EFFECTS OF SELECTED MEDICINAL PLANT EXTRACTS ON MITOCHONDRIAL FUNCTION

A THESIS SUBMITTED

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IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF
MASTER OF PHILOSOPHY IN BIOCHEMISTRY

INTEGRI PROCEDAMUS

TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY,
UNIVERSITY OF GHANA, LEGON

DECEMBER, 2015
DECLARATION

I declare that this thesis, with the exception of cited references, is my original research work and no part of this thesis has previously been submitted for the award of a degree or any other qualification at this University or any other institution. This work was done under the supervision of Dr. Augustine Ocloo and Dr. Regina Appiah-Opong at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and the Noguchi Memorial Institute for Medical Research (NMIMR).

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Mitochondria are organelles present in the eukaryotic cell that produce about 90% of the energy required by the cell through oxidative phosphorylation. They also play vital roles in other metabolic processes in the cell. They have unique features that make them sensitive to a number of xenobiotics. Thus, they serve as primary or secondary targets for these xenobiotics. Medicinal plants contain a range of phytochemicals which also have the potential to interfere with mitochondrial function. Therefore, there is the need to study the effects of these medicinal plants, especially those in Ghana, on mitochondrial function. This study aimed to determine the effects of *Taraxacum officinale*, *Morinda citrifolia* and *Millettia thonningii* on mitochondrial function. The antioxidant properties, phenolic content and phytochemistry of the extracts were determined. Effects of the extracts on cell viability and mitochondrial respiratory chain activity were also determined. All the extracts significantly stimulated basal respiration and also caused an increase in complex IV respiration. The extracts showed strong antioxidant activities and all of them contained phytochemicals which have been shown to have antioxidant properties. *M. thonningii* partially blocked the effect of rotenone while Noni juice partially blocked the effect of antimycin A. The extracts, however, had weak cytotoxic effects on the Jurkat leukemia cells and the MCF7 breast cancer cells.
DEDICATION

I dedicate this work to my parents, Mr. A.P. Achampong and Mrs Victoria Achampong and my brothers, Mr. Yaw Poku Achampong, Mr. Akwasi Kyere Achampong and Mr. Kofi Duro Achampong for their outstanding support and encouragement.
ACKNOWLEDGEMENTS

I am very thankful to the Almighty God for granting me the strength to complete this thesis. His goodness, endless love and daily blessings endure forever.

I would also like to express my heartfelt thanks to my supervisors for their excellent supervision, valuable suggestions and encouragement. Their guidance and good-natured support helped me greatly throughout my work.

Besides my supervisors, I would also like to thank the members of the Scientific and Technical Committee (STC) and the members of staff at the Department of Biochemistry, Cell and Molecular Biology for improving my research work with their insightful comments.

A special thanks to Isaac Tuffour, Godwin Dziwornu and Bright Azumah for their dedication, patience and enthusiasm in assisting me during sample collection and analyses of the extracts.

Finally, I am very grateful to the technical staff at the Clinical Pathology Department at the Noguchi Memorial Institute of Medical Research and the technical staff at the Department of Biochemistry, Cell and Molecular Biology. Their technical assistance is deeply appreciated.
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ABBREVIATIONS

ADP – Adenosine diphosphate
Ant A – Antimycin A
Asc – Ascorbate
ATP – Adenosine triphosphate
BHT – Butylated hydroxytoluene
CPMR – Centre for Plant Medicine Research
Cyt c – Cytochrome c
DMEM – Dulbecco’s Modified Eagle Media
DNA – Deoxyribonucleic acid
DNA pol-γ – DNA polymerase-γ
DPPH – 2,2-diphenyl-1-picrylhydrazyl
EC₅₀ – Fifty percent effective concentration values of the extracts
ER – Endoplasmic recticulum
ETC – Electron transport chain
Ext – Extract
FBS – Fetal Bovine Serum
G – Glutamate
GAE – Gallic acid equivalent
HIF-1 – Hypoxia-inducible factor-1
IC₅₀ – Fifty percent inhibitory concentration values of the extracts
LAN5 – Human neuroblastoma cells
LLC – Lewis lung carcinoma cells
M – Malate
Mc – Morinda citrifolia
MCF7 – Human breast cancer cells
MCF-7/AZ – Human breast cancer cells
MELAS – Mitochondrial encephalomyopathy and stroke-like episodes
MERRF – Myoclonic epilepsy and ragged red fiber disease
MPTP – Mitochondria permeability transition pore
Mt – Millettia thonningii
mtDNA – mitochondrial DNA
MTT – [3 - (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]
Nj – Noni juice
NSAIDs – Non-steroidal anti-inflammatory drugs
OXPHOS – Oxidative phosphorylation
PUFAs – Polyunsaturated fatty acids
ROS – Reactive Oxygen Species
Rot – Rotenone
RPMI – Roswell Park Memorial Institute
SEM – Standard error of the mean
SkQ – Plastoquinonyl decyltriphenylphosphonium ion
SkQ1 – 10-(6’-plastoquinonyl) decyltriphenylphosphonium
Sol – Solvent
Succ – Succinate
TCA – Tricarboxylic acid
To – *Taraxacum officinale*
USNCI – United States National Cancer Institute
CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

1.1.1 Background

Mitochondria are important organelles present in the eukaryotic cell. They produce about 90% of the energy needed by the cell in the form of adenosine triphosphate (ATP) through a process known as oxidative phosphorylation (OXPHOS) (Nadanaciva et al., 2007). In addition, mitochondria also play essential roles in other metabolic processes in the cell such as apoptosis, heme synthesis, fatty acid β-oxidation and calcium metabolism (Scatena et al., 2007).

Not only are mitochondria important organelles but they also have unique properties that enable them to carry out their functions and also make them sensitive to a number of chemical compounds. They have a double membrane which consists of the outer and the inner membrane. The cholesterol-rich outer membrane delineates the entire organelle while the cardiolipin-rich inner membrane encloses the matrix. In addition, mitochondria are the only organelles in animal cells that contain their own DNA and possess an alkaline (negatively charged) interior (Wallace and Starkov, 2000).

These unique properties provide both primary or secondary targets for drugs and other xenobiotics (Wallace and Starkov, 2000). Consequently, a number of drugs produce their pharmacological effects through their interaction with the mitochondria. The immunosuppressant, Cyclosporin A, for instance, is known to prevent the induction of the
mitochondria permeability transition pore (MPTP) and hence provide protection against ischemic reperfusion injuries (Smith et al., 2012). Idebenone is another drug which acts as an antioxidant. It increases complex I activity of the electron transport chain (ETC) and protects against neurodegeneration and cardiomyopathy (Smith et al., 2012). Methylene blue is known to increase complex IV activity in Alzheimer’s models (Smith et al., 2012).

However, some of these compounds exhibit their toxic effects by interfering with mitochondrial function. Valproic acid, widely used as a drug in the treatment of epilepsy and other seizures, is a substrate for the fatty acid β-oxidation pathway, which takes place primarily in the mitochondrion. The toxicity of valproate is considered to be due primarily to its interference with mitochondrial β-oxidation (Silva et al., 2008). Antiretroviral drugs such as zidovudine have also been shown to induce mitochondrial toxicity (Lewis et al., 2003). Zidovudine inhibits the polymerase function of DNA polymerase-γ (DNA pol-γ)—the enzyme that replicates mtDNA. This inhibition of pol-γ leads to the depletion of mtDNA and thereby causes mitochondrial dysfunction (Lewis and Dalakas, 1995). Well-characterized drugs associated with adverse events to which oxidative stress may contribute include non-steroidal anti-inflammatory drugs (NSAIDs), antipsychotics and analgesics (Deavall et al., 2012).

The toxicity produced by some of these drugs on the mitochondria subsequently leads to bioenergetic failure and mitochondrial dysfunction (Wallace and Starkov, 2000). Impaired mitochondrial function leads to uncoupling of oxidative phosphorylation, excessive production of reactive oxygen species (ROS) and ATP depletion. Mitochondrial dysfunction has been associated with a range of clinical conditions including diabetes, obesity, Parkinson’s disease,
Alzheimer’s disease, cancer, lactic acidosis and retinopathy (Scatena et al., 2007; Wallace, 2013). Thus, although mitochondria serve as important primary targets for drugs, some medications produce toxicity. Therefore, in drug development, including the development of medicinal plant products, it is very important to study the effect of the compound being produced on mitochondrial function in order to predict and prevent the serious side effects of the compounds and the herbal preparations. Gohil et al. (2010) have screened a large array of drugs that are already in clinical use and identified several of them to interfere with mitochondrial function.

Medicinal plant extracts contain a large array of phytochemicals, which include alkaloids, flavonoids, polyphenols and tannins. These phytochemicals just like the synthetic compounds and drugs have the potential to interfere with mitochondrial function (Wallace and Starkov, 2000; Forbes-Hernandez et al., 2014). Phyllanthus urinaria, for instance, has been found to inhibit complex I and complex II of the ETC and significantly induces a decline in mitochondrial respiration (Huang et al., 2014). The dichloromethane extract of the seeds of Millettia thonningii has been shown to inhibit complex I activity and this inhibition is believed to account for its molluscicidal and schistosomicidal activities (Lyddiard and Whitfield, 2001). Similarly, the ethanolic extract of Paulinia pinnata has been found to inhibit complex II activity, which could account for its reported toxicity in fish (Ocloo et al., 2015). Even though there have been some studies on some medicinal plants on their effects on mitochondria, there is little information currently available on the effects of medicinal plants in sub-Saharan Africa on mitochondrial function.
1.1.2 Problem Statement

About 80% of the population in sub-Saharan Africa rely on medicinal plants for various ailments (WHO, 2000). Although there have been some studies on the efficacy and toxicity of the plant extracts, there is still a lot more to be done. Medications for many diseases produce toxicity on the mitochondria and this toxicity results in mitochondrial dysfunction and subsequently in diseased conditions. Mitochondria pharmacology and toxicology have advanced and many compounds are currently being studied for their effect on mitochondria because of the importance of the organelle and its sensitivity to several compounds. Yet, a lot remains to be done on medicinal plants, especially, those in Ghana, to determine their effects on mitochondria.

1.1.3 Justification

Because of their unique properties and the important roles they play in cells, mitochondria are important therapeutic targets. Mitochondria serve as primary and secondary targets for drugs which produce their therapeutic effects by interacting with mitochondrial function. Therefore, understanding mitochondrial function in normal and pathological states is vital for developing the therapeutic potential of mitochondria in preventing and treating diseases. An understanding of mitochondrial function would also lead to the elucidation of the mechanisms of natural, pharmaceutical and environmental chemicals which induce mitochondrial toxicity by interfering with mitochondrial bioenergetics. Research on mitochondrial function is therefore, very crucial in pharmacological and toxicological analysis of drugs including herbal preparations.

A number of drugs have been assessed for their pharmacological and toxicological effects on mitochondrial function. However, there is limited information on the pharmacological and
toxicological effects of medicinal plants, particularly those in Ghana, on mitochondrial function. Such information would be very vital in identifying novel therapeutics as well as short term and long term toxicity associated with these medicinal plants.

1.1.4 Aim

The aim of the study was to investigate the effects of *Taraxacum officinale*, *Morinda citrifolia* and *Millettia thonningii* on mitochondrial function.

1.1.4.1 Specific objectives: The specific objectives were to:

1. Directly determine the effects of aqueous leaf extracts of *T. officinale*, *M. citrifolia* and *M. thonningii* as well as *M. citrifolia* fruit juice (Noni juice) on mitochondria complexes I, II, III and IV activity *in situ* in permeabilized cardiac fibers.

2. Indirectly assess the effects of the aqueous leaf extracts of *T. officinale*, *M. citrifolia* and *M. thonningii* as well as Noni juice on mitochondrial function in leukemia (Jurkat) and breast cancer (MCF7) cell lines using the MTT assay.

3. Determine the antioxidant activities, phenolic content and phytochemistry of the leaf extracts of *T. officinale*, *M. citrifolia* and *M. thonningii* and Noni juice.
1.2 LITERATURE REVIEW

1.2.1 Mitochondria: The Organelle

Mitochondria are double membrane-bound organelles found in the cytoplasm of almost all eukaryotic cells. They are motile and highly dynamic and exist in different shapes and sizes. They may be $0.5 - 1 \mu m$ in diameter and up to $7 \mu m$ long (Krauss, 2001). They may appear spherical, elongated, or even branched (Wallace and Starkov, 2000). The number of mitochondria found in a particular cell depends on the organism, the tissue type and the energy requirements of that cell. The number may vary from a few mitochondria in, for instance, a rat thymus lymphocyte, to a massive and dynamically fluctuating network of single interconnected mitochondria in a human fibroblast (Wallace and Starkov, 2000). In addition, tissues with a high capacity to perform aerobic metabolic functions such as liver, brain, cardiac and skeletal muscles contain the largest number of mitochondria and are most susceptible to mitochondrial pathologies and to drugs acting on mitochondria (Szewczyk and Wojtczak, 2002).

