MODULATION OF MITOCHONDRIAL-MEDIATED APOPTOSIS BY PURIFIED FRACTIONS OF *Calliandraportoricensis* BENTH

BY

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ABSTRACT

Apoptosis is downregulated in all forms of cancers. The mitochondrion has been implicated in the apoptotic process and, recently has been targeted in cancer therapy because of its role in cancer progression. Medicinal plants are used in the treatment of cancer, in particular, *Calliandraportoricensis*(CP)in the management of urinary obstruction in elderly males. There is paucity of information to justify the use of the plant in ethnomedicine. This study was designed to investigate the modulatory effects of CP on mitochondrial-mediated apoptosis in cancer cells and rats.

Root barks of *CP* (UIH-22466)were extracted with methanol by cold maceration to obtain crude methanol extract (MECP), which was fractionated using vacuum liquid chromatography to obtain methanol fraction (MFCP). Inhibition of mitochondrial lipid peroxidation (mLP), mitochondrialATPase (mATPase) activity and mitochondrial Permeability Transition (mPT) were determined in MFCP by spectrophotometry. Prostatic LNCaP, DU-145, lung adenocarcinoma and healthy VERO cells were used to assess cell proliferation. Cell cycle analysis was evaluated by flow cytometry. Levels of pro-apoptotic Bax, anti-apoptotic Bcl-2, Cytochrome C Release (CCR) and activation of caspases 3(C3) and 9 (C9) were determined by ELISA, while mitochondrial integrity was evaluated by Fluorescent Intensity Ratio (FIR). Thirty-five male Wistar rats (70-80 g) were divided into five groups of seven animals and each group was orally administered distilled water (control), 25, 50, 100 and 200 mg/kg of MFCP, respectively for 30 days. Rat liver mitochondria were isolated by differential centrifugation and mPT evaluated spectrophotometrically. The MFCP was purified by thin layer and column chromatography. Proton

NMR,^{13}C and liquid chromatography mass spectroscopy were carried out on the partially purified compound. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$

The MECP and MFCP induced mPT pore opening at 10, 20, 40 and 60 µg/mL by 1.1, 2.8, 4.5, and 13.8 folds, and 2.7, 4.5, 6.8, 7.4 folds, respectively compared with control. The MFCP enhanced mATPase activity and inhibited mLP maximally at 100 µg/mL and 120 µg/mL by 3.5 folds and 93.1%, respectively compared with control. Opening of mPT pore by MFCP was observed after treatmentat 100 and 200 mg/kg by 2.6 and 3.3 folds, respectively. The MFCP inhibited proliferation of prostatic LNCaP, DU-145, lung adenocarcinoma and VERO cells with IC_{50} values of 2.4± 0.2, 3.3± 0.2, 3.6± 0.2 and 17.9± 1.6 µg/mL, respectively. The growth inhibition by MFCP correlated with a 3-fold decreased expression of Bcl-2 and a 4-fold increase in Bax levels at 10 µg/mL in LNCaP cells. Furthermore, MFCP caused a 3.5-fold reduction in FIR at 10 µg/mL and induced CCR by 4.2 folds at the same concentration relative to control. The MFCP-induced CCR is associated with activation of C3 and C9 at 10 µg/mL by 4.2 and 5.1 folds, respectively which prompted cancer cells to arrest at S phase (by up to 64.3%). The NMR data revealed the presence of gallic-acid and xanthone in MFCP. Studies have shown that xanthone inhibits growth of cancer cells.

Methanol fraction of *Calliandraportoricensis* exhibited anticancer and antiproliferative activities via mitochondrial-mediated apoptosis.

Keywords: Mitochondrial permeability transition, Prostate cancer cells, *Calliandra portoricensis*-induced apoptosis

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CERTIFICATION

I certify that this work was carried out by Oyebode, OlubukolaTitilope in the Department of Biochemistry, College of Medicine,University of Ibadan, Nigeria.

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ABBREVIATIONS

NMR Nuclear Magnetic Resonance

mPT	mitochondrial Permeability Transition
MMP	Mitochondrial membrane potential
APAF-1	Apoptosis protease activating factor -1
TNFR	Tumour Necrosis Factor Receptor
DISC	Death Inducing Signalling Complex
PCD	Programmed Cell Death
FAK	Focal Adhesion Kinase
ICAD	Inhibitor of Caspase Activated DNase
FADD	Fas Associated Death Domain
IMM	Inner Mitochondrial Membrane
IAP	Inhibitors of Apoptosis Proteins
AIF	Apoptosis Inducing Factor
AR	Androgen Receptor
PCV	Packed Cell Volume
TBARS	Thiobarbituric Acid Reactive Species
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone
ELISA	Enzyme Linked Immunosorbent Assay
CASPASE	Cysteine-dependent Aspartate Specific Proteases
TLC	Thin Layer Chromatography

LC-MS	Liquid chromatography –mass spectrometry
MS	Mass Spectroscopy
VDAC	Voltage dependent Anion Channel
ANT	Adenine Nucleotide Translocase
CyP-D	Cyclophilin D
AST	Aspartate Aminotransferase
ALT	Alanine Amino transferase
EGTA	Ethylene-glycol tetraacetic acid
2,4-DNP	2,4-dinitrophenol
BAX	Bcl-2 Associated X Protein
DMSO	Dimethylsufoxide
PI	Propidium Iodide
OMM	Outer Mitochondrial Membrane
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid
PT	Permeability Transition
JC-1	Tetraethylbenzimidazolylcarbocyanine iodide
HRP	Horse-radish peroxidase
pNA	p-nitroaniline
PE	Phycoerythrin
PS	Phosphatidylserine
MFCP	Methanol fraction of *Calliandraportoricensis*

FITC	Fluorescein Isothiocyanate
LDL	Low Density Lipoprotein
SMAC	Second Mitochondria-derived Activator of Caspases
DIABLO	Direct IAP binding protein with low pI
BID	BH_3 Interacting Death Agonist

APPENDIX

CHAPTER ONE

1.0 INTRODUCTION

The menace called cancer is taking a heavy toll on humanity. This is as a result of high systemic toxicity and drug resistance which limit the successful outcomes of clinical chemotherapies. In this regard, the International Agency for Research on Cancer (IARC) has predicted that global new cases of cancers are expected to rise from 11 million to 16 million by 2020, with developing countries contributing to 70% of them. She also notes that there are more cases of prostate cancer being diagnosed in males, while breast cancer is more prevalent in females (International Agency for Research on Cancer, 2015).

Thus, development of newer anti-cancer therapies which have the ability to kill malignant cells while producing minimal or no toxicity to normal cells as opposed to irradiation and deleterious chemotherapeutic agents are therefore encouraged. In search for a potent cancer cure, mitochondria came into focus of contemporary research given the fact that programme cell death is evaded in all forms of cancer.

Programmed cell death otherwise called apoptosis is a genetically physiological process that allows a cell to commit suicide by design (Duprez *et al.*, 2009; Wyllie, 2010). Essentially, the survival of multicellular organisms is dependent on this vital process in order to eliminate damaged or infected cell that may interfere with normal cell function (Gupta *et al.*, 2009; Galluzzi *et al.*, 2010). The two major apoptotic pathways are the mitochondrial-mediated or intrinsic pathway and the receptor or extrinsic pathway (Elmore, 2007 ; Favaloro *et al.*, 2012).

The extrinsic pathway is mediated by a sub-group of Tumor Necrosis Factor receptors (TNFR) superfamily that includes TNFR, Fas and TRAIL. Activation of these so called death receptors leads to the recruitment and activation of initiator caspases 8 and 10 (Portt *et al.*, 2011). The process involves the formation and activation of complexes such as the Death Inducing Signaling Complex (DISC) which leads to the activation of effector /executioner caspases 3, 6 and 7. The active executioner caspases are responsible for the cleavage of a number of so-called death substrates that lead to the well-known characteristic phenotypes of an apoptotic cell including DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and biochemical changes (Hengartner, 2000; Adams, 2003; Portt *et al.*, 2011).

The intrinsic apoptosis is initiated by the stress-mediated release of cytochrome c from the mitochondria that results in the formation of the apoptosome. The apoptosome then activates initiator caspase, typically caspase 9, which leads to the activation of the executioner caspases 3,6 and 7, the same apoptotic response as observed for the extrinsic pathway (Cooper, 2012). In response to apoptotic stimuli, pro-apoptotic members of the Bcl-2 protein family (Bax and Bak) become activated and act on the mitochondria to induce the release of cytochrome c and other pro-apoptotic proteins by the mitochondria (Galluzzi *et al.*, 2010).

A crosstalk has been shown to occur via the caspase 8 cleavage of the pro-apoptotic Bcl-2 member Bid, to its activated tBid form which leads to the release of cytochrome c via permeabilization of outer mitochondrial membrane (Lopez and Tait, 2015). This demonstrates the central role of the mitochondria in the highly regulated and complex process of many forms of programmed cell death (PCD) (Nagley *et al.*, 2010).

Natural compounds including diterpenoids, sesquiterpenes, flavonoids, alkaloids, and polyphenolics have been reported to have wide spectrum of biological effects, including antifungal, antihelmintic, antimicrobial, anti-inflammatory, antitrypanosomal, and antiproliferative effects on various cancer types (Martin, 2006; Millimouno *et al.*,2014)

1.2 RATIONALE

Attempts to understand the molecular structure of the mitochondrial permeability transition (mPT) pore has gained momentum since its discovery but the protein composition of the pore remained an unsolved riddle as models explaining the recent advances in mPT pore structure are still emerging. Although the exact structure of the mPT pore is not known, its existence has been confirmed by the numerous chemicals which interact it. The importance of the pore is underscored by the release of cytochrome c upon the opening of the pore which eventually results in the activation of caspases and subsequently leads to cell death/ apoptosis. This feature of the pore has made it an important pharmacological target in diseases associated with dysregulated apoptosis.

Currently, a number of chemicals have been shown to interact with the pore via modulation of mitochondrial –mediated apoptosis. Studies have shown that there are bioactive agents isolated from natural sources that act as mitochondrial membrane disruptors (Martin, 2006). These agents include epigallocatechingallate (EGCG) in green tea, quercetin in onions, resveratrol in grapes, curcumin in turmeric and genistein in soybeans, lycopene in tomatoes etc. (Millimouno *et al.*, 2014).

Interestingly, some of these chemicals are undergoing clinical trials for the treatment of diseases such as cancer and ischaemic reperfusion injury. *Calliandra portoricensis*, a member of the mimosaea family, is used in combination with *Plumbago zeylanica* in the traditional treatment of prostate tumour. The active principle of *P. zeylanica* "plumbagin"- a naphtoquinone derivative has been identified and shown to have anticancer and antiproliferative activities in different

cancer cell lines (Subramaniya *et al.*, 2011) even though it is highly toxic and is not considered for clinical trials.

However, there is paucity of knowledge on the bioactive agent(s) present in *C. portoricensis* and the mechanism of its role in traditional method of prostate cancer treatment. It is not known whether fractions of *C. portoricensis* contain potent phytochemicals that modulate apoptosis via opening of the mPT pore. It is against this background that the influence of certain fractions of *C. portoricensis* on mitochondrial-mediated apoptosis was assessed.

1.3 OBJECTIVES

Downregulation of apoptosis has been documented in all forms of cancers. Given that apoptosis is tightly regulated, it is important to confirm downstream events in order to ensure that the process was not aborted. Bioactive agents that induce the opening of the mPT pore, thereby activating the intrinsic pathway of apoptosis, are useful in disease conditions where apoptosis is downregulated while those that inhibit pore opening are important chemotherapeutic tools in diseases characterised by excessive cell death e.g ischeamic reperfusion injury. Hence, the mPT pore is a platform that paves way for modulating the intrinsic pathway of apoptosis.

2.1 GENERAL OBJECTIVE

This study is therefore aimed at examining the influence of certain fractions of *C. portoricensis* on mitochondrial-mediated apoptosis.

2.2 SPECIFIC OBJECTIVES

1. To demonstrate the in *vitro* effects of the crude methanol extract of root bark of *C. portoricensis* and its various solvent fractions (chloroform, ethylacetate, and methanol) on mPT pore of rat liver.

2. To determine the effect of crude extract and its various solvent fractions (chloroform, ethylacetate, methanol) on mitochondrial ($\mathbf{F_oF_1}$) ATPase activity.

3. To determine the effect of the crude extract and its various solvent fractions (chloroform, ethylacetate, methanol) on $FeSO_4$-induced mitochondrial lipid peroxidation.

4. Assessment of the efficacy of the most potent fraction in the induction of cytochrome c release and activation of caspases.

5. To determine the toxic effects of the most potent fraction on vital organs such as the liver, kidney and prostate via the histology of the organs in normal wistar strain rats and its effects on haematological parameters.

6. To determine the anticancer and anti-proliferative properties of the most potent fraction and its isolated compound on mitochondrial-mediated apoptosis in some cancer cell lines.

7. To identify molecular targets of the most potent fraction with respect to mitochondrial-mediated apoptosis in prostate cancer cell lines.

CHAPTER TWO

LITERATURE REVIEW

2.0 APOPTOSIS

Apoptosis was a term introduced in 1972 by Kerr, Wyllie, and Currie in 1972 to describe and distinguish a morphological form of cell death with regulated, endogenously driven and energy-dependent mechanisms (Kerr and Wyllie, 1972; Kerr, 2002; Wyllie, 2010). The concept of the mechanisms involved in the process of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (Horvitz, 1999). Apoptosis occurs physiologically during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. It is a process employed by multicellular organisms to remove infected, damaged, and unwanted cells so that the whole can better survive. It also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or harmful agents (Norbury and Hickson, 2001)

2.1 MORPHOLOGICAL FEATURES OF A DYING CELL

The early phase of apoptosis is characterised by cell shrinkage which results in a smaller cell size, a dense cytoplasm and more tightly packed organelles and chromatin condensation referred to as Pyknosis (Kerr *et al.*, 1972; Hacker, 2000). Extensive plasma membrane blebbing occurs followed by separation of cell fragments into apoptotic bodies during a process called budding."

Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and enclosed within an intact plasma

membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells or neoplastic cells and degraded within phagolysosomes (Hacker, 2000).

Essentially, apoptosis or removal of apoptotic cells is not associated with any inflammatory reaction because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue as they are quickly phagocytosed by surrounding cells thus, likely preventing secondary necrosis. This also prevents engulfing cells from producing anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka *et al.*, 2003).

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, extensive protein cross-linking (Nemes *et al.*, 1996), DNA breakdown and phagocytic recognition that together result in the biochemical features that differentiates them from healthy cells (Hengartner, 2000). Their DNA is degraded by Ca^{2+}-and Mg^{2+}-dependent endonucleases , resulting in DNA fragment (Bortner *et al.*, 1995). The expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells is achieved by the flipping out of the normal inward-facing phosphatidylserine of the cell's lipid bilayer for expression on the outer layers of the plasma membrane (Bratton *et al.*, 1997). Although, externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, recent studies have shown that other proteins including Annexin I and calreticulin can also be exposed on the cell surface during apoptotic cell clearance.

Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis

(Arur *et al*., 2003) while calreticulin is a protein that binds to an LDL receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (Gardai *et al*., 2005).

2.2 CASPASES: THE DEMOLITION MACHINERY

Caspases are a unique and closely related set of proteases, so called because of the cysteine at their active site and the tightly defined four-amino-acid motif (including aspartate at positions 1 and 4) at their target site. Caspases exist as inactive zymogens or proenzymes. The procaspase is proteolytically cleaved during apoptosis to generate a small subunit and a large subunit. The two cleaved fragments form a heterotetramer, which is the active form of the enzyme. Activation of caspases is a downstream event in apoptosis pathways and blocking caspase activity has been shown to eliminate almost all programmed developmental cell death in *Caenorhabditis elegans* (Yuan *et al.*,1993). Hence, activation of caspases must be and is indeed under tight control.

The caspases form a cascade in which initiator caspases are activated by death stimuli arising either at the cell membrane as a result of cytokine–receptor binding, or within the cell, in relation to internally determined signals, often generated in the micro-environment of particular organelles. Thus, caspases 8 and 10 are activated when specific extracellular ligands of the tumour necrosis factor family bind to their receptors while caspase 9 is activated at the mitochondrial membrane (Wyllie, 2010). These initiator caspases activate (by cleavage at their specific target sites) a set of effector caspases, notably caspases 3, 6 and 7, which then cleave proteins in many cell compartments leading to caspase activation and is responsible for the biochemical phenotype observed during apoptosis (Thornberry and Lazebnik, 1998 ; Shi, 2002).

Caspase substrates also include cytoskeleton proteins (Ndozangue-Touriguine *et al.*,2008) and the focal adhesion kinase (FAK) (Wen *et al.*, 1997), whose cleavage accounts for the loss of substratum contact and the loss and rounding up of the dying cell. Some caspase substrates form part of complex pathways with several interacting members e.g the cytoplasmic chaperone inhibitor of caspase activated DNAse (ICAD), whose cleavage releases a nuclease from its anchor within the cytoplasm and permits its unfolding to reveal a nuclear localisation signal (Nagata, 2000). Caspases may also be responsible for the release of nucleotides from apoptotic cells that serve as homing signals for the macrophages that ultimately engulf them (Elliott *et al.*, 2009). Thus, the activation of caspases is central to the observed molecular events that occur in apoptosis.

2.3 EXTRINSIC PATHWAY

The extrinsic pathway of apoptosis involves transmembrane receptor-mediated interactions. These involve ligation of death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily with their cognate ligands (Locksley *et al.*,2001). A common feature of the TNF receptor family is a similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit, 1998). The death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. The best-characterized ligands and their corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5 (Chicheportiche *et al.*, 1997; Suliman *et al.*, 2001; Rubio- Moscardo *et al.*, 2005).

Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein Fas associated death domain(FADD) and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TNF-related associated binding domain (TRADD) (Hsu *et al.*, 1995 ; Wajant, 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain resulting in the formation of a death-inducing signaling complex (DISC) which leads to the auto-catalytic activation of procaspase-8 (Kischkel *et al.*, 1995). Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective (Scaffidi *et al.*, 1999).

2.4 INTRINSIC PATHWAY/MITOCHONDRIAL-MEDIATED PATHWAY

It has been widely accepted that the permeability of the mitochondrial inner membrane is extremely low; thus, the discovery of a nonspecific permeability transition with a threshold of 1.5 kDa suggested the existence of a pore called the mitochondrial permemability transition (mPT) pore that was responsible for this transition (Halestrap *et al.*, 1997).

Opening of the mPT pore facilitates the free passage of protons across the inner mitochondrial membrane (IMM), leading to a dissipation of the mitochondrial membrane potential, an essential component of the proton motive force (PMF). This sudden event does not only prevent ATP synthesis, an important process in all cells driven by PMF, but also activates its hydrolysis, causing the breakdown of cytosolic ATP that is generated via glycolysis. Energy metabolism is further impaired, thereby resulting in a continuous cycle of increasing Ca^{2+} deregulation and mPT pore opening (Bernardi and Di Lisa, 2015) .

Molecular events sequel to prolonged mitochondrial permeability transition include collapse of mitochondrial membrane potential, increase in phosphate concentration which is an index of enhancement of ATPase activity and cytochrome c release (Kroemer *et al.*, 2007; Rasola and Bernardi, 2011).

The mPT pore has therefore become a strategic target for various pharmacological intervention and drug development because the consequent release of cytochrome c upon the opening of the pore is a point of no return for apoptosis, a form of programmed cell death that is downregulated in all forms of cancers (Halestrap and Brenner, 2003; Kroemer *et al.,2007).* The stimuli that initiate mitochondrial–mediated events include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals which bring about changes in the inner mitochondrial membrane thus results in an opening of the mitochondrial permeability transition (mPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens *et al.*, 2004; Elmore, 2007; Wang and Youle, 2009).

The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Du *et al.*, 2000; Garrido *et al.*, 2006). These proteins activate the caspase-dependent mitochondrial pathway .The release of cytochrome c from the intermembrane space into the cytosol is considered a point of no return for apoptosis to take place. This is because once released, cytochrome *c* binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" (Chinopoulos *et al.*, 2014; Lopez and Tait, 2015).

The clustering of procaspase-9 in this manner leads to caspase-9 activation and subsequent activation of effector caspases (Olszewska and Szewczyk, 2013). The Smac/DIABLO and HtrA2/Omi have been shown to promote apoptosis by inhibiting IAP activity (inhibitors of apoptosis proteins) (van Loo *et al.*, 2002; Schimmer, 2004). Additional mitochondrial proteins have also been identified that interact with and suppress the action of IAP. However, gene knockout experiments suggest that binding to IAP alone may not be enough evidence to label a mitochondrial protein as "pro-apoptotic" (Ekert and Vaux, 2005).

The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell is committed to die. The AIF translocates to the nucleus and causes DNA fragmentation and condensation of peripheral nuclear chromatin (Joza *et al.*, 2001) This early form of nuclear condensation is referred to as "stage I" condensation (Joza *et al.*, 2001). Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments (Li *et al.*, 2001). AIF and endonuclease G both function in a caspase-independent manner. The CAD is subsequently released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation (Enari *et al.*, 1998) also referred to as "stage II" condensation (Susin *et al.*, 2000).

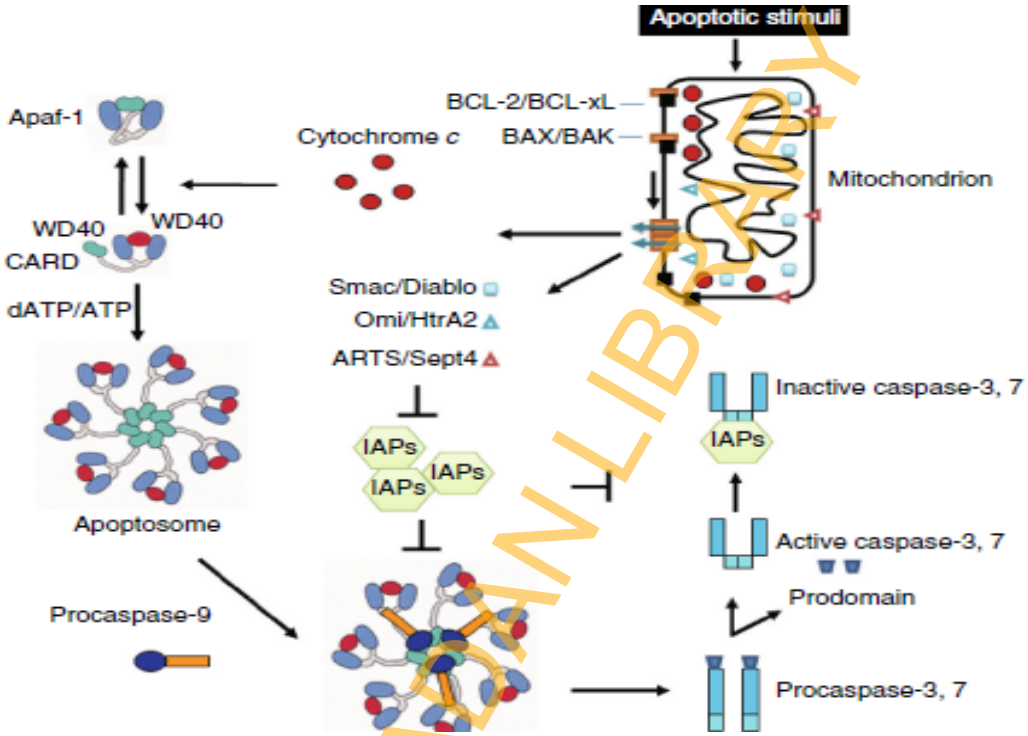

Figure 1: Mitochondrial -mediated pathway of apoptosis (Xiong *et al.*, 2014)

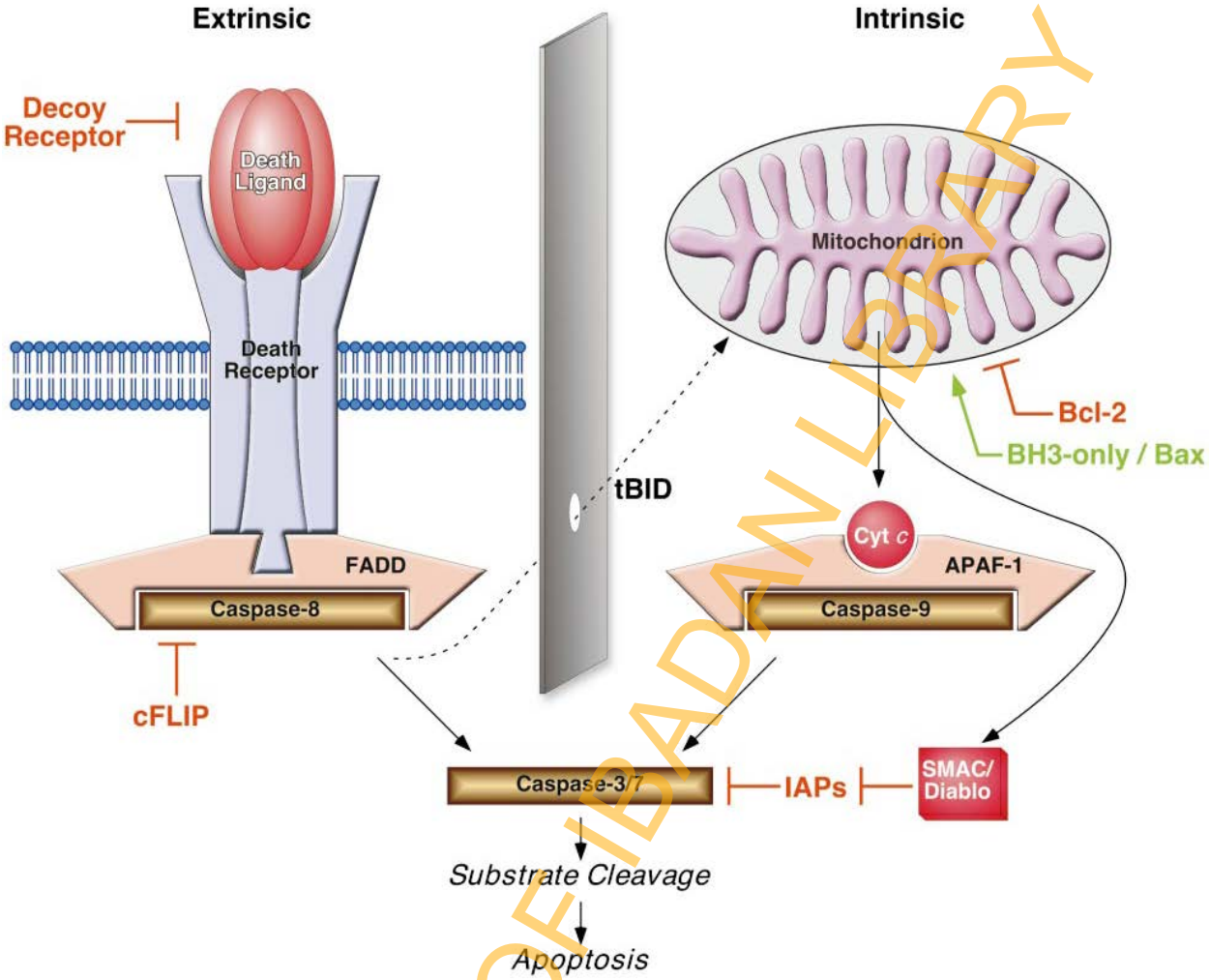

Figure 2: Cross talk between Extrinsic and Intrinsic apoptotic pathways (Roy and Nicholson, 2000)

2.5 PATHOLOGIC APOPTOSIS

Various stimuli have been shown to trigger apoptosis, however not all cells will die in response to the same stimulus. Studies have shown that cell death dysregulation is a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome (ALPS) (Worth *et al.*, 2006), AIDS, ischemia, and neurode-generative diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease. Some conditions are characterized by insufficient apoptosis whereas others feature excessive apoptosis (Elmore, 2007 ; Wang and Youle, 2009). Tumor cells can acquire resistance to apoptosis and fuel the pathway to formation of tumours by the expression of anti-apoptotic proteins such as Bcl-2 or by the down-regulation or mutation of pro-apoptotic proteins such as Bax. The expression of both Bcl-2 and Bax is regulated by the *p53* tumor suppressor gene (Miyashita *et al.*, 1994). Certain forms of human B-cell lymphoma have over-expression of Bcl-2 and this is one of the first and strongest lines of evidence that failure of cell death contributes to cancer (Vaux *et al.*, 1988).

Besides, apoptosis suppression in cancer can also involve evasion of immune surveillance by diminishing the response of the death receptor pathway to FasL produced by T-cells. The mechanisms involved include downregulation of the Fas receptor on tumor cells (Smyth *et al.*, 2001), expression of non-functioning Fas receptor (Cheng *et al.*, 1994; Elnemr *et al.*, 2001). Studies have also shown that some tumor cells are capable of a Fas ligand-mediated "counterattack" that results in apoptotic depletion of activated tumor infiltrating lymphocytes (Koyama *et al.*, 2001).

Pathological conditions that feature excessive apoptosis (neurodegenerative diseases, AIDS, ischemia, etc.) may benefit from artificially inhibiting apoptosis (Nicholson, 2000).

Autoimmune diseases results from infection with the human immunodeficiency virus (HIV) (Li *et al.*, 1995). This virus infects $CD4^+$ T-cells by binding to the CD4 receptor. The virus is subsequently internalized into the T-cell where the HIV Tat protein is thought to increase the expression of the Fas receptor, resulting in excessive Apoptosis of T cells. Alzheimer's disease, a neurodegenerative condition, is thought to be caused by mutations in certain proteins such as APP (amyloid precursor protein) and presenilins.

2.6 CONTROL AND REGULATION OF APOPTOSIS INTERPLAY OF BCL-2 FAMILY MEMBERS

The Bcl_2 family share common features with the B-cell lymphoma oncogene whose discovery led eventually to the identification of most of the other family members, but at the molecular level this family is remarkably diverse. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk (Adams and Cory, 2007). These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. The Bcl-2 family of proteins can either be pro-apoptotic or antiapoptotic. They define thresholds for apoptosis by governing mitochondrial membrane permeability and are united through their possession of homologous domains responsible for protein– protein interactions amongst the family members (Danial, 2004; Adams and Cory, 2007; Hotchkiss *et al.*, 2009).

The Bcl_2 itself and its closest relatives (e.g. Bcl-XL) possess four such domains, BH-1, 2 and 4, defining a hydrophobic groove within the molecule, and BH3, a short (8–12 amino acid) region that binds within that groove. These members of the family support cell survival, whilst the shared BH domains permit interaction with two powerful pro-death molecules, Bax and Bak, through the formation of heterodimers. Bax and Bak (which possess the BH1-3 domain but not BH4) can also form homo-oligomers and, in this configuration, can create a wide diameter pore through cell membranes (Kuwana *et al.*, 2002).

In the mitochondrial membrane, studies have shown that such pores allow escape of apoptogenic molecules from the intermembrane space to the cytosol. Amongst the escaping molecules are cytochrome c and dATP, which together activate caspase 9, held by its association with a monomeric protein (with remarkable, seven-fold symmetry) called Apaf-1. The concentrations of Bax or Bak relative to the Bcl_2-$BclX_L$ or other pro-survival family members thus determine the probability that this dramatic rise in mitochondrial permeability will occur, activating the intrinsic pathway. It is therefore thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome *c* release from the mitochondria via alteration of mitochondrial membrane permeability (Delbridge and Strasser, 2015).

The remaining members of the Bcl_2 family possess only the BH3 domain as their region of homology with the rest of this extended family. These proteins (bid, bad, bim and others) all promote death. Although their precise mode of action is still disputed, it is thought that their high affinity binding via the BH3 domain to the hydrophobic groove in Bcl_2 and Bcl-X_L is responsible for their action. (Willis *et al.*, 2007; Adams and Cory, 2007).

A rise in cell concentration of BH3-only proteins will therefore create conditions in the immediate vicinity of the mitochondrial outer membrane that favour formation of bax/bak oligomer formation and the permeablization of the outer mitochondrial membrane. This widely diverse family of "BH3- only" proteins appears to provide signals in response to a variety of injuries. Thus, they act as sensors of "danger" or "stress" conditions.

2.6.1 EXTENDED ROLES OF THE BCL_2 FAMILY

Amplification of the intrinsic/Fas pathway of apoptosis in cases where apoptotic stimuli does not result in cell death is mediated by the caspase-8 cleavage of Bid to its truncated form, tBid (Li *et al.*, 1998; Esposti, 2002) which oligomerizes on the outer mitochondrial membrane leading to release of apoptogenic proteins (Igney and Krammer, 2002). Serine phosphorylation of Bad is associated with 14-3-3, a member of a family of multifunctional phosphoserine binding molecules. When Bad is phosphorylated, it is trapped by 14-3-3 and sequestered in the cytosol but once Bad is unphosphorylated, it will translocate to the mitochondria to release cytochrome c (Zha *et al.*,1996).

Bad can also heterodimerize with Bcl-Xl or Bcl-2, neutralizing their protective effect and promoting cell death (Yang *et al.*, 1995). When not sequestered by Bad, both Bcl-2 and Bcl-X_L inhibit the release of cytochrome c from the mitochondria although the mechanism is not well understood. Reports indicate that Bcl-2 and Bcl-XL inhibit apoptotic death primarily by controlling the activation of caspase proteases (Newmeyer *et al.*, 2000). An additional protein designated "Aven" appears to bind both Bcl-Xl and Apaf-1, thereby preventing activation of procaspase-9 (Chau *et al.*, 2000). There is evidence that overexpression of either Bcl-2 or Bcl-X_L will downregulate the other, indicating a reciprocal regulation between these two proteins.

Puma and Noxa are two members of the Bcl_2 family that are also involved in pro-apoptosis. Puma plays an important role in *p53*-mediated apoptosis. It was shown that, in vitro,overexpression of Puma is accompanied by increased Bax expression, Bax conformational change, translocation to the mitochondria, cytochrome *c* release and reduction in the mitochondrial membrane potential (LiLiu *et al.*, 2003).

Noxa is also a candidate mediator of *p53*-induced apoptosis. Studies show that this protein can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 (Oda *et al.*, 2000). Since both Puma and Noxa are induced by *p53*, they might mediate the apoptosis that is elicited by geno-toxic damage or oncogene activation. The Myc oncoprotein has also been reported to potentiate apoptosis through both *p53*-dependent and –independent mechanisms (Meyer *et al.*,2006).

