSH molecules are gained, which can again act as antioxidants scavenging reactive oxygen species in the cell (Halprin and Ohkwara, 1967).

In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress (Halprin and Ohkawara, 1967). Glutathione peroxidase is a cytoplasmic selenoprotein that reduces H_2O_2 as well as other hydroperoxides to water by oxidizing reduced glutathione (GSH) to oxidized form (GSSG) (Michiels *et al.,* 1994). Glutathione Peroxidase GPx is a tetrameric enzyme containing selenium. It is not only

responsible for the decrease of H_2O_2, but also transforms lipoperoxide and other organic hydroperoxide into their corresponding hydroxylated compounds, which are less reactive. For this, GPx uses reduced glutathione (GSH) as a proton donor (H^+), which, after the reaction, becomes oxidized glutathione (GSSG) (Roberts and Sindhu, 2009).

SOD function is catalyse the dismutation of superoxide anions (O^2•) into hydrogen peroxide (H_2O_2), which is less reactive than the previous species. H_2O_2 also has oxidizing ability and easily diffuse across cell membranes and cytosol. To reduce this action, the presence of CAT and GPx enzymes is required, which are able to neutralize this reactive species, producing water (H_2O) and molecular oxygen (Kotani and Taniguchi, 2011).

2.2.5 Relationship within Skeletal Muscle Calcium Homeostasis, Oxidative Stress and Energy Depletion

Being the site of most ATP generation, the mitochondrion is at the core of cellular energy metabolism. Calcium acts within the cellular organelle at several levels as a key regulator of the mitochondrial function and stimulation of ATP synthesis. Deregulation of mitochondrial Ca^{2+} homeostasis has been known to play a key role in several pathologies. Like, mitochondrial matrix Ca^{2+} overload can lead to enhanced generation of reactive oxygen species, triggering of the permeability transition pore, and cytochrome *c* release, leading to apoptosis (Brookes *et al*., 2004).

The mitochondrion is a double membrane-bound organelle found in most eukaryotic cells. They are key components in mechanisms of cell growth, cell proliferation and apoptosis (Karbowski and Neutzner, 2012). Because mitochondria are involved in diverse and important cellular functions, the breakdown or dysfunction of mitochondrial processes may result in a number of disorders or diseases. Many of these disorders are caused by defective

degradation of damaged or non-functional mitochondria and a subsequent lack of *de novo* synthesis of new replacement mitochondria (Huang and Manton, 2004).

Mitochondrial permeability transition (MPT) refers to a sudden increase in the permeability of the inner mitochondrial membrane. MPT is mediated by the opening of a complex termed the mitochondrial permeability transition (MPT) pore (MPTP), which is responsible for the osmotic influx of water into the mitochondrial matrix, resulting in swelling of mitochondria and dissipation of the mitochondrial membrane potential (Bonora *et al.,* 2016). MPTP is a protein that is formed in the inner membrane of the mitochondria under certain physiological conditions, whose opening allows increase the permeability of the mitochondria to molecules of less than 500D (Lemasters *et al.,* 2009). Factors that enhance the opening of the MPTP include high level of calcium ion (possible by Ca^{2+} binding to and activating Ca^{2+} binding sites on the matrix of the mpt) (Ichas and Mazat, 1998), dissipation of the transmembrane potential of the mitochondria, presence of free radical (which could be a result of Ca^{2+} overload) (Brustovetsky *et al.,* 2003), fatty acids (Gar-cia *et al.,* 2000), and inorganic phosphate (Nicholls and Brand, 1980). However all the other factors cannot open the pore without high concentration of Ca^{2+}, but Ca^{2+} alone can induce mpt (Gunter *et al.,* 1994). Molecules such as Mg^{2+} that can compete with Ca^{2+} for the binding site on the matrix can inhibit the opening of the MPT (Doczi *et al.,* 2010).

Upon MPTP induction, the mitochondrial membrane permeability is increased causing depolarization of the mitochondria and a loss of transmembrane potentials ($\Delta\psi$). When this happens, proton and some molecules are able to flow across the outer membrane of the mitochondria uninhibited (Schnider *et al.,* 1996; White and Reynold, 1996). This also interferes with the production of ATP producing an energy deficit in the cell and this also prevents the production of ATP that fuels the activities of ion pumps such as Na^{+}/Ca^{2+} exchanger (which are necessary in the cell to get rid of excess Ca).

Consequent to MPT opening, ROS are also produced. The opening can allow the exit of antioxidant molecules like the glutathione, reducing the ability of the cell to neutralize ROS. More ROS may also be produced through the loss of components of the electron transport chain (such as cytochrome *c*) leading to loss of electrons from the chain which can reduce molecules to form free radicals (Luetjens *et al.,* 2000).

Finally, MPT can become permeable to molecules of low molecular weight (less than 1500Daltons), which draws in water into the mitochondria by increasing the organelle's osmolar load (Bŭki *et al.,* 2000). This leads to swelling of the organelle and may lead to the rupture of the outer membrane and release of cytochrome *c* which can make the cell go through apoptosis ("commit suicide) by activating pro-apoptotic factors (Bŭki *et al.,* 2000).

The release of Ca^{2+} through the MPT can place further stress on neighboring mitochondria, and can activate harmful calcium- dependent proteases such as calpain system.

2.2.6 Markers of Muscle Degeneration

Markers of muscle damage include creatine kinase (CK), myoglobulin (Mb), ALT, AST, and lactate dehydrogenase (LDH) (Clarkson *et al.,* 2005). An ideal marker of skeletal muscle injury should: be absolutely muscle specific, have a broad diagnostic window to allow early and late diagnosis, be highly sensitive, be barely detectable in normal patients, not damage skeletal muscle fibers, be stable, with rapid measurement, easy and cost effective and be present in high concentration in the skeletal myocytes leading to high serum entry after fiber damage. However, a single marker cannot meet up with all these characteristics, hence; there may be need for a combination of markers (Sorichter *et al.,* 1999).

2.2.6.1 Creatine Kinase

Creatine kinase (CK) is an enzyme of around 82 kDa that is found in both the cytosol and mitochondria of tissues where energy demands are high. It catalyzes the reversible phosphorylation of creatine to phosphocreatine and of ADP to ATP (Brancaccio *et al.,* 2010; Sayers and Clarkson, 2003). It is therefore important in the regeneration of cellular ATP (Figure5).

$$\text{Phosphocreatine} + \text{MgADP}^{-} + \text{H}^{+} \Longleftrightarrow \text{MgATP}^{2-} + \text{Creatine}$$

CK forms the core of an energy network known as the phosphocreatine (PCr) circuit (Figure 5). In this circuit, the cytosol isoenzymes are closely coupled to glycolysis and produce ATP for muscle activity. The MtCK version is closely coupled to the electron transport chain and can use mitochondrial ATP to regenerate PCr, which readily returns to the cytosol to resupply cytosolic PCr. This shuttle system is critical for the production and maintenance of energy supply and is involved in the metabolic feedback regulation of respiration (Saks, 2008). Hence, the skeletal muscle has the high levels of CK that can account for as much as 20% of the soluble sarcoplasmic protein in specific muscles (Saks, 2008).

Until mid-1990s, serum CK level was key in the diagnosis of myocardial infarction. Subsequently, the diagnostic role has been replaced, to a certain extent, by the muscle protein troponin. However, raised levels of serum CK are still closely associated with cell damage, muscle cell disruption, or disease. These cellular disturbances can lead to a leak of CK from cells into blood serum (Totsuka *et al.,* 2002). Measurement of serum CK activity and determination of isoenzyme profiles is still an important indicator of the occurrence of muscle cell necrosis and tissue damage due to disease or trauma (Brancaccio *et al.,* 2007). Myofibrillar CK-MM is bound to the M-line of the sarcoplasmic reticulum of myofibrils and

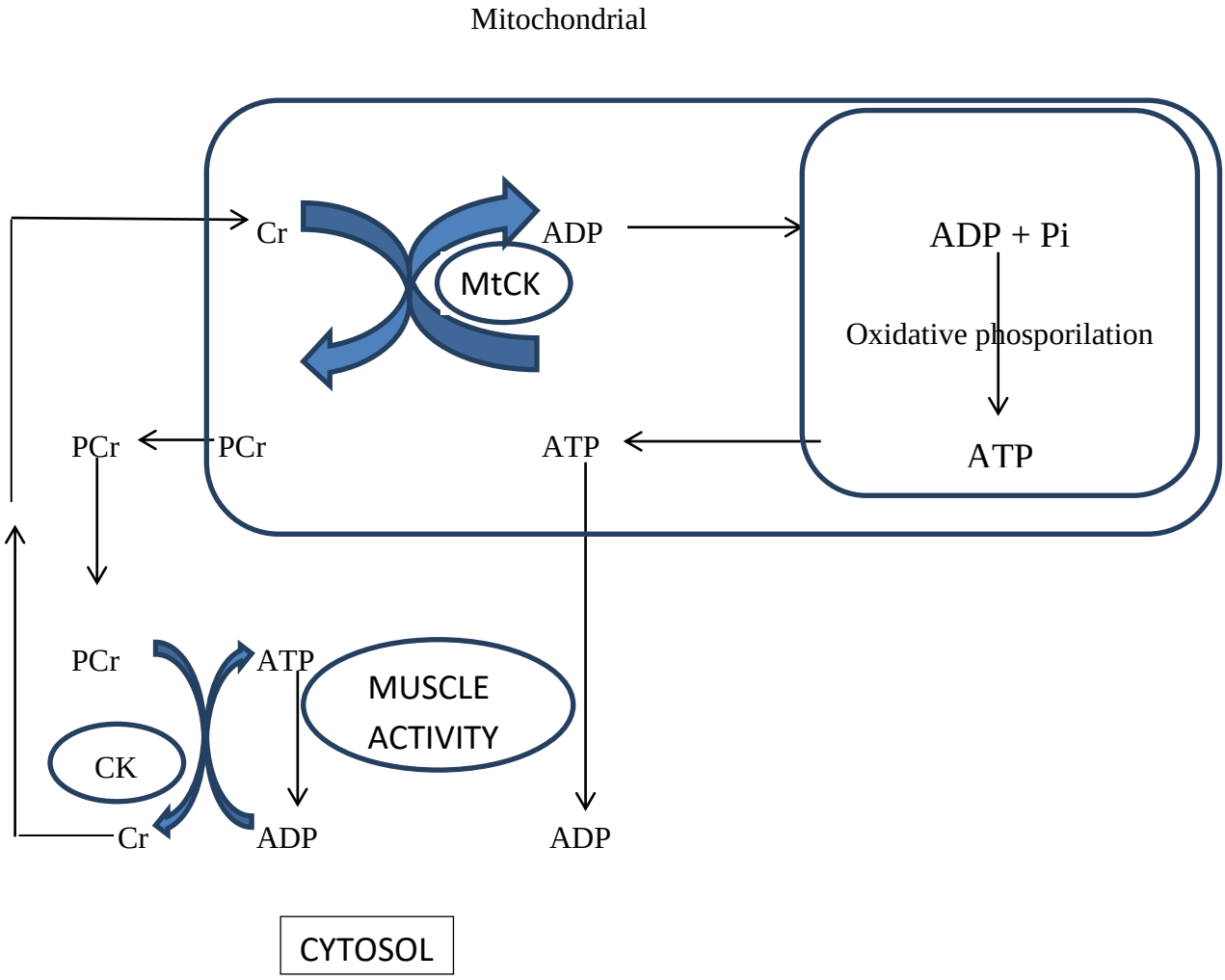


Figure 5: **Phosphocreatine (PCr) Circuit Showing the Rephosphorylation of Creatine (Cr) in Mitochondria Using ATP Derived from Oxidative Phosphorylation**

Source: Saks (2008).

is also found in the space of the I-band sarcomeres providing support for muscle energy requirements (Heled *et al.,* 2007). Thus, the enzyme is normally confined to the muscle cell.

In general terms, high serum CK in some ethnic groups may reflect a genetic condition of naturally increased levels of CK muscle tissue activity, which is not related to exercise frequency or muscle disturbance (Brewster *et al.,* 2006). It has been proposed that higher than normal levels of tissue CK activity may augment the availability of cellular energy and improve myofibril contraction responses (Brewster *et al.,* 2006). Therefore, high levels of serum CK, in the absence of muscle damage or other pathological conditions, may reflect the level of enzyme- tissue activity of the individual. Serum CK levels alone may however not provide a fully accurate reflection of structural damage to muscle cells (Mohaupt *et al,* 2009: Magal *et al.,* 2010).

Studies of serum CK response to exercise in aging human skeletal muscle have produced variable results. A review by Fell and Williams (2008) on the effect of aging on skeletal muscle in athletes suggests that aging can lead to greater exercise-induced damage and a slower repair and adaptation response. Muscle mass and function gradually decline with age, and cell apoptosis may have a role in age-related sarcopenia (Siu, 2009). Lower levels of plasma CK in older female subjects have been attributed to a decline in circulating neutrophils with age which may, in part, be due to reduced oestradiol levels and endogenous antioxidant status (Arnett *et al.,* 2000). Circulating neutrophils produce oxidants such as superoxide free radicals, which increase cell damage and leakage. Therefore, an increased serum CK could be related to optimal functionality of the cell, which may decline with age, and is not simply a marker of less damage. Free radial production appears to moderate signalling for adaptation of skeletal muscle in response to exercise (McArdle *et al.,* 2002),

and this response may be attenuated in older muscle, rendering it less adaptive to exercise stress.

Gunst *et al*., 1998, examined CK activity and glutathione concentrations in serum from 200 healthy subjects and a series of 38 patients with multiple organ failure, muscle wasting, and low serum CK. Overall, low serum glutathione concentrations correlated with low serum CK, and in the organ failure group, low serum CK was accompanied by very low serum glutathione concentrations. They noted that low serum CK in the circulation associated with glutathione depletion could not be restored by thiol-reducing compounds in the CK assay.

2.2.6.2 Lactate Dehydrogenase

Lactate dehydrogenase A (LDHA) is a cytosolic enzyme, predominantly involved in anaerobic and aerobic glycolysis (Valvona, 2016). Under an anaerobic condition (such as during exercise), humans undergo fermentation during which pyruvate gets catalyzed into lactic acid by the enzyme lactate dehydrogenase (in a reversible reaction). This reaction also concomitantly oxidizes NADH to NAD^+.

Lactate dehydrogenase is a cytosolic enzyme that catalyzes the anearobic interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. It converts pyruvate, the final product of glycolisis, to lactate in the absence of oxygen or in short supply of oxygen, and it performs the reverse reaction during the Cori cycle in the liver. LDH is expressed extensively in body tissues such as blood cells and heart muscle. Because it is released during tissue damage, it is considered as a marker of common injuries and disease such as heart failure (Nelson and Cox, 2005).

LDH exist in two subunits. The M (muscle) and H (heart) subunits both of which are have the same active sites but differs by replacement of alanine (in the M chain) with glutamine (in the

H chain), hence the H subunit binds fater than the M subunit (Eventoff *et al.*,1977; Sorichter *et al.,* 1999). It has five isoenzymes (LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5) distributed within the tissue in a manner reflecting there metabolic requirements (Sorichter *et al.,* 1999).

Activity of LDH is increased with higher in periods of high energy requirements (like period of extreme skeletal muscle output) due to increase substrate (ADP, AMP and Pi). In other words, LDH activity is usually regulated by the relative concentration of its substrate (pyruvate and NADH) (Spriet *et al.,* 2000).

2.2.6.3 Aspartate Transaminase (AST)

AST is similar to alanine transaminase (ALT) because they are both associate to the liver but differ because ALT is majorly found in the liver with negligible quantities in other organs like the kidney, heart and skeletal muscle while AST is found in more significant amount in liver, heart muscle, skeletal muscle, kidneys, brain and red blood cells. Hence ALT is considered most appropriate as an indicator of liver damage rather other organs while AST might be relevant in other organs mentioned. Moreover, AST was the first marker used for the laboratory diagnosis of muscle injury (Ladue *et al*., 1954), especially myocardiac infaction, but because it lacks muscle fiber specificity, it is recently having only a minor scientific or clinical significance in the diagnosis of muscle fiber injury (Sorichter *et al*., 1999). Its use in diagnosis of myocariac infarction has been superseded by use of cardiac troponin (Gaze, 2007).

2.2.6.4 Alanine Transaminase (ALT)

Alanine transaminase (ALT) (EC 2.6.1.2) also called alanine aminotransferase is a transaminase enzyme that catalysis the reversible transfer of an amino group from L-alanine to α-ketoglutarate, producing pyruvate and L-glutamate. It is and was formerly called serum glutamate-pyruvate transaminase (SGPT) or serum glutamic-pyruvic transaminase (SGPT)

(Karmen *et al*., 1955). It is found in the plasma and many other tissues but most commonly in the liver.

L-glutamate + Pyruvate ↔ α- ketoglutarate + L-alanine

2.3 Malnutrition

Malnutrition refers to deficiencies, excesses or imbalances in a person's intake of energy and/or nutrients. The term covers 2 broad groups of conditions: One is 'undernutrition'—which includes stunting (low height for age), wasting (low weight for height), underweight (low weight for age) and micronutrient deficiencies or insufficiencies (a lack of important vitamins and minerals). The other is overweight, obesity and diet-related non-communicable diseases (such as heart disease, stroke, diabetes and cancer) (WHO, 2016). Malnutrition is a medical condition caused by improper or insufficient diet (Nikolaos, 2011). The term "severe malnutrition" is often used to refer specifically to protein-energy malnutrition (PEM) which is often associated with micronutrient deficiency (Nikolaos, 2011).Two known forms of protein-energy malnutrition are kwashiorkor and marasmus, and they commonly coexist (Nikolaos, 2011) (Plate 1).

Kwashiorkor is mainly caused by inadequate protein intake resulting in a low concentration of amino acids (Nikolaos, 2011). The main symptoms are oedema, wasting, liver enlargement, hypoalbuminaemia, steatosis, and possibly depigmentation of skin and hair (Nikolaos, 2011). Kwashiorkor is identified by swelling of the extremities and belly, which is deceiving of actual nutritional status (Chowdhury *et al*., 2009). The activity of the Ca^{2+}-ATPase has been found to be at least 10% less in the erythrocytes of kwashiorkor patients than in normal erythrocytes (Olorunshogo, 1989). The implication of this finding is that there will be a raised intracellular Ca^{2+} level in kwashiorkor as a result of the defective Ca^{2+} pump (Bababunmi *et al*., 1994).

Plate 1: Photograph of a Nurse Attendant and Children with Different Degree of Protein- Energy Malnutrition at a Nigerian Orphanage in the Late 1960s.

Image courtesy of Dr. Lyle Conrad and the CDC Public Health Image Library.

Marasmus (wasting away) is caused by an inadequate intake of both protein and energy. The main symptoms are severe wasting, leaving little or no oedema, minimal subcutaneous fat, severe muscle wasting, and non-normal serum albumin levels (Nikolaos, 2011). Marasmus can result from a sustained diet of inadequate energy and protein, and the metabolism adapts to prolong survival (Nikolaos, 2011). It is traditionally seen in famine, food restriction, or anorexia (Nikolaos, 2011). Conditions are characterized by extreme wasting of the muscles and a gaunt expression (Chowdhury *et al.*, 2009).

According to the World Health Organization (2016), malnutrition affects people in every country. Around 1.9 billion adults worldwide are overweight, while 462 million are underweight. An estimated 41 million children under the age of 5 years are overweight or obese, while some 159 million are stunted and 50 million are wasted. Adding to this burden are the 528 million or 29% of women of reproductive age around the world affected by anaemia, for which approximately half would be amenable to iron supplementation. As a consequence to the fact that many families cannot afford or access enough nutritious foods like fresh fruit and vegetables, legumes, meat and milk, while foods and drinks high in fat, sugar and salt are cheaper and more readily available, leading to a rapid rise in the number of children and adults who are overweight and obese, in poor as well as rich countries. Hence it is quite common to find under-nutrition and overweight within the same community, household or even individual (e.g being both overweight and micronutrient deficient).

Malnutrition has shown to be an important concern in women, children, and the elderly. Because of pregnancies and breastfeeding, women have additional nutrient requirements (Nubé *et al.*, 2003). Children can be at risk for malnutrition even before birth, as their nutrition levels are directly tied to the nutrition of their mothers (Sue *et al.*, 2008). Breastfeeding can reduce rates of malnutrition and mortality in children (Bhutta *et al.*, 2008),

and educational programs for mothers could have a large impact on these rates (UNICEF, 2012). The elderly have a large risk of malnutrition because of unique complications such as changes in appetite and energy level, and chewing and swallowing problems (Wellman *et al.,* 1997). Adequate elderly care is however essential for preventing malnutrition, especially when the elderly cannot care for themselves.

The frequency of occurrence as calculated by the Food and Agricultural Organization of the United Nations (2015) was 793 million undernourished amounting to 13% of the population. Moreover, death resulting from nutrient deficiencies was calculated to be 406,000 by Mortality and Causes of Death Collaborators (2016).

2.3.1 Classification of Malnutrion

There are two models for the classifications of malnutrition. These are the “Gómez classification” (Table 1) and “Waterlow classification” (Table 2). The Gomez classification is as shown in Table Gomez's original classification is still used today; it provides a way to compare malnutrition within and between populations. The classification has been criticized for being "arbitrary" and for not considering overweight as a form of malnutrition. Also, height alone may not be the best indicator of malnutrition; children who are born prematurely may be considered short for their age even if they have good nutrition (Gueri *et al.,* 1980).

In a paper titled "Classification and Definition of Protein-Calorie Malnutrition", John Conrad Waterlow established a new classification for malnutrition (Waterlow, 1972). Instead of using just weight for age measurements, the classification established by Waterlow combines weight-for-height (indicating acute episodes of malnutrition) with height-for-age to show the stunting that results from chronic malnutrition (Watts and Geoff, 1982). An advantage of this classification over the Gomez classification is that weight for height can be examined even if ages are not known (Waterlow, 1972). This classification is however shown in Table. These

Table 1: Gómez Classification

Degree of PEM	% of desired body weight for age and sex
Normal	90% - 100%
Mild: Grade I (1st degree)	75% - 89%
Moderate: Grade II (2nd degree)	60% - 74%
Severe: Grade III (3rd degree)	< 60%

Source: Chowdhury *et al.,* 2009.

Table 2: Waterlow Classification

Degree of PEM	Stunting (%) Height for age	Wasting (%) Weight for height
Normal: Grade 0	>95%	>90%
Mild: Grade I	87.5 - 95%	80 - 90%
Moderate: Grade II	80 - 87.5%	70 - 80%
Severe: Grade III	< 80%	<70%

Source: Waterlow, 1972.

classifications of malnutrition are commonly used with some modifications by WHO (Grover *et al.,* 2009).

2.4 *Moringa oleifera*

Moringaoleifera is an angiosperm belonging to the family Moringaceae (Dalziel, 1956). It is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan where it is used in folk medicine (Fahey, 2005), it is however now widely distributed all over the world (Lockelt and Grivetti, 2000) (Plate 2).Its English names include horseradish tree, drumstick or ben oil seed tree. In Nigeria, it is locally known as 'Zogale gandi' in Hausa, 'Ewe igbale' in Yoruba, 'Okwe oyibo' in Igbo (Dalziel, 1956) and the Fulanis call it "Gawara" (Ramachandran *et al.,* 1980). It is a fast growing, drought resistant tree native to the southern foot hills of the Himalayas in north western India. However it is now cultivated in all regions of the world.

The leaves of this plant contain a profile of important trace elements, and are a good source of proteins, vitamins, beta-carotine, amino acids and various phenolics (Anwar *et al.*, 2007). Several biological properties ascribed to various parts of this plant have been reviewed in the past (Anwar *et al.,* 2007). These include its use as an antioxidant, anticarcinogenic, antiulcer, antibacterial, and antifungal. Phytochemical analyses have shown that its leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids as well as antioxidants (Amaglo *et al.,* 2010).

Studies on proximate and phytonutrient analysis of the leaf by Bamishaiye *et al.,* 2011 also showed that it has high percentage of carbohydrate and protein and compared favourably with other high protein/ carbohydrates food crops. It is however a potential leaf source of food that is suitable for fortification of foods and its use as nutritional supplements is highly promising (Bamishaiye *et al.*, 2011). Dietary consumption of its part is therein promoted as a

(a) **(b)**

Plate 2: Photograph of *M. oleifera* leaf (a) and its Plantation (b)

strategy of personal health preservation and self-medication in various diseases (Siddhuraju and Becker, 2003).

So much evidence has been gathered about the high medicinal value of moringa (Basara *et al.,* 2011), and is multifunctionality (Anwar *et al.,* 2007; Oduro *et al.,* 2008). The plant has high nutritional value (Thurber and Fahey, 2009), therapeutic uses and prophylactic properties (Fuglie 2001; 2000; Fahey, 2005). It is propagated sexually through seeds and vegetatively through stem cuttings (Fuglie, 2001). While the stem cutting method is easy and successful for tree propagation, the recent introduction of moringa as a field crop for biomass production requires propagation through seed (Nouman *et al.,* 2012). However, seed viability of the plant is low when compared to other field and horticultural crops (Croft *et al.,* 2012).

It usually grows in hot dry land in tropical insular climate, and is only slightly affected by drought (Morton, 1991).The leaves are tripinnate and a rich source of amino acids, protein, vitamin A and C, calcium, potassium and natural antioxidants (Mehta *et al.,* 2011; Kanchan *et al.,* 2012). The phytochemical components of the leaf revealed to be rich in flavonoids, saponins, tannins, phenolic acids (Vinoth *et al.,* 2012). The Moringa oleifera leaves also has anti-inflammatory (Sharma and Vaghela, 2011), anticataleptic (Anu *et al.,* 2012), antioxidant (Ranira *et al.,* 2005), antimicrobial (Anthonia, 2012), antihypertensive (Danji *et al.,* 2002), hypocholesterolemic (Ghasi *et al.,* 2000), antifungal (Chuang *et al.,* 2007), radioprotective (Rao *et al.,* 2001) properties. Analgesic effect of ethanolic leaf extract of Moringa oleifera has also been reported (Bhattacharya *et al.,* 2014). Ethanolic extract of Moringa oleifera has also been reported to have exhibited a significant ($p < 0.05$) dose dependent CNS depressant and muscle relaxant activity (Bhattacharya *et al.,* 2014).

A study by Aja *et al*., (2014) using Gas Chromatography- mass spectrometer (GC- MS) revealed the presence of 16 chemical constituents of *M. oleifera* leaf. These chemical compounds were: 9-octadecenoic acid, L-(+)-ascorbic acid- 2,6- dihexadecanoate, 14–methyl-8-hexadecenal, 4- hydroxyl-4-methyl-2-pentanone, 3-ethyl-2, 4-dimethylpentane, phytol, octadecamethyl-cyclononasiloxane, 1, 2-benzene dicarboxylic acid, 3, 4-epoxyethanone, N-(-1-methylethyllidene)-benzene ethanamine, 4, 8, 12, 16-tetramethylheptadecan-4-olide, 3-5-bis (1, 1-dimethylethyl)-phenol, 1-hexadecanol, 3, 7, 11, 15-tetramethyl-2 hexadecene-1-ol, hexadecanoic acid and 1, 2, 3-propanetriyl ester-9 octadecenoic acid.

Various researches have demonstrated that nutrition plays a crucial role in the prevention of chronic diseases; this is because most of them can be related to diet. Thus, the concept of regarding food as being "functional food" not only necessary for living but also as a source of mental and physical well-being, contributing to the prevention and reduction of risk factors for several diseases or enhancing certain physiological functions (López-Varela *et al.*, 2002). A functional food is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of wellbeing and health or reduction of the risk of a disease. The beneficial effects could be either maintenance or promotion of a state of wellbeing or health and/or a reduction of risk of a pathologic process or a disease (Roberfroid, 1999). Whole foods represent the simplest example of functional food (Krishnaswamy, 1996) and hence the concept of "Nutraceutical" which is a term coined by Stephen DeFelice, in 1979 (DeFelice, 1992). It is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease (Dillard and German, 2000).

2.5 Jasmonate

Jasmonate (JA) and its derivatives are lipid-based hormone signals that regulate a wide range of processes in plants, ranging from growth and photosynthesis to reproductive development. In particular, JAs are critical for plant defense against herbivory and plant responses to poor environmental conditions and other kinds of abiotic and biotic challenges (Farmer and Ryan, 1990).

Jasmonates are oxygenated fatty acid, synthesized in plants via the octadecanoid pathway (Creelman and Mullet, 1997). Briefly they are synthesized from linolenic acid residing in chloroplast membranes. Synthesis is initiated with the conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA), which then undergoes a reduction and three rounds of oxidation to form (+)-7-iso- jasmonic acid. Only the conversion of linolenic acid to OPDA occurs in the chloroplast all subsequent reactions however occur in the perixisome (Katsir *et al.,* 2008). Members of the jasmonate family include jasmonic acid (Katsir *et al.,* 2008); methyl jasmonate (Demole *et al.,* 1962); jasmine (Thomas *et al.,* 1997). Methyl-JA was first identified as a component of the essential oil of several plant species, while JA was first obtained from a fungal culture filtrate (Creelman and Mullet, 1997).

Biochemical investigations showed that jasmone, menthone, menthol, and methyl jasmonate are highly selective stimulators of the SR Ca^{2+}-ATPase in mammalian skeletal muscle, but they have no effect on the properties of the phospholipid bilayer (Starling *et al.,* 1994).

Methyl jasmonate (Figure 6) is a volatile organic compound used by the plant for defence and developmental processes like seed germination, root growth, flowering, ripening and senescense (Joumaa *et al*., 2002) demonstrated that methyl jasmonate concentrations of up to 100 μM reduced the time required to load the SR with calcium, whereas this effect was less marked at higher concentrations. This report is similar to those previously reported on the

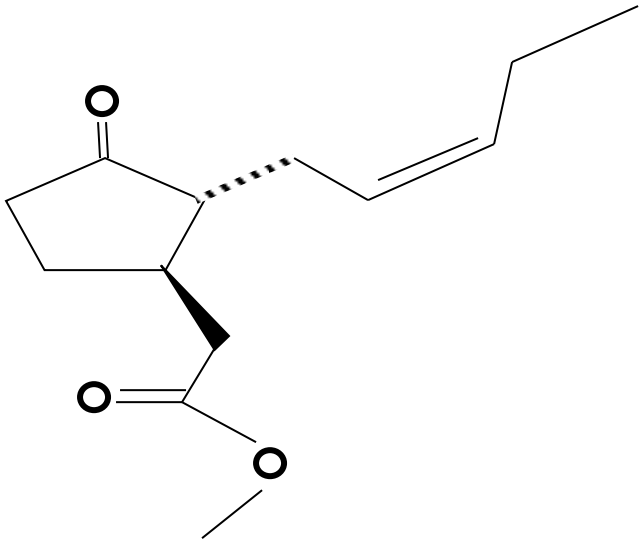


Figure 6: Structure of Methyl Jasmonate

Source: Aurélie *et al*., 2010.

ATPase activity of the purified Ca^{2+}-ATPase from rabbit fast skeletal muscle (Starling *et al.*, 1994), except that in Joumaa *et al*. (2002) experiment, methyl jasmonate concentrations were 100-fold lower. The fact that comparable effects were obtained for calcium loading in both types of muscle suggests that the application of methyl jasmonate resulted in an increase of ATPase activity in slow muscle, with a maximum response at 100 μM.

They observed that the characteristics of the twitch generated by short electrical stimulation were modified by methyl jasmonate, and their results indicated that methyl jasmonate had distinct dose-dependent effects on the contraction of skeletal soleus muscle cells (Bewaji and Bababunmi, 2008). Although the excitation-contraction coupling mechanism was not affected by methyl jasmonate, the shift in the activation curve to more positive values, resulting from exposure to methyl jasmonate, could be related to changes in membrane potential and/or the depolarization rate and/or Ca^{2+} sensitivity for contractile proteins.

The jasmonate molecule contains two chiral centers located at C3 and C7 generating four possible stereoisomers, since either chiral center can have an R or S absolute configuration. The mirror image isomers, (3R, 7S) and (3S, 7R), have their side chains in a *cis* orientation. These isomers are known as (+)-JA and (-)-7-iso-JA or (+)-*trans*-JA and (-)-epi-JA. The enantiomers, (3R, 7R) - and (3S, 7S)-JA or (-)-JA and (+)-JA, have their side chains in the *trans*configuration. Because of increased steric hindrance, the *cis* orientation is less stable and will epimerize to the more stable *trans* configuration. This occurs via a keto-enol tautomerization involving the C6 ketone and the C7 proton to form the corresponding diastereomers (Creelman and Mullet, 1997). Commercially available synthetic Methyl-JA used in many experiments is composed of a 9:1 ratio (-)-Methyl-JA:(-)-7- iso-Methyl-JA.

2.6 Vitamin E (γ- Tocopherol)

Vitamin E is a fat soluble antioxidant (Herrera *et al.,* 2001; Packer *et al.,* 2001) (poorly soluble in aqueous solution) refers to a group of compounds that includes the tocophenols and tocotrienols (Brigelius-Flohè *et al.,* 1999), out of which γ- tocopherol is the most commonly found in food (Traber, 1998) and is the most biologically active form (Brigelius-Flohè *et al.,* 1999; Reboul *et al.,* 2006). γ- tocopherol can however be found in corn oil, soybean oil, margarines, papayas, leafy vegeTables, walnuts etc. it was first discovered in 1922 by Herbert McLean Evans and Katharine Scott Bishop (Evans *et al.,* 1922) and it was first isolated in its pure form by Gladys Anderson Emerson (Oakes, 2007) and thereafter first synthesized by Paul Karrer (National Research Council, 1987).

The Food and Nutrition Board (FNB) of the U.S. Institute of Medicine updated Estimated Average Requirements (EARs) and Recommended Dietary Allowances (RDAs) for vitamin E. The current EAR for vitamin E for women and men ages 14 and up is 12 mg/day while the RDA is 15 mg/day. RDA for pregnancy equals 15 mg/day while that of a lactating woman equals 19 mg/day. For infants up to 12 months the Adequate Intake (AI) is 4–5 mg/day and for children ages 1–13 years the RDA increases with age from 6 to 11 mg/day. FNB also sets Tolerable Upper Intake Levels (ULs) for vitamins and minerals. In the case of vitamin E the UL is 1,000 mg/day (Institute of Medicine, 2000).

High-dosage supplementation may slightly increase mortality (Miller *et al.*, 2005; Bjelakovic *et al.,* 2008). Deficiency can cause myopathies (Brigelius-Flohè *et al.,* 1999), skeletal myopathies (Institute of Medicine, 2000; Kowdley *et al.,* 1992), red blood cell destruction (Whitney and Sharon, 2011), impairment of the immune system (Institute of Medicine, 2000; Kowdley *et al.,* 1992) and spinocerebellar ataxia (Traber and Atkinson, 2007).

2.7 Carnitine

L-Carnitine (β-hydroxy-γ-trimethylammonium butyrate) is a nutrient that is derived from the amino acids lysine and methionine (Rebouche, 2004). Its name is derived from the fact that it was first isolated from meat (carnus). It is not considered a dietary essential because it is synthesized in the body. The body produces carnitine in the liver and kidneys and stores it in the skeletal muscles, heart, brain, and other tissues. But its production may not meet the needs under certain conditions such as increased energy demands and therefore it is considered a conditionally essential nutrient.

Carnitine is a hydrophilic quatinary amine (Figure 7) that plays its essential role in energy metabolism with its major role in the transfer of long-chain fatty acids into the mitochondria for subsequent β-oxidation (Roe and Ding, 2001). The carnitines exert a substantial antioxidant action, thereby providing a protective effect against lipid peroxidation of phospholipid membranes and against oxidative stress induced at the myocardial and endothelial cell level (Claudio, 2002). Although L-carnitine has been marketed as a weight-loss supplement, there is no scientific evidence to show that it improves weight loss. Somestudies however show that oral carnitine reduces fat mass, increase muscle mass, and reduce fatigue. All of these effects may indirectly contribute to weight loss (University of Maryland Medical Center, 2011).

In 2011, researchers using L-carnitine L-tartrate supplementation for six months in a well-controlled study demonstrated not only increased muscle carnitine in subjects without carnitine deficiencies, but also an impact on muscle metabolism and performance; however, measurements of lipid oxidation were not taken in this study, and further research is however needed (Wall *et al.*, 2011).

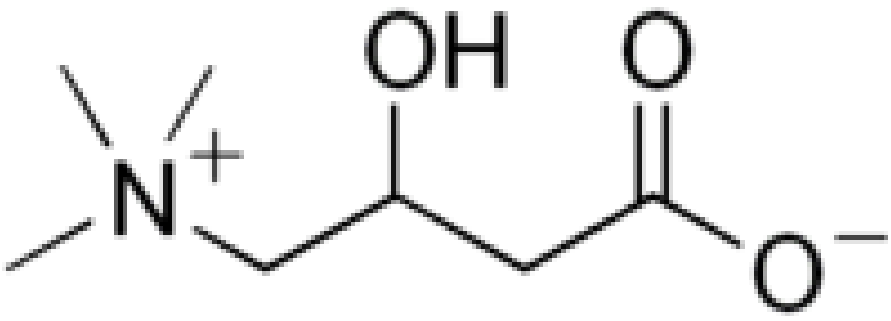


Figure 7: **Structure of Carnitine (4- Trimethylamino- 3-hydroxybutyrate)**

Source: Voet *et al*., 1999

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental Animals

A total of 175 albino rats (*Rattus norvergicus*) with an average weight of 165g were procured from the animal holding unit of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. All the animals were fed commercially prepared feed and clean water *ad libitum* for one week to acclimatize. Ethical approval for the use of animal was obtained for the research from the University Ethical Review Committee, University of Ilorin, Ilorin with an approval number of URC/ASN/2017/908.