Despite the structural diversity of mitochondria and their wide variability in number in different tissues, all mitochondria share several fundamental properties regardless of the cell type (Wallace and Starkov, 2000). They contain two phospholipid bilayer membranes: the outer membrane and the inner membrane (Figure 1.1). The two membranes are distinct in appearance and chemical composition. The smooth outer membrane encloses the entire organelle and is structurally similar to other cell membranes (Scatena et al., 2007). It is rich in cholesterol and contains protein structures called porins which allow molecules up to 14 kDa to pass through them (Wallace and Starkov, 2000; Scatena et al., 2007). Hence, the outer mitochondrial membrane is widely permeable to nutrient molecules, ions, ATP and ADP molecules (Lejay et
The inner membrane is more complex in structure than the outer membrane. It is folded into convoluted structures called cristae which increase the surface area of this membrane (Marín-García, 2013). The inner membrane encloses a compartment called the matrix, where the mitochondrial DNA and other soluble enzymes are located.

Thus in animal cells, the mitochondria are the only organelles that contain their own DNA. The mitochondrial genome consists of a small circular chromosome that contains a total of 37 genes. Thirteen of these genes encode proteins that are components of the ETC (Scatena et al., 2007). The ETC, located in the inner membrane, is the main enzymatic machinery of oxidative phosphorylation (OXPHOS). Enzymes found in the matrix include the enzymes of the citric acid cycle with the exception of succinate dehydrogenase, which is bound to the inner membrane and also forms part of complex II of the ETC (Krauss, 2001). Enzymes of the β-oxidation pathway are also located in the matrix. Unlike the outer membrane, the inner membrane contains very
little or no cholesterol. However, it is rich in cardiolipin, a phospholipid that binds the proteins of the electron transport chain (Scatena et al., 2007). The inner membrane is a rigid barrier and is selectively permeable to certain metabolites (Marín-García, 2013). The inner membrane and the outer membrane are separated by the intermembrane space which plays a vital role in oxidative phosphorylation (Lejay et al., 2007).

1.2.2 Mitochondria in Health and Diseases

Mitochondria are the main sites of oxygen metabolism and their main physiological function is the generation of energy in the form of adenosine triphosphate (ATP) (Smith et al., 2012). Thus, they are referred to as the energy “powerhouses” of the cell. Mitochondrial respiration is very important for normal cell life, especially cells of the heart, brain and skeletal muscle, which have high energy demands (Guzun et al., 2009). Mitochondria make approximately 85–90% of the cell’s ATP by oxidative phosphorylation. OXPHOS is a process that couples the oxidation of substrates to ATP synthesis (Chance et al., 1979; Shigenaga et al., 1994). This process relies on a series of enzyme complexes (I–V) (Smith et al., 2012) known as the electron transport chain (Figure 1.2).

The ETC is composed of enzymes and low molecular weight redox intermediates (coenzymes) that transport electrons from respiratory substrates to molecular oxygen (Szewczyk and Wojtczak, 2002). Complex I (NADH dehydrogenase) catalyzes the transfer of two electrons from NADH to ubiquinone via flavin mononucleotides, producing NAD$^+$ and four protons, which are pumped into the intermembrane space (Salway, 2004). Electrons released by the oxidation of FADH$_2$ or succinate are transferred to complex II (succinate dehydrogenase) which is devoid of proton pumping activity (Smith et al., 2012; Gaude and Frezza, 2014). Complex II
represents a link between the tricarboxylic acid (TCA) cycle and the mitochondrial respiratory chain.

Figure 1.2: Schematic diagram of the electron transport chain showing the enzyme complexes and the substrates involved in oxidative phosphorylation. Source: Cole et al., 2011

Complex III (coenzyme Q–cytochrome c reductase) catalyzes the electron transfer from reduced ubiquinone or coenzyme Q 10 to cytochrome C and this is followed by the pumping of four protons into the intermembrane space. The Q cycle facilitates electron transport and proton translocation in complex III (Krauss, 2001). Cytochrome C oxidase (complex IV) is the terminal complex of the respiratory chain and it catalyzes the reduction of oxygen into water and four protons, which are pumped into the intermembrane space (Gaude and Frezza, 2014). The energy released by the electrons during their transfer is used to pump the protons from the matrix into the intermembrane space. This creates an electrochemical proton gradient across the inner
membrane. This gradient drives the synthesis of ATP by ATP synthase (complex V) from Adenosine diphosphate (ADP) and inorganic phosphate utilizing the back flow of protons through the ATP synthase complex into the matrix (Forbes-Hernández et al., 2014). This gradient makes the matrix alkaline and negatively-charged.

In addition to their well-known role in oxidative phosphorylation, mitochondria play essential roles in metabolic processes such as the tricarboxylic acid (TCA) cycle, apoptosis, urea generation, heme synthesis, fatty acid β-oxidation and calcium metabolism (Scatena et al., 2007; Murphy et al., 2009; Duchen and Szabadkai, 2010; Wallace et al., 2010; Forbes-Hernández et al., 2014). Mitochondria are also known to play crucial roles in growth and development, reactive oxygen species production and clearance, oxygen and hypoxia sensing, the control of cell cycle, monitoring of cell differentiation and the innate immune system (Lejay et al., 2007; Smith et al., 2012; Forbes-Hernández et al., 2014).

In general, abnormality in any of the processes in which mitochondria are involved can be referred to as mitochondrial dysfunction (Brand and Nicholls, 2011). Impaired mitochondrial function leads to uncoupling of oxidative phosphorylation, excessive production of reactive oxygen species (ROS) and ATP depletion. ROS are molecules that are chemically reactive due to the incomplete reduction of oxygen (O₂) (Perry et al., 2013). They include superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH’). Defects in the transfer of electrons cause accumulation of electrons in the electron transport chain and enhance ROS production, a condition that contributes to diseased conditions. The principal sites for ROS production are complexes I and III of the respiratory chain (Beyer, 1992; Cadenas and Davies, 2000).
Mitochondria are not only the main site of ROS generation in the cell, but are also the primary target for ROS. The noxious action of ROS mainly consists of the peroxidation of lipids, especially phospholipids of biological membranes, and oxidative damage to proteins and DNA (Halliwell and Gutteridge, 1990; Lenaz et al., 1999; Cadenas and Davies, 2000). ROS-mediated damage to biomolecules can have direct effects on the components of the ETC. OXPHOS enzymes and mtDNA are particularly vulnerable to free radicals within the mitochondria (Forbes-Hernández et al., 2014). Direct damage to mitochondrial proteins declines their affinity for substrates or coenzymes and, therefore, decreases their functional efficiency (Liu et al., 2002).

Owing to its close proximity to the electron transport chain and its lack of histones, mtDNA is less protected from radical damage than nuclear DNA (Duchen, 2004). Mitochondrial DNA mutations cause impaired respiratory chain functioning. Mild mtDNA mutations affect caloric metabolism and result in metabolic abnormalities such as diabetes and obesity and/or affect the most energy-demanding organs such as the brain and lead to late-onset degenerative diseases, such as Parkinson’s disease and Alzheimer’s disease (Krieger and Duchen, 2002; Schon and Manfredi, 2003; Wallace, 2013). Severe mtDNA mutations, like myoclonic epilepsy and ragged red fiber disease (MERRF) (Wallace et al., 1988; Shoffner et al., 1990) and mitochondrial encephalomyopathy and stroke-like episodes (MELAS) (Goto et al., 1990), cause progressive multisystem diseases, frequently resulting in premature death. MtDNA mutations are also found in a wide array of human cancers including colon, breast, lung, prostate, liver, pancreatic, kidney, thyroid, brain and ovarian cancer and are usually associated with bioenergetic defects (Chatterjee et al., 2006). Dysfunctional mitochondria contribute to the metabolic reprogramming
of cancer cells and also modulate a plethora of cellular processes involved in tumorigenesis (Gaude and Frezza, 2014). Because mitochondrial dysfunction contributes to various human pathologies, mitochondria are important therapeutic targets.

Mitochondria are organelles with unique structural and functional characteristics that make them important targets for selective delivery of drugs as well as xenobiotic-induced bioenergetic failure (Szewczyk and Wojtczak, 2002). The most common target of mitochondrial toxicants is the inner membrane. The regulated permeability of the inner membrane could be compromised by xenobiotics either by increasing the permeability of the inner membrane or by inhibiting transport proteins embedded within it (Wallace and Starkov, 2000). Many drugs (e.g. adriamycin-like anthraquinones) have a very high affinity for cardiolipin present in the inner membrane and these drugs preferentially bind to and concentrate in the inner mitochondrial membrane (Wallace and Starkov, 2000). Mitochondria also have alkaline and negatively charged interior which promote the accumulation of lipophilic compounds of cationic character and weak acids in their anionic form (Nicholls and Ferguson, 1992).

1.2.3 Mitochondria as Primary and Secondary Targets for Drugs and Other Xenobiotics

Mitochondria have long been known to be sensitive to a number of chemical compounds including drugs, nutrients and hormones. Therefore, a number of natural, commercial, pharmaceutical and environmental chemicals manifest toxic effects by interfering with mitochondrial bioenergetics ultimately leading to mitochondrial failure and/or dysfunction (Wallace and Starkov, 2000). The unique structural and functional properties of mitochondria provide a number of primary and secondary targets for drugs and other xenobiotics (Wallace and
Starkov, 2000). These properties also offer opportunities for discovery of novel mitochondria targeted pharmacological agents and insight into the potential toxicity of many xenobiotics.

Some of the xenobiotics primarily target mitochondria and directly affect mitochondrial function. The ETC can be directly affected by the alteration of a single complex or indirectly by the generation of free radicals by agents that disrupt the ETC. Metformin is a drug widely used for the treatment of non-insulin-dependent diabetes. It is known to slowly accumulate within mitochondria and directly inhibit complex I (Owen et al., 2000). Inhibition of complex I by Metformin restrains hepatic gluconeogenesis while increasing glucose utilization in peripheral tissues. This inhibition can also account directly for the modest inhibition of fatty acid oxidation that occur following Metformin treatment as well as lactic acidosis, an occasional side effect (Owen et al., 2000).

Lipophilic cations accumulate preferentially within the negatively charged mitochondrial matrix and several pharmacological strategies take into consideration this remarkable property for targeting mitochondria in the treatment or prevention of many diseases (Frantz and Wipf, 2010). Antioxidants have been specifically introduced into mitochondria within the intact cell by covalently coupling antioxidant moieties with the lipophilic triphenylphosphonium cation (Smith et al., 1999; Coulter et al., 2000; Murphy and Smith, 2000). Recently, new types of compounds called SkQs have been synthesized based on mitochondrial targeting by cationic conjugates (Figure 1.3). They consist of a plastoquinone (an antioxidant moiety), a penetrating cation, and a decane or pentane linker. SkQs have been found to have a strong effect on myocardial infarction
or stroke. They also inhibit development of age-related diseases and traits such as cataract, retinopathy, glaucoma and osteoporosis (Skulachev et al., 2009).

![SkQ1](image)

**Figure 1.3:** Structure of SkQ1, an SkQ derivative. It comprises a plastoquinone, a penetrating cation and a decane or pentane linker and is known to have therapeutic effects on mitochondria associated disorders.

**Source:** Skulachev et al., 2009

Some xenobiotics have their primary targets at other cellular locations and their interactions with mitochondria are secondary. For instance, Cyclosporin A, is a powerful immunosuppressive agent and it targets T cells by blocking the transcription of cytokine genes in activated T cells (Rovira et al., 2000). However, it is also known to prevent the opening of the mitochondrial permeability transition pore (MPTP) through its interaction with Cyclophilin D, a cyclosporine-binding protein which confers sensitivity of the pore complex to Cyclosporin A (Duchen, 2004). MPTP is known to contribute to ischemia reperfusion and Cyclosporin A provides protection against ischemic reperfusion, especially during brain injury.

### 1.2.4 Plant Phytochemical Constituents and their Potential Effect on the Mitochondria

Medicinal plants have been used for many centuries in most parts of the world for the treatment of diseases and infections. Their therapeutic effects have also been confirmed by a number of scientific studies. Plant medicine is increasingly gaining popularity in both the developed and the
developing world. Medicinal plant extracts are normally consumed as crude herbal preparations. They have been used to treat ailments such as hypertension, atherosclerosis and arrhythmia (Ocloo and Dongdem, 2012).