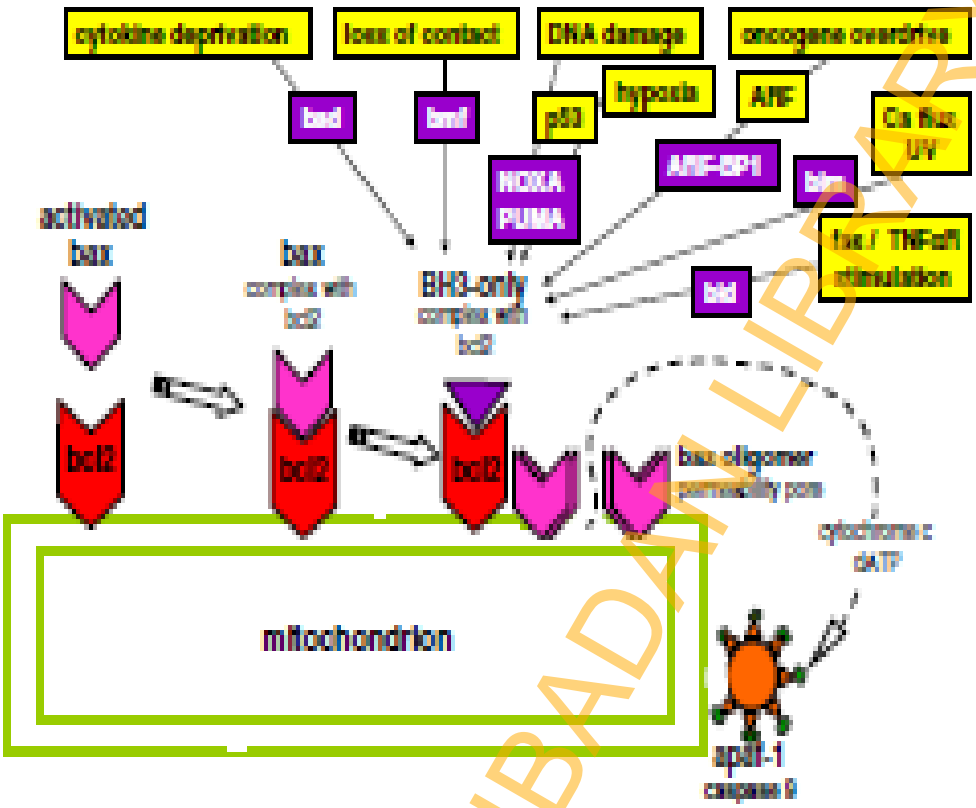

Figure 3: The three major mammalian factions of the Bcl-2 family (Wylie, 2010)

2.7 APOPTOSIS VERSUS NECROSIS

The death pathway employed by a cell is dependent on the type of stimuli, the degree and duration of stimuli. At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis but these same stimuli can result in necrosis, an accidental demise, at higher doses (Elmore, 2007). Necrosis is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death as opposed to apoptosis (Levin *et al.*, 1999).

Apoptosis and necrosis are two processes that can occur independently, sequentially, as well as simultaneously (Hirsch *et al.*, 1997; Zeiss, 2003). Besides, the nature of the cell death signal, the tissue type, extent of ATP depletion, availability of caspases ,the developmental stage of the tissue and the physiologic milieu (Fiers *et al.*, 1999; Zeiss, 2003) also has a role to play in this important cell decision. (Leist *et al.*, 1997; Denecker *et al.*, 2001)

Although the mechanisms and morphological features of apoptosis and necrosis differ, evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the "apoptosis-necrosis continuum" by Zeiss in 2003. Necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells.

Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of

ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane (Kerr, 1972; Majno and Joris, 1995; Trump *et al.*, 1997).

This loss of cell membrane integrity results in the release of the cytoplasmic contents into the surrounding tissue, sending chemotatic signals with eventual recruitment of inflammatory cells.

2.8 MITOCHONDRIAL PERMEABILITY TRANSITION: AN HISTORICAL PERSPECTIVE

The discovery of the permeability transition (PT) dates back to almost 40 years , when Robert Haworth and Douglas Hunter demonstrated a reversible opening of a channel in the inner mitochondrial membrane that could be induced by Ca^{2+}, phosphate, arsenate or oleic acid, resulting in matrix swelling as a result of the opening of a conductance channel referred to as mitochondrial Permeability Transition (mPT) pore (Hunter *et al.*, 1976; Haworth and Hunter, 1979). Further, they showed that this process required the energized uptake of Ca^{2+} through a ruthenium red-sensitive mechanism (now known to be the mitochondrial calcium uniporter (MCU), the genetic identity of which was recently discovered (De Stefani *et al.*, 2011; Mallilankaraman *et al.*, 2012) and that permeability transition resulted in the uncoupling of oxidative phosphorylation.

The interest on the mPT pore was dramatically raised when it was discovered that it could be inhibited by a fungal peptide, cyclosporin A(CsA), following its interaction with a mitochondrial chaperone, the peptidyl-prolyl cis-trans isomerase cyclophilin D (CyP-D) (Crompton *et al.*, 1988; Halestrap and Davidson, 1990)

2.9 MITOCHONDRIAL PERMEABILITY TRANSITION: PHYSIOLOGICAL ROLE AND CONSEQUENCES

It has been widely accepted that the permeability of the mitochondrial inner membrane is extremely low; thus, the discovery of a non-specific permeability transition with a threshold of 1.5 kDa suggested the existence of a pore (called the mPT pore) that was responsible for this transition (Halestrap *et al.*, 1997).

Mitochondrial permeability transition is defined as the collapse of the chemiosmotic gradient across the inner mitochondrial membrane (IMM) mediated by opening of a large conductance pore referred to as the mPT pore. (Elrod and Molkentin, 2013) Indeed, the mPT pore is a voltage and Ca^{2+}-dependent, CsA-sensitive, high conductance channel, whose prolonged opening leads to a sudden increase in the permeability of the inner mitochondrial membrane to solutes with molecular mass up to 1500 Da (Bernardi *et al.*, 2006). Consequently, a sequence of events which leads to a bio-energetic catastrophe occurs. These include equilibration of the proton gradient which causes mitochondrial depolarization, followed by respiratory inhibition and generation of reactive oxygen species (ROS), massive release of matrix Ca^{2+} and swelling of mitochondria leading to breaches in the outer mitochondrial membrane that induce the release of intermembrane proteins. Thus, mPT pore opening is traditionally linked to mitochondrial dysfunction and prompts the demise of the cell (Grimm and Brdiczka, 2007; Rasola and Bernardi, 2007; Zorov *et al.*, 2009), and its dysregulation is a crucial step in the pathogenesis of a variety of diverse diseases including ischemia-reperfusion damage (Grimm and Brdiczka, 2007; Javadov *et al.*, 2009), lysosomal storage diseases (Sano *et al.*, 2009), liver damage (Soriano *et al.*, 2004), many acute and chronic disorders of the central nervous system (Rasola

and Bernardi, 2011), collagen VI myopathies (Bernardi and Bonaldo, 2013) and cancer (Rasola *et al.*, 2010).

These detrimental effects on energy conservation and cell viability are only seen for long-lasting openings of the mPT pore (Petronilli *et al.*, 2001) while short-term openings which have been shown both in isolated mitochondria and in situ (Hüser and Blatter, 1999) to be involved in physiological regulation of Ca^{2+} and reactive oxygen species (ROS) homeostasis (Zorov *et al.*, 2014). As a result, reversible openings of the mPT pore provide mitochondria with a fast mechanism for Ca^{2+} release (Bernardi and Petronilli, 1996; Bernardi and Stockum, 2012).

2.10 MITOCHONDRIAL PERMEABILITY TRANSITION (mPT) PORE: MANY SUSPECTS, NO CULPRITS

The initial clue for the existence of the mPT pore came from the very early studies of Haworth and Hunter, which suggested that a hydrophilic channel was responsible for the permeability transition induced by polyethylene glycol (PEG) polymers of size up to 1.5 kDa (Harworth and Hunter, 1979). Crompton and Costi in 1988 confirmed this idea by showing how, in its opened state, the mPT pore channel should obtain a diameter of 2–2.6 nm (Crompton, 1990). Later, the mitochondrial mega channel (MMC), a channel in the innermembrane endowed with high-conductance (≈1–1.3 nS), and all the key regulatory features of the mPT pore was discovered through electrophysiological experiments performed by Zoratti's group. These features include inhibition by CsA, adenine nucleotides, Mg^{2+}, acidic pH, and reducing agents; induction by $Ca^{2+}$and voltage sensitivity (Kinnally *et al.*, 1989; Szabo and Zoratti, 1991). Taken together, these observations leave little doubt that the MMC is the mPT pore (Szabo and Zoratti, 1992). Over the years, models explaining the mPT pore components have been put forward.

2.10.1 CONFORMATIONAL CHANGE IN ADENINE NUCLEOTIDE TRANSLOCASE (ANT) HYPOTHESIS

The ANT is a-32 kDa inner membrane transporter responsible for the import of ADP into the mitochondrial matrix in exchange for ATP and as such is an integral component of the mitochondrial ATP synthesis machinery (Klingenberg, 2008). The finding that bongkrekic acid, an inhibitor of ANT by locking it in the "m" confirmation, was capable of delaying permeability transition, while a different inhibitor of ANT that locks it in the "c" confirmation (atractyloside) could sensitize opening in response to Ca^{2+}, suggested that ANT could be a structural component of the mPT pore (Lê Quôc and Lê Quôc, 1988). Halestrap and his colleague further developed this hypothesis stating that the effects of both CsA and the inhibitory action of ADP on pore opening could be attributed to direct binding of ANT to CypD, an already known inhibitor of the pore. (Halestrap and Davidson, 1990). This theory held until mice lacking *Ant1*and *Ant2* (*Ant1/2–/–*) were generated and mitochondria isolated from double-null hepatocytes were shown to still undergo permeability transition (CsA-sensitive) and remained susceptible to cell death initiated by various agents (Kokoszka *et al.*, 2004). This finding suggested that ANT was not a structural component of the mPT pore, although it should be noted that *Ant1/2–/–* mitochondria required a higher concentration of Ca^{2+} to induce opening and that ANT ligands (such as ADP) lost inhibitory capacity on pore-open probability. However, there is evidence that ANT may represent a major site of oxidant stress and thiol modulation of mPT pore function (Halestrap *et al.*, 1997). Collectively, studies on ANT suggest that it is not a requisite component of the mPT pore, but it clearly regulates mPT pore activity.

2.10.2 CONTACT –SITE HYPOTHESIS

The first evidence that VDAC (referred to as porin in the older literature) may be the outer membrane pore-forming component of the mPT pore came in 1985 when the Le Quocs characterized a mitochondrial swelling event that appeared to require the outer mitochondrial membrane (OMM). They found mitoplasts (mitochondria stripped off their outer mitochondrial membrane)) did not respond in the classic swelling assay.(Le Quoc and Le Quoc, 1985). They suspected VDAC was involved possibly by migrating to the IMM to permit permeability transition. Other reports also supported the VDAC hypothesis through anti-VDAC antibody blockade of permeability transition, (Shimizu *et al* , 2001) and inhibition of the mPT pore by VDAC phosphorylation (Javadov *et al.*, 2009). However, as with ANT, a genetic approach in the mouse eventually dispelled the notion that VDAC was a necessary component of the permeability pore. Baines *et al* (2007) demonstrated that deletion or knockdown of all three *Vdac* genes (*Vdac1*, *2*, *3*) did not disrupt mPT pore function and furthermore did not alter necrotic or apoptotic cell death (Baines *et al.*, 2007). In fact, deletion of *Vdac2* enhanced cell-death propensity (Cheng *et al.*, 2003; Baines *et al.*, 2007).

The initial involvement of the VDAC in the mPT pore structure suggested that it might not be a common pore but rather a more complex and highly organized structure that included contact sites between the inner mitochondrial membrane and the outer mitochondrial membrane , a multimolecular mPT pore model was proposed that included proteins of all mitochondrial compartments as a result of their aggregation at “contact sites” (Zamzami and Kroemer, 2001). Following this model, mPT pore components included CyP-D in the matrix, the adenine nucleotide translocator (ANT) in the inner membrane, the mitochondrial creatine kinase in the

intermembrane space, the porin/voltage-dependent anion channel (VDAC) in the outer membrane, the isoform II of hexokinase (HK II) and the peripheral benzodiazepine receptor (PBR, now termed TSPO), together with Bcl-2 family proteins, externally associated on the mitochondrial surface. Despite its popularity, rigorous genetic analyses ruled out the possibility that any of these proteins could be part of the core of the mPT pore as a CsA-sensitive permeability transition could be easily detected in the absence of ANT (Kokoszka *et al.*, 2004) VDAC (Krauskopf *et al.*, 2006; Baines *et al.*, 2007)] as well as of TSPO (Šileikyte *et al.*, 2014). However, the same genetic analyses demonstrate that CyP-D is a crucial regulator of the mPT pore. Indeed, deletion of CyP-D renders the mPT pore insensitive to CsA and doubles the threshold of Ca^{2+} required to open the pore in the presence of phosphate anion (Pi) (Baines *et al.*, 2005; Basso *et al.*, 2005). Hence, these proteins have been categorized in the broad group of activity regulators.

2.10.3 THE MITOCHONDRIAL PHOSPHATE CARRIER HYPOTHESIS

Inorganic phosphate carrier (PiC) (*SLC25A3* gene) is the primary transporter of inorganic phosphate (P*i*) into the mitochondrial matrix by proton co-transport or in exchange for hydroxyl ions (Palmieri, 2012) . Historically, P*i* is a potent modulator of pore opening with countless studies suggesting a definitive relationship between matrix P*i* content and mPT pore activation.(Crompton *et al.*, 1988;Varanyuwatana and Halestrap, 2012)

An alternative model is the formation of the mPT pore by the Pi carrier following its interaction with CyP-D and ANT and due to the fact that phosphate greatly influences mPT pore opening. Besides, this interaction is strengthened by mPT-inducing agents whereas mPT-blocking agents diminished this interaction (Leung *et al.*, 2008). In the same year, based on a genetic screening, another group discovered that PiC overexpression induces mitochondrial dysfunction and apoptosis (Alcalá *et al.*, 2008). These results, together with the earlier finding that a non-specific pore is generated in liposomes by reconstituting the inorganic phosphate carrier (PiC) (Schroers *et al.*,1997) identified PiC as a strong candidate for the core-forming element of the mPT pore.

This idea was well accepted until 2014 when the same group performed PiC silencing experiments and found that knockdown of up to 70% of this carrier does not lead to any significant alteration in the Ca^{2+} threshold for the mPT, suggesting that either a small amount of PiC is required in the mPT pore structure or that PiC is not a component of this structure (Varanyuwatana and Halestrap, 2012). Moreover, genetic deletion of the PiC further confirmed the idea that this protein is essential for mPT pore formation (Kwong *et al.*, 2014), while studies on Ppif−/− mice (Ppif is the unique gene encoding CyP D in the mouse) have also earlier demonstrated that this protein is an important modulator which sensitizes the mPT pore to Ca^{2+} and confers sensitivity to CsA, but not an essential pore component (Basso *et al.*, 2005; Baines *et al.*, 2005).

2.10.4 CYCLOPHILIN D: THE ONLY DEFINED mPT PORE REGULATOR

CyP-D belongs to cyclophilins known as peptidyl-prolyl cis–trans isomerases (PPIases), a family of proteins that catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds and possess chaperone activity to regulate protein folding. Halestrap and Davidson were the first to identify a matrix-localized PPIase as the target of CsA and further proposed a unified model of interaction with adenine nucleotide translocase (ANT) to modulate permeability. The effect of CsA on the mammalian mPT pore is best described as "desensitization" in the sense that the mPT pore can still occur but becomes more resistant to Ca^{2+}, Pi and other inducers (Bernardi, 2013). This consideration is important because CsA can desensitize but not block the mPT pore, and therefore lack of sensitivity to CsA does not necessarily imply that the mPT pore is not involved in the event being studied; and different cells express different levels of CyPD, and obviously only CyP D-expressing mitochondria can respond to CsA (Li *et al.*, 2012).

To illustrate the importance of the isomerase domain, Baines *et al* (2005) showed that an isomerase deficient mutant of cyclophilin D (R96G) was unable to rescue mitochondrial swelling or ROS-induced cell death in *Ppif*–/– mouse embryonic fibroblasts (MEFs), yet the wildtype (WT) version fully rescued (Baines *et al.*, 2005). These results suggest that the isomerase domain of CypD is necessary for modulation of the mPT pore.

There are seven major cyclophilin isoforms found in subcellular compartments including the cytoplasm (CyP-D, CyP-NK, and CyP-40), endo (sarco) plasmic reticulum (CyP-B, CyP-), nucleus (CyP-E) and mitochondria (CyP-D) (Lee and Kim, 2010). Notably, individual cyclophilins can have distinct effects on cell survival under pathological conditions. Studies performed on various cancer models and tissue samples from patients demonstrated that overexpression of CyP-A stimulates cancer cell growth (Lee and Kim, 2010). On the other hand, expression of CyP-D, a soluble matrix protein, is associated with mPT pore opening and cell death during ischemia/reperfusion in the heart and brain. CyP-D is a nuclear encoded protein widely expressed in all mammalian tissues. It contains a mitochondrial targeting presequence which is cleaved after its translocation into the matrix (Halestrap *et al.,* 1997). In addition to its role in pore opening, CyP-D has been shown to catalyze folding of newly imported proteins in the matrix of mitochondria (Matouschek *et al.*, 1995).

Furthermore, its cysteine residue at position 203 appears to have critical importance, especially regarding the sensitivity of mPT pore to reactive oxygen species (ROS) (Nguyen *et al.,* 2011). Nonetheless, mtCypD is a mitochondrial matrix protein; thus, it is unable to generate a pore, and its depletion does not deny the existence of an MPT but rather dramatically increases the threshold for Ca^{2+} induction (Baines *et al.,* 2005).

2.10.5 ROLE OF CYCLOPHILIN D IN mPT PORE OPENING

Existing studies provide strong evidence that CyP-D plays a regulatory role in mPT, and understanding the mechanism(s) of CyP-D activation and its interaction with the mPT pore complex is important in developing new pharmacological agents to modulate mitochondria-mediated cell death. Importantly, the translocation of CyP-D from the matrix to the IMM and its interaction with a target protein to induce pore opening in response to oxidative stress can occur through both direct and indirect mechanisms. Thus, accumulating data suggest that activation of CyP-D and its interaction with the mPT pore complex can occur through different mechanisms including (i) post-translational modification of the protein, (ii) direct interaction with an active target protein, and/or (iii) indirectly via binding to a matrix protein (Crompton *et al.*, 1998).

Oxidative stress can induce conformational changes of the target protein by chemical modification and/or alterations in the inner membrane topography due to increased matrix swelling bringing about direct binding of CyP-D to a target protein in the IMM. This is evident in most, if not all, previous studies that were focused on ANT andVDAC as target proteins interacting with CyP-D to initiate the pore opening (Crompton *et al.*, 1998)

Also, oxidative stress sensitizes the mPT pore to Ca^{2+} by antagonizing adenine nucleotide binding and enhances CyP-D binding to the ANT (McStay *et al.*, 2002). Chemical modifications of three cysteine residues (Cys56, Cys159 and Cys256) in ANT in response both to oxidative stress and thiol reagents were shown to be associated with a conformational change of the exchanger (Majima *et al.*, 1993).

CyP-D can be activated in the matrix due to post-translational modification, which may facilitate its translocation to the IMM and initiate mPT. Moreover, CyP D can undergo post-transitional modifications (phosphorylation, nitrosylation, acetylation, etc.) on specific site(s) which would increase its activity to interact with a target protein.

However, at present, there are rather few studies directly showing post translational modifications of CyP-D. Recent studies also discovered that acetylation of CyP-D due to inhibition of the mitochondrial isoform of sirtuins, SIRT3 increased interaction of CyP-D with ANT (Shulga and Pastorino, 2010). In addition, significant fraction of GSK-3beta has been shown to be co-localized with CyP-D in mitochondria, suggesting thus a potential regulatory role for GSK-3 beta in pore opening. Active GSK is shown to phosphorylate CyP-D in an ERK1/2-dependent manner, and phospho-CyP-D Ser/Thr promoted depolarization of mitochondria and pore opening (Rasola *et al.,* 2010). Conversely, pharmacological inhibition of GSK-3beta prevented the phosphorylation of CyP-D, which may lead to the inhibition of the mPT in murine tubular epithelial cells (Bao *et al.,* 2012).

Direct evidence for CypD modulation of energy homeostasis can be found in the previously mentioned direct interactions with complex V (ATP synthase) and modulation by hexokinase II , a regulator of the mPT pore that binds within the IMM (Beutner *et al.,* 1998). These proteins are intrinsically linked to ATP production and thus are potential sites of CypD regulation of cellular metabolism.

Indirect binding of CyP-D to a target protein(s) in the IMM can occur through its interaction with other proteins in the matrix. Most recent studies demonstrated that in response to oxidative stress induced by brain ischemia/reperfusion injury, p53, a tumor suppressor protein, accumulates in the mitochondrial matrix and triggers mPT pore opening and necrosis by physical interaction with CyP-D (Vaseva *et al.*, 2012). Likely, p53 triggers translocation of CyP-D to the IMM and therefore facilitates the pore opening through interaction with a pore protein(s).

Notably, binding of CyP-D to a matrix protein in cancer cells may have an opposite effect, leading to inhibition of the mPT pore. Also, it has been demonstrated that abundant expression of Hsp60 in mitochondria of tumor cells is associated with increased levels of the Hsp60-CyP-D complexes and reduced mPT pore opening (Ghosh *et al.*, 2010).

2.10.6 The mPT PORE and MITOCHONDRIAL ATPase

The FoF_1 ATP synthase is a multi-subunit enzymatic complex that couples proton translocation across the inner membrane to ATP synthesis. The proton-translocating domain (F_O)of the ATP synthase is embedded in the inner membrane and is connected to the F_1 catalytic domain through the central and peripheral stalks (Jonckheere, 2012). Since the earliest characterization of the mPT pore, ATP synthase has been postulated as a possible component of the pore itself (Hunter *et al.*, 1976).

The mitochondrial ATPsynthase is a large multiprotein complex composed by 15 sub-units, and it is formed by the catalytic, soluble F_1 domain, which protrudes into the matrix, and by the inner membrane F_0 domain which allows the translocation of protons; the two domains are connected by a lateral and a central stalk (Yoshida *et al.*, 2001). The ATP synthase is a rotary enzyme: in

respiring mitochondria, rotation of the subunit in the central stalk, caused by proton flow across the F_O subunit induces conformational changes in the F_1 subunit that elicit ATP synthesis, while the lateral stalk acts as a stator (Rees *et al.,* 2009). CypD was initially found to modulate ATP synthase activity by binding to the oligomycin-sensitivity conferring protein (OSCP) and delta subunits of the peripheral stalk (Giorgio *et al.,* 2009; Giorgio *et al.,* 2013), which triggers mPT pore opening through dimerization of the F_0F_1 ATP synthase and purified dimers of the F_1F_0 ATP synthase reconstituted into lipid bilayers recapitulated channel activity similar to that of the mPT pore (Giorgio *et al.,* 2013), hence they concluded that the pore is formed by dimers of the F_0F_1 ATP synthase. This interaction resulted in reduced activity (both hydrolase and synthase that can be restored by displacing mt CypD with CsA while anti apoptotic Bcl-X_L, a known mPT-inhibitor interacts with ATP synthase and promotes its activity (Alavian *et al.,* 2011).

Recently, it was shown that gene silencing of isomers of the c-subunit of the ATP synthase inhibited mPT pore opening (Bonora *et al.,* 2013). Alavian *et al* (2011) proposed that the c-subunit itself can generate pore-forming activity in proteoliposomes, although in this state, it lacks regulation by CypD. However, when the whole ATP synthase is isolated and reconstituted, it then creates a pore that is regulated by CypD, and concluded that previously reported work (Giorgio *et al.,* 2013) showing the interaction between OSCP and CypD.This may account for the regulation of the c-subunit pore by CypD, Ca^{2+} and CsA (Alavian *et al.,* 2014). Bernadi and his colleague (Bernardi and Di Lisa, 2015) considers that the mPT pore-mitochondrial mega channel (MMC) is readily formed from ATP synthase dimers but not monomers as reported by Giorgio *et al.,* 2013; and supports the proposal that inactivation of the“dimerization” subunits e

and g in Saccharomyces cerevisiae increases resistance of the mPT pore to Ca^{2+} (Carraro *et al.*, 2014).

They favor the idea that the pore forms at the interface between two monomers in the dimeric enzyme, and hypothesize that the OSCP is a "negative" modulator, whose effect can be counteracted by binding of the "positive" effector CyPD (which indeed decreases the threshold Ca^{2+} required for pore opening). Removal of OSCP, or CyPD binding to OSCP, would induce similar conformational effects on the rigid stalk proteins, leading to increased probability of mPT opening at the IMM. This is a working hypothesis that awaits experimental testing.

Data from Pinton's laboratory reveals that silencing c subunit expression completely blocks mPT induction by calcium and oxidants whereas c subunit overexpression dramatically enhances mPT induction. Silencing the c subunit does not affect ATP synthesis, suggesting that mPT inhibition is not due to the accumulation of ADP in the mitochondrial matrix. Furthermore, silencing α subunit expression does not lead to any significant alteration in mPT activity, suggesting that the c subunit of the mitochondrial ATP synthase is a central component of the mPT pore (Morciano *et al.*, 2015). Together, these studies provide compelling evidence that the ATP synthase is required for proper mPT pore function.

However, further work is required to reconcile two potential models: ATP synthase dimers forming the mPT pore versus ATP synthase monomers alongside the c subunit ring forming the pore (Giorgio *et al.*, 2013; Alavian *et al.*, 2014). Although, these exciting results support a novel model for the mPT pore, a caveat is that the F_OF_1 ATP synthase cannot explain regulation by all known mPT pore effectors, such as the ANT binding agents bongkrekic acid or atractyloside,

which can dramatically effect mPT pore activity through altering the conformational states of ANT Moreover, it also remains possible that the ATP synthase alters the activity of an unidentified component that is the actual pore itself. *In vivo* genetic loss-of-function analyses of the various ATP synthase components will be helpful in resolving this issue.

A final issue is the uncertain therapeutic potential of the F_0F_1 ATP synthase as a target because agents that block the c-subunit pore activity should also reduce ATP production, although in certain disease situations this later effect might also be a benefit (such as with select neurodegenerative disorders). Finally, genetically testing this newly hypothesized role of the F_0F_1 ATP synthase as the mPT pore will be very challenging for similar reasons, but will be absolutely critical in fully validating this model.

Although, the molecular composition of the pore remains uncertain till now, the emergence of Spastic Paraplegia 7 (SPG7), a mitochondrial AAA-type membrane protease which forms a 6-stave barrel is fast gaining acceptance (Biasutto *et al.,* 2016).

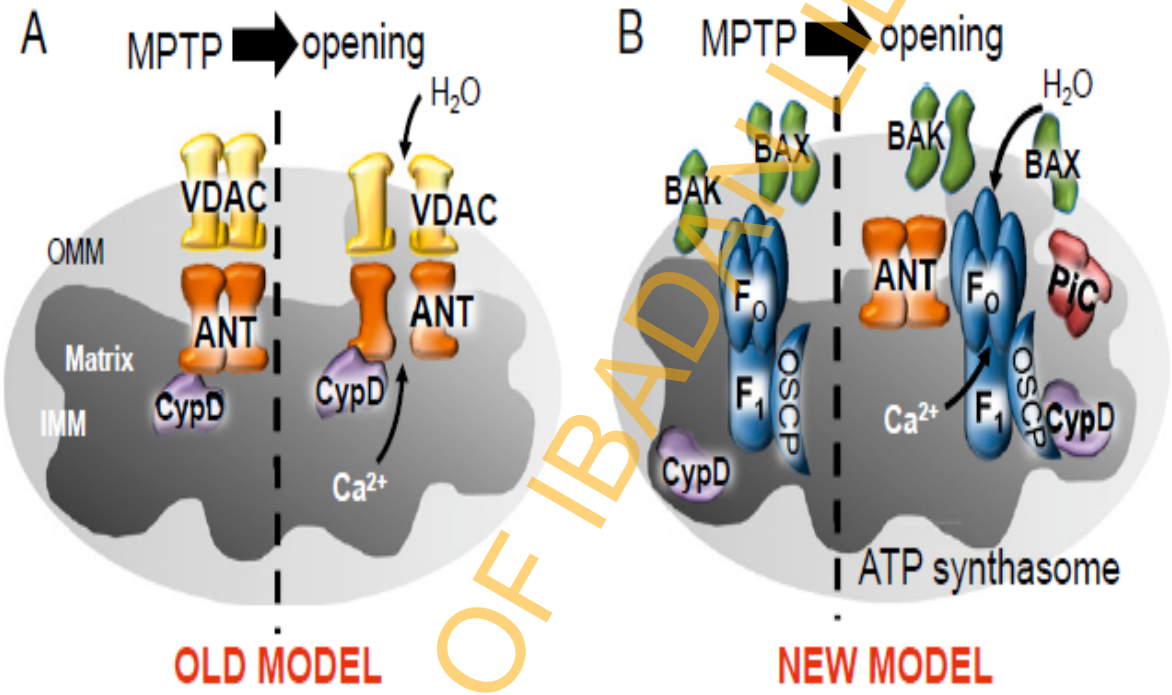

Figure 4: Evolving models of mPT pore (Karch and Molkentin, 2014)

2.11 THE mPT PORE IN TUMOUR DEVELOPMENT

Evasion of apoptosis is recognized as one of the hallmarks of cancer and is required for the development and progression of the pathology. The mPT pore can initiate cell death; thus, it may play a role in cancer. Studies in cancer cell lines and tumor models have shown that different mPT pore proteins are overexpressed (Brenner and Grimm, 2006).

2.12 PHYTOCHEMICALS AND APOPTOSIS

In spite of substantial progress in the development of anticancer therapies, the incidence of cancer is still increasing worldwide. Recently, chemoprevention by the use of naturally occurring dietary substances is considered as a practical approach to reduce the ever-increasing incidence of cancer. The intervention of multistage carcinogenesis by modulating intracellular signaling pathways may provide molecular basis of chemoprevention with a wide variety of dietary phytochemicals. It has been estimated that by making modifications in the diet, more than two-thirds of human cancers could be prevented (Sarkar *et al.*, 2004). The defect in apoptosis mechanism is recognized as an important cause of carcinogenesis.

A dysregulation of proliferation alone is not sufficient for cancer development as suppression of apoptotic signalling is also required. Experimental evidence indicates that dietary constituents, particularly phytochemicals can modulate the complex multistage process of carcinogenesis at each of the three recognized stages of initiation, promotion, and malignant progression. As examples, promising dietary chemopreventive compounds include epigallocatechin gallate (EGCG) in green tea, quercetin in onions and tomatoes, resveratrol in grapes, curcumin in turmeric, sulforaphane and other isothiocyanates (ITCs) in cruciferous vegetables, genistein in soybeans, organosulfur compounds in garlic, and lycopene in tomatoes, among many others

(Chen and King, 2005) . Generally, the growth rate of preneoplastic or neoplastic cells exceeds that of normal cells due to dysregulation of their cell-growth and cell-death machineries. Therefore, an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, preinitiated or neoplastic cells from the body is by induction of apoptosis or cell cycle arrest by human acceptable doses of dietary chemopreventive compounds. Resveratrol selectively target tumor cells with presumably dysfunctional cell cycle checkpoints and spare normal tissue (Gautam, *et al.*, 2000). Beta-carotene, a carotenoid in orange vegetables, induces apoptosis preferentially in various tumor cells from human prostate, colon, breast and leukemia. Many more examples of dietary substances inducing apoptosis of cancer cells are available. Conversely, normal cells are largely resistant to the induction of apoptosis by beta-carotene (Palozza, 2005).

Currently, several dietary components reportedly possess chemopreventive activity, and approximately 40 are being studied clinically for efficacy in chemoprevention trials (Kelloff *et al.*, 1999). Dietary polyphenols from tea reduce prostate cancer in the TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse model of prostate cancer, induce apoptosis in skin tumors of mice exposed to ultraviolet radiation, and protect against chemically induced hepatic tumors in mice (Lu *et al.*, 2000; Gupta *et al.*, 2001). Recently, extracts of tomatoes and associated phytochemical lycopene have been shown to induce apoptosis in prostate cells *in vitro* and *in vivo* (Kim *et al*, 2003).

Silymarin, a flavonoid antioxidant from milk thistle, has been shown to cause prominent caspases-9 and -3 activation as well as PARP cleavage, accompanied by a strong apoptotic death and growth inhibition of leukemia cells (Zhong, 2006). Curcumin induces mitochondrial swelling and collapses the mitochondrial membrane potential (MMP), resulting in apoptosis in numerous cell types (Kim *et al.,* 2003). Beta-carotene, a carotenoid found in carrots, can induce release of cytochrome c from mitochondria and alter MMP in different tumor cell lines derived from leukemia, colon adenocarcinoma and melanoma cell (Palozza *et al.,* 2003; Martin, 2006).

In addition to rodent and *in vitro* studies, human trials have also revealed induction of apoptosis, and a subsequent improved clinical outcome, by numerous dietary components. These results support the idea that apoptosis is a novel molecular target for chemoprevention because of its capacity to slow the progression of, reverse, or inhibit carcinogenesis, ultimately with fewer manifestations of clinically invasive disease.