3.1.2 Nutritional Supplements

Methyl jasmonate and adenosine triphosphate were products of Sigma Chemical Company, UK. Vitamin E (as d-alpha tocopherol acetate) and Acetly L-carnitine were products of Bulk supplements.com, Henderson. All other chemicals and reagents were of analytical grade.

3.1.3 Feed Materials

Fresh *M. oleifera* leaves was harvested by falling a whole tree from Adewole Estate Ilorin, thereafter cutting out the leaves and dried in a hot air oven drier (Lampfield hot air oven) at 60^{o}C. The dried leaves were then blended to powder with an electronic blender (Marlex Electroline). DL- methionine and Vitamin- Mineral mix (Miavit GmbH Germany) were purchased from Olufunmilayo farm limited, Offa garage road, Ilorin. Other feed components (corn, soybean, corn chaff, soy oil, refined sugar) and Golden penny soy bean oil (ROM oil mill limited, Oyo, Nigeria) were purchased raw and prepared (as described in the appendix). The components of the control and test meal are as shown in Table 3.

3.1.4 Solvents and Chemicals

All the solvents and chemicals materials used were of analytical grade and were procured from notable and reliable sources. These include; creatinine kinase assay kit, sucrose, sodium dodecylsulphate (SDS), serum albumin assay kit and Ascorbic acid etc.

3.2 Methods

3.2.1 Experimental Design

The experiment was done in three phases. In phase one, preliminary evaluation of test feeds was done while in phase two evaluation of the effects of the test diets on markers of muscle degeneration was done. A third phase of the research involved the toxicological study of the *M. oleifera* leaf-based diet and chemical characterization of the leaf.

In each set of the experiment, 5 randomly picked animals were positive control (they were fed 25% protein all through). The remaining animals were fed 4% iso-caloric low protein diet for four weeks for malnutrition to manifest and was muscle degeneration induced. Anthropometric parameters were obtained from all the animals including the control weekly. After the first four weeks three animals were randomly selected from each group and sacrificed for analysis. The remaining animals were subdivided into treatment groups and treated for 4 weeks after which serum and organ homogenates were used for analysis. The test feed and formulations were administered orally.

3.2.2 Feed Formulation

The composition of the test diets was coined from Sunmonu and Oloyede (2007) with slight modification (Table 3)

Table 3: Components of Control and Test Feed

Diet composition	**Control diet (25% soy-based)**	**Test diet (4% soy- based**	**Test diet (4% moringa leaf-based)**	**Test diet (25%** MoLD**)**	**Test diet (12.5%** MoLD**)**
		(g/kg)			
Soy bean	250	40	---	---	----
Moringa leaves	---	---	40	250	125
Corn starch	516	100	100	516	516
Soy bean oil	40	40	40	40	40
Cellulose	40	400	400	40	165
Sucrose	100	366	366	100	100
DL-methionine	4	4	4	4	4
*Vitamin/	50	50	50	50	50
Mineral mix					

*****Vitamin/ Mineral mix: Vitamin A 4,000,000 i.u; Vitamin D3, 800,000 i.u; Tocopherols, 400 i.u; Vitamin K_3 800 mg, Folacin, 200 mg; Thiamine, 600 mg; Riboflavin 1,800 mg; Niacin, 6000 mg; Calcium pathothenate, 4 mg; Biotin, 8 mg; Manganese, 30,000 mg, Zinc, 20,000 mg; Iron, 8,000 mg; Choline chloride 80,000 mg; Copper, 2,000 mg; Iodine, 480 mg; Cobalt, 80 mg; Selenium, 40 mg; BHT, 25,00 mg Anticaking agent 6000 mg.

MoLD = *M. oleifera* leave based diet

3.2.3 Proximate Analysis

The proximate analysis of all the feeds were done Proximate analysis of a food sample determines the total protein, fat, carbohydrate, ash, and moisture reported as the percentage composition of the product.

3.2.3.1 Determination of Protein Content of Feed

The protein content was determined by the Kjeldhal method reported by James (1995). The total nitrogen was determined and multiplied by the factor, 6.25 to obtain the protein concentration or content.

Principle

The Kjeldahl Nitrogen determination, developed in 1883, depends upon the fact that most organic N compounds are converted into $(NH_4)_2SO_4$ when heated with concentrated

H_2SO_4; the exceptions are $–NO_2$ and –N=N– groups, which if present in any quantity should be previously reduced to the amine.

Principle

Digestion: $RNH_2 + 2H_2SO_4 \xrightarrow{Se} (NH_4)_2SO_4 + CO_2 + H_2O$

Distillation: $(NH_4)_2SO_4 + NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O$

Absorption: $3NH_3 + H_3BO_3 \longrightarrow (NH4)_3BO_3$

Titration: $(NH_4)_3BO_3 + 3HCl \longrightarrow H_3BO_3 + 3NH_4Cl$

Procedure

2 g of sample was weighed accurately into a micro Kjedhal flask together with 5g of Na_2SO_4, 0.5 g of $CuSO_4$, 25 ml of concentrated H_2SO_4 and a tablet of selenium as catalyst.

Digestion were carried out on moderate heat from the heating mantle for about $1^1/_2$ hours.The solution were green after heating. It was cooled and the solution diluted with distilled water poured into a 250 ml flask and made up to mark with distilled water.

To distil, the Kjedhal distillation apparatus were set up, 5 ml NaOH were used to distil 5 ml of the sample, 5 ml of boric acid were used in the receiving flask to trap the liberated ammonia, as ammonia were trapped the original colour of boric acid (purple) will change to green colour of $(NH_4)_3BO_3$.When the green solution is up to 5 ml in the receiving flask, it will then be titrated against 0.01 ml HCl. At the end point, boric acid will once more be produced as well as ammonium chloride giving us the original colour of boric acid.

Calculation:

$$\text{Total nitrogen in g/100 ml} = \frac{14\times10^{-5}\times100\times V\times V_T}{V_{used}\times W}$$

Protein in g/100 ml = Total Nitrogen × 6.25

Where $14x10^{-5}$ = Nitrogen Index

V = Volume of HCl used in titration

V_T= Total volume of distillate collected

V_{used} = Volume of distillate used in titration

W = Weight of sample used for digest

6.25 = Protein conversion factor for food materials.

3.2.3.2 Determination of Ash Content

The method described by Pearson (1973) was employed. Dry clean porcelain crucible was ignited in a muffle furnace for one minute. The heated crucible will then be cooled and weighed W_1. Sample was placed in the crucible and weighed W_2. The crucible was gently heated on a Bunsen burner until smoking ceased after which it was transferred to a muffle furnace and heated at 600^{o}C to white ashes .The crucible was then removed and placed in desiccators to cool, after which it was weighed to constant weight W_3

Calculation:

Weight of Crucible = W_1

Weight of Crucible + Sample = W_2 (g)

Weight of Ash = W_3 (g)

$$\text{Ash \% content} = \frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$$

Weight of ash = W_3-W_1×100

Weight of sample = W_2-W_1

$$\text{Ash content} = \frac{W_3\text{-}W_1\times 100}{W_2\text{-}W_1}$$

3.2.3.3 Determination of Lipid Content of Feed

The crude lipid was determined by the continuous solvent extraction method in a Soxhlet apparatus as described by James (1995).Soxhlet extractor was fixed with a reflux condenser and a round bottom flask containing chloroform and methanol in the ratio 2:1 half filled. The

round bottom flask placed inside a curved shaped burner or heater. A known amount in of the sample was weighed W_1 (g). The sample was plugged with filter paper and weighed W_2 (g). This was then placed in an extractor solvent (chloroform and methanol at 40-60^oC) to the siphon. This set up was placed on a heating mantle and allowed to extract for six hours after which the apparatus was detached. The thimble removed, dried in the oven until a constant weight was obtained at 60-100^oC. This was then transferred into a desiccator to cool after which the final weight W_3 (g) was taken. The weight of the extracted lipid was obtained from the weight gain in the flask after extraction and oven drying.

Calculation:

Weight of filter paper = W_1

Weight of filter paper + dry sample = W_2

Weight of filter extraction + weight of sample = W_3

$$\% \text{ Crude} = \frac{\text{Weight loss of sample}}{\text{Weight of sample}} \times 100 = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

3.2.3.4 Determination of Crude Fibre Content

The crude fibre was determined by the Weende method described by both Pearson (1976) and James (1995).

A known weight of the sample (2g) was defatted with petroleum ether for 8hrs. It was boiled under reflux for exactly 30 minutes with 200 ml of 1.25% H_2SO_4, filtered and washed with boiling water until the washings are no longer acidic. The residue was boiled in a round bottom flask with 200 ml of 1.25% NaOH for another 30 minutes. This was filtered into a previously weighed crucible and the crucible with the sample (residue) was dried in the oven at 100^oC. It will then be left in a desiccator to cool and weighed again (C_2) before being

incinerated in a muffle furnace at about 600^{o}C for 3hours, placed in a desiccator to cool and weighed again (C_3).

Calculation:

Weight of Fibre = (C_2 - C_3) g

$$\%\text{Fibre} = \frac{C_2 - C_3}{\text{Weight of Original Sample}} \times 100$$

3.2.3.5 Determination of Moisture Content of Feeds

The moisture content of the samples was determined by the methods described by Pearson (1976) and James (1995).

Empty dry crucibles were oven dried, left in a desiccator and the weighed (C_1). 2 g of samples were added into the dried crucibles and the new weight taken as (C_2) and thereafter taken into the oven wee it is left to dry to a constant weight (C_3). The percentage moisture was determined using the following formula:

$$\frac{C_1 - C_2}{C_1} \text{ x } 100$$

3.2.3.6 Determination of Carbohydrate

The carbohydrate content was determined by estimation using the arithmetical difference method described by Pearson (1976) and James (1995). The carbohydrate content was calculated and expressed as the nitrogen free extract.

The sum of the percentage moisture, ash, crude lipid, crude protein and crude fibre was subtracted from100%.

i.e Carbohydrate= 100 - (% Moisture + % Ash + % Protein + % Lipids + %Fibre).

3.2.4 Phytochemical Screening

3.2.4.1 Total Flavonoids Determination

This was determined using Aluminum chloride colorimetric method for flavonoids determination (Chang *et al*., 2002). *M. oleifera* leaf extracts (0.5 ml) was mixed (in triplicates) with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water. It was left at room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solution. Flavonoid content is expressed in terms of quercetin equivalent (mg g^{-1} of extracted compound).

3.2.4.2 Determination of Total Phenol Content

The total phenol content of the extracts was determined using the method reported by Singleton *et al*. (1999). Appropriate dilutions of the extracts (0.5 ml) were oxidized with 2.5mL of 10% Folin–Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45℃ and the absorbance measured at 765 nm with spectrophotometer. The total phenol content was subsequently calculated using gallic acid as standard.

3.2.4.3 Determination of Alkaloids

This was done by measuring 0.5 g of the sample and dissolved in 96% ethanol -20% H_2SO_4 (1:1). The filtrate (1 ml) was added to 5 ml of 60% tetraoxosulphate (VI), and allowed to stand for 5 min. Then, 5 ml of 0.5% formaldehyde will be added and allowed to stand for 3 h. The reading was taken at absorbance of 565 nm (Oloyede, 2005).

3.2.4.4 Determination of Saponins

This was evaluated by measuring 0.5 g of the extract, which was thereafter added to 20 ml of 1M NaCl and boiled for 4 h. After cooling, it was filtered and 50 ml of petroleum ether was added to the filtrate for ether layer and evaporated to dryness. 5 ml of acetone ethanol was added to the residue and 0.4 ml of each into 3 different test tubes. Ferrous sulphate reagent (6 ml) was added to each followed by 2 ml of Concentrated H_2SO_4. Mixed after 10 min and read the absorbance at 490 nm (Oloyede, 2005).

3.2.4.5 Determination of Tannins

This was evaluated measuring 0.2 g of extract into a 50 ml beaker. 20 ml of 50% methanol was added and covered with para film and placed in a water bath at 77-80^o for one hour. This was shaken thoroughly to ensure a uniform mixing and then filtered using a double layered whatman No 41 filter paper into a 100 ml volumetric flask. 20 mls of water, 2.5 ml folin-Ciocalteu reagent and 10 ml of 17% Na_2CO_3 were added and mixed properly. The mixture was made up to mark with water, mixed well and allowed to stand for 20 minutes. A bluish-green colour was developed at the end of range 0-10 ppm.The absorbance of tannin acid standard solution as well as sample were read after colour development on a spectrophotometer at wave length of 760 nm (A.O.A.C, 1984).

3.2.4.6 Terpenoids Determination

Total terpenoids content was determined according to the procedure described by Chang *et al.* (2002). Briefly, 0.5 mg/ml of each extracts was dissolved in methanol. Then, 100 µl of the extract was mixed with 100 µl of 5% sulphuric acid solution. Themixture was then heated for 45 minutes at 60ºC and cooled in cold water to the ambient temperature. Five percent of glacial acetic acid (2.25 ml) was added to extract and the absorbance read at 548 nm, using a spectrophotometer. Eucalyptol (0.5 mg/ml in methanol) was used as a standard.

3.2.5 Induction of PEM

Muscle degeneration was induced by feeding the animals with low protein (4%) iso-caloric diet *ad libitum* for four weeks (a slight modification of the method used by Nadia *et al.* (1999) (Appendix 1).

3.2.6 Animal Grouping

Phase 1: preliminary study

- Group 1- Control
- Group 2- malnourished with 4% soy meal- based diet
- Group 3- malnourished with 4% soy meal- based diet + vitamin E (40 mg/kg body weight).
- Group 4- malnourished with 4% *M. oleifera* leave based diet (MoLD).

After 4 weeks, three animals from each group were sacrificed then the remaining animals were subdivided into treatment groups as follows:

- Group 2a- treated with 25% soy
- Group 2b- treated with 25% MoLD
- Group 3a- were treated with 25% soy supplemented with vitamin E
- Group 3b- treated with 25% soy alone
- Group 4- treated with 25% MoLD.

Phase two and three

Fourty-six animals were used for this stage out of which six animals were grouped into control groups while the remaining were malnourished with 4% soy- based diet in the first four weeks. Three of the malnourished animals were sacrificed after four weeks and the remainng were grouped into the four treatment groups as stated below:

- Control group
- Untreated malnourished group (BT)
- Group treated with 25% soy + methyl jasmonate + vitamin E + Carnitine (Soy-based)
- Treated with 25% MoLD
- Treated with 12.5% MoLD

  Methyl jasmonate = 100 µg/kg body weight

  Vitamin E = 20 mg/kg body weight

  Carnitine = 3 mg/kg body weight

3.2.7 Anthropometric Measurements

Anthropometric measurements that were taken include;

- the length (cm) using a non-elastic tape rule
- weight (g) of the rats were measured using a 0.01g sensitive weighing balance
- circumference of the head and thigh using veneer caliper.

3.2.8 Preparation of Tissues

The rats were sacrificed under anaesthesia and blood collected through jugular puncture. Blood samples were collected into plain sample bottles and some into EDTA coated bottle to prevent clotting (for serum and haematological analysis respectively) while the skeletal muscle from the hind limbs were quickly extracted into iced cold solutions of 250 mM sucrose buffer(250 mM sucrose, 10 mM tris, pH 7.4).

3.2.8.1 Preparation of Tissue Homogenates

The tissues (liver and skeletal muscle) were washed with the same buffer to remove blood and fur. The tissues were homogenized in a Teflon homogenizer. The buffer was the

homogenizing medium. The suspensions of tissue homogenate were stored in aliquot units in Eppendoff tubes and stored in the freezer. The homogenates were kept frozen overnight to ensure maximum release of the enzymes (Ngaha *et al*., 1989). Note that skeletal muscle homogenate was used immediately on the day of sacrifice for mitochondrial functioning and permeability tests. The stored homogenates were used for Ca- ATPase assay within three days of full freezing to prevent loss of enzyme activity.

3.2.8.2 Preparation of Serum

Blood sample collected in the plain sample bottle were allowed to stand at room temperature for 30 mins to clot. Serum was thereafter prepared by centrifuging the blood samples at 3000 rpm for 5 minutes (Ogbu and Okechukwu, 2001) and serum collected by pipetting, labeled and refrigerated for further use.

3.2.8.3 Isolation of the Mitochondrial

This was done using the method described by Schnider (1957) modified by Johnson and Lardy (1967)

Procedure

The skeletal muscle and liver were quickly excised from the sacrificed rat and washed in buffer C (210 mM manitol, 70 mM sucrose, 5 mM Hepes- KOH and 1 mM EGTA pH 7.4), homogenized and centrifuged at 4^0C in refrigerated centrifuge. The nuclear fraction and cellular debris were sedimented by centrifuging at 2300 rpm for 5 minutes twice. Supernatant obtained were centrifuged further to remove the remaining nuclear debris. The supernatant were centrifuged at 13,000 rpm for 10 minutes to get the mitochondrial. The supernatant were discarded and the mitochondrial pellet obtained was washed twice with buffer D at 12,000 rpm for 10 minute. Mitochondrial obtained were suspended in MSH buffer and dispensed in

eppendoff tubes for the reading to be taken in a spectrophotometer. All assays were done in an ice cold condition and on fresh samples.

3.2.9 Protein Determination

The protein concentration in the tissue homogenates were determined using Biuret method described by Gornall *et al.* (1949) using bovine serum albumin as the standard protein.

Aim: The aim is to determine the number of peptide bonds present in the test sample

Principle: A purple coloured complex is obtained when compound containing peptide bonds are treated with copper sulphate. This is as a result of the coordination of Cu^{2+} with unshared pair of electrons of the peptide chains. The intensity of the colour formed is proportional to the number of peptide bonds in solution.

Procedure

The test was done in triplicates. The content of the test media is as shown in Table 2. The content of the test tubes were mixed thoroughly at intervals and allowed to stand for 20 minutes after which the optical density of the test samples was read against blank at wavelength of 520 nm using spectrophotometer.

A standard protein curve was then plotted by plotting the values of the absorbance against different concentrations of BSA (standard protein).

3.2.10 Determination of Serum Albumin

Serum albumin concentration was quantified by the method described by Doumas *et al.* (1971). The measurement was based on quantitative binding of the albumin to the indicator 3,3,5,5- tetrabromo- m- cresol sulphonephthalein (bromo- cresol green BCG). The albumin-

BCG- complex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration of the albumin in the sample.

3.2.11 Biomarkers of Muscle Degeneration

3.2.11.1 Creatine Kinase Activity

Method: Creatine kinase activity is determined in a coupled enzyme system utilizing pyruvate kinase (PK) and lactate dehydrogenase (LDH). The procedure was described by Tanzer and Gilvarg (1959). One unit is defined as the conversion of one micromole of creatine to creatine phosphate per minute at 25°C and pH 8.9 under the specified conditions.

Reagents

Reagent solution: the required amount of solution were prepared containing: 8.5 mM ATP, 1.22 mM NADH, 2.0 mM PEP, 15.0 µ/ml LDH, 7.0 µ/ml PK, 28.0 mM $MgSO_4.7H_2O$, and 26.0 mM Glutathion (reduced) all adjusted to pH 7.4.

Buffered Creatine: this contained 0.40 M Glycine containing 53.2 mM creatine and 62 mM potassium carbonate. The pH was adjusted to 8.9 with NaOH.

Enzyme diluent: this contained freshly prepared 5 mM glycine at pH 9.0

Enzyme: Prepare 1 mg/ml in 5 mM glycine, pH 9.0. Dilute to 0.1-10 µg/ml in glycine buffer.

Table 4: Protocol for Standard Protein Determination

Reagents (ml)	**Blank (1)**	**2**	**3**	**4**	**5**	**6**	**7**
1% BSA (10 mg/ml)	-	0.1	0.2	0.3	0.4	0.5	0.6
Distilled water	1.0	0.9	0.8	0.7	0.6	0.5	0.4
Biuret reagent	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Reacting volume	5.0	5.0	5.0	5.0	5.0	5.0	5.0

Procedure

The spectrophotometer was adjusted to 25°C and 340 nm. 0.7 ml of reagent solution was added to 2.22 ml of buffered creatine in the cuvette and then incubated in spectrophotometer at 25°C for 3-5 minutes to achieve temperature equilibrium and establish blank rate, if any. 0.1 ml of diluted enzyme was added and decrease in A_{340} for 5-8 minutes was recorded. An initial lag period may occur. The $\Delta A_{340}$was determined from linear portion of the curve.

Calculation

$$\text{Units/ mg} = \frac{\Delta A_{340}/\text{ min}}{6.2 \text{ x mg enzyme/mg reaction mixture}}$$

3.2.11.2 Lactate Dehydrogenase Assay

The assay kit used was a product of Fortress Diagnostics Limited, United Kingdom.

Principle

The test is a quantification of lactate dehydrogenase (LDH). Lactate dehydrogenase catalyses the conversion of pyruvate to lactate. During this process, NADH is oxidised to NAD. The rate of decrease in NADH is directly proportional to the activity of LDHand is determined by the measurement of the rate of absorbance change at 340 nm due to reduction.

$$\text{Pyruvate + NADH} \xrightarrow{\text{LDH}} \text{L-Lactate + NAD}$$

Reagents

R1 – buffer/substrate (contained tris buffer pH 7.5 (50 mmol/l) and pyruvate 0.6 mmol/l)

R2- contained NADH 0.18 mmol/l

Procedure

1. The working reagent was prepared by mixing five portions of R1 with a portion of R2
2. 1000 µl of the working reagent and 20 µl of sample was pipetted together in the test tube, mixed thoroughly and left to incubate at assay temperature for one minute.
3. The spectrophotometer was set to a wave length of 340 nm and blanked against air
4. The initial absorbance was read and repeated at exactly 1, 2 and 3 minutes.
5. The LDH activity was calculated using the following formular

   Calculation

   U/L = 8095 X ΔA 340 nm/min

   U= unit

   L= liter

   ΔA= change in absorbance

3.2.11.3 Aspartate Aminotransferase Activity (AST)

The assay kit used was a product of Pointe Scientific medical test company, USA.

Significance

The activity of aspartate transaminase (AST) in the serum and liver homogenate were determined following the method reported by Reitman and Frankel (1957) as modified by Schmidt and Schmidt (1963). AST catalyses the formation of oxaloacetate from L- aspartate and α-ketoglutarate. The oxaloacetate generated is unstable and it is spontaneously decarboxylated to form pyruvate. The absorbance of the red coloured complex formed from the reaction of pyruvate with ρ-nitrophenyl hydrazine was then read on a spectrophotometer at 546 nm.

Principle

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + NAD^+ + H_2O$$

Aspartate aminotransferase (AST) catalyses the transfer of the amino group from L-aspartate to α -Ketoglutarate to yield oxalacetate and L-glutamate. The oxalacetate undergoes reduction with simultaneous oxidation of NADH to NAD^+ in the malate dehydrogenase (MDH) catalysed indicator reaction.

The resulting rate of decrease in absorbance at 340 nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

Materials

AST (SGOT) Reagents R1 and R2 are mixed in the ratio 5:1 (i.e 5 parts of R1 reagent with 1 part R2 reagent) to make the working reagent. After mixing, reagent contains: L-aspartic acid >200 mM, α-ketoglutaric acid 12 mM, LDH (microbial) > 1000 U/L, MDH (microbial) >800 U/L, NADH >0.18 mM, buffer, pH 7.8 ± 0.1, sodium azide 0.25%, Stabilizers.

Procedure

1. 1.0 ml of the working reagent was pipetted into tubes and pre-warm at 37°C for five minutes.
2. 0.100 ml (100 µl) of sample reagent was added, mix and incubate at 37°C for one minute.
3. After one minute, the absorbance was read and recorded at 340 nm against water blank.

4. The reading was repeated every minute for the next two minutes.
5. The average absorbance difference/minute was calculated (ΔAbs/Min.).
6. The ΔAbs/Min. multiplied by the factor 1768 yielded the results in IU/L

Calculation

One international Unit (IU/L) is defined as the amount of enzyme that catalyses the transformation of one micromole of substrate per minute under specified conditions.

$$\text{AST (IU/L)} = \frac{\Delta\text{Abs/Min} \times 1.10 \times 1000}{6.22 \times 0.10 \times 1.0} = \Delta\text{Abs/min} \times 1768$$

Where ΔAbs/Min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

1.10 = Total reaction volume (ml)

6.22 = Millimolar absorptivity of NADH

0.10 = Sample Volume (ml)

3.2 = Light path in cm.

3.2.11.4 Alanine Aminotransferase Activity (ALT)

The assay kit used was a product of Pointe Scientific medical test company, USA.

Significance of the test

The activity of glutamate–pyruvate transaminase (ALT) in the serum and liver homogenate were determined following the method reported by Reitman and Frankel (1957) as modified by Schmidt and Schmidt (1963). ALT catalyses the reaction between L-alanine and α-

ketoglutarate to produce L-glutamate and pyruvate. The method measures spectrophotometrically the absorbance of the red coloured complex formed from the reaction between pyruvate and 2,4-dinitrophenylhydrazine.

Principle

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^{+} + H2O$$

ALT catalyses the transfer of the amino group from L-alanine to α-ketoglutarate resulting in the formation of pyruvate and L-glutamate. Lactate dehydrogenase catalyses the reduction of pyruvate and the simultaneous oxidation of NADH to NAD^{+}. The resulting rate of decrease in absorbance is directly proportional to ALT activity.

Materials

ALT (SGPT) Reagents R1 and R2.

Reagent Preparation

The working reagent was prepared by mixing 5 parts of R1 reagent with 1 part R2 reagent.

After combining R1 and R2 the reagent contains: L-alanine 500mM, α-ketoglutaric acid 15mM, LDH(microbial) >2000IU/L, NADH >0.18mM, Buffer 100mM, pH 7.5±0.1, Sodium azide 0.2%, Stabilizers.

Procedure

1. 1.0ml of the working reagent was pipetted into tubes and pre-warm at 37°C for five minutes.
2. 0.10 ml (100µl) of sample was added to the reagent, mix and incubated at 37°C for one minute.

3. The spectrophotometer was zeroed with water at 340nm.
4. After one minute, the absorbance was read and recorded. Reading was repeated every minute for the next two minutes.
5. Mean absorbance difference/minute was calculated (ΔAbs./Min.).
6. The ΔAbs./Min. multiplied by the factor 1768 yield results in IU/L.

Calculation

One international Unit (IU/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

$$\text{ALT (IU/L)} = \frac{\Delta\text{Abs/Min} \times 1.10 \times 1000}{6.22 \times 0.10 \times 1.0} = \Delta\text{Abs/min} \times 1768$$

Where ΔAbs/Min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

1.10 = Total reaction volume (ml)

6.22 = Millimolar absorptivity of NADH

0.10 = Sample Volume (ml)

1.0 = Light path in cm

3.2.11.5 Alkaline Phosphatase Activity (ALP)

The assay kit used was a product of Pointe Scientific medical test company, USA.

Significance

Alkaline phosphatase (ALP) was assayed using the method described by Wright *et al.* (1972), which employs the use of ρ-nitrophenyl phosphate as substrate. In this method the amount of phosphate ester that is split within a given period of time is a measure of the phosphatase

enzyme activity. ρ-Nitrophenyl phosphate (ρ-NPP) is hydrolysed to q-nitrophenol and phosphoric acid at pH of 10.1. The ρ-nitrophenol confers a yellowish colour on the reaction mixture and its intensity, measured spectrophotometrically at 400 nm gives the measure of the enzyme activity.

Principle

$$\rho\text{-NPP} + H_2O \xrightarrow{\text{Alkaline Phosphatase}} \rho\text{ -Nitrophenol} + H_3PO_4$$

ρ-Nitrophenyl phosphate is hydrolysed to ρ-nitrophenol and inorganic phosphate. The rate at which the p-NPP is hydrolysed, measured at 405 nm, is directly proportional to the alkaline phosphatase activity.

Materials

Alkaline Phosphatase R1 and R2 Reagent. 5 Parts of R1 Reagent was mixed with 1 part R2 Reagent. After combining R1 and R2 as directed, the reagent contains: AMP Buffer (pH 10.45), ρ-NPP ≤ 16mM, Magnesium ions ≥ 1.0mM, activators and preservatives.

Procedure

1. The working reagent was prepared by mixing 5 parts of R1 Reagent with 1 part R2 Reagent. 0.1 ml of reagent was pipette into tubes and pre-warm at 37°C for five minutes.
2. 0.025 ml (25 µl) of sample was transferred into reagent, mixed and incubated at 37°C for one minute.
3. The spectrophotometer was zeroed with water at 405 nm.
4. After one minute, the absorbance was and recorded and readings repeated every minute for the next two minutes.
5. The average absorbance difference per minute was then calculated. (ΔAbs/Min)

6. The ΔAbs/Min multiplied by the factor 2187 will yield results in IU/L.
7. Samples with values above 1000 IU/L should be diluted with an equal volume of saline, re-assayed and the results multiplied by two.

Calculations

One international Unit (IU/L) is defined as the amount of enzyme that catalyses the transformation of one micromole of substrate per minute under specified conditions.

$$(IU/L) = \frac{\Delta Abs/Min \times 1000 \times 1.025}{18.75 \times 1 \times .025} = \Delta Abs/min \times 2187$$

Where ΔAbs/Min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

1.025 = Total reaction volume (ml)

18.75 = Millimolar absorptivity of ρ-nitrophenol or extinction coefficient of 1 mole of ρ-nitrophenol in alkaline solution of 1ml volume of 1cm light path at 400 nm.

1. 025 = Sample Volume (ml)

1 = Light path in cm

3.2.12 Ca^{2+}-ATPases Assay

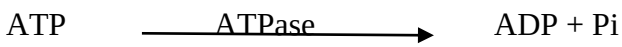
Ca^{2+}-ATPase assay were conducted on the skeletal muscle tissue homogenate of test and control animal groups after the 4th and 8th weeks.

Aim

The aim of the assay is to determine ATPase (particularly Ca^{2+} ATPase) activities spectrophotometrically as this enzyme is specifically implicated in muscle degeneration.

Principle

The ATPaseassay is a membrane assay that indirectly measures the activity of efflux transporters. ATP Binding Cassette or efflux transporters mediate the transport of substrates across cell membranes against a concentration gradient. ATP cleavage is tightly linked to substrate translocation, as the energy for the substrate translocation is derived from ATP hydrolysis. ATP hydrolysis yields inorganic phosphate (Pi), which can be measured by a simple colorimetric reaction. The amount of Pi liberated is directly proportional to the activity of the transporter (Glavinas *et al.,* 2008).



The activities of enzymes were determined in tissues of animal treated with the different formulations and controls and compared.

Procedure

The procedure of Runner *et al*. (1977) modified by Bewaji (2004) were used. Constituents of the reacting medium for the different types of ATPases is as shown in Tables 5 and 6. Homogenate were added last to start the reaction at regular interval of 10 seconds after which the reacting media were incubated at 37^{o}C for 30 minutes with constant shaking. The reaction was stopped with 0.2 ml (i. e 200 µl) of sodium dodecyl sulphate (SDS). 2 ml of reagent C were added for colour development in 20 minutes and absorbance read at 820 nm against blank using a spectrophotometer.

The values of absorbance were extrapolated on the standard phosphate curve to determine the amount of phosphate released. The following formula was used to determine the specific enzyme activity.

Table 5: Constituents of the Media for the Assay of Mg^{2+}, Ca^{2+}, and Na^{+}/P^{+}-Atpases

	Mg^{2+}- ATPase (mM)	Ca^{2+}- ATPase (mM)	Na^{+}/P^{+}- ATPases (mM)
NaCl	100	-	100
KCl	20	120	20
$MgCl_2$	5	5	5
$CaCl_2$	-	0.2	-
Tris buffer (pH 7.4)	30	30	30
ATP	1	1	1
Vanadate	0.05	-	-

Source: Bewaji, 2004.

Table 6: Protocol for ATP Dependence Ca^{2+}- ATPase Activity Determination

Tubes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Enzyme blank**	**ATP blank**
Ca^{2+} Buffer (µL)	500	500	500	500	500	500	500	500	500	500
$CaCl_2$ (µL)	100	100	100	100	100	100	100	100	100	100
Distilled water (µL)	380	370	360	350	340	330	310	290	300	390
Tissue homogenate (µL)	10	10	10	10	10	10	10	10	-	10
ATP	10	20	30	40	50	60	80	100	100	-

Note that the volume of Ca^{2+} buffer, homogenate and distilled water could vary.

Source: Bewaji, 2004.

$$\text{Specific enzyme activity} = \frac{\mu M\ Pi}{\text{Mg Protein x time taken}}$$

Where µM Pi = µM phosphate released into the reacting medium by the enzyme

3.2.13 Markers of Oxidative Stress

3.2.13.1 Catalase (CAT) Activity

Catalase activity was determined by the method described by Beers and Sizer (1952).

Principle

The disappearance of peroxide was followed spectrophotometrically at 240nm. One unit decompose one micromole of H_2O_2 per minute at 25^0C and pH 7.0

Procedure:

Prior to use, the enzyme source was diluted in 0.05M phosphate buffer (pH 7.0) to obtain a rate of 0.03 - 0.07 ΔA/min. the assay mixture was constituted as shown in Table 7:

Decrease in absorbance was monitored at 240nm for 2 min immediately after the addition of the appropriately diluted enzyme source.

Calculation: Change in absorbance (ΔA_{240}/min) was calculated from the initial 45 seconds.

CAT activity was obtained from the following expression:

$$\text{Catalase activity (Units/mg)} = \frac{\Delta A_{240}/\text{min X } 1000}{43.6 \text{ x mg protein/ml reaction mixture}}$$

Table 7: Protocol for Catalase Activity Determination

Reagent	Volume
Distilled water	1.9 ml
Hydrogen peroxide (0.059 M)	1.0 ml
This were incubated in spectrophotometer for 5 min to achieve temperature equilibration and to establish blank rate	
Enzyme source (appropriately diluted)	0.1 ml

3.2.13.2 Determination of Superoxide Dismutase (SOD) Activity

SOD activity was determined by the method of Misra and Fridovich, (1972).

Principle

The ability of superoxide dismutase to inhibit the auto oxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for dismutase. Superoxide radical (O_2^{-}) generated by xanthine oxidase reaction causes the oxidation of epinephrine to adenochrome and the yield of adenochrome produced increases per O_2^{-}introduced with increasing pH (Valerino and McCormack, 1971) and also increase with increasing concentration of epinephrine. These results led to the proposal that auto oxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide radical and hence can be inhibited by SOD.

Water-soluble tetrazolium, the sodium salt of 4-[3-(4 iodophenyl) -2-(4- nitrophenyl)-2H-5-tetrazolio]-1,3- benzene disulfonate, was used as a detector of superoxide radical generated by xanthine oxidase and hypoxanthine, in the presence of a range of concentrations of superoxide dismutase. A major advantage of the assay is that one reaction mixture is prepared and aliquotted into wells, avoiding pipetting errors and variable xanthine oxidase activity between samples.

Materials

i. SOD assay buffer

ii. SOD standard (0, 0.75,1, 1.5, 3, 5, 6 µ/ml)

iii. Enzyme solution

iv. Indicator solution

v. Dilution buffer

Tissue Samples

The supernatant collected was used in the assay. Dilutions were in 10 folds (i.e 0.5 g tissue in 5 ml phosphate buffer)

Serum was used directly without any pretreatment and dilution was in 5 folds.

Assay Procedure

Working Reagent was prepared by mixing the Enzyme reagent, the indicator solution and 30 ml of Assay buffer. Then,

1. 20 µl of sample, 20 µl of assay buffer (blank) and 20 µl of standards was added to wells of a microplate in duplicates.
2. 200 µl of the working reagent prepared above was added to each well, and mix thoroughly
3. The plate was incubated at 37 °C for 20 min.
4. The absorbance was read at 450 nm using a microplate reader.

Calculation

SOD activity (% Inhibition) was calculated using the following equation

For standards

SOD activity (% Inhibition) = $\{[(A_{Blank}) - (A_{standard})]/ (A_{blank})\}$ x 100

For sample

SOD activity (% Inhibition) = $\{[(A_{Blank}) - (A_{standard})]/ (A_{blank})\}$ x 100

Calibration Curve

A Calibration curve was drawn by plotting the % inhibition obtained above for each standard (on Y-axis) against SOD standard concentration in U/ml (on X-axis). SOD concentration was then extrapolated for each sample in U/ml from the calibration curve.

3.2.13.3 Determination of Glutathione - S-Transferase Activity

Glutathione S-transferase (GST) activity was dertermined according to the method of Habig *et al*. (1977). The assay kit used was a product of Sigma Chemical Company, USA.

Principle

The assay is based on the fact that all GST demonstrate a relatively high activity with 1-chloro-2, 4-dinitrobenzene as the second substrate. Consequently, the conventional assay for GST activity utilizes 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. When this substance is conjugated with reduced glutathione, its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

Materials

i. Substrate Solution

ii. Buffer

iii. Reduced Glutathione (Reconstitute by adding 4ml Buffer)

Procedure

The assay reaction was performed at room temperature (25 °C). All reagents were allowed to equilibrate to room temperature.

An enzyme cocktail was prepared by mixing the following reagent in proportion stated below;

- 980 µl of buffer solution

- 10 µl of Glutathione solution

- 10 µl of Substrate (CDNB) Solution

The solution might become slightly cloudy upon the addition of CDNB to the solution. This cloudiness disappears when the solution is completely mixed.