Extracts from medicinal plants contain a variety of phytochemical compounds (Figure 1.4). Phytochemicals are compounds found in plants that are not required for normal functioning of the body, but have beneficial effects on health or play roles in amelioration of diseases (Ayoola et al., 2008). The phytochemical constituents of a plant often determine the physiological action on the human body (Pamplona-Roger, 1998). Phytochemicals, like many xenobiotics, have the potential to interfere with mitochondrial function (Wallace and Starkov, 2000; Forbes-Hernandez et al., 2014). An isoflavonoid, 4-O-methylalpinumisoflavone, isolated from Lonchocarpus glabrescence, was found to inhibit HIF-1 activation by inhibiting mitochondrial respiration and protein synthesis (Liu et al., 2009). In another study, whole plant extract of Phyllantus urinaria was found to induce ROS production at the mitochondrial inner membrane by inhibiting complex I and complex II and thus significantly inducing a decline in mitochondrial respiration (Huang et al., 2014). Due to its interaction with the ETC and subsequent generation of free radicals, P. urinaria disrupted mitochondrial function in Osteosarcoma 143B cells and this resulted in mitochondrial dysfunction (Huang et al., 2014). Phytochemicals can be classified as phenolics, flavonoids, alkaloids, terpenoids, saponins, tannins and glycosides (Kennedy and Wightman, 2011; Forbes-Hernandez et al., 2014). Some of these compounds are known to have antioxidant properties.
Figure 1.4: Representative chemical structures of some phytochemicals
Antioxidants are phytochemicals that protect cells from oxidative damage caused by free radicals. They inhibit the formation of free radicals by scavenging species that initiate peroxidation or chelating metal ions and making them unable to generate reactive species (Atawodi, 2005). They also stabilize the respiratory complexes and increase availability of electrons, consequently, resulting in better coupling of substrate oxidation to phosphorylation (Martin et al., 2002). Antioxidants reduce the risk of disorders such as cancer (Surh and Fergusson, 2003), cardiovascular diseases and ischemia/reperfusion injury (Marczin et al., 2003). The major therapeutic application of mitochondria-targeted therapies so far has been as antioxidants to block mitochondrial oxidative damage (Smith et al., 2012). Antioxidants include nutrient antioxidants: vitamins A, C and E, the minerals: copper, zinc and selenium, and polyphenols.

Liposoluble vitamins such as vitamins A and E are known to influence mitochondrial functionality. Vitamin A is a group of organic molecules that includes retinol, retinal and retinoic acid. They have been shown to have antioxidant, anti-proliferative properties as well as pro-apoptotic activity related to mitochondrial pathways (Bushue and Wan, 2010). Vitamin E is a group of lipophilic chain breaking antioxidants composed of naturally occurring α-, β-, γ- and δ-tocopherols and α-, β-, γ and δ tocotrienols that are synthesized exclusively by photosynthetic organisms (Nowak et al., 2012). The antioxidant properties of vitamin E and its capacity of preventing either polyunsaturated fatty acid (PUFAs) oxidation or other events driven by free radicals are well known (Forbes-Hernández et al., 2014).
Polyphenols are the most abundant dietary antioxidants (Scalbert et al., 2005). They include flavonoids, phenolic acids and phenolic alcohols. Flavonoids are the largest group of phenols. They are reported to be beneficial against inflammation, atherosclerosis, hypertension and neurodegeneration (Ossola et al., 2009; Perez-Vizcaino et al., 2009; Chirumbolo, 2010; Ishizawa et al., 2011). One principal example of flavonoids is quercetin. It has been shown to act as an antidote to rotenone, up-regulating the complex I activity in damaged or normal dopaminergic neurons in neurotoxin-induced hemi parkinsonian rats (Karuppagounder et al., 2013). Quercetin supplementation also reverses inhibition of respiratory chain complexes (complexes II, IV and V) induced by 3-nitropropionic, restores ATP levels, attenuates mitochondrial lipid peroxidation and prevents mitochondrial swelling in an induced model of Huntington’s disease (Sandhir and Mehrotra, 2013). One common phenolic acid is caffeic acid. Caffeic acid is able to significantly attenuate oxidative damage and impairment in ETC activity (Forbes–Hernandez et al., 2014). One of the most common phenolic alcohols is hydroxytyrosol (3,4-dihydroxyphenylethanol). It enhances mitochondrial function, increasing activity and protein expression of mitochondrial complexes I, II, III and V, thus increasing oxygen consumption (Forbes–Hernandez et al., 2014).

Tannins are complex high molecular compounds. They include water-soluble polyphenols that are present in many plant foods. They have been reported to exert physiological effects such as the acceleration of blood clotting, reduction in blood pressure, decrease in the serum lipid level and modulation in immunoresponses (Chung et al., 1998). The effects of tannins are often depicted as beneficial for cell survival, such as prevention against oxidative insults and protection against cancer (Liu et al., 2004). However, their actions are complex and often seemingly antagonistic or paradoxical. For example, although some tannins are described as
antioxidant agents for protecting against cytotoxic insults to cells, others have been reported to be prooxidants and apoptotic (Liu et al., 2004).

Terpenoids are a class of hydrocarbons that consist of terpenes attached to an oxygen-containing group. They are also known as isoprenoids and include naturally-occurring aromatic compounds. They exhibit various important pharmacological activities such as anti-inflammatory, antioxidant, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and antibacterial activities (Mahato and Sen, 1997; Grassmann, 2005). A combination of terpenes i.e. camphene and geraniol, was found to prevent oxidant-antioxidant imbalance and its downstream apoptotic events during nimesulide-induced hepatotoxicity (Singh et al., 2012).

Alkaloids are naturally occurring organic nitrogen-containing bases. Many alkaloids possess anesthetic properties. Others are used as cardiac or respiratory stimulants (Hérouart et al., 1988). Some alkaloids have been identified to act on the ETC with consequent decrease of ATP levels. Recently, it has been reported that an alkaloid, capsaicin, can induce oxidative stress by alteration in the mitochondrial electron transport chain (Urra et al., 2013). It selectively inhibits complexes I and III with consequent ATP depletion in pancreatic cancer cells (Urra et al., 2013).

Saponins constitute a complex and chemically diverse group of compounds. They derive their name from their ability to form stable, soaplike foams in aqueous solutions (Shi et al., 2004; Man et al., 2010). They contain a carbohydrate moiety attached to a triterpenoid or steroid. Saponins make the sarcolemma permeable by destroying cholesterol-containing membranes. The structure and function of mitochondria remain intact after treatment with saponin (Veksler et al.,
Saponins decrease blood lipids, lower cancer risks, blood glucose response and cholesterol levels (Shi et al., 2004).

Cardiac glycosides are organic compounds containing sugars that act on the contractile force of the cardiac muscle. Their primary target is the $\text{Na}^+/\text{K}^+$-ATPase. They have been commonly used in the treatment of congestive heart failure (Gheorghiade et al., 2004). Unfortunately, the therapeutic use of cardiac glycosides in treating heart failure is limited by their adverse side effects, including cardiac arrhythmias (Ferrier, 1977). They have been used traditionally as arrow poisons, abortifacients, emetics, diuretics, and heart tonics (Newman et al., 2008).

Some phytochemicals have been shown to contain precursors for novel pharmaceuticals. However, the biologically active substances present in medicinal plants have been structurally defined and standardized for only a few of the plants (Shaw et al., 1997). Furthermore, many herbal remedies used today have not undergone careful scientific assessment, and some have the potential to cause serious toxic effects and major drug-to-drug interactions (Mashour et al., 1998). Pharmacological and toxicological evaluations of medicinal plants are therefore essential not only for drug development but also for ascertaining the mechanisms of short-term and long-term toxicity associated with these medicinal plants. Thus, considering the important role of mitochondria in pharmacology and toxicology, they offer an opportunity for pharmacological and toxicological evaluation of medicinal plants.

A number of pharmaceuticals such as meclizine, phenformin, artemisinin and pentamidine have been screened for their pharmacological and toxicological effects on mitochondrial function and
energy metabolism (Gohil et al., 2010). However, there is paucity of information on the effect of herbal extracts, particularly those in Ghana, on mitochondrial function. Additionally, although there is very little or no knowledge of the chemical constituents of most of the herbal mixtures produced from these medicinal plants, the use of herbal mixtures is becoming increasingly popular in Ghana. Such knowledge would aid in the discovery of novel pharmaceuticals as well as identifying medicinal plant products with potential long- and/or short-term toxicity (Ocloo and Dongdem, 2012).

1.2.5 The Selected Medicinal Plant Extracts or Products

1.2.5.1 Taraxacum officinale

*Taraxacum officinale* is a popular medicinal plant in Ghana and most parts of the world. It is a herbaceous perennial plant of the family Asteraceae (Özcan et al., 2012). It is most commonly known as Dandelion (Figure 1.5). It grows to a height of about 12 inches and has culinary and medicinal uses. The leaves are serrated or deeply toothed and are used in salads and tea (Modaresi and Resalatpour, 2012; Oseni and Yussif, 2012). They are high in fiber, potassium, iron, calcium, magnesium, phosphorus, vitamins A and C, the B vitamins: thiamine and riboflavin, and proteins (Schmidt, 1979; Jackson, 1982). The leaves have also been reported to be rich in saponins, phenolics, terpenoids and vitamin D (Yarnell and Abascal, 2009). Several flavonoids including caffeic acid, chlorogenic acid, ferulic acid, luteolin, and luteolin 7-glucoside have also been isolated from *Taraxacum officinale* (Schütz et al., 2005; Shen et al., 2006; Xu and Xu, 2008). The leaves also contain silicon, sodium, zinc, manganese and copper (Cordatos, 1992).
Figure 1.5 Picture showing the aerial view of the *Taraxacum officinale* plant

*T. officinale* is a plant with enormous medicinal values. It has been used in folk medicine in the treatment of hepatic disorders, inflammation, breast and uterine cancers (Mir et al., 2013). It is considered a blood purifier in some communities and is used for ailments such as eczema, jaundice, fever, eye problems and gastrointestinal problems (Oseni and Yussif, 2012). Various parts of the plant have also been used to treat poor digestion, water retention, anemia, hypertension and hepatitis (Dearing et al., 2001; Mahesh et al., 2010; Awortwe et al., 2013). In traditional Chinese medicine, Dandelion has been used for ailments ranging from digestive disorders to complex disorders such as uterine, breast and lung tumors (Sigstedt et al., 2008). It is also acclaimed by traditional Chinese medicine as a non-toxic herb with choleric, diuretic, antirheumatic and anti-inflammatory properties (Mir et al., 2013).

Recently, pharmacological profiling of *T. officinale* has shown diuretic, choleric, anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-allergic, anti-hyperglycemic and anti-thrombotic activities (Ahmad et al., 2000; Schütz et al., 2005). The ethanolic fraction of the
aqueous extract of *T. officinale* has been reported to have good antioxidant properties (Tettey *et al.*, 2014). Furthermore, the ethanolic fraction of the aqueous extract of *T. officinale* leaves partially but significantly blocked the inhibitory effect of rotenone and produced a better coupling effect (higher respiratory control ratio) (Ocloo *et al.*, 2015). Hence, *T. officinale* offers an opportunity to be explored as a natural energy booster.

### 1.2.5.2 *Morinda citrifolia*

*Morinda citrifolia* (Rubiaceae), commonly known as Noni, is a small evergreen shrub which attains a height of 3–10 m at maturity (Figure 1.6). It grows widely throughout the Pacific and is one of the most significant sources of traditional medicines among Pacific island societies (Chunhieng *et al.*, 2005). The fruits have an ovoid form and appear transparent when ripe. The mature fruit has a pungent odour. A number of chemical compounds have been identified in the Noni plant such as potassium, vitamin C, terpenoids, alkaloids, anthraquinones, carotene, vitamin A, flavone glycosides, linoleic acid, ursolic acid, rutin and proxeronine (Peerzada *et al.*, 1990; Higa and Fuyama, 1993; Farine *et al.*, 1996).

The Noni fruit is a rich source of antioxidants, vitamins and minerals and is known to increase energy and vitality. Phenolic compounds such as flavonoids are the most active and common antioxidants present in the fruit (Nijveldt *et al.*, 2001). During the last decade, Noni, mostly marketed as a fermented juice (Figure 1.6), has become a widely traded food supplement worldwide, based on health claims related to some of its compounds, in particular, flavonoids. Noni juice is especially known for its medicinal properties: antibacterial, analgesic, anti-congestive, antioxidant, anti-inflammatory, astringent, laxative, sedative and hypo-tensor
activities (Singh et al., 1984; Singh, 1986). The decoction or infusion of roasted mature unripe fruits has been recommended to relieve the symptoms of nausea and vomiting (Ekpalakorn et al., 1987).

Various parts of the Noni plant have been used traditionally as folk remedy to treat a broad range of diseases including diabetes, hypertension and cancer (Hirazumi et al., 1996; Wang et al., 1999). In addition, other pharmacological activities reported for the Noni plant include analgesic, anti-inflammatory, antioxidant, anti-tumor, hepatoprotective, cardioprotective and antifungal (Pandy et al., 2012).