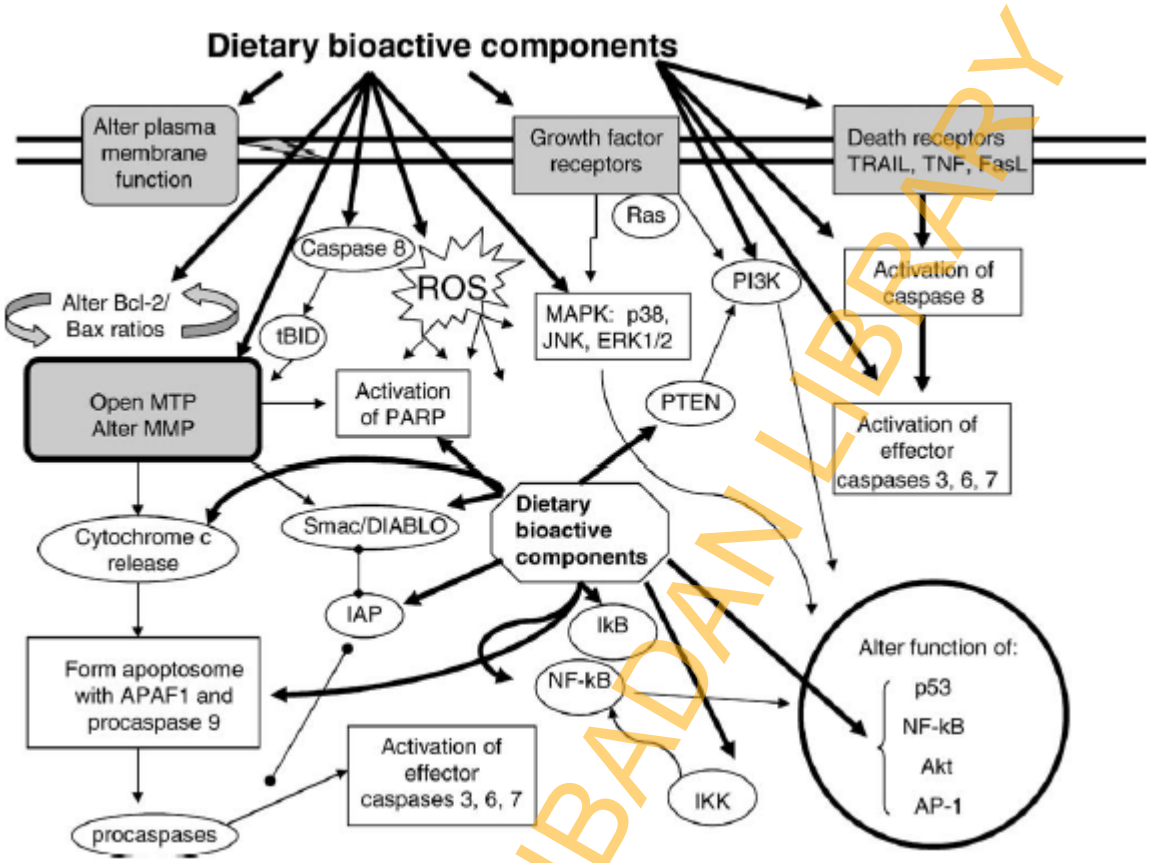

Figure 5: Potential mechanistic targets of bioactive agents in apoptosis (Martin,2006)

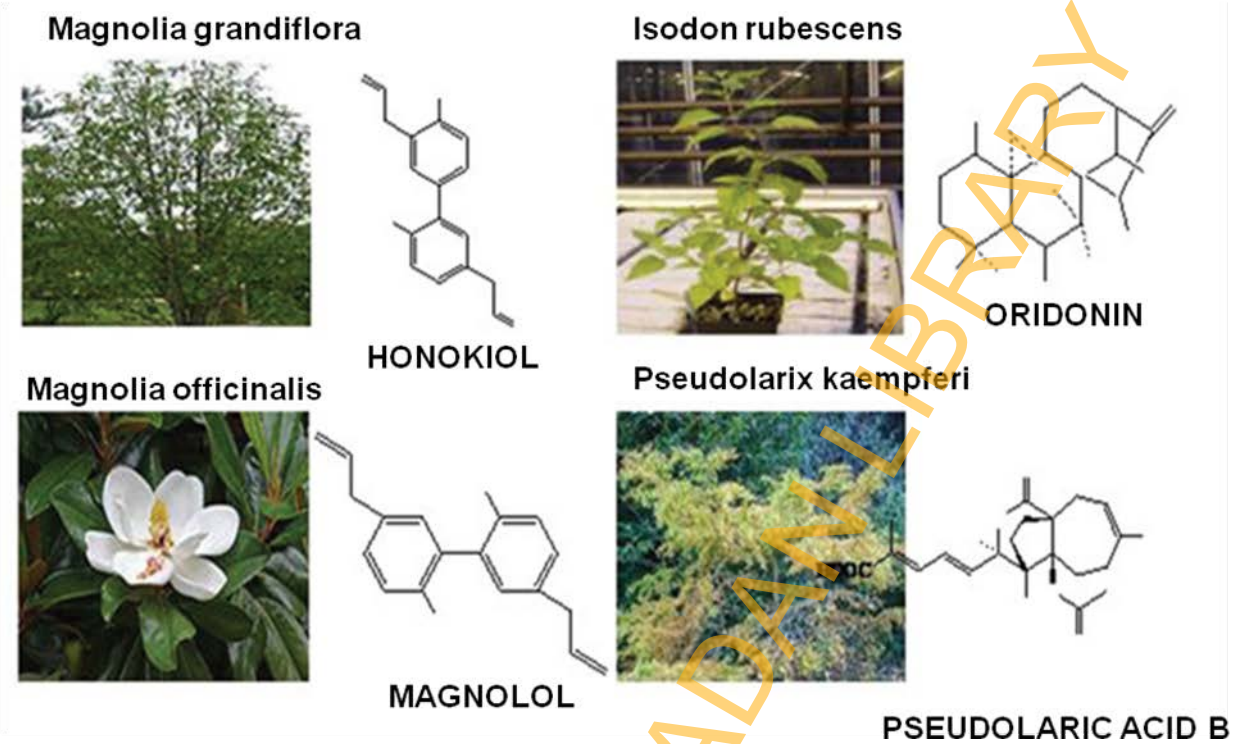

Figure 6: Chemical structure of the promising natural compounds and major natural sources.

(Millimouno *et al.*, 2014)

2.13 CELL CYCLE AND APOPTOSIS

Cell division cycle describes a series of events that occur in a cell leading to its division and duplication. It is essentially an identical replication whose progression and regulation by molecules are well defined. Defects in apoptotic pathway disturb the balance between proliferation and programmed cell death, allowing cells with genetic abnormalities to survive (Evan and Vousden, 2001). Thus, tumor growth is a result not only of uncontrolled proliferation, but also of reduced cell death. Proteins that sense cellular damage or aberrant growth signals arrest the cell cycle so that the damage can be repaired or apoptosis can be induced (Meikrantz and Schlegel, 1995). However, malfunction of this system leads to cancer development by allowing cells to proliferate.

Interestingly, oncogenes have a dual role; they can either induce proliferation or apoptosis. Cancer cell survival therefore depends on cells having a selection pressure toward additional changes in apoptotic pathways (Alenzi, 2004). Deregulated cell-cycle control and reduced apoptosis are an efficient combination for carcinogenesis (Sjöström and Mäkelä, 2006).

Mutations in cell-cycle checkpoint genes are thought to both contribute to carcinogenesis and affect the responses of tumor cells to chemotherapy and radiotherapy (Alenzi, 2004).

2.13.1 PHASES OF THE CELL CYCLE

Cycling takes place in cells with a nucleus and basically eukaryotic cell cycle can be divided in two major phases, interphase and mitosis. The interphase is characterized by accumulation of material and nutrients and subsequently doubles its genome while the mitophase involves the processes during which the cell splits into two distinct but identical daughter cells (identical DNA material, identical genome). The mitophase ends with the cytokinesis when the orginal (mother) cell is completely divided and the two daughter cells are on their own (Behl and Ziegler, 2013). There are two general major sequences in the cell cycle, the phases before and the phases after mitosis (M), called G-phases, where G stands for gap. The gap phases are used to monitor the intracellular but also the extracellular conditions to make sure that everything is in order before actually proceeding to the next cycle phase (Malumbres and Barbacid , 2001).

The G_1-phase directly follows cell division and is frequently also called post-mitotic pre-synthesis phase. The cell starts to grow, the content of the cell (cytoplasm) with the functional machineries (organelles) is formed. In addition, the synthesis of mRNA takes place, histone proteins and the enzymes of the DNA replication machinery necessary for the next phase are generated. In a constantly dividing cell the G_1-phase regularly takes approximately 3 h depending on the particular cell type. The S- or synthesis phase is characterized by the process of DNA replication (doubling of the cellular genome) and a major effort to produce histone proteins that are finally needed for the packaging of the genomic DNA. On average the S-phase takes about 7 h. The G_2-, premitotic or post-synthetic phase is the time when the cell prepares to split off in two cells, the actual division. As part of a certain tissue cells loosen up the direct contact to

neighbouring cells, they usually round up and increase in general size. The synthesis of RNAand proteins concerns the main players needed for the mitosis and may take up to 4 h.

Finally, in the M-or mitosis-phase, division occurs: the doubled DNA organized in chromosomes is separated, the cellular nucleus divides (karyokinesis) as does the rest of the cell (cytokinesis). The M-phase itself may take approximately 30–60 min and can itself be divided into five phases (pro-, prometa-, meta-, ana-, telophase) (Alberts *et al.*, 2007). In proliferating tissue with cells undergoing constant divisions after mitosis then the next G_1-phase occurs. Fully differentiated cells having special functions and roles in the tissue permanently remain in the G_1-phase which is then called G_0- or quiescence-phase. G_0 represents a specialized resting and quiescent state of the cell. Nerve cells, muscle cells and red blood cells (erythrocytes) are the most prominent examples of cells in G_0-phase. G_0 is not only the state for differentiated cells so that they can fulfill their tasks in the tissue. Cells also enter G_0 when the extracellular microenvironment is not in favor for further cycling, for instance when growth factors or nutrients are lacking that are necessary for the S-phase. It should be noted that not all cells in G_0 are on the road to cell death. Upon appropriate stimulation (growth factors) some cell types may re-enter the cell cycle. Damage to the cellular DNA and other significant changes provoke the quiescent state of the cell. It is important to note that the status of senescence and of quiescence are quite different, since once a cell is entering the senescence process this is a point of no return, ultimately leading to controlled cell death (apoptosis) while quiescence on the other hand is reversible. Constant cycling triggered by uncontrolled growth input is a hallmark of cancer (Vermeulen *et al.*,2003).

Some cells enter a G_0 stage known as quiescence which can be considered a place of rest. A cell in G_0 has exited the cell cycle, and is neither dividing nor preparing to divide; however the cell is still alive nor actively metabolizing, it has simply stopped dividing. The cell may re-emerge from this quiescent stage back into the cell cycle, given the right signals from its microenvironment. Some cells permanently exit the cell cycle, moving to a post-mitotic state. There is no coming back from this path; it is usually associated with mature cells that have differentiated, i.e. taken their adult form. Therefore a cell, at the basic level, has three choices facing it; continue to grow and divide by staying in the cell cycle, take a temporary break by entering G_0, or permanently exit the cell cycle into the post-mitotic state. (Malumbres and Barbacid , 2001; Behl and Ziegler, 2013).

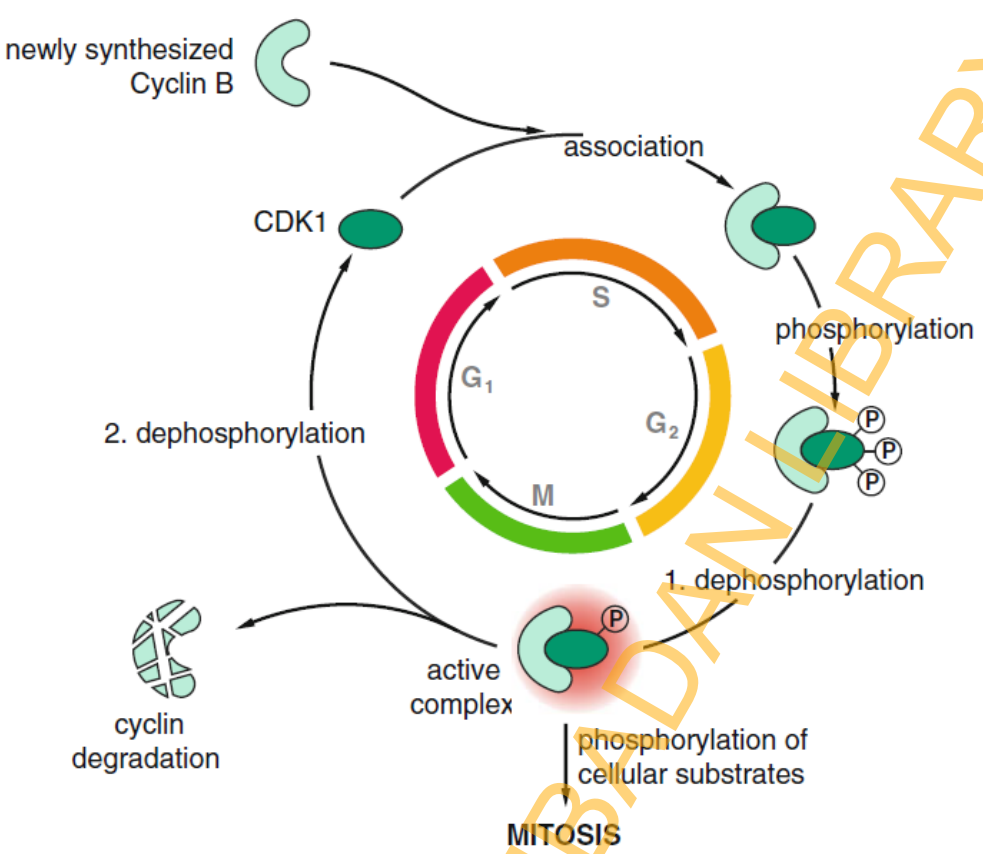

Figure 7: Molecular Basis of Cell Cycle Regulation and Apoptosis (Vermeulen *et al.*, 2003)

Many of the genes that control the killing and engulfment processes of programmed cell death have been identified, and the molecular mechanisms underlying these processes have proven to be evolutionarily conserved (Metzstein *et al.,* 1998). Alterations of various cell signalling pathways can result in dysregulation of apoptosis and lead to cancer. The *p53* tumor suppressor gene is a transcription factor that regulates the cell cycle and is the most widely mutated gene in human tumorigenesis (Wang and Harris, 1997).The critical role of *p53* is evident by the fact that it is mutated in over 50% of all human cancers. *p53* can activate DNA repair proteins when DNA has sustained damage, can hold the cell cycle at the G1/S regulation point on DNA damage recognition, and can initiate apoptosis if the DNA damage proves to be irreparable (Pietenpol and Stewart, 2002). Tumorigenesis can occur if this system goes awry, however, if the *p53* gene is damaged, then tumor suppression is severely reduced.

Meikrantz and Schlegel (1995) demonstrated that the cell cycle and apoptosis may be linked, and provided arguments to support such a link. This is because apoptosis is almost present in proliferating cells and molecules acting on cells in late G1 phase are also required for apoptosis. Besides, passage of a cell from late G1 to the S phase of the cell cycle is controlled by p53 and cdk. Finally, artificial manipulation of the cell cycle (e.g., retroviral transduction) could either abolish or potentiate apoptosis.

2.14 *CALLIANDRA PORTORICENSIS*

Calliandra is a genus of flowering plants in the subfamily *Mimosaceae* under the family *Leguminoseae* (Pea Family). There are over 200 species of *Calliandra* plants native to tropical and subtropical areas of Asia, Africa, Australia and the Americas. The flowers of the *Calliandra* are round and thin- like needles; however they are soft and wispy, earning them the common name "Powder Puff Plant". *Calliandra portoricensis* is a distinctive ornamental shrub and medicinal plant. Its local names in Nigeria are '*Tude*' in Yoruba, '*Ule*' in Igbo and '*Oga*' in Hausa.

It is referred to as *Calliandra portoricensis* Benth and has for a long time been the drug of choice among herbalists in the Southern States of Nigeria for gastrointestinal problems and convulsions. When used as a chewing stick, the stem is locally employed as an analgesic for toothache. It is used in Nigeria folklore medicine as a laxative/worm expeller and an abortificient in human beings (Ayensu, 1978). The plant has also been reported to have anticonvulsant, (Adesina, 1982; Akah and Nwaiwu, 1988) antidiarrheal, antispasmodic, antipyretic, antirheumatic and analgesic (Aguwa and Lawal, 1988) activities in humans. In addition, *C. portoricensis* has also been reported to exhibit anticholigenic, antacid, antiulcer, molluscidal and ovucidal activities in laboratory animals (Aguwa and Lawal, 1988).

Though the genus *Calliandra* consists of many species distributed worldwide, the species growing in Nigeria are *Calliandra haematocephala* and *Calliandra portoricensis.*

Calliandra haematocephala has been extensively evaluated for its chemical constituents. Three acylated quercetin rhamnosides were recently reported from the leaves and stem of *C.*

haematocephala and their structures were established as quercitrin 2"-*O*-caffeate, quercitrin 3"-*O*-gallate and quercitrin 2",3"-di-*O*-gallate (Moharram *et al.*, 2006).

Also, 17 known compounds were reported for the first time from the genus *Calliandra* ,they are gallic acid, methyl gallate, myricitrin, quercitrin, myricetin 3-*O*-â-D-4C1-glucopyranoside, afzelin, isoquercitrin, myricetin 3-*O*-(6"-*O*-galloyl)-â-Dglucopyranoside, myricitrin 2"-*O*-gallate, quercitrin 2"-*O*gallate, afzelin 2"-*O*-gallate, myricitrin 3"-*O*-gallate,afzelin 3"-*O*-gallate 1,2,3,4,6-penta-*O*-galloyl-â-D-4 C1-glucopyranose, myricitrin 2",3"-di-*O*-gallate and quercetin 3-*O*-methyl ether (*Moharram et al., 2006)*. Moreover caffeic acid, betulinic acid were previously reported. (Nia *et al.*, 1999). Compounds myricitrin, quercitrin, myricitrin 2"-*O*-gallate, quercitrin 2"-*O*-gallate, myricitrin 3"-*O*gallate, and myricitrin 2",3"-di-*O*-gallate, exhibited moderate to strong radical scavenging properties on lipid peroxidation, hydroxyl radical, superoxide anion generation and DPPH radical in comparison with that of quercetin as a positive control *in vitro* (Moharram *et al.*,*2006).*

Phytochemical screening of the leaves, stem and root extracts of *Calliandra portoricensis* reveal the presence of saponins, glycosides, steroids, fatty acids and digitalis glycosides in the plants (Orishadipe *et al.*, 2010). Analysis of gas chromatography-mass spectrometry hexane fraction of the root of *Calliandra portoricensis* indicated that the extract is rich in fatty acids /methyl esters which have been implicated in the plant's antimicrobial activities (Orishadipe *et al.*, *2010).*

Histopathological studies of acute and chronic effect of *Calliandra portoricensis* leaf extract on the stomach and pancreas of adult Swiss albino mice (Ofusori and Adejuwon, 2011) suggest

that chronic administration of *the plant's* leaf extracts may inhibit the proper function of stomach and pancreas. Besides, the leaves and roots of the plant has been shown to have protective effects (antidote) in wistar rats challenged with snake venom (Onyeama *et al.*, 2012) . Epiafzelechin isolated from the stem bark of *the plant* have displayed antimicrobial effects (Iftikhar *et al.*,2013). *C. portoricensis* has also shown to possess antisickling property (Amujoyegbe *et al.*, 2014)

Figure 8: Leaves and dried root bark of *Calliandra portoricensis*

2.15 Apoptosis and Cancer

Cancer is a process that results from the accumulation of somatic mutations in the progeny of a normal cell, leading to a selective growth advantage in the mutated cells and ultimately to uncontrolled proliferation. The most frequent human cancers arise in epithelial tissues such as the skin, colon, breast, prostate or lung, and collectively lead to several million deaths per year (Blanpain, 2013)

Cancer involves unregulated growth of cells. It is characterized by cells that continue to replicate instead of differentiating. Some types of cancer cells often travel to other parts of the body through blood circulation or lymph vessels (metastasis), where they begin to grow (Hanahan and Weinberg, 2000).

The hallmarks of cancer include uncontrolled cell division, apoptosis evasion, non-dependence on growth regulation, angiogenesis, immortality, invasion and metastasis. The terrain of cancer treatment has metamorphisized over the last four decades. Surgery and radiotherapy are no longer the only effective way to fight tumour growth. A complex scenario where the molecular features of tumours seem to be the chiefstone of any therapy is now emerging (Urruticoechea *et al.,* 2010). Therapies that spare normal cells while targeting transformed cells are encouraged (Martin, 2006) in order to minimize associated systemic toxicity. Within the complex body ecosystem, cancer cells mutate and face selective pressures as they change and adapt to their environment. The evolving cancer cell escapes the apoptotic machinery which causes cells to self-destruct, corrupt and co-opt otherwise loyal surrounding cells and migrate to distant parts of the body (Fernald and Kurokawa, 2013).

2.16 PROSTATE CANCER

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Prostate cancer is predicted to be the leading cause of cancer-related death in men over the next decade in the United States (Siegel *et al.*, 2013). It is the most common non-cutaneous malignancy diagnosed in men. Approximately one in six men will be diagnosed with prostate cancer in their lifetime (Brandt and Tareen, 2016). It begins when healthy cells in the prostate change and grow uncontrollably, forming a mass called a tumor. A tumor can be cancerous or benign.

The prostate gland is found only in males. It is just below the bladder and in front of the rectum. The tube that carries urine (the urethra) runs through the center of the prostate. The prostate contains cells that make some of the fluid (semen) that protects and nourishes the sperm.

In younger men, it is about the size of a walnut, but it can be much larger in older men. The growth is fueled by male hormones (called androgens) such as testosterone. There are several types of cells in the prostate, but nearly all prostate cancers start in the gland cells. This kind of cancer is known as adenocarcinoma (American Cancer Society, 2016)

Prostate specific antigen (PSA) is a protein produced by cells in the prostate gland. The PSA is detected using a blood test. Higher-than-normal levels of PSA are found in men with prostate cancer, as well as other non-cancerous prostate conditions. Those conditions include benign prostatic hyperplasia (BPH), which is an enlarged prostate, and prostatitis, which is inflammation or infection of the prostate. More than 95% of prostate cancers are a type called adenocarcinomas. (American Cancer Society, 2016).

2.17 Androgen Receptor and Prostate Cancer

The normal development and maintenance of the prostate is dependent on androgen acting through the androgen receptor (AR). AR remains important in the development and progression of prostate cancer (Heinlein and Chang, 2004). Standard therapy relies on removing, or blocking the actions of, androgens. In most cases, this therapy results in a regression of the cancer because the prostate and most primary prostate tumors depend on androgens for growth and the avoidance of apoptosis. However, a portion of the cancers eventually relapse, at which point they are termed "androgen refractory" and can no longer be cured by conventional therapy of any type.

Androgens such as testosterone are synthesized primarily by the Leydig cells in the testes, under the regulation of luteinizing hormone (LH) produced by the anterior pituitary gland. The LH secretion is in turn regulated by gonadotropin-releasing hormone (GnRH). Once produced, testosterone mostly circulates bound to serum sex hormone-binding globulin (SHBG) and albumin. Only the free form enters prostate cells. Intracellularly, testosterone is converted into a more potent 5α-reduced metabolite of testosterone, 5α-dihydrotestosterone (DHT), which promotes the growth and survival of prostate cells. The DHT binds to the AR with high affinity, displaces heat-shock proteins from the AR, drives the interaction between the N and C termini of the AR, and binds importin-α to translocate the AR into the nucleus In the nucleus, receptor dimers bind to androgen response elements (AREs) in the promoter regions of target genes, such as prostate-specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2), *etc*, to which they recruit various coregulatory proteins to facilitate transcription, leading to responses such as growth and survival (Grossmann *et al.*, 2001).

Male sexual differentiation fails to occur in the absence of androgens or without a functioning AR. A complete loss of AR function in males results in complete androgen insensitivity syndrome (Brinkmann, 2001). The role of the AR in the development and progression of prostate cancer has led to increasing interest in this nuclear receptor. The development and progression of prostate cancer depends on androgenic stimulation (Heinlein and Chang, 2004), as such, prostate cancer is treated by depriving tumors of androgens such as DHT and testosterone or blocking their actions. However, the effect of this type of treatment is transient, as patients relapse after developing a castration-resistant form of the disease that is usually due to increased levels of AR expression or mutations that cause the AR to be resistant to antiandrogens. Many studies have focused on providing new insights into the mechanisms of AR action in prostate cancer (Tan *et al.*, 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.0 COLLECTION OF FRESH ROOTS of *Calliandra portoricensis*

Freshly harvested roots *of Calliandra portoricensis* were obtained from Oje market, Ibadan, Oyo State, Nigeria. Samples were authenticated and identified at the Herbarium, Department of Botany, University of Ibadan, Ibadan, Oyo State and a specimen Voucher No.UIH-22466 was deposited in the Herbarium. The roots were washed, the bark was peeled and air-dried for three weeks in the laboratory after which they were powdered with industrial machine and weighed.

3.1. PREPARATION OF CRUDE METHANOL EXTRACT OF *Calliandra portoricensis* (MECP)

Seven-kilogramme air-dried, powdered root bark of *Calliandra portoricensis* were extracted with sufficient methanol (Sigma Aldrich Chemical Co. St Louis USA) in all- glass jars at room temperature for seventy-two hours. The filtrate was decanted, filtered and concentrated under reduced pressure using a rotary evaporator (Stuart). The crude methanol extract was heated over a water bath at 40^0C to obtain a solvent free extract and thereafter lyophilized to obtain a portion that could be weighed (200g).

3.2 PARTITIONING OF CRUDE METHANOL EXTRACT OF *C. Portoricensis* USING VACUUM LIQUID CHROMATOGRAPHY

3.2.1 Packing of the Chromatographic column

The prewashed sintered glass Buchner was further cleaned with concentrated H_2SO_4 to remove impurities from the sieve. The column was then packed three-quarters full with silica gel 60 (0.040–0.063mm, MERCK). The sample slurry was then placed on a conical Buchner flask and

connected to the vacuum pump. The pump was switched on and n-hexane solvent was applied to the column. This was done to further pack the column.

3.2.2 Preparation of the Sample Slurry

Silica gel 60 (0.040–0.063mm, MERCK) 8g was added to 12g of the methanol extract sample. The gel-sample mixture was stirred until a homogenous mixture was obtained. The mixture was air-dried to obtain a powdery form.

3.2.3 Loading of sample on the column

The sample was applied to the top of the column with the pump switched on, the first solvent system, 100% n-hexane (Sigma Aldrich Chemical Co. St Louis USA) was added to the column to elute the n-hexance fractionat a flow rate of 25mL/min. Thereafter, the marc was eluted again with chloroform until exhaustion of the chloroform fraction in the column. The column was further eluted with ethylacetate (100%) and lastly with methanol (100%). The fractions obtained were concentrated at 40^0C using rotary evaporator and transferred into pre-weighed all-glass sample vials and labelled. These various solvent fractions were concentrated using a rotary evaporator to obtain solvent-free n-hexane (HFCP), chloroform (CFCP), ethylacetate (EFCP) and methanol (MFCP) fractions, respectively. All the solvent-free fractions were stored in the refrigerator until used.

Thin Layer Chromatography (TLC) of the fractions were carried out in order to assess the purity and also to identify the phytochemicals present in each of the fractions gotten from the solvent systems. Weight of each fraction was determined as follows:

$$Weight\ of\ fraction = Weight\ of\ Sample\ and\ bottle - weight\ of\ bottle\ only$$

Percentage (%) yield of the extracts was calculated as follows:

$$Percentage\ yield\ of\ the\ extract = \frac{(X - Y)}{Z}\ X\ 100$$

Where weight of fraction and dish, dish only and fraction only were X, Y and Z respectively.

3.3 EXPERIMENTAL ANIMALS

Wistar strain albino rats (70) weighing between 70-80g were used for mitochondrial and acute toxicity studies. They were obtained from the Preclinical Animal House, University of Ibadan, Ibadan, Nigeria. The animals were kept in cages and acclimatized for two weeks prior to the commencement of the experiments. They had access to food and water *ad libitum*.

3.4 PHYTOCHEMICAL SCREENING

Phytochemical analysis was carried out qualitatively to detect various natural products present in the crude methanol extract and the n-hexane, chloroform, ethylacetate and methanol fractions. Phytochemical screening was performed using standard procedures (Sofowora, 1993; Trease and Evans, 1989)

3.4.1 Test for anthraquinones

A weight of 0.5 g each of the methanol extract, n-hexane, chloroform, ethylacetate and methanol fractions was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1ml of 20% ammonia was added. The resulting solution was observed for colour changes. Observation of a rose pink colour in the aqueous layer indicated the presence of anthraquinone.

3.4.2 Test for terpenoids (Salkowski test)

To 0.5 g each of the methanol extract, n-hexane, chloroform, ethylacetate and methanol fractions was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration at the interface indicated the presence of terpenoids.

3.4.3 Test for flavonoids

A portion of the extract and fractions was heated with 10 ml of ethylacetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of 20% ammonia solution. A yellow colouration indicated the presence of flavonoids.

3.4.4 Test for saponins

The ability of saponin to produce frothing in aqueous solution and to hemolyse red blood cell was used as screening test for saponin. Each of the plant fractions and extract (0.1g each) were dissolved in water in a test tube and the mixture was shaken vigorously. Frothing which persisted on warming was taken as preliminary evidence for the presence of saponin.

3.4.5 Test for tannins

About 0.5 g each of the samples was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. Brownish green or a blue-black colouration indicated the presence of tannin.

3.4.6 Test for alkaloids

Fractions and extract (0.1g each) were dissolved in 5mls of 1% hydrochloric acid (aqueous) in a steam bath. The solutions were sieved through filter paper. Dragendoff's reagent was added to 1ml of the filtrate. The observation of yellowish brown colour indicated the presence of alkaloid.

3.4.7. Test for steroids

Acetic anhydride (2mls) was added to the plant fractions and extract (0.5g each) followed by the addition of 2ml of concentrated H_2SO_4. The colour change from violet to blue or green in the same sample indicated the presence of steroids.

3.4.8 Test for cardiac glycosides

The plant extracts (0.5ml each) and 0.4ml of glacial acetic acid are mixed with 10% ferrous chloride and 0.5ml of concentrated sulphuric acid (H_2SO_4). The presence of blue colour indicated the presence of cardiac glycoside.

3.5 DETERMINATION OF HEMATOLOGICAL PARAMETERS

Haemoglobin, packed cell volume, red blood cell and white blood cell counts were determined by the method described by Jain (1986).

3.5.1 Hemoglobin concentration

This was determined as described by Jain (1986) using cyanomethaemoglobin method.

Principle

Methaemoglobin reacts with potassium cyanide to form cyanomethaemoglobin which has maximum absorption at 540nm. The colour intensity measured at 540nm is proportional to the total hemoglobin concentration.

Reagent

Drabkin's reagent

Sodium hydrogen carbonate ($NaHCO_{3)}$ 1g (Hopkins and Williams Ltd, England), 0.2g of potassium ferricyanide-$K_3Fe[CN]_6$ (BDH Chemicals Ltd., England) and 0.05g of potassium cyanide- KCN (BDH Chemicals Ltd., England) were dissolved in distilled water and made up to 1 litre in a flat-bottomed flask.

Procedure

Blood - 0.02ml was added to 4ml of Drabkin's reagent. The solution was allowed to stand for 10 minutes and the absorbance of the resultant solution was read using UV-visible SPECTRUMLAB spectrophotometer at 540nm with the Drabkin's reagent as reference.

Concentration of haemoglobin expressed in g/100mL was calculated as shown below:

$$\text{Hb conc. (g/100ml)} = \frac{\text{Abs. of test solution}}{\text{Abs. of standard solution}} \times \text{Hb conc. of the Std. Solution} \times \text{Dil. factor}$$

Dilution factor = 201, Hb concentration of standard= 0.0572g/ml

3.5.2 Packed Cell Volume (PCV)

The packed cell volume was determined by filling a capillary tube with blood. One end of the tube was sealed using plasticin and the tube centrifuged in a microhematocrit centrifuge for 5 minutes. The PCV percentage was read directly from a graphic reader.

3.5.3 Red Blood Cell Count

Red blood cell count was determined using the hemocytometer method. It involves using the conventional method of filling the capillary tube with blood. One end of the tube was sealed with plasticin and the tube centrifuged in a microhematocrit centrifuge for 25 minutes. The percentage of red blood cell count was then measured after separation using haematocytometer.

Reagent

Haymen's Solution

Mercury chloride (0.25g) (Hopkins and Williams Ltd. England) and 0.25g of sodium sulphate (Hopkins and Williams Ltd. England) were dissolved in distilled water and made up to 100ml in

a flat-bottomed flask. This solution was isotonic with blood, and prevented clot formation and coagulation.

Procedure

By means of rubber tubing attached to the end of the red cell pipette, blood was drawn to the 0.5 mark on the pipette. Keeping the pipette nearly horizontal, Haymens solution was drawn to the 101 mark, care being taken to prevent overshoot. Holding the pipette horizontal, the rubber tubing was removed without squeezing. The pipette was then rotated for about 1 minute to ensure thorough mixing and dilution of the fluid in the bulb.

Diluted blood was then introduced carefully into the counting chamber with clean cover slip in place. Red cell counting was done using 'x 40' objective of microscope. Counting was carried out in 5 sets of small squares containing 80 small squares. Only the overlapping red cells at the bottom and right hand side were not counted.

Blood volume was calculated as follows:

Volume of diluted blood over each small square = 1 /4000 mm^3 x 80 = 1 /50 mm3

Volume of diluted blood over 80 small square = 1 /4000 mm^3

If x red cells were present in 1/50 mm^3 of diluted blood, the 50x red cells would be in $1mm^3$ of diluted blood and 50x times 200 (10, 000x) red cells would be present in $1mm^3$ of undiluted blood.

3.5.4 Total White Blood Cell Count

White blood cells were counted using hematocytometer, white cell pipette and white cell diluting fluid.

Principle

The glacial acetic acid lyses the red cells while the gentian violet slightly stains the nuclei of the leucocyte. The blood is diluted to 1:20 in a white blood cell pipette with the diluting fluid and the cells are counted under low power microscope using a counting chamber.

Reagent

White cell diluting fluid: This contained 2% acetic acid and 2ml of methyl violet dye

Procedure

The procedure followed was similar to that used to determine red blood cell count. The ratio of dilution was 1:20 and each of the 4 white cell counting areas was bounded by a single thoma line. The multiplication factor was 50 when all the 4 l counting areas were used.

White cell count was calculated as shown below:

White cell count/ mm^3= Cell counted x 20 (1:20) x 10 (0.1mm depth) x 4 number of square millimeter counted or the sum of the cells counted x 50= Total leucocytes/mm^3

3.6 PREPARATION OF SERUM

The Wistar strain albino rats that were orally administered MFCP for 30 days were sacrificed after the last dose by cervical dislocation. They were quickly opened up and blood was collected

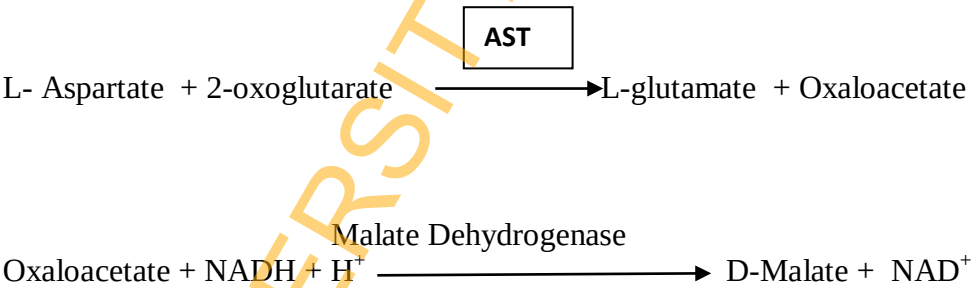
from the heart. The collected blood was drawn into plane sample bottles for serum preparation. The blood was allowed to clot and after 30 minutes, the blood samples were centrifuged at 3,000rpm for 20 minutes. The serum obtained was separated from the clotted blood by using pasteur pipette and serum obtained was used to assay for alanine transaminase (ALT) and aspartate transaminase (AST) activities.

3.7 ASSAY OF SERUM LIVER ENZYMES

3.7.1 Determination of Serum Aspartate Aminotransferase (AST) Activity

Principle

α-ketoglutarate reacts with L-aspartate in presence of AST to form oxaloacetate and L-glutamate. The increase in oxaloacetate is determined in an indicator reaction catalyzed by malate dehydrogenase. The conversion of NADH to NAD^+, at 340 nm is proportional to the activity of AST in serum and is determined kinetically as rate of decrease in absorbance (Reitman and Frankel, 1957).



Reagents

The assay kits (Randox Laboratories Limited, Diamond Road, Crumlin, County Antrim BT29 4QY, United Kingdom) for quantitative *in vitro* determination of AST and ALT activities were purchased.

TABLE 1: COMPOSITION OF REAGENT FOR DETERMINATION OF AST ACTIVITY

Content	Initial concentration of solution
Reagent 1	
Phosphate Buffer	100mmol/L, pH 7.4
L-Aspartate	100mmol/L
α-ketoglutarate	2mmol/L
Reagent 2	
2, 4-dinitrophenylhydrazine	2mmol/L

Procedure

Reagent 1 (0.5ml) was pipette into both sample and blank test tubes and serum sample (0.1ml) was added to the test tubes for the sample. Distilled water (0.1ml) was added to the blank test tube. The test tubes were vortexed and incubated for 30 minutes at 37^0C. Reagent 2 (0.5ml) was added to both blank and sample test tubes. The mixture was mixed and allowed to stand for 20 minutes at 25^0C.Then, 5ml of 0.4M sodium hydroxide was added and the absorbance read at 546nm after 5 minutes against reagent blank.

3.7.2 Determination of Alanine Amino Transferase (ALT) Activity

Alanine amino transferase is measured by monitoring the concentration of pyruvate hydrazone that is formed with 2, 4-dinitrophenyl hydrazine.