For blank

- 1 ml of the above solution was added to a cuvette, mixed and allowed to equilibrate for 1 minute

- The initial absorbance was read, then read again after 1 min, 2 min, and 3 minutes

- The Absorbance after 3 minutes was the final reading

- The Change in Absorbance of Blank was calculated using the following equation

$$(\Delta A_{340})/\text{min} = \frac{A_{340}\,(\text{final read}) - A_{340}\,(\text{initial read})}{\text{Reaction time (min)}}$$

For sample

- 950 µl of the above solution was added to a cuvette, then 50 µl of sample, mixed well and allowed to equilibrate for 1 minute

- The initial absorbance was then read again after 1 min, 2 min, and 3 minutes

- The Absorbance after 3 minutes was the final reading

• The Change in Absorbance of Sample solution (i.e (ΔA_{340})/min) was calculated using the following equation:

$$(\Delta A_{340})/\text{min} = \frac{A_{340}\,(\text{final read}) - A_{340}\,(\text{initial read})}{\text{Reaction time (min)}}$$

Calculations

Subtract the (ΔA_{340})/minute of the blank from the (ΔA_{340})/minute of the sample.

This rate was then used for the calculation of the GST specific activity.

GST specific activity:

$$\frac{(\Delta A_{340})/\text{min} \times V\,(\text{ml}) \times \text{dil}}{\varepsilon\text{mM}\,(9.6) \times \text{Venz (ml)}} = \mu\text{mol/ml/min}$$

Where:

dil = the dilution factor of the original sample

V = the reaction volume: · for test in 1 ml cuvette = 1 ml

Venz = the volume of the enzyme sample tested (0.05 ml)

εmM ($mM^{-1}cm^{-1}$) = the extinction coefficient for CDNB conjugate at 340 nm.

The extinction coefficient of CDNB at 340 nm = 9.6 $mmol^{-1}cm^{-1}$

3.2.13.4 Determination of Reduced Glutathione (GSH)

The levels of reduced glutathione (GSH) in the samples were determined by the method of Ellman (1959) as described by Bulaj *et al*. (1998). Glutathione (-glutamylcysteinylglycine or

GSH) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells. GSH is required as a coenzyme by a variety of enzymes including glutathione peroxidase, glutathione S-transferase and thioltransferase. GSH also plays a major role in drug metabolism, calcium metabolism, the -glutamyl cycle, cell membrane and blood platelet functions. GSH is crucial to a variety of life processes, including the detoxification of xenobiotics, maintenance of the –SH level of proteins, thiol-disulfide exchange, removal of hydroperoxides and free radicals, and amino acid transport across membranes. Physiological values for the concentration of intracellular GSH generally range from 1 to 10 mM.

Principles

These spectrophotometric procedures are based on the method of Ellman 1959, who reported that 5,5'-dithiobis- (2,-nitrobenzoic acid) is reduced by SH groups to form 1 mole of 2-nitro-5- mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow color which when measured at a wavelength of 412 nm can be used to measure SH groups.

Materials

- ProteinPrecipitation reagent
- GSH dilution buffer
- GSH Standard (2 mM)
- GSH chromogen

Standard Curve Preparation

2 mM GSH standard provided was used to prepare series of standards as indicated in the table in the Appendix II. This was prepared immediately prior to use.

Assay Procedure

1. 100 µl of standard and samples was placed into a micro-cuvette.
2. 880 µl of GSH dilution buffer was added to the micro-cuvette.
3. 20 µl GSH Chromogen was added to the tube and mixed well
4. The absorbance of resulting solution was measured at 412 nm within 5 minutes

Calculation

A standard curve was plotted using the Absorbance value for each Standard versus the GSH concentration for each Standard. The equation of the line was found using a linear fit method. The Concentration of GSH in each sample was then extrapolated from the Graph.

3.2.13.5 Malondialdehyde Assay (MDA)

The concentration of malondialdehyde was quantified according to the procedure described by Reilly and Aust (1999) for assessment of lipid peroxidation.

The assay kit was a product of Oxford Biomedical research (Colorimetric TBARS Microplate

Assay KitProduct Number: FR40).

Principle

Small amounts of malondialdehyde (MDA) are produced during lipid peroxidation and these are able to react with thiobarbituric acid (TBA) to generate a pink coloured complex (chromophore) which in an acidic solution absorbs light maximally at 532 nm.

Materials

i. Protein Precipitation reagent
ii. MDA Indicator solution
iii. MDA Standard (10 mM)
iv. Sample pre-treatment

Preparation of Tissue

0.5 g tissue was homogenized in 2.5 ml protein precipitation reagent, mixed and centrifuged at 3,000 rpm for 5 minutes. The supernatant was used for the assay.

Standard Curve Preparation

10 mM MDA Standard was diluted to 1:500 in distilled water by adding 20 µl of 10 mM MDA to 9.98 ml dilute H_2O to make 20 µM MDA Standard Stock. This was prepared immediately prior to use.

Assay procedure for free MDA

1. Preparation of Standards and Samples: Each of the following reagents was added into glass test tubes and mix well.
    - Standards: 200 µl of standard and 200 µl of Indicator Solution.
    - Samples: 200 µl of sample and 200 µl of Indicator Solution. –
2. After the standards, samples and blanks have been mixed; they were allowed to react for 45 minutes at room temperature.
3. 300 µl of each was transfered into the microplate wells and the absorbance of the resulting solution measured at 532 nm. The resulting pink color is stable for several hours at room temperature.

Calculation

Standard curve was plotted using the A_{532} OD value for each Standard versus the MDA concentration for each. The equation of the line was found using a linear fit method. The MDA concentration in the sample can be calculated from the equation of the graph and is expressed µM as:

$$\text{MDA (units/mg protein)} = \frac{\text{absorbance x volume of mixture}}{E_{532} \text{ x volume of sample x mg protein}}$$

Where E_{532} is molar absorbtivity at 532 nm = 1.56×10^5

3.2.14 Mitochondrial Membrane Functionality and Permeability Assessment (Marker of Energy Depletion)

3.2.14.1 Assay of Mitochondrial Membrane Swelling

This was done using spectrophotometrically. The change in the absorbance at wavelength of 540 nm for 30 minutes was observed. Decrease in the absorbance of the mitochondrial suspension indicates mitochondrial swelling (Chinda *et al*., 2014). Absorption spectrophotometer was used to take the absorbance.

3.2.14.2 Assay for Mitochondrial Change in Membrane Potential

The method was described by China *et al.* (2014). It was done by staining the mitochondria (20 µl) with the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocanine iodide (JC-1). The green fluorescent (JC-1 monomer) was excited at wavelength of 485 nm and emission detected at 590 nm while the red fluorescent (JC-1 aggregates) was excited at 485nm and the emission detected at 530 nm using fluorescent microplate reader (SpectraMax Germini Molecular Device).

A decrease in the red/green fluorescent intensity ratio indicates depolarization of the mitochondrial membrane (Chinda *et al*., 2014).

$$\text{i.e. membrane potential } (\Delta\Psi) = \frac{\text{Red fluorescent emission}}{\text{Green fluorescent emission}}$$

3.2.14.3 Assay for Mitochondrial ROS Production

The mitochondrial ROS level was detected using the excitation and emission wave lengths.This was done by the method described by Apaijai *et al*. (2013) and Palee *et al*. (2011) which employs the use of fluorescent dye and fluorescent spectrophotometer. Mitochondrial ROS production in the skeletal muscle and liver was measured by staining the mitochondria with dichlorohydrofluoresce in diacetate (DCFDA) dye for 25 minutes, after which a fluorescent microplate reader (spectraMax Germini Molecular Device) was used to detect the ROs level using excitation wavelength of 480 nm and emission wavelength of 530 nm.

Procedure

20 µl of the mitochondrial fractions were placed in the holes of the microplate and then 10 µl of the fluorescent dye was added and left for 25 minutes and read in a fluorescent microplate reader (spectraMax Germini Molecular Device)

3.2.15 Haematological Analysis

Haematological parameters that were analyzed are red blood cell count, white blood cell count and blood haemoglobin. These were analyzed using automated haematological analyzer. The analyzer uses whole blood samples to produce values for the parameter. It uses two fields, the cell packs (which functions as a detergent and self-rinses the system to avoid introduction of errors) and the stromatolyzer (which works on the cells).

Principle: The red cells are counted and lysed by the stromatolyser releasing the haemoglobin and estimates its concentration photometrically. The machine assumes that all nucleated cells are white and counts them into their different forms, i.e. lymphocytes and neutrophils.

The blood sample collected into the EDTA coated bottle were used for this analysis

3.2.16 Toxicological Studies

3.2.16.1 Liver Function Indices

AST, ALT and ALP assays are a done in phase one of this research.

3.2.16.1.1 Serum Albumin Concentration

Principle: The procedure described by Doumas *et al.* (1971) was used for the assay of the serum albumin concentration. This metho determines the albumin concentration by quantitatively measuring the binding of albumin to 3, 3', 5, 5'- tetrabromo creol sulphonapthalein (bromocresol grrn, BCG). The albumin-BCG complex formed, absorbs maximally at 630 nm. The absorbance is directly proportional to the albumin concentration in the serum sample.

Procedure: the sample (0.1 ml of the serum or standard control) was added to 3.0 ml of BCG reagent, mixed and incubated for 5 minutes at room temperature. The absorbance was thereafter measured with spectrophotometer against blank at 630 nm. The blank was prepared by substituting the sample with distilled water.

Calculation:

Albumin concentration (g/dl) = absorbance of sample x Concentration of standard / absorbance of standard

3.2.16.1.2 Serum Bilirubin Concentration

Princilpe: this was determined by monitoring the intensity of blue coloured complex formed between diazonized sulphanilic acid and bilirubun (Daumas *et al.*, 1985).

Procedure: the sample (0.2 ml of serum or standard control) was added to 0.2 ml of sulphanilic/ hydrochloric reagent. One drop (0.05 ml) of sodium nitrite and 1ml of sodium benzoate were then added and mixed thouroughly. The resulting solution was incubated for10 minutes at room temperature (≤ 27^{o}C). The reaction was terminated by adding 1 ml of 0.1 M sodium hydroxide and then allowed to atand for an additional 25 minutes at room temperature. The absorbance was read at 578 nm against blank.

Calculation

Total billirubin (mg/dl) = ΔAsample x concentration of standard (mg/dl) / ΔAstandard

3.2.16.2 Kidney Function Indices

3.2.16.2.1 Serum Urea Concentration

Serum urea concentration was determined uing the method described by Tietz *et al.* (1995). The principle of this method is based on the formation of green coloured complex (2, 2 - dicarboxylindophenol) by the reaction of ammonia librated from the urea in the presence of urease enzyme with salicyclate and hypochlorite in the reagent. The absorbance of the green coloured complex is directly proportional to the concentration of urea.

Procedure

The sample (0.01 ml of serum sample or standard control) was added to 1 ml of urease prepared in phosphate buffer containing 6.4 mM sodium salicyclate, 5 mM sodium nitroprusside, 1.5 mM EDTA, 18mM sodium hypochorite and 750 mM sodium hydroxide. The mixture was incubated for 10 minutes at room temperature. Absorbance of test sample was read against blank (prepared by replacing the sample with distilled water) at 600 nm.

Calculation:

Urea concentration (g/dl) = A_{sample} x concentration of standard / $A_{standard}$

3.2.16.2.2 Serum Creatinine

The assay kit used was a product of Fortress Diagnostics Limited, U.K.

Principle: Concentration of serum creatinine was determined by the method described by Tietz (1995). In an alkaline solution, creatinine reacts with picric acid to form a coloured complex the amount of which is directly proportional to the concentration of creatinine.

Procedure: The sample (0.1 ml of either the ample or standard control) was added to 1ml of 5ml picric acid prepared in 0.32 M NaOH. The mixture was then mixed and absorbance was read after 30 seconds against blank at 492 nm (A_1). At exactly 2 minutes later, the absorbance of the sample and the standard was taken again (A_2). The concentration of serum creatinine was the calculated as:

Creatinine (mg/dl) = ΔAsample x concentration of standard (mg/dl) / ΔAstandard

Principle

Creatinine reacts with picric acid in an alkaline medium to form a deep yellow colour complex, the amount of which is directly propotional to the level of creatinine in the sample.

Reagents

Sodium hydroxide (0.32 mol/l), picric acid (35 mmol/l) and standard (177 µmol/dl). The working reagent was prepared by mixing equal volume of NaOH and picric acid solutions as required for the number of test samples.

Procedure

1. The spectrophotometer was set to 492 nm and blanked with distilled water.
2. 100 µl of standard and sample were poured in different test tube.

3. 1 ml of working reagent was added to the content of each test tube, mixed well and read the absorbance after 30 seconds and again after 120 seconds
4. The concentration of creatinine was calculated from the following formular:

   Calculation

   $$\text{Creatinine conc} = \frac{\text{Change in absorbance of sample } (\Delta Abs)}{\text{Change in the absorbance of standard}} \times 177 = \mu mol/l$$

   Or $$\text{Creatinine conc} = \frac{\text{Change in absorbance of sample } (\Delta Abs)}{\text{Change in the absorbance of standard}} \times 2 = mg/dl$$

3.2.17 Statistical Analysis

All data were presented as mean ± standard deviation with alleast three replicates. Statistical analyses were carried out using Duncan Multiple Range Test (Montgomery, 1976). This was complemented with Student's t- test (Dixon and Massey 1969). Statistical significance was taken at $p < 0.005$**.**

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition of Formulated Feeds

The results of the proximate analysis done on the formulated diets are as presented in Table 8.There was no significant difference in the % moisture composition of the feeds except for the 12.5% MoLD. Moreover, there were no significant difference in the percentage crude protein of the groups with equal protein or *M. oleifera* leaf (i.e crude protein of 25% soy-based (12.95) and 25% MoLD (13.77). Only a reduced percentage *M. oleifera* leaf inclusion gave a significantly lower percentage crude protein (i.e 4% soy based (4.29) and 4% MoLD (4.12) when compared with the control. Lower percentage inclusion of *M. oleifera* leaf gave a significantly higher crude ash content compared with the control. There was no significant difference in the crude fibre content of 25% soy (3.50%) and 25% MoLD (5.50%) while the low protein (4%) iso-caloric diets had significantly higher crude fibre content. The isocaloric diets (i.e all the four feeds) prepared had no significant difference in their carbohydrate and energy values. The same trend was observed in other proximate parameter (i.e moisture andcrude lipid).

4.2 Phytochemical Screening of *M. oleifera* Leaf

The result of the phytochemical sreening on methanolic leaf extract of *M. oleifera* is as shown on Table 9. From the the result, it was observed that the methanolic extract of *M. oleifera* leaf is most abundant in total phenol then flavonoids followed by tannins and then terpenoids. Its concentration of alkaloids was least followed by saponins.

Table 8: Percentage Proximate Composition of Formulated Feeds

	% moisture	% crude protein	%ash	% crude fibre	% crude lipid	% carbohydrate	Energy value (Kcal/g)
25% soy-based diet	7.77 ± 0.53^{a}	12.95 ± 0. 20^{a}	0.67 ± 0.16^{a}	3.50 ± 0.00^{a}	7.34 ± 2.58^{a}	67.78 ± 2.58^{a}	420.00 ± 12.23^{a}
25% MoLD	9.73 ± 0.90^{a}	13.77 ± 0.23^{a}	2.67 ± 1.17^{a}	5.50 ± 0.00^{ab}	5,94 ± 1.28^{a}	62.89 ± 1.44^{a}	399.00 ± 16.67^{a}
12.5% MoLD	4.57 ± 0.56^{b}	8.18 ± 0.00^{b}	3.59 ± 0.59^{b}	5.50 ± 0.00^{a}	6.66 ± 2.17^{a}	71.32 ±3.30^{a}	397.00 ± 14.73^{a}
*4% soy- based diet	12.33 ± 0.83^{a}	4.29 ± 0.123^{c}	0.50 ± 0.00^{a}	12.00 ± 0.00^{c}	6.00 ± 0.00^{a}	64.88 ± 0.90^{a}	380.00 ± 0.00^{a}
*4% MoLD	13.00 ± 2.00^{a}	4.12 ± 0.44^{c}	5.00 ± 0.00^{b}	11.00 ± 0.00^{c}	10.00 ± 0.00^{a}	56.88 ± 2.16^{b}	386.00 ± 0.00^{a}
**_Moringa_ leaf	7.73 ± 1.71	11.36 ± 0.12	10.33 ±0.33	8.50 ± 0.00	8.98 ±0.01	53.09 ± 1.27	369.60 ± 23.76

Values with different superscript down the same column are significantly different ($p < 0.05$)

*Low protein iso- caloric diets

**values were not compared with other data

Table 9: Phytochemical Constituents of Methanolic Extract of *M. oleifera* Leaf

Phytochemicals	(mg/g)
Terpenoid	2.91± 0.01
Tannins	9.20 ± 0.05
Flavonoid	12.35 ± 0.11
Total Phenol	42.34 ± 0.02
Alkaloid	$30.86 \times 10^{-3} \pm 0.21$
Saponin	$55.22 \times 10^{-3} \pm 0.32$

Values are expressed as mean ± SEM of three replicates

4.3 Preliminary Evaluations and Induction of Muscle Atrophy (Phase One)

4.3.1 Effects of Low Protein Iso-Caloric Diet on Anthropometric Parameters

All the formulated low protein iso-caloric diets produced significant weight reduction in the experimental rats with the leastreduction observed in the group malnourished with 4% soy-based diet supplemented with vitamin E (Figure 7). However, after feeding the animals with the treatment feeds all the treatment groups were significantly increased (Figure 8).

4.3.2 Effects of Low Protein Iso-Caloric Diets on Haematological Indices

Tables 10 and 11 show the heamatological parameters of the malnourished animals before and after treatment. All the heamatological indices including red blood cell (RBC), white blood cells (WBC), heamoglobin (HGB) and pack cell volume (PCV) were observed to be significantly lower than the control before treatment. The least reduction was in the blood parameters of the animals in the group treated with only 25% soy-based diet (Table 12). After treatment, the parameters were significantly increased (Table 13). The parameters of the groups treated with 25% *M. oleifera* leaf-based diet was not significantly different from the control while those of the group treated with Vitamin E supplemented diet were significantly higher than the control.

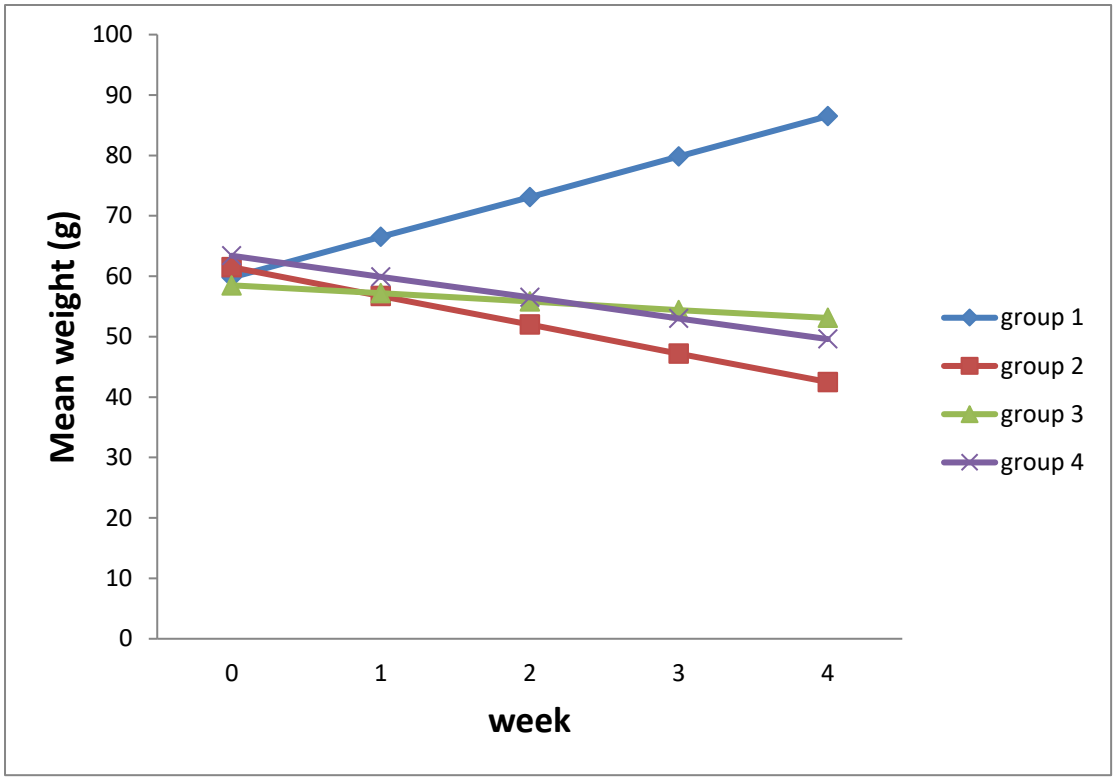


Figure 8: Weight of Rats Fed with Low-protein Isocaloric Diet for 28 Days

Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4% soy- based diet + vit.E, Group 4 = malnourished with 4% *M. oleifera*

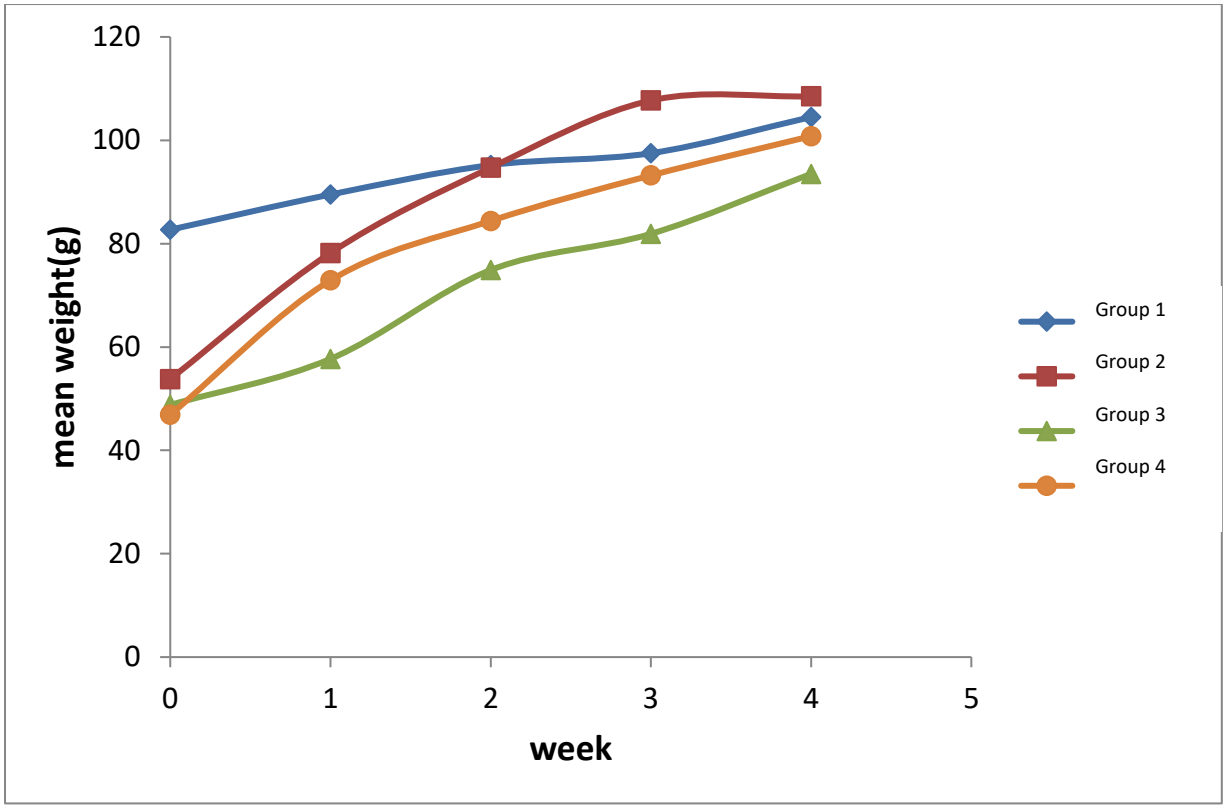


Figure 9: Weight of Malnourished Rats Treated with Formulated Diets for 28 Days

Group 1= control; Group 2 = treated with 25 % soy-based diet; Group 3 = treated with 25% soy + vitamin E; Group 4 = 25% *M. oleifera*

Table 10: Haematological Parameters of Rats Fed Low-Protein Isocaloric Diet for 28 Days

	Group 1	Group 2	Group 3	Group 4
RBC ($x10^6$/µl)	7.46 ± 0.01^a	6.16 ± 0.08^b	6.22 ± 0.02^b	6.36 ± 0.07^b
HGB (g/dl)	12.70 ± 0.15^a	10.10 ± 0.05^c	11.20 ± 0.15^b	11.50 ± 0.10^b
PCV (%)	48.40 ± 0.20^a	35.00 ± 0.25^c	39.00 ± 0.30^b	38.10 ± 0.40^b
WBC ($x10^3$/µl)	17.80 ± 0.35^a	12.30 ± 0.10^b	13.05 ± 0.12^b	12.90 ± 0.05^b

Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4% soy + Vit.E, Group 4 = malnourished with 4% MoLD

Results are expressed as mean of three replicates ± SEM; Values with different superscript across the same row are significantly different at $p < 0.05$

RBC = Red blood cells, WBC = White blood cells, HGB= haemoglobin, PCV = Packed cell volume

Table 11: Haematological Parameters of Malnourished Rats Treated with Formulated Diets for 28 Days

	RBC(X 10^6/µl)	WBC(10^3/µl)	HGB(g/dl)	PCV (%)
Group 1	6.75 ± 0.03 [a]	14.75 ± 0.38 [a]	11.60 ± 0.10 [a]	40.20 ± 0.10 [a]
Group 2	7.03 ± 0.01[b]	18.70 ± 0.50[ab]	12.30 ± 0.10 [a]	48.65 ± 0.33[c]
Group 3	7.45 ± 0.03 [d]	21.30 ± 0.40 [b]	12.00 ± 0.15 [a]	47.50 ± 0.10[bc]
Group 4	6.66 ± 0.06 [a]	14.50 ± 3.20 [a]	11.60 ± 0.95 [a]	45.10 ± 5.20[bc*]
*BT	6.16 ± 0.08[c]	12.30 ± 0.10[c]	10.1 0 ± 0.05[b]	35.00 ± 0.25[d]

Results are expressed as mean of three replicates ± SEM; Values with different superscript across the same column are significantly different at $p < 0.05$

RBC = Red blood cells, WBC = White blood cells, HGB= haemoglobin, PVC = Packed cell volume

Group 1= control Group 2 = 25 % soy, Group 3 = 25%soy + vitamin E Group 4 = 25%*M. oleifera*

*BT = Before treatment

4.3.3 Effects of Low Protein Iso-Caloric Diets on Serum Albumin

Serum concentration of albumin was significantly ($p < 0.05$) reduced in experimental rats following feeding with the three low protein iso-caloric diets. The addition of vitamin E to the low protein iso- caloric diet fed to the group three animals did not prevent the complication / malnourishment resulting from low protein and thus, the most significant reduction in the serum albumin concentration was observed in the group malnourished with 4% soy supplemented with Vit. E. Serum albumin concentration however increased significantly after feeding the malnourished animals with the formulated treatment feeds, with the most significant increase in the group treated with 25% MoLD followed by the group supplemented with Vitamin E, the control and the groups treated with 25% soy alone were not significantly different from each other. This is shown in Table 12.

4.3.4 Effects of Low Protein Iso-Caloric Diets on Calcium Atpase Activity (Preliminary Study)

Skeletal muscle Calcium ATPase activity reduced in all the malnourished groups except the group treated with 4% *M. oleifera* leaf-based diet (Figure 9). However, after feeding with the treatment feeds, calcium ATPase activities in all the treatment groups was raised than before treatment and significantly different from each other. Group 2a treated with only 25% soy-based diet had an increase in Ca^{2+} ATPase activity lower than the control, while group 2b animals treated with 25% soy, supplemented with vitamin E (i.e 25% soy-based diet + vitamin E) had an increase not significantly different from the control (Figure 10). Similarly, group 3b animals treated with only 25% soy- based diet alone had an increase in calcium ATPase activity significantly lower than the control were those treated with supplemented diets (group 3a) was not significantly different from the control (Figure 11). Animals in group 4 treated with 25% *M. oleifera* leaf-based diet had an increase in activity significantly higher than the control (Figure 12).

Table 12: Concentration of Albumin in the Serum of Rats Fed Low-Protein Isocaloric Diet for 28 Days

	Group1	Group2	Group3	Group4
Albumin (g/l) before treatment	3.690 ± 0.05 [a]	1.407 ± 0.00 [c]	0.352 ± 0.00 [d]	1.583 ± 0.01 [b]
*Albumin (g/l) after treatment	14.39 ± 0.05 [a]	13.08 ± 0.06[a]	18.64 ± 0.07 [b]	23.06 ± 0.13 [c]

Group 1= control; Group 2 = 25 % soy; Group 3 = 25% soy + vitamin E Group; 4 = 25% *M. oleifera*

Results are expressed as mean of three replicates ± SEM; Values with different superscripts along the rows are significantly different ($p < 0.05$)

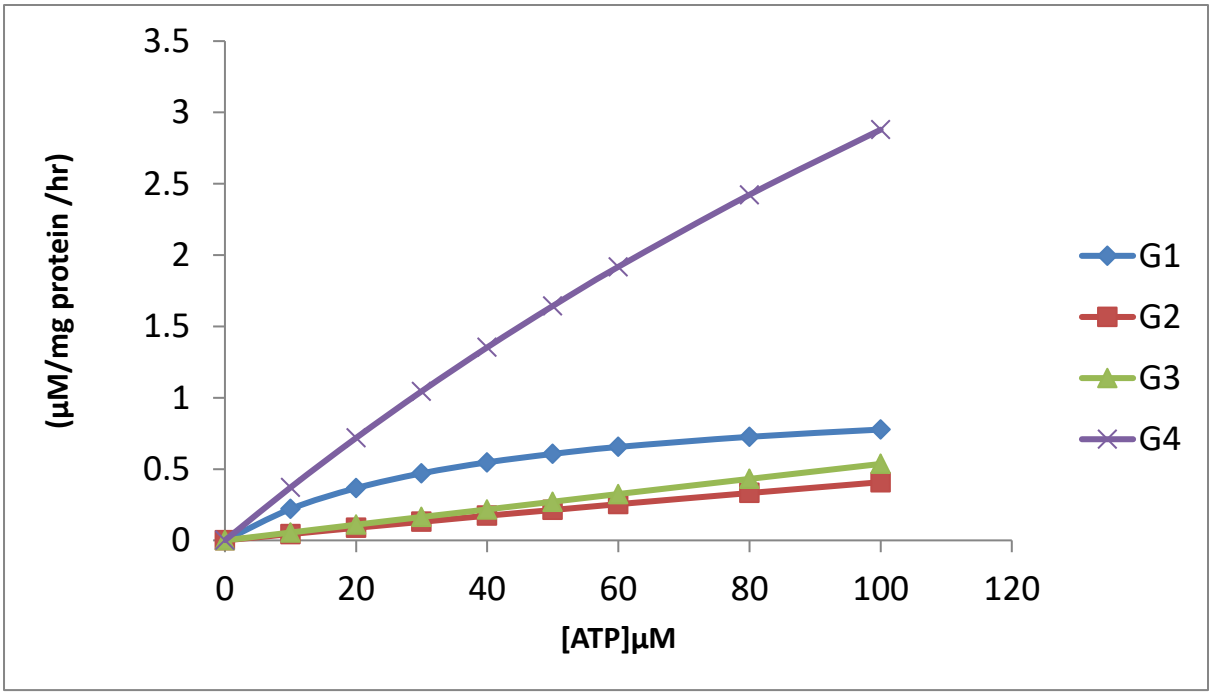


Figure 10: Ca^{2+}ATPase Activity in the Skeletal Muscle of Rats Fed Low-protein Isocaloric Diet for 28 Days

Group 1= control animals, Group 2= malnourished with 4% soy, Group 3= malnourished with 4%soy + Vitamin E, Group 4 = malnourished with 4% *M. oleifera*

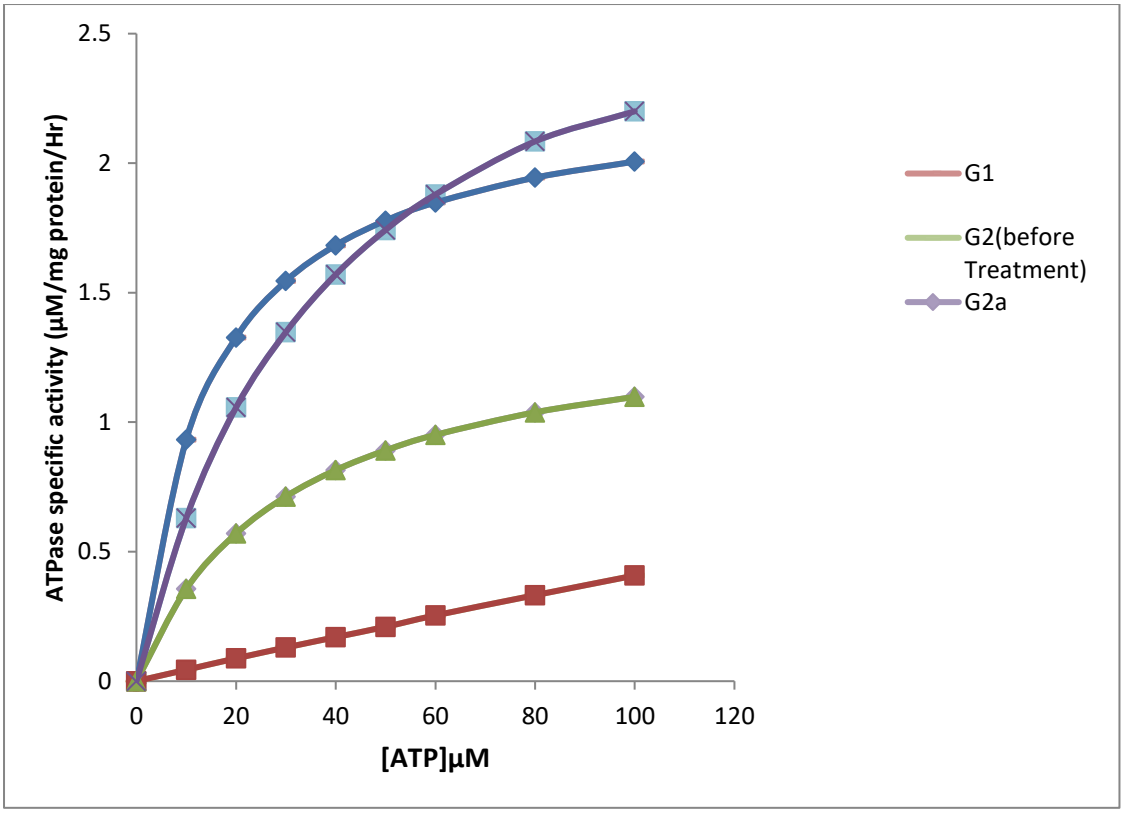


Figure 11: Activity of Ca^{2+}ATPase in the Skeletal Muscle of Group 2 Rats Treated with the Formulated Diets for 28 Days

Group 1= control animals, Group 2a= treated with 25% soy, Group 2b= treated with 25% MoLD

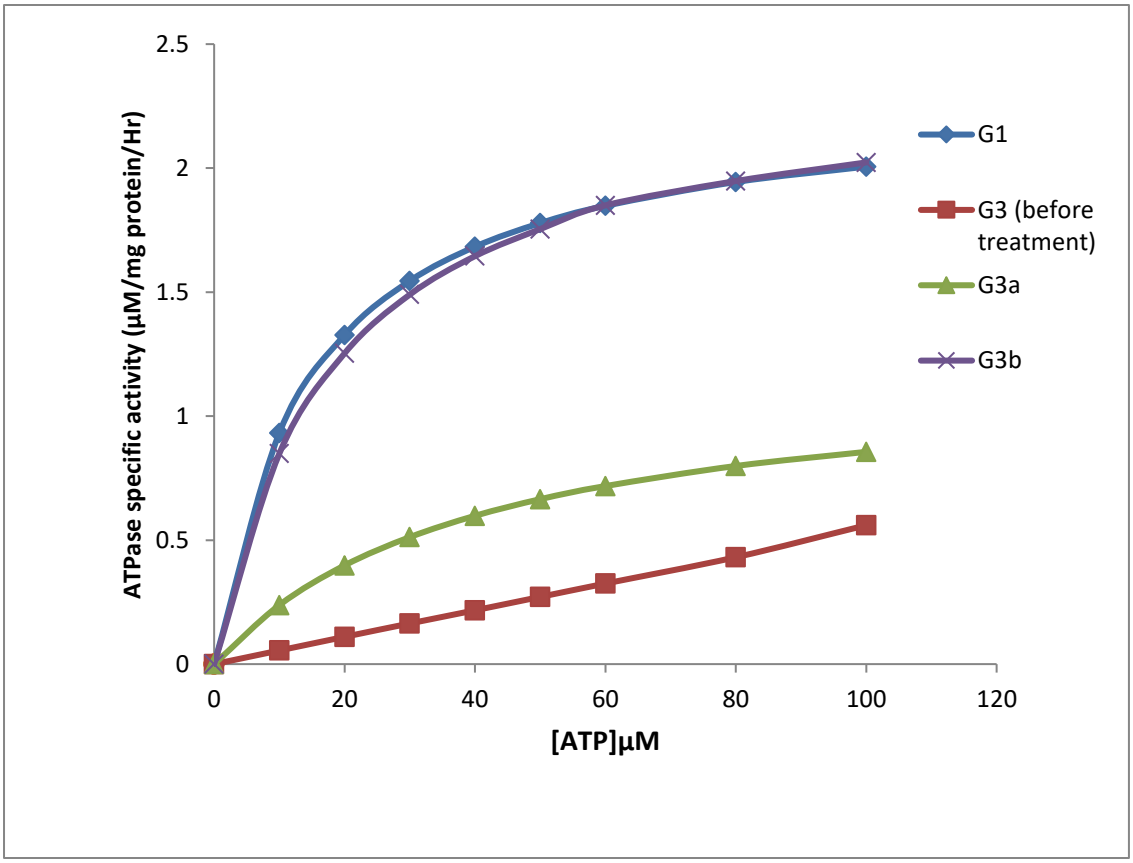


Figure 12: Activity of Ca^{2+}ATPase in the Skeletal Muscle of Group 3 Rats Treated Formulated Diets for 28 Days

Group 1= control animals, Group 3a = treated with 25%soy alone, Group 3b = treated with 25% soy + vitamin E

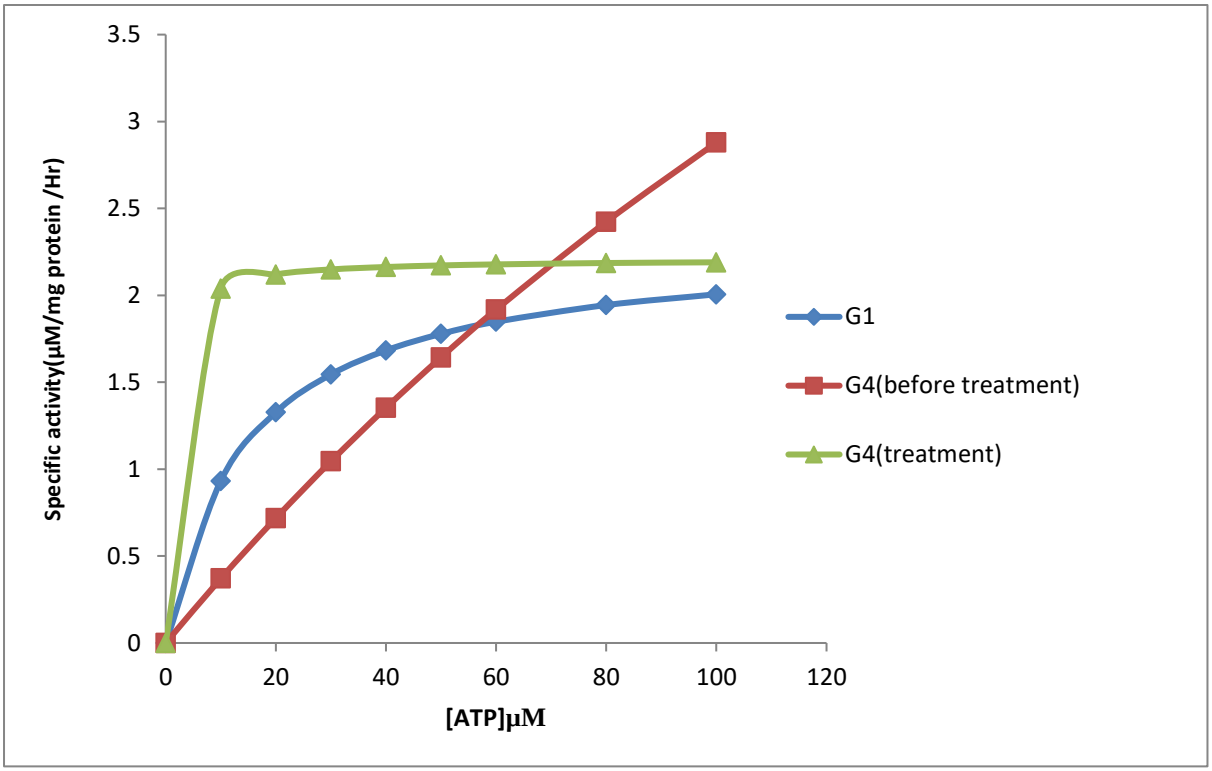


Figure 13: Activity of Ca^{2+}ATPase in the Skeletal Muscle of Group 4 rats Treated 25% *M. oleifera* Leaf-based Diet for 28 Days

Group 1= control animals, Group4= treated with 25% MoLD

4.4 Evaluation of the Effect of Formulated Diets on Markers of Muscle Degeneration (Phase Two Studies)

This phase was designed considering the recommendations from the results of the preliminary study (phase one) and some shortcomings identified in its design. Some of the observation from and modifications of the previous phase is as follows:

1. Malnourishing the experimental animals with 4 % soy-base diet gave the best results and thus this feed formulation was used in all the other phases as a model for inducing protein energy malnutrition.
2. It was observe that supplementing the low- protein iso-caloric diet with vitamin E did not prevent the factors affected by malnutrition from being affected. Although, supplementing 25% soy-based diet with vitamin E gave a very good result.
3. Only improved diet (e.g feeding the malnourished animals only 25% soy-based diet) will not improve all the factors affected by malnutrition (especially Ca^{2+} ATPase). Thus, the 25% soy-based treatment group received feed supplements (methyl jasmonate; a specific positive modulator of Ca^{2+} ATPase, vitamin E: an antioxidant and carnitine: a supplement againt energy depletion).
4. Moreover, more markers of muscle degeneration was assayed in the second phase.