Figure 1.6 Pictures show a. Morinda citrifolia leaves, b. Noni fruit juice

The traditional medicinal uses of Noni have been supported by recent scientific findings. Wang and colleagues have shown that phytochemical components from Morinda citrifolia are associated with antibacterial and antiviral activities in humans (Wang et al., 2002). Analyses
sponsored by Tahitian Noni Juice, the main global provider of Noni juice, showed that Noni juice enhances the efficacy of anticancer drugs such as Prednisolone and Taxol (Mian-ying et al., 2002) and hence Noni juice could be potentially used in combination with anticancer drugs. The ethanol precipitable fraction (ppt) of noni juice, corresponding to a polysaccharide-rich substance composed of glucuronic acid, galactose, arabinose, and rhamnose, has been found to have immunomodulatory and anti-tumor effects against Lewis lung carcinoma (LLC) (Chan-Blanco et al., 2006). The synergistic effects of Noni juice with the anticancer drugs will therefore require the use of lower doses of the anticancer drugs to achieve the same or even better results in cancer patients (Mian-ying et al., 2002). Despite the existence of scientific evidence supporting the use of Noni in treating various diseases, there is limited information on its effects on mitochondrial function even though the fruit juice is touted as an energy booster.

1.2.5.3 *Millettia thonningii*

*Millettia thonningii* (Figure 1.7) is a deciduous plant that belongs to the family Papilionaceae. It is indigenous to tropical West Africa but also grows in tropical climates all over the world. It can grow to a height of 20 m. It has a grey bark with leaves arranged alternatively on the branches. *Millettia thonningii* has been used in folk-medicine for the treatment of inflammatory, chronic and several pathogenic diseases (Banzouzi et al., 2008). It is also reported to have therapeutic effect against dysmenorrhea and amenorrhea. The leaf extract has been used for the treatment of diarrhea and dysentery in Nigeria. However, the leaf juice is said to be lethal to the Bulinus snail, a water snail which is a vector for *Schistosoma cercariae* which causes schistomiasis (Irvine, 1961; Abbiw, 1990).
Figure 1.7 Picture of the Millettia thonningii plant

The seed extracts have also demonstrated molluscicidal activity toward schistosome-transmitting snails (Evans et al., 1986) and their eggs (Tang et al., 1995). Three isoflavonoids have been isolated from M. thonningii seeds. These are robustic acid, alpinumisoflavone and dimethylalpinumisoflavone (Lyddiard and Whitfield, 2001). When tested against rat liver mitochondria, the dichloromethane extract of the seeds of M. thonningii demonstrated inhibitory activity towards complex I of the electron transport chain in a rotenone-like manner (Lyddiard and Whitfield, 2001). This inhibition is believed to account for the molluscicidal activity of the extract. The leaves of M. thonningii would be analyzed to determine their effect on mitochondrial activity.
1.2.6 Experimental Manipulation of Mitochondrial Function

Assessment of mitochondrial function is fundamental to the study of intracellular energy metabolism. Standard procedures of organelle isolation based on differential centrifugation of tissue or cell homogenates allow precise in vitro characterization of the functional properties of mitochondria (Frezza et al., 2007). However, this method has some disadvantages. Large quantities of tissues are required for optimal yield. Additionally, normal mitochondrial interactions are disrupted in isolated mitochondria. To overcome these limitations, selective permeabilization of cells, skinned muscle fibers and tissue homogenates allow the analysis of the mitochondria within an integrated cellular system (Kuznetsove et al., 2008).

Saponin is a cholesterol-specific detergent that can be used to achieve permeabilization. It has high affinity for cholesterol and interacts with cholesterol-rich membranes such as the plasma membrane (Glauert et al., 1962; Kuznetsov et al., 2008). The cholesterol content of intracellular organelles like mitochondria or endoplasmic reticulum (ER) is considerably lower than the cholesterol content in the plasma membrane. The treatment of the muscle fibers with saponin, therefore, causes a selective perforation of the plasma membrane, leaving the mitochondria intact (Korn, 1969; Comte, 1976; Kuznetsov et al., 2008). This subsequently induces a loss of membrane integrity (permeabilization), so that the barrier between the intracellular space and surrounding medium disappears. The cytosol is washed out and the intracellular space is equilibrated with the experimental incubation medium (Kuznetsov et al., 2008). This allows direct access of exogenous substrates to the mitochondria.
Mitochondrial function can be indirectly assessed by measuring cell viability using the MTT assay. MTT is a yellow water-soluble tetrazolium salt dye. It is converted to a purple water-insoluble formazan product (Figure 1.8) by the succinate dehydrogenase system of an active mitochondrion by the reductive cleavage of its tetrazolium ring (Slater et al., 1963). When cells die, they lose the ability to convert MTT into formazan. The amount of formazan formed can be determined spectrophotometrically at 570 nm and this can be used to estimate the number of active mitochondria as well as viable cells (Denizot & Lang, 1986). Formazan, however, accumulates as an insoluble precipitate inside cells. Therefore, it is solubilized with an acidified organic solvent (eg. isopropanol) before the absorbance is read.

![Figure 1.8 Chemical reaction showing the reduction of MTT to formazan](http://www.biotek.com/assets/tech_resources/10479/figure1.jpg)

Antioxidants are chemical compounds that scavenge free radicals and protect cellular biomolecules from damage. The capacity of plant extracts to interfere with mitochondrial function can be determined by analyzing the antioxidants present in the plant extracts. The
Antioxidant activities of the extracts can be assessed by measuring the free radical scavenging capacities using the DPPH method. This method is an antioxidant assay based on electron-transfer (Huang et al., 2005). DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals (Molyneux, 2003). The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution at about 520 nm. In the presence of an antioxidant molecule, DPPH is reduced, giving rise to pale yellow ethanol solution (Figure 1.9) (Huang et al., 2005). In the DPPH method, the antioxidant activity of an extract is determined from the effective concentration of the extract that causes 50% loss of DPPH activity (EC$_{50}$); the lower the EC$_{50}$ value, the higher the antioxidant activity and vice versa.

![Chemical reaction showing the reduction of the DPPH radical in the presence of an antioxidant](http://www.damocos.co.kr/damo/language/english/lab_paper3.php)

**Figure 1.9** Chemical reaction showing the reduction of the DPPH radical in the presence of an antioxidant

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Bournemouth, U.K.) with the exception of MgCl$_2$.6H$_2$O, sucrose, imidazole and KH$_2$PO$_4$. MgCl$_2$.6H$_2$O was purchased from Scharlau (Barcelona, Spain) while sucrose was obtained from Roth (Karlsruhe, Germany). KH$_2$PO$_4$ was obtained from Merck (Frankfurt, Germany) while imidazole was purchased from Fluka (Belgium).

2.1.2 Plant Materials

The leaves of the three medicinal plants were harvested at different locations. *Taraxacum officinale* Weber leaves were harvested at Adabraka, Accra, Ghana while *Morinda citrifolia* Linn leaves were harvested at Ayikuma in the Greater Accra Region of Ghana. *Millettia thonningii* (Schum. & Thonn) Baker leaves were harvested near the Department of Botany, University of Ghana, Legon, Accra, Ghana. Noni juice was purchased from the Royal Noni Factory at Dzorwulu, Accra, Ghana. The identity of each plant specimen was confirmed by Mr. Prosper Avekor at the Department of Botany, University of Ghana, Legon. The voucher specimen numbers for *T. officinale*, *M. citrifolia* and *M. thonningii* are GC45929, ASMC1014 and CSRPM/406 respectively.
2.2 METHODS

2.2.1 Preparation of Plant Materials

The leaves of *T. officinale, M. citrifolia and M. thonningii* were air-dried for 3 weeks and then pulverized with a blender. Aqueous extracts were prepared from the pulverized samples by adding 500 ml of distilled water to 50 g of the pulverized sample (10 % of mixture prepared). The resulting mixture was heated at 80 °C for 1 hour, cooled to room temperature and centrifuged at 4500 x g for 20 minutes. The supernatant was decanted. The pellet was re-suspended in 500 ml distilled water. The heating and centrifugation of the extracts were repeated and the supernatant obtained was again decanted, collected and added to the previous one. The leaf extracts and Noni juice were freeze-dried in a Supermodulo freeze-dryer (Thermo Electron Corporation, USA).

2.2.2 Analysis of the Effect of the Extracts on Mitochondrial Complexes

2.2.2.1 Preparation of tissues for permeabilization

Male ICR mice between the ages of 3 – 5 months with an average weight of 28.75 g were obtained from the Centre for Plant Medicine Research (CPMR), Akuapem-Mampong, Ghana. The animals were acclimatized in the laboratory for 24 hours and then sacrificed.
The myocardial tissues were isolated using a pair of scissors and forceps. The tissues were placed in ice-cold isolation buffer (2.77 mM CaK$_2$EGTA, 7.23 mM K$_2$EGTA, 5.77 mM Na$_2$ATP, 6.56 mM MgCl$_2$.6H$_2$O, 20 mM taurine, 15 mM Na$_2$Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES). They were then transferred to a plastic petri dish containing a little amount of the ice-cold isolation buffer. Fat and connective tissues were removed. The myocardial tissue was dissected into muscle strips under a dissecting microscope (Figure 2.1). These strips were further dissected into thin muscle fibers.

### 2.2.2.2 Permeabilization of mouse cardiac tissues

Permeabilization of the fibers was carried out as described previously (Kuznetsov et al., 2008).

The fiber bundles were permeabilised by transferring them into a vial containing 2 ml of isolation buffer and 50 μg/ml of saponin and mixed gently on a rocker-shaker at 4 ºC for 20
minutes. The permeabilized fibers were washed by transferring them into another vial containing the respiration medium (0.5 mM EGTA, 3 mM MgCl$_2$.6H$_2$O, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM HEPES, 110 mM sucrose, 1 g/l fatty acid free BSA) and mixed gently at 4 °C for 5 minutes to wash out the saponin. This step was repeated three more times.

### 2.2.2.3 Measurement of oxygen consumption

Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Figure 2.2) (Strathkelvin Instruments Limited, Scotland) in a sealed chamber at 37 °C.

![Figure 2.2](image-url) **Figure 2.2** Setup for measuring oxygen consumption; this consists of electrodes, an oxygen meter, a computer, a water bath, syringes and a timer. The Strathkelvin 782 software was used for data collection and analyses.

The Clark-type oxygen electrode consists of a gold or platinum cathode and silver/silver chloride anode separated by a potassium chloride (KCl) electrolyte solution. The two-half cells are separated from the experimental assay media by an oxygen permeable membrane. When a voltage is applied across the two half-cells, oxygen diffuses through the membrane and is
reduced at the cathode to the hydroxyl radical (OH\(^{-}\)). The KCl electrolyte is buffered to remove the OH\(^{-}\) produced at the cathode. The reduction of each oxygen molecule is accompanied by 4 electrons of current flow in the circuit.

The size of the signal generated by the electrode is proportional to the flux of oxygen molecules to the cathode. This oxygen flux is also proportional to the partial pressure of oxygen (PO\(_2\)) of the respiration media, the permeability of the membrane, the temperature and the surface area of the cathode (Strathkelvin Instruments Ltd, 2012). During the respiration assays, the output signal from the oxygen meter was electronically collected every second by the Strathkelvin 782 system software. The data files were converted to absolute values, based on an oxygen content of 210 \(\mu\)mol of O\(_2\) and on the amount of mitochondrial protein used.

For oxygen consumption measurements, permeabilized fibers (2.5 - 4 mg) were added to 500 \(\mu\)l of a continuously stirred respiration medium. The fibers were incubated with 2 \(\mu\)l of the extracts (10mg/ml) for 2 minutes. A step-by-step functional analysis on the respiratory chain enzyme complexes was done using specific substrates and inhibitors in a substrate–inhibitor titration (Kuznetsov et al., 2008) (Figure 2.3).

In this titration, 1.25 \(\mu\)l of malate (0.8 M) and 2.5 \(\mu\)l of glutamate (2 M) (complex I substrates) were first added to the oxygraph chambers to measure complex I respiration. This was followed by the addition of 5 \(\mu\)l of 0.5 M ADP to obtain maximal mitochondrial respiration. Complex I inhibition was achieved by the addition of 0.25 \(\mu\)l of rotenone (0.01 M). Subsequently, 5 \(\mu\)l of 1 M succinate was added to stimulate complex II respiration. An aliquot of 0.25 \(\mu\)l of 0.02 M
antimycin A was then added to inhibit complex III respiration. This was followed by the measurement of complex IV respiration by the addition of 1.25 µl of 0.2 M TMPD (N,N,N’,N’-tetramethyl-p-phenylenediaminedihydrochloride) and 1.25 µl of 0.8 M ascorbate (artificial substrates of complex IV). The efficacy of the preparation was evaluated by confirming mitochondrial integrity through the addition of 1.25 µl of cytochrome C (4 mM).

Figure 2.3 A representative screenshot of respiratory traces of permeabilized mitochondria in situ using Strathkelvin oxygen system. The red and blue traces represent the rates of oxygen consumption in the two oxygen electrodes.