TABLE 2: COMPOSITION OF REAGENT FOR ALT ACTIVITY

Content	Initial concentration of solution
Reagent 1	
Phosphate Buffer	100mmol/L, pH 7.4
L-Alanine	200mmol/L
α-ketoglutarate	2mmol/L
Reagent 2	
2, 4-dinitrophenylhydrazine	2mmol/L

Procedure

Reagent1 (0.5mL) was pipette into both sample and blank test tubes. Serum sample (0.1mL) was added to the test tubes for the samples only while 0.1ml of distilled water was added to the blank test tube. The test tubes were vortexed and incubated for 30 minutes at 37^0C. Reagent 2 (0.5mL) was added to both blank and sample test tubes. After mixing, the tubes were allowed to stand for 20 minutes at 25^0C. 5ml of 0.4M sodium hydroxide added, mixed and the absorbance read at 546nm after 5minutes against the reagent blank.

3.8 TISSUE PREPARATION FOR HISTOPATHOLOGY

Liver, prostate and kidney were used for histopathology. They were quickly removed and trimmed and were placed in 10% formalin for about five days for proper fixation, dehydrated by ascending grades of isopropyl alcohol for an hour. The dehydrated organs were cleared in xylene and transferred into two changes of liquid paraffin wax. The tissue sections were stained in Ehrlich's hematoxylin for eight minutes, washed in tap water and dipped in acid alcohol to remove excess stain. These were counter stained in 10% aqueous eosin, incubated and mounted for photomicrography.

3.9 ISOLATION OF RAT LIVER MITOCHONDRIA

The rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) based on differential centrifugation technique.

Reagents

Buffer C (Homogenizing Buffer): (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH pH 7.4, 1mM EGTA)

HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (Sigma Aldrich Inc.,USA) (0.12g) was dissolved in 70ml of distilled water, mannitol (Sigma Aldrich Inc., USA)(3.83g), sucrose (Sigma Aldrich Inc., USA) (2.4g)and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (Sigma Aldrich Inc., USA) (0.038g) were then added and adjusted to pH 7.4 with KOH (Sigma Aldrich Inc., USA). The solution was then made up to 100mL in a standard volumetric flask and then stored in the refrigerator.

Buffer D (Washing Buffer): (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, 0.5% BSA)

HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (Sigma Aldrich Inc., USA) 0.12g was dissolved in 70mL of distilled water , mannitol (Sigma Aldrich Inc., USA)(3.83g), sucrose (Sigma Aldrich Inc., USA) (2.4g) and bovine serum albumin (Sigma Aldrich Inc., USA)(0.5%) were then added and adjusted to pH 7.4 with KOH (Sigma Aldrich Inc., USA). The buffer was made up to 100ml mark and stored in the refrigerator.

Mannitol-Sucrose-HEPES (MSH) Buffer: **(210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH pH 7.4)**

HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (Sigma Aldrich Inc., USA) (0.12g)was dissolved in 60ml of distilled water, mannitol (Sigma Aldrich Inc., USA)(3.83g), sucrose (Sigma Aldrich Inc., USA) (2.4g) were weighed and dissolved in the solution containing HEPES-KOH pH (7.4) the pH was adjusted to 7.4 with KOH and made up to 100ml in a flat-bottomed flask**.**

0.25M Sucrose

Sucrose (BDH Chemical, Poole, England) (85.6g) was dissolved in distilled water and made up to 1 litre in a standard volumetric flask. This solution was kept in the refrigerator.

Procedure

The animals were sacrificed by cervical dislocation. Livers were rapidly excised and trimmed.The livers were blotted with blotting paper and weighed. After weighing, the livers were washed with buffer C, minced with a pair of scissors and a 10% suspension of the liver in ice-cold buffer C was immediately prepared. It was then homogenized on ice with Potter Elvehjem glass homogenizer.

The homogenate was centrifuged at 2,3000rpm twice in an MSE centrifuge at 4^0C for 5 minutes to sediment nuclear fraction and cell debris.The supernatant obtained was centrifuged at 13,000rpm for 10 minutes to obtain the mitochondrial pellet. The mitochondrial pellet obtained was washed twice in buffer D and centrifuged at 12,000rpm for 10 minutes each time. The mitochondrial fraction was resuspended in MSH buffer and dispensed into Eppendorf tubes as

aliquots and used within six hours. All experiments were carried out on ice to preserve the mitochondrial integrity.

Mitochondria used to assay for mitochondrial ATPase activity was prepared using the procedure described above except that ice-cold sucrose (0.25M) was used as buffer for the preparation of mitochondria and suspended in the sucrose medium.

3.10 MITOCHONDRIAL PROTEIN DETERMINATION

Mitochondrial protein content was estimated according to the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Principle

Phenol groups reduce phospho-18-molybdictungstic complex (a mixture of several molecular forms such as $3H_2O.P_2O_5.9MoO_3$. and $3H_2O.P_2O_5.10WO_38MoO_3$) giving a blue colour at alkaline pH. Tyrosine and (or) tryptophan present in the protein are responsible for the reduction of this phospho-18-molybdictungstic complex giving rise to the blue colouration.

Reagents

Reagent A: 2% Na_2CO_3 in 0.1M NaOH

Sodium trioxocarbonate (IV) (2g) and sodium hydroxide pellets (both from BDH Chemicals Ltd., Poole, England) (4g) were dissolved in a small amount of distilled water in a 100ml standard volumetric flask and then made up to the 100ml mark with distilled water.

Reagent B: 2% Na-K-Tartarate

Sodium potassium tartarate (BDH Chemicals Ltd., Poole, England)(2g) was dissolved in 70ml of distilled water and made up to the 100ml mark with distilled water.

Reagent C: 1% $CuSO_4.5H_2O$

Copper (II) tetraoxosulphate(VI)pentahydrate (BDH Chemicals Ltd., Poole, England) (1g) was dissolved in about 50mL of distilled water and made up to the 100ml mark with distilled water.

Reagent D: Alkaline Copper Solution

This was prepared fresh before use. Reagents A, B, and C were mixed together in ratio 100:1:1 respectively. The tartarate solution was added first to prevent cloudiness of the solution.

Reagent E: Folin-Ciocalteau Reagent

Sodium tungstic ($Na_2WO_4.2H_2O$) (100g) and sodium molybdate ($Na_2MoO_4.2H_2O$) (25g) were dissolved in about 700ml of distilled water in a round bottomed quick-fit flask. 100ml of concentrated HCl and 50ml of 85% phosphoric acid were added to the mixture and refluxed for 10 hours in an all-glass apparatus. To the resulting mixture were added 150g of lithium sulphate (Li_2SO_4), 50ml of water and a few drops of bromine. The mixture was boiled for 15 munites in a fume chamber without condenser to remove excess bromine. The mixture was later cooled, diluted to 1litre, filtered and stored in a black container at 4^0C in a refrigerator because it is photolytic. The resultant solution is a 2N solution which is usually diluted to 1N with distilled water just before use. The reagent is golden yellow in colour, if it has a greenish tint, it is unsatisfactory for use and may be regenerated by boiling with a few drops of bromine.

STANDARD PROTEIN CURVE

Bovine Serum Albumin (BSA) (Sigma Aldrich Inc., USA) 10ml of 4mg/ml was prepared by dissolving 40mg of BSA in 10ml of distilled water. This stock solution (1ml) was diluted with distilled water twenty times (by adding 19mls of distilled water) to prepare a 200µg/ml solution. The assay was carried out in triplicates.

TABLE 3: PROTOCOL FOR PROTEIN DETERMINATION

Test tubes in triplicate	Blank	1	2	3	4	5
Standard BSA solution (µl)	-	100	200	300	400	500
Distilled water (µl)	1000	900	800	700	600	500
Reagent D (ml)	3.0	3.0	3.0	3.0	3.0	3.0
Allow to stand for 10 minutes						
Reagent E (ml)	0.3	0.3	0.3	0.3	0.3	0.3

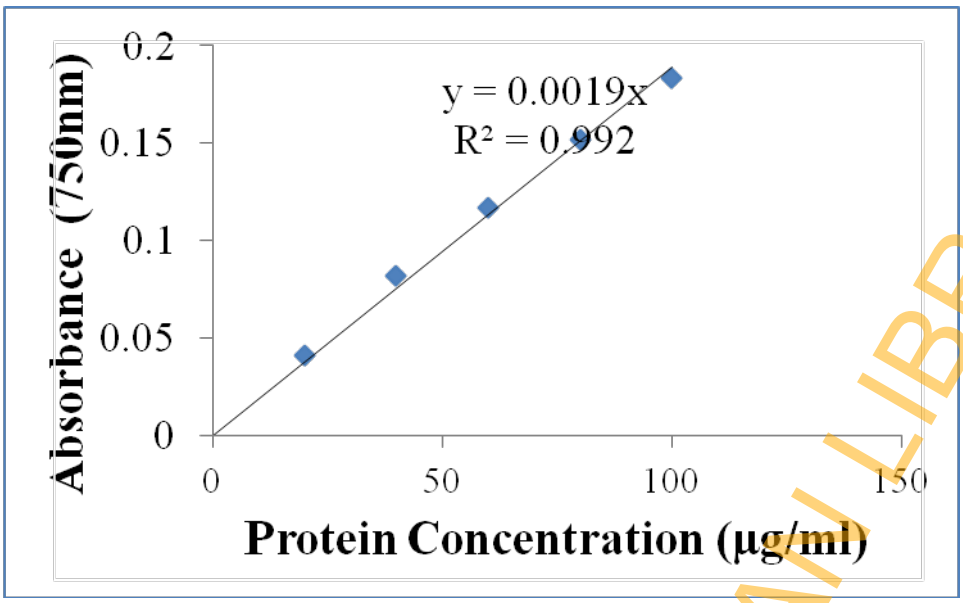


Figure 9: Standard Protein Curve

3.11 ESTIMATION OF MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE OPENING IN RAT LIVER MITOCHONDRIA

Principle:

When mitochondrial membrane is permeabilized and the mitochondrion swells, the refractive index changes in such a way that they scatter less light which can be detected as a decrease in absorbance measured at 520nm in a spectrophotometer.

Mitochondria undergoing calcium induced permeability transition (PT) show colloidosmotic large amplitude swelling which results in a decrease in photometric absorption at 540nm. Membrane permeability transition was assessed by measuring the swelling of mitochondria based on the principle that as mitochondria swell, with the concomitant release of inner mitochondrial proteins, the refractive index changes and less light is scattered (Lapidus and Sokolove, 1992). This is detected as a decrease in light absorption measured spectrophotometrically. Changes in the redox state of respiratory chain components may cause complications. To avoid this, the wavelength of the incident light should be at the isobestic point for the cytochromes (520nm) or 540nm as has been severally used on isolated mitochondria (Lapidus and Sokolove, 1992).

Reagents for inducing mitochondrial swelling

200µM Rotenone

Rotenone (Sigma Aldrich Inc., USA) (0.8mg) was dissolved in 8ml of distilled water and made up to the 10ml mark. Because it is photosensitive, it is stored in a dark (or amber) bottle.

4mM Spermine

Spermine (Sigma Aldrich Inc., USA) (8.094mg) was dissolved in water and made up to 10ml mark. The solution was stored in a dark (or amber) bottle.

12mM $CaCl_2.2H_2O$

Calcium chloride dihydrate (Sigma Aldrich Inc., USA) (17.64mg) was dissolved in 8ml of distilled water and made up to 10ml with distilled water.

250mM Sodium succinate

Sodium succinate (Sigma Aldrich Inc., USA) (675.3mg) was dissolved in 8ml of distilled water and made up to 10ml mark.

Procedure for mitochondrial swelling assay

Mitochondria were incubated in the presence of 0.8µM rotenone and the swelling buffer for 3.5 minutes before the addition of 250mM sodium succinate. When exogenous calcium was used as triggering agent, mitochondria were incubated in the MSH buffer and rotenone for 3 minutes, 12mM $CaCl_2$ was then added and after 30 seconds, 250mM sodium succinate was also added to energize the mitochondria. The change in absorbance was read as a light scattering effect using a CamSpec M106 spectrophotometer at 540nm. To assay for the inhibitory effect of spermine, mitochondria were incubated with MSH buffer, 8µM rotenone and 4mM spermine for 3 minutes. Calcium was added after 3 minutes and sodium succinate was added 30 seconds later. The change in absorbance was spectrophotometrically measured.

3.12 ASSAY FOR MITOCHONDRIAL ATPase ACTIVITY

Mitochondrial ATPase activity was determined according to the method described by Lardy and Wellman (1953). Each test tube for the reaction contained 65mM Tris-HCl buffer pH 7.4, 0.5mM KCl, 1mM ATP and 25mM sucrose in a reaction volume of 2ml. Distilled water or test compound was added accordingly. The reaction was started by the addition of the mitochondrial fraction and allowed to proceed for 30 minutes with constant shaking at 37^0C. The reaction was stopped with the addition of 8ml of 10% trichloroacetic acid to the test tube content. This was then centrifuged in an MSE centrifuge at low speed. The supernatant was kept for phosphate determination.

Reagents

0.1M Tris-HCl (pH 7.4)

Tris (hydroxyl methyl) aminomethane (Sigma Aldrich Inc., USA) (1.21g) was dissolved in 60ml of distilled water and the pH was adjusted to 7.4 after which the final volume was made up to 100ml with distilled water. This was stored in the refrigerator.

0.2M Sucrose

Sucrose (Sigma Aldrich Inc., USA) (6.85g) was dissolved in about 60ml of distilled water and made up to the 100ml mark with distilled water.

5mM KCl

Potassium chloride KCl (BDH Chemicals, England) 37.3mg was dissolved in 60ml of distilled water and made up to 100ml mark with distilled water.

50mM ATP (pH 7.4)

Disodium salt of ATP (Sigma Aldrich Inc., USA) (0.2756g) was dissolved in a small quantity of distilled water, the pH was adjusted to 7.4 and the whole volume made up to 10ml with distilled water. This was stored at -20^{0}C.

3.12.1 Determination of Mitochondrial inorganic phosphate concentration

Principle

This was performed according to the method of Bassir (1963). In this method, inorganic phosphate, in the presence of molybdic acid gives a yellow colour. The yellow colour can be reduced by ascorbic acid to a blue colour and the intensity of the colour formed is directly proportional to the concentration of inorganic phosphate.

Reagents

1mM Na_2HPO4

Disodium hydrogen phosphate (Sigma Aldrich Inc., USA) (1.43mg) was dissolved in 6mL of distilled water and the volume made up to 10mL mark with distilled water.

1.25% Ammonium molybdate in 6.5% H_2SO_4

Ammonium molybdate (Hopkins and Williams Ltd., England) (6.25g)was dissolved in about 450ml of 32.5% H_2SO_4 and the whole volume made up to 500ml mark with 32.5% H_2SO_4, that was prepared by adding 32.5ml of concentrated H_2SO_4 to a small volume of distilled water and made up the volume to 500ml. The molybdate solution was stored at 25^{0}C in a plastic bottle.

9% Ascorbic acid

Ascorbic acid (BDH Chemicals, Poole, England) (9g) was dissolved in about 80ml of distilled water and the volume made up to 100ml mark with distilled water.

2, 4-Dinitrophenol

2,4 Dinitrophenol (BDH Chemicals, Poole, England,) 0.0092g in 40mls of ethanol and made up to 50ml mark with ethanol.

TABLE 4: PROTOCOL FOR DETERMINATION OF INORGANIC PHOSPHATE CONCENTRATION

Test tubes in duplicates	Blank	1	2	3	4	5
1mM Na_2HPO_4 (µl)	-	20	40	60	80	100
Distilled water (µl)	1000	980	960	940	920	900
1.25% Ammonium molybdate (ml)	1	1	1	1	1	1
9% Ascorbate (ml)	1	1	1	1	1	1

Absorbance was read at 660nm after incubation at room temperature for 30 minutes. The standard curve was obtained by plotting the absorbance against the corresponding phosphate concentration using distilled water as the blank.

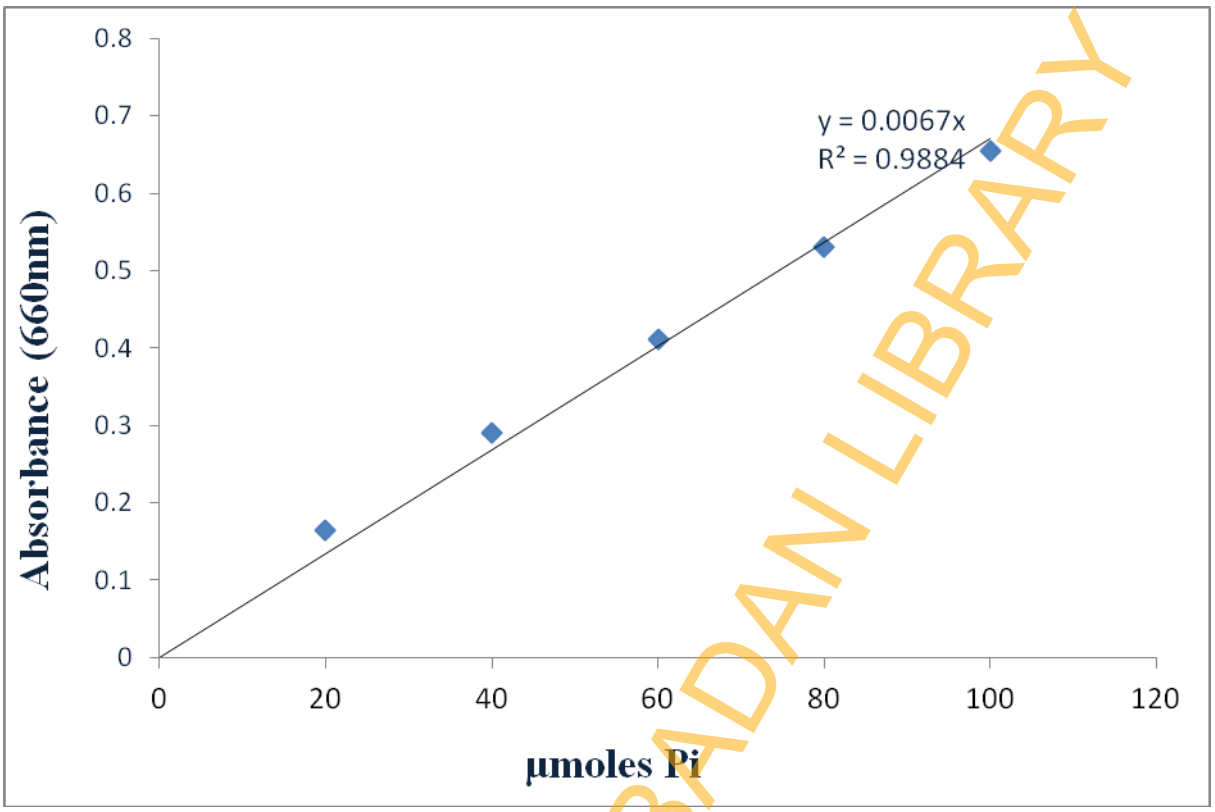


Figure 10: Standard Phosphate Curve

3.13 LIPID PEROXIDATION ASSAY

Principle

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using mitochondria as lipid-rich media, as described by Ruberto *et al.*, (2000). Small amount of malondialdehyde are produced during peroxidation process and this can react with thiobarbituric acid to generate a pink coloured product, which will absorb light at 532nm and is readily extractable into organic solvents such as butan-1-ol.

Reagents

0.8% Thiobarbituric acid

Thiobarbituric acid (BDH Chemicals, Ltd, Poole, England) (0.8g) was dissolved dissolved in 50ml of distilled water and the resulting solution was made up to 100ml mark in a flat-bottomed flask using distilled water.

1.1% Sodium dodecyl sulphate

Sodium dodecyl sulphate (BDH Chemicals, Ltd, Poole, England) (1.1g) was dissolved in 80ml of distilled water and the solution was made up to 100ml mark in a flat-bottomed flask using distilled water. Equal volumes of TBA abd SDS were mixed and used in the assay.

60µM Ferrous Sulphate

Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Sigma Aldrich Co. St Louis, USA) (0.973g) was dissolved in a little quantity of distilled water and the solution was made up to 50ml.

20% Acetic acid

Glacial acetic acid (BDH Chemicals, Ltd, Poole, England) (20ml) was added to 80 ml of distilled water.

Procedure

Mitochondria (1mg/ml) obtained from rat liver (0.5mL) was added to 0.1 mL of the various concentrations of the extract and fractions (100 - 800 µg/mL). The volume was then made up to 1.0 mL with distilled water. Thereafter, 0.05ml 60µM of $FeSO_4$ was added and the mixture was incubated at 37^0C for 30 minutes. Then, 1.5 mL of 20% acetic acid was added, followed by 1.5 mL of TBA in SDS. The resulting mixture was vortex mixed and heated at 95^0C for 1 hour. After cooling, 5 mL of butanol was added and the mixture was centrifuged at 3000 rpm for 10 minutes. The blank contained the sample and the reagents only. Distilled water was used to make up the volume. The absorbance of the organic upper layer was measured at 532 nm and the percentage inhibition was calculated with the formula:

$$Percentage\ inhibition\ of\ lipid\ peroxidation = Ao - A1/Ao\ x\ 100$$

Where: Ao= the absorbance of the control; A1= the absorbance of the sample

3.14 CELL CULTURING

Cell culture media generally consist of an appropriate source of energy and compounds which regulate the cell cycle in addition to nutrients. The medium also maintains pH and osmolality. The cells are thawed as rapidly as possible in contrast to the optimal slow rate of freezing. This is to minimize ice crystal formation which may damage the cells and the UV light of the Biosafety Hood was turned on for at least 30 min before the commencement of the experiment. The culture media EMEM (Eagles Minimal Essential Medium), Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone Laboratories,USA) or RPMI-(Rosewell Park Memorial Institute) 1640 (Gibco-BRL,USA) supplemented with 10% Fetal Bovine Serum (FBS), 4mM Glutamine and 1% penicillin –streptomycin (100U/mL penicillin and 100µg/mL streptomycin) and trypsin (0.53mM EDTA) were warmed at 37^0C in the water bath. Cryo vials of cells (healthy kidney VERO, lung adenocarcinoma (A549) and Prostatic LNCaP and DU145 cells) were removed from the liquid nitrogen tank using gloves and handling tong. The vial's cap were checked to be sure that they were still tightly screwed and the labels also were checked for proper identification. The vials were immersed in a 37^0C water bath and gently shaken until thawed. The contents of the vial were transferred to sterile 15ml tubes containing 7ml warmed culture medium. The cells were washed by pelleting with gentle centrifugation (Eppendorf 5702 R centrifuge) at 1200 rpm for 5 mins. The medium was discarded and the thawed cell resuspended in warmed fresh culture medium in a 100mm dish .the cells were immediately cultured in 37^0C, humidified incubator with a 5% CO_2 environment.

3.14.1 Subculturing /Passaging

Principle

Anchorage dependent cell lines growing in monolayers need to be sub-cultured at regular intervals to maintain them in exponential growth. When cells are near the end of exponential growth (about 70-90% confluent) they are ready to be sub-cultured. Sub-culturing involves breakage of both intercellular and intracellular cell-surface bonds. It may require the digestion of their protein attachment bonds with proteolytic enzyme preparations such as tyrpsin-EDTA.

Procedure

First, the hood was sprayed with 70% ethanol after the UV light in the hood was switched on. The growth medium and trypsin-EDTA solution was brought to 37^0C in a water bath and also sprayed with 70% ethanol. All materials going into the hood were sprayed with 70% ethanol in order to prevent contamination. The cultured cells were monitored under microscope for density and once detected to be full, the medium was sucked out of the culture plate and 1mL of trypsin added to digest the cells. The trypsin solution was allowed to cover the surface of the cells and placed in the incubator for about 3minutes. The cells were checked under the microscope again to see if the cells were already detached from the dish.

Detached cells were seen floating under the microscope. The dish containing cells wasplaced back into the hood and 7ml of complete growth media added to inactivate the trypsin, followed by repeated pipetting in order to dislodge crumping cells. After cells have been dissociated into a single cell suspension, they were diluted to the appropriate concentration (usually 1 mL of dislodged cells in suspension is added to 7 ml of fresh growth medium) transferred into fresh culture vessels (100mm dish) and appropriate growth medium where they reattach, grow and

divide. This ratio (cells to fresh growth medium) is subject to the seeding density that is needed for the required assay. Cells were maintained in a humidified incubator at 37^0C and an atmosphere of 95% air and 5% CO_2. The growth media was changed every three days and cell density monitored and sub-cultured when the cells in the dish attain 70% confluency.

3.14.2 Cell Plating

Depending on the assays, cells were seeded into 96-well and 6-well plates. Number of cells seeded per well is dependent on type of assay required. Similarly, incubation time also differed depending on assay-type, variations include 24hr and 72 hours. Seeded cells were treated with the indicated concentrations of the different extracts and fractions of *Calliandra portoricensis*. The cell suspension for plating must be thoroughly mixed by pipetting up and down severally to get an evenly distributed mix.

3.14.3 Cell Counting

From the evenly distributed monolayer suspension, 10µL are placed in haemocytometer (counting chamber) grid and number of cells present were counted under an inverted microscope and multiplied by 1000 to estimate number of cells per ml. The use of trypan blue dye is important at this stage in order to distinguish viable from non-viable cells. Non-viable cells stain blue while viable cells remained opaque with the dye. The total number of cells in the 10µL cell suspension was then extrapolated by the calculation.

Total no of cells per ml = no of cells counted/4(no of chambers in haemocytometer) X Dilution factor (if applicable)

= Y x10^4 cells/mL

After plating, treatment of cells with varying concentrations of methanol fraction of *Calliandra portoricensis (*MFCP) was done usually after twenty four hours.

3.15 ASSESSMENT OF CELL VIABILITY USING THE 5-[3-(CARBOXYMETHOXY) PHENYL]-3-(4,5-DIMETHYL-2-THIAZOLYL)-2-(4-SULFOPHENYL)-2H-TETRAZOLIUM SALT (MTS) ASSAY

Principle

The MTS assay is a metabolic viability assay that relies on the reduction of the colourless or weakly coloured tetrazolium salt (MTS) to coloured derivatives known as formazans to distinguish live cells from their dead counterparts. Under most standard culture conditions, it requires incubation of a reagent and population of viable cells which convert a substrate to a coloured product that can be detected with a plate reader. When cells die, they lose their ability to convert MTS (composed of MTS and an electron coupling agent) into a soluble purple formazan product which is quantified spectrophotometrically by measuring absorbance at 490nm. The color intensity is directly proportional to the number of viable cells; therefore relative cell numbers can be determined based on optical absorbance from each well of the microtiter plate. Thus, viable cells with active metabolism convert the substrate to purple coloured forms.

MTS is negatively charged and does not readily penetrate cells and as such are typically used with an intermediate electron acceptor, phenazine methosulphate (PMS) that can transfer electrons from the cytoplasm or plasma membrane to facilitate reduction of the tetrazolium salt.

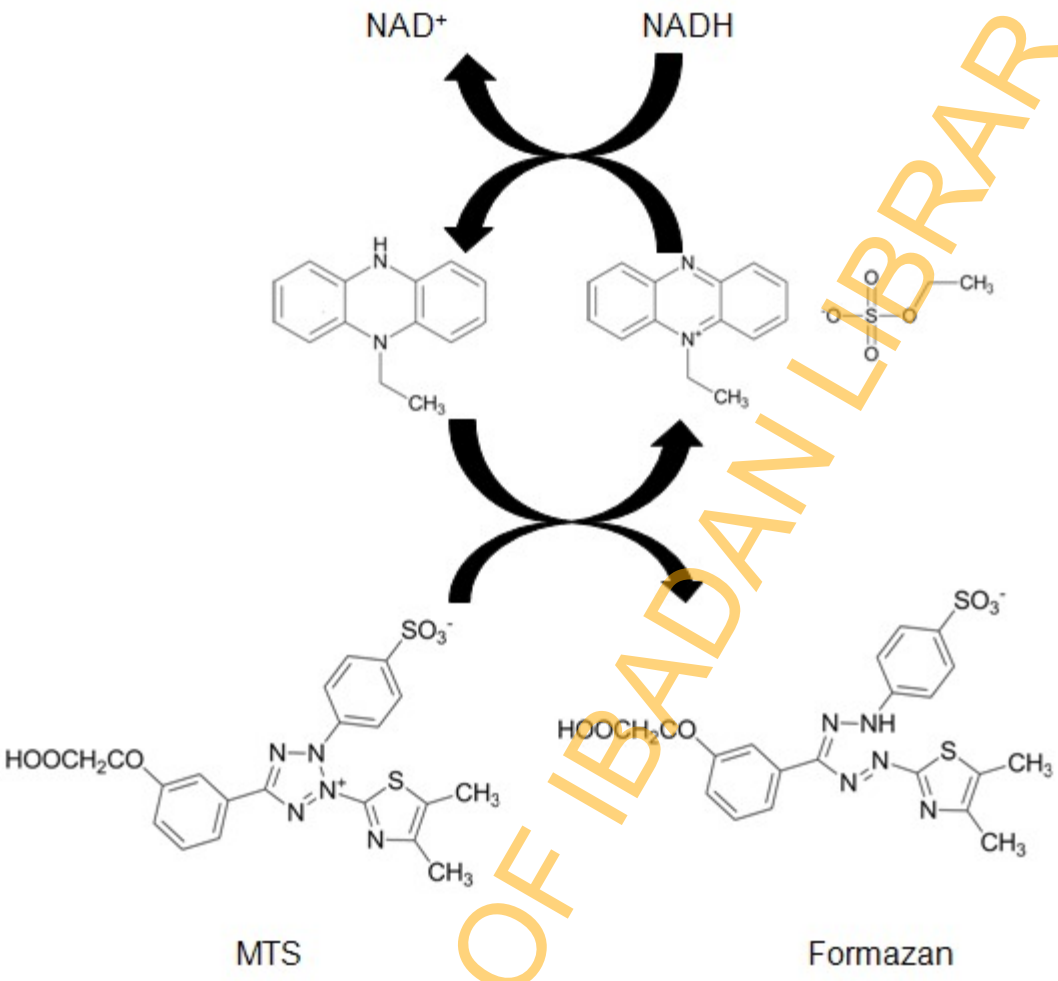


Figure 11: Intermediate electron acceptor Phenazine MethoSulphate (PMS) transfers electron from NADH in the cytoplasm to reduce MTS in the culture medium into an aqueous soluble formazan. (Riss *et al.*, 2013)

PROCEDURE

The human prostate cancer cell lines LNCaP and DU-145, the human lung cancer cells (A549) and normal kidney VERO cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were grown in RPMI-1640 –L-Gln (Gibco-BRL, USA) and EMEM (Hyclone Laboratories, USA) culture media for LNCaP and DU-145 cells, respectively. All culture media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (100U/ml penicillin and 100µg/ml streptomycin). Cells were maintained in an incubator at 37^0C in a humidified 5% CO_2 atmosphere. The cells in 100mm dishes were later passaged into 96-well plates and cells were seeded with density 4500 /100µl in 96- well plates and incubated for 24 hrs to achieve confluence. After incubation, they were treated with varying concentrations of methanol fraction of *C. portoricensis* (1µg/mL -2000µg/mL) and incubated for 72 hours at 37^0C in a humidified incubator. After treatment, cells in each well were incubated with 20µL of MTS reagent for 2-4 hrs at 37^0C. The absorbance of the soluble formazan crystals formed by MTS was measured at 490 nm using a microtitre plate reader (Thermo Labsystems) spectrophotometer. Cell viability was expressed as percentage of MTS reduction. The cell number was determined using a hemocytometer. Maximum inhibitory concentrations of *C.portoricensis* that killed 50% of the cell population (IC_{50}) were calculated using Graphpad Prism software.

3.16 DETERMINATION OF THE EFFECT OF MFCP ON MITOCHONDRIAL MEMBRANE POTENTIAL

Principle

Tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is a membrane permeable lipophilic cationic dye that accumulates in energized mitochondria and used to measure/probe mitochondrial membrane potential. This dye can exist in two different states: aggregates (590 ±17.5nm) and monomers (530 ± 15 nm) and each state is characterized by a different emission spectra. Normal healthy mitochondria take up JC-1 dye leading to formation of aggregates resulting in high red fluorescence. The formation of JC-1 aggregates is reversible and occurs during transition of mitochondria from polarized to depolarized state due to apoptosis or other physiological events. This leads to leaking of dye out of the mitochondria into the cytoplasm, resulting in a fluorescence emission shift that favours emission of green fluorescence. JC-1 dye does not accumulate in mitochondria with depolarized mitochondrial membrane potential but remains in the cytoplasm as monomers resulting in green fluorescence. Therefore a decrease in the aggregate fluorescent count is indicative of depolarization whereas an increase is indicative of hyperpolarization.

FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone) is an ionophore and uncoupler of oxidative phosphorylation. Thus, it eliminates mitochondrial membrane potential and JC-1 staining. This was used as a depolarization control.

Procedure

Cells (1.5 x 10^4 cells /well) were seeded in a black 96-well plate. They were allowed to attach overnight and treated with different concentrations of MFCP diluted in complete media for 72 hours. Four (4) hours prior to completion of treatment, 11µL of 10mM FCCP was added to depolarized control wells while 100µL of 40µM JC-1 dye was added thirty minutes prior to the end of the treatment. Varying concentrations of MFCP (2.5, 5 and 10µg/mL) were incubated with JC-1 dye for twenty minutes. Cells were washed twice with 100µL 1X Dilution buffer containing MFCP. The last wash was left in the wells and the plate transferred to the microplate reader and fluorescence readings taken at 475± 20nm (for simultaneous aggregate and monomer excitation) while emission wavelength was set at 530 ± 15nm (monomer species) and 590 ± 17.5nm (aggregate emission). A ratio between the aggregate and momoner measurements was plotted after substraction of background signal (non-stained cells).

3.17 DETERMINATION OF CYTOCROME C RELEASE USING ABCAM CYTOCHROME C HUMAN ELISA KIT

Principle

The principle of sandwich ELISA is the quantification of a specific protein through its containment in a sandwich of specific antibodies conjugated to the colorimetric 3,3',5,5'-tetramethylbenzidine (TMB) substrate, whose intensity is proportional to the protein quantity and is specific for the protein being assayed for. A secondary biotin-linked antibody specific to the protein (cytochrome c) recognizes and binds to the primary antibody, forming a "sandwich" of specific antibodies around the desired protein in the cell lysate i.e. several secondary antibodies

will bind to primary antibody, hence enhancing the signal. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible.

The streptavidin-HRP (Horse-radish peroxidase) complex was used to bind the biotin-linked secondary antibody through its streptavidin portion. The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes colour during reaction. The HRP domain reacts with the added TMB substrate, which is converted by the enzyme-HRP to elicit a chromogenic signal forming a colored product that was measured at 450 nm by a plate reader (ChroMate-4300, FL, USA) after which the reaction was terminated by the addition of stop solution which is usually acidic.

Preparation of Cytosolic Lysate

Cells that have been pretreated with different concentration of MFCP were harvested and centrifuged for 15 minutes at 1200rpm.They were washed once in cold PBS and resuspended in lysis buffer to a concentration of 1.5 x 10^6 cells/mL. They were incubated for an hour at 25^0C with gentle shaking and centrifuged at 200g for 15 minutes. The supernatant was diluted 50-fold in 1X Assay Buffer (component of ABCAM kit) as directed by the manufacturer. Supernatant was aliquoted and stored at -70^0C.