4.4.1 Effect of Formulated Diets on Parameters of Malnutrition

4.4.1.1 Effect of Formulated Diets on Antropometric Parameters

The weight of the animals revealed a significant reduction before treatment similar to what was observed in the preliminary study. The weight of the treated animals however significantly increased after treatment with the lowest increase observed in the group treated with 12.5% *M. oleifera* leaf-based diet (Figure 14).

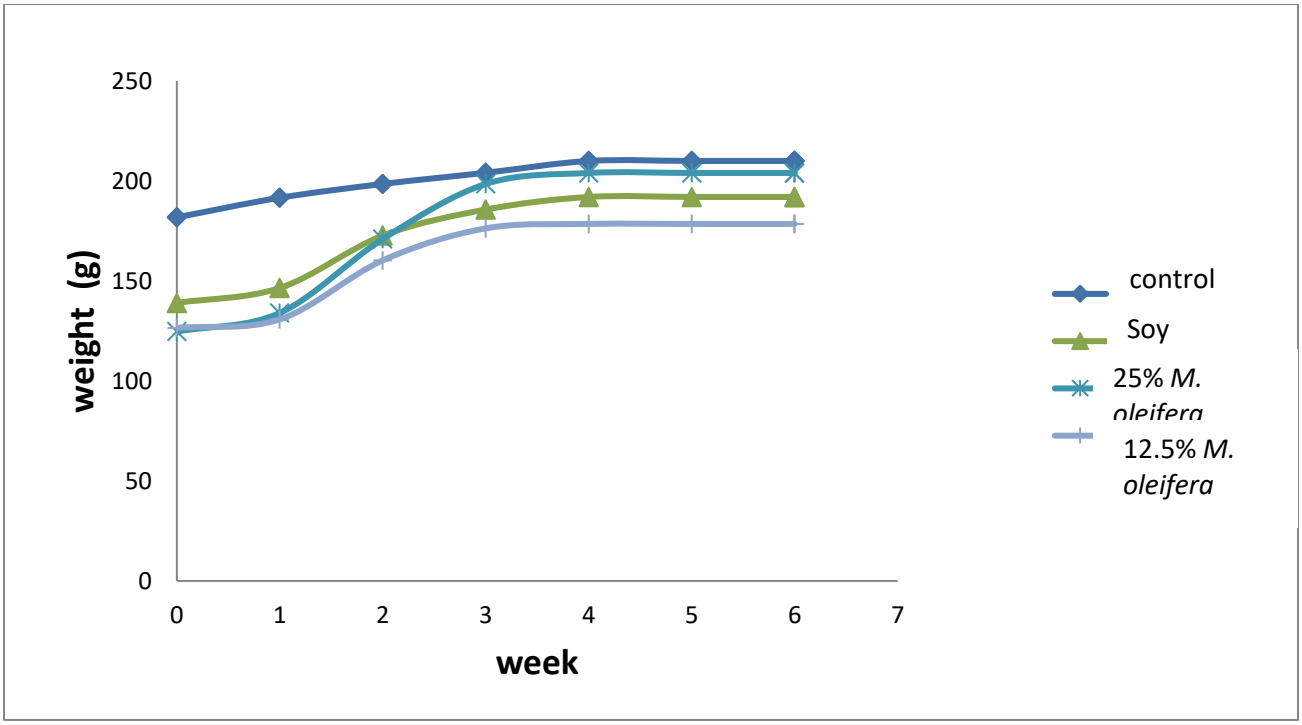


Figure 14: Weight of Malnourished rats Treated with Formulated Diets for 28 Days

4.4.1.2 Effect of Formulated Diets on Haematological Parameters

Similar to the result observed in the preliminary study, all the haematological parameters includingwhite blood cell (WBC), re boold cell (RBC), heamoglobin (HGB), packed cell volume (PCV) and lymphocyte (LYM), in all the malnourished were significantly reduced when compared with the control before treatment. There was however no significant difference between the control and all the treatment groups after maintaining them on the formulated diets except for the group treated with 12.5% MoLD whose WBC and LYM were not significantly different from the untreated group (Table13).

4.4.1.3 Effect of Formulated Diets on Serum Albumin Concentration

The effects of the formulated feeds on serum albumin are shown in Figure 15. Serum albumin concentration was significantly ($P < 0.05$) low in the animals induced with muscle degeneration before treatment. There was an elevation in the albumin concentration in the serum of all the treatment groups with no significant ($p < 0.05$) difference compared with the control.

Table 13: Effect of Treatment Feeds on Blood Parameters

	WBC ($x10^{-3}$/µl)	RBC ($x10^{-6}$/µl)	HGB (dL)	HCT%	LYM (X10-3/µL)
Before treatment	5.25 ± 0.15^{b}	2.73 ± 0.94^{b}	3.65 ± 0.95^{b}	17.55 ± 4.55^{b}	4.90 ± 0.00^{d}
Control	18.05 ± 1.35^{a}	7.59 ± 0.43^{a}	9.00 ± 0.30^{a}	44.1 ± 4.70^{a}	13.90 ± 4.00^{b}
Soy based	10.40 ± 2.90^{a}	6.82 ± 0.24^{a}	8.35 ± 0.95^{a}	41.45 ± 2.65^{a}	8.80 ± 2.10^{c}
25% MoLD	20.85 ± 2.25^{ab}	7.08 ± 0.47^{a}	8.35 ± 0.95^{a}	42.75 ± 1.95^{a}	17.80 ± 2.00^{a}
12.5% MoLD	5.05 ± 0.45^{b}	6.29 ± 0.26^{a}	7.20 ± 0.40^{a}	35.35 ± 1.15^{a}	5.50 ± 0.5^{cd}

Results are expressed as mean of three replicates ± SEM; Values with different superscripts down the column are significantly different ($p<0.05$)

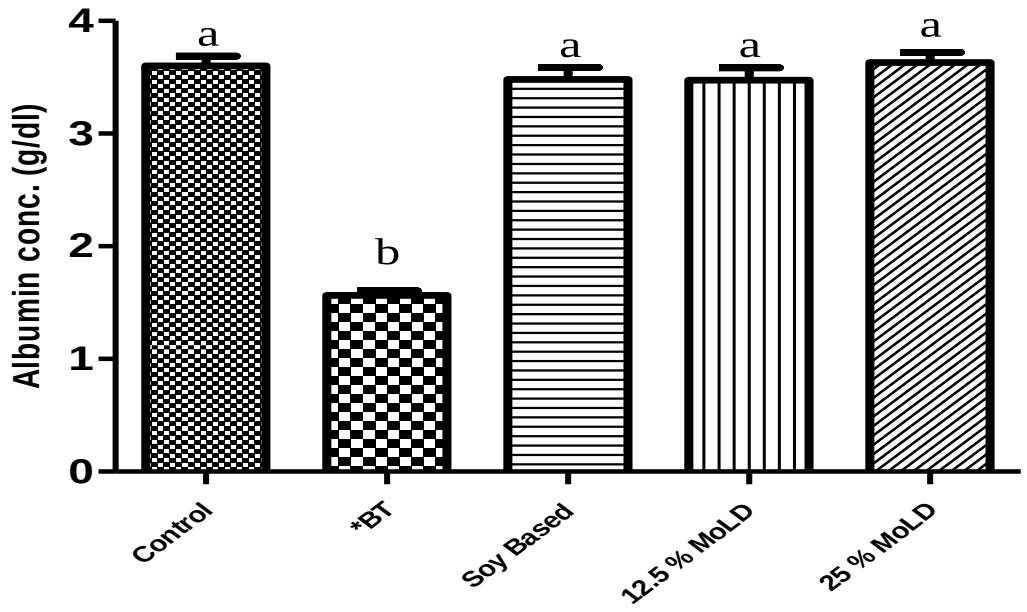


Figure 15: Concentration of Albumin in the Serum of Malnourished Rats Treated with the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

4.4.2 Effect of Formulated Diets on Markers of Muscle Degeneration

4.4.2.1 Effect of Formulated Diets on Creatine Kinase Activity

Creatine kinase (CK) activity in the skeletal muscle of untreated malnourished animal was significantly ($p < 0.05$) lower than the control and other treatment groups. There was significant elevation of the activity of the enzyme in the skeletal muscle of the treatment groups. There was no significant ($p < 0.05$) difference in the activity of the enzyme in the skeletal muscle of animals fed with 25% and 12.5% MoLD when compared with the control while those fed with 25% soy-based diet had significantly lower activity than the control animals (Figure 16). A correspondingly opposite result was observed in the serum of the animals (Figure 17). In the serum of the malnourished animals (i.e untreated animals) there was an elevation of CK activity. There was a significant reduction in the enzymes activity in all the treatment groups. There was no significant difference in the activity of serum CK in the animals treated with 12% *M. oleifera* leaf-based diet and the control, while that of group treated with 25% soy-based supplemented diet was significantly higher than the control and 25% *M. oleifera* leaf-based diet.

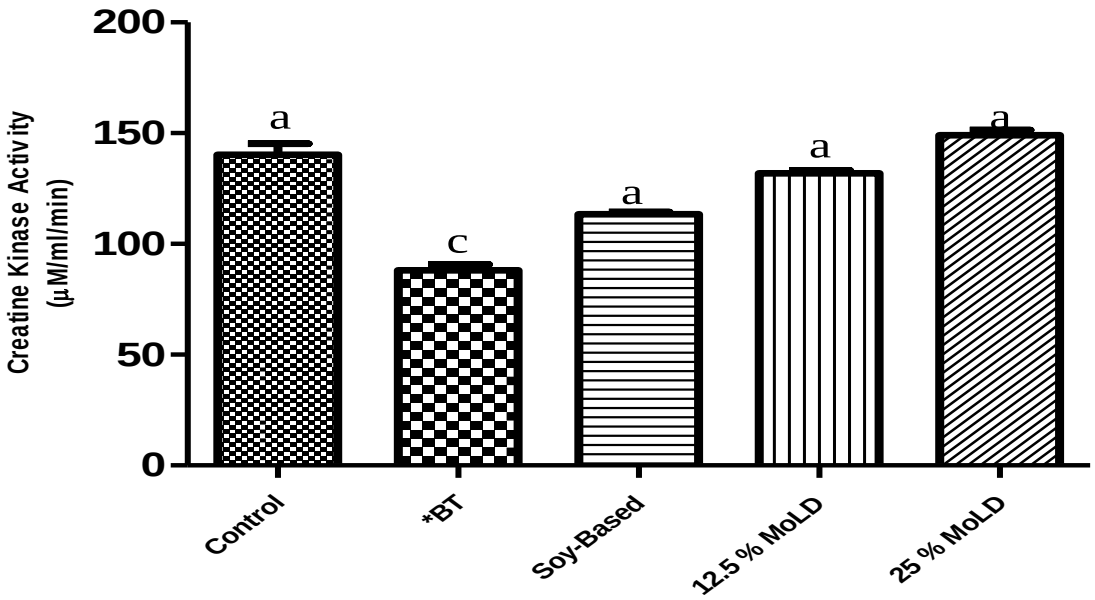


Figure 16: Activity of Creatine Kinase Activity in the Skeletal Muscle of Malnourished Rats Treated with the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

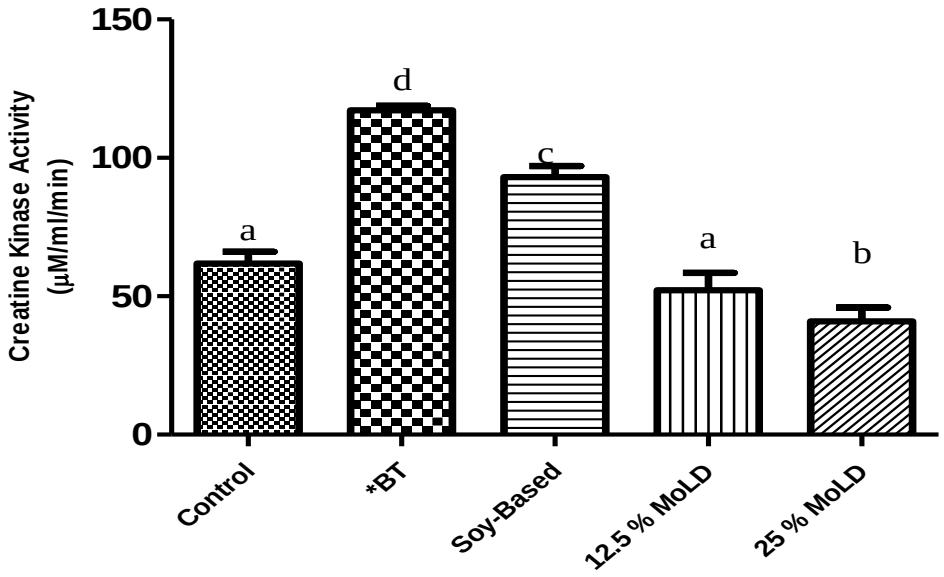


Figure 17: Activity of Creatine Kinase activity in the Serum of Malnourished Rats Fed with Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment

4.4.2.2 Effect of Formulated Diets on Lactate Dehydrogenase

LDH activity in the serum and skeletal muscle of the animals before treatment was significantly higher than those of treatment and control groups. In the skeletal muscle, LDH activity of the control group was not significantly different from the groups treated with *M. oleifera* leaf-based diet (25% and 12.5%), while the groups treated with soy-based diet was significantly higher than the control but lower than the untreated group.

Likewise in the serum, there was a significant elevation in LDH activity in the untreated group. LDH activity in the soy-based treated animals was significantly higher than the two *M. oleifera* leaf-based diet (25% and 12.5%) whose activities were not significantly different from each another (Figures 18 and 19).

4.4.2.3 Effect of Formulated Diets on Aspartate Transaminase Activity (Ast) of Malnourished Rats Treated with the Formulated Diets

The activity of the AST in the serum was significantly higher in the serum of the untreated animals when compared with the control. In the serum of the control, 25% and 12.5% *M. oleifera* leaf-based diet treated animals, there was no significant difference in the activity of the enzyme. AST activity was however significantly increased in the serum of animals treated with 25% soy- based diet (Figure 20). A correspondingly reverse AST activity was observed in the skeletal muscle of the treated and untreated animals (Figure 21)

4.4.2.4 Effect of Formulated Diets on the Activity of Serum Alanine Transaminase (ALT) of Malnourished Rats Treated With the Formulated Diets

Serum ALT activities of animals fed 25% and 12.5% *M.oleifera* leaf-based diet were not significantly different from the control. Contrarily, serum ALT activity in the untreated animals and those treated with 25% soy-based diet where significantly higher (Figure 22).

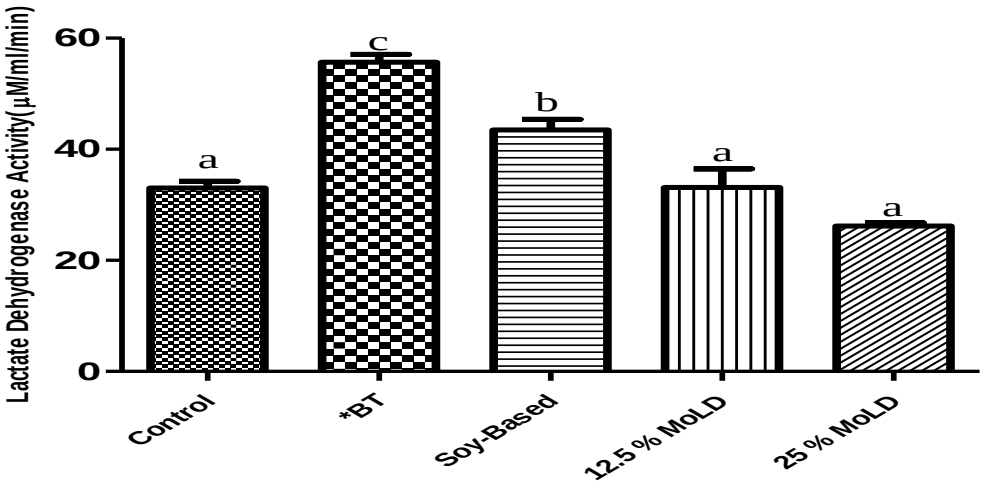


Figure 18: Activity of Lactate Dehydrogenase in the Skeletal Muscle of Malnourished Rats Treated for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

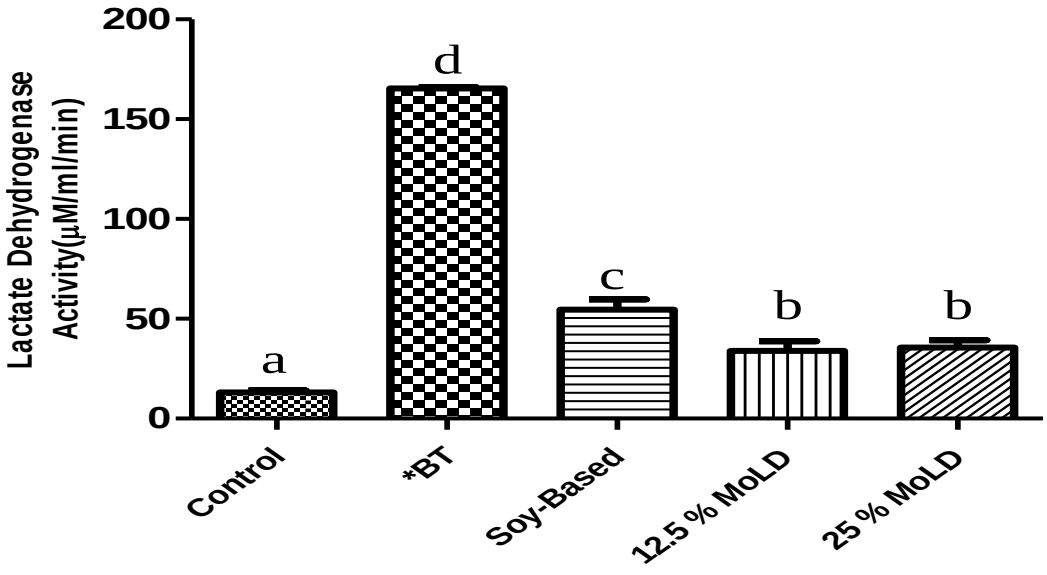


Figure 19: Activity of Lactate Dehydrogenase in the Serum Muscle of Malnourished Rats Fed the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

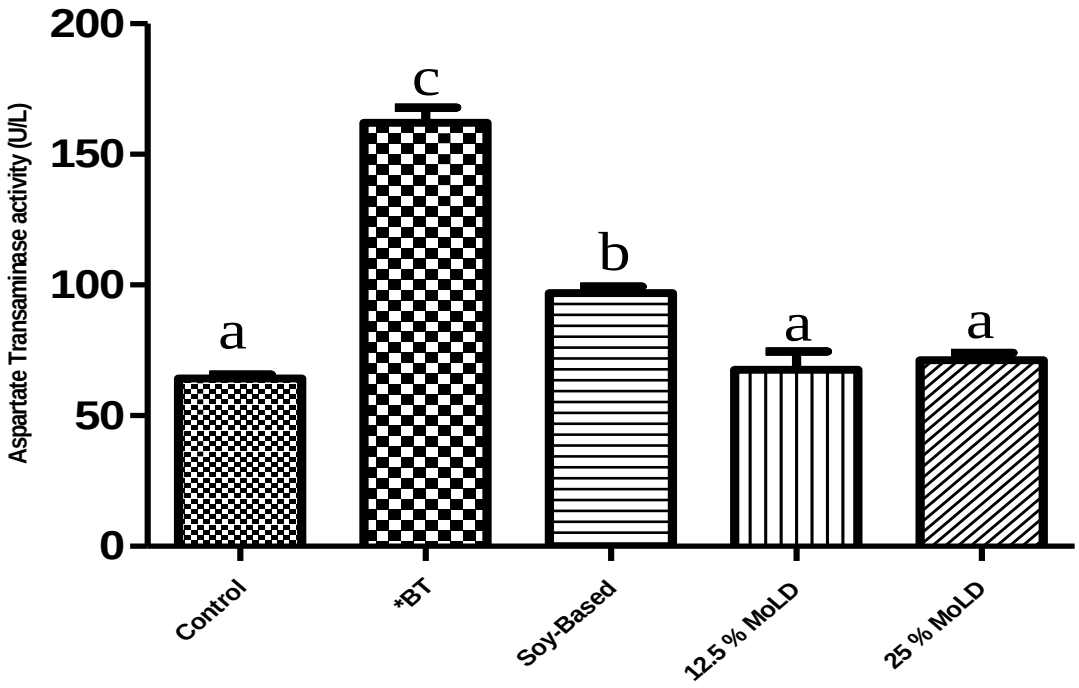


Figure 20: Activity of Serum AST of Malnourished Rats Fed the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

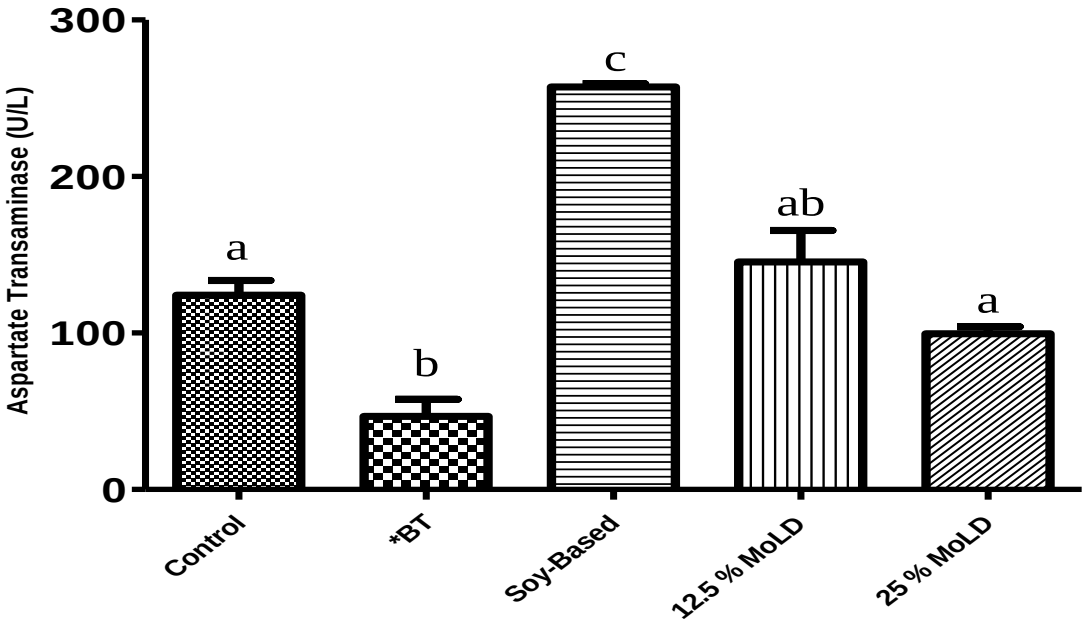


Figure 21: Activity of Skeletal Muscle AST of Malnourished Rats Fed the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

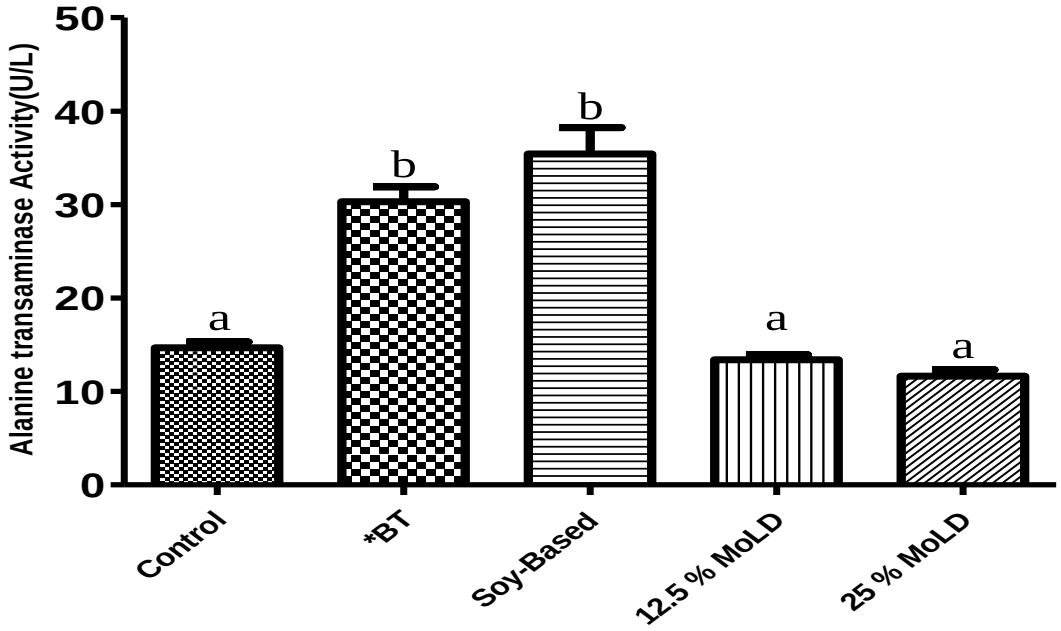


Figure 22: Activity of Serum Alanine Transaminase (ALT) of Malnourished Rats Fed with the Formulated Diets Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

4.4.3 Effect of Formulated Feeds on Calcium ATPase Activity in the Cytosolic and Mitochondrial Skeletal Muscle

The activity of calcium ATPase was viewed as function of its V_{max} and Km. Table 14 shows the effect of formulated feeds on the activity of the skeletal muscle Ca^{2+} ATPase. There was a reduction in both the cytosolic and mitochondrial Ca^{2+} ATPase of the untreated animals while the treated groups had significant ($p < 0.05$) increase in all treatment groups. The highest increase in activity was seen in the groups treated with feeds supplemented with methyl jasmonate, vitamine E and carnitine (i.e soy-based group). While there was no significant ($p < 0.05$) difference in the activity of groups treated with *M. oleifera* leaf-based diet and the control. A reduced activity was observed in the groups treated with 12.5% *M. oleifera* leaf-based diet though significantly ($p < 0.05$) higher than the untreated goup.

4.4.4 Effects of Formulated Diet on Oxidative Stress Markers

4.4.4.1 Effect of Formulated Feeds on Superoxide Dismutase

The activity of superoxide dismutase in the skeletal muscle of malnourished rats fed with the treatment feeds and formulations is shown in Figure 23. Malnourished untreated rats showed significant ($p < 0.05$) decrease in superoxide dismutase activity when compared with activity of the control and other treated groups. Meanwhile, the enzyme activity in the skeletal muscle of the animals fed with supplemented soy-based was significantly higher than the control and those fed with both 25% and 12.5% *M. oleifera* leaf-based diet.

4.4.4.2 Effect of Treatment Feeds on Catalase Activity

The activity of catalase enzyme in the skeletal muscle of the malnourished animals fed with formulated diets is shown in Figure 24. There was no significant difference in the activity of catalase in the skeletal muscle of the untreated and treated animals.

Table 14: V_{max} of Calcium Adenosine Triphosphatase in the Skeletal Muscle of Rats Treated with Formulated Diets for 28 Days

	Vmax (µM/mg protein/Hr) (Cytosolic)	Vmax (µM/mg protein/Hr) (Mitochondria)
Control	0.274 ± 0.62^{a}	0.633 ± 0.07^{a}
Soy based diet	0.248 ± 0.23^{b}	0.680 ± 0.05^{b}
25% MoLD	0.234 ± 0.10^{c}	0.639 ± 0.07^{a}
12.5% MoLD	0.194 ± 0.09^{d}	0.269 ± 0.05^{c}
Before treatment	0.101 ± 0.13^{e}	0.111 ± 0.09^{e}

Values are expressed as mean of three replicates ± SEM; data with different supercripts along the coloumn are significantly different ($p < 0.05$)

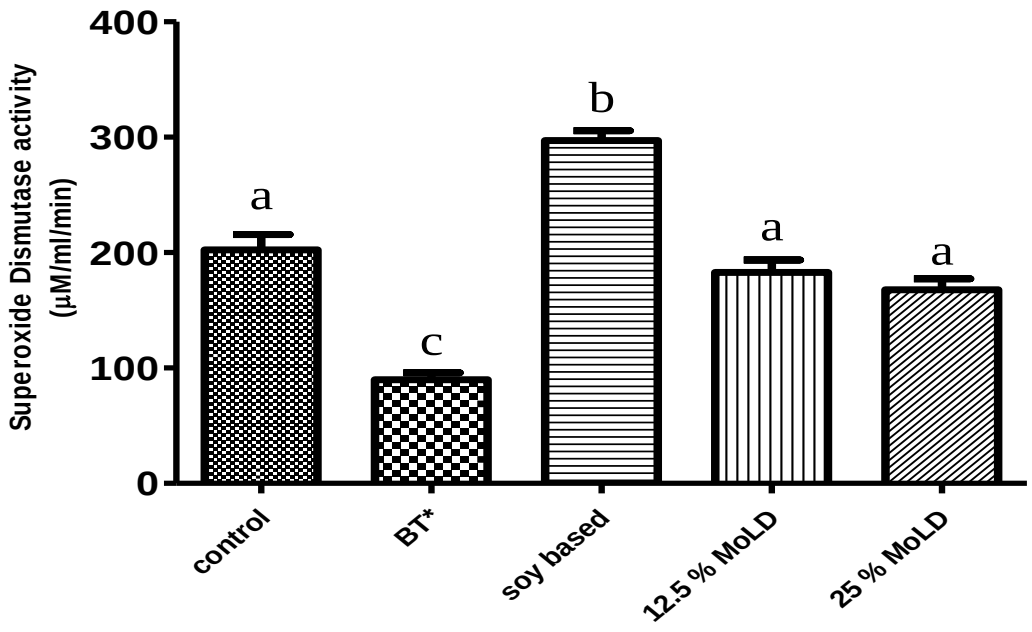


Figure 23: Activity of Superoxide Dismutase Skeletal Muscle of Malnourished Rats Treated with the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

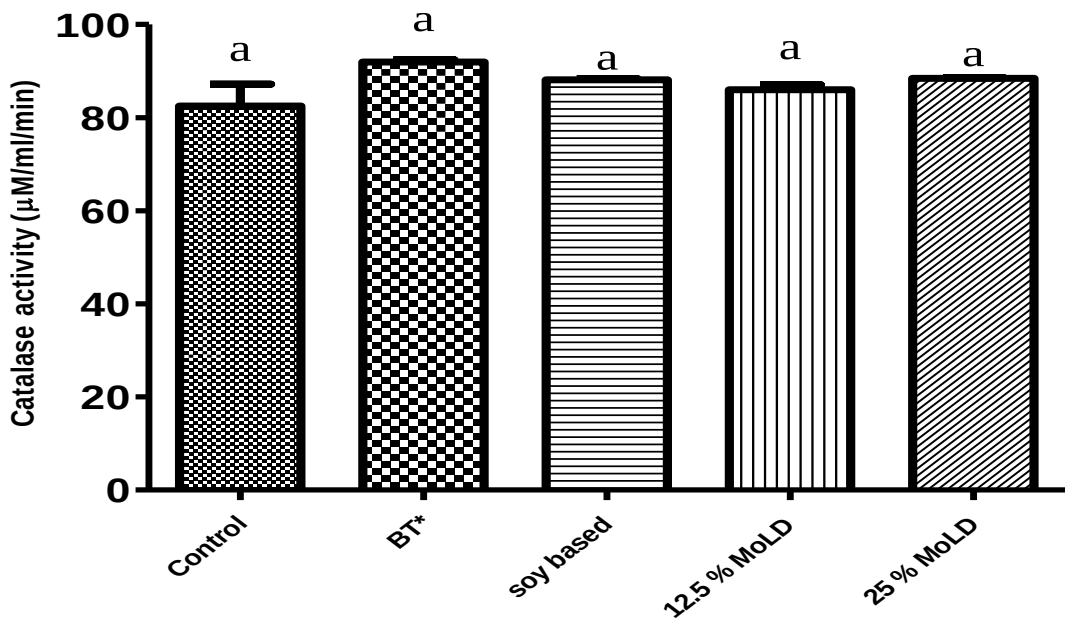


Figure 24: Activity of Catalase in the Skeletal Muscle of Malnourished Rats Fed with the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

4.4.4.3 Effect of Treatment Feeds on Glutathione –S- Transferase Activity

Figure 25 shows the activity of gluthathione-s-transferase in the skeletal muscle of malnourished rats fed with the treatment feeds. There was a significant ($p < 0.05$) reduction in the activity of the enzyme in the untreated animals, likewise those treated with soy-based supplemented diet, and 12.5% MoLD. However, the emzyme activity increased in the control and 25% MoLD.