2.2.3 Determination of the Effects of the Extracts on Cell Viability

The cytotoxic effect of the extracts was assessed using tetrazolium based colorimetric [3 - (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay (Ayisi et al., 2011). The cell lines used in this study were the MCF7 breast cancer and Jurkat leukemia cell lines. The MCF7 cells were maintained in DMEM supplemented with 10% FBS while the Jurkat cells were maintained in RPMI 1640 supplemented with 10% FBS. The cells were
incubated at 37 °C in the presence of 5% CO₂. They were then seeded (1 x 10^5 cells/ml) into 96-well plates pre-treated with varying concentrations (0 – 1000 µg/ml) of the crude extracts. Curcumin was used as the positive control. The experiment was performed in triplicates.

After incubating the cells with the extracts for 72 hours, 20 µl of 2.5 mg/ml MTT solution was added to each well and the incubation continued for 4 hours. The reaction was then stopped with 150 µl of acidified isopropanol after which the plate was incubated in the dark at room temperature overnight. Optical density was read using Tecan Infinite M200 (Austria) plate reader at a wavelength of 570 nm. Percent cell survival was evaluated and IC₅₀ values were determined. Cytotoxicity was determined by the concentration of the extracts at which cellular proliferation was inhibited by 50% (IC₅₀).

2.2.4 Determination of Antioxidant Activities of the Extracts

The antioxidant activities of the extracts and the Noni juice were assessed by measuring the free radical scavenging capacities. The free radical scavenging activities of the extracts and the Noni juice were assessed using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method with slight modification (Blois, 1958). In this method, a concentration of 10 mg/ml of each extract was prepared by dissolving the dry extract in distilled water. An amount of 10 mM butylated hydroxytoluene (BHT) was used as the positive control. Varying concentrations of the extracts ranging from 0 – 5 mg/ml were prepared. The reaction mixture was made up of 100 µl of plant extract or BHT and 100 µl of 0.05 mM DPPH in 96–well plates. Triplicate experiments were performed. The samples were incubated in the dark at room temperature for 20 minutes. The absorbance was then read at 517 nm using a microplate reader (Tecan Infinite M200, Austria).
The free radical scavenging capacity of each extract was calculated as the percent DPPH radical scavenging effect using the formula:

\[
\% \text{ DPPH Scavenging effect} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100,
\]

where \(A_0\) is the absorbance of the blank and \(A_1\) is the absorbance of the extract. The \(EC_{50}\) (extract concentration to produce 50\% reduction of DPPH) values were determined from a plot of \% DPPH scavenging effect versus log concentration of extract; the lower the \(EC_{50}\) value, the higher the antioxidant activity and vice versa.

### 2.2.5 Determination of Total Phenolic Content of Extracts

Total phenolic content of the extracts and Noni juice was determined using the Folin-Ciocalteau assay (Marinova \textit{et al.}, 2005) with slight modification. The Folin–Ciocalteau reagent is a mixture of tungstates and molybdates (Jadhav \textit{et al.}, 2012). Polyphenols in plant extracts react with the Folin-Ciocalteau reagent to form a blue complex (a phosphotungsticphosphomolybdenum complex) that can be quantified by visible-light spectrophotometry (Schofield \textit{et al.}, 2001). The blue color produced has a maximum absorption in the region of 750 nm and is proportional to the total quantity of phenolic compounds present.

In the determination of the total phenolic content, a concentration of 5 mg/ml of each extract was prepared. In a 96–well plate, an aliquot of 10 µl of extract was added to 790 µl of distilled water in each well. To this solution, 50 µl of Folin-Ciocalteau reagent was added. The resulting solution was thoroughly mixed and incubated in the dark for 8 minutes. This was followed by the addition of 150 µl of 7 \% \(Na_2CO_3\) and further incubation for 2 hours in the dark at room
temperature. The experiment was performed in duplicates. The absorbance was read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). Gallic acid was used as the standard phenolic compound. A gallic acid calibration curve was plotted and used to determine the phenolic content of the extracts (Appendix IVC).

2.2.6 Phytochemical Analysis

The crude extracts and Noni juice were screened for the presence of six phytochemicals: alkaloids, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. The alkaloids were tested as described (Sofowora, 1993) with slight modification. The method described by Evans (2002) for detecting the presence of flavonoids was also slightly modified and used. Tests for the other phytochemicals were carried out as described (Mir et al., 2013).

2.2.6.1 Determination of the presence of alkaloids in the extracts

An amount of 0.1 g of each of the crude extracts was weighed into 5 ml of 2 M hydrochloric acid (HCl) solution. The solution was stirred, warmed and filtered. The filtrate from each extract was then divided into three portions. Dragendorff’s reagent was added to one portion of each test solution while Mayer’s reagent was added to another portion. Wagner’s reagent was added to the third portion of the test solutions. The presence of a yellowish or reddish brown precipitate indicated the presence of alkaloids.

2.2.6.2 Determination of the presence of glycosides

An aliquot of 5 ml of aqueous extract containing 0.1 g of the extract was mixed with 2 ml of glacial acetic acid (CH₃COOH) containing 1 drop of iron chloride (FeCl₃). Concentrated sulphuric acid (H₂SO₄) (1 ml) was carefully added to the above mixture such that the
concentrated H$_2$SO$_4$ was underneath the mixture. The appearance of a brown ring indicated the presence of the glycoside constituent.

### 2.2.6.3 Determination of the presence of flavonoids

An amount of 0.1 g of each of the crude extracts was added to 5 ml of 80 % (v/v) ethanol. The resulting solution was filtered. The filtrate for each extract was divided into two portions. To one portion of the filtrate, magnesium turnings were added. This was followed by the addition of 0.5 ml of concentrated HCl. The solution was monitored for 10 minutes to observe any colour change. Concentrated HCl (5 ml) was added to the other portion and the solution was warmed for 5 minutes. The observation of a light pink colour was an indication of the presence of flavonoids.

### 2.2.6.4 Determination of the presence of saponins

An amount of 0.5 g of each extract was boiled together with 5 ml of distilled water for 30 seconds and then filtered. An aliquot of 2.5 ml of the filtrate was mixed with 1.25 ml of distilled water and shaken vigorously to obtain a stable persistent froth. Three drops of olive oil was added to the froth. The formation of an emulsion indicated the presence of saponins.

### 2.2.6.5 Determination of the presence of tannins

In detecting the presence of tannins, an amount of 0.25 g of the extract was boiled in 10 ml of distilled water for 30 seconds and then filtered. This was followed by the addition of 3 drops of 0.2 % FeCl$_3$ to the filtrate. The observation of a brownish green or a blue black colouration indicated the presence of tannins.
2.2.6.6 Determination of the presence of terpenoids

An aliquot of 5 ml of aqueous extract containing 0.1 g of the extract was mixed with 2 ml of chloroform (CHCl₃). To the aqueous extracts, 3 ml of concentrated H₂SO₄ was carefully added to the mixture to form a layer. The presence of terpenoids was indicated by an interface with a reddish brown coloration.

2.2.7 Data Analysis

The data for the antioxidant and the cell viability assays and mitochondrial respiration measurement were analyzed as described below:

The EC₅₀ values obtained in the DPPH assay were evaluated from a plot of % DPPH scavenging effect versus log concentration of extract using GraphPad Prism 5.0 version (GraphPad Prism Software Inc., San Diego, CA). The percent DPPH radical scavenging effect was calculated using the formula:

\[
\% \text{ DPPH Scavenging effect} = \left(1 - \frac{A_1}{A_0}\right) \times 100,
\]

where \(A_0\) is the absorbance of the blank and \(A_1\) is the absorbance of the extract.

The IC₅₀ values obtained in the MTT assay were determined from a plot of percent cell viability versus concentration of the extract using Microsoft Excel. The percent cell viability was calculated using the formula:
% Cell viability = \( \frac{A_1}{A_0} \times 100 \)

where \( A_0 \) is the absorbance of the blank and \( A_1 \) is the absorbance of the extract.

Data from the respiration assay was analyzed using the Strathkelvin 782 system, which converted respiration rates into absolute values in Microsoft Excel. Each plant extract was evaluated five times and values recorded as mean ± SEM. Comparisons between means were performed and significance was evaluated by one-factor analysis of variance using Microsoft Excel. Probability value of \( P < 0.05 \) was used as the criteria for significant differences.

The respiratory control ratio (RCR) was calculated as state 3/state 2, as a measure of mitochondrial coupling. State 3 respiration was stimulated by the addition of 0.5 M ADP while State 2 respiration was stimulated by the addition of 0.08 M malate and 2 M glutamate.

The leak respiration was also determined from the G+M–stimulated respiration (state 2 respiration) in the absence of ADP. Leak respiration measures proton leak across the inner mitochondrial membrane.
CHAPTER THREE

3.0 RESULTS

3.1 EFFECTS OF THE SELECTED PLANT EXTRACTS ON MITOCHONDRIAL FUNCTION IN SAPONIN-PERMEABILIZED CARDIAC FIBERS

The effects of the extracts on the mitochondrial complexes were investigated by measuring the rates of oxygen consumption in the presence or absence of the extracts and various substrates and inhibitors in saponin-permeabilized mouse cardiac fibers. Figures 3.1 to 3.4 show the effects of the selected extracts on mitochondrial respiratory chain activity.

The first pair of bars represents the incubation of the permeabilized fibers in the presence of only the solvent or the extracts. This respiration was observed to be minimal. Complex I respiration was stimulated by the addition of glutamate and malate (G+M) and is represented by the second pair. The third pair represents the activation of oxidative phosphorylation by the addition of ADP. The fourth pair indicates inhibition of complex I by rotenone, a known complex I inhibitor. Complex II respiration was stimulated by the addition of succinate and is indicated by the fifth pair. The sixth pair represents inhibition of complex III by antimycin A, a known inhibitor of complex III. The seventh pair represents the addition of ascorbate and N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD) to stimulate respiration through complex IV. Ascorbate and TMPD are artificial electron donors to complex IV. The last pair of bars represents the addition of cytochrome C which is a test for the intactness of the outer mitochondrial membrane.
3.1.1 Effects of *Taraxacum officinale* on Mitochondrial Respiratory Chain Activity

In the presence of the *T. officinale* extract alone, a significant minimal respiration was observed while no respiration was observed in the presence of the solvent alone (Figure 3.1). The subsequent addition of the substrates and inhibitors produced non-significant changes. Compared to the solvent, the G+M–stimulated respiration appeared to have been unaffected by the *T. officinale* extract. The extract seemed to have non-significantly decreased the ADP-stimulated respiration, rotenone-inhibited respiration, succinate-stimulated respiration and antimycin A-inhibited respiration compared to the solvent. The ascorbate+TMPD–stimulated respiration, however, increased non-significantly in the presence of the *T. officinale* extract compared to the solvent. The addition of cytochrome C appeared to have caused a non-significant increase in respiration in the presence of the *T. officinale* extract compared to the solvent.
Figure 3.1 Effects of *Taraxacum officinale* and solvent on respiration rates; Values are means ± SEM (n=5): 2 µl of solvent, 2 µl of 10 mg/ml of extract; White bars indicate respiration rates in the presence of the solvent (sol); Black bars represent respiration rates in the presence of the extracts (ext); * - Values significantly different from control (p<0.05).
3.1.2 Effects of *Morinda citrifolia* on Mitochondrial Respiratory Chain Activity

In the presence of the solvent alone, no respiration was observed. However, in the presence of the *Morinda citrifolia* extract alone, a significant minimal respiration was observed (Figure 3.2). The *M. citrifolia* extract also caused a significant increase in the complex IV respiration compared to the solvent. On the other hand, in the presence of this extract, non-significant decreases in respiration were observed with the addition of G+M, ADP, succinate and antimycin A. Compared to the solvent, the rotenone-inhibited respiration appeared to have been unaffected in the presence of the *M. citrifolia* extract. The addition of cytochrome C appeared to have caused a non-significant increase in respiration in the presence of the *M. citrifolia* extract compared to the solvent.
Figure 3.2  Effects of *Morinda citrifolia* and solvent on respiration rates; Values are mean ± SEM (n=5): 2 µl of solvent, 2 µl of 10 mg/ml of extract; White bars indicate respiration rates in the presence of the solvent (sol); Black bars indicate respiration rates in the presence of the extracts (ext); * - Values significantly different from control (p<0.05).
3.1.3 Effects of Noni Juice on Mitochondrial Respiratory Chain Activity

There was no respiration in the presence of the solvent alone while a significant minimal respiration was observed in the presence of the Noni juice extract alone (Figure 3.3). The subsequent addition of the substrates and inhibitors produced non-significant changes. The Noni juice extract appeared to have non-significantly increased the antimycin A–inhibited respiration and the ascorbate+TMPD–stimulated respiration. However, the Noni juice extract appeared to have non-significantly decreased the G+M–stimulated respiration, ADP-stimulated respiration, rotenone-inhibited respiration and succinate-stimulated respiration compared to the solvent. The addition of cytochrome C appeared to have caused a non-significant increase in respiration compared to the solvent.
Figure 3.3 Effects of Noni juice and solvent on respiration rates; Values are mean ± SEM (n=5): 2 µl of solvent, 2 µl of 10 mg/ml of extract; White bars represent respiration rates in the presence of the solvent (sol); Black bars represent respiration rates in the presence of the extracts (ext); * - Values significantly different from control (p<0.05).
3.1.4 Effects of *Millettia thonningii* on Mitochondrial Respiratory Chain Activity

Similar to the trend observed with the other extracts, the *Millettia thonningii* extract alone appeared to have stimulated a significant minimal respiration compared to the solvent (Figure 3.4). Non-significant changes were observed with the addition of the substrates and inhibitors. The G+M–stimulated respiration appeared not to have been altered by the *M. thonningii* extract compared to the solvent. The *M. thonningii* extract appeared to have non-significantly increased the ADP–stimulated respiration, rotenone–inhibited respiration and ascorbate+TMPD–stimulated respiration. The succinate–stimulated respiration and the antimycin A–inhibited respiration, however, appeared to have non-significantly declined in the presence of the *M. thonningii* extract compared to the solvent. The addition of cytochrome C caused a non-significant increase in respiration in the presence of the *M. thonningii* extract compared to the solvent.
Figure 3.4 Effects of *Millettia thonningii* and solvent on respiration rates; Values are mean ± SEM, (n=5): 2 µl of solvent, 2 µl of 10 mg/ml of extract; White bars indicate respiration rates in the presence of the solvent (sol); Black bars represent respiration rates in the presence of the extracts (ext); * - Values significantly different from control (p<0.05).
3.1.5 Effects of the Plant Extracts on Respiratory Control Ratio (RCR) and Leak Respiration

The RCR values obtained for the extracts are indicated in Table 3.1. *Millettia thonningii, Morinda citrifolia* and *Taraxacum officinale* appeared to have caused higher RCR values than the solvent and appeared to have non-significantly increased coupling. However, the RCR in presence of the Noni juice was lower than the solvent indicating that the Noni juice appeared to have non-significantly decreased coupling.