Procedure

The microtitre plate was washed twice with approximately 400µL 1X wash buffer (component of ab19521 kit). The cell lysate (100µL) was added to the wells of microtiter plates that were precoated with Cytochrome c specific primary antibodies. Samples and standards were added to wells along with a biotinylated cytochrome c detection antibody (50µL) and the microtitre plates were then incubated at 25^0C for 2 hours. Following washing with wash buffer, 100µL of a streptavidin –HRP conjugate is added to all wells and incubated for one hour. Later, 100µL of TMB substrate is added after washing and incubated at 25^0C in order to ensure colour development. The stop solution was added after 10 minutes and absorbance read at 450nm.

3.18 DETERMINATION OF CASPASE-9 ACTIVITY (USING THE CASPASE -9 COLORIMETRIC ASSAY KIT)

Principle

Caspase activation underlies most of the phenotype of apoptosis. Caspase specific kits utilize a tetrapeptide coupled to a colorimetric /flouorimetric reporter system to assess proteolytic activity in cell lysates. Caspase-9 belongs to the initiator family of caspases whose cleavage recognition motif/sequence is a tetrapeptide LEHD (Leucine, Glutamine, Histidine, Aspartate) substrate conjugated to p-nitroaniline, a color reporter molecule. The assay is based on the spectrophotometric detection of the chromophore (pNA) after cleavage from labelled substrate LEHD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtitre plate reader at 400 or 405 nm. Comparison of the absorbance of pNA from an induced sample with an uninduced control allows determination of the fold increase in Caspase -9 activity.

Ac-LEHD-pNA $\xrightarrow{\text{Activated Caspase -9}}$ LEHD + pNA (released chromophore)

Procedure

Prostate LNCaP cells that have been pretreated with different concentrations of MFCP were harvested from the incubation medium after 24 hours using trypsin. Cells (2 $x10^6$) were counted and pelleted along with a control (no induction of apoptosis). They were resupended in 50µL of chilled lysis buffer (Biovision Incorporated, CA, USA) and incubated on ice for 10 minutes. The cells were centrifuged for 1 minute at 10,000g and supernatants or cytosolic extracts transferred to fresh tubes and put on ice for immediate use or stored at -80^0C for future use. Protein concentration was assayed for using the Bradford method.

Cell lysis buffer (50µL) was added to cytosolic lysate at protein concentration of 100µg for each assay, after which 50µl of 2X reaction buffer {containing 10mM (Dithiothreitol) DTT} was equally added to each sample. 5µL of 4mM LEVD-pNA substrate (200µM) final concentration was equally added to all samples and incubated for 37^0C for 2 hours. The absorbance (colour intensity from LEVD-pNA cleavage) were read at 400 or 405 nm while background reading from cell lysates and buffers were subtracted from the reading of both induced and non induced samples before calculating fold increase in Caspase-9 activity.

3.19 DETERMINATION OF CASPASE-3 ACTIVITY USING CASPASE -3 COLORIMETRIC ASSAY KIT

Principle

Caspase-3 specific kits is based on spectrophotometric detection of the chromophore p-nitranilide (pNA) after cleavage from the labelled substrate DEVD. Activation of caspases initiates apoptosis in mamamalian cells. The activation of caspase-3 in cell lysates enables recognition of the sequence DEVD. The assay is based on the spectrophotometric detection of the chromophore p- nitroaniline (pNA) after its cleavage from the labelled substrate DEVD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 400 or 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in Caspase-3 activity.

Ac-DEVD-pNA $\xrightarrow{\text{Activated Caspase -3}}$ DEVD + pNA (released chromophore)

PROCEDURE

Cells that have been pretreated with different concentrations of MFCP were harvested from the incubation medium after 24 hours by trypsination.They were collected by centrifuging the cell suspension at 250g for 10mins. Cells (1-5 x 10^6) were pelleted along with a control culture without induction .They were resupended in 50µL of chilled lysis buffer (Biovision Incorporated ,CA,USA) and incubated on ice for 10 minutes. They were centrifuged for 1 minute at 10,000g and supernatants or cytosolic extracts transferred to fresh epperndorf tubes and put on ice for immediate use or stored at -80^0C for future use. Protein concentration was assayed for using the Bradford method and 50-200µg protein was diluted to 50µL with cell lysis buffer for each assay, after which 50µL of 2X reaction buffer (containing 10mM DTT) was added to each sample. Then, 5µL of 4mM DEVD-pNA substrate (200µM) final concentration was equally added to all samples and incubated at 37^0C for 2 hours.

The absorbance (color intensity from DEVD-pNA cleavage) were read at 400 or 405 nm while background reading from cell lysates and buffers were subtracted from reading of both induced and non-induced samples before calculating fold increase in Caspase-3 activity.

3.20 DETERMINATION OF PROTEIN CONCENTRATION IN PROSTATIC CELL LYSATES BY BRADFORD ASSAY

Principle

Protein content of the lysates was determined using the Bradford method. The Bradford protein determination procedure utilizes the absorbance shift of the dye Coomassie Brilliant Blue G-250 when under acidic conditions, which converts the red coloured dye to a reduced blue form, the absorbance of which is measured at 595 nm.

Procedure

A stock solution of BSA (1mg/mL) was serially diluted with distilled water up to 0.0625mg/mL and added into wells while lysates (10µL) were added into different wells. Bradford solution (200µL) was then added into all wells and mixed gently.The solution was incubated for 15 mins at 25^0C after which absorbance at 595nm was taken.

3.21 FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS IN PROSTATIC LNCaP CELLS FOLLOWING TREATMENT WITH METHANOL FRACTION OF *C. portoricensis.*

Principle

Annexin V-FITC apoptosis detection relies on the property of cells to lose membrane asymmetry in the early phase of apoptosis which precedes loss of membrane integrity. Cells translocate membrane phosphatidyl serine (PS) from the inner leaflet of the plasma membrane to the cell surface after apoptosis is initiated. Once on the outer leaflet, PS can easily be detected by staining with a fluorescent conjugate of Annexin V, a 35-36 kDa Ca^{2+} - dependent protein that has a high affinity for PS. Annexin V may be conjugated to fluorochromes such as FITC and Phycoerythrin (PE), or to biotin or tagged with EGFP (Enhanced Green Fluorescent Protein). These formats retain their high affinity for PS and thus serve as sensitive probes for flow cytometric analysis of cells undergoing apoptosis. Propidium Iodide (PI) is a standard flow cytometric viability probe used to distinguish between viable and non-viable cells. It binds cellular DNA in cells where cell membrane integrity has been totally compromised. Viable cells with intact membrane exclude PI while membranes of dead and damaged cells are permeable to it. Basically, dual staining of Annexin V and PI distinguish live cells which undergo early apoptosis evident by increase in annexin binding ($Annexin^{+}/PI^{-}$) from cells that are in the later stages of cell death or already dead which stain positive to both dyes. ($Annexin^{+}/PI^{+}$). This enables identification of different populations within the cells.

Procedure

Prostate LNCaP cells were treated with varying concentrations of MFCP and PPF 3. Adherent and flowing cells (5 X 10^5) were collected by centrifugation and resuspended in 500µL 1X binding buffer. 5µL of Annexin V-FITC and propidium iodide were added and incubated at 25^0C for 5 min in the dark, after which quantification by flow cytometry was done. Annexin V-FITC binding were analysed by flow cytometry (Excitation = 488nm, Emission = 530nm) using FITC signal detector (usually FL1) while PI staining by the phycoerythrin emission signal detector (usually FL2).

3.22 DETERMINATION OF PRO-APOPTOTIC BAX LEVELS IN PROSTATIC LNCaP CELLS TREATED WITH MFCP USING ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Principle

The simple step ELISA employs an affinity tag labelled capture antibody and a reporter conjugated detector antibody which immunocaptures the sample analyte in solution. The entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well.

3.22.1 **Preparation of Cytosolic** Lysate

Growth media were removed from cells and rinsed twice in PBS (phosphate buffered saline). The cells were solubilised by addition of 1mL chilled 1X Cell extraction buffer, PTR (component of ABCAM kit) directly on the plate. The cells were scrapped using a cell scraper into microcentrifuge tubes and the lysate incubated on ice for 15 minutes. After centrifugation at 18,000g for 20 minutes at 4^0C, they were transferred into clean tubes and the pellets discarded. The cytosolic lysates were aliquoted and stored at -80^0C . Samples were diluted 4-fold in 1X Cell Extraction Buffer and protein concentration was quantified using Bradford protein assay method.

3.22.2 **Preparation of Mitochondrial Lysate**

Mitochondria of human prostatic LNCaP cell line were isolated by conventional differential centrifugation as described by Clayton and Shadel (2014). Prostate LNCaP cells were harvested, washed once with cold PBS and re-suspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1.5mM $MgCl_2$, (Hypotonic Buffer) and 1 mM EDTA, 70 mM sucrose, 210 mM mannitol. After incubating in an ice-bath for 10 min, the cell suspension was homogenized with 15 strokes in a 2 mL glass homogenizer. The samples were centrifuged twice at 1,500× *g* at 4°C for 5 min to remove nuclei and cell debris. The supernatants were centrifuged at 15,000× *g* for 15 min to separate the mitochondrial and cytosolic fractions. The mitochondria-enriched fractions were suspended in appropriate buffer containing 210 mM mannitol, 70 mM sucrose and 5 mM Tris-HCl (pH 7.5) .

Procedure

Samples/Standards (50µL) were added to the wells followed by the addition of 50µL antibody mix/cocktail (Component of ABCAM kit). The microplate was sealed and incubated for an hour at room temperature (25^0C) on a plate shaker set to 400rpm, the wells were washed thrice to remove unbound material. Complete removal of liquid at each step was ensured for good performance. After the last wash, the micro titre plate was inverted and blotted against a clean paper towel to remove excess liquid. Thereafter, 100 µL of TMB substrate was added and incubated for 10minutes in the dark during which it was catalyzed by Horse Radish Peroxidase (HRP) generating a blue colour. This reaction was then stopped by the addition of 100µL stop solution completing the colour change from blue to yellow. Intensity of colour generated was proportional to the amount of bound analyte and the intensity was measured at 450nm.

3.23 DETERMINATION OF ANTI-APOPTOTIC BCL_2 LEVELS IN PROSTATIC LNCaP CELLS TREATED WITH MFCP USING ENZYME LIKNED IMMUNOSORBENT ASSAY

Principle

The sandwich ELISA measures the amount of antigen between two layers of antibody. The antigen to be measured must contain at least two antigenic sites capable of binding to anibody since at least two antibodies act in the sandwich. It employs an affinity labelled antibody which immunocapture the sample analyte in solution. An anti-tag antibody precoated on the well immobilizes the capture antibody/analyte/detector antibody via immunoaffinity.

3.23.1 Preparation of Cytosolic Lysate

Cells that have been pretreated with MFCP for 24 hours were harvested and rinsed twice in cold PBS. They were solubilised by the addition of 1mL chilled 1X Cell Extraction Buffer PTR (component of ABCAM kit). The cells were scraped into microcentrifuge tubes and the cytosolic lysate was incubated on ice for 15 minutes. They were centrifuged at 18,000g for 20 minutes at 4^0C. The supernatants were transferred to clean tubes and the pellets discarded. The cytosolic lysates were aliquoted and stored at -80^0C . Samples were diluted 4-folds in 1X Cell Extraction Buffer PTR and protein concentration was quantified using Bradford protein assay method.

Procedure

All reagents, working standards and samples were prepared as directed by the manufacturer. Samples/standards (50 µL) were added to appropriate wells followed by addition of 50 µL of antibody cocktail to each well. The plates were sealed and incubated for an hour at 25^0C on a plate shaker set to 400rpm. The wells were washed thrice to remove unbound material. Complete removal of liquid at each step was also ensured for good performance. After the last wash the microplate was inverted and blotted against a clean paper towel to remove excess liquid. After this, 100µL of TMB substrate was added and incubated for 10minutes in the dark during which it is catalyzed by HRP, generating a blue colour. This reaction was then stopped by the addition of 100µL stop solution completing any colour change from blue to yellow. The intensity of developed colour was measured at 450nm.

3.24 ANALYSIS OF CELL CYCLE DISTRIBUTION IN CELL LINES TREATED WITH METHANOL FRACTION OF *C. portoricensis*

Principle

The amount of cellular DNA content in a cell changes during cell cycle. Propidium iodide (PI) is the most widely used fluorescent dye for staining DNA in whole cells or isolated nuclei. PI intercalates into the DNA helix of fixed and permeabilized cells. Given that PI stains both double-stranded RNA (dsRNA) and DNA (dsDNA), cells are treated with RNase to ensure that PI staining is DNA specific. PI does not cross the intact plasma membrane of viable cells. However, PI can readily enter dead cells, cells in late stages of apoptosis, or fixed cells that have damaged plasma membranes. The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNAthat is added to a suspension of permeabilized single cells or nuclei. The stained cells has incorporated an amount of dye proportional to the amount of DNA, the cells' emitted fluorescent signal yield an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered as measurement of the cellular DNA content. Single cell populations are then analysed for DNA content which is directly proportional to the fluorescence of the nucleic acid stain.

Procedure

Prostate LNCaP cells (1×10^6) were treated with MFCP at its maximum inhibitory concentration (IC_{50}), five times its maximum inhibitory concentration ($5IC_{50}$) and 0.1% DMSO. Adherent cells were collected by centrifugation and were fixed in 70% ethanol at 4°C overnight. Cell cycle progression was determined by flow cytometry following staining with propidium iodide using a BD C6 Accuri flow cytometer.

3.25 PURIFICATION, ISOLATION AND IDENTIFICATION OF BIOACTIVE AGENTS FROM *Calliandra portoicensis* ROOT BARK

3.25.1 THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography (TLC) is a technique used to analyze and separate mixtures. Different compounds in a sample mixture travel at different rates due to differences in their attraction to the stationary phase, and because of differences in solubility in the solvent. By modifying the solvent system, the separation of components (measured by R_f value) can be adjusted (Sweety, 2012)

Procedure

Fractions obtained using Vacuum Liquid Chromatography (VLC) were subjected to TLC by using the coated plates (Whatman KC 18F silica gel 60 F254, 0.25mm). Several solvent systems were used to elute the TLC plate. Visualization of the spots was achieved under UV (λ_{max} 254 and 366nm) and with the aid of chromogenic reagents specific for each phytochemical. Samples were dissolved in appropriate solvent and applied, using capillary pipette, on spots made on the TLC plate. This was carefully placed inside the chromatography tank with the appropriate solvent system and covered. As the mobile phase ascends the plate, the mixtures were resolved into bands or spots. After developing the thin layer plate, the plate was removed from the tank after the solvent front had reached about 1cm from the top of the plate. The plate was then air-dried, viewed under the UV light, marked to show the compound that fluoresced and then sprayed with the appropriate chromogenic reagent.

3.25.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROSCOPY (HPLC-MS)

Liquid chrmomatography –mass spectrometry (LC-MS) otherwise known as High Perofrmance Liquid chromatography mass spectroscopy (HPLC-MS) is a powerful tool in analytical chemistry. It has the ability to separate, identify and quantify the components that are present in any sample that can be dissolved in a liquid and also analyse the materials by mass. It is used for the separation and analysis of non-volatile or thermally unstable compounds. It combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (Parasuraman *et al.,* 2014). Generally its application is directed towards specific detection and potential identification of chemicals in the presence of other chemicals.

This technique allow for the structural elucidation of unknown molecules through fragmentation.

Principle

A sample solution is injected into a column of porous material (stationary phase and a liquid (mobile phase) pumped at high pressure through the column. The separation of sample is based on differences in the rate of migration through the column arising from different partition of the sample between the stationary and mobile phase.

Depending on the partition behaviour of different components, elution at different time takes place. Essentially, a pump is use to provide a continuous flow of a solvent into which a dissolved sample is introduced. Once the sample is in the solvent flow, it travels through an analytical column. The compounds present in the sample mixture are then separated depending on their affinity for the coated particles and the retention time i.e time it takes for a compound to pass from the injector to the detector. The retention time of the compound of interest may then be compared to a reference material. Mass spectroscopy works by ionizing chemical compounds to generate charged molecules or molecular fragments by measuring their mass to charge ratios.

Procedure

HPLC-MS was operated on onstrument of WATERS 2767, with Shimadzu Shim- Pack, PRC-ODS 20*250MM, 15u, two connected in series. Solvent A used for HPLC was water/0.05% (TFA) and solvent B was acetonitrile. The elution condition was a linear radient increase of solvent B from 5% to 100% over a period of 20 minutes at a flow rate of 30ml/min.

3.26 ISOLATION OF POTENT COMPOUNDS FROM *N*-DI-TERT-BUTYL BICARBONATE (BOC-ANHYDRIDE) -PROTECTED MFCP

Principle

The protection of a functional group can be in the chemistry of poly functionalised molecules, when a reaction has to be carried out in a part of the compounds without the rest perturbing the molecule. Di-*tert*-butyl dicarbonate ($C_9H_{18}O_3$) is a reagent widely used in organic synthesis (Cheraiet *et al.*, 2012). Since this compound can be regarded formally as the acid anhydride derived from a *tert*-butoxycarbonyl (Boc) group, it is commonly referred to as "Boc anhydride." This pyrocarbonate reacts with amines to give *N*-*tert*-butoxycarbonyl or so-called Boc derivatives.

These carbamate derivatives do not behave as amines, which allow certain subsequent transformations to occur that would be incompatible with the amine functional group. The Boc can later be removed from the amine using acids. Thus, Boc serves as a protective group (Kocienski, 2004).

It is in the nature of amino-protecting groups that they are electron withdrawing. Substituting a hydrogen atom at an acylnitrogen by electron-withdrawing Boc-group can therefore be expected to decrease the electron density on nitrogen. Milder nucleophiles are therefore required to release the BOC-protected amine from acylcarbamates than for cleavage of the original amides.

Procedure

Di-tert-butyl bicarbonate (BOC-anhydride) (20g) was added to MFCP (10g) dissolved in 1M NaOH (480mls) and acetonitrile (120mls) in a one- litre round-bottom flask. The resulting solution was constantly stirred at 25^0C for 48 hours. Progress of the reaction was monitored by TLC, which indicates complete disappearance of precursor amines.

Work up was done by equilibrating a mixture of Dichloromethane and water supplemented with 10% methanol. This was done thrice in separating funnel until exhaustion. Organic layers were combined, filtered and dried over Na_2SO_4 and concentrated at 40^0C. The TLC of BOC-Protected MFCP (BocMFCP) was done to determine the success of the experiment. Preparatory TLC of BOC-protected MFCP was carried out to isolate the bioactive compound (Sub-BocMFCP).

Subfraction-Boc-MFCP(PPF 4) was chromatographed on a silica gel column with DCM-MeOH in a gradient form to yield 33 fractions which were pooled to give 6 fractions These fractions were concentrated under reduced pressure at 40^0C.

SPECTROSCOPIC MEASUREMENT

One dimensional (^{1}D) NMR and 13C-NMR spectra pulse sequences were recorded in deuterated solvents only on Bruker AM-300 or 400 MHz spectrometers. Chemical shifts were measured in parts per million (ppm) (δ) and coupling constants (J) were given in Hz.

STATISTICAL ANALYSIS

Mean, standard deviation and 95% confidence interval were computed. Comparison between groups was performed using the Student's't' test. Multiple comparisons were performed using the Duncan Multiple Range Test. Graphpad Prism software was used to produce dose response curves for cell viability assays.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT 1: DETERMINATION OF PHYTOCHEMICAL COMPOSITION OF THE ROOT BARK OF *C. portoricensis*

INTRODUCTION

Naturally occurring compounds including flavonoids, sesquiterpenes, alkaloids and polyphenols found in fruits, vegetables and medicinal plants possess a variety of anticancer properties that target multiple cellular signaling pathways in cancer cells (Martin, 2006). These phytochemicals have exhibited a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anitallergic and vasodilatory actions (Millimouno *et al.*, 2014). Many studies have suggested that flavonoids in particular exhibit biological activities, including antiallergenic, antiviral, antiinflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000).

Calliandra portoricensis is used in combination with *Plumbago zeylanica for* the treatment of prostate tumour locally in Africa. There are incidentally several claims about its potency as an anti-tumour. However, these claims have not been established in the laboratory. The aim of this experiment was to determine the phytochemicals present in the root bark of the *C.portoricensis* in order to have insight into the constituents of the plant that may be responsible for its traditional use in prostate enlargement therapy.

PROCEDURE

The root bark of *C. potoricensis* was collected, extracted and partitioned as earlier explained on pages 59 and 60. The plant extracts and fractions were screened for the presence of some secondary metabolites such as saponins, tannins, steroids, phlobatannins, alkaloids, flavonoids, terpenoids, and cardiac glycosides as described on pages 61 - 63.

RESULTS

Table 5 shows the results of qualitative phytochemical screening of the various fractions of the root bark of *C. portoricensis*. Altogether, *C. portoricensis* contains alkaloids, saponins, tannins, phenols, antraquinones, steroids, flavonoids and cardiac glycosides

The cold maceration method employed in this study showed that the crude methanol extract yielded 3.7%. When partitioned, the methanol fraction of the extract was 35.4%, while that of n-hexane, chloroform, ethylacetate fractions were 0.2%, 3.1%, and 12.6% respectively. This showed that the methanol fraction had the highest percentage yield while n-hexane fraction had the smallest yield.

CONCLUSION

The root bark of *C. portoricensis* contains phytochemicals which are beneficial to humans. The reported role of *C. portoricensis* in traditional treatment of protate tumour may be as a result of the presence of some of these phytochemicals.

Table 5: Phytochemicals of the various fractions of the root bark of *C.portoricensis*

Chemical Constituents	MECP	MFCP	CFCP	EFCP
Alkaloids	-	+	+	+
Antraquinones	+	-	-	+
Cardiac glycosides	+	-	+	-
Flavonoids	+	+	+	+
Phenols	+	+	+	-
Reducing Sugar	-	-	-	-
Saponins	+	+	-	-
Steroids	+	+	+	-
Tanins	+	+	+	+

MECP: Crude m2ethanol extract of *Calliandra portoricensis*, **CFCP**: Chloroform fraction of *Calliandra portoricensis*, **EFCP**: Ethylacetate fraction of *Calliandra portoricensis*

MFCP: Methanol fraction of *Calliandra portoricensis*

EXPERIMENT 2: EVALUATION OF THE EFFECT OF CALCIUM AND SPERMINE ON RAT LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

INTRODUCTION

Increased matrix calcium in combination with a triggering agent, which may be elevated calcium levels, inorganic phosphate concentration, increased temperature, oxidative stress or chemicals induce the inner mitochondrial membrane to undergo permeability transition. This allows entry/efflux of small (<1500 dalton) solutes (Lapidus and Sokolove, 1992). The permeability transition is Ca^{2+}-dependent and cyclosporin A (CsA)-sensitive. It is accompanied by uncoupling/collapse of the membrane potential, oxidation of pyridine nucleotides, loss of small matrix solutes, matrix swelling and permeability transition. The permeability transition reflects irreversible opening of a pore in the inner mitochondrial membrane (Lapidus and Sokolove, 1992). Cyclosporin A inhibits pore opening possibly by interacting with its intracellular receptor, cyclophilin, a peptidyl-prolyl cis-trans isomerase. Studies have shown that spermine has protective effect on the mitochondria (Lapidus and Sokolove, 1994; Yu *et al.,* 2016). This is achieved by inhibition of the inner membrane permeability transition through selective prevention of the opening of the high-conductance state of the pore (Elustondo *et al.,* 2015) Given the fact that mitochondria are delicate organelles, their isolation should be carried out with care and maintained at 4^0C. This experiment was carried out to ascertain the integrity of the mitochondria by maintaining low temperature and pH of the homogenate in order to prevent activation of proteases and phospholipases.

PROCEDURE

The rat liver mitochondria were isolated from normal healthy male Wistar albino rats essentially according to the method of Johnson and Lardy, (1967), as a described on pages 73 and 74. Briefly, the animals were sacrificed by cervical dislocation and the livers were rapidly excised and trimmed to remove excess tissue. The livers were homogenized and mitochondria isolated by differential centrifugation. The protein content of the mitochondria was determined according to the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as standard. Mitochondrial permeability transition was determined as described on page 79. In summary, mitochondrial swelling in the absence of a triggering agent (Ca^{2+}) involved preincubation of the mitochondria subsequently in the presence of MSH buffer (swelling buffer) and 0.8µM rotenone for $3^1/_2$ minutes after which 5mM succinate was added to energize the reaction**.** Thereafter, 4mM spermine was added immediately following addition of calcium and rotenone and followed by the addition of mitochondria. This is to ascertain if the integrity of the membrane after induction by the triggering agent (calcium).

In the presence of a triggering agent (Ca^{2+}), preincubation of the mitochondria was carried out in the presence of MSH buffer (swelling buffer) and 0.8µM rotenone for 3minutes after which 120mM $CaCl_2$ was added. Inhibition of calcium-induced opening in the presence of spermine involved pre-incubation of mitochondria in the presence of MSH buffer, 0.8µM rotenone and 4mM spermine for 3 minutes after which Ca^{2+}, the triggering agent was added and 30 seconds later, 5mM succinate was added. Mitochondria permeability transition was quantified at 540nm for 12 minutes at 30 seconds interval in M106 spectrophotometer. A significant decrease in absorbance of the liver mitochondria is an indication of permeability transition.

RESULTS

The data presented in Figure 12 showed that there was no significant change in the volume of intact mitochondria respiring on succinate in the presence of rotenone over a period of twelve minutes. As seen from the figure, addition of calcium caused highly significant increases (10.8folds) in mitochondria swelling which was almost totally reversed by spermine, a standard inhibitor of the opening of the mPT pore, thus showing that the mitochondria used in this investigation were intact.

CONCLUSION

Calcium induced mitochondrial permeability transition (mPT) pore opening in isolated rat liver while spermine inhibited this inductive effect. This indicated that the mPT pore of the control animal was intact and not uncoupled *ab initio*, therefore suitable for further use.

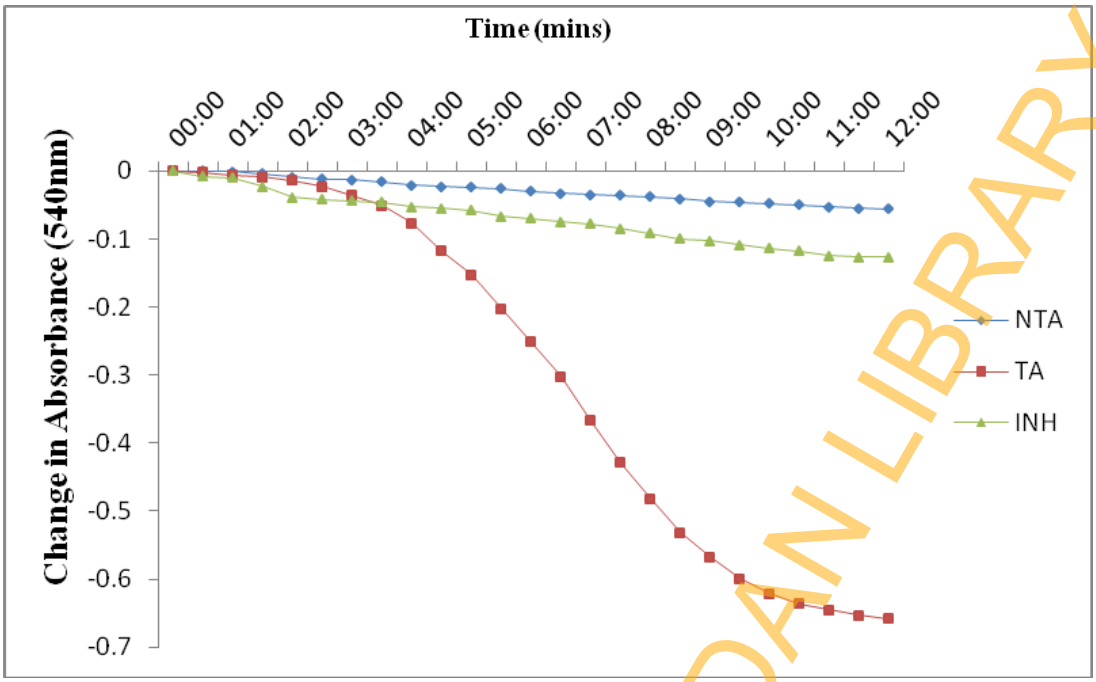


Figure 12: Calcium-induced opening of mitochondrial membrane permeability transition pore in normal liver mitochondria respiring in the presence of succinate-rotenone and its inhibition by spermine. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

EXPERIMENT 3: EFFECTS OF SOLVENT FRACTIONS OF *CALLIANDRA PORTORICENSIS* ROOT BARK ON RAT LIVER MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN THE ABSENCE OF CALCIUM

INTRODUCTION

Mitochondria are cellular organelles that regulate commitment to and execution of apoptosis. The intrinsic apoptotic pathway culminates in the permeabilization of the mitochondrial outer membrane and dismantling of the cell. Apoptosis of cancer cells is a favourable outcome when administering chemotherapeutic treatment. However, drug resistance and systemic toxicity has increased search for alternatives (Fernald and Kurokawa, 2013 ; Millimouno *et al.*, 2014). Unlike pharmaceutical drugs, selected natural compounds induce apoptosis by targeting multiple cellular signaling pathways including modulation of mitochondrial membrane permeability transition. Examples in this regard include betulinic acid and resveratrol (Martin, 2006; (Millimouno *et al.*, 2014). Hence, the aim of this experiment was to determine the effect of the fractions of the extract of *C.portoricensis* on mitochondrial permeability transition.

PROCEDURE

Low ionic strength mitochondria were isolated from normal healthy male Wistar strain albino rats using the method of Johnson and Lardy (1969) according to the method described on page 69. Mitochondrial swelling assay was carried out as described on pages 79.

RESULTS

The effects of various concentrations of methanol extract of *C. portoriecnsis* (MECP) on mPT pore in the absence of calcium are shown in Figure 13. According to the results, addition of different concentrations of the methanol extract (10 µg/mL,20 µg/mL,40 µg/mL and 60 µg/mL) to succinate–energized mitochondria resulted in highly significant opening of the mPT pore by by 1.1, 2.8, 4.5, 13.8 folds, respectively when compared with control. In this regard the effect was concentration-dependent. Maximum induction (13.8 folds) was obtained at the highest concentration of the extract (60 µg/mL) which was greater than the induction of pore opening caused by calcium (10.8folds)

Figure 14 shows the effects of chloroform fraction on mPT pore in the absence of calcium. Addition of different concentrations (10-120 µg/mL) of chloroform fraction (CFCP) has no effect whatsoever on the mPT pore in the absence of calcium. Similarly, ethylacetate fraction (EFCP) had no effect on the mPT pore at all concentrations tested (10-120 µg/mL) as shown in Figure 15. However, addition of different concentrations of methanol fraction (MFCP) to succinate-energized mitochondria resulted in significant opening of the mPT pore by 2.7, 3.9, 9.7, 11.2, 12.5, 14.5, 15.1 folds at 10, 20, 40, 60, 80, 100, and 120 µg/mL). The inductive effect (11.2, 12.5, 14.5, 15.1 folds) observed at higher concentrations (≥ 60 µg/mL) were greater than the induction of pore opening caused by calcium alone (10.8 folds) (Figure 16).

Overall, the pattern of induction by the various concentrations of the extract was concentration-dependent.

CONCLUSION

Of all the fractions of crude methanol extract of *C.portoricensis*, methanol fraction (MFCP) induced mitochondrial permeability transition pore opening in a concentration-dependent manner.

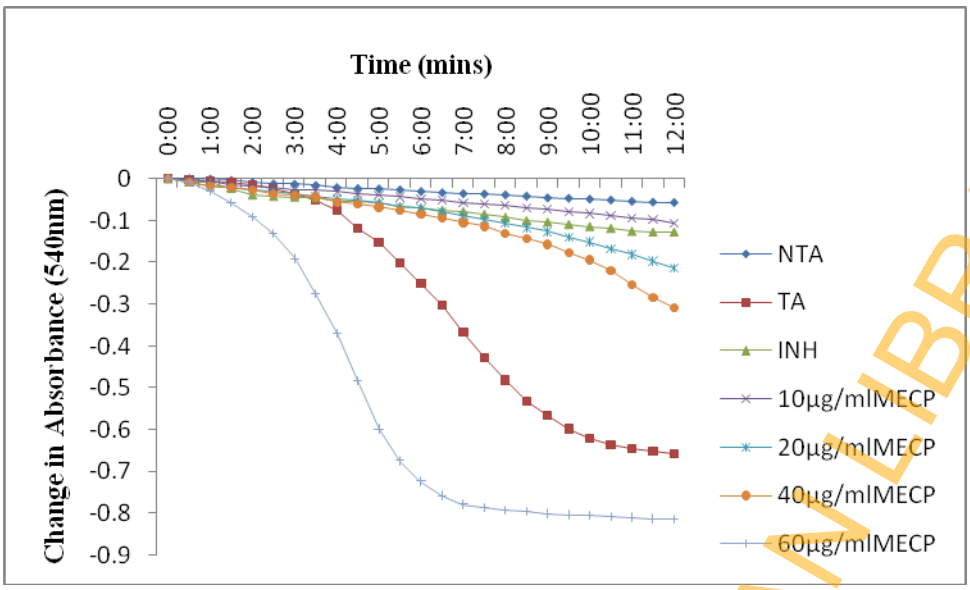


Figure 13: Effects of various concentrations of methanol extracts of *C. portoricensis* (MECP) on the mitochondrial membrane permeability transition pore in the presence of succinate but with no triggering agent, calcium. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

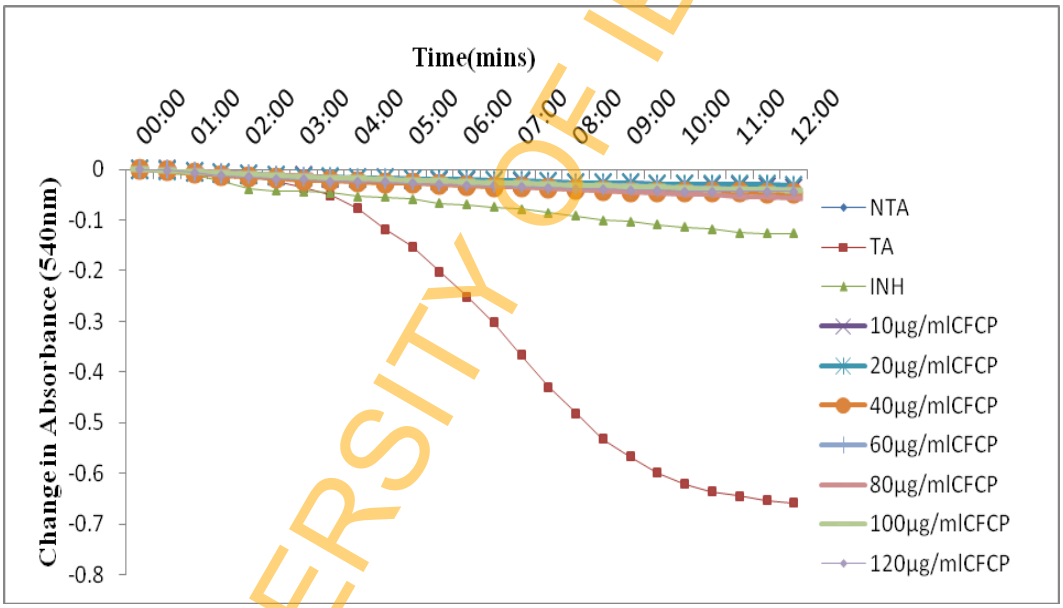


Figure 14: Effects of various concentrations of chloroform fraction of *C. portoricensis* (CFCP) on the mitochondrial membrane permeability transition pore in the absence of calcium. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

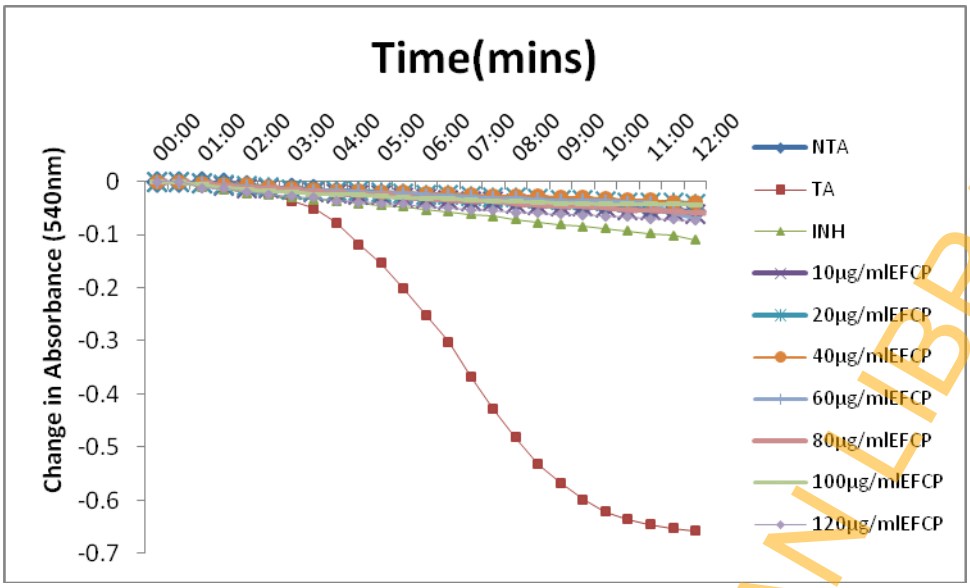


Figure 15: Effects of various concentrations of ethylacetate of *C. portoricensis* (EFCP) on the mitochondrial membrane permeability transition pore in the absence of calcium. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine).