4.4.4.4 Effect of Treatment Feeds on Reduced Glutathione Concentration

The concentration of reduced glutathione in the skeletal muscle of malnourished animals fed with the formulated diets are as shown in Figure 26. The concentration of reduced glutathione was significantly lower in the skeletal muscle of the untreated animal as well as the animals fed 12.5% MoLD. Reduced glutathione concentration was significantly increased in the animals treated with 25% *M. oleifera* leaf-based diet, supplemented soy-based diet and the control.

4.4.4.5 Effect of Formulted Feeds on Malonyldialdehyde Concentration

Figure 27 shows the concentration of malonyldialdehyde (MDA) in the skeletal muscle of malnourished animals treated with the formulated diets and supplements. The concentration of MDA in the skeletal muscle of untreated animals was significantly higher than those of the control and other treatment groups. Meanwhile, the concentration in the 25% soy-based suplimented diets, 12.5% and 25% MoLD were not significantly different from each other.

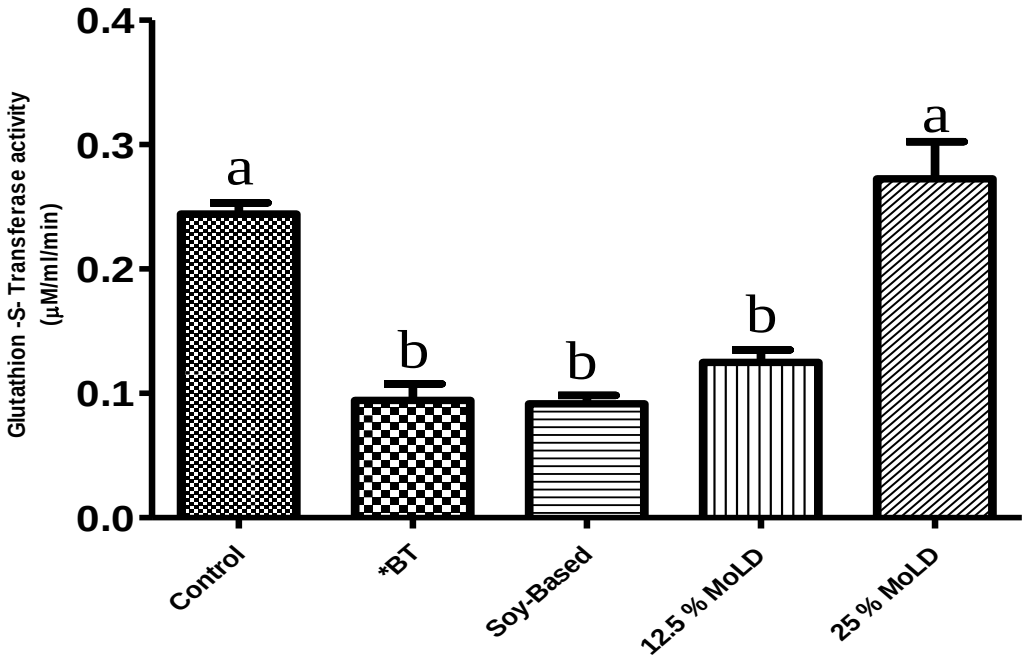


Figure 25: Activity of Glutathion –S- Transferase in the Skeletal Muscle of Malnourished Rats Fed with Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

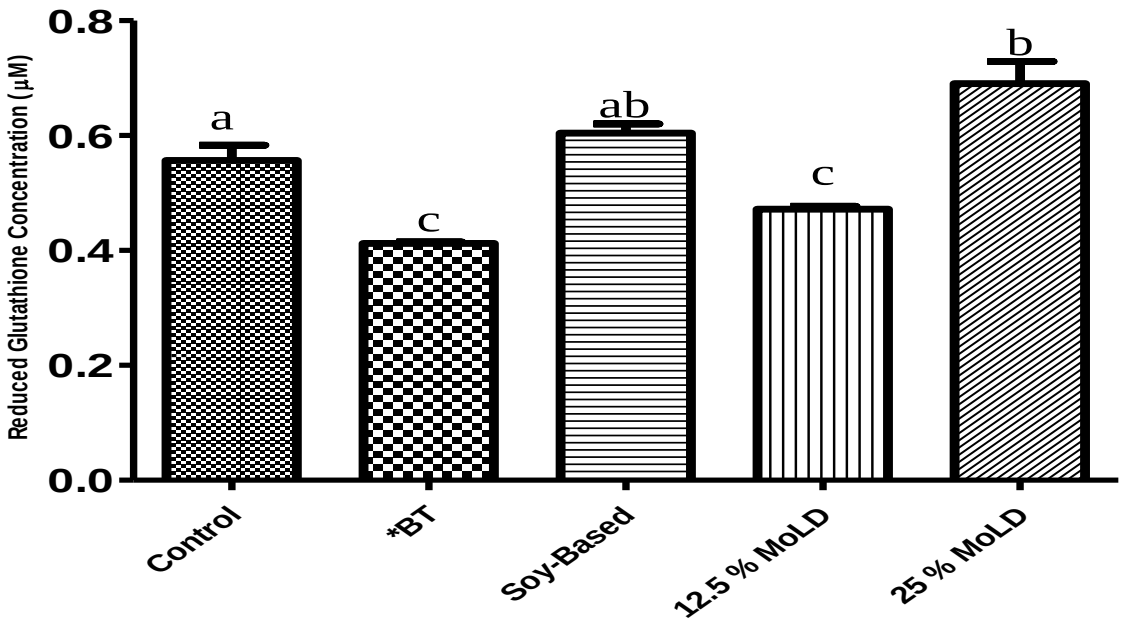


Figure 26: Reduced Glutathione Concentration in the Skeletal Muscle of Malnourished Rats Treated with the Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

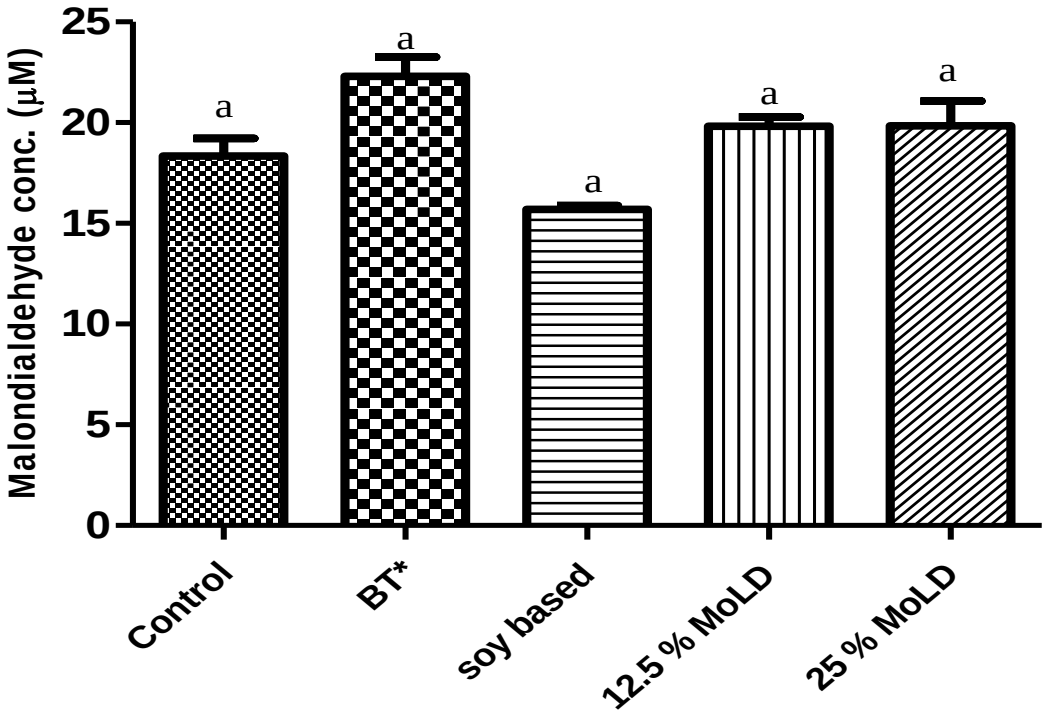


Figure 27: Malondialdehyde Concentration in the Skeletal Muscle of Malnourished Rats Treated with Formulated Diets for 28 Days

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

4.4.5 Evaluation of the Functionality and Permeability of the Mitochondrial Membrane of the Skeletal Muscle (As a Marker of Energy Depletion)

4.4.5.1 Effect of Formulated Feeds on Mitochondrial Swelling

In both the liver and skeletal muscle, the level of mitochondrial swelling was significantly higher in the liver and skeletal muscle of the untreated animals while those of all the treatment groups and the control were not significantly different from each other (Figures 28 and 29).

4.4.5.2 Effect of Treatment Feeds on Mitochondrial Membrane Potential

Figures 30 and 31 show the skeletal muscle and liver mitochondrial membrane potentials of malnourished animals fed with the formulated feeds. There was no significant difference in the mitochondrial membrane potentials of the skeletal muscle of all the treatment groups and the control. However, the membrane potential in the liver of the animals fed with 12.5% *M. oleifera* leaf-based diet was significantly higher than the control and other treatment groups.

4.4.5.3 Effect of Formulated Feeds on Mitochondrial Reactive Oxygen Species (ROS)

Figure 32 shows the level of ROS in the skeletal muscle mitochondria of malnourished rats fed with the formulated feeds and supplements. The level of ROS generated in the skeletal muscle of animals treated with 12.5% *M. oleifera* leaf-based diet was significantly higher than those of the control and other treatment groups while that of the control and other treatment groups were not significantly different from each other. However, in the liver, ROS generation was only significantly higher in the soy- based diet treated animals (Figure 33).

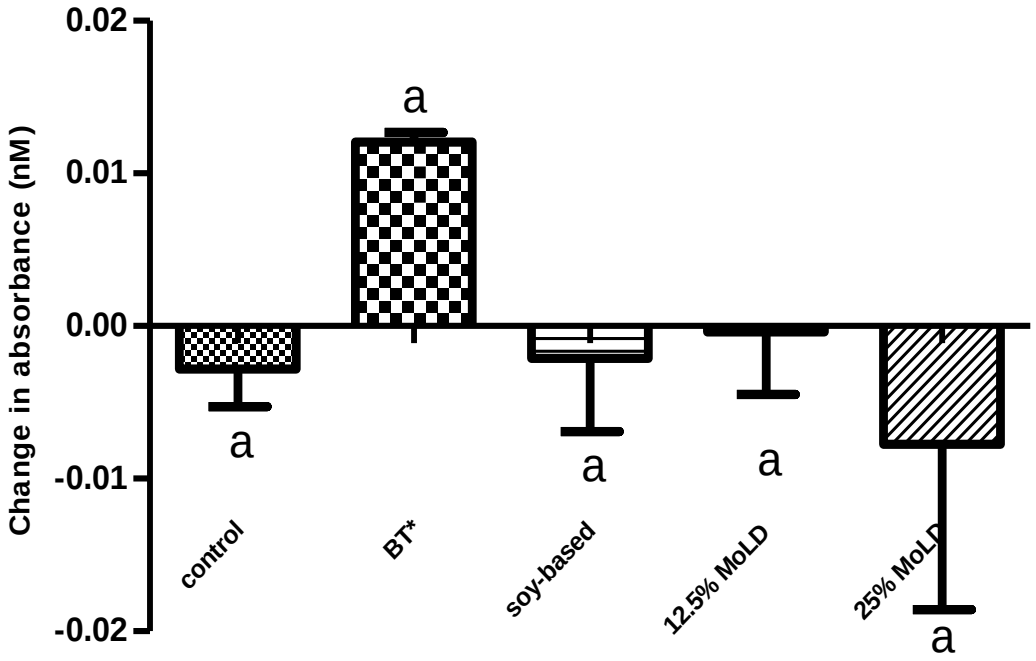


Figure 28: Mitochondrial Membrane Swelling in the Liver of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

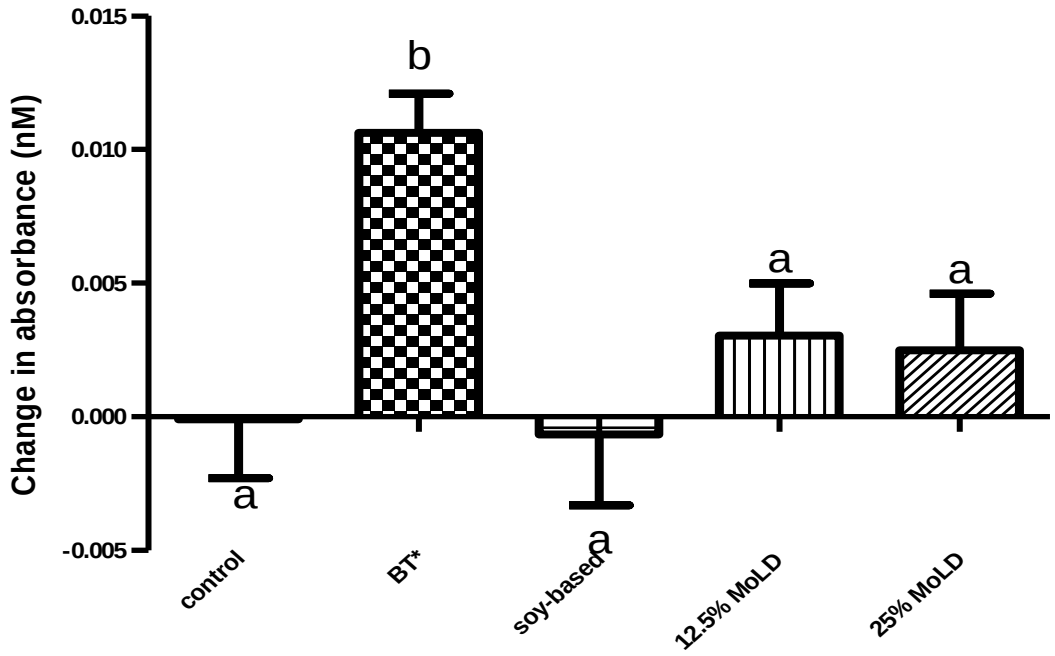


Figure 29: Mitochondrial Membrane Swelling in the Skeletal Muscle of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

*BT = before treatment.

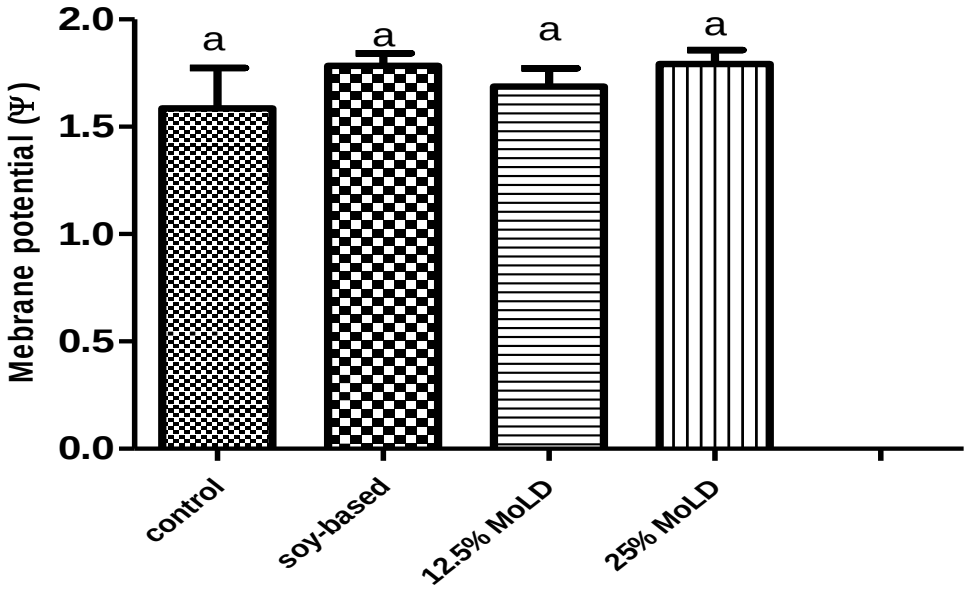


Figure 30: Mitochondrial Membrane Potential in the Skeletal Muscsle of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

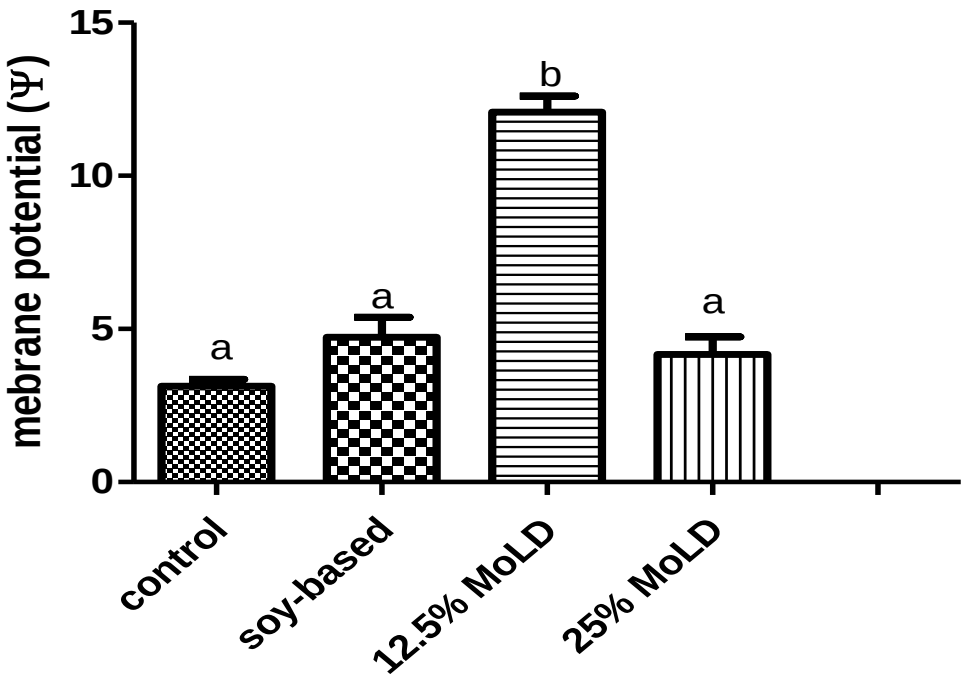


Figure 31: Mitochondrial Membrane Potential in the Liver of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

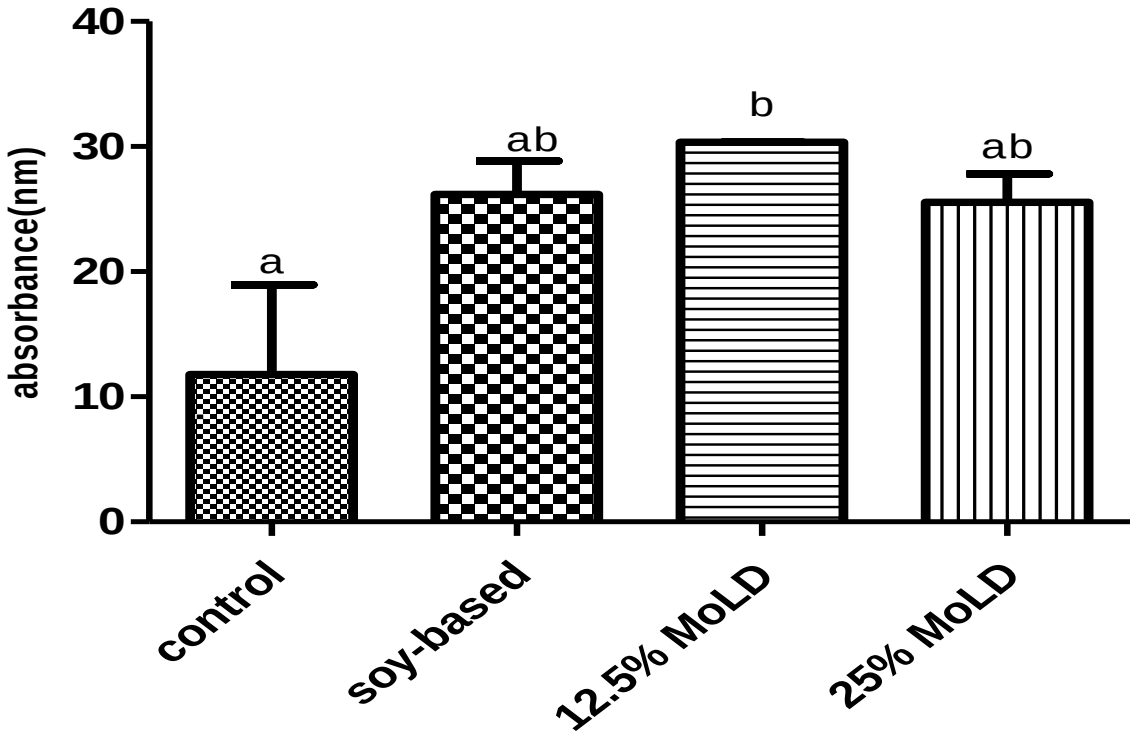


Figure 32: Mitochondrial ROS in the Skeletal Muscle of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) from each other

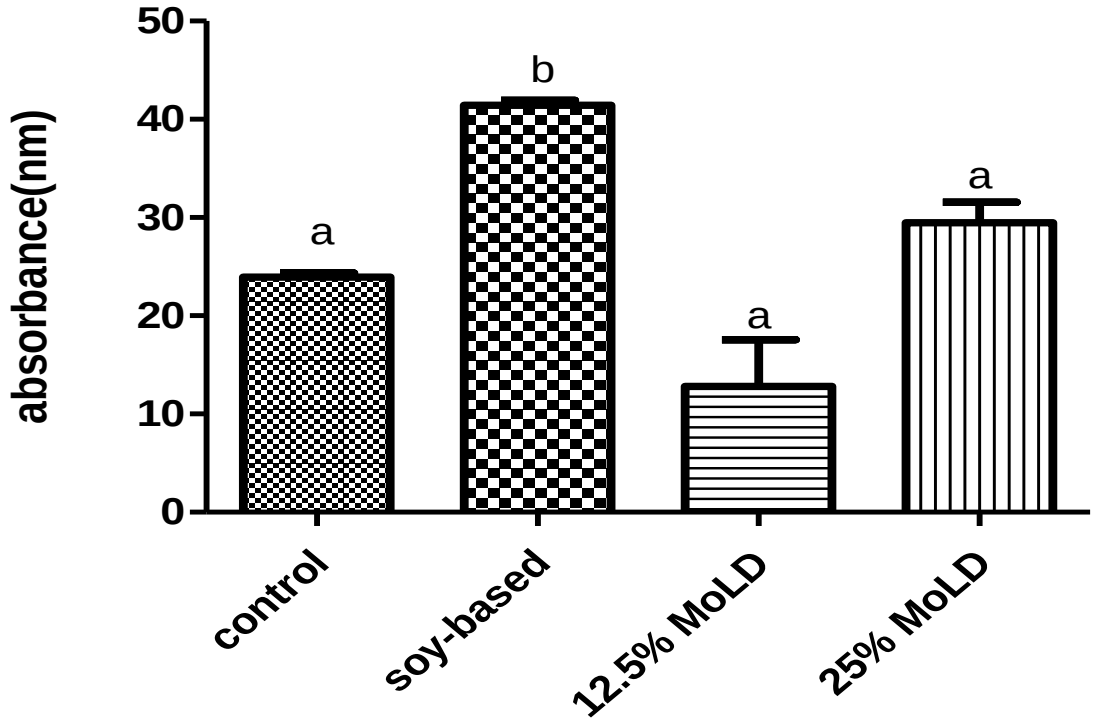


Figure 33: Mitochondrial ROS in the Liver of Rats Fed with Formulated Diets

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control are significantly different ($p < 0.05$) significantly different from each other

4.5 Toxicological Studies

4.5.1 Effect of Formulated Feeds on Liver Function Indices

Table 15 presents the activities of indices of liver function.All the treatment groups showed no significant difference in the activity of aspartate amino transferase (AST) when compared with the control. For the serum alkaline phosphatase (ALP), there was no significant difference in the activity of the animals treated with 25% MoLD and the control. There was however a significant increase in the activity of the enzyme in the animals treated with 25% soy-based diet and 12.5% MoLD when compared with the control and with the 25% MoLD fed rats.

Moreover, there was no significant difference in the concentration of serum albumin and bilirubin in all the treatment groups and the control.

4.5.2 Effect of Treatment Feeds on Kidney Function Indices

The results of three parameters of kidney function are as represented in Table 16. Urea concentration in the group's feded with soy-based diet was significantly higher than the control and other treatment groups while that of the animals treated with 25% and 12% MoLD were not significantly different from the control. There was no significant difference in the concentration of Na^+, K^+ and Ca^{2+} in the serum and muscle of the control and all the treatment groups.

Table 15: Effect of Formulated Diets on Liver Function Indices of the Experimental Rats

	ALT(U/L)	AST (U/L)	ALP (U/L)	Serum albumin (g/l)	Bilirubin (mg/dl)
Control	29.31 ± 3.96[a]	4.747 ± 0.86[a]	51.28 ± 4.40[a]	3.60 ± 0.88[a]	3.00 ± 5.10[a]
Soy based	40.59 ± 3.14[b]	11.57 ± 0.89[b]	76.07 ± 5.66[b]	3.48 ± 0.11[a]	2.55 ± 3.42[b]
25% MoLD	22.41 ± 1.22[a]	3.03 ± 0.18[a]	43.64 ± 5.47[a]	3.63 ± 0.09[a]	1.85 ± 2.08[b]
12.5% MoLD	41.02 ± 3.68[ab]	4.81 ± 2.1[a]	101. 30 ± 2.51[c]	3.47 ± 0.11[a]	2.98 ± 4.07[bc]

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control down the column are significantly different ($p < 0.05$) from each other

*BT = before treatment.

Table 16: Effect of Formulated Diets on Kidney Function Indices of the Experimental Rats

	Urea (mg/dl)	Creatinine (µmol/L)	Na^+(mmol/L)	K^+(mg/dl)	Ca^{2+}(mmol/L)
Control	24.45 ± 0.086^a	1.45 ± 0.003^a	185.90 ± 2.55^a	6.32 ± 0.11^a	10.12 ± 0.07^a
Soy based	38.24 ± 2.827^b	33.760 ± 0.145^b	182.70 ± 4.67^a	5.83 ± 1.40^a	9.619 ± 0.35^a
25% MoLD	23.79 ± 1.282^{ab}	1.012 ± 0.145^a	183.10 ± 2.09^a	5.63 ± 0.28^a	13.05 ± 1.42^{ab}
12.5% MoLD	20.34 ± 3.929^a	1.350 ± 0.915^a	180.10 ± 3.41^a	4.95 ± 0.68^a	10.22 ± 0.36^a

Values are expressed as mean ± SEM of three replicates. Bars with superscripts different from the control down the column are significantly different ($p < 0.05$) from each other

*BT = before treatment.

CHAPTER FIVE

5.0 DISCUSSION

The use of plants as food and therapeutic agents dates back to the time immemorial, starting with the instinctive use by animals (Stojanoski, 1999). *M. oleifera* being a medicinal food plant has been reportedly used in literature and from ethno botanical survey for different purpose. In the preliminary phase of this study, 25% inclusion (protein replacement) of *M. oleifera* leaf in diet (MoLD) (i.e 25% *M. oleifera* leaf-based diet) produced a therapeutic feed whose proximate analysis (Table 8) showed no significant difference with the control diet (produced with 25% soy protein (standard). These similarities could be linked to the fact that the percentage crude protein of the dried leaf was high, comparable to results of previous studies reported in literature (Teixeir *et al.,* 2014; Fuglie and Sreeja, 2016). The high percentage crude protein likewise the carbohydrate and energy value of the Moringa leaf (11.36%) recorded in this study is comparable to the results of other studies like that of Teklit (2015) and Aja *et al.* (2013). Isitua *et al.* (2015) even reported a higher protein content of *M. Oleifera* leaf of 24.1% and carbohydrate of 55.97% similar to what is presented in this report. Hence supporting the conclusions of Bamishaiye *et al.* (2011) that *M. oleifera* leaf can be a potential source of carbohydrate and protein and thus the recommendation of its use as a food supplement.

The fact that the percentage protein of *M. oleifera* leaf could be as high as that of many known protein, could have been responsible for the similarities observed in the standard protein feeds (prepared with soy) and those replaced by *M. oleifera* leaf.However, low protein iso-caloric diet prepared from both *M. oleifera* leaf and soy bean both produced similar negative effects on the animals' anthropometric parameter (Figures 8 & 9),

heamatology (Table 10 & 11), serum protein as well as calcium ATPase activity (Figures 9-12).

The morphological changes such as loss of weight (Figure 8) observed in the test animals may be as a result of malnutrition. These changes are often reversed by improved nutrition and thus the increased weight observed after maintaining with treatment feeds (Figure 9 & 14). Weight loss is an important prognostic factor in determining the overall survival of some physiological conditions like cancer (Shamberger. 1984). Cancer cachexia is associated with depletion of both adipose tissue and skeletal muscle mass which may be due to increased muscle catabolism or reduced protein synthesis or a combination of both. The worst prognostic impact is felt by the loss of visceral protein and lean body mass depletion rather than adipose depletion and this is assessed by serum albumin concentration and creatinine-height index) (Nixon *et al*., 1980)

The result of this study indicated that all the adverse morphological changes observed in the test animals were improved with the intake of the formulated (improved) diets (Figure 8, 9 &14) though with different capacities. In the first phase, animals fed only 40% soy- based diet had the lowest morphological improvement when compared with those fed vitamin E supplemented diet and those fed *M. oleifera*- based diet (Figures 8 & 9). Protein energy malnutrition (PEM) causes mirads of morphological changes which includes; muscle wasting especially in the thigh and buttocks (Grover and Looi, 2009), alopecia (loss of fur), oedema, anaemia, infections (Oyegbemi *et al*., 2008) and the subject becomes apathetic and lethargic (Grover and Looi, 2009) among other symptoms. The leaves of *M. oleifera* is a source of both macro- and micronutrients such as β-carotene, protein, vitamin C, calcium, and potassium (Siddhuraju and Becker, 2003) hence their use as an antioxidant (Verma, 2009). Higher growth pattern (Figure 9) as well as haematological parameters (Tables 10 & 11)

observed in animals fed vitamin E supplemented died (Group 3) and those fed 25%MoLD (Group 4) may have been because of the antioxidant properties they have in common, added to other nutritional qualities in *M. oliefera* leaf, thus the highest improvement was observed in *M. oleifera*- based diet fed animals which could be a result of other Phytochemical constituents (Table 9).

The reduction in the serum albumin concentration (Table 12) during malnutrition could also be a sign of low protein. Bolarinwa *et al*. (1991) reported a significant reduction in the levels of total protein in protein-calorie malnourished rats. A similar correlation was drawn between total protein levels and severity of protein energy malnutrition (Laditan, 1976). Serum albumin level is an indicator of liver function status, hence reduction in its concentration following the malnutrition suggests liver damage (Grant and Kuachmar, 1987). Impaired intestinal absorption of protein may provide the liver with inadequate supply of amino acids to synthesize serum proteins (such as albumin), leading to a drop in serum protein level. Also, liver damage may impair the synthesis of serum proteins in the liver thereby leading to low serum levels (Grant and Kuachmar, 1987).

The results of the preliminary phase indicated no need for multiple use of feed for induction of muscle degeneration by malnutrition, and hence only the feed formulated with 4%soy bean was used to induce muscle degeneration in the phases two and three of this research. Hence, markers of muscle degenerations were generally assessed to confirm induction. Moreover, supplementing the low protein isocaloric diet with vitamin E did not prevent the factors affected by malnutrition and atrophy.

Effects of formulated diets on markers of muscle degeneration (Phase two studies)

The significantly high activity of CK in the serum of malnourished animals (Figure 17) with a correspondingly low concentration in the skeletal muscle (Figure 16), as well as high

concentration of LDH in both serum and muscle (Figure 18 & 19) is a positive indicator of muscle degeneration in the malnourished animals. Damage to the skeletal muscle has been reported to be manifested by weakness, and an increase in the circulation of muscle proteins such as creatine kinase (CK), lactate dehydrogenase (LDH), and myoglobin (Mb) (Clarkson, and Hubal, 2002; Proske and Morgan, 2001).

Subnormal activity of creatine kinase (CK) in serum has also been observed in a variety of clinical conditions which may be as a result of diminished efflux of the muscle enzyme into serum from reduced physical activity caused by illness or advanced age or may result from reduced muscle mass accompanying muscle wasting or cachectic states (Barnert, 1985). Delanghe *et al*. (1986) also found low serum CK activity in intensive care patients (the majority of whom had severe infections or septicaemia) and the activity was reversed with clinical improvement of the patients. The diagnostic role of CK has been almost replaced by the muscle protein troponin. However, high levels of serum CK are still closely associated with cell damage, muscle cell disruption, or disease. These cellular disturbances can cause CK to leak from cells into blood serum (Totsuka *et al*., 2002).

LDH activity is usually expressed in tissues undergoing lipid peroxidation. The activities of CK and LDH are usually clinically supported by AST, ALT and sometimes ALP activity in the serum. The high activities of these enzymes in the serum with corresponding decrease in skeletal muscle of malnourished animals in addition to the CK activity and LDH concentration confirm the induction of muscle degeneration before treatment feeds were administered. These effects were subsequently corrected by the administration of formulated (treatment) feeds. Higher activities of AST, ALT and ALP observed in the animals fed with soy-based diets supplemented with methyl jasmonate, vitamin E and carnitine were indicative of possible damage and hence the need for the toxicological study.

The mechanism of muscle damage was postulated to involve malfunctioning of calcium ion homeostasis system which results in calcium overload and then subsequently, oxidative stress among other factors in the mitochondria leading to energy depletion (Bababunmi, 2002). Hence, the assay of calcium ATPase, oxidative stress markers and analysis of mitochondrial functionalities.

In the preliminary study as well as the 2nd phase of this study, Vitamin E supplemented dietalso resulted into a much significant increase in activity of Ca^{2+} ATPase (Figure 10 &11 and Table 14) (which could mechanistically lead to reduction of oxidative stress similar to those of *M. oleifera* leaf-based diet (Figure 13). This might imply some relationship between the two feeds probably because of some uniformity in their antioxidant properties. A pioneering study has demonstrated association between an immobilized rodent skeletal muscle with increased level of oxidative stress, which could partially be arrested by vitamin E supplementation (Agten *et al*., 2011; Min *et al*., 2011). Meanwhile, calcium ATPase activity and oxidative stress are both key factors affected by muscle atrophy (Mc Ardle and Jacson, 1994).

Kinetic data combined with detailed information about an enzyme's structure and its catalytic mechanisms, provide some of the most powerful clues to the enzyme's biological function(s) and may suggest ways to modify it for therapeutic purpose (Voet *et al*., 1999). In the preliminary study, Ca^{2+} ATPase activities in the skeletal muscle of the group fed an un-supplemented soy-based diet (i.e 25% soy based diet alone) (Figure 11 & 12) had only slight increase in Ca^{2+} ATPase activity, giving an insight that only improved diet with no added values cannot fully reverse the factors affected by atrophy. The higher Ca^{2+} ATPase activity in animals fed *M. oleifera* leaf-based diet and those supplemented with vitamin E might be as a result of their antioxidant properties. Similar trend of enzyme activity was observed in the

2nd phase of the study (Table 14). However, the highest activity observed in the soy-based supplemented diet might be a resultant effect of methyl jasmonate on Ca^{2+} ATPase which is a positive modulator of the enzyme (Bewaji and Bababunmi, 2008). Increased activity observed in *M. oleifera* leaf- based diet fed animals could also be traced to their phytonutrients components and hence the analysis of phytochemicals reported in Table 9.

The significant reduction in the activity of the enzyme observed in animals fed a reduced percentage *M. oleifera* inclusion (i.e 12 % *M. oleifera*) and the un-treated animals might be as a result of a reduction in the synthesis or inactivation of the enzyme during the malnourished condition. Since the main function of the Ca^{2+} ATPase is to pump out Ca^{2+} from the cell thereby keeping the concentration of Ca^{2+} low (Carafoli, 1991), impaired or reduced activity of the enzyme will result in an unhealthy high concentration of Ca^{2+} within the cell which might finally result in cell death. Subsequent significant increase in the activity of Ca^{2+} ATPase across some of the groups after treatment could be as a result of activation or increase synthesis of the enzyme during treatment and however implies that the effect of malnourishment on Ca^{2+} ATPase is reversible by replenishment of feed and especially the *M. oleifera* leaf-based diet and/or supplemented diets.

The enzymatic markers; superoxide dismutase (Figure 23), catalase (Figure 24) and glutathione –S- transferase (Figure 25) of oxidative stress showed increased activities in the treatment groups with a conversely significantly lower activity in the untreated group. High concentration of reduced glutathione (Figure 26) which is a molecule of the antioxidant system in the group fed with *M. oleifera* leaf-base diet (especially 25% inclusion), corresponding with a reduce concentration of MDA (Figure 27) signals an increase in the oxidant system of the treated animals *vis-a-vis* oxidative stress. Long-term elevation of ROS

activity can act via redox signaling mechanisms to alter muscle gene expression, causing protein loss that diminishes muscle mass (atrophy) (Reid, 2005).

With the mitochondria being the central point of energy metabolism, its functionality and permeability evaluation (the cellular power house which depicts the energy generation of the cell) reflected the extent of energy depletion,. The treatment diets, 25% *M. oleifera* leaf-based diets gave normal level of ROS in the skeletal muscle mitochondrial (Figure 32) and low ROS concentration in the liver mitochondria (Figure 33) while the swelling of the mitochondrial membrane was highly reversed both in the skeletal muscle (Figure 28) and in the liver (Figure 29) mitochondrial by the treatment feeds most especially by 25% *M. oleifera* leaf-based diet. These two factors together with the mitochondrial membrane potentials which were on a normal level in the muscle (Figure 30) and in the liver (Figure 31) except in the animals treated with 12% *M. oleifera* leaf-based diet reflect good functionality in the mitochondrial membrane which may imply a reversal of energy depletion which may have been caused by muscle degeneration.