Figure 3.5 shows a graph of the effects of the extracts on the leak respiration. None of the extracts significantly altered the leak respiration. Compared to the solvent, the *T. officinale* and *M. thonningii* extracts appeared to have caused slightly lower leak respirations while the *M. citrifolia* and Noni juice extracts appeared to have caused much lower leak respirations compared to the solvent.
Table 3.1 Effects of plant extracts on mitochondrial respiratory control ratio (RCR)

<table>
<thead>
<tr>
<th>Extracts</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>2.82 ± 0.16</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em></td>
<td>3.23 ± 0.80</td>
</tr>
<tr>
<td><em>Morinda citrifolia</em></td>
<td>3.15 ± 0.37</td>
</tr>
<tr>
<td>Noni juice</td>
<td>2.49 ± 0.70</td>
</tr>
<tr>
<td><em>Millettia thonningii</em></td>
<td>3.52 ± 0.83</td>
</tr>
</tbody>
</table>
Figure 3.5 Effects of the extracts on leak respiration (proton leak across the inner membrane)
3.2 EFFECTS OF PLANT EXTRACTS ON CELL VIABILITY

Graphs showing the effects of the extracts on the Jurkat leukemia cells and the MCF7 breast cancer cells are presented in Figure 3.6 and Figure 3.7 respectively. Curcumin, the positive control, had IC$_{50}$ values of 11.4 µM (Figure 3.6A) and 74.24 µM (Figure 3.7A) in the Jurkat leukemia cells and the MCF7 breast cancer cells respectively. However, the IC$_{50}$ values of the extracts obtained in the Jurkat leukemia cells were greater than 100 µg/ml although dose-dependent responses were observed (Figure 3.6). No IC$_{50}$ value was obtained for Noni juice in the Jurkat leukemia cells. At the highest concentration tested, Noni juice inhibited growth of Jurkat cells by about 40%. Additionally, no IC$_{50}$ value was obtained for any of the extracts in the MCF7 breast cancer cells and flat curves were observed (Figure 3.7). The IC$_{50}$ values have been summarized in Table 3.2.
Figure 3.6 Dose – response curves showing the effects of extracts on cell viability in Jurkat leukemia cells: A. Curcumin (positive control) B. *Taraxacum officinale* (To) C. *Morinda citrifolia* (Mc) D. Noni juice (Nj) E. *Millettia thonningii* (Mt); Data represent mean ± SD of n = 3.
Figure 3.7 Effects of extracts on MCF7 breast cancer cell viability: A. Curcumin (positive control)  B. *Taraxacum officinale* (To)  C. *Morinda citrifolia* (Mc)  D. Noni juice (Nj)  E. *Milletia thomningii* (Mt); Data represent mean ± SD of n = 3.
Table 3.2 IC$_{50}$ of plant extracts and Curcumin (positive control)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Jurkat leukemia cells</th>
<th>MCF7 breast cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>11.4 µM</td>
<td>74.74 µM</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em></td>
<td>485 µg/ml</td>
<td>-</td>
</tr>
<tr>
<td><em>Morinda citrifolia</em></td>
<td>933.33 µg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Noni juice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Millettia thonningii</em></td>
<td>930 µg/ml</td>
<td>-</td>
</tr>
</tbody>
</table>

IC$_{50}$: Fifty percent inhibitory concentration values of the plant extracts and the positive control (Curcumin)
3.3 ANTIOXIDANT ACTIVITIES OF THE SELECTED PLANT EXTRACTS

The results of the DPPH scavenging activities of the extracts and the Noni juice are as presented in Figure 3.8 and Table 3.3. Figure 3.8 shows the DPPH scavenging activities of the extracts and the standard (BHT) over the concentration range of 0 – 5 mg/ml. The EC$_{50}$ values are summarized in Table 3.3. As shown in Figure 3.8, all the extracts scavenged DPPH radicals in a dose-dependent manner. The EC$_{50}$ value for *Millettia thonningii* (0.21 mg/ml) was lower compared to that of BHT (0.43 mg/ml). *Taraxacum officinale* also had a lower EC$_{50}$ value of 0.31 mg/ml compared to the standard, BHT. *M. citrifolia* and Noni juice had higher EC$_{50}$ values of 0.60 mg/ml and 1.98 mg/ml respectively compared to BHT. Among the extracts, *M. thonningii* had the lowest EC$_{50}$ value while Noni juice had the highest EC$_{50}$ value.
Figure 3.8: Antioxidant activities of BHT and extracts: A. Butylated hydroxyl toluene (BHT) B. Taraxacum officinale C. Morinda citrifolia D. Noni juice E. Millettia thonningii; Radical scavenging capacity of each extract calculated as the percent DPPH radical scavenging effect; Each data point is mean ± SD of n = 3.
Table 3.3 EC$_{50}$ of plant extracts and standard (BHT)

<table>
<thead>
<tr>
<th>Extract</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT</td>
<td>0.43</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em></td>
<td>0.31</td>
</tr>
<tr>
<td><em>Morinda citrifolia</em></td>
<td>0.60</td>
</tr>
<tr>
<td>Noni juice</td>
<td>1.98</td>
</tr>
<tr>
<td><em>Milletia thonningii</em></td>
<td>0.21</td>
</tr>
</tbody>
</table>

EC$_{50}$: Fifty percent effective concentration values of the plant extracts and the standard (BHT)
3.4 PHENOLIC CONTENT IN THE SELECTED PLANT EXTRACTS

The phenolic content of the four extracts were determined from the gallic acid calibration curve and are indicated in Table 3.4. *Millettia thonningii* had the highest phenolic content (23880.38 ± 0.14 mg GAE/100 g), and was followed by *Morinda citrifolia* (7338.92 ± 0.17 mg GAE/100 g) and then *Taraxacum officinale* (7127.98 ± 0.16 mg GAE/100 g) and finally, Noni juice, which had the lowest phenolic content (3036.69 ± 0.17 mg GAE/100g).
Table 3.4 Phenolic content of the extracts

<table>
<thead>
<tr>
<th>Extract (5 mg/ml)</th>
<th>[Phenolics] mg GAE/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Taraxacum officinale</em></td>
<td>7127.98 ± 0.16</td>
</tr>
<tr>
<td><em>Morinda citrifolia</em></td>
<td>7338.92 ± 0.17</td>
</tr>
<tr>
<td>Noni juice</td>
<td>3036.69 ± 0.17</td>
</tr>
<tr>
<td><em>Millettia thonningii</em></td>
<td>23880.38 ± 0.14</td>
</tr>
</tbody>
</table>
3.5 PHYTOCHEMICAL CONSTITUENTS OF THE SELECTED PLANT EXTRACTS

Qualitative tests were performed to determine the presence of alkaloids, cardiac glycosides, flavonoids, saponins, tannins and terpenoids in the extracts. Table 3.5 shows the results from the phytochemical analysis. All the plant extracts tested positive for the presence of saponins. All except Noni juice tested positive for the presence of tannins. Only *Morinda citrifolia* and *Taraxacum officinale* tested positive for the presence of flavonoids while only *Morinda citrifolia* tested positive for the presence of alkaloids. Terpenoids and glycosides were found to be absent in all the four extracts.
Table 3.5 Qualitative screening of the phytochemicals in the extracts

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Extract</th>
<th>Taraxacum officinale</th>
<th>Morinda citrifolia</th>
<th>Noni juice</th>
<th>Millettia thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = present; - = absent
CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSION

Mitochondria are very important and unique organelles that are sensitive to a number of xenobiotics including drugs and phytochemicals. Some of these xenobiotics exhibit their pharmacological effects and/or their toxicity by interfering with mitochondrial function. Medicinal plants since ancient times have made important contribution to health care and they are known to contain several phytochemicals. In the present study, the extracts of three medicinal plants [Taraxacum officinale Weber, Morinda citrifolia Linn and Millettia thonningii (Schum. & Thonn) Baker] were analyzed to determine their antioxidant activities, phenolic content, phytochemistry as well as their effects on mitochondrial respiration and cell viability. The data obtained indicated that all the extracts showed strong antioxidant activities and contained phytochemical compounds which are known to have antioxidant properties. In addition, all the extracts affected mitochondrial respiratory chain activity. However, they had very weak cytotoxic effects on the cancer cell lines tested.

Effects of the Selected Plant Extracts on Mitochondrial Respiratory Chain Activity

This study investigated the effects of extracts of T. officinale, M. citrifolia and M. thonningii on mitochondrial respiratory chain activity in permeabilized mouse cardiac fibers using a substrate-inhibitor titration. All the extracts appeared to have some effect on mitochondrial respiratory chain activity. This observation agrees with previous findings that plant xenobiotics interfere with mitochondrial function (Forbes-Hernandez et al., 2014).
All the extracts significantly stimulated basal respiration (Figure 3.1 to Figure 3.4) indicating that they may contain phytochemical compounds that act as substrates and donate electrons to the enzyme complexes stabilizing their activities. An increase of 5 – 20 % in respiration was observed in the presence of the extracts with the addition of cytochrome C indicating that the outer mitochondrial membrane remained intact throughout the respiration assays (Figure 3.1 to Figure 3.4).

Effects of the extracts on respiratory chain enzyme complexes

*T. officinale* appeared to have caused a decrease in complex I respiration and this suggests that it may contain phytochemical compounds that inhibit complex I activity (Figure 3.1). *T. officinale* also appeared to have decreased the rotenone-inhibited respiration. This result does not agree with the previous finding by Ocloo *et al.* (2015) where the aqueous extract of *T. officinale* caused an increase in the rotenone-inhibited respiration and blocked the effect of rotenone. Complex II respiration decreased in the presence of the *T. officinale* extract suggesting that this extract may contain phytochemical compounds that inhibit complex II activity. A similar finding was made by Ocloo *et al.* (2015) where the aqueous extract of *T. officinale* caused a decrease in complex II respiration. The *T. officinale* extract, however, caused an increase in complex IV activity and thus may contain phytochemicals that donate electrons to complex IV stabilizing its activity. This observation is consistent with the findings of Ocloo *et al.* (2015) where the aqueous extract of *T. officinale* also caused an increase in complex IV activity. The discrepancy between the results obtained in this study and that obtained by Ocloo *et al.* (2015) may be due to varied chemical constituents in the *T. officinale* extracts due to differences in plant location, time of harvesting and method of preparation.
M. citrifolia appeared to have caused a decrease in complex I respiration indicating that it may contain phytochemical compounds that inhibit complex I activity (Figure 3.2). M. citrifolia also appeared to have caused a decrease in complex II respiration suggesting that it may contain phytochemical compounds that inhibit complex II activity. However, M. citrifolia was the only extract that caused a significant increase in complex IV respiration and this indicates that M. citrifolia may contain phytochemical compounds that donate electrons to complex IV and stimulate its activity.

Noni juice appeared to have caused a decrease in complex I respiration suggesting that it may contain phytochemical compounds that inhibit complex I activity (Figure 3.3). However, it appeared not to have altered complex II respiration. Noni juice was the only extract that caused an increase in the antimycin A–inhibited respiration. This suggests that it may contain compounds that donate electrons downstream of complex III to complex IV and block the effect of antimycin A. Complex IV respiration also increased in the presence of Noni juice indicating that the Noni juice extract may contain phytochemical compounds that stimulate complex IV activity by donating electrons to complex IV.