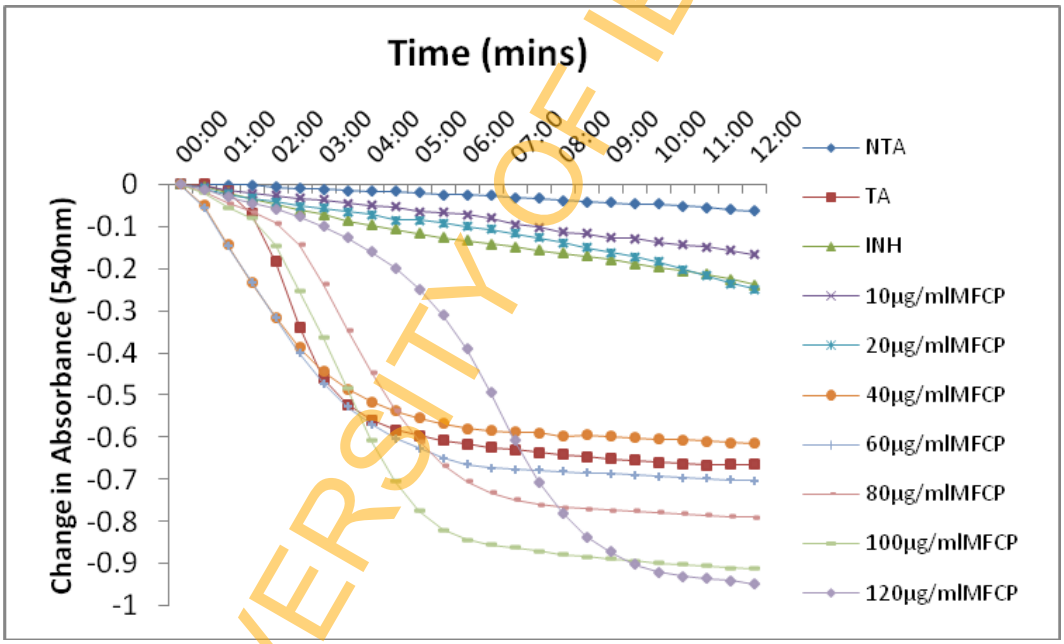


Figure 16: Effects of various concentrations of methanol fraction of *C. portoricensis* (MFCP) on the mitochondrial membrane permeability transition pore in the absence of calcium. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine).

EXPERIMENT 4: EFFECTS OF SOLVENT FRACTIONS OF *CALLIANDRA PORTORICENSIS* ROOT BARK ON RAT LIVER MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN THE PRESENCE OF CALCIUM.

INTRODUCTION

Dysregulation of Ca^{2+} homeostasis has long been implicated to play an important role in cell injury. Many chemicals and radicals promote the mPT. Typically the .effect of such inducers is to decrease the threshold amount of Ca^{2+} needed to cause mPT pore opening. In pathological settings where the mPT contributes to cell killing, Ca^{2+} may have several roles. First, increased Ca^{2+} alone and its uptake into mitochondria may cause mPT onset. Second, other stressors may decrease the threshold for the Ca^{2+}-induced mPT such that Ca^{2+} need not change but is still permissive for mPT onset. Lastly, stressors and increased Ca^{2+} may act synergistically to induce the mPT (Lemasters *et al.*,2009) The response of the mitochondria to addition of calcium depends on the amount of calcium added and the level of mitochondrial calcium. Nicholls (1978) first reported that mitochondria would buffer extramitochondrial calcium at pCa of about 6.1 until the calcium in the matrix reached a level of ~60nmol Ca/mg protein. He showed that when calcium was added to isolated mitochondria they would accumulate extramitochondrial Ca until they came to this set point and that, when EGTA was added to lower calciun below that set point, mitochondria would release calcium (via a Ca efflux pathway such a Na-Ca or H-Ca exchanger) and return to the same extramitochondrial calcium set point **.** The aim of this experiment was to determine the effect of fractions of the *C. portoricensis* on mitochondrial permeability transition on addition of extra mitochondrial calcium.

PROCEDURE

Mitochondrial suspension was incubated for 3 minutes in a glass cuvette at 30^0C before the addition of calcium, a standard triggering agent. 5mM sodium succinate was added 30 seconds after to energize the mitochondria. The light scattering effect was measured at 540nm over a period of 12 minutes at 30 seconds intervals in a CamSpec M106 spectrophotometer.

RESULTS

Figure 17 shows the pattern of inductive effect of varying concentrations of MECP on calcium-induced opening of the pore. Addition of calcium further increased the opening of the pore. In this regard, MECP showed a maximum inductive effect of 16.5 folds at 60 µg/mL and minimum inductive effect of 14.3 folds at 10 µg/mL while the triggering agent gave a 10.8 fold increase in mPT pore opening.

However, addition of calcium had no significant effect on the mPT pore of mitochondria treated with CFCP as represented in Figure 18. The effects of ethylacetate fraction on mPT pore in the presence of calcium are shown in Figure 19. Again, EFCP had no effect whatsoever on calcium-induced opening of the mPT pore at all concentrations tested.

Figure 20 shows the effect of MFCP on calcium-induced opening of the mPT pore. According to the results, MFCP potentiated calcium–induced opening of the pore. In this regard, the maximum inductive effect of 13.7 folds was obtained at the highest concentration (120 µg/mL) while a minimum inductive fold of 2.7 was obtained at 10 µg/mL. Calcium alone had an inductive fold of 10.5. Taken together, pattern of inductive effect was also concentration- dependent.

CONCLUSION

MECP and MFCP potentiated calcium- induced opening of the pore while EFCP and CFCP had no effect whatsoever at all concentrations tested.

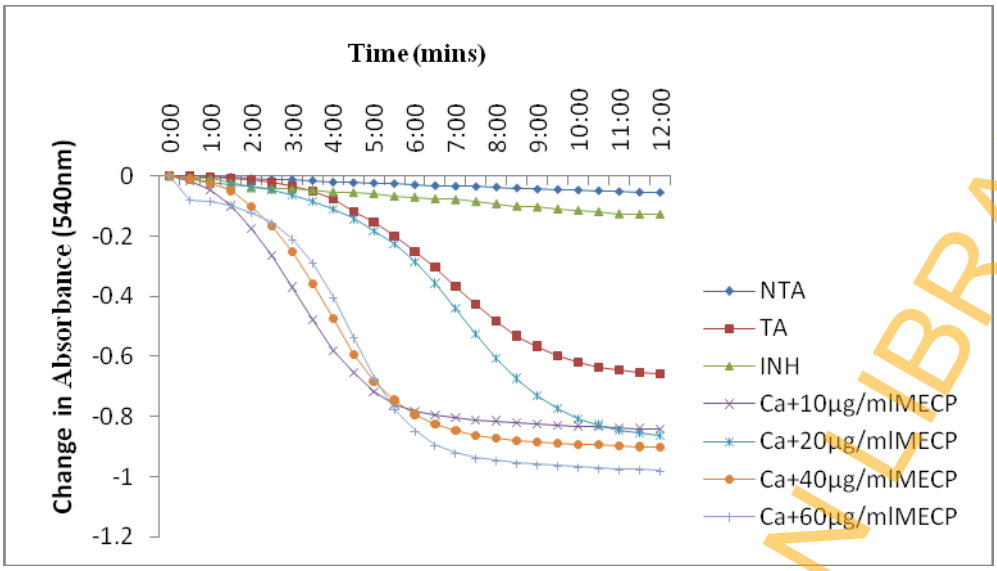


Figure 17: Effects of various concentrations of methanol extracts of *C. portoricens*is (MECP) on calcium-induced opening of mitochondrial membrane permeability transition pore.
NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

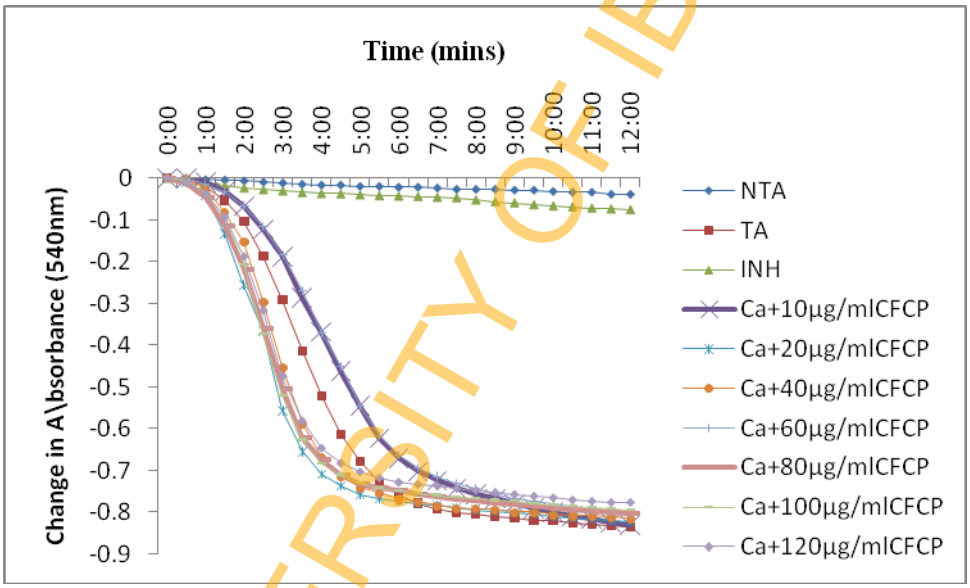


Figure 18: Effects of various concentrations of chloroform fraction of *C. portoricensis* (CFCP) on the mitochondrial membrane permeability transition pore in the presence of calcium.
NTA: No triggering agent TA: Triggering agentINH: Inhibitor (spermine)

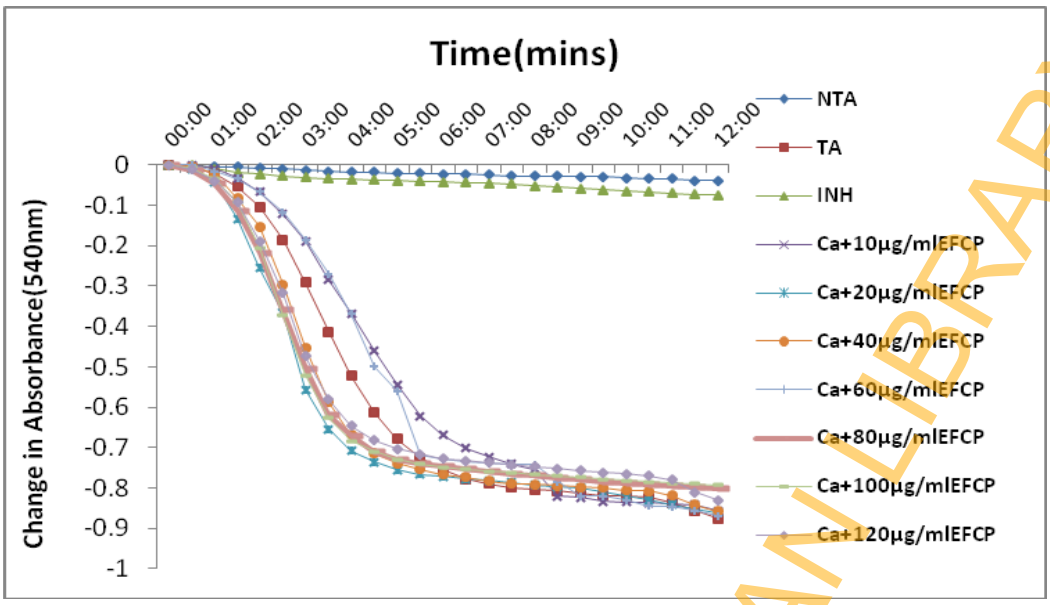


Figure 19: Effects of various concentrations of ethylacetate fraction of *C. portoricens*is (EFCP) on calcium-induced opening of mPT pore. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

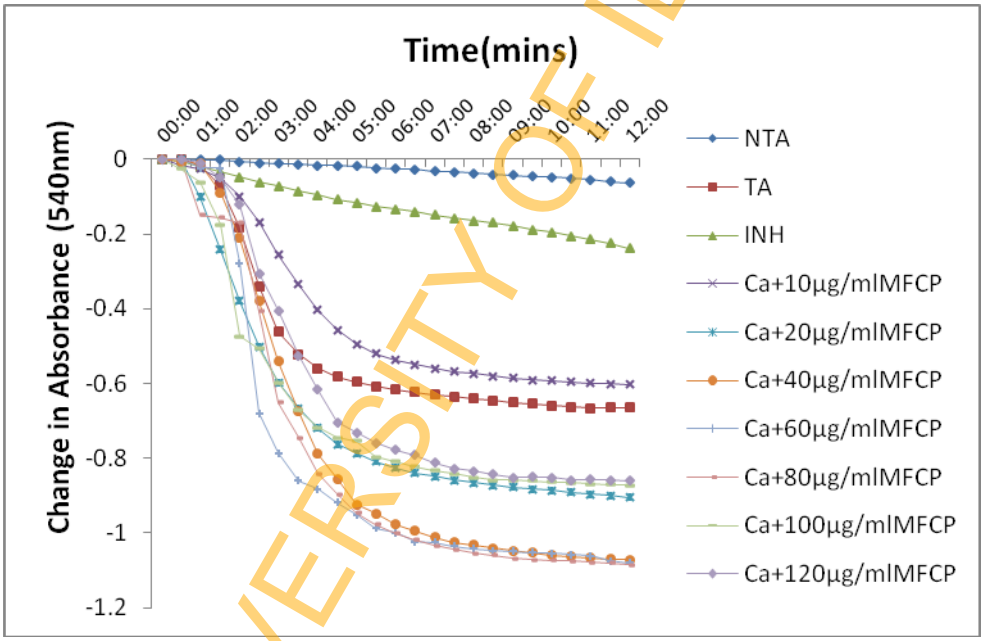


Figure 20: Effects of various concentrations of methanol fraction of *C. portoricens*is (MFCP) on calcium-induced opening of mPT pore. NTA: No triggering agent TA: Triggering agent INH: Inhibitor (spermine)

EXPERIMENT 5: EFFECTS OF SPERMINE ON METHANOL FRACTION OF *C. PORTORICENSIS* – INDUCED OPENING OF MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN THE PRESENCE OF CALCIUM.

INTRODUCTION

The mitochondrial permeability transition (mPT) pore is a large channel of the mitochondrial inner membrane, the opening of which is the central event in many types of stress-induced cell death. The mPT pore opening is induced by elevated concentrations of mitochondrial calcium (Elustondo *et al*., 2015). It is inhibited not only by cyclosporin A (CsA), a cyclic undecapeptide which acts by binding cyclophilin D (CypD), universally considered to be a regulatory component of the mammalian mPT pore complex (Broekemeier *et al.*,1989 ; Gutiérrez-Aguilar *et al.*, 2014) but also by Mg^{2+}, ADP, ATP and low pH . It has been demonstrated that spermine and other polyamines can delay calcium-induced swelling of isolated mitochondria, suggesting that their role as inhibitors of the mPT pore opening is reversible on both a short (ms) and a long (seconds) time scale, as shown directly by electrophysiological observation and as well as indirectly by monitoring mitochondrial polarization and tracer fluxes (Al-Nasser and Crompton, 1986**;** Blanchet *et al.*, 2014). The aim of this experiment was to determine the effect of spermine, a standard inhibitor of pore opening on the potent methanol fraction–induced opening of the mPT pore in the presence of calcium.

PROCEDURE

Mitochondrial swelling involved preincubation of the mitochondria in the presence of MSH buffer (swelling buffer), 0.8μM rotenone for 3 minutes before addition of methanol fraction and calcium after which 5mM succinate was added to energize the reaction.

Addition of 4mM spermine was done immediately following addition of rotenone and just before the addition of mitochondria.

RESULTS

Figure 21 shows the effects of spermine on methanol fraction –induced opening of the pore in the presence of spermine. As seen from the results, MFCP induction of mPT pore opening was reversible by spermine in the presence of calcium at all concentrations tested. This is in line with what obtains in calcium-induced opening, which is also reversible by spermine.

CONCLUSION

Like calcium-induced opening, MFCP-induced pore opening was reversible by spermine

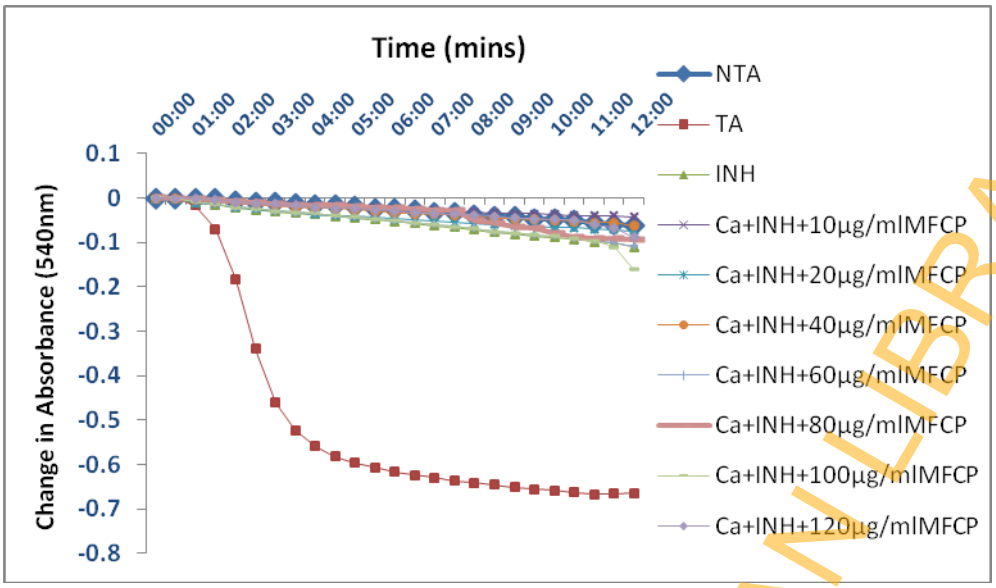


Figure 21: Effects of spermine on methanol fraction-induced opening of mitochondrial membrane permeability transition pore in the presence of calcium

NTA- Non-triggering agent TA- Triggering agent (calcium) INH- Inhibitor (spermine)

EXPERIMENT 6: EFFECTS OF SOLVENT FRACTIONS OF *CALLIANDRA PORTORICENSIS* ROOT BARK ON MITOCHONDRIAL ATPase ACTIVITY

INTRODUCTION

The human mitochondrial (mt) ATP synthase, or complex V (EC 3.6.3.14) is the 5th multi subunit oxidative phosphorylation (OXPHOS) complex (Schapira, 2006). It uses the energy created by the proton electrochemical gradient to phosphorylate ADP to ATP) (Zeviani and Donato, 2004). Intact mitochondrial coupling membrane is a prerequisite for formation of the proton motive force required by the ATP synthase for production of ATP. Inner mitochondrial membrane permeabilization often results in destruction of membrane potential and enhancement of mitochondrial ATPase activity (Dietze *et al.*, 2001). The permeability of the inner mitochondrial membrane uncouples oxidative phosphorylation, causing the proton-translocating ATPase to reverse direction and so actively hydrolyse ATP. Under such conditions, intracellular ATP concentrations rapidly decline, leading to the disruption of ionic and metabolic homeostasis (Halestrap *et al.*, 1998; Crompton, 1999). Many chemo-preventive agents have been shown to induce apoptosis by directly inhibiting mitochondrial respiration (Hail and Lotan, 2004). This event encourages the hydrolysis of ATP rather than its synthesis. In order to ascertain that status of the mPT pore of mitochondria treated with different fractions of *C. portoricensis,* this experiment was designed to assess the effect of the solvent fractions of *C. portoricensis* on mitochondrial ATPase activity using concentration of inorganic phosphate released as an index of enhancement and 2,4 dinitrophenol as a classical inducer of ATPase activity.

PROCEDURE

Mitochondria were isolated from rat liver in 0.25M sucrose by differential centrifugation as earlier described on pages 73 and 74. Mitochondria ATPase activity was assayed by the method of Lardy and Wellman (1953) as described on pages 80-82 and as modified by Olorunsogo and Bababunmi (1979) by measuring the rate of release of inorganic phosphate during the bydrolysis of ATP.

In each test tube for the reaction, 65mM Tris-HCl (pH 7.4), 0.5mM KCl, 1mM ATP and 25mM sucrose was added in a total volume of 2ml. Distilled water or test compound or dinitrophenol was added accordingly. The reaction was started by the addition of the mitochondrial fraction (0.5mg protein) and allowed to proceed for 30 minutes at 27^0 C with constant shaking. The reaction was stopped with the addition of 8ml of 10 percent trichloroacetic acid to the test tube content. The mixture was then centrifuged in an MSE centrifuge at low speed and the supernatant was used for phosphate determination. The resulting solution was allowed to stand for 20 minutes and absorbance measured spectrophotometrically at 660nm. The blank (zero time tube) was prepared by adding the solution of ATP after the additionof 8ml of a solution of 10% trichloroacetic acid. Mitochondrial protein was determined by the Lowry method. The experiments were carried out in triplicates.

RESULTS

The effects of different solvent fractions on mitochondrial F_0F_1 ATPase activity are shown in Figure 22. Mitochondria ATPase activity was enhanced by methanol extract (MECP) in a concentration –dependent manner. A maximum enhancement of 2.6 fold was observed at 100 µg/mL. This enhancement was close to that of 2, 4 DNP, a standard uncoupler of oxidative phosphorylation. Similarly, potent methanol fraction (MFCP) elevated the hydrolysis of ATP to ADP and inorganic phosphate maximally with 3.7 folds at 100 µg/mL. Levels of inorganic phosphate released were also close to that of 2, 4-DNP, a standard uncoupler. Conversely, ethylacetate (EFCP) and chloroform fractions (CFCP) did not show significant enhancement of ATPase activity at all concentrations tested.

CONCLUSION

MECP and MFCP enhanced latent mitochondrial($F_0F_{1)}$ ATPase activity.

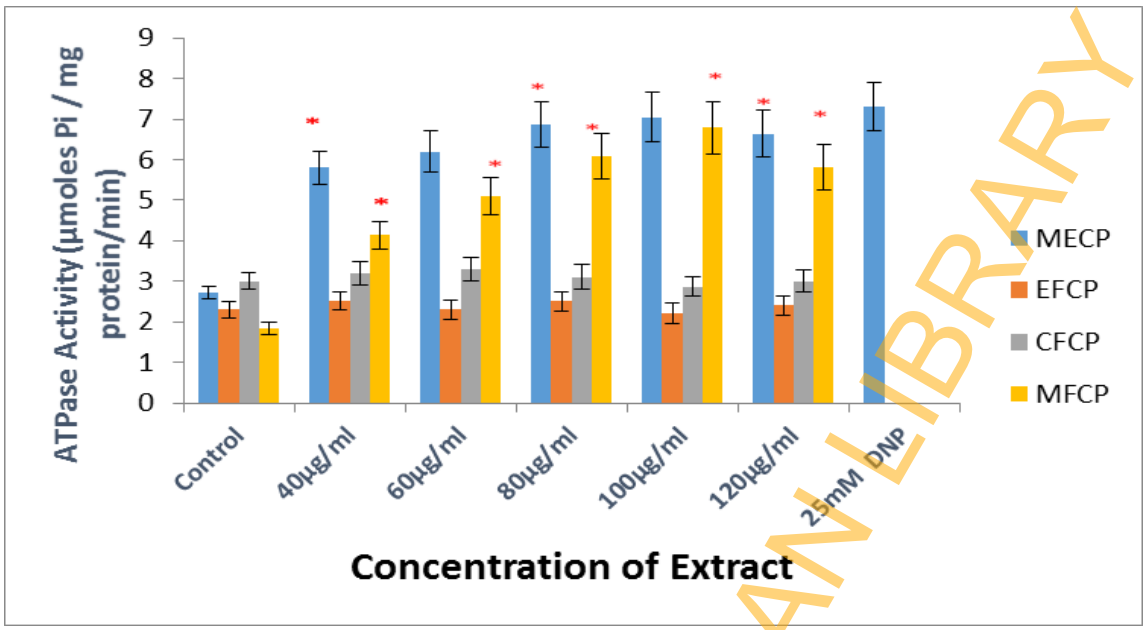


Figure 22: Effect of fractions of Methanol extract of *Calliandra portoricensis* on mitochondrial ATPase activity.

EXPERIMENT 7: EFFECTS OF SOLVENT FRACTIONS OF THE ROOT BARK OF *CALLIANDRA PORTORICENSIS* ON Fe^{2+}-INDUCED MITOCHONDRIAL LIPID PEROXIDATION

INTRODUCTION

Natural compounds including flavonoids are able to reduce oxidative stress, one of the mechanisms through which they mediate protective effects against cancer development. In addition, *in vitro* and *in vivo* studies have suggested that flavonoids, such as epigallocatechin-3-gallete (EGCG), quercetin, and curcumin, act by induction of apoptosis. Several natural compounds inhibit cell proliferation and angiogenesis (Hail, 2005 ; Kuno *et al.,* 2012).

In fact, numerous phenolic compounds have been shown to display anti-proliferative and cytotoxic effects towards several tumor cells, presenting toxic effects that specifically target cancer cells rather than normal cells (Lepley *et al.*, 1996 ; Agullo *et al.,* 1997, Scalbert *et al.,* 2005). The aim of this experiment was to determine the effects of fractions of *Calliandra portoricensis* on Fe^{2+} - induced lipid peroxidation.

PROCEDURE

Mitochondrial lipid peroxidation was determined by thiobarbturic acid reactive species (TBARS) assay as described by Ruberto *et al.,* (2000) to measure the rate of mitochondrial lipid peroxide formed in the assay medium. Liver mitochondria (1mg/ml) was added to varying concentrations of the fractions of C. *portoricensis* in test tubes. Iron sulphate (0.07M) was added to induce lipid peroxidation and the mixture incubated for 30 minutes at 37^0C. Then, 20% acetic acid and 0.8% thiobarbituric acid in 1.1% sodium dodecyl sulphate (SDS) was added and the resulting

mixture vortexed and then heated at 95^0C for 60 minutes. After cooling, butan-1-ol was added to the resulting mixture and centrifuged at 3000 rpm for 10 mins. The absorbance of the upper organic layer was measured at 532nm**.**

RESULTS

The effects of fractions of *C. portoricensis* on Fe^{2+}- induced lipid peroxidation are shown in figure 23. As seen from the figure, all fractions of *C.portoricensis* inhibited the generation of mitochondrial lipid peroxides. In this regard, methanol extract (MECP), chloroform (CFCP), ethylacetate fraction (EFCP) and methanol fraction (MFCP) inhibited Fe^{2+}-induced lipid peroxidation maximally at 800 µg/mL with percentage inhibition of 90%, 59% , 41% and 93%, respectively. From the results, MFCP strongly inhibited generation of lipid peroxides as well as MECP. CFCP showed moderate inhibition while EFCP had the least potency. Overall, the effect of the fractions on inhibition of mitochondrial lipid peroxidation was concentration-dependent.

CONCLUSION

All fractions of *C.portoricensis* inhibited iron -induced intra-mitochondrial peroxide production.

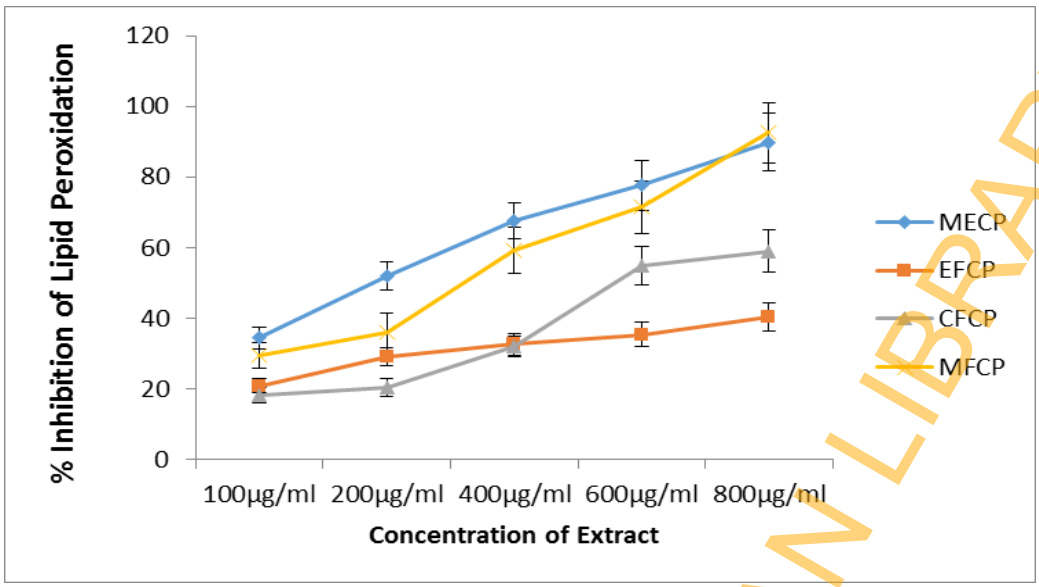


Figure 23: Inhibition of Fe^{2+} - induced lipid peroxidation by fractions of methanol extract of *C. portoricensis* root bark

EXPERIMENT 8: *IN VIVO* EFFECTS OF VARIOUS DOSES OF METHANOL FRACTION (MFCP) ON RAT LIVER MITOCHONDRIAL PERMEABLILITY TRANSITION PORE IN THE ABSENCE AND PRSENCE OF CALCIUM

INTRODUCTION

Epidemiological studies indicate that dietary habits contribute to at least one third of all human cancers (Willett, 1995). This suggests that certain dietary components can exacerbate or interfere with carcinogenesis. Apoptosis is likely to be a crucial mechanism in the chemopreventive properties associated with such dietary factors (Watson *et al.*, 2000). Food contains several promising chemopreventive compounds (Riboli and Norat, 2003; Stan *et al.*, 2008) . Bioavailability of individual compounds of interest at the target site is one of the important challenges/parameters to determine the therapeutic efficiency of the target drug (Aqil *et al.*, 2013). Given the observed effects of methanol fraction in *vitro*, it was considered pertinent to determine the effects of various doses of MFCP *in vivo*.

PROCEDURE

Healthy untreated rats (control) having a mean weight of 100g were purchased from the Animal house of Faculty of Basic Medical Sciences, University of Ibadan. They were allowed to acclimatize for one week. They were fed *ad libitum* with water and rat chow throughout the period of the experiment. The animals were divided into groups and were orally administered with MFCP for 21days and 30 days.

Dose Regimen:

Control: received distilled water

25mg/kg: received 25mg/kgbw of MFCP

50mg/kg: received 50mg/kgbw of MFCP

100mg/kg: received 100mg/kgbw of MFCP

200mg/kg: received 200mg/kgbw of MFCP

400mg/kg: received 200mg/kgbw of MFCP

For oral administration of MFCP for 30 days, the highest dosage group was 200mg/kg. This may be due to toxicity of the extract because no animal survived the 400mg/kg dose.

After the last day of administration, the animals were sacrificed by cervical dislocation and the livers excised, minced, homogenized and then centrifuged to isolate the low ionic strength mitochondria according to the method described by Johnson and Lardy (1967). The protein content of the mitochondria was determined according to Lowry *et al.*, (1951) using Bovine Serum Albumin as standard. Assay for mitochondrial swelling was carried out according to the method described on page 79.

RESULTS

The effect of calcium and spermine on mitochondria of control animals following oral administration of doses of methanol fraction for 21 and 30 days is shown in figure 24. There was no significant change in the volume of mitochondria respiring on succinate in the presence of rotenone in the mitochondria of control animals after oral administration of varying doses of methanol fraction for 21 and 30 days.

Figures 25 and 26 show the profile of changes in absorbance of mitochondria at various doses of MFCP following oral administration for 21 and 30 days, respectively in the absence of calcium. These results show that after twenty one (21) days of oral administration of MFCP, there was no significant effect on mPT pore in the absence of calcium at all doses administered. However, after 30 days of administration, MFCP induced mPT pore opening in the absence of calcium at doses of 100 and 200 mg/kg with induction folds of 2.6 and 3.3, respectively. However, there was no induction of mPT pore opening at lower doses of 25mg/kg and 50 mg/kg.

Figures 27 and 28 show the profile of changes in absorbance of mitochondria at various doses of MFCP following oral administration for 21 and 30 days respectively, in the presence of calcium. The results revealed that calcium potentiated mPT pore opening of rat liver at doses of 200 and 400mg/kg after 21 days of oral administration. In this regard, the induction folds of 10.6 and 10.9 were obtained at doses 200mg/kg and 400mg/kg, respectively while calcium gave a 10.5 fold increase. Oral administration of lower doses of 25, 50 and 100mg/kg had an inductive effect of 10.1, 10.2, 10.4 folds, respectively. Overall, the inductive effect was concentration-dependent.

After thirty days of administration, (Figure 28) the effect of mPT induction was greatly potentiated by MFCP in the presence of calcium at doses 100 and 200 mg/kg with induction folds of 17.6 and 19.0 respectively. Induction of mPT pore opening was also observed at doses 25 and 50mg/ kg with inductive folds of 16.8 and 17.0 while calcium gave a 17.1- fold increase.