Phytochemical study

It is generally believed that crude extract from medicinal plants are more biologically active than isolated compounds because of their synergistic effects (Jana and Shekhawat, 2010) and hence the use of whole *M. oleifera* leaf in the formulation of feed (Table 8). Moreover, from ethno botanical survey, it is most commonly used as food in the whole leaf were it is used to make vegetable soups, puddings, used to garnish food like rice, salads and porridges, as tea or even eaten raw. Phytochemical screening of the leaf (Table 9) revealed that it contained high concentration of flavonoid, terpenoids, taninis, total phenol, alkaloids and saponin is similar to the work of Cowan (1999) which revealed the presence of alkaloids, tannins, flavonoids, steroids, glycosides and saponins. The presence of flavonoid and phenol in high

concentration (Table 9) further shows it antioxidant potentials which are very important in disease control. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds (Lin *et al.,* 1998) and thus among the greatest source of natural antioxidants (Wanasundara and Shahidi, 1994).

Ascorbic acid, alkaloids and other phenolic compounds found in plants are important antioxidants. They act as electron donors for important enzymes in human (Akinmoladum *et al.,* 2007). They help in stabilizing plasma components and has been shown to be an effective scavenger of superoxide radical anion (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($O^{\cdot}$) and reactive nitrogen oxide (NO) (Okwu and Ighodaro, 2010).

The presence of a trace amount of alkaloid in the leaf of *M. oleifera* can also be an added factor responsible for its medicinal values as alkaloids have commonly been found to possess antimicrobial properties due to their ability to intercalate with the DNA of microorganisms (Kasalo *et al*., 2010) as well as a potent antioxidant property. The major active nutraceutical ingredients in plants are polyphenols (e.g flavonoids) and alkaloids (Pu *et al.*, 2013). As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Tapas *et al*., 2008).

Similar to the work of Makkar and Becker (1996), there was a negligible amount of saponin and a similar quantitative amount of phytate in leaf of *M. oleifera* extract (Table 11). This is also similar to what is obtainable in soybean meal (Makkar and Becker, 1996). Saponin lowers body cholesterol by making them unavailable for absorption or by causing increased feacal excretion of bile acid, by so doing, indirectly eliminating cholesterol (Oakenful and Sidhu, 1989; Sidhu and Oakenfull, 1986). The presence of saponin in *M. oleifera* leaf can be

linked to the ability of the leaf-based diet to increase Ca^{2+} ATPase activity as the presence of membrane cholesterol has been reported to increase conformational thermostability of integral proteins like the Ca^{2+} ATPase in a natural membrane (Ortega *et al.,* 1996). Cholesterol has also been shown to modulate Ca^{2+}ATPase from sarcoplasmic reticulum (Chang *et al*., 1998). A study also revealed that dietary sources of saponins offer preferential chemopreventive properties in lowering the risk of human cancers as it can inhibit or kill cancer cells without affecting the normal cells (Rao, 1996).

Terpenoid was also present in the leaf of *M. oleifera* analyzed in this study (Table 9). Terpenoids are essential phyto-constituent in human diet. They are also synthesized through the mevalonate / HMG-CoA reductase pathway. Vitamin A is an example of terpene also produced from consumed carotenoids which are important in energy generation; hence the reinstatement of the mitochondrial functions (*vis a vis* energy depletion). During the metabolism of terpenes, they may be converted to cholesterol. The availability of cholesterol may however increase thermostability of the Ca^{2+}ATPase (Ortega *et al*., 1996), hence the boost in activity of the enzyme observed in Figure 10, Figure 13 and Table 14. Similar to the suggestive conclusion of the report of Lambe and Bewaji (2017), MoLD would be able to elicite all the observed effects in the treated animals because of the presence of these wide range of phytochemicals and their properties.

Toxicological study

Toxicity evaluation of the formulated diets through the assement of the liver and kidney function indices revealed that *M. oleifera* leaf-based diet is non toxic at either level of inclusion (25% and 12%) although 25% MOLD was more effective. This report is in agreement with the report of (Aminu *et al*., 2011) who also reported *M. oleifera* leaf to be non- toxic.The activities of ALP, AST, ALT estimated in the serum was to assess

hepatocellular toxicity (liver injury). Serum activities of AST, ALT and ALP (Table 15) showed no significant differences with the control in the animals fed 25% *M. oleifera* leaf-based diet. Being the major enzymes used in liver function test, the reduced activity of the enzymes in the serum shows that consumption of the leaf-based diet poss no toxicity to the liver. This is in conformity with reports from some other research like Afzal *et al.* (2014).

The insignificant levels of serum urea and creatinine concentration with the control (Table 16) might be as a result of a normal protein metabolism which produces its metabolites in the normal range. In the same track, other kidney function indices (Table 16) also showed that *M. oleifera* leaf-based diet has no toxic effects of the kidney functionality at 25% and 12.5% diet inclusion. Kidney function indices such as serum urea, creatinine, and electrolyte can be used to evaluate the functionality of the nephron. An elevated value indicates defective functional state (Yakubu *et al.,* 2003). The insignificant difference in the serum and tissue electrolyte between the treatment groups and the control shows a proper electrolyte handling of the kidney, hence a normal functional kidney, and thus no toxicity.

Summary of findings

The findings of this research are summarized as follows:

ix. *M. oleifera* leaf contained terpenoids (2.91 mg/g), tannins (9.2 mg/g), flavonoids (12.5 mg/g), total phenols (42.34 mg/g), alkaloids (30.86 µg/g) and saponins (55.22 µg/g);

x. The crude protein (13.77%) and carbohydrate (62.89%) contents of 25% MoLD were not significantly different ($p < 0.05$) from the control feed;

xi. Activities of creatinine kinase, lactate dehydrogenase, aspartate aminotransferase and alanine transaminase in the muscle and serum of the animals fed 25% MoLD were not significantly ($p < 0.05$) different from the control;

xii. There was significant ($p < 0.05$) increase in the cytosolic and mitochondrial Ca^{2+}-ATPase activities in the skeletal muscle of rat fed 25% MoLD;

xiii. 25% MoLD significantly ($p < 0.05$) increased the activities of superoxide dismutase, glutathione-S-transferase and reduced glutathione concentration. It also reduced malondialdehyde concentration and had no significant effect on catalase activity;

xiv. Mitochondrial membrane swelling and reactive oxygen species were significantly reduced in the animals fed 25% MoLD, whereas the membrane potentials were significantly increased; and

xv. Serum aspartate aminotransferase, alanine transaminase and alkaline phosphatase activities of 25% MoLD fed animals, as well as serum albumin, urea and creatinine concentrations were not significantly different from controls in the toxicological study.

CONCLUSION

In conclusion, the results of this study revealed that 25% *M. oleifera* leaf diet inclusion can prevent muscle degeneration by positively affecting the three postulated factors (Ca^{2+} homeostasis, oxidative stress and energy depletion).This may be attributed to its phytochemical constituents *vis-a-vis* its antioxidant properties, effects on Ca^{2+} ATPase, mitochondrial membrane permeability and functionality. These properties qualify *M. oleifera* leaves as a nutraceutical and an important food plant in combating muscle degenerative diseases, particularly those related to nutrition.

RECOMMENDATIONS

Based to the findings from this research, the following recommendations were made

1. *M. oleifera* can serve as a nutraceutical in combating muscle degenerative diseases
2. There is need for local awarenessprograms aimed at promoting the consumption of *M. oleifera* leaf-based diet
3. Generally, *M. oleifera* leaf can be incorporated into food diets packaged as aid for people in areas of low food supplies or those suffering from malnutrition like refugees, internally displaced persons or those affected by famine or war.

REFERENCES

Adebayo, A. O., Enyedi, A., Verma, A. K., Filoteo, A. G. and Penniston, J. T. (1995). Two residues that may ligate Ca^{2+} in transmembrane domain six of the plasma membrane Ca^{2+}-ATPase. *Journal of Biological Chemistry*; 270, 27812-27816

Adhihetty, P. J., Ljubicic, V., Menzies, K. J. and Hood, D. A. (2005). Differential susceptibility of subsarcolemmal and intermyofibrillar mitochondria to apoptotic stimuli. *American Journal of Physiology. Cell Physiology*; 289:C994–C1001.

Afzal, S., Fouzia, Y., Smita, A., Mashuir, R., Khairul, I., Ekhtear, H., Shakhawoat, H., Md Rezaul, K., Farjana, N., Zahagir, A. S. and Khaled, H. (2014). Protective effects of Moringa oleifera Lam. leaves against arsenic-induced toxicity in mice. *Asian Pacific Journal of Tropical Biomedicine*; 4(Suppl 1): S353–S358.

Agten, A., Maes, K., Smuder, A., Powers, S. K., Decramer, M. and Gayan-Ramirez, G. (2011). N-Acetylcysteine protectsthe rat diaphragm from the decreased contractility associated with controlled mechanicalventilation. *Critical Care Medicine*; 39:777–782.

Aja, P. M., Ibiam, U. A., Uraku, A. J., Orji, O.U., Offor, C. E. and Nwali, B. U. (2013). Comparative Proximate and Mineral Composition of Moringa oleifera Leaf and Seed. *Global Advanced Research Journal of Agricultural Science* (ISSN: 2315-5094) Vol. 2(5) pp. 137-141.

Aja, P. M., Nwachukwu, N., Ibiam, U. A., Igwenyi, I. O., Offor, C. E. and Orji, U. O. (2014). Chemical Constituents of *Moringa oleifera* Leaves and Seeds from Abakaliki, Nigeria. *American Journal of Phytomedicine and Clinical Therapeutics.* [2][3]310-321

Akinmoladum, A. C., Ibukun, E.O., Akinsinlola, B. L., Onibon, T. R., Rinboboye, A. O., Obuofor, E. M. and Farombi, E. O. (2007). Chemical Constituents and Antioxidant Activity of Alstonia boonei. *African Journal of Biotechnology*, 6 (10): 1197-1201.

Amaglo N. K., Bennett R. N., Lo Curto R. B., Rosa E. A. S., Lo Turco V., Giuffrid A., Lo Curto A., Crea F. and Timpo G. M. (2010). Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. *Food Chemistry*.122, 1047–1.

American Society of Parenteral and Enteral Nutrition (2002). The Clinical Guidelines Task Force, Guidelines for the use ofparenteral and enteral nutrition in adult and pediatric patients. *Journal of Parenter Enteral Nutrition*; 26:S1.

Ames, B. N.; Shinenaga, M. K. and Hagen, T. M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences.* U.S.A. 90, 7915 – 7922.

Aminu, A. A., Ezzedin M. A., Umar, A. K., Muhammad, I. S., Umar, U. P. and Najume D. G. I. (2011). Toxicity Evaluation of Moringa Oleifera Leaves. *International Journal of Pharmaceutical Research and Innovation*, Vol. 4: 22-24.

Amthor, H., Otto, A., Vulin, A., Rochat, A., Dumonceaux, J., Garcia, L., Mouisel, E., Hourdé, C., Macharia, R., Friedrichs, M., *et al*. (2009). Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity. *Proceedings of the National Academy of Sciences*. USA 106, 7479–7484.

Anger, M., Samuel, J. L., Marotte, F., Wuytack, F., Rappaport, L., and Lompre, A.M. (1994). In situ mRNA distribution of sarco (endo) plasmic reticulum Ca2+-ATPase isoforms during ontogeny in the rat. *Journal of Molecular Cell Cardiology*; 26:539–550.

Anthonia, O. O. (2012). Evaluation of Antimicrobial properties and nutritional potentials of Moringa oleifera Lam.leaf in South Western Nigeria. *Malaysian Journal of Microbiology;* 8(2). 59-67.

Anu, E. J., Shyamjith, M. and Shankar, B. K. (2012). Acute effect of Ethanolic Extract of Moringa oleifera on Haloperidol Induced Catalepsy in Mice Models. *Drug Intervention Today;* 4(10). 543-545.

Anwar, F., Latif, S., Ashraf, M. and Gilani, A. H. (2007). Moringa oleifera: a food plant with multiple medicinal uses. *Phytotherapy Research*, vol. 21, no. 1, pp 17–25.

AOAC, (1990). Official Methods of the Association of Official Analytical Chemists. 15th Ed. Association of Official Analytical Chemists, Arlington, VA. USA. pp: 777.

AOAC, (2005). Official Methods of Analysis. 17th Ed. *Association of Analytical Chemists*, Gaithersburg, MD.

Apaijai, N., Pintana, H. and Chattipakan, S. (2013). Effects of vildagliptin versus sitagliptin, on cardiac function, heart rate variability and mitochondrial function in obese insulin-resistant rats. *British Journal of Phamarcology*; 169(5): 1048–1057.

Armstrong, R. B. (1996). Properties, distribution, and functions of mammalian skeletal muscle fibers. In: Exercise Bioenergetics and Gas Exchange. Edited by Cerretelli P and Whipp B. J. Amsterdam: *Elsevier/North-Holland Biomedical Press*, p. 137–46.

Arnett, M. G. Hyslop, R., Dennehy, R. H. and Schneider, C. M. (2000). Age-related variations of serum CK and CK MB response in females. *Canadian Journal of Applied Physiology*, vol. 25, no. 6, pp. 419–429.

Aurélie, G., Lucie, D., Robin, L., Edward, E. and Farmer (2010) Jasmonate Biochemical Pathway: Structures of Jasmonate and its Precursors Gene Expression Laboratory, Plant Molecular Biology, Faculty of Biology and Medicine, University of Lausanne, 1015 Lausanne, Switzerland. *Science Signal.*, Vol. **3**, Issue 109, p. cm3

Bababunmi, E. A. (2002). Formulation and method for treating skeletal muscle degeneration caused by malnutrition and disease. *United States Patent* 6887499

Bababunmi, E. A. & Bewaji, C.O. (2002). Oxidative stress and antioxidant therapy in wasting conditions associated with malnutrition, HIV/AIDS, tuberculosis and malaria. *Biokemistri*, vol. 12. No 3

Bababunmi, E. A. (2002). Formulation and methods for treating skeletal muscle degeneration caused by malnutrition and disease. *United States Patent No.* US 006465021B2.

Bababunmi, E. A.; Olorunsogo, O. O. and Bewaji, C. O. (1994). Pathological and chemical effectors of the erythrocyte calcium pumping protein: A review. *Tropical Journal of Health Science.* 1, 33 - 47.

Baehr, L. M., Furlow, J. D. and Bodine, S. C. (2011). Muscle sparing in muscle RING finger 1 null mice: response to synthetic glucocorticoids. *Journal of Physiology*. 589, 4759–4776.

Bamishaiye, E. I. Olayemi, F. F. Awagu, E. F. and Bamshaiye. O. M. (2011). Proximate and Phytochemical Composition of Moringa oleifera Leaves at Three Stages of Maturation. *Advance Journal of Food Science and Technology* 3(4): 233-237.

Bannai, S. (1984). Induction of cystine and glutamate transport activity in human fibroblasts by diethyl maleate and other electrophilic agents. *The Journal of Biological Chemistry*. 259 (4): 2435–40.

Bannai, S. and Tateishi, N. (1986). Role of membrane transport in metabolism and function of glutathione in mammals. *The Journal of Membrane Biology*. 89 (1): 1–8.

Barnert, J. and Behr, W. (1985). Acute viral hepatitis and low values for serum creatine kinase [Letter]. *Clinical Chemistry*; 31:1909-1910.

Baro, M. and Deubel, T. F. (2006). Persistent Hunger: Perspectives on Vulnerability, Famine, and Food Security in Sub-Saharan Africa. *Annual Review of Anthropology* 35: 521.

Basara, S. M. A., Iftikhar, M. N. and Afzal, I. (2011). Potential of Moringa (Moringa oleifere) Leaf Extract as Priming Agent for hybrid Maize Seeds. *International Journal of Agriculture and Biology* ISSN 1814-9596.

Beers, R. F. And Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry;* 195(1):133-40

Bewaji, C. O. (1993). Studies on Vanaium Interactions with the Membrane-bound Calcium-pumping ATPase from Porcine Erythrocytes. *Nigerian Journal of Biochemistry*, volume 8, pp. 18-22.

Bewaji, C. O. (1996a). Comparison of the effects of thapsigargin, 2, 5-di-(tert-butyl)-1, 4-hydroquinone and cyclopiazonic acid on calcium release from rat liver endoplasmic reticulum. *Nigerian Journal of Biochemistry and Molecular Biology;* 11, 27 – 30.

Bewaji, C. O. and Bababunmi, E. A. (2008). Jasmonate: Regulation of Ca2+- ATPase and role in calcium homeostasis. *International Journal of Biomedicine and Health Science*; Volume 4, Issue 4

Bewaji, C. O. and Dawson, A. P. (1995). Thapsigargin protects human erythrocyte Ca2+-ATPase from proteolysis. *Cell Calcium* 17, 14 - 20.

Bhattacharya, A., Agrawal, D., Sahu, P. K., Kumar, S., Mishra, S. S. and Patnaik, S. (2014). Analgesic effect of ethanolic leaf extract of Moringa oleifera on albino mice. *Indian Journal of Pain;* 28:89-94.

Bhattacharya, A., Lustgarten, M., Shi, Y., Liu, Y., Jang, Y. C., Pulliam, D., Jernigan, A. L. and Van Remmen, H. (2011). Increased mitochondrial matrix-directed superoxide production by fatty acid hydroperoxides inskeletal muscle mitochondria. *Free Radical Biology and Medicine;* 50:592–601.

Bhattacharya, M. R., Naik, D., Pratap, K. S., Sanjay, K. and Sudhanshu, S. M. (2014). CNS Depressant and Muscle Relaxant effect of Ethanolic Leaf Extract of Moringa Oleifera on Albino Rats. *International Journal of PharmTech Research* CODEN (USA): IJPRIF ISSN: 0974-4304 Vol.6, No.5, pp 1441-1449.

Bhutta, Z. A.; Ahmed, T.; Black, R. E.; Cousens, S.; Dewey, K.; Giugliani, E.; Haider, B. A.; Kirkwood, B. Et Al (2008). What Works? Interventions For Maternal and Child Undernutrition and Survival. *The Lancet* 371 (9610): 417.

Bjelakovic, G., Nikolova, D., Gluud, L. L. and Simonetti, R. G. (2008). Antioxidant supplements for prevention of mortality in healthy participants with various diseases. *Cochrane database of systematic review* (2); Ncd007176.

Blaauw, B., Canato, M., Agatea, L., Toniolo, L., Mammucari, C., Masiero, E., Abraham, R., Sandri, M., Schiaffino, S. and Reggiani, C. (2009). Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. *FASEB Journal*. 23, 3896–3905.

Bobe, R., Bredoux, R., Wuytack, F., Quarck, R., Kovacs, T., Papp, B., Corvazier, E., Magnier, C. andEnouf, J. (1994). The Rat Platelet 97-Kda Ca^{2+}Atpase Isoform Is the sarcoendoplasmic reticulum Ca^{2+}ATPase 3 protein. *Journal of Biological Chemistry*; 269:1417–1424.

Bodine, S. C. (2013). Disuse- induced Muscle Wasting. *International journal of Biochemistry and cell Biology,* 23(10): 2200-8

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K., *et al*. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294, 1704–1708.

Bolarinwa, A. F., Ajayi, F. F., Alak, O. O. and Akande, O.O. (1991). Effect of malnutrition on basal and induced gastric acid secretion. *Nigerian Journal of. Physiological Sciences*; 5: 144-148.

Bonaldo, P. and Sandri, M. (2013). Cellular and molecular mechanisms of muscle atrophy. *Disease Model and Mechanism:* vol. 6 no. 1 25-39

Bonaldo, P. and Sandri, M. (2013). Cellular and molecular mechanisms of muscle atrophy. *Department of Biomedical Sciences,* University of Padova, 35131 Padova, Italy.

Bonora, M., Morganti, C., Morciano, G., Giorgi, C., Wieckowski, M. & Pinton, P. (2016). Comprehensive analysis of mitochondrial permeability transition pore activity in living cells using fluorescence-imaging-based techniques. *Nature Protocols*. Volume: 11, Pages: 1067–1080. Susan C.Kandarian

Booth, F. W. (1982). Effect of limb immobilization on skeletal muscle. *Journal of Applied Physiology*; 52: 1113–1118.

Boron, W. F., and Boulpaep, E. L. (2009). Medical Physiology: A Cellular and Molecular Approach. 2nd Edn. Philadelphia, PA: Saunders *Elsevier*

Brancaccio, P., Lippi, G. And Maffulli, N. (2010). Biochemical markers of muscular damage. *Clinical chemistry Lab Medicine,* 48(6):757-67.

Brandt, P., Neve, R. L., Kammesheidt, A., Rhoads, R. E. And Vanaman, T. C. (1992). Analysis of the tissue-specific distribution of mRNAs encoding the plasma membrane calcium-pumping ATPases and characterization of an alternately spliced form of PMCA4 at the cDNA and genomic levels. *Journal of Biological Chemistry;* 267, 4367-4385

Brewster, L. M., Mairuhu, G., Sturk, A., van Montfrans, G. A. (2007) Distribution of creatine kinase in the general population: implications for statin therapy. *Aericanm Heart Journal*; 154:655.

Brigelius-Flohé, R. and Traber, M. G. (1999). Vitamin E: function and metabolism. *FASEB Journal*; 13 (10): 1145–1155.

Brini, M., Call, T., Ottolini, D. and Carafoli, E. (2013). Intracellular Calcium Homeostasis and Signaling. In Banci, Lucia (Ed.). *Metallomics and the Cell. Metal Ions in Life Sciences. 12. Springer.*

Brodin, P., R. Falchetto, T. Vorherr& E. Carafoli. (1992). Identification of two domains which mediate the binding of activating phospholipids to the plasma-membrane Ca^{2+} pump. *European Journal of Biochemistry*; 204, 939-946.

Brookes, P. S. and Darley-Usmar, V. M. (2004). Role of calcium and superoxidedismutase in sensitizing mitochondria to peroxynitrite-induced perme-ability transition. *American Journal of Physiology, Heart Circulation Physiology:* 286: H39–H46.

Brookes, P. S., Yoon, Y., Robotham, J. A., Anders, M.W. and Sheu, S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *The American journal of Physiology*. Volume 287 no 4.

Brustovetsky, N., Brustovetsky, T., Purl, K. J., Capano, M., Crompton, M. and Dubinsky, J. M. (2003). Increased susceptibility of striatal mitochondria to calcium-induced permeability transition. *The Journal of Neuroscience: the official journal of the Society for Neuroscience* 23 (12): 4858–4867.

Buck, M. and Chojkier, M. (1996). Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *EMBO Journal*; 15:1753-1765.

Büki, A., Okonkwo, D. O., Wang, K. K. and Povlishock, J. T. (2000). Cytochrome c release and caspase activation in traumatic axonal injury. *The Journal of Neuroscience: the official journal of the Society for Neuroscience* 20 (8): 2825–2834.

Bulaj, G., Kortemme, T. and Goldenberg, D. P. 1998. Ionization: Reactivity relationships for cysteine thiols in polypeptides. *Biochemistry*, 37: 8965–8972

Byers, T. (1993). Vitamin E supplements and coronary heart disease. *Nutrition Review*. 51, 333 – 345.

Cable, M. B. and Briggs, F. N. (1988). Allosteric regulation of cardiac sarcoplasmic reticulum Ca-ATPase: A comparative study. *Molecular and Cell Biochemistry*. 82, 29-36

Carafoli, E. (1987). Intracellular calcium homeostasis. *Annual Review of Biochemistry*; 56, 395-433

Carafoli, E. (1991). Calcium pump of the plasma membrane. *Physiological Reviews*; 71 (1): 129 –153.

Carafoli, E. (1994). Biogenesis: Plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB Journal* 8, 993-1002.

Carafoli, E. and Stauffer, T. (1994). The plasma membrane calcium pump: functional domains, regulation of the activity, and tissue specificity of isoform expression. *Journal of Neurobiology;* 25, 312-324

Carreira, R. S., Lee, Y., Ghochani, M., Gustafsson, A. B. and Gottlieb, R. A. (2010). Cyclophilin D is required for mitochondrial removal by autophagy in cardiac cells. *Autophagy*. 6:462–472.

Cavalier-Smith, T. (1978). Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. *Journal of Cell Science;* 34:247–278.

Champeil, P., le Maire, M., Andersen, J. P., Guillain, F., Gingold, M., Lund, S. and Møller, J. V. (1986). Kinetic characterization of the normal and detergent-perturbed reaction cycles of the sarcoplasmic reticulum calcium pump. Rate-limiting step(s) under different conditions. *Journal of Biological Chemistry*; 261, 16372-16384.

Chance, B., Sies, H. and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiology Review*; 59:527–605.

Chang, C. C., Yang, M. H., Wen, H. M. and Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*; 10: 178-182.

Chinda, K., Sanit, J., Chattipakorn, S. and Chattipakorn, N. (2014). Dipeptidyl peptidase-4 inhibitor reduces infarct size and preserves cardiac function via mitochondrial protection in ischaemia-reperfusion rat heart. *Diabetics and Vascular Diseases Research*. 11:75–8.

Christopher, D., John, B. W., and Allan, W. (2008). Nutrition in pediatrics; basic science, clinical application. *Hamilton*: BC Deccker. Pp. 127-141.

Chuang, P. H., Lee, C. W., Chou, J. Y., Murugan, M., Sheih, B. J. and Chen, H. M. (2007). Antifungal activity of crude extract and essential oil of Moringa oleifera lam. *Bioresource Technology*; 98:232-236.

Clapham, D. E. (2007). Calcium Signaling. *Cell*. 131 (6): 1047–1058.

Clarkson, P. M., and Hubal, M. J. (2002). Exercise-induced muscle damage in humans. *American Journal of Physiology and Medical Rehabilitation.* 81:S52–S69,

Claudio, C. (2002) Composition for the Prevention and Treatment of Osteoporosis Due To Menopause Syndrome, *US Patent* 6,335,038, Column 3.

Cong, H., Sun, L., Liu, C. and Tien, P. (2011). Inhibition of atrogin-1/MAFbx expression by adenovirus-delivered small hairpin RNAs attenuates muscle atrophy in fasting mice. *Human Gene Therapy*. 22, 313–324.

Couto, N., Malys, N., Gaskell, S. J. and Barber, J. (2013). Partition and turnover of glutathione reductase from Saccharomyces cerevisiae: a proteomic approach. *Journal of Proteome Research*. 12 (6): 2885–94.

Cowan, M. M., (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review;* 12: 564-582.

Creelman, R. A. and Mullet, J. E. (1997). Annual Review of Plant Physiology. *Plant Mololecular Biology*: 48:355–381,

Croft, M., Bickster, A., Manson, J. and Burnette (2012). Vacuum Sealing vs Refrigeration: Which is the most effective way to store seeds? *Echo Asia notes*. A Regional Supplement to Echo Development notes.

Csibi, A., Cornille, K., Leibovitch, M. P., Poupon, A., Tintignac, L. A., Sanchez, A. M. and Leibovitch, S. A. (2010). The translation regulatory subunit eIF3f controls the kinase-dependent mTOR signaling required for muscle differentiation and hypertrophy in mouse. *PLoS ONE* 5, e8994.

Dalle-Donne, A., Scaloni, D., Giustarini, E., Cavarra, G., Tell, G., Lungarella, R., Colombo, R. and Rossi, A. M. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrometry Reviews*, 24, pp. 55-99

Dalziel, J. M. (1956). Useful Plants of West Tropical Africa. *Crown Agents of Oversea Government and Administration,* London. Page 23.

Dangi, S., Jolly, C. and Narayanan, S. (2002). Antihypertensive activity of total alkaloids from the leaves of Moringa oleifera. *Pharmaceutical Biology*; 40:144-150.

David R. T. (2007). Loss of skeletal muscle mass in aging: Examining the relationship of starvation, sarcopenia and cachexia. Division of Geriatric Medicine, Saint Louis University Medical Center, 1402 South Grand Blvd. M238, Saint Louis, MO 63104, USA.

Davis, T. M., Pongponratan, E., Supanaranond, W, Pukrittayakamee, S., Helliwell, T., Holloway, P. and White, N. J. (1999). Skeletal muscle involvement in falciparum malaria: Biochemical and ultrastructural study. *Clinical Infectious Diseases*. 29(4), 831 – 835.

Davis, T. M., Supanaranond, W., Pukrittayakamee, S., Holloway, P., Chubb, P. and White, N. J. (2000). Progression of skeletal muscle damage during treatment of severe falciparum malaria. *Acta Tropical*. 76(3), 271 – 276.

DeFelice, S. L. (1992). Nutraceuticals: Opportunities in an Emerging Market. *Scrip Magazine*; 9:14–5.

Delanghe, J, de Buyzere, M, de Scheerder, I, Van den Abeele, A. M., Vandenbogaerde, J. and Wieme R. (1986). Significance of low creatine kinase in intensive care patients [Letter]. *Clinical Chemistry*; 32:713-714.

Demaurex, N and Nunes, P. (2016). The role of STIM and ORAI proteins in phagocytic immune cells. *American Journal of Physiology-cell Physiology*. 310 (7); C496- C508.

Demole, E., Lederer, E. and Mercier, D. (1962). Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant caractéristique de l'essence de jasmin. *Helvetica Chimica Acta*; 45: 675–85.

Deval, C., Mordier, S., Obled, C., Bechet, D., Combaret, L., Attaix, D. and Ferrara, M. (2001). Identification of cathepsin L as a differentially expressed message associated with skeletal muscle wasting. *Biochemistry Journal*; 360, 143–150.

Dillard, C.J. and German, J. B. (2000). Phytochemicals: Nutraceuticals and human health. *Journal of the Science of Food and Agriculture*; 80:1744–56.

Ding, W.X. and Yin, X. M. (2012). Mitophagy: mechanisms, pathophysiological roles and analysis. *Biological Chemistry*; 393:547–564.

Dipolo, R and Beaugé, L (2006). Sodium/Calcium Exchanger: Influence of Metabolic Regulation on Ion Carrier Interactions. *Physiological Reviews* 86 (1): 155–203.

Dixon, W., and Massey, F., Jr. (1969). Introduction to Statistical Analysis. New York: McGraw-Hill, 638pp.

Doczi, J., Turiák, L., Vajda, S., Mándi, M., Töröcsik, B., Gerencser, A. A., Kiss, G., Konràd, C., Adam-Vizi, V. andChinopoulos, C. (2010). Complex contribution of cyclophilin

D to Ca^{2+}- induced permeability transition in brain mitochondria, with relation to the bioenergetic state. *Journal of Biological Chemistry* 286 (8): 6345–6353.

Dodd, S. L., Gagnon, B. J., Senf, S. M., Hain, B. A. and Judge, A. R. (2010). Ros-mediated activation of NF-kappaB andFoxo during muscle disuse. *Muscle & Nerve*.; 41:110–113.

Doumas, B. T., Watson, W. A. and Biggs, H. G. (1971). Albumin Standards and the Measurement of Serum Albumin with Bromocresol Green. *Clinica Chimica Acta.*, 31: 87.

Drury, R. A. and Wallington, E. A. C. (1980). Coreleton's Histological technique 4th edition. Oxford, Oxford University Press.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics:* 82: 70-77

Elwess, N. L., Filoteo, A. G., Penniston, E. A. and Penniston, J. T. (1997). Plasma membrane Ca^{2+} pump isoforms 2a and 2b are unusually responsive to calmodulin and Ca^{2+}. *Journal of Biological Chemistry*; 272, 17981-17986

Enyedi, A., Verma, A. K., Heim, R., Adamo, H. P., Filoteo, A. G., Strehler, E. E. and Penniston, J. T. (1994). The Ca^{2+} affinity of the plasma membrane Ca^{2+} pump is controlled by alternative splicing. *Journal of Biological Chemistry*; 269, 41-43.

Evans, H. M. and Bishop, K. S. (1922). On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science*; 56 (1458): 650–651.

Eventoff, W., Rossmann, M. G., Taylor, S. S., Torff, H. J., Meyer, H., Keil, W. and Kiltz, H. H. (Jul 1977). Structural adaptations of lactate dehydrogenase isozymes. *Proceedings of the National Academy of Sciences of the United States of America.* 74 (7): 2677–81.)

Fahey, J. W. (2005). Moringa oleifera: A Review of the medical evidence for its Therapeutic, and prophylactic properties. Part 1. *Trees for life journal*. 1: 5.

Farmer, E. E. and Ryan, C. A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proceeding of the National Academy Sciences of the United States;* A87 (19): 7713–7716.

Filoteo, A. G., Enyedi, A. and Penniston, J. T. (1992). The lipidbinding peptide from the plasma membrane Ca2+ pump binds calmodulin and the primary calmodulin-binding domain interacts with lipid. *Journal of Biological Chemistry;* 267, 11800-11805

Food and Agricultural Organization of the United Nations (2015). The state of food insecurity in the world.

Franco, J. R. and Lansman, J. B. (1990). Calcium entry through stretch-inactivated ion channels in mdx myotubes. *Nature*; vol. 344, no. 6267, pp. 670–673.

Frost, R. A. and Lang, C.H. (2005). Skeletal muscle cytokines: regulation by pathogen-associated molecules and catabolic hormones. *Current Opinion in Clinical Nutrition Metabolic Care;* 8(3):255–63.

Fuglie, L. J. (2001). The Miracle Tree: The Miracle Tree: *The Multiple Attributes of Moringa*, 172

Fuglie, L. J. and Sreeja, K. V. (2016). Cultivation of moringa available at http://moringafarms.com/161/cultivation of moringa.

Furuno, K., Goodman, M. N. and Goldberg, A. L. (1990). Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. *Journal of Biological Chemistry;* 265, 8550–8557.

Fuster, G., Busquets, S., Almendro, V., López-Soriano, F. J., and Argilés, J. M. (2007). Antiproteolytic effects of plasma from hibernating bears: a new approach for muscle wasting therapy? *Clinical Nutrition* 26 (5) 658-61.

Garcia de la Asuncion, J., del Olmo, M. L., Sastre, J.,Millan, A.,Pellin, A.,Pallardo, F. V. and Vina, J. (1998). AZT treatment induces molecular and ultrastructural oxidative damage to muscle mitochondria: Prevention by antioxidant vitamins. *Journal of Clinical. Investigation.* 102, 4 – 9.

García-Ruiz, C., Colell, A., París, R. and Fernández-Checa, J. C. (2000). Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. *FASEB Journal* 14 (7): 847–858.

Gaze, D. C. (2007). The role of existing and novel cardiac biomarkers for cardioprotection. *Current Opinion in Investigational Drugs.* 8 (9): 711–7.

Ghasi, S., Nwebodo, E. and Ofili, J. O. (2000). Hypocholesterolemic effects of crude extract of leaf of *Moringa oleifera* lamm in high fat diet fed Wister rats. *Journal of Ethnopharmacology*; 69:21-25.

Glavinas, H., Méhn, D., Jani, M. Oosterhuis, B. Herédi-Szabó, K. and Krajesi, P. (2008). Utilization of membrane vesicle preparations to study drug-ABC transporter interactions. *Expert Opinion on Drug Metabolism and toxicology*, 4:721-732.

Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A., Goldberg, A. L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences.* U. S. A.; 98:14440–14445.

Gordon, J. N., Green, S. R. and Goggin, P. M. (2005). Cancer cachexia. *Quarterly Journal of Medicine;* 98(11):779–88.

Gornall, A. G., Bardawill, C. J. and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry*, 177: 751-766.

Grant, G. H. and Kachmar, J. F. (1987). Fundamental of clinical Chemistry, (Tietz, N.W, ed.), 3rd Edition. W.B. Saunders Company, Philadelphia. pp. 298- 320.

Greeb, J. and Shull, G. E. (1989). Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skeletal muscle. *Journal of Biological Chemistry*; 264, 18569- 18576.

Grover, Z. and Looi C. (2009). Protein Energy Malnutrition. *Pediatric Clinics of North America* 56 (5): 1055–1068.

Grumati, P., Coletto, L., Sabatelli, P., Cescon, M., Angelin, A., Bertaggia, E., Blaauw, B., Urciuolo, A., Tiepolo, T., Merlini, L., *et al*. (2010). Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. *Nature Medicine*. 16, 1313–1320.

Grune T, Merker K, Sandig G, Davies K. J. (2003). Selective degradation of oxidatively modified proteinsubstrates by the proteasome. *Biochemical and Biophysical Research Communication;* 305:709–718.

Guerini, D., Foletti, D., Vellani, F. and Carafoli, E. (1996). Mutation of conserved residues in transmembrane domains 4, 6, and 8 causes loss of Ca^{2+} transport by the plasma membrane Ca^{2+} pump. *Biochemistry*; 35, 3290-3296.

Gunst, J. J., Langlois, M. R., Delanghe, J. R., De Buyzere, M. L. and Leroux-roels, G. (1998). Serum creatine kinase activity is not a reliable marker for muscle damage in conditions associated with low extracellular glutathione conditions. *Clinical Chemisry;* 44:5.

Gunter, T. E., Gunter, K. K., Shue, S.S. and Gavin, C. E. (1994). Mitochondrial calcium transport: Physiological and pathological relevance. *The American journal of physiology* 267 (2 pt 1): C13- C339.

Grover, Zubin E. and Looi C. (2009). "Protein Energy Malnutrition". *Pediatric Clinics of North America* 56 (5): 1055–1068.