M. thonningii appeared to have had no effect on complex I respiration (Figure 3.4). This is contrary to the findings of a previous study where M. thonningii seeds inhibited complex I respiration (Lyddiar and Whitfield, 2001). M. thonningii was the only extract that caused an increase in the rotenone-inhibited respiration suggesting that it may contain phytochemicals that donate electrons downstream of complex I to complexes II, III and IV and block the effect of rotenone.
M. thonningii appeared to have caused a decrease in complex II respiration suggesting that it may contain phytochemical compounds that inhibit complex II activity. The leaves of M. thonningii are known to have molluscicidal activity (Harrison et al., 2011). The inhibition of complex II activity by the M. thonningii extract, therefore, might partly explain the mechanism involved in the molluscidal activity of the leaves. M. thonningii stimulated complex IV activity suggesting that M. thonningii may contain phytochemical compounds that donate electrons to complex IV and stabilize its activity.

**Effects of the extracts on ATP synthesis**

The ADP-stimulated respiration increased only in the presence of the M. thonningii extract suggesting that this extract may contain phytochemicals that stimulate ATP synthesis (Figure 3.4). The other extracts, however, appeared to have decreased the ADP-stimulated respiration indicating that they may contain compounds that inhibit ATP synthesis (Figure 3.1 to Figure 3.3). Previously, the aqueous extract of T. officinale appeared to have had no effect on ADP-stimulated respiration (Ocloo et al., 2015).

**Effects of the extracts on respiratory control ratio (RCR)**

Respiratory control ratio is the ratio of state 3 respiration to state 2 respiration. It is a way of measuring coupling efficiency. Compared to the solvent, all the extracts except Noni juice appeared to have increased coupling (Table 3.1). Compared to the solvent, Noni juice appeared to have decreased coupling and this casts doubt on its effect as an energy booster. Similar to these results, the study by Ocloo et al. (2015) showed that the aqueous extract of T. officinale also increased coupling.
Effects of the extracts on leak respiration

Oxidative phosphorylation is incompletely coupled since protons can leak across the inner membrane. Leak respiration is an indirect measure of proton leak across the inner membrane. It is also an indirect estimate of coupling efficiency. It is measured as mitochondrial respiration in the absence of ADP but in the presence of reducing substrate(s) (Gnaiger, 2012). A high leak respiration indicates a high uncoupling. None of the extracts significantly altered the leak respiration compared to the solvent (Figure 3.5) suggesting that there was a low proton leak across the inner mitochondrial membrane.

Cytotoxicity of the Selected Plant Extracts

The succinate dehydrogenase system of the mitochondrion converts MTT to formazan in viable cells (Slater et al., 1963). Therefore, the cytotoxicity of the selected plant extracts was determined as an indirect measure of the effects of the extracts on mitochondrial function using the MTT assay. The extracts appeared to show dose dependent cytotoxic effects on the Jurkat leukemia cells (Figure 3.6). However, a plant extract, according to the US National Cancer Institute, is considered to have active cytotoxic effect if the IC$_{50}$ value is 30 μg/ml or less (Geran et al., 1972; Lee and Houghton, 2005). All the extracts had IC$_{50}$ values greater than 100 μg/ml. Therefore, the extracts had weak cytotoxic effects on the Jurkat leukemia cells and they lack the potential to significantly alter mitochondrial function. The extracts also appeared to have had no cytotoxic effect on the MCF7 breast cancer cells (Figure 3.7) and this also suggests that mitochondrial function was unaffected by the extracts.
Anecdotal information on the anticancer activity of *Taraxacum officinale* has been reported previously (Sigstedt *et al.*, 2008). The aqueous leaf extract of *T. officinale* has also been shown to reduce the growth of MCF-7/AZ breast cancer cells in a dose-dependent manner (Sigstedt *et al.*, 2008) and this inhibitory effect is ascribed to the presence of phenolic compounds in the Dandelion leaves (Schütz *et al.*, 2006). Contrary to these findings, *T. officinale* showed weak cytotoxic effects on the cell lines used in this study. It is also possible that *Taraxacum officinale* acts as a pro-drug, thus *in vitro* assays alone may be inappropriate for investigation of its anticancer property.

There have also been reports on the anticancer properties of Noni juice. The fruit juice has been reported to possess significant antitumour activity against LLC peritoneal carcinomatosis (Hirazumi *et al.*, 1996). Additionally, the methanol extract of the fruit exhibited cytotoxic activity against breast cancer (MCF7) and neuroblastoma (LAN5) cell lines (Arpornsuwan and Punjanon, 2006). Another study showed that the ethanol precipitated polysaccharide-rich substance of Noni juice possessed immunomodulatory and antitumour activity against Sarcoma 180 ascites tumour in mice (Furusawa *et al.*, 2003). However, in this study, Noni juice had a weak cytotoxic effect on the cancer cell lines. This result does not agree with the observations in the previous studies. Probably, the difference in cytotoxic effect is due to the methods of preparation of the juice, since the preparation is quite elaborate. Noni fruit juice is processed by fermentation over two months during which the light intensity, pH and temperature are carefully monitored. This may or may not be followed by pasteurization. In addition, this study did not investigate methanolic extracts of the fruit which has been reported to possess cytotoxic activity.
Although there have been reports on the anticancer activities of the *T. officinale* and Noni juice extracts, the results obtained in this study do not support these claims. There is paucity of information on the anticancer properties of the *M. citrifolia* and *M. thonningii* leaves. The reason for the weak cytotoxic effects of the extracts on the cell lines is unclear.

**Antioxidant Activities and Phenolic Contents of the Selected Plant Extracts**

Free radicals such as reactive oxygen species (ROS) are normal by-products of respiration. However, overload of free radicals may lead to oxidative damage and the imbalance of cellular redox homeostasis contributes to the pathogenesis of diseases such as diabetes, atherosclerosis, immunosuppression and neurodegeneration (Hensley *et al*., 2000; Gupta *et al*., 2012). Antioxidants are chemical compounds that include vitamins, minerals and nutrients that scavenge free radicals and protect cellular DNA, proteins and lipid membranes (Osawa *et al*., 1990). In addition, antioxidants have been implicated in the maintenance of human health and the prevention and treatment of some diseases (Halliwell and Gutteridge, 1981).

Of the plant extracts analyzed, *M. thonningii* showed the strongest antioxidant activity (Table 3.2). Noni juice, on the other hand, showed the weakest antioxidant activity among the four extracts. However, Noni juice is considered a good source of polyphenols and antioxidants (Bramorski *et al*., 2010; De-Lu *et al*., 2013). *M. citrifolia* leaves are well known for their strong antioxidant activity and they have been shown to be safe in acute, subacute, and subchronic oral toxicity tests on mice (West *et al*., 2007; Serafini *et al*., 2011). The results obtained in this study confirm the strong antioxidant activity of the *Morinda citrifolia* leaves. *T. officinale* has been shown to be an easily accessible source of natural antioxidants (Amin *et al*., 2013). In a previous
study, *T. officinale* effectively scavenged free radicals released in the liver in rats with CCl₄ induced hepatotoxicity (Gulfraz *et al.*, 2014). Dandelion also reduced the extent of atherosclerosis by reducing oxidative stress in cholesterol-fed rats (Choi *et al.*, 2010). The results obtained in this study agree with these previous findings and confirm the antioxidant properties of *T. officinale*.

Phenols have been shown to have antioxidant activities towards free radicals. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Jung *et al.*, 2003). Hence measuring the concentration of phenolic hydroxyl groups in the plant extracts is another way of confirming the antioxidant capacity. Additionally, a high linear correlation has been observed between DPPH radical scavenging activity and total phenolic content (Ghafar *et al.*, 2010). Li *et al.* (2009) also identified significant positive correlations between the antioxidant effects and the presence of phenolic compounds in *Radix angelicae sinensis*. The results obtained in this study agree with these previous findings. Intriguingly, *Millettia thonningii* which showed the strongest antioxidant activity had the highest phenolic content while Noni juice which showed the weakest antioxidant activity had the lowest phenolic content in the present study (Table 3.3).

**Phytochemical Constituents of the Selected Plant Extracts**

Studies on medicinal plants have indicated the presence of phytochemicals such as phenolics, flavonoids and tannins (Hill, 1952). These phytochemicals often determine the physiological action of the medicinal plants on the human body and they are known to have beneficial effects
on health (Pamplona-Roger, 1998; Ayoola et al., 2008). Some of the phytochemicals are antioxidants and thus attenuate the adverse effects of reactive oxygen species. In the current study, the plant extracts were screened for the presence of six classes of phytochemicals which are commonly found in most plants. These phytochemicals include alkaloids, flavonoids, glycosides, saponins, tannins and terpenoids. Each extract contained at least one of these phytochemicals.

The aqueous extract of *Millettia thonningii* leaves was found to contain saponins and tannins in the present study (Table 4.4). Saponins and tannins are phytochemicals with known antioxidant properties (Chen et al., 2014; Forbes-Hernández et al., 2014). A study by Borokini and Omotayo (2012) identified these two phytochemicals as well as glycosides, alkaloids, flavonoids and terpenoids in the leaves of *M. thonningii*. In another study, isoflavonoids were identified in the deuterochloroform extract of hexane defatted seeds of *M. thonningii* (Lyddiard and Whitfield, 2001).

Saponins, tannins and flavonoids were identified in the aqueous extract of *T. officinale* leaves (Table 3.4) in this study and these are known components of the plant (Yarnell and Abascal, 2009). Flavonoids are polyphenols with antioxidant properties (Pietta, 2000; Forbes-Hernández et al., 2014). In previous studies, *T. officinale* leaves have been reported to be rich in phenolics as well as other antioxidants such as vitamin A, vitamin C, zinc and copper (Schmidt, 1979; Jackson, 1982; Yarnell and Abascal 2009).
*M. citrifolia* leaves contained alkaloids, saponins, flavonoids and tannins (Table 3.4). These have been identified in previous studies in the *M. citrifolia* plant (Singh *et al.*, 2012; Pandy *et al.*, 2014). Some alkaloids have been shown to have antioxidant properties (Maiza-Benabdesselam *et al.*, 2007). Other constituents which have been identified in the *Morinda citrifolia* products include vitamin A, vitamin C, rutin and flavone glycosides (Higa and Fuyama, 1993; Farine *et al.*, 1996). Noni juice is known to be a rich source of antioxidants such as flavonoids (Nijveldt *et al.*, 2001). Studies on the chemical composition of Noni juice have also indicated that phenolic compounds predominant in the juice act as free radical scavengers and prevent several diseases (Dixon *et al.*, 1999; Chan-Blanco *et al.*, 2006). Contrary to these findings, only saponins were identified in the juice.

The extracts contained different classes of phytochemicals and appeared to affect mitochondrial function. However, the phytochemical analysis done in this study was qualitative and only identified the presence of these phytochemical compounds in the extracts. Because no quantitative analysis was done, the relationship between the phytochemistry of the extracts and their observed effects on mitochondrial function is unclear. *Millettia thonningii*, for instance, had the highest antioxidant activity and the highest phenolic content and it exhibited the presence of saponins and tannins. Saponins and tannins, as mentioned earlier, have antioxidant properties. Hence, it was not surprising that the *M. thonningii* extract appeared to have stimulated complex IV activity and removed the inhibition from complex I by rotenone. However, because the concentrations of the saponins and tannins in the extract were not determined, it is difficult to tell if these effects are as a result of these phytochemicals alone or a combination of other factors.
Additionally, comparing the four extracts, they all had a similar effect on complex IV by stimulating complex IV activity. They also increased basal respiration in the absence of substrates. However, the effects of the extracts on complexes I, II and III were different. Each of the extracts contained saponins. *T. officinale*, *M. citrifolia* and *M. thonningii* contained tannins as well. *T. officinale* and *M. citrifolia* contained flavonoids in addition to saponins and tannins and alkaloids were also detected in *M. citrifolia*. The amounts of each of these phytochemicals that contributed to the antioxidative effect observed on the mitochondrial complexes were not determined in this study. The varied effects on complexes I, II and III may be as a result of different concentrations of the phytochemicals in each of the extracts. For instance, although four phytochemicals were identified in *M. citrifolia* and only two were identified in *M. thonningii*, the latter had a higher antioxidant activity probably due to the presence of higher concentrations of the phytochemicals identified in this extract. No clear relationship was observed between the phytochemicals identified in each extract and their observed effects on mitochondrial function.