CONCLUSION

MFCP induced the opening of the mPT pore at higher doses (100 and 200mg/kg) following administration for 30 days.

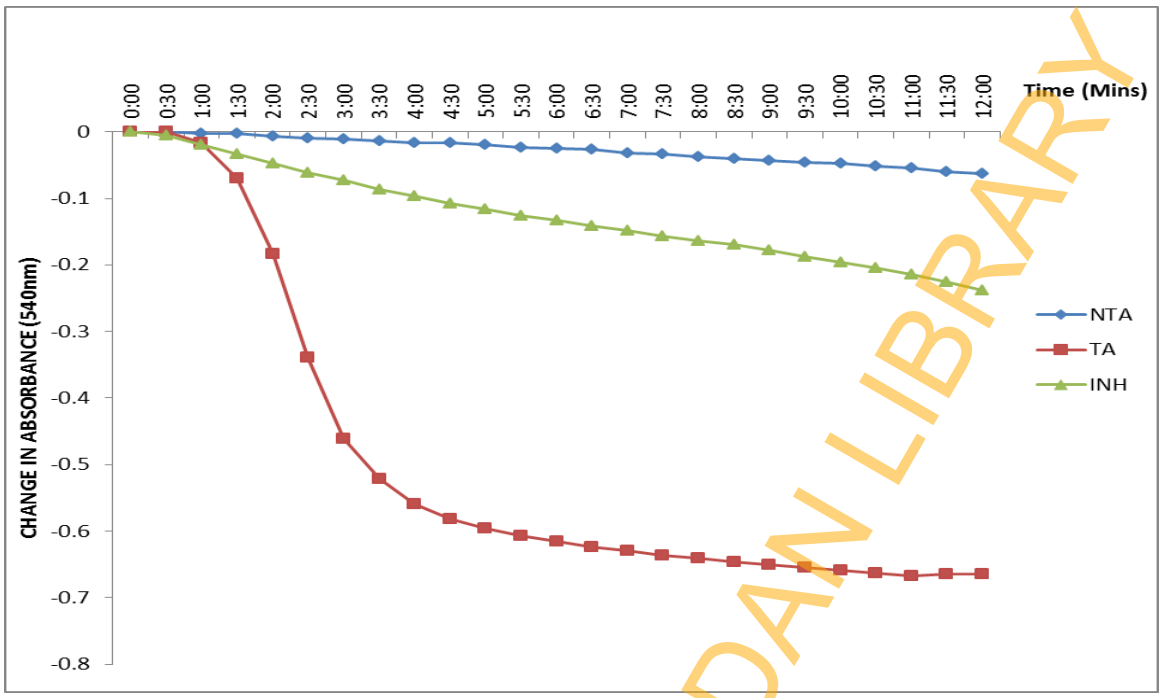


Figure 24: Change in absorbance (540nm) of reversal of calcium-ion induced mitochondrial permeability transition pore opening by Cyclosporine A (Control group – 21 & 30 days)

NTA- Non triggering agent TA-Triggering agent (Calcium) INH- Inhibitor-Spermine

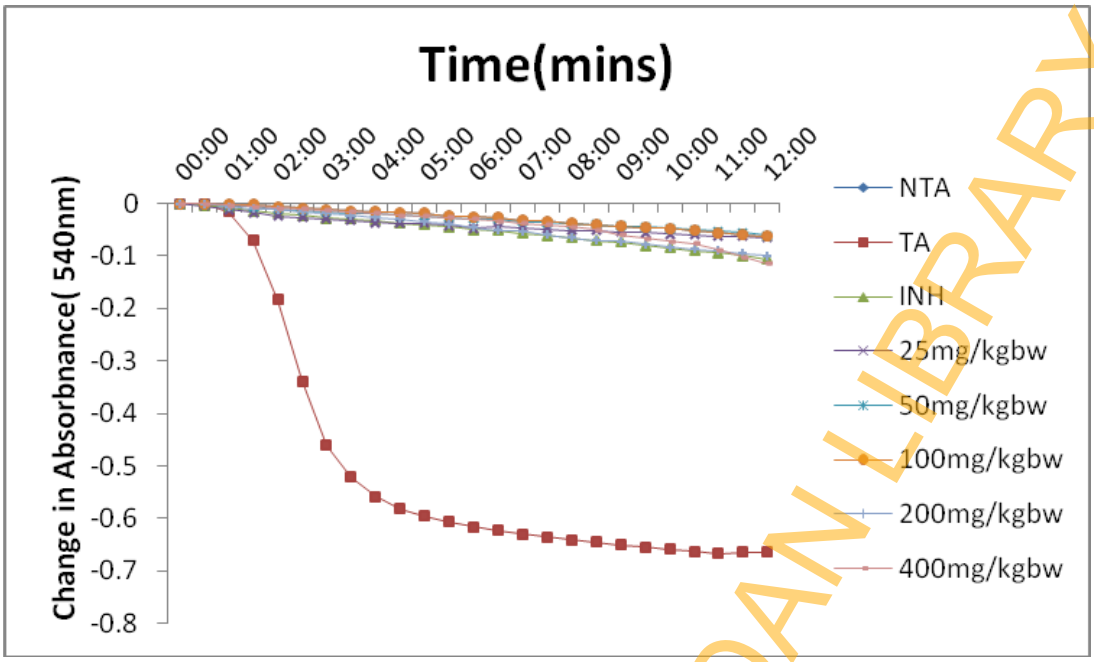


Figure 25: Change in absorbance of mitochondria at various doses (25, 50, 100, 200 and 400mg/kg) of MFC P (21days) over a period of 12 minutes in the absence of calcium

NTA- Non triggering agent TA-Triggering agent (Calcium) INH- Inhibitor-Spermine

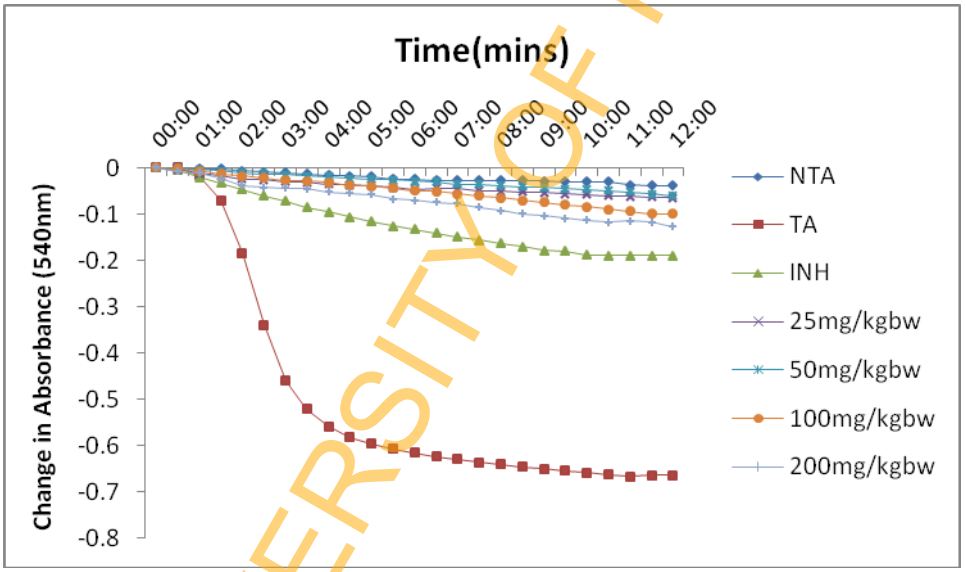


Figure 26: Change in absorbance of mitochondria at various doses (25, 50, 100 and 200mg/kgbw) of MFCP (30 days) over a period of 12 minutes in the absence of calcium

NTA- Non triggering agent TA-Triggering agent (Calcium) INH- Inhibitor-Spermine

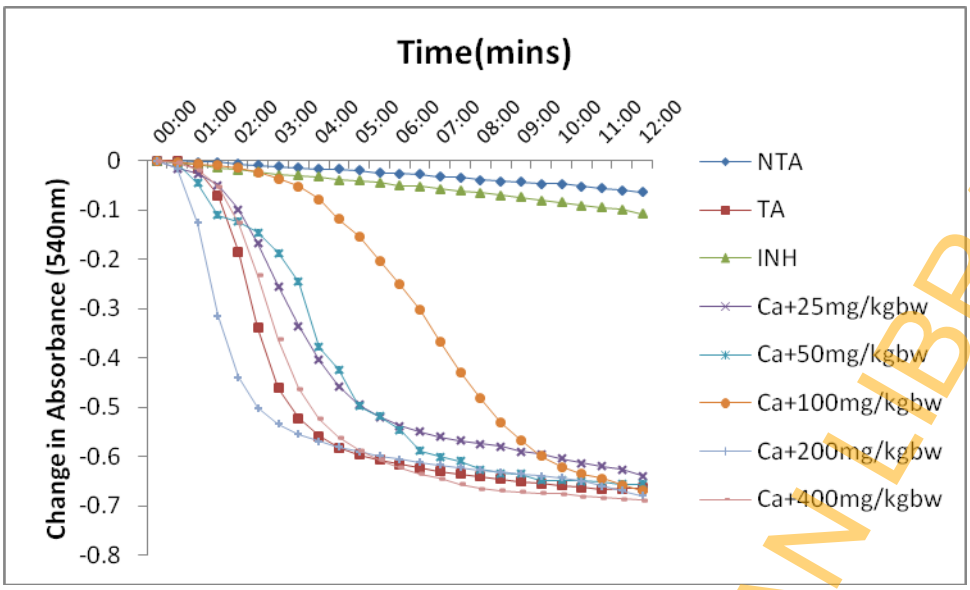


Figure 27: Changes in absorbance of mitochondria at various doses (25, 50, 100,200 and 400mg/kgbw) of MFCP (21 days) over a period of 12 minutes in the presence of calcium

NTA- Non triggering agent TA-Triggering agent (Calcium) INH- Inhibitor-Spermine

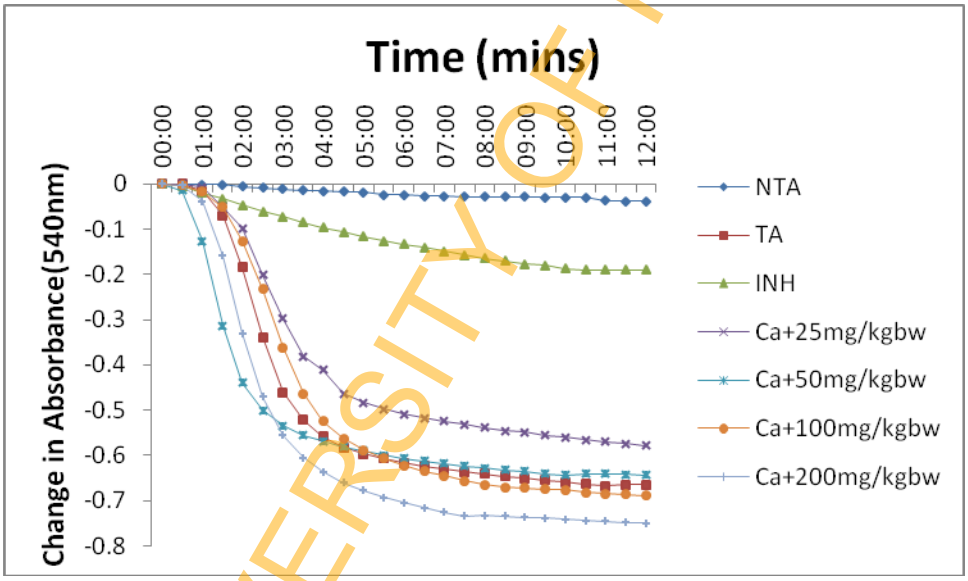


Figure 28: Changes in absorbance of mitochondria at various doses (25, 50, 100 and 200mg/kgbw) of MFCP (30 days) over a period of 12 minutes in the presence of calcium

NTA- Non triggering agent TA-Triggering agent (Calcium) INH- Inhibitor-Spermine

EXPERIMENT 9: EFFECT OF MFCP ON HEMATOLOGICAL, LIVER ENZYMATIC PROFILES AND HISTOLOGICAL PROFILES OF RATS AFTER TWENTY ONE AND THIRTY (21 & 30) DAYS OF ORAL ADMINISTRATION OF DOSES OF METHANOL FRACTION

INTRODUCTION

Aspartate aminotransferase (AST) is an enzyme found mainly in the heart and liver cells. It is released into the blood stream when the heart or liver is damaged. Increased levels may be associated with liver and heart disease. Alanine aminotransferase (ALT) is found predominantly in the liver and also in kidney, heart and skeletal muscle (Fujii, 1997). Generally most ALT elevations are caused by liver damage. Liver function are assumed by measuring the level of Alanine Aminotransferase (ALT) and Aspartate aminotransferase (AST). These transaminases are enzymes involved in transfer of an amino acid group from a 2-amino to a 2-oxoacid. Alanine aminotransferase (ALT) is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from alanine to α-Ketoglutarate to form pyruvate and glutamate. Significantly elevated levels of these enzymes often suggest the existence of other medical problems such as viral hepatitis, diabetes, congestive heart failure, liver damage, bile duct problems, infectious mononucleosis, or myopathy, (Travlos *et al.*, 1996).

White blood cells (WBC) play a vital role in the body's immune defense against diseases. The number of WBC may be reduced or increased depending on the disease condition or reaction occurring in the body which could be normal or abnormal. Microscopy is routinely relied upon as a primary endpoint measurement for epidemiological studies, intervention studies and clinical

trials. Complete blood counts, particularly WBC can be performed using new generation automated hematology analyzers (Bossche *et al*., 2007). Red blood cells (RBC) are concave shaped cells and are filled with Hemoglobin, the protein that transports oxygen and carbon dioxide throughout the body. The hematocrit is a measurement of the proportion of blood that is made of RBCs. The hemoglobin concentration is a measure of total amount of hemoglobin in the blood. This experiment was carried out in order to detect any toxicity that may be associated with oral administration of methanol fraction of *C. portoricensis.*

PROCEDURE

Alanine aminotransferase and aspartate aminotransferase were measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine using Randox Kit and absorbances were measured at 546nm (Reitman and Frankel, 1957). Blood samples were drawn from the experimental animals used for the mPT *in vivo* study with capillary tubes into EDTA bottles and immediately kept in the refrigerator to prevent lysis of the blood cells. The blood samples were then centrifuged at 3000rpm for 10 minutes in order to separate serum for the liver function test. Hematological parameters were obtained from the plasma of the experimental animals with the use of automated hematology analyzers.

RESULTS

The i*n vivo* effects of oral administration of MFCP (21days) on haematological parameters are shown in Figure 29. After 21 days of administration, the packed cell volume of the animals increased maximally at 50mg/kg when compared with control. Similar effects were also observed for white blood cell (WBC) and Red Blood Cell (RBC) counts as well as haemoglobin

(Hb) levels. A decrease in the levels of WBC, RBC, PCV and Hb were observed at doses higher than 50mg/kg.

Figure 30 shows the *in vivo* effect of oral administration of MFCP (30 days) on haematological parameters. Interestingly, there were significant declines ($P>0.05$) in the hematological parameters (PCV, WBC, RBC and Hb) at all the dosage groups (25, 50, 100 and 200mg/kgbw) when compared to the control group. Cell counts were adversely affected at the highest dose (200mg/kg) compared with control and other treatment groups (25, 50 and 100mg/kg).

The *in vivo* effect of oral administration of MFCP (21 days) on serum aminotransferases are depicted in Figure 31. According to the results, there were significant increases in the serum ALT and AST activity at 200 and 400 mg/kg dosage groups when compared to the control rats after twenty one days of administration. However, at all doses of methanol fraction administered (25, 50, 100, 200 mg/kg), serum AST and ALT levels were significantly elevated when compared with the control after 30 days of oral administration of MFCP. The data is depicted in Figure 32.

Photomicrographs of liver sections are depicted in Figure 33. As doses of methanol fraction administered increased, a moderate to severe diffuse vacuolar degeneration of hepatocytes was observed while control animals had very mild diffuse vacuolation.

Figure 34 shows the photomicrographs of kidney sections of wistar rats following oral administration of methanol fraction for 30 days. No visible lesions were observed in kidney of animals that received 25, 50 and 100mg/kg MFCP. However, protein casts were abundant in kidney of animals that received the highest dose of 200mg/kg.

Photomicrographs of prostate sections of animals orally administered methanol fraction for 30 days are shown in figure 35. As seen from the figure, glandular secretions were observed in the prostate of animals administered 50mg/kg and 100mg/kg while distortion of the entire gland was observed at the highest dose of 200mg/kg. In particular, at 50mg/kg, lumen of the glandular units appeared shrunken but the epithelial glands appeared normal while necrotic debris were observed in the prostate of animals that received the highest dose of 200mg/kg. No visible lesion was observed in the prostate of control animals.

CONCLUSION

MFCP altered levels of serum aminotransferases and haematological profiles at all the dosage groups administered with MFCP for 30 days. This suggests that highdoses of methanol fraction could be hepatotoxic.

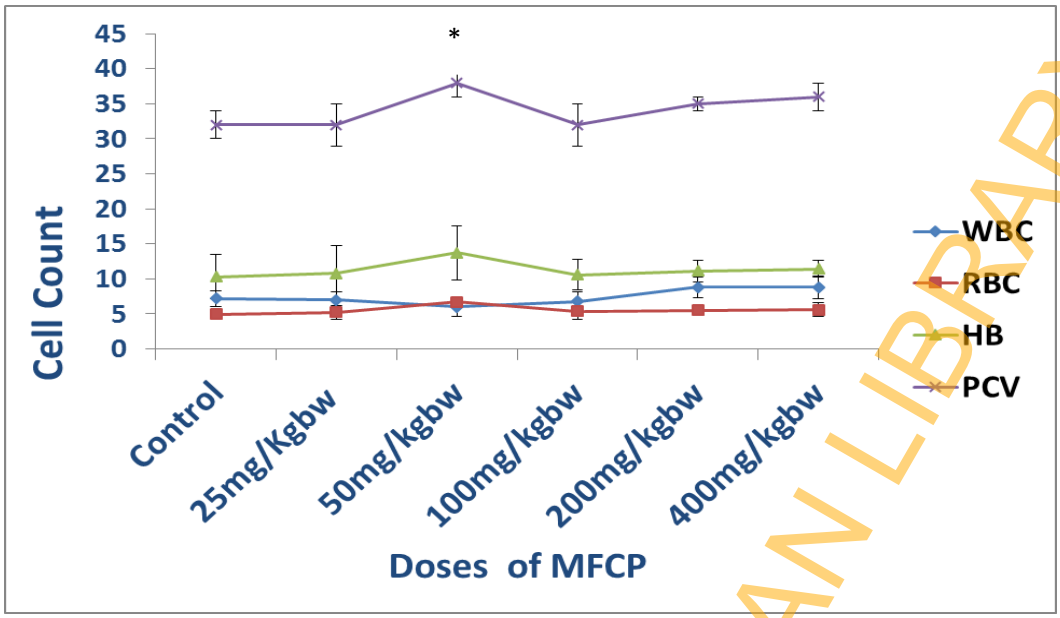


Figure 29: *In vivo* effect of oral administration of MFCP on Haematological parameters (21days)

ALT- Alanine aminotransferase AST- Aspartate aminotransferase

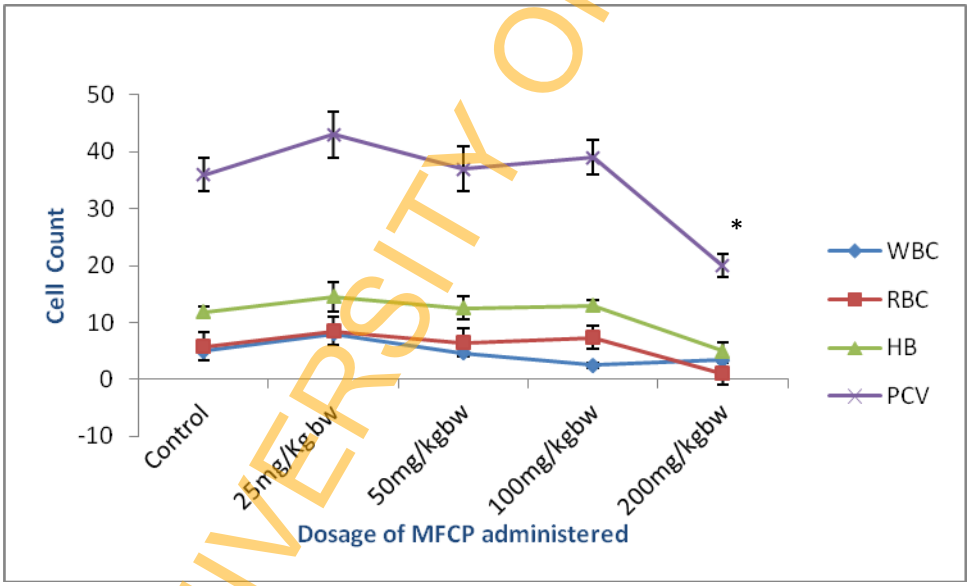


Figure 30: *In vivo* effect of oral administration of MFCP on Haematological parameters (30 days).

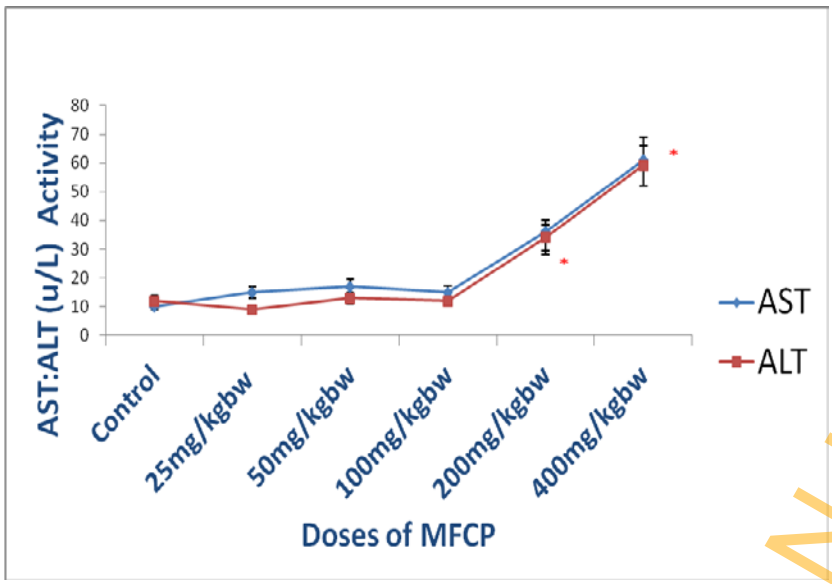


Figure 31: *In vivo* effect of oral administration of MFCP on serum aminotransferases (21 days)

ALT- Alanine aminotransferase AST- Aspartate aminotransferase

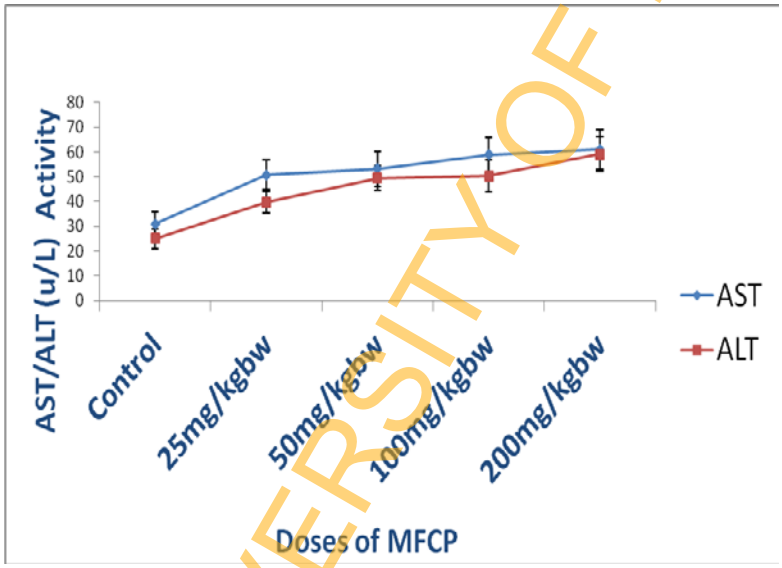


Figure 32: *In vivo* effect of MFCP on liver enzymatic profile (30 days)

ALT- Alanine aminotransferase AST- Aspartate aminotransferase

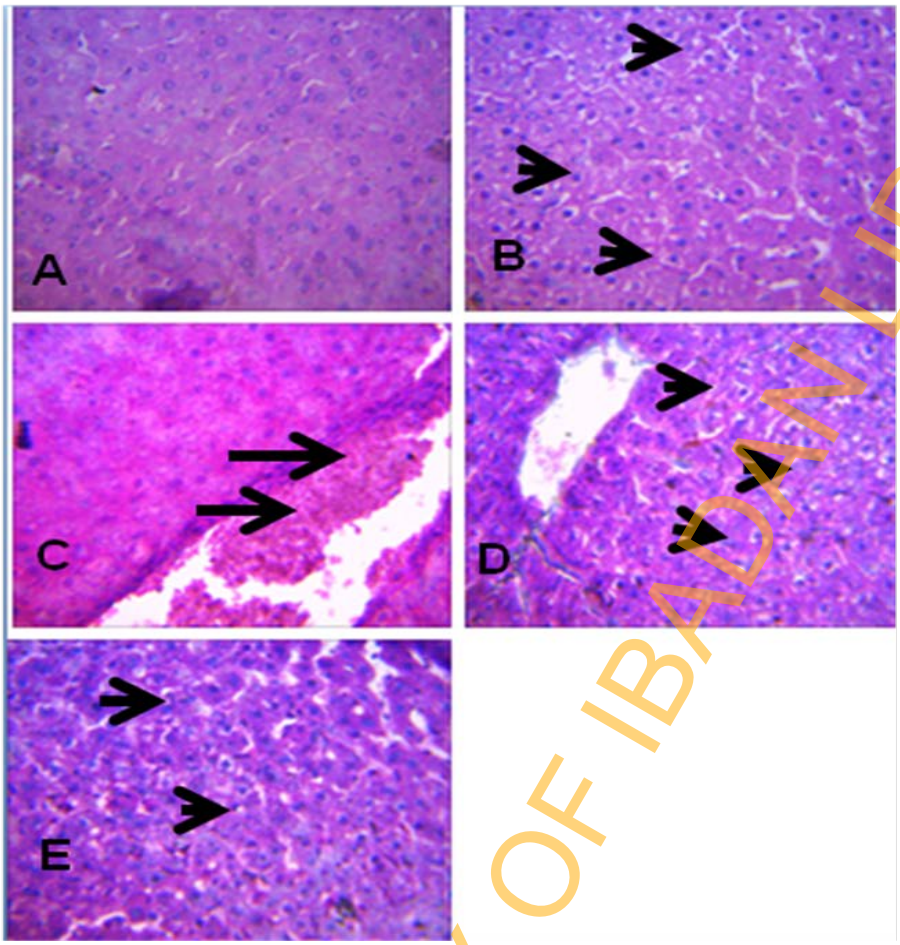


Figure 33: Photomicrographs of liver sections of animals orally administered MFCP for 30days. A- Control B-25mg/kgbw. C--50mg/kgbw. D – 100mg/kgbw E- 200mg/kgbw. Mag x400

A- Very mild diffuse vacuolation of hepatocytes B- Moderate vacuolar degeneration of hepatocytes C- Further vacuolation of hepatocytes D – Severe degeneration of hepatocytes

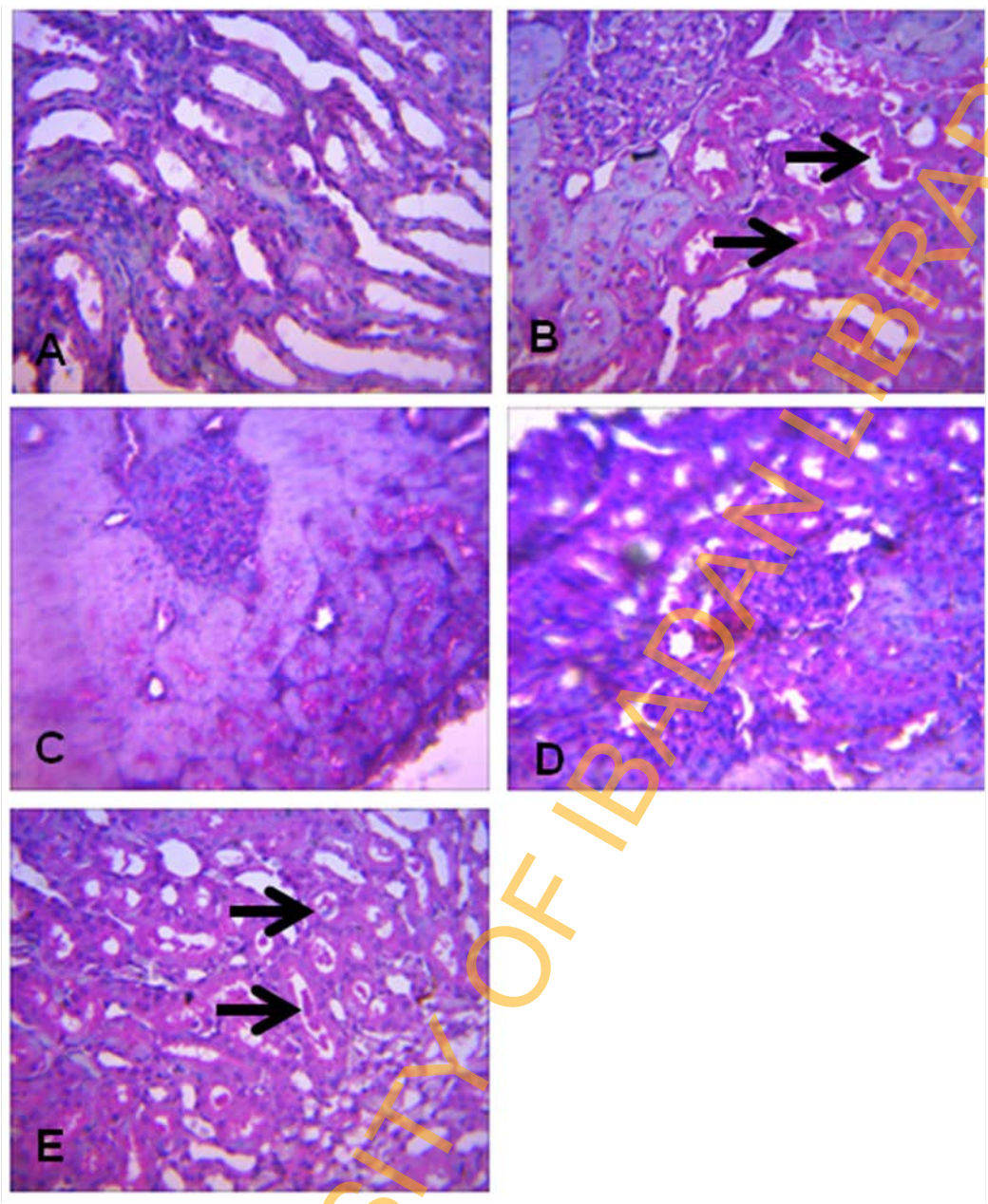


Figure 34: Photomicrographs of kidney sections of animals orally administered MFCP for 30days. A- Control B-25mg/kgbw . C-50mg/kgbw. D – 100mg/kg bw E- 200mg/kgbw. Mag x400

A-No visible lesions seen B – slightly few lesions observed C- No visible lesions D- Protein casts were observed

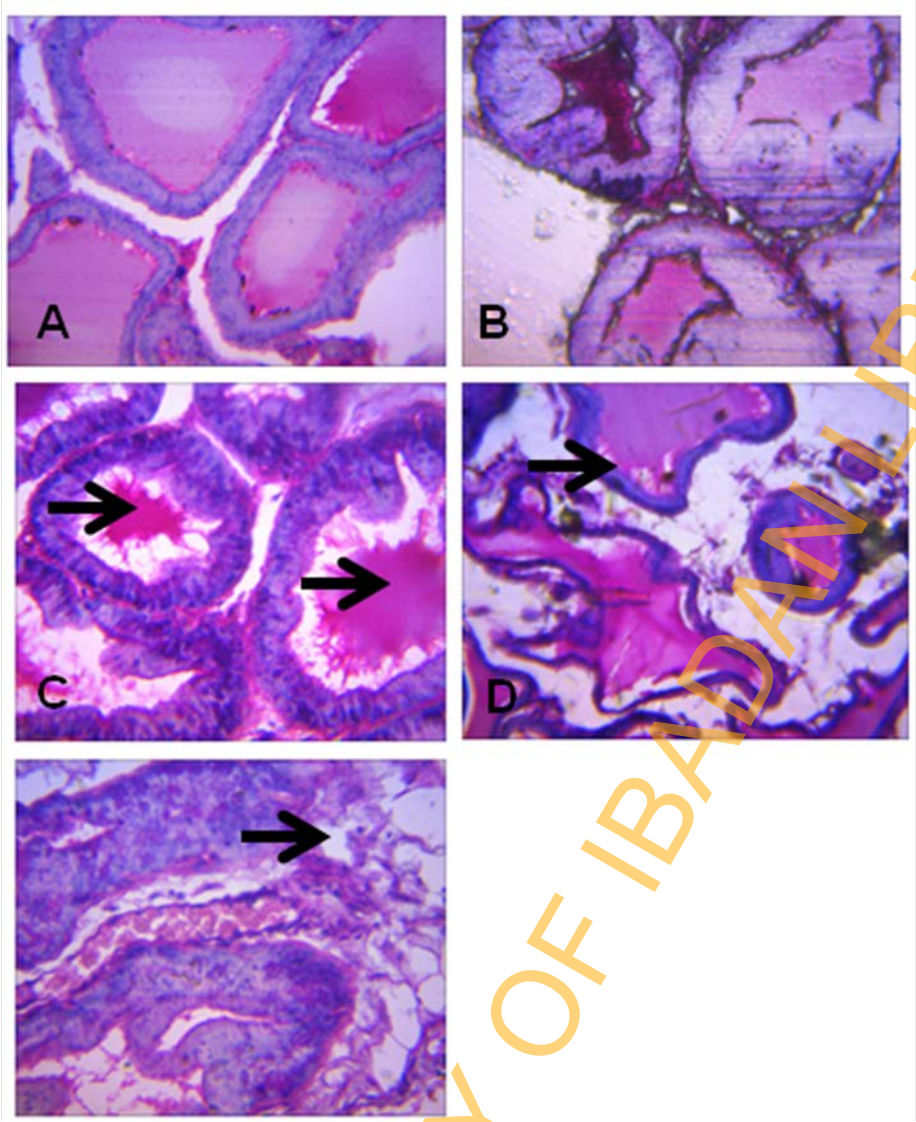


Figure 35: Photomicrographs of prostate sections of animals orally administered MFCP for 30days. A- control B-25mg/kgbw . C-50mg/kgbw. D – 100mg/kg bw E- 200mg/kgbw. Mag x400

No visible lesions seen B- Glandular secretions seen C- Shrinkage of lumen of glandular units D- Necrotic debris/distortion of the entire gland

EXPERIMENT 10: ASSESSMENT OF CYTOTOXIC EFFECTS OF FRACTIONS OF *C. PORTORICENSIS* IN PROSTATE CANCER CELL LINES

INTRODUCTION

Cell-based assays are often used for screening collections of compounds to determine ffects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss *et al.*, 2013). They are also widely used to assess the success of cryopreservation techniques, toxicities of substances, signal transduction events, trafficking of cellular components or monitor organelle function. Regardless of the type of cell-based assay being used, it is important to know how many viable cells at the end of the experiment. The parameters that define cell viability are diverse. They include redox potential of cell population, integrity of cell membrane, activity of cellular enzymes, etc (Berridge *et al.*, 2005). The 5-[3-(carboxymethoxy) phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium salt(MTS) metabolic viability assay relies on the reduction of the MTS, a tetrazolium salt which is reduced to a colored formazan product. The amount of formazan product is directly proportional to the number of living cells; therefore, cell proliferation or death can be quantified (Riss *et al.*,2013). The aim of this experiment was to determine the optimum concentration of methanol fraction of *C. portoricensis* that would inhibit the growth/ proliferation of prostatic tumour cells LNCaP and DU-145. The effects of other fractions of *C. portoricensis* were also confirmed in prostatic cell lines.