Habig, W. J., Pabst, M. J. and Jacoby, W. B. (1974). Glutathione S-transferase, the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry:* 249: 7130-7139

Haddad, F., Zaldivar, F. P., Cooper, D. M. and Adams, G. R. (2005). IL-6 induced skeletal muscle atrophy. *Journal of Applied Physiology*; 98(3):911–7.

Hall, Z. W. and Ralston, E. (1989). Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science*, Vol. 244, Issue 4908, pp. 1066-1069.

Halliwell, B. and Gutteridge, J. M. C. (1989). Free radicals in Biology and Medicine. 2nd edn. Clarendon Press, Oxford.

Halprin, K. M., Ohkawara, A. (1967). The measurement of glutathione in human epidermis using glutathione reductase. *The Journal of Investigative Dermatology*. 48 (2): 149–52.

Handy, D. E. and Loscalzo, J. (2012). Redox regulation of mitochondrial function. *Antioxidants Redox Signaling*; 16:1323–1367.

Hasselgren, P. O. and Fischer, J. E (1997). The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. *Annals of Surgery*; 225: 307–316.

Hayyan, M., Hashim, M. A., Al Nashef, I. M. (2016). Superoxide Ion: Generation and Chemical Implications. *Chemical Review*; 116 (5): 3029–3085.

Heled, Y., Bloom, M. S., Wu, T. J., Stephens, Q. and Deuster, P. A. (2007). CM-MM and ACE genotypes and physiological prediction of the creatine kinase response to exercise. *Journal of Applied Physiology,* vol. 103, no. 2, pp. 504–510.

Herrera, E. and Barbas, C. (2001). Vitamin E: action, metabolism and perspectives. *Journal of Physiology and Biochemistry*. 57 (2): 43–56.

Hilfiker, H., Guerini, D. and Carafoli, E. (1994). Cloning and expression of isoform 2 of the human membrane Ca^{2+} ATPase. *Journal of Biological Chemistry*; 269, 26178-26103

Holton, M. L., Wang, W., Emerson, M., Neyses. L. and Armesilla, A. L. (2010). Plasma membrane calcium ATPase proteins as novel regulators of signal transduction pathways. *World Journal of Biological Chemistry;* 1(6): 201-208

Huang, H. and Manton, K. G. (2004). The role of oxidative damage in mitochondria during aging. *Frontiers in Bioscience*; 9:1100-17.

Ichas, F. and Mazat, J. P. (1998). From calcium signaling to cell death: Two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochimica et Biophysica Acta*1366 (1–2): 33–50.

Ingalls, C. P., Warren, G. L. and Armstrong, R. B. (1999). Intracellular Ca^{2+} transients in mouse soleus muscle after hindlimb unloading and reloading. *Journal of Applied Physiology;* 87:386–390.

Institute of Medicine (2000). Vitamin E; Dietary reference intake for Vitamin C, Vitamin E, Selenium and Carotenoids. *The National Academies Press*. Washington, DC: Pp. 186-283

Isitua, C. C., Lozano, M. J. S., Jaramillo, C. J. and Dutan, F. (2015). Phytochemical and nutritional properties of dried leaf powder of Moringa oleifera Lam. from machala el oro province of Ecuador. *Asian Journal of Plant Science and Research:* 2015, 5(2):8-16.

Jagoe, R. T. and Goldberg, A. L. (2001). What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Current Opinion in Clinical Nutrition Metabolic Care;* 4: 183–190.

Jagoe, R. T., Redfern, C. P., Roberts, R. G., Gibson, G. J. and Goodship, T. H. (2002). Skeletal muscle mRNA levels for cathespin B, but not components of the ubiquitin-proteasome pathway, are increased in patients with lung cancer referred for thoracotomy. *Clinical Science*; 102:353–61.

James, C. S., (1995). Analytical chemistry of food. Champmann and Hall, NewYork.

Jana, S. and Shekhawat, G.S. (2010). Phytochemical analysis and antibacterial screening of in vivo and in vitro extracts of Indian medicinal herb: Anethum graveolens. *Reserch Journal of Medicinal Plants:* 4: 206-212.

Javesghani, D., Magder, S. A., Barreiro, E., Quinn, M. T. and Hussain, S. N. (2002). Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. American Journal of Respiration. *Critical Care Medicine*; 165:412–418.

Jensen, T. P., Buckby L. E. and Empson R. M. (2004). Expression of plasma membrane Ca^{2+} ATPase family members and associated synaptic proteins in acute and cultured organotypic hippocampal slices from rat. *Brain Research. Developmental Brain Research*.152 (2): 129–136.

Johnson, D. and Lardy, H. A. (1967). Isolation of liver or kidney mitochondria. *Methods in Enzymology* 10: 94–96

Joumaa, W. H., Bouhlel, A., Meme, W. and Leoty, C. (2002). Methyl jasmonate-induced stimulation of sarcoplasmic reticulum Ca2+-ATPase affects contractile responses in rat slow-twitch skeletal muscle. *J. Pharmacol. Exp. Ther.* 300; 638 – 646.

Kanchan, P. U., Vinod, D. R. and Vijay, B. M. (2012). Antimigraine activity study of Moringa oleifera leaf juice. *International Journal of Green Pharmacy*. 6: 204-207.

Kandarian, S. C. and Stevenson, E. J. (2002). Molecular events in skeletal muscle during disuse atrophy. *Exercise and Sport Sciences Reviews;* 30: 111–116.

Kaplowitz, N. (1981). The importance and regulation of hepatic glutathione. *The Yale Journal of Biology and Medicine.* 54 (6): 497–502.

Karbowski, M. and Neutzner, A. (2012). Neurodegeneration as a consequence of failed mitochondrial maintenance. *Acta Neuropathologica Communitions*; 123:157-71.

Karmen, A. (1955). A note on the spectrometric assay of glutathione-oxalacetic transaminase in human blood streem. *The Journal of Clinical Investigation*. 34 (1): 131–3.

Karmen, A., Wroblewski, F. and Ladue, J. S. (1955). Transaminase activity in human blood. *The Journal of Clinical Investigation.* 34 (1): 126–31.

Kasolo, J. N., Gabriel, S., Bimenya, L.O., Joseph, O. and Ogwal-Okeng, J. W. (2010). Phytochemicals and uses of Moringa oleifera leaves in Ugandan rural communities. *Journal Medicinal Plants Research*; 4(9): 753-757.

Katsir, L., Chung, H. S., Koo, A. J. K., and Howe, G. A. (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. *Current Biology*. 11: 428–435.

Keeton, T. P. and Shull, G. E. (1995). Primary structure of rat plasma membrane Ca^{2+}-ATPase isoform 4 and analysis of alternative splicing patterns at splice site A. *Biochemical Journal*. 306, 779-785.

Keeton, T. P., Burk, S. E. and Shull, G. E. (1993). Alternative splicing of exons encoding the calmodulin-binding domains and C-termini of plasma membrane Ca^{2+}-ATPase isoforms 1,2,3, and 4. *Journal of Biological Chemistry;* 268, 2740-2748.

Kettelhut, I. C., Pepato, M. T., Milgliorini, R. H., Medina, R. and Goldberg, A. L. (1994). Regulation of different proteolytic pathways in skeletal muscle in fasting and diabetes mellitus. *Brazilian Journal of Medical and Biological Research;* 27(4):981-993]

Kim, E., DeMarco, S. J., Marfatia, S. M. Chishti, A. H. M. Sheng, M. and Strehler, E. E. (1998). Plasma membrane Ca^{2+} ATPase isoform 4b binds to membrane-associated guanylate kinase (MAGUK) proteins via their PDZ (PSD-95/Dlg/ZO-1) domains. *Journal of Biological Chemistry* 273, 1591-1595

Klionsky, D. J., Abeliovich, H., Agostinis, P., Agrawal, D. K., Aliev, G., Askew, D. S., Baba, M., Baehrecke, E. H., Bahr, B. A., Ballabio, A., *et al*. (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 4, 151–175.

Klip, A., Reithmeier, R. A. F., and MacLennan, D. H. (1980). Alignment of the Major Tryptic Fragments of the Adenosine Triphosphatase from Sarcoplasmic Reticulum. *Journal of Biological Chemistry*. Volume 255(no. 14), 6562–6568.

Kondo, H., Miura, M. and Itokawa, Y. (1991). Oxidative stress in skeletal muscle atrophied by immobilization. Acta. *Physiologica Scandinavica*. 142: 527–528, 1991.

Kondo, H., Nakagaki, I., Sasaki, S., Hori, S. and Itokawa, Y. (1993). Mechanism of oxidative stress in skeletal muscleatrophied by immobilization. *American Journal of Physiology*; 265:E839–E844.

Kotani, K. and Taniguchi, N. (2011). The association between reactive oxygen metabolites and metabolic syndrome in asymptomatic Japanese men. *Journal of Clinical Medicine Research*; 3(5): 247-51.

Kowdley, K. V., Mason, J. B., Meydani, S. N., Cornwall, S., Grand, R. J; Mason, M. and Cornwall, G. (1992). Vitamin E deficiency and impaired cellular immunity related to intestinal fat malabsorption. *Gastroenterology*. 102 (6): 2139–42.

Krishnaswamy, K. (1996). Indian functional food: Role in prevention of cancer. *Nutrition Reviews.* 54:127–31.

Kroemer, G., Marino, G. and Levine, B. (2010). Autophagy and the integrated stress response. *Molecular Cell*.40:280–293.

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, T., Yoshimori, T., Ohsumi, Y., Tokuhisa, T. and Mizushima, N. (2007). Autophagy-dependent viaral recognition by plastocytoid dendritic cells. *Science,* 315, pp.1398-1401

Kumar, A., Bhatnagar, S. and Paul, P. K. (2012). TWEAK and TRAF6 regulate skeletal muscle atrophy. *Current Opinion in Clinical Nutrition and Metabolic Care*; 15, 233–239.

Laditan, A. A. (1976). Some clinical data of prognostic importance in Protein Calorie Malnutrition (PCM). *Tropical Geographycal Medicine*; 28: 216-219.

Ladue, J. S., Wroblewski, F. and Karmen, A. (1954). Serum glutamic oxaloacetic transaminase activity in human acute transmural myocardial infarction. *Science*.120 (3117): 497–9.

Ladue, J. S., Wroblewski, F. and Karmen, A. (1991).Serum glutamic oxaloacetic transaminase activity in human acute myocardial infaction. *Clinincal Chemistry*; 37:78-82.

Lambe, M. O. and Bewaji, C. O. (2017). Protective role of Moring oleifera leaf-based diet on protein – energy malnutrition –induced skeletal muscle degeneration. *International Journal of Scientific Reports*.Vol. 3, No. 2.

Larsson, L., Edström, L., Lindegren, B., Gorza, L. and Schiaffino, S. (1991). MHC composition and enzyme-histochemcal and physiological properties of a novel fast twitch motor unit type. *The American Journal of Physiology:* 261 (1 pt 1): C93–101.

Lecker, S. H., Goldberg, A. L. and Mitch, W. E. (2006). Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology*. 17, 1807–1819.

Lecker. H. S., Jagoe, T. R.., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, R. S., Mitch, E. W. and Goldberg, L. A. (2004). Multiple type of skeletal muscle atrophy involve a common program of changes in gene expression. *The Journal the federation of American society for experimental biology (FASEB).* Vol. 18 no. 1 39-51.

Lee, D. and Goldberg, A. (2011). Atrogin1/MAFbx: what atrophy, hypertrophy, and cardiac failure have in common. *Circulation Research*; 109, 123–126.

Lemaster, J. J., Theruvath, T. P., Zhoung, Z. and Nieminen, A. L. (2009). Mitochondrial calcium and the permeability transition in cell death. *Biochemical et Biophysical Acta - Bioenergetics* 1787 (11): 195- 1401.

Levine and Kroemer, (2008). Autophagy in the Pathogenesis of Disease. *Cell*. Volume 1, issue 1, p27-42.

Li, Y. P., Chen, Y., Li, A. S. and Reid, M. B. (2003). Hydrogen peroxide stimulates ubiquitin-conjugating activity andexpression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *American Journal of Physiology Cell Physiology*; 285:C806–C812.

Lin, J. K., Lin, C. H., Ling, Y. C., Lin-Shian, S. Y. and Juan, I. M. (1998). Survey of catechins, gallic acid and methylxantines in green, oolong, puerh and black teas. *Journal of Agriculture and Food Chemistry*. 46:3635–42.

Lockett, C. T. and Grivetti, L. E. (2000). Food-related behaviors during drought: a study of rural Fulani, northeastern Nigeria. *International Journal of Food Sciences and Nutrition;* 51:91–107.

Lockett, C. T., Calvert, C. C. and Grivetti, L. E. (2000). Energy and micronutrient composition of dietary and medicinal wild plants consumed during drought. Study of rural Fulani, Northeastern Nigeria. *International Journal of Food Sciences and Nutrition;* 51:195–208.

López-Varela, S., González-Gross, M. and Marcos, A. (2002). Functional foods and the immune system: A review. *European Journal of Clinical Nutrition.* ; 56:S29–33.

Luetjens, C. M., Bui, N. T., Sengpiel, B., Münstermann, G., Poppe, M., Krohn, A. J., Bauerbach, E., Krieglstein, J. and Prehn, J. H. (2000). Delayed mitochondrial dysfunction in excitotoxic neuron death: Cytochrome c release and a secondary increase in superoxide production. *The Journal of neuroscience: The official journal of the Society for Neuroscience* 20 (15): 5715–5723.

Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T. and Thompson, C. B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, 120, pp. 237–248

Mackenzie, C. (2015). The Action of Muscle: Including Muscle Rest and Muscle Re-education. England: Paul B. Hoeber. P. 1.

Magal, M. Dumke, C. L. Urbiztondo, Z. G. et al., (2010). Relationship between serum creatine kinase activity following exerciseinduced muscle damage and muscle fibre composition. *Journal of Sports Sciences*, vol. 28, no. 3, pp. 257–266.

Makkar, H. P. S. and Becker, K. (1996). Nutrional value and antinutritional components of whole and ethanol extracted Moringa oleifera leaves. *Animal Feed Science and Technology:* volume 63, issue1-4, pages 211- 228.

Mc Ardle, A., and Jackson, M.J. Intracellular mechanisms involved in damage to skeletal muscle. *Basic Appl. Myol.*, 4 (1): 43-50, 1994.

McArdle, A., Vasilaki, A. and Jackson, M. (2002). Exercise and skeletal muscle ageing: cellular and molecular mechanisms. *Ageing Research Reviews:* vol. 1, no. 1, pp. 79–93.

McCarthy, J. J., Mula, J., Miyazaki, M., Erfani, R., Garrison, K., Farooqui, A. B., Srikuea, R., Lawson, B. A., Grimes, B., Keller, C., *et al*. (2011). Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* 138, 3657–3666.

McCloud, A. (2011). Build Fast Twitch Muscle Fibers. Complete strength Training

McClung, J. M., Judge, A. R., Powers, S. K. and Yan, Z. (2010). p 38 MAPK links oxidative stress to autophagy-relatedgene expression in cachectic muscle wasting. American Journal of Physiol. *Cell Physiology*; 298:C542–C549.

McCracken, Thomas (1999). New Atlas of Human Anatomy. China: Metro Books. pp. 1–120.

Mehta J, Shukla A, Bhukariya V, Charde R. (2011). The magic remedy of *Moringa oleifera*: An overview. *International journal of Biomedical and advanced research.* 2: 215-227.

Meléndez, A. and Levine, B. (2009). Autophagy in C. elegans, WormBook, Ed. The C. elegans Research Community, WormBook.1.147.1

Michiels, C. Raes, M. Toussaint, O. and Remacle, J. (1994). Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radical Biology and Medicine:* vol. 17, no. 3, pp. 235–248.

Miller, E. R., Pastor-Barriuso R., Dalal, D., Riemersma, R. A., Appel, L. J., Guallar, E., Pastor-Barriuso, D., and Guallar, R. A. (2005). Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine*. 142 (1): 37–46.

Miller, R. J. (1991). The control of neuronal Ca2+ homeostasis. *Progress in Neurobiology*; 37, 255-285

Min, K., Smuder, A. J., Kwon, O. S., Kavazis, A. N., Szeto, H. H. and Powers, S. K. (2011). Mitochondrial-targetedantioxidants protect skeletal muscle against immobilization-induced muscle atrophy. *Journal of Applied Physiology*; 111:1459–1466.

Misra, H. P., and Fridovich, I. (1972). The role of superoxide anion in the autooxidation of epinephrine and simple assay for superoxide dismutase. *Journanl of Biological Chemistry*: 247, 3170-3175.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T. and Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Molecular Biology of the Cell*; 15, 1101–1111.

Mohaupt, M. G. Karas, R. H. Babiychuk, E. B. et al., (2009). Association between statin-associated myopathy and skeletal muscle damage. *Canadian Medical Association Journal;* vol. 181, no. 1-2, pp. E11–E18.

Monteith, G. R. and Roufogalis, B. D. (1995). The plasma membrane calcium pump - a physiological perspective on its regulation. *Cell Calcium* 18, 459-470.

Montgomery, D. C. Introduction to StatisticalQuality Control. Arizona State University. Sixth Edition, pp 646-652. John Wiley & Sons, Inc.

Moresi, V., Williams, A. H., Meadows, E., Flynn, J. M., Potthoff, M. J., McAnally, J., Shelton, J. M., Backs, J., Klein, W. H., Richardson, J. A., et al. (2010). Myogenin and class II HDACs control neurogenic muscle atrophy by inducing E3 ubiquitin ligases. *Cell* 143, 35–45.

Morton, J. F. (1991). The Horseradish tree, Moringa pterygosperma (Moringaceae) – a boon to arid lands. *Economic Botany*; 45: 318-333.

Moss, D. W. and Henderson, M. B. (1994). Enzymes In: Tiezt Textbook of Clinical Chemistry Burtis CA, Ashwood ER, eds. 2nd Edition WB Saunders USA. 735-896.

Moylan, J. S., Smith, J. D., Chambers, M. A., *et al*. (2008). TNF induction of atrogin-1/MAFbx mRNA depends on Foxo4 expression but not AKT-Foxo1/3 signaling. *American Journal of Physiology Cell Physiology*; 295:986-993.

Mueller, R. G., Neville, K., Emerson, D. and Lang, G. E. (1975). Depressed apparent creatine kinase activity in sera with abnormally high alkaline phosphatese activity. *Clinical Chemistry*; 21: 268

Murray, S., Schell, K., McCarthy, D. O. and Albertini, M. R. (1997). Tumor growth, Weight loss and cytokines in SCID mice. *Cancer Letters*; 111:111 –5.

Nadia, B., Claude, R., Joseph, J. H. and Brigitte, R. A. (1999). Low- Protein Isocaloric Diet During Gestation Affects Brain Development And Alter Permanently Cerebral Cortex Blood Vessels In Rat Offspring. *The American Society for Nutritional Sciences. The Journal of Nutrition.*

Nanji, A. A. and Blank, D. (1981). Low serum creatine kinase activity in patients with alcoholic liver disease [Letter]. *Clinical Chemistry*; 27:1954.

National Research Council, (1987). Vitamin E, in Vitamin Tolerance of Animals. Subcommittee on vitamin tolerance, committee on animal nutrition. *National Academy of Science.*

Nethery, D., Callahan, L. A., Stofan, D., Mattera, R., DiMarco, A., and Supinski, G. (2000). PLA2 dependence of diaphragm mitochondrial formation of reactive oxygen species. *Journal of Applied Physiology:* vol. 89, no. 1, pp. 72–80.

Ngaha, E. O., Akanji M. A. and Madusolomo, M. A. (1989). Studies on correlation between chloroquine–induced tissue damage and serumchanges in rats. *Experimentia*. 45: 143.

Nicholls, D. G. and Brand, M. D. (1980). The nature of the calcium ion efflux induced in rat liver mitochondria by the oxidation of endogenous nicotinamide nucleotides. *The Biochemical Journal*: 188 (1): 113-118.

Nikolaos, K. (2011). Clinical Nutrition in Practice. *John Wiley & Sons.* pp. 37.

Nixon, D. W. Heymsfield, S. B and Cohen, A. E (1980). Protetn calorie undernutrition inhospitalized cancer patients. *American Academy of Aging Medicine*; 68: 683V-690

Nordgren, M. and Fransen, M. (2014). Peroxisomal metabolism and oxidative stress. *Biochimie*, vol. 98, no. 1, pp. 56–62.

Nouman, W., Siddiqui, M. T., Basra, S. M. A, Afzal, I. and Rehman, H. (2012). Enhancement of emergence potential and stand establishment of Moringa oleifera Lam. by seed priming. *Turkish Journal of Agriculture and Forestry.* 36: 227-235.

Oakenful, D. and Sidhu, G. S. (1989). Saponins. In: Cheeke, P.R. (Ed.), Toxicants of Plant Origin. Academic Press, New York, 2: 78-113.

Oakes, E. H. and Emersons, G. A. (2007). Encyclopedia of World Scientists. P. 211.

Oduro, I., Ellis, W. O. and Owusu, D. (2008). Nutritional potential of two leafy vegeTables: Moringa oleifera and Ipomoea batatas leaves. *Scientific Research and Essay*. 3(2): 57-60.

Ogbu, S. I. and Okechukwu, E. I. (2001). The effect of storage temperature prior to separation on plasmaandserumpotassium. *Journal of Medical Laboratory Science:* 10: 1-4.

Okwu, E. D. and Ighodaro, U. B., (2010). GC-MS evaluation of bioactive compounds and antibacterial activity of the oil fraction from the leaves of *Alstonia boonei*. De Wild. *Der PharmaChemica:* 2(1): 261-2.

Oloyede, O. I. (2005). Chemical Profile of Unripe Pulp of Caricapapaya. *Pakistan Journal of Nutrition;* 4 (6): 379-381.

Ortego, A., Santiago-Garcia, J., Mas-Oliva, J. and Lepock, J. R. (1996). Cholesterol increases the thermal stability of the Ca2+/Mg2+-ATPase of cardiac microsomes. *Biochemical et Biophysical Acta:* 1283 (1); 45-50

Packer L, Weber, S. U, Rimbach, G, Weber; Rimbach (2001). Molecular aspects of α-tocotrienol antioxidant action and cell signaling. Journa*l of Nutrition.* 131 (2): 369S–73S.

Palee S, Weerateerangkul P, Surinkeaw S, Chattipakorn S & Chattipakorn N (2011). Effect of rosiglitazone on cardiac electrophysiology, infarct size and mitochondrial function in ischaemia and reperfusion of swine and rat heart. *Experimental physiology*; 96, 778–789.

Paolo, B. and Marco, S. (2013). Cellular and molecular mechanisms of muscle atrophy. *Disease Model and Mechanism*: vol. 6 no. 1 25-39.

Papp, B., Enyedi, A., Paszty, K., Kovacs, T., Sarkadi, B., Gardos, G., Magnier, C., Wuytack, F. and Enouf, J. (1992). Simultaneous presence of two distinct endoplasmic-

reticulum-type calcium-pump isoforms in human cells. *Biochemistry Journal*. 288, 297 - 302.

Park, Y. E., Hayashi, Y. K., Bonne, G., Arimura, T., Noguchi, S., Nonaka, I. and Nishino, I. (2009). Autophagic degradation of nuclear components in mammalian cells. *Autophagy* 5, 795–804.

Patterson, M., Sney, D. J., and Friel, D. D. (2007). Depolarization-Induced Calcium Responses in Sympathetic Neurons: Relative Contributions from Ca^{2+} Entry, Extrusion, ER/Mitochondrial Ca^{2+} Uptake and Release, and Ca^{2+} Buffering. *Journal of General Physiol*ogy: 129 (1): 29–56.

Pearson, D. (1976). Chemical Analysis of Foods. Churchill Livingstone, Edinburgh, UK. pp: 7-14.

Pedersen, P. L. & Carafoli, E. (1987). Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. *Trends in Biochemical Sciences* 12, 146-150.

Peng, T. I. and Jou, M. J. (2010). Oxidative stress caused by mitochondrial calcium overload. *Annals of the New York Academy of Sciences*; 1201:183–188.

Penniston, J. T. (1983). Plasma membrane Ca^{2+}-ATPases as active Ca^{2+} pumps. In: Calcium and Cell Function. Ed: Cheung, W. Y., Academic Press, New York, NY, Vol IV, 99-147

Penniston, J. T. and Enyedi, A. (1998). Modulation of the plasma membrane Ca^{2+} pump. *Jounal of Membrane Biology*; 165, 101-109.

Powers, S. K., Hudson, M. B., Nelson, W. B., Talbert, E. E., Min, K., Szeto, H. H., Kavazis, A. N. and Smuder, A. J. (2011a). Mitochondria-targeted antioxidants protect against mechanical ventilation-induced diaphragmweakness. *Critical Care Medicine*; 39:1749–1759.

Powers, S. K., Smuder A. J and, Criswell D. S. (2011b). Mechanistic links between oxidative stress and disuse muscle atrophy. *Antioxidant Redox Signal;* 15:2519–2528.

Powers, S. K., Wiggs, M. P., Duarte, J., Zergeroglu, A. M. and Demirel, H. A. (2012). Mitochondrial signaling contributes todisuse muscle atrophy. *American Journal of Physiology. Endocrinology Metabolism;* 303:E31–E39.

Proske, U. and Morgan, D. L. (2001). Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *Journal of Physiology*. 537:333–345.

Pu, F., Ren, X. L. and Zhang, X. P. (2013). Phenolic compounds and antioxidant activity in fruits of six Diospyros kaki genotypes. *European Food Research and Technology*: vol. 237, no. 6, pp. 923–932.

Qu, X., Zou, Z., Sun, Q., Luby-Phelps, K., Cheng, P., Hogan, R. N., Gilpin, C. and Levine, B (2007). Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell*, 128, pp. 931–946

Raffaello, A., Milan, G., Masiero, E., Carnio, S., Lee, D., Lanfranchi, G., Goldberg, A. L. and Sandri, M. (2010). JunB transcription factor maintains skeletal muscle mass and promotes hypertrophy. *Journal of Cell Biology*. 191, 101–113.

Ramachandran, N., Munteanu, I., Wang, P., Aubourg, P., Rilstone, J. J., Israelian, N., Naranian, T., Paroutis, P., Guo, R., Ren, Z. P., Nishino, I., Cabrol, B., Pe;;issier, J. F., Minetti, C., Udd, B., Fardeau, M., Tailor, C. S., Mahuran, D. J., Kissel, J. T., Kalimo., H, Levy, N., Manolson, M. F., Ackerley, C. A. and Minassian, B. A. (2009). VMA21 deficiency causes aquautophagic myopathy by compromising V-ATPase activity and lysosomal acidification. *Cell* 137, 235–246.

Rambold, A. S. and Lippincott-Schwartz, J. (2011). Mechanisms of mitochondria and autophagy crosstalk. *Cell Cycle*; 10:4032–4038.

Ranira, G., Rimi, H., Kaushik, R. and Debajani, G. (2005). Effect of Moringa oleifera in Experimental model of Alzheimer‟s disease: Role of antioxidants. *Annals of Neurosciences*; 12(3). 36-39.

Rao, A. V. (1996). Anticarcinogenic properties of plant saponins. Second International Symposium on the role of soy in preventing and treating chronic.

Rao, A. V., Devi, P. U. and Kamath, R. (2001). In vivo radioprotective effect of Moringa oleifera lamm. *Indian Journal of Experimental Biology*; 39:858-863.

Ravasco, P., Monteiro-Grillo, I., Vidal, P. M. and Camilo, M. E. (2003). Nutritional Deterioration in Cancer: The Role of Disease and Diet. *Clinical Oncology*15 (8): 443–450.

Rebouche, C. J. (2004). Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Annals of New York Academic Science*; 1033:30–41.

Reboul, E., Richelle, M., Perrot, E., Desmoulins-Malezet, C., Pirisi, V., Borel, P., Richelle, P., and Desmoulins-Malezet, P. B. (2006). Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry*.54 (23): 8749–8755.

Reggiori, F., Komatsu, M., Finley, K., andSimonsen, A. (2012). Autophagy: more than a nonselective pathway. *International Journal of Cell Biology*; 2012:219625.

Reid, M. B. Role of Oxidative Stress in Skeletal Muscle Weakness. University of Kentucky Medical Center, Lexington, KY. 110th Abbott Nutrition 2 Research Conference.

Reid, M. B, (2005). Response of the ubiquitin-proteasome pathway to changes in muscle activity. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*; 288:R1423.

Reitman, S. and Frankel, S. (1957). Colometric methods for aspartate and alanine aminotransferase. *American Journal of Clinical Pathology*; 28: 55-60

Ricardo, J. G., Alberto, B. and Juan, J. P. (2014). Biochemistry of oxidative stress; pathophysilogy and clinical aspects. *Springer link*: volume 16.

Rice-Evans, C. (2001). Flavonoid antioxidants. *Current Medicinal Chemistry*. 8, 797–807.

Rice-Evans, C. A. and Bruckdorfer, K. R. (1992). Free radicals, lipoproteins and cardiovascular dysfunction. *Molecular Aspects Medicine*. 13, 1 – 111.

Rice-Evans, C. A. and Diplock, A. T. (1993). Current status of antioxidant therapy. *Molecular Aspects Medicine;* 15, 293 – 376.

Roberfroid, M. B. (1999). What is beneficial for health? The concept of functional food. *Food and Chemical Toxicology;* 37:1034–41.

Robert, W. J. and Susan, C. K. (2004). The molecular basis of skeletal muscle atrophy. *American Journal of Physiology - Cell Physiology:* Vol. 287 no. 4, C834-C843

Roberts, C. K. and Sindhu, K. K. (2009). Oxidative stress and metabolic syndrome. *Life Sciences*. 84(21-22): 705-12.

Roe, C. and Ding, J. (2001). Mitochondrial fatty acid oxidation disorders. *The Metabolic and Molecular Bases of Inherited Disease*: McGraw-Hill; New York: pp. 2297–2326.

Romanello, V. and Sandri, M. (2010). Mitochondrial biogenesis and fragmentation as regulators of muscle protein degradation. *Curr. Hypertens. Rep*. 12, 433–439.

Romanello, V., Guadagnin, E., Gomes, L., Roder, I., Sandri, C., Petersen, Y., Milan, G., Masiero, E., Del Piccolo, P., Foretz, M., et al.(2010). Mitochondrial fission and remodelling contributes to muscle atrophy. *EMBO Journal.* 29, 1774–1785.

Rosenberg, I. H. (1989). Summary comments. *American Journal of Clinical Nutrition*; 50: 1231–3.

Runner, P., Gazzotti, P., and Carafoli, E. (1977). A Lipid Requirement For The (Ca^{2+} Mg^{2+})-Activated Atpase Of Erythrocyte Membranes. *Archieve of Biochemistry and Biophysics.* 179, 578-583

Sacheck, J. M., Hyatt, J. P., Raffaello, A., Jagoe, R. T., Roy, R. R., Edgerton, V. R., Lecker, S. H. and Goldberg, A. L. (2007). Rapid disuse and denervation atrophy involve transcriptional changes similar to those of muscle wasting during systemic diseases. *FASEB Journal*; 21, 140–155.

Saks, V. (2008). The phosphocreatine-creatine kinase system helps to shape muscle cells and keep them healthy and alive. *Journal of Physiology,* vol. 586, no. 12, pp. 2817–2818.

Sandri M. (2008). Signaling in Muscle Atrophy and Hypertrophy. *Physiology*; 23: 160-170.

Sartorelli, V. and Fulco, M. (2004). Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. *Science:* STKE, re11.

Sartori, R., Milan, G., Patron, M., Mammucari, C., Blaauw, B., Abraham, R. and Sandri, M. (2009). Smad2 and 3 transcription factors control muscle mass in adulthood. *America. Journal of. Physiology. Cell Physiology:* 296, C1248–C1257.: 10893434

Sayers, S. P. and Clarkson, P. M. (2003). Short-term immobilization after eccentric exercise. Part II: creatine kinase and myoglobin. *Medicine and Science in Sports and Exercise*: vol. 35, no. 5, pp. 762–768.

Schaible, U. E. and Kaufmann, S. H. E. (2007). Malnutrition and infection: complex mechanisms and global impacts. *PLoS Medicine*: 4 (5): e115.

Schatzmann, H. J. (1966). ATP-dependent Ca^{2+} extrusion from human red cells. *Experientia* 22, 364-368

Schinder, A. F.; Olson, E. C.; Spitzer, N. C.; Montal, M. (1996). Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *The Journal of neuroscience:* 16 (19): 6125–6133.

Schmidt, E. and Schmidt, F. W. (1963). Determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Enzyme Biological Clinic:* 3:1-5.

Shamberger, R. J. (1984). Cancer cachexia. *Nutrition and Cancer*. p. 353. Plenum

Sharma, R. and Vaghela, J. (2011). Anti-Inflammatory Activity of Moringa oleifera leaf and pod extracts against Carrageenan Induced Paw Edema in Albino Mice. *Pharmacologyonline*; 1. 140-144.

Shull, G. E. and Greeb, J. (1988). Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. *Journal Biological Chemistry*; 263, 8646-8657.

Siddhuraju, P. and Becker, K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry*; vol. 51, no. 8, pp. 2144–2155.

Sidhu, G. S. and Oakenfull, D. G. (1986). A mechanism for the hypocholesterolaemic activity of saponins. *British Journal of Nutrition*; 55(3): 643-9

Siems, W., Capuozzo, E., Lucano, A., Salerno, C. and Crifo, C. (2003). High sensitivity of plasma membrane ion transport ATPases from human neutrophilstowards 4-hydroxy-2, 3-trans-nonenal. *Life Science*; 73: 2583–2590.

Sies, H. (1991) Oxidative Stress. II: Oxidants and Antioxidants. Academic Press, London and New York.

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology;* 299: 152-178.

Smuder, A. J., Kavazis, A. N., Hudson, M. B., Nelson, W. B. and Powers, S. K. (2010). Oxidation enhances myofibrillarprotein degradation via calpain and caspase-3. *Free Radical Biology and Medicine*; 49:1152–1160.

Sorichter, S., Puschendorf, B. and Mair, J. (1999). Skeletal muscle injury induced by eccentric muscle action. Muscl protein as markers of muscle fiber injury. *Exercise Immunology Review*. Vol. 5:5-21.

Spriet, L.L., Howlett, R. A. and Heigenhauser, G. J. (2000). An enzymatic approach to lactate production in human skeletal muscle during exercise. *Med Sci Sports Exerc*. 32 (4): 756–63.)

Starling AP, Hughes G, East JM and Lee AG (1994) Mechanism of stimulation of the calcium adenosine triphosphatase by jasmone. *Biochemistry* 33: 3023-3031.

Stauffer, T. P., Guerini, D. andCarafoli, E. (1995). Tissue distribution of the four gene products of the plasma membrane Ca^{2+} pump. A study using specific antibodies. *The Journal of Biological Chemistry*; 270, 12184-12190.

Stauffer, T. P., Hilfiker, H., Carafoli, E. and Strehler, E. E. (1993). Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes. The Journal of Biological Chemistry; 269, 32022 (1994). *Journal of Biological Sciences*; 268, 25993-26003.

Stefanie, A., Thomas, K., Dietmar, P., Eva, O., Alexander, R., Lisa, Z., Ilja, R., Marius, E. M., Christoph, G. and Stephen, P. (2015). Skeletal Muscle Depletion and Markers for Cancer Cachexia Are Strong Prognostic Factors in Epithelial Ovarian Cancer. *PLoS One*: 2015; 10(10)

Stojanoski, N. (1999). Development of health culture in Veles and its region from the past to the end of the 20th century. Veles: *Society of science and art.* 13–34.

Strehler, E. E. (1991). Recent advances in the molecular characterization of plasma membrane Ca^{2+} pumps. *Journal of Membrane Biology*: 120, 1-15.

Strehler, E. E. (1996). Sodium-calcium exchangers and calcium pumps. In: Principles of Medical Biology. *JAI Press, Greenwich,* CT, Vol 4, 125-150

Strehler, E. E., Scarisbrick, I. A. and DeMarco, S. J. (1998). The plasma membrane calcium ATPase 2b binds to membrane cytoskeleton-associated PDZ domain proteins. *Society for Neuroscience;* 24, 1801

Sue, H., Harold, A. and Juan, A. R. (2008). The Challenge of Hunger and Malnutrition. *Copenhagen Consensus Challenge Paper*.

Sunmonu, T. O. and Oloyede, O. B. (2007). Biochemical assesement of the effect of crude oil contaminated catfish (Claris gariepinus) on the hepatocytes and performance of rat. *African Journal of Biochemistry Research*; vol. 1 (5), pp 083- 089.

Tada, M., Ohmori, F. and Kinoshita, N. (1978a). Cyclic Adenosine 31, 51-momophosphate regulation of active calcium transport across membrane of sarcoplasmic reticulum; role of the 22,000 dalton phosphoprotein phospholamban. *Advance cyclic nucleotide research*, 9 (inpress)

Tanzer, M. L. and Gilvarg, C. (1959). Creatine and Creatine Kinase Measurement. *Journal of Biological Chemistry*; 234: 3201-.

Tapas, A. R, Sakarkar, D. M. and Kakde, R. B. (2008). Review article flavonoids as nutraceuticals: A review. *Tropical Journal of Pharmaceutical Research*; 7:1089–99.

Teixeira, E. M. B., Carvalho, M. R. B., Neves, V. A., Silva, M. A. and Arantes-Pereira, L. (2014). *Food Chemistry*; 147:51-54.

Teklit G. A. (2015). Chemical Compositions and Nutritional Value of Moringa Oleifera Available in the Market of Mekelle. *Journal of Food and Nutrition Sciences*. Vol. 3, No. 5, pp. 187-190.