In a previous study, a positive linear relationship between antioxidant activity and anticancer effect of the water extracts of five herbal plants (*Lobeliae chinensis, Rheum officinale, Sanguisorba officinalis, Agrimonia pilosa* and *Paris polyphylla*) was identified by comparing the percentage free radical scavenging capacity and percentage growth inhibition on A549 and MCF-7 cells (Li *et al.* 2007). This suggested that the antioxidants present in the herbal water extracts might have contributed to their anticancer effects on A549 and MCF-7 cells. In this study, no such relationship between antioxidant and anticancer activity was observed. Even though the extracts exhibited prominent antioxidant activities, their growth inhibitory effects on the Jurkat leukemia cells and the MCF-7 cells were generally weak.
4.2 CONCLUSION

All the extracts significantly stimulated basal respiration. Additionally, they all appeared to have caused an increase in complex IV respiration although only *Morinda citrifolia* stimulated a significant increase. All the extracts showed strong antioxidant activities and all of them contained phytochemicals which have been shown to have antioxidant properties. *M. thonningii* partially blocked the effect of rotenone. Noni juice partially blocked the effect of antimycin A. Finally, all the extracts had weak cytotoxic effects on the Jurkat human leukemia and the MCF7 human breast cancer cell lines.

4.3 RECOMMENDATIONS

Further studies could be done to isolate the individual components of the extracts to determine which components contribute to the strong antioxidant activities of the extract. The effects of each of these components on complex IV activity could also be studied. Quantitative analyses could be done to determine the concentration of each phytochemical in the extracts and the effects of their various concentrations on mitochondrial function. Additionally, the study could be conducted *in vivo* by administering the extracts to animals and then analyzing the activities of the respiratory complexes since the approach used in this study is limited by the ability of the constituents to cross the mitochondrial membrane. The effects of the extracts on other tissues such as the skeletal muscle could be investigated. Finally, the cytotoxic effects of the extracts could be investigated on other cell lines.
REFERENCES


# APPENDICES

## Appendix I

Data on the measurement of oxygen consumption in the presence of the solvent and the extracts; values are mean ± standard error

<table>
<thead>
<tr>
<th>Solvent</th>
<th>T. officinale</th>
<th>M. citrifolia</th>
<th>Noni juice</th>
<th>M. thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ext/Sol</td>
<td>-0.02 ± 0.08</td>
<td>0.20 ± 0.084</td>
<td>0.27 ± 0.11</td>
<td>0.38 ± 0.22</td>
</tr>
<tr>
<td>G+M</td>
<td>1.81 ± 0.39</td>
<td>1.78 ± 0.58</td>
<td>1.55 ± 0.33</td>
<td>1.48 ± 0.91</td>
</tr>
<tr>
<td>ADP</td>
<td>5.10 ± 1.16</td>
<td>4.26 ± 0.31</td>
<td>4.74 ± 0.74</td>
<td>3.86 ± 2.54</td>
</tr>
<tr>
<td>Rot</td>
<td>2.43 ± 0.45</td>
<td>2.01 ± 0.54</td>
<td>2.43 ± 0.69</td>
<td>2.31 ± 1.58</td>
</tr>
<tr>
<td>Succ</td>
<td>6.96 ± 1.26</td>
<td>6.51 ± 0.85</td>
<td>5.89 ± 0.88</td>
<td>6.91 ± 1.30</td>
</tr>
<tr>
<td>Ant A</td>
<td>2.52 ± 0.91</td>
<td>2.43 ± 0.99</td>
<td>2.45 ± 0.85</td>
<td>2.89 ± 1.09</td>
</tr>
<tr>
<td>Asc+TMPD</td>
<td>7.12 ± 1.04</td>
<td>8.71 ± 1.85</td>
<td>8.95 ± 1.66</td>
<td>8.56 ± 1.63</td>
</tr>
<tr>
<td>Cyt C</td>
<td>7.22 ± 1.68</td>
<td>8.72 ± 1.19</td>
<td>8.15 ± 1.41</td>
<td>8.84 ± 2.02</td>
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</table>
**Appendix II**

Data on the effects of the extracts on cell viability

**Appendix IIA**

Absorbance and percent cell viability values of the extracts on Jurkat leukemia cells; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>[Extract] µg/ml</th>
<th>T. officinale</th>
<th>M. citrifolia</th>
<th>Noni juice</th>
<th>M. thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.41 ± 0.00</td>
<td>0.41 ± 0.00</td>
<td>0.41 ± 0.00</td>
<td>0.41 ± 0.00</td>
</tr>
<tr>
<td>62.5</td>
<td>0.42 ± 0.04</td>
<td>0.40 ± 0.04</td>
<td>0.41 ± 0.01</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>125</td>
<td>0.36 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>0.40 ± 0.04</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>250</td>
<td>0.29 ± 0.02</td>
<td>0.32 ± 0.02</td>
<td>0.44 ± 0.10</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>500</td>
<td>0.20 ± 0.02</td>
<td>0.30 ± 0.03</td>
<td>0.39 ± 0.04</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>1000</td>
<td>0.13 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.26 ± 0.05</td>
<td>0.20 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>62.5</td>
</tr>
<tr>
<td>125</td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>1000</td>
</tr>
</tbody>
</table>
Appendix IIIB

Absorbance and percent cell viability values of the extracts on MCF7 cells; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>[Extract] µg/ml</th>
<th>T. officinale</th>
<th>M. citrifolia</th>
<th>Noni juice</th>
<th>M. thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.17 ± 0.00</td>
<td>1.17 ± 0.00</td>
<td>1.17 ± 0.00</td>
<td>1.17 ± 0.00</td>
</tr>
<tr>
<td>62.5</td>
<td>1.13 ± 0.02</td>
<td>1.19 ± 0.06</td>
<td>1.22 ± 0.16</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>125</td>
<td>1.12 ± 0.02</td>
<td>1.25 ± 0.09</td>
<td>1.23 ± 0.17</td>
<td>1.07 ± 0.12</td>
</tr>
<tr>
<td>250</td>
<td>1.12 ± 0.06</td>
<td>1.20 ± 0.13</td>
<td>1.22 ± 0.09</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>500</td>
<td>1.14 ± 0.02</td>
<td>1.26 ± 0.09</td>
<td>1.24 ± 0.11</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>1000</td>
<td>1.14 ± 0.01</td>
<td>1.26 ± 0.14</td>
<td>1.21 ± 0.12</td>
<td>1.17 ± 0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Cell viability</th>
<th>0</th>
<th>100.0</th>
<th>100.0</th>
<th>100.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>96.7</td>
<td>102.1</td>
<td>104.6</td>
<td>97.4</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>95.7</td>
<td>106.7</td>
<td>105.8</td>
<td>91.8</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>95.5</td>
<td>102.5</td>
<td>104.3</td>
<td>85.5</td>
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</tr>
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<td>500</td>
<td>97.6</td>
<td>107.7</td>
<td>106.6</td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>98.0</td>
<td>107.8</td>
<td>103.6</td>
<td>100.3</td>
<td></td>
</tr>
</tbody>
</table>
Appendix IIC

Absorbance and percent cell viability values of curcumin (positive control) on the Jurkat leukemia cells and MCF7 breast cancer cells; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>[Curcumin] µM</th>
<th>Mean Absorbance</th>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.26 ± 0.00</td>
<td>100.0</td>
</tr>
<tr>
<td>6.25</td>
<td>0.16 ± 0.03</td>
<td>71.1</td>
</tr>
<tr>
<td>12.5</td>
<td>0.09 ± 0.01</td>
<td>45.7</td>
</tr>
<tr>
<td>25</td>
<td>0.02 ± 0.04</td>
<td>32.7</td>
</tr>
<tr>
<td>50</td>
<td>0.02 ± 0.02</td>
<td>25.7</td>
</tr>
<tr>
<td>100</td>
<td>0.02 ± 0.01</td>
<td>27.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Curcumin] µM</th>
<th>Mean Absorbance</th>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.03 ± 0.00</td>
<td>100.0</td>
</tr>
<tr>
<td>6.25</td>
<td>0.91 ± 0.02</td>
<td>88.5</td>
</tr>
<tr>
<td>12.5</td>
<td>0.90 ± 0.15</td>
<td>87.8</td>
</tr>
<tr>
<td>25</td>
<td>0.70 ± 0.06</td>
<td>68.2</td>
</tr>
<tr>
<td>50</td>
<td>0.64 ± 0.07</td>
<td>62.8</td>
</tr>
<tr>
<td>100</td>
<td>0.37 ± 0.04</td>
<td>36.4</td>
</tr>
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</table>
Appendix III
Data on the antioxidant activities of the extracts

Appendix IIIA
Absorbance values of the extracts from the DPPH assay; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>Mean Absorbance</th>
<th>Extract [mg/ml]</th>
<th>Log [Extract]</th>
<th>BHT</th>
<th>T. officinale</th>
<th>M. citrifolia</th>
<th>Noni juice</th>
<th>M. thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06</td>
<td>0</td>
<td>-2</td>
<td>1.19 ± 0.03</td>
<td>1.04 ± 0.13</td>
<td>1.09 ± 0.08</td>
<td>1.02 ± 0.03</td>
<td>0.93 ± 0.16</td>
</tr>
<tr>
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<td>0.01</td>
<td>-3</td>
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<td>1.07 ± 0.13</td>
<td>1.09 ± 0.05</td>
<td>1.06 ± 0.04</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
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<td>1.20 ± 0.01</td>
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<td>1.08 ± 0.07</td>
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<td>0.92 ± 0.13</td>
</tr>
<tr>
<td>0.06</td>
<td>0.06</td>
<td>-1.22</td>
<td>1.16 ± 0.01</td>
<td>1.00 ± 0.09</td>
<td>1.10 ± 0.02</td>
<td>1.09 ± 0.01</td>
<td>0.86 ± 0.10</td>
</tr>
<tr>
<td>0.06</td>
<td>0.19</td>
<td>-0.72</td>
<td>1.08 ± 0.02</td>
<td>0.82 ± 0.08</td>
<td>0.97 ± 0.01</td>
<td>1.04 ± 0.01</td>
<td>0.66 ± 0.09</td>
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<td>-0.25</td>
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<td>0.72 ± 0.01</td>
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</tr>
<tr>
<td>0.06</td>
<td>1.67</td>
<td>0.22</td>
<td>0.50 ± 0.05</td>
<td>0.27 ± 0.01</td>
<td>0.46 ± 0.13</td>
<td>0.52 ± 0.08</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>0.06</td>
<td>5</td>
<td>0.70</td>
<td>0.11 ± 0.02</td>
<td>0.30 ± 0.03</td>
<td>0.44 ± 0.04</td>
<td>0.22 ± 0.07</td>
<td>0.32 ± 0.07</td>
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</table>
Appendix IIIB

Data on percent antioxidant activities of the extracts

<table>
<thead>
<tr>
<th>% Antioxidant activity</th>
<th>Log [Extract]</th>
<th>BHT</th>
<th>Extracts</th>
<th>T. officinale</th>
<th>M. citrifolia</th>
<th>Noni juice</th>
<th>M. thonningii</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>-3.6</td>
<td>3.1</td>
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</tr>
<tr>
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<td>-1.70</td>
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<td>1.3</td>
<td>-5.8</td>
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</tr>
<tr>
<td>0.06</td>
<td>-1.22</td>
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<td>4.2</td>
<td>-1.1</td>
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<td>10.6</td>
<td>-1.7</td>
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<td>0.56</td>
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<td>58.4</td>
<td>34.3</td>
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<td>90.9</td>
<td>71.4</td>
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<td>78.7</td>
<td>66.1</td>
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</table>
Appendix IV

Data on the phenolic contents of the extracts

Appendix IVA

Varying concentrations and absorbance values of gallic acid used in plotting the gallic acid calibration curve; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>[Gallic acid] mg/ml</th>
<th>Mean Absorbance</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>0.08</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>0.16</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>0.31</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>0.63</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>1.25</td>
<td>0.53 ± 0.00</td>
</tr>
<tr>
<td>2.5</td>
<td>0.86 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>1.81 ± 0.19</td>
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</table>
Appendix IVB

Absorbance values of the extracts from the Folin-Ciocalteau assay; absorbance values are mean ± standard deviation

<table>
<thead>
<tr>
<th>Extracts  (5mg/ml)</th>
<th>Mean absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. officinale</em></td>
<td>0.18 ± 0.00</td>
</tr>
<tr>
<td><em>M. citrifolia</em></td>
<td>0.19 ± 0.00</td>
</tr>
<tr>
<td>Noni juice</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td><em>M. thonningii</em></td>
<td>0.47 ± 0.01</td>
</tr>
</tbody>
</table>

The [phenolic content] mg/ml was calculated using the equation of the calibration curve:

\[ y = 0.0581x + 0.3461 \]

where \( y \) = absorbance of the extract

and \( x \) = [phenolic content] mg/ml of the extract

Hence, [phenolic content] mg/ml = \((y - 0.0581)/0.3641\)

[Phenolics] mg GAE/100 g = \((100 \times 1000/ [E]) \times [\text{phenolic content}] \) mg/ml

Hence, [Phenolics] mg GAE/100 g = \((100 \times 1000/ [E]) \times (y - 0.0581)/0.3641\)
Appendix IVC

Calibration curve for gallic acid

\[ y = 0.3461x + 0.0581 \]

\[ R^2 = 0.9977 \]