Tepikin, A. V., Voronina,S. G.,Gallacher, D. V.and Petersen, O. H.(1992). Pulsatile Ca^{2+} extrusion from single pancreatic acinar cells during receptor-activated cytosolic Ca2+ spiking. *The Journal of Biological Chemistry;* 267, 14073-14076.

Thomas, K., KatjaBandemer, and Wilhelm, B. (1997). Biosynthesis of cis-Jasmone: a pathway for the inactivation and the disposal of the plant stress hormone jasmonic acid to the gas phase? *Helvetica ChimicaActa*; 80 (3): 838–850.

Thomas, D. R. (2002). Distinguishing starvation from cachexia. *Geriatric Clinic of North America;* 18: 883–92.

Thurber, M. D. and Fahey, J. W. (2009). Adoption of Moringa oleifera to combat under-nutrition viewed through the lens of the "Diffusion of Innovations" theory. *Ecology of food and nutrition*; 48(3): 212-225

Tintignac, L. A., Lagirand, J., Batonnet, S., Sirri, V., Leibovitch, M. P. and Leibovitch, S. A. (2005). Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *The Journal of Biological Chemistry;* 280, 2847–2856.

Toescu, E. & Petersen, O. H. (1995). Region-specific activity of the plasma membrane Ca^{2+} pump and delayed activation of Ca^{2+} entry characterize the polarized, agonist-evoked Ca^{2+} signals in exocrine cells. *The Journal of Biological Chemistry;* 270, 8528-8535

Totsuka, M. Nakaji, S. Suzuki, K. Sugawara, K. and Sato, K. (2002). Break point of serum creatine kinase release after endurance exercise. *Journal of Applied Physiology*: vol. 93, no. 4, pp. 1280– 1286.

Traber, M. G. (1998). The biological activity of vitamin E. *The Linus Pauling Institute.*

Traber, M. G. and Atkinon, J. (2007). Vitamin E, antioxidants and nothing more. *Free Radical Biology and Medicine;* 43 (1); 4-15.

Twig, G., Elorza, A., Molina, A. J, Mohamed, H., Wikstrom, J. D., Walzer, G., Stiles, L., Haigh, S. E, Katz, S., Las, G., et al. (2008a). Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO Journal*. 27:433–446.

Uchida, K. (2000). Role of reactive aldehyde in cardiovascular diseases. *Free Radical Biology and Medicine*; 28, pp. 1685-1696

UNICEF. "Introduction to Nutrition". *UNICEF*. 2012.

Upadhye, K., Rangari, V. and Mathur, V. (2011). Evaluation of antinociceptive activities of fresh leaf juice and ethanolic extract of Moringa oleifera lamm. *Asian Journal of Pharmaceutical and Clinical Research*. 4:114-116.

Valerino, D. M and McCormack, J. J. (1971). Xanthine Oxidase-mediated oxidation of epinephrine. *Biochemical Pharmacology*; 20(1): 47 – 55.

Valvona, C. J., Fillmore, H. L., Nunn, P. B. and Pilkington, G. J. (2016). The regulation and function of lactate dehydrogenase A: therapeutic potential in brain tumor. *Brain pathology;* 26(1):3-17.

Vávrová, L., Kodydková, J., Zeman, M., et al. (2013). Altered activities of antioxidantenzymes in patients with metabolic syndrome. *Obesity Facts*; 6(1): 39-47.

Verboomen, H., Wuytack, F., De Smedt, H., Himpens, B. and Casteels, R. (1992). Functional difference between SERCA 2a and SERCA 2b Ca^{2+}-pumps and their modulation by phospholamban. *Biochemistry Journal*; 286, 591 - 596.

Verma, A. K., Enyedi, A., Filoteo, A. G., Strehler, E. E. andPenniston, J. T. (1996). Plasma membrane calcium pump isoform 4a has a longer calmodulin-binding domain than 4b. *The Journal of Biological Chemistry*; 271, 3714-3718.

Verma, A. R., Vijayakumar, M., Mathela, C. S. and Rao, C. V. (2009). In vitro and in vivo antioxidant properties of different fractions of Moringa oleifera leaves. *Food Chemistry and Toxicology*; 47:2196–201.

Vinoth, R., Manivasagaperumal, R. and Balamurugan, S. (2012). Phytochemical analysis and antibacterial activity of Moringa oleifera lam. *International Journal of Research in Biological sciences*; 2: 98-102.

Voet, D., Voet, J. G. and Pratt, C. W. (1999). Lipid metabolism. *Fundamentals of Biochemistry:* John Wiley and sons, Inc. NewYork, pp 569.

Wanasundara, U. N. and Shahidi, F. J. (1994). Canola extract as an alternative natural antioxidant for canola oil. *Journal of the American Oil Chemist's Society*; 71(8): 817-822.

Waterlow, J. C. (1972). Classification and Definition of Protein-Calorie Malnutrition. *British Medical Journal:* 3 (5826): 566–569.

Wellman, N. S., Weddle, D. O., Kranz, S. and Brain, C. T. (1997). Elder Insecurities: Poverty, Hunger, and Malnutrition. *Journal of the American Dietetic Association*: 97 (10): S120–S122.

Wearne, J., Moore, R. W. and Caplan, B. (1975). Does alkaline phosphatase affect serum creatine kinase values [Letter]. *Clinical Chemistry*; 21:1343.

Weiss, N., Andrianjafiniony, T., Dupre-Aucouturier, S., Pouvreau, S., Desplanches, D. and Jacquemond, V. (2010). Altered myoplasmic Ca (2+) handling in rat fast-twitch skeletal muscle fibres during disuse atrophy. *Pflugers Arch.*; 459:631–644.

White, R. J.; Reynolds, I. J. (1996). Mitochondrial depolarization in glutamate-stimulated neurons: An early signal specific to excitotoxin exposure. *The Journal of Neuroscience.* 16 (18): 5688–5697.

Whitney, E. and Sharon, R. (2011). Understanding Nutriton. Peggy Williams, ed. (twelfth edition) Cengage Lerning, Califonia; Wadsworth.

World Health Organization (2012). WHO, nutritiom experts take action on malnutrition

World Health Organization (2016). WHO Online Question and Answer: what is Malnutrition?

Wright, P. J., Leathwood, P. D. and Plummer, D. T. (1972). Enzymes in rat urine: alkaline phosphate. *Enzymologia,* 42 (4): 317.

Wu, K. D., Lee, W. S., Wey, J., Bungard, D., Lytton, J. (1995). Localization and Quantification of Endoplasmic Reticulum Ca^{2+}-ATPase Isoform Transcripts. *American Journal of Physiology, Cell Physiology*; 269:C775–C784.

Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. andCasteels, R., (1994). A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *The Journal of Biological Chemistry*; 269:1410–1416.

Yakubu, M. T., Bilbis, L. S., Lawal, M. and Akanji, M. A. (2003). Evaluation of selected parameters of rat liver and kidney function following repeated administration of yohimbine. *Biokemistri*: 15(2): 50-56.

Zvaritch, E., James, P., Vorherr, T., Falchetto, R., Modyanov, N. and Carafoli, E. (1990). Mapping of functional domains in the plasma membrane Ca^{2+} pump using trypsin proteolysis. *Biochemistry*: 29, 8070-8076

APPENDIX I

PREPARATION OF SOLUTIONS

(1) Preparation of 0.25M sucrose buffer solution

$$\text{Molarity (M)} = \frac{\text{mass (g)}}{\text{Molar mass (g/mol)}}$$

Molar mass of sucrose= 342.3g/mol.

$$0.25\text{M} = \frac{\text{mass}}{342.3}$$

Reacting mass = 342.3 x 0.25 = 85.575g

Procedure

85.57g of sucrose was weighed into a 1L beaker about 600ml of distilled water was added and mixed till all dissolved. The mixture was quantitatively poured into 1L volumetric flask and made-up to 1000ml mark point. This as kept in a deep freezer for subsequent use.

(2) Preparation of Sucrose- Tris (10mM) buffer solution

10mM tris =

Molar mass of tris = 121.1g/mol.

Reacting mass = 121.1 x 0.25= 1.1211g

Procedure

85.57g of sugar was dissolved in little quantity of distilled water in a beaker.1.121g of tris was added and stired until all totally dissolved. The pH of the solution was adjusted to 7.4 and transferred into 1000ml volumetric flask were it was made up to 1L mark point with distilled water. The solution was pured into a plastic container and refrigerated.

(3) Biuret reagent

1.5 copper II sulphate($CuSO_4.H_2O$)(BDH Chemical Limited,Poole England) and 6.0g of sodium potassium tartarate($NaKC_4O_6.4H2O$ or NaK.C4H4O6)(BDH Chemical Limited,Poole England)were dissolved in distilled waaterto make500ml in a 1L standard volumetric flask.with constant shaking, add 1g patassiumIOdide(KI)(BDH Chemical Limited,Poole England)and 300ml of 10% sodium hydroxide(NaOH)solution(10% weight by volume). The resulting solution was then made up to 1L mark point with distilled water. The sodium potassium tartarate acts as a stabilizer.

(4) 1% Bovine albumin (BSA)

1% BSA was prepared by dissolving 1g of BSA (BDH Chemical Limited, Poole England) in 100mldistilled water.

1% BSA= 10mg/ml BSA i.e0.1g BSAdissolvedin 100ml distilled water

(5) 1mg/ml Tissue (liver) homogenate

The optical density of the liver homogenate was read at 540nm using the spectrophotometer during standard protein determination. The absorbance value was extrapolated on the standard protein curve and used to determine its concentration. The concentration of protein in the tissue was reduced to1mg/ml by appropriately diluting the homogenate with 0.25Msucrose solution.

(6) Reagents for phosphate determination

Reagent A

9.0ml of concentrated sulphuric acid (BDH Chemical Limited, Poole England) was added to 2.0g of ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$)] (Mayer and beker limited, Dagenham, England) in a clean beaker. About 60ml distilled water was added to generate and accelerate

the dissolution of the ammonium molybdate so that it could be easily dissolved in the acid. After it has completely dissolved, the remaining distilled water was added to make up to the 310 mark point in the measuring cylinder. The reagent was stored in a labelled plastic container (since it is highly reactive with glass wares) and refrigerated.

Reagent B (9% L- Ascobic acid)

9% ascorbic acid (BDH Chemical Limited,Poole England) was prepared by dissolving of powdered 9% ascorbic acid in a small quantity of distilled water, shaken to dissolve and then made up to the 100ml mark point on the volumetric flask. It was stored in a labelled plastic container and refrigerated.

Reagent C (reagent A: reagent B; 4:1)

This was prepared by mixing reagents A and B in ratio 4:1. It is always freshly prepared immediately before use because, degeneration may occur if kept at room temperature for a long time (about 12 hours).

1mM standard phosphate solution

The phosphate standard stock solution i.e 1mM sodium dihydrogen orthophosphate duodecahydrate ($NaH_2PO_4.12H_2O$) was prepared by dissolving 0.142g of the salt in a little amount of distilled water and made up to 1L.

(7) Reagents for ATPase assay

Sodium/ Potassium ATPase buffer

200mM NaCl(BDH Chemical Limited,Poole England), 40mM KCl (BDH Chemical Limited,Poole England), 4mM$MgCl_2.6H_2O$(Kermel chemical limited)and tris (BDH Chemical Limited,Poole England).this was prepared by dissolving 11.69g of NaCl, 2g of KCl, 8.13g of $MgCl_2$ and 4.85g tris in about 200ml distilled waer in a beaker. The pH of the

solution was adjusted to 7.4by adding conc HCl using a pH meter.the content of the beaker was poured into a 1L standard volumetric flask and made up to 1000ml. The buffer was stored in a plastic container and refrigerated.

Calcium ATPase buffer

240mM KCl (Kermel chemical limited), 4mMM$gCl_2.6H_2O$ (Kermel chemical limited) and 40mM Tris, which was prepared by dissolving 17.89g of KCl,0.81g $MgCl_2.6H_2O$ and 4.85g tris in little distilled water, the pH adjusted to 7.4 and made up to 1000ml with distilled water. The buffer was stored in a labelled plastic and refrigerated

10mM CaCl

1.47g of $CaCl.2H_2O$ (BDH Chemical Limited, Poole England) was dissolved in 100ml distilled water. The solution was preserved in a labelled container and refrigerated.

10mM ATP solution

This was prepared by dissolving 0.55 of ATP (Sigmal- Aldrich, Inc. USA) in little distilled water and made up to 100ml mark point in a standared volumetric flask. This was immediately transferred into a labelled container and refrigerated. The solution was always kept frozen to prevent non-enzymatic hydrolysis of the ATP.

5% SDS

5g of Sodium Dodecyl Sulphate (Sigmal- Aldrich,Inc. USA) was dissolved in little distilled water in a beaker, transferred into a 100ml volumetric flask and made up to the mark point. The solution was oured into a labelled container and kept at room temperature.

(8) Preparation of Feed Supplements

100µl/kg body weight Methyl jasmonate: was prepared by dissolving 100µl methyl jasmonate (sigma product japan M.W= 224.0g/mol) per milliliter of distilled water and then vortexed at high rpm with vortex mixer (XH-C, Jinotech instrument).

(9**) Preparation of feed materials**

(i) **Corn starch**: yellow maize was soaked for two days and grinded with a mechanical grinder, sieved with fine mucelain cloth and left to settle for about twelve hours. The seimented corn paste was then poured back into the fine cloth, tighed and drained off water. The drained corn paste was thereafter airdried and blended back into powdered corn powder ready for use. This was kept in an air tight container for subsequent use.

(ii) **Soy bean powder:** soy bean grain was soaked for less than 10 minutes. The seed coat was then removed by scrubing against the palm or mortal and pestle. The coatless seeds were thereafter dried and blended into fine powder which was kept in air tight container for subsequent use.

(iii) **Vitamin/miniral mix:** (Miavit V.T.A powder) a product of Miavit GmbH Germany purchased from olufunmilayo farms limited, Offa garage rod, Ilorin. The constituents of the vitamin/ mineral mix is as follows:

s/n	Constituents	Quantity
1.	Vitamin A	40.000.000IU
2.	Vitamin D_3	10.000.000IU
3.	Vitamin E	40.000mg
4.	Vitamin B_1	4.000mg
5.	Vitamin B_2	2.000mg
6.	Vitamin B_6	5.000mg
7.	Vitamin $_{12}$	50.000mg
8.	Vitamin K_3	3.500mg
9.	Vitamin C	40.000MG

10.	CALPAIN	15.000mg
12	Nicotinamide	50.000mg
13	Folic acid	1.000mg

(iv) **Corn chaff (fibre):** the corn chaff was dried and grounded to fine particles and kept for use.

(v) **Feed preparation:** the feeds were prepared by weighing each component of feed as shown in Table 9 into a clean bowl and mixed properly. Water was then added in bits till the mixture formed a uniform paste which was thereafter made into pellets by pressing over a mesh. The pellets were then oven dried at 40^0C. After drying, the feeds were packed tightly into polythene bags and kept in the freezer till use.

(10)10% formalin: was prepared by measuring 10ml of formaldehyde solution and making it up to 100ml mark point with distilled water in a 100ml volumetric flask.

(11) 0.059M H_2O_2: this was prepared by taking 2.006 of stock solution of H_2O_2, and making it up to 1000ml mark point with distilled water in a 1000ml volumetric flask.

(12) 0.05M Phosphate buffer (pH 7.0): 4.3g of K_2HPO_4 and 4.2g of KH2PO4 was weighed and dissolved in distilled water, mixed up to dissolve well and made up to 1000ml mark point in a standard volumetric flask. The pH was then checked and adjusted to pH 7.0.

(13) Carbonate buffer (pH 10.2): 7.15g of $Na_2CO_3.10H_2O$ and 2.1g of $NaHCO_3$ was dissolved in 400ml of distilled water and then made up to 500ml. the pH was checked and adjusted to 10.2.

The formular used in the calculation of the ratio of acid: base in a buffer is as follows

$pH = pKa + \log [base]/[acid] \leftrightarrow$ $pH = pKa + \log [A^-]/[HA]$

(14) 0.3M of Adrenaline: 0.0137g of adrenalin (epinephrine) was dissolved in 200ml of distilled water and made up to 250ml. This solution was prepared just before use.

(15) Reagent for MMPT

Buffer C: Homogenizing buffer

3.83g mannitol (210mM), 2,4g sucrose (70mM), 0.12g Hepes (5mM) and 0.0338g EGTA (1mM) was weighed and all dissolved in 100ml distilled water. pH wa adjusted to 7.4.

Buffer D: Washing buffer

3.83g mannitol (210mM), 2,4g sucrose (70mM), 0.12g Hepes (5mM) and 0.5g BSA (50%) was weighed and all dissolved in 100ml distilled water. pH wa adjusted to 7.4.

Swelling buffer

3.83g mannitol (210mM), 2,4g sucrose (70mM) and 0.12g Hepes (5mM) was weighed and all dissolved in 100ml distilled water. pH wa adjusted to 7.4.

The pH was very important... adjust with KOH and if needed Hcl.

Other reagents

1. 3µM $CaCl_2$ -0.01764g of $CaCl_2$ was weighed and dissolved in 10ml of water.
2. 5mM Sodium succinate- was prepared by weighing 0.6753g and dissolved in 10ml of water.
3. 0.8µM Rotenone- wa prepared by weighing 0.0081g in 5ml of 10% ethanol + 5ml of water.
4. 4mM Spermine- weigh 0.01393g in 10ml of water. (Stored in dark container).

APPENDIX II

Dermination of protein concentration in rat liver homogenate

The protocol for standard protein determination has been described in chapter two. The concentration of the liver homogenate was then calculated as follows;

The optical density of test tube 8 which contain liver homogenate (crude or the mitochondrial fragment) as the protein source was extrapolated on the standard protein curve. This value represents the amount of protein in the homogenate.

Determination of the amount of inorganic phosphate (Pi) realeased in the tissue (liver)

The absorbance at 820nm for each sample at substrate (adenosine triphosphate) concentration was determined in triplicates. The absorbance was used to calculate the amount of inorganic phosphate (Pi) released when extrapolated on the standard phosphate curve.

The Pi released was as a result of ATP breakdown to ADP and Pi by the enzyme adenosine triphosphatase (ATPase) present in the membrane of the tissue (liver homogenate).

ATP $\xrightarrow{\text{ATPase}}$ **ADP + Pi**

These amount of Pi released were determined in the presence and absence of antisickling agents (moduretic and nifedipin) and antihypertensive agent (hyroxyurea) by the enzyme from the liver.

The Specific Activities of the Enzymes

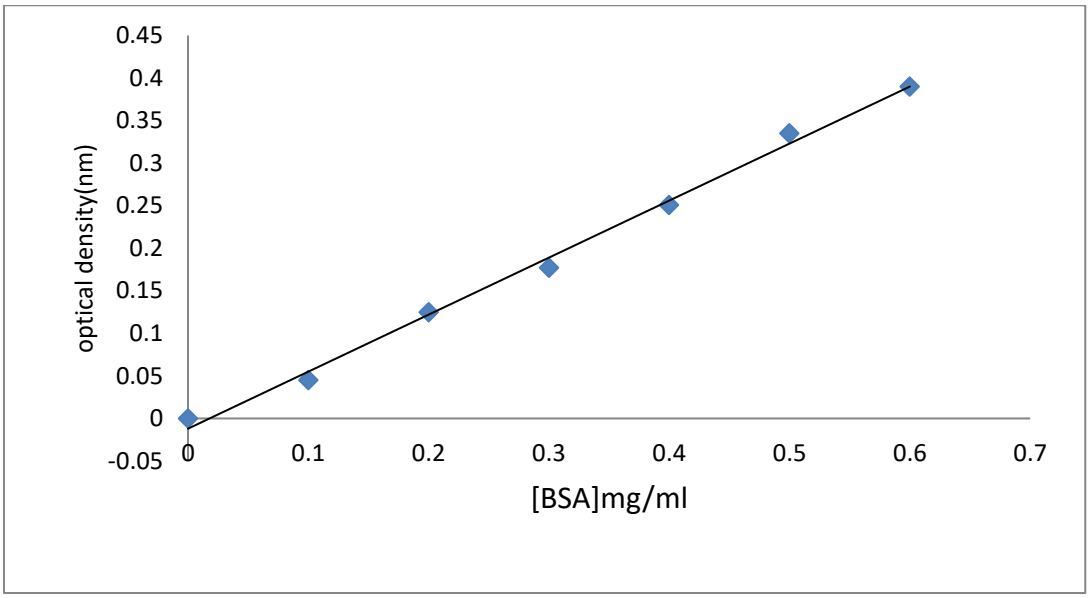
The specific activity of the enzymes was determined using the formular bellow;

$$\text{Specific activity (umole Pi/ mg Pr. /hr)} = \frac{\mu\text{mole Pi}}{\text{Mg Protein x time (hour)}}$$

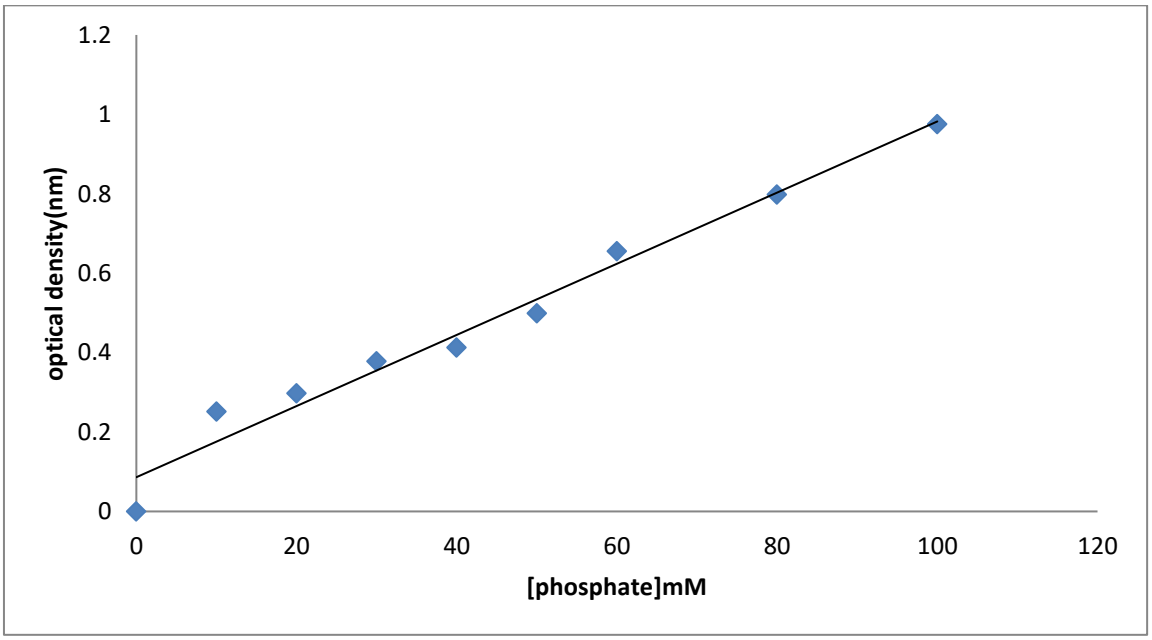
This is then used to plot the drug dependence and Michaelis - Menten plots.

APPENDIX III (Standard Curves)

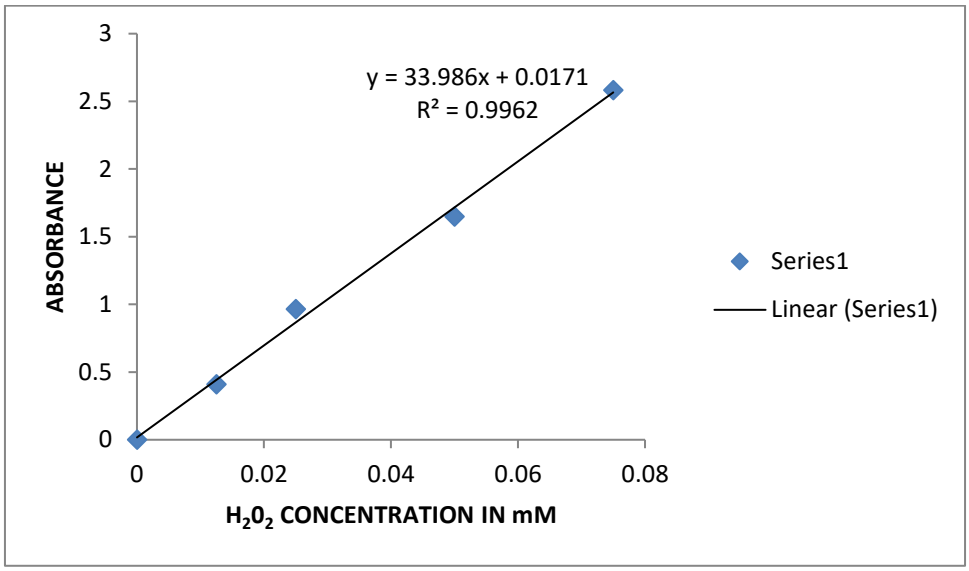
III-A: Standard curve for protein determination



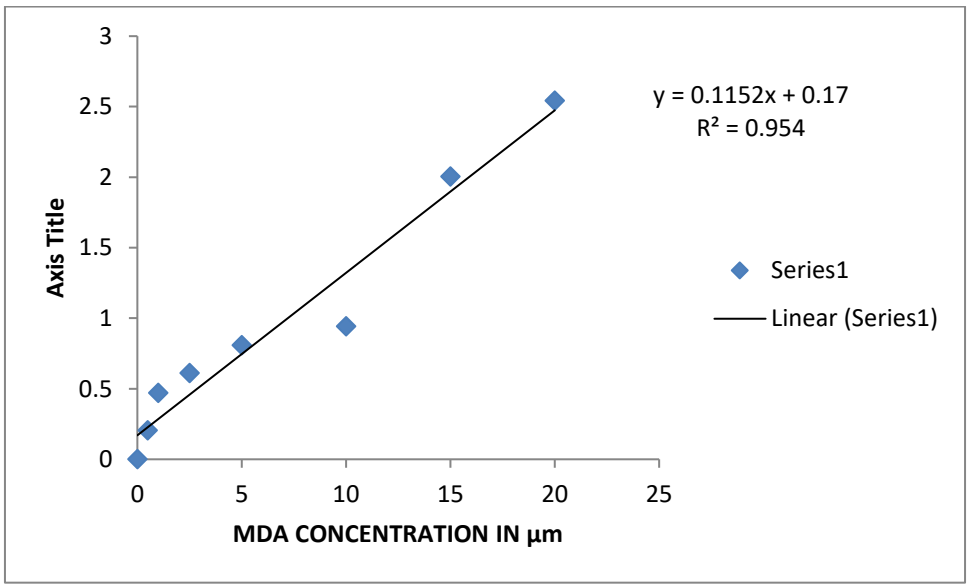
III-B: The standard curve for inorganic phosphate determination

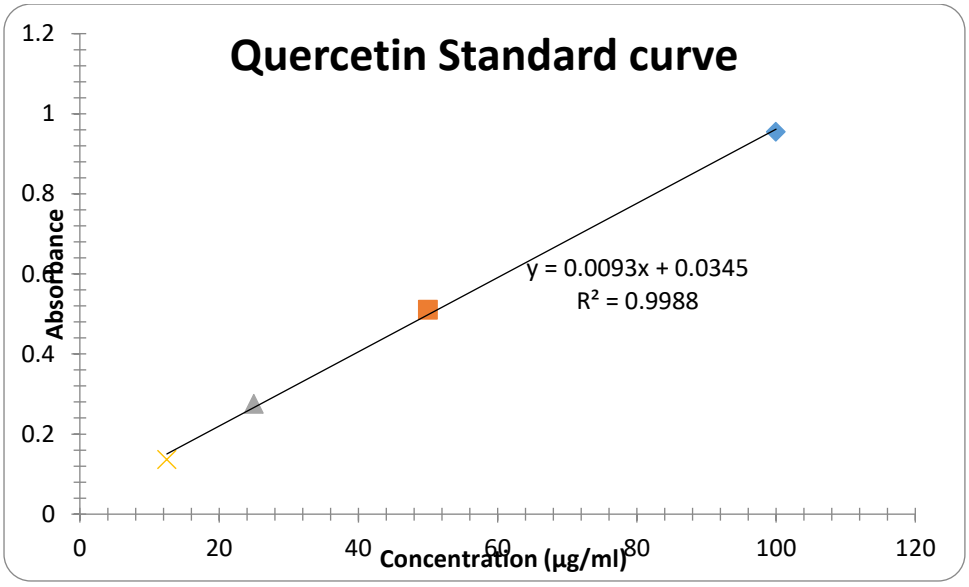


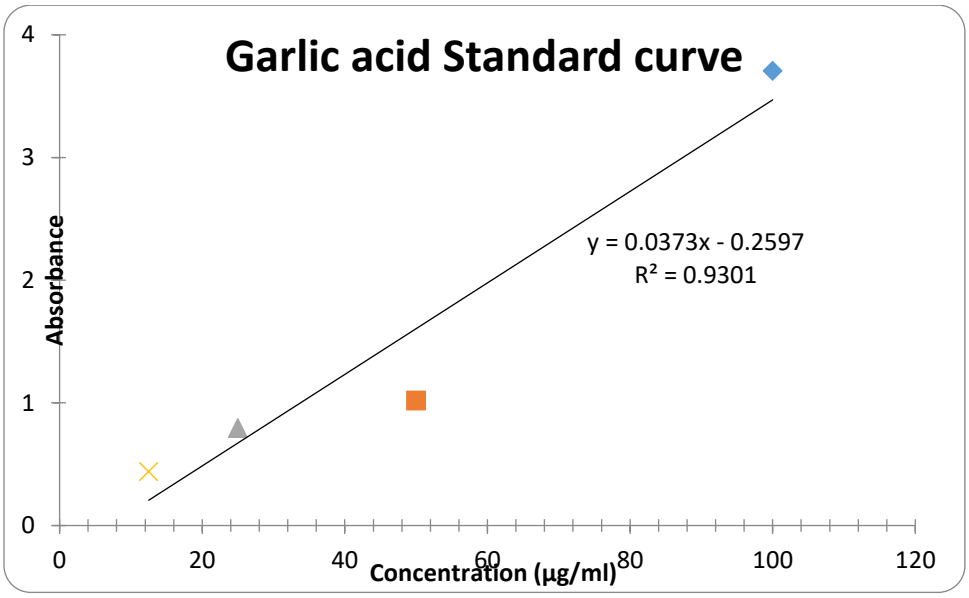
III- C: The standard curve for H_2O_2 concentration

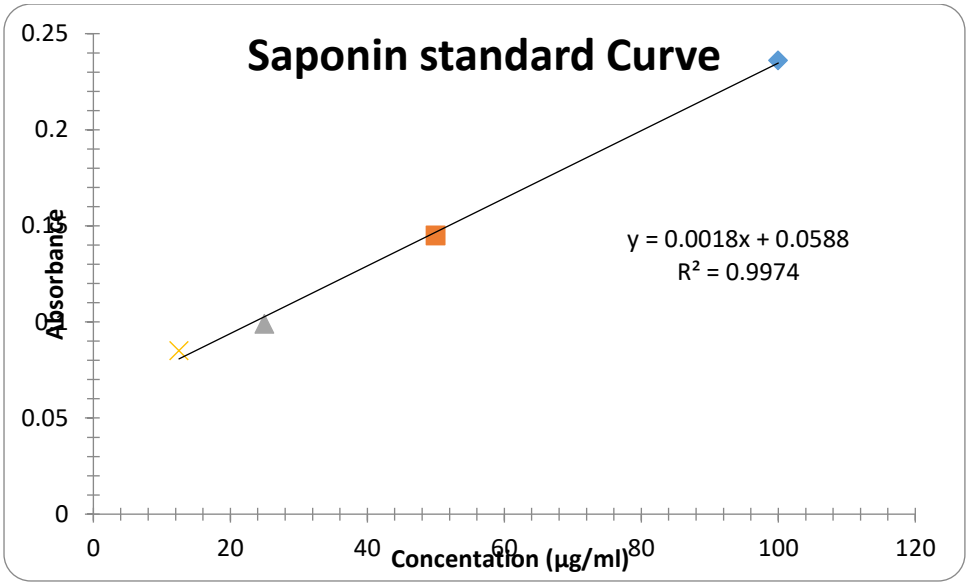


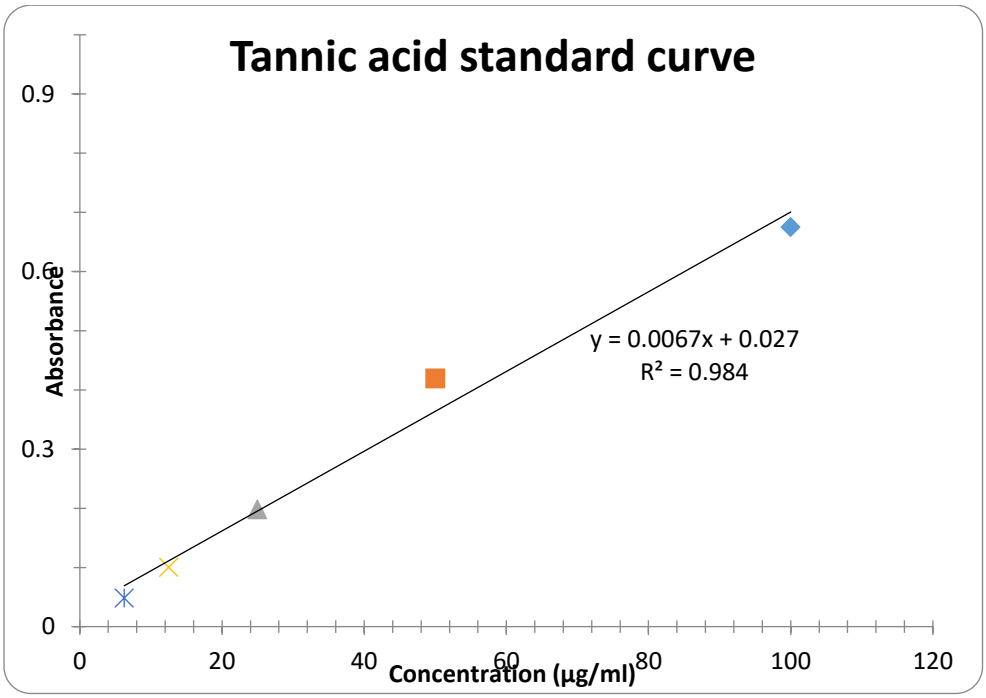
III- D: MDA Concebtration Standard curve



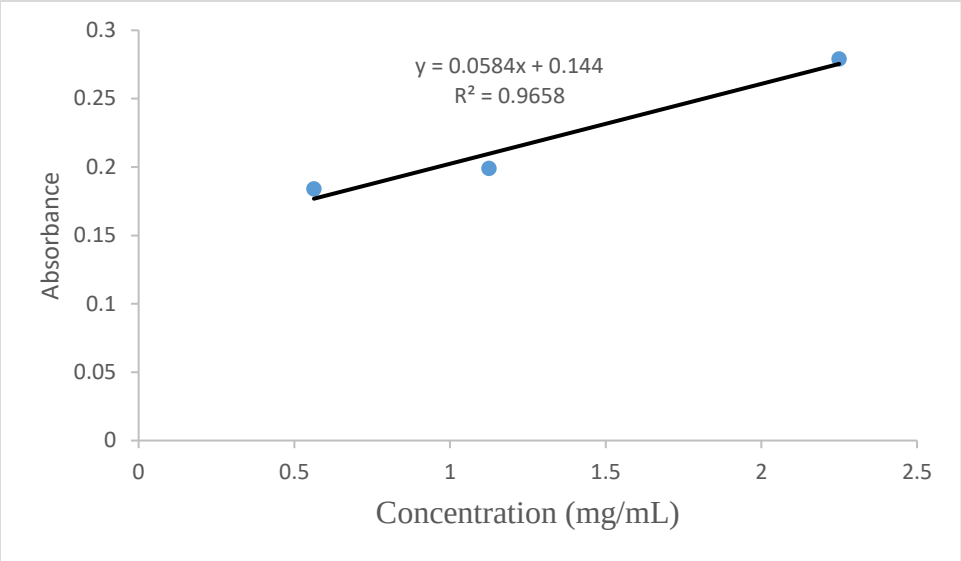

Quercetin Standard curve
Absorbance
Concentration (µg/ml)
y = 0.0093x + 0.0345
R² = 0.9988
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80
100
120


Garlic acid Standard curve
Absorbance
Concentration (µg/ml)
y = 0.0373x - 0.2597
R² = 0.9301
0
1
2
3
4
0
20
40
60
80
100
120


Saponin standard Curve
y = 0.0018x + 0.0588
R² = 0.9974
Absorbance
Concentation (µg/ml)
0.25
0.2
0.15
0.1
0.05
0
0
20
40
60
80
100
120


Tannic acid standard curve
y = 0.0067x + 0.027
R² = 0.984
Absorbance
Concentration (µg/ml)
0.9
0.6
0.3
0
0
20
40
60
80
100
120

Standard curve for terpenoid



Constituents GSH standard curve

Standard	GSH Conc(mM)	Vol of distilled water(µl)	Source(µl)
S1	0	1000	-
S2	0.0625	500	500 of S3
S3	0.125	500	500 0f S4
S4	0.25	500	500 of S5
S5	0.5	500	500 of S 6
S6	1	500	500 of stock (2 mM solution)

Standard curve preparation

Standard	MDA Conc (µM)	Vol of distilled water (µl)	Volume of 20µM MDA Stock (µl)
S0	0	400	-
S1	0.5	390	10
S2	1.0	380	20
S3	2.5	350	50
S4	5.0	300	100
S5	10.0	200	200
S6	15.0	100	300
S7	20	-	400