PROCEDURE

Prostate carcinoma cells (LNCaP & DU-145) in 100mm dishes were passaged into 96- well plates and seeded at 4500 cells/well for cell viability assay. After 24 hours, seeded cells were treated with different concentrations of MFCP, EFCP and CFCP. Cells were then incubated for 72hrs at 37^0C in a humidified incubator after which 20µL of MTS reagent was added. Results were reported as a percentage of viable cells where absorbance given by suspension of control cells was considered as 100 %.

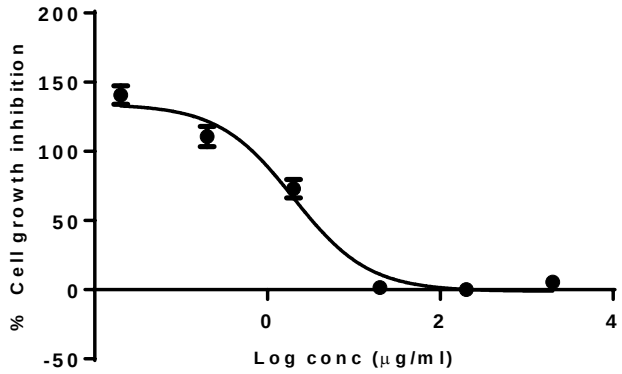
RESULTS

Figure 36a and 36b depict the cell viability data at different concentrations of MFCP on prostate carcinoma cells LNCaP and DU-145. Treatment of LNCaP and DU-145 cells with MFCP for 72 hours decreased cell viability with IC_{50} values of 2.37 and 3.34µg/mL, respectively. A lower IC_{50} indicates higher potency. In this regard, higher potency was observed with LNCaP cells with IC_{50} of 2.37 µg/mL.

Analysis of cell growth inhibition data of chloroform (CFCP) and ethylacetate (EFCP) fractions on LNCaP cells are shown in Figures 37a and 37b. CFCP and EFCP decreased cell viability at very high concentrations with half maximum inhibitory concentration (IC_{50}) values of 95 µg/mL and 221 µg/mL, respectively.

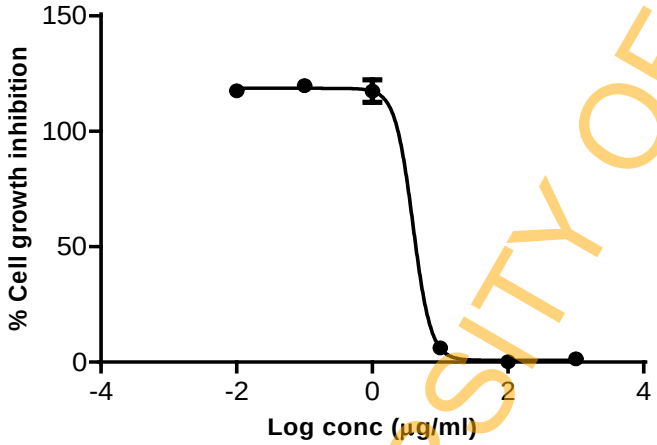
CONCLUSION

MFCP has the highest potency with respect to reducing number of viable cells.



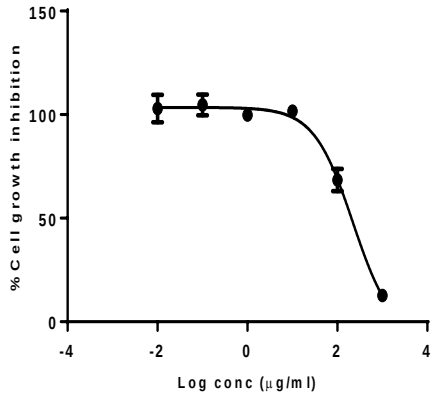
IC_{50} = 2.37 **µg/ml** ± 0.16

Fig 36a: The cell viability data of prostate LNCaP cells treated with different concentrations of MFCP for 72h. Percentage cell viability was plotted against concentrations in the logarithmic scale. The IC_{50} values were determined using a four-parameter dose response (variable slope) equation in GraphPad Prism



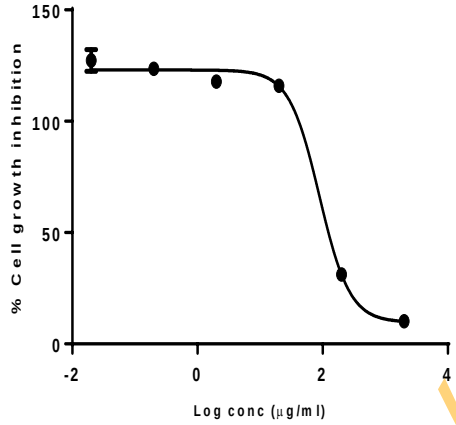
IC_{50} = 3.34 µg/ml ± 0.19

Fig 36b: The cell viability data of prostate DU-145 cells treated with different concentrations of MFCP for 72h. Percentage cell viability was plotted against concentrations in the logarithmic scale. The IC_{50} values were determined using a four-parameter dose response (variable slope) equation in GraphPad Prism



IC_{50} = 221.3 ± µg/ml ± 10.3

Fig 37a: Cytotoxic effect of ethylacetate fraction (EFCP) of *C .portoricensis* on prostate cancer cells (LNCaP)



IC_{50} = 95.2 µg/ml ± 6.8

Fig 37b: Cytotoxic effect of chloroform fraction (CFCP) of *C. portoricensis* on prostate cancer cells (LNCaP)

EXPERIMENT 11: ASSESSMENT OF CYTOTOXIC EFFECTS OF METHANOL FRACTIONS OF *C. PORTORICENSIS* IN LUNG ADENOCARCINOMA AND KIDNEY VERO CELL LINES

INTRODUCTION

One of the hall marks of cancer cells is the ability to overproliferate (Hanahan and Weinberg, 2000). Evidence abound showing that medicinal plants may have inhibitory effects on the proliferation of cancer cells. In order to determine whether methanol fraction is specific for inhibition of growth of prostate cancer cells and to determine its potency against normal cells, lung adenocarcinoma cells (A549) and healthy kidney VERO cells were treated with different concentrations of MFCP.

PROCEDURE

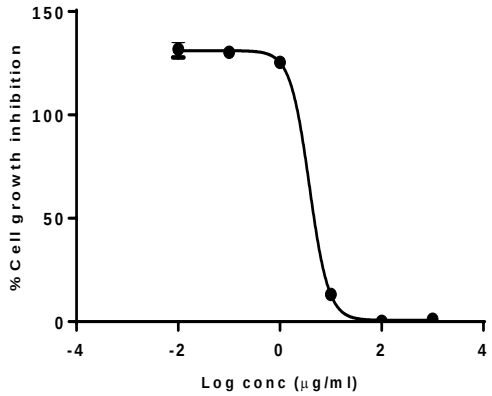
Lung adenorcarcinoma and healthy kidney VERO cells in 100mm dishes were later passaged into 96- well plates and seeded at 4500 cells/well for cell viability assay. After 24 hours, seeded cells were treated with different concentrations of MFCP. Cells were then incubated for 72hrs at 37^0C in a humidified incubator after which 20µL of MTS reagent was added. Cell viability was measured according to the method described on pages 88 and 89.

RESULTS

The cell viability data at different concentrations of MFCP on lung adenocarcinoma and healthy kidney VERO cells are depicted in Figures 38a and 38b. The results showed that MFCP clearly inhibited the proliferation of lung adenocarcinoma cells with IC_{50} value of 3.64 µg/mL but healthy kidney VERO cells were not affected by lower concentrations of methanol fraction (MFCP) as observed in its higher IC_{50} value of 17.86 µg/mL.

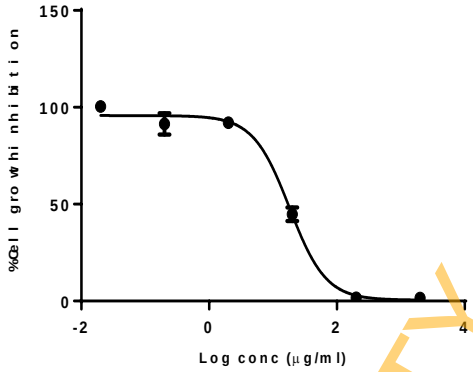
CONCLUSION

The MFCP inhibited the growth/ proliferation of lung adenocarcinoma cells. However, it is less \toxic to healthy kidney VERO cells.



IC_{50} = 3.64 µg/ml ± 0.18

Fig 38a: Cell viability data of lung adenocarcinoma cells treated with different concentrations of MFCP for 72h.



IC_{50} = 17.86 µg/ml ± 1.69

Fig 38b: Cell viability data of healthy kidney VERO cells treated with different concentrations of MFCP for 72h.

EXPERIEMENT 12: CYTOTOXIC ACTIVITIES OF PARTIALLY PURIFIED FRACTIONS OF *C. PORTORICENSIS* ON PROSTATE CANCER CELLS

INTRODUCTION

In recent years, a substantial body of evidence has demonstrated a wide range of pharmacological activities for a number of medicinal herbs (Borgia *et al.,*1981; De las Heras *et al.,* 1998). Thin layer chromatography (TLC) is frequently used for the analysis of botanical raw materials. The visualization of the entire pattern of compounds present in a herbal drug is important in the quality and stability testing of the herbal preparations. The aim of this experiment was to detect and monitor active compounds in the fractions of *C.portoricensis* and identify bioactivity-guided compounds in the potent MFCP via evaluation of antiproliferative activity of isolated compounds viz a viz comparison with the parent methanol fraction (MFCP).

PROCEDURE

The fractions of methanol extract of *C.portoricensis* were obtained as described earlier. The fractions were spotted on TLC plates and the best solvent system was chosen based on separation of the constituents of the fractions. The dissolved fractions were spotted on the plates and placed in the chromatographic tanks containing appropriate solvent system as the eluent. As the solvent ascended the plates, the resolution of the constituents was made and the elution was terminated at about 1cm from the end of the plates. The plate was air dried and activated where necessary, after which the plates were labeled and then sprayed with anisaldehyde and ninhydrin stains. Partially purified compounds were isolated using Preparatory TLC as described on page 109. Antiproliferative activities of the purified fractions were done as described earlier.

RESULTS

Figure 39 showed the reaction of different fractions of *C.portoricensis* when sprayed with chromogenic stains ninhydrin and anisaldehyde, respectively. MFCP developed a purple coloration to the ninhydrin stain which suggested the presence of primary amines. All fractions (MFCP, CFCP, and EFCP) of *C. portoricensis* tested positive to the anisaldehyde stain which revealed the presence of terpernes.

The TLC pattern of the potent MFCP when spotted on a silica gel glass plate in a solvent system of DCM: MeOH: TEA is also depicted in the same figure. Two (2) UV active compounds were isolated using preparatory TLC. They were named partially purified fractions 1 and 2 (PPF 1 & 2).

Comparison of cytotoxic activities of partially purified fractions of methanol fraction (MFCP) and its parent fraction on LNCaP prostate cancer cells was depicted in Table 6. Partially purified fractions 1 and 2 decreased cell viability with IC_{50} values of 6.32 and 5.74 µg/mL respectively. However, treatment of prostatic LNCaP cells with equal concentrations of partially purified fractions 1&2 (PPF 3) gave an IC_{50} value of 4.16 µg/mL.

The cytotoxic effect obtained when addition of equal concentrations of PPF 3 were exposed to LNCaP cells (IC_{50} =4.16 µg/mL) was higher compared with their individual cytotoxic activities (IC_{50} =6.32 µg/mL & 5.74 µg/mL) relative to that of the parent methanol fraction (IC_{50} =2.37 µg/mL) .

Table 7 showed the comparison of cytotoxic activities of partially purified fraction 3 (PPF 3) of methanol fraction (MFCP) and its crude form on prostatic DU-145 cells. Similar effects was observed when prostatic DU-145 cells were exposed to addition of equal concentrations of PPF1

and 2 which gave an IC_{50} value of 6.77 µg/mL when compared those of 7.23 g/mL and 7.01 µg/mL for partially purified fractions 1 and 2, respectively. Again, the addition of equal concentrations of PPF 3 to DU-145 cells gave a lower IC_{50} of 6.77 µg/mL relative to their individual inhibitory activities (IC_{50} = 7.23 µg/mL & 7.01 µg/mL) relative to the parent methanol fraction (IC_{50}= 3.34µg/mL).

CONCLUSION

Purification of MFCP did not enhance potency with respect to reduction of viable cell numbers. However, combination of PPF-1 & 2 (PPF-3) produced a better inhibitory effect with respect to reduction of viable cell numbers. All fractions of *C. portoricensis* contain terpenes while methanol fraction alone contains primary amine.

Fig 39: TLC pattern of most potent methanol fraction of *Calliandra portoricensis* and detection of active compounds by use of Ninhydrin and Anisaldehyde stains

Table 6: Comparison of cytotoxic activities of partially purified fractions of methanol fraction MFCP) and its crude form on LNCaP prostate cancer cells

Partially purified fractions	IC_{50} (µg/ml) (Partially purified) (LNCaP)	IC_{50} (µg/ml) (CRUDE) (LNCaP)
PPF 1	6.32 ± 0.34	2.37 ± 0.16
PPF 2	5.74 ± 0.29	2.37 ± 0.16
PPF 1 + PPF 2 = PPF 3	4.16 ± 0.34	2.37 ± 0.16

Table 7: Comparison of cytotoxic activities of partially purified fractions of methanol fraction (MFCP) and its crude form on hormone refractory (DU-145) prostate cancer cells

Purified fractions	IC_{50} (µg/ml) (Partially purified) (DU-145)	IC_{50} (µg/ml) (CRUDE) (DU-145)
PPF 1	7.23 ± 0.63	3.34 ± 0.19
PPF 2	7.01 ± 0.26	3.34 ± 0.19
PPF 1 + PPF 2 = PPF 3	6.77 ± 0.26	3.34 ± 0.19

EXPERIMENT 13: DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL OF PROSTATE CANCER CELLS EXPOSED TO METHANOL AND PARTIALLY PURIFIED FRACTIONS OF *C. PORTORICENSIS*

INTRODUCTION

The supply of energy by the mitochondrion depends on the maintenance of the chemiosmotic gradient also known as Proton Motive Force (PMF) across its inner membrane (Mitchell, 1979). The PMF has two components, the mitochondrial membrane potential, which arises from the net movement of positive charges across the inner membrane and the pH gradient. Mitochondrial membrane potential ($\Delta\Psi_m$) is a reliable indicator of cell's health and functional status (Mathur *et al.*, 2000). It is highly interlinked to many mitochondrial processes. The $\Delta\Psi_m$ controls ATP synthesis, generation of ROS, mitochondrial calcium sequestration, protein import and mitochondrial membrane dynamics (Kroemer *et al.*, 2007; Tait and Green, 2010; Kühlbrandt, 2015). Conversely, $\Delta\Psi_m$ is controlled by ATP utilization, mitochondrial proton conductance, respiratory capacity and mitochondrial calcium. The JC-1 dye (5, 5, 6, 6-tetracholoro-1, 1, 3, 3-tetraethylbenzimidazolyl- iodide) accumulates in the mitochondrial matrix of healthy cells owing to their high membrane potential. At low concentrations (due to low $\Delta\Psi_m$), JC-1 is predominantly a monomer that yields fluorescence with emission of 530± 15nm. At high concentrations, the dye aggregates yielding a red to orange coloured emission (590nm±17.5nm) (Perry *et al.*, 2011). Any event that dissipates the $\Delta\Psi_m$ prevents its accumulation in the mitochondria and causes a fluorescent emission shift that favours formation of green monomers instead of red aggregates. Hence, a decrease in the aggregate fluoresecent count is indicative of hyperpolarization (Solaini *et al.*, 2007)

The FCCP (carboxycyanide tri-4-fluoro-methoxy) phenyl hydrazone) is an uncoupler of oxidative phosphorylation and was used as a depolarization control in this experiment. Treatment of cells with FCCP eliminates membrane potential and JC-1 staining. The aim of this experiment was to determine the effect of MFCP in mitochondrial membrane potential in prostate cancer cell lines.

PROCEDURE

Prostate LNCaP (1.5 x 10^4 cells /well) cells were seeded at on a dark 96-well plate. They were allowed to attach overnight and were treated with different concentrations of MFCP diluted in complete media for 72 hours. Four (4) hours prior to completion of treatment, 11µL of 10mM FCCP were added to depolarized control wells while 100µL of 40µM JC-1 dye was added thirty minutes prior to the end of the treatment. Incubation of cells with different concentrations of MFCP, washing and flouresent count determination were carried out as described on pages 94.

RESULTS

The fluorescence intensity ratio of LNCaP cells exposed to FCCP and DMSO is shown in Figure 40. Vehicle (DMSO)–treated control LNCaP cells predominantly exhibited red aggregate forms of JC-1 dye with minimal green fluorescence. This is indicated in the cells' high fluorescent intensity ratio. As expected, FCCP-treated cells displayed a fluorescence emission shift which favoured increase in the percentage of cells that exhibited JC-1 momomers (green fluorescence). This is also evident in the cells' decrease in fluorescent intensity ratio.

The JC-1 Assay results for LNCaP cells treated with MFCP and PPF 3 are shown in Figures 41 and 42, respectively. The results show that incubation of LNCaP cells with 2.5, 5, 10 µg/mL of both parent and partially purified fraction 3 (PPF 3) for 72 hours caused a significant decrease in mitochondrial membrane potential i.e an increase in green/red fluorescent intensity ratio which is indicative of mitochondrial membrane depolarization.

CONCLUSION

MFCP and PPF-3 dissipated mitochondrial membrane potential in prostatic LNCaP cells.

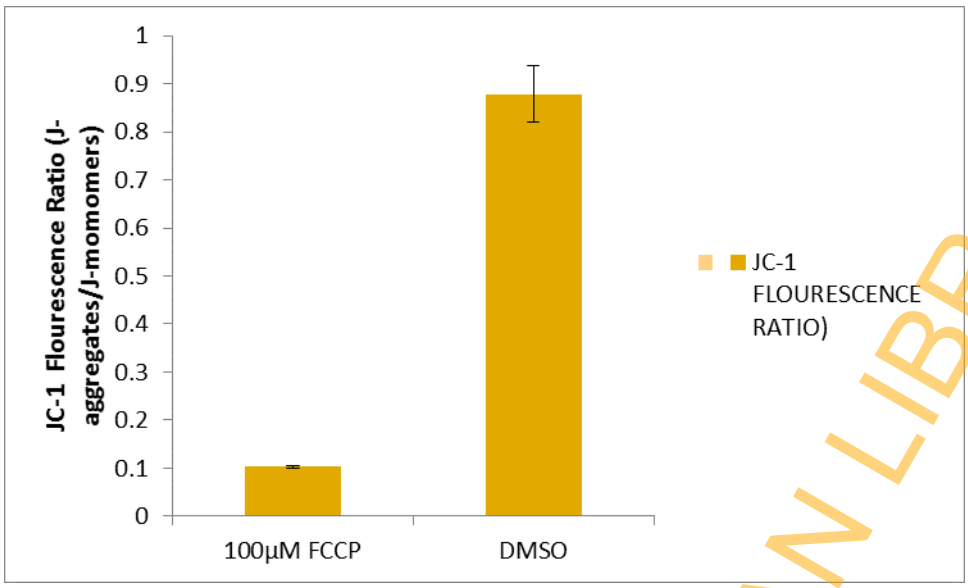


Figure 40: JC-1 Assay result in LNCaP cells treated with FCCP and DMSO. LNCaP cells were seeded at 1.5 x 10^4 cells per well on a 96 well plate for 4 hours with 100µM FCCP or vehicle/diluent control (DMSO). Cells were read on a Biotek Multilabel Plate Reader. Mean and standard deviation is plotted for 3 replicates from each condition.

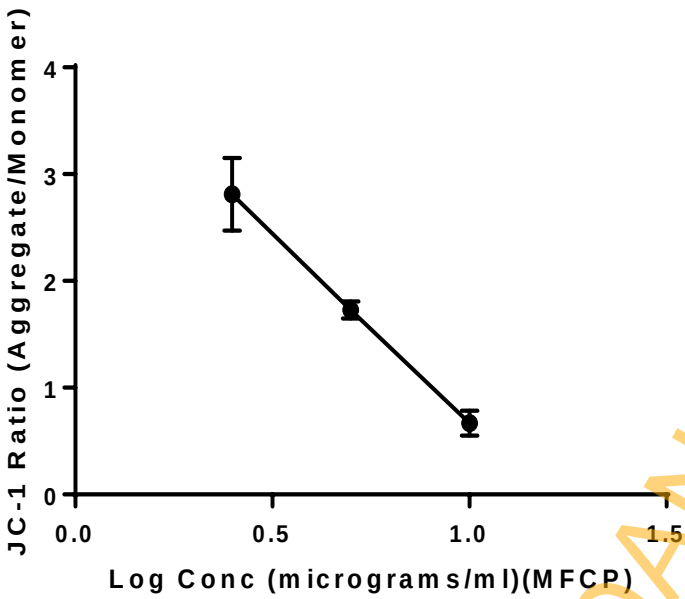


Figure 41: Mitochondrial membrane Potential (JC-1Assay result) in LNCaP cells treated with MFCP

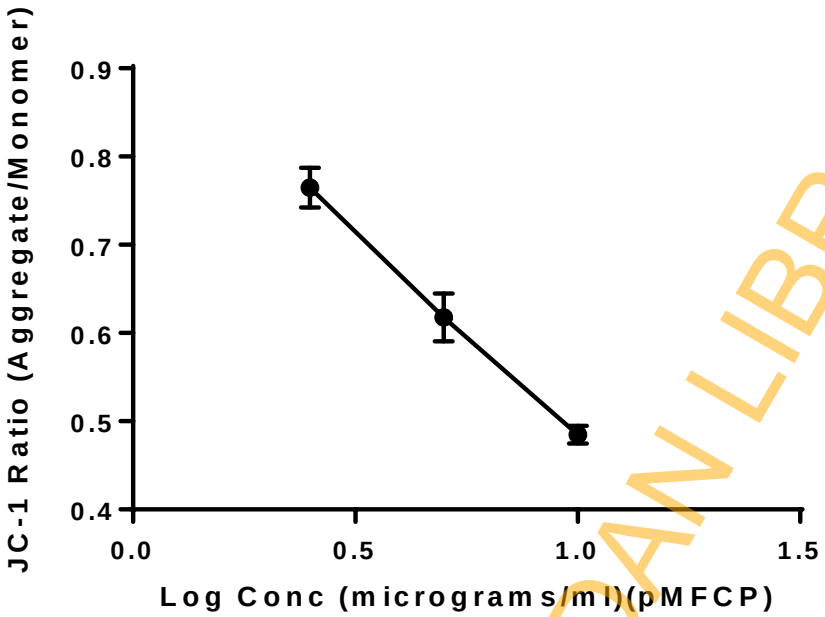


Figure 42: Mitochondrial membrane potential (JC-1 Assay result) in LNCaP cells treated with PPF-3.

EXPERIMENT 14: DETERMINATION OF CYTOCHROME C RELEASE IN CANCER CELLS EXPOSED TO METHANOL AND PARTIALLY PURIFIED FRACTION OF *C. PORTORICENSIS*

INTRODUCTION

Apoptotic cell death is a fundamental feature of virtually all cells. It is indispensable during normal development, tissue homoeostasis and regulation of immune system (Hassan *et al.,* 2014) Dysregulation of apoptosis has grave consequences and contributes to half of all human diseases (Biasutto *et al.,* 2016). Studies have shown that mitochondria accelerate and contribute to many forms of apoptosis. Hence, mitochondria have become a major checkpoint of this important process (Fernald and Kurokawa, 2013). Mitochondria outer membrane permeabilization provokes the release of cytochome c and causes it to redistribute from mitochondria to the cytosol during apoptosis in intact cells (Brenner and Moulin, 2012; Lopez and Tait, 2015). The release of cytochrome c activates caspases and ultimately cell death (Eleftheriadis *et al.,* 2016). This is an important event in mitochondrial-mediated apoptosis. Hence, this experiment was carried out to investigate if the collapse of mitochondrial membrane potential by MFCP is accompanied by release of the apoptogenic protein, cytochrome c.

PROCEDURE

Cytosolic lysates from LNCaP cells exposed to 2.5, 5, 10 µg/mL MFCP and its partially purified fraction were prepared according to the method described on page 95. Quantitative measurement of cytochrome c in cell lysates was determined by the use of ELISA technique.as described on page 96.

RESULTS

The induction of Cytochrome c release in LNCaP lysates following 24 hour exposure to varying concentrations of MFCP and PPF 3 are shown in Figures 43 and 44

As seen from the figure, increase in cytosolic levels of cytochrome c was observed in lysates of cells treated with MFCP and its partially purified fraction (PPF 3) when compared with uninduced cells. The MFCP induced cytochrome c release 3.6, 4.0 and 4.2 folds at 2, 5 and 10 µg/mL. However, there was no significant release of cytochrome c in LNCaP cells treated with 2.5 µg/mL of the partially purified fraction (PPF 3), although a maximum inductive fold of was obtained at 10 µg/mL relative to control.

CONCLUSION

The MFCP and PPF-3 induced the release of cytochrome c, a pro-apoptotic factor, from the mitochondria to the cytosol in prostatic LNCaP cells.

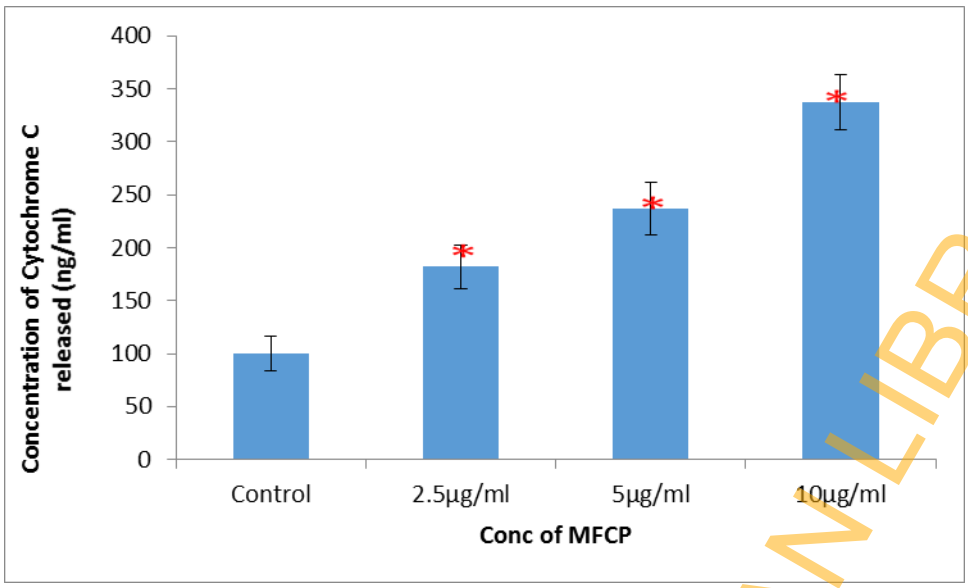


Fig 43: Induction of Cytochrome c release in LNCaP lysates following 24 hour exposure to varying concentrations of MFCP (2.5, 5, &10µg/ml)

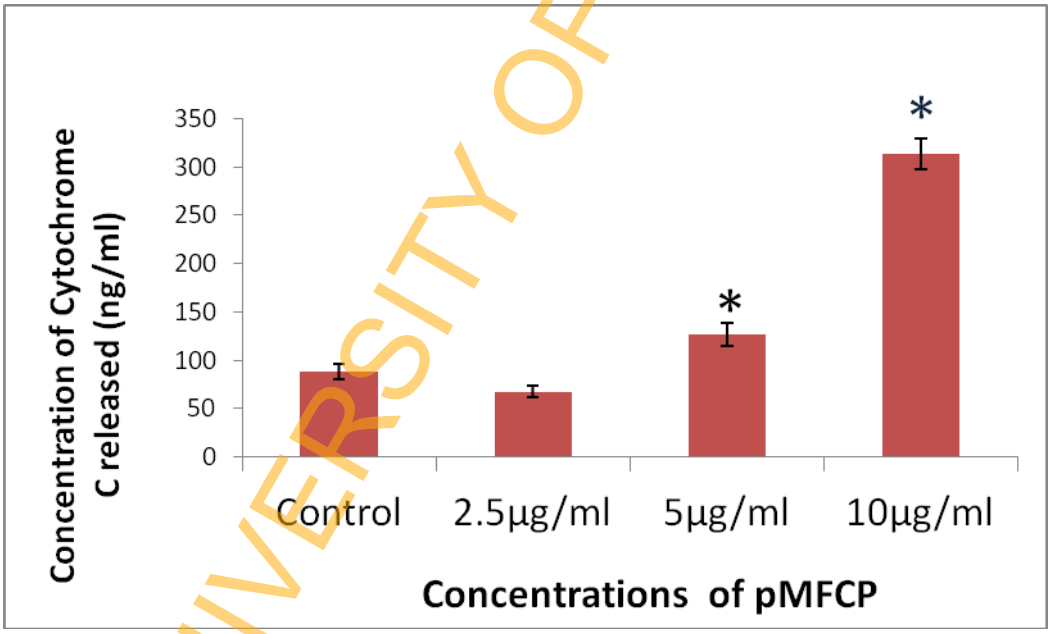


Fig 44: Induction of Cytochrome c release in LNCaP lysates following 24 hour exposure to varying concentrations of MFCP (2.5, 5 &10µg/ml)

EXPERIMENT 15: INDUCTION OF CASPASES 3 AND 9 ACTIVITIES BY PROSTATE CANCER CELLS EXPOSED TO METHANOL FRACTION OF *C. PORTORICENSIS*

INTRODUCTION

Effector caspases are responsible for initiating the hallmarks of the degradation phase of apoptosis; including DNA fragmentation, cell shrinkage and membrane blebbing (Woo *et al.*, 1998; Parrish *et al.*, 2013).

Caspase activation occurs as a result of growth factor withdrawal, exposure to radiation or chemotherapeutic agents, or initiation of the Fas/Apo-1 receptor-mediated cell death process. Cytosolic cytochrome c combines with monomeric Apoptosis Protease Activating Factor-1(APAF-1) to assemble the apoptosome in the presence of dATP. Apoptosome becomes a platform on which pro-caspase 9 is recruited and activated (Elmore *et al.*, 2007; Cain *et al.*, 2000; Chai and Shi, 2014). Activated procaspase-9 activates executioner caspases3, 6 and 7 (Brentnall *et al.*, 2013). Active caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic disassembly of dying cells (McIlwain *et al.*, 2013). The biological substrates of caspases include poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), lamins, topoisomerases, protein kinase C, sterol regulatory element binding proteins (SREBP) (Thornberry and Lazebnik, 1998; Shalini *et al.*, 2015). Caspase-3 specifically cleaves at the C-terminal side of the aspartate residue of the amino acid sequence DEVD (Asp-Glu-Val-Asp) while caspase 9, an initiator caspase cleaves at the C- terminal side of the amino sequence LEHD (Glu-Asp). In order to confirm that cytochrome c is a point of no return for the apoptotic process, initiator caspase-9 which is specific for the mitochondrial pathway and executioner caspase-3 were assayed for.

PROCEDURE

Prostatic LNCaP cells were incubated with MFCP /vehicle (0.1% DMSO) for 24 h. Caspase-9 and 3 activities were assayed using a colorimetric substrate, Ac-LEHD-pNA and Ac-DEVD-pNA, respectively using Biovision Caspase kit (Biovision Incorporated, CA, USA). Assay was carried out according to manufacturer's instruction as described on page 99. Cleavage of the C-terminal peptide bond by the enzyme-released p-nitroaniline was measured at 405 nm.

RESULTS

Induction of Caspases 9 and 3 activities in lysates of LNCaP cells following 24 hour exposure to varying concentrations of MFCP are depicted in figures 45 and 46. Treatment of LNCaP cells with 2.5, 5 and 10 µg/mL for 24 hrs caused significant increase in caspases-3 and 9 activation. The hydrolytic enzyme activity of caspase-3 towards their substrates was elevated by 2.1, 3.6, and 4.5 folds at 2.5, 5 and 10 µg/mL, respectively when compared to control.

Similarly, MFCP induced the activity of caspase -9 by 1, 2.5 and 3.64 fold when compared with uninduced cells. In this regard, increase in caspase- 3 & 9 activities correlated with increase in concentration of the methanol fraction.

CONCLUSION

Taken together, the release of cytochrome c might be an important event in MFCP-induced caspase –mediated apoptosis

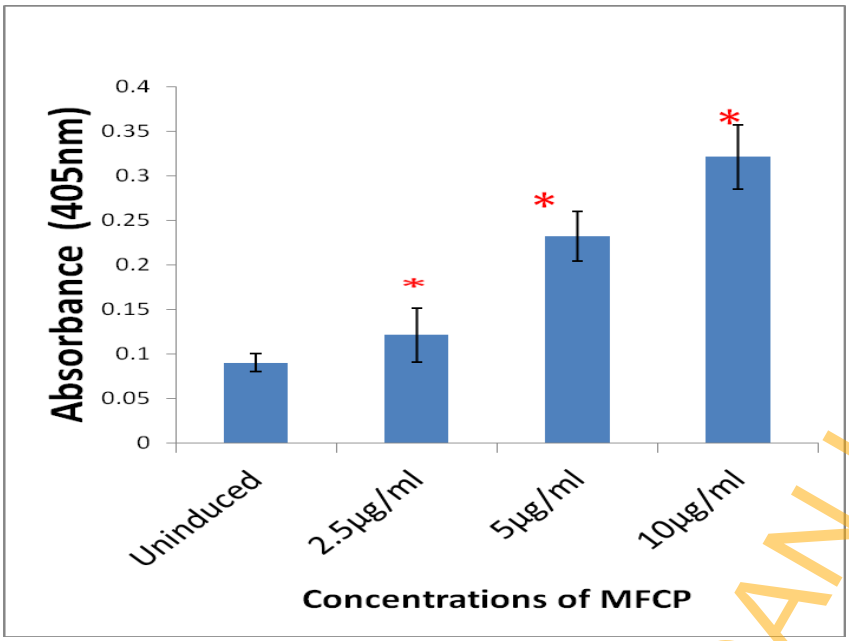


Fig 45: Induction of Caspases -9 activity in prostate cancer cell lysates following 24 hour exposure to varying concentrations of MFCP(2.5,5,&10µg/ml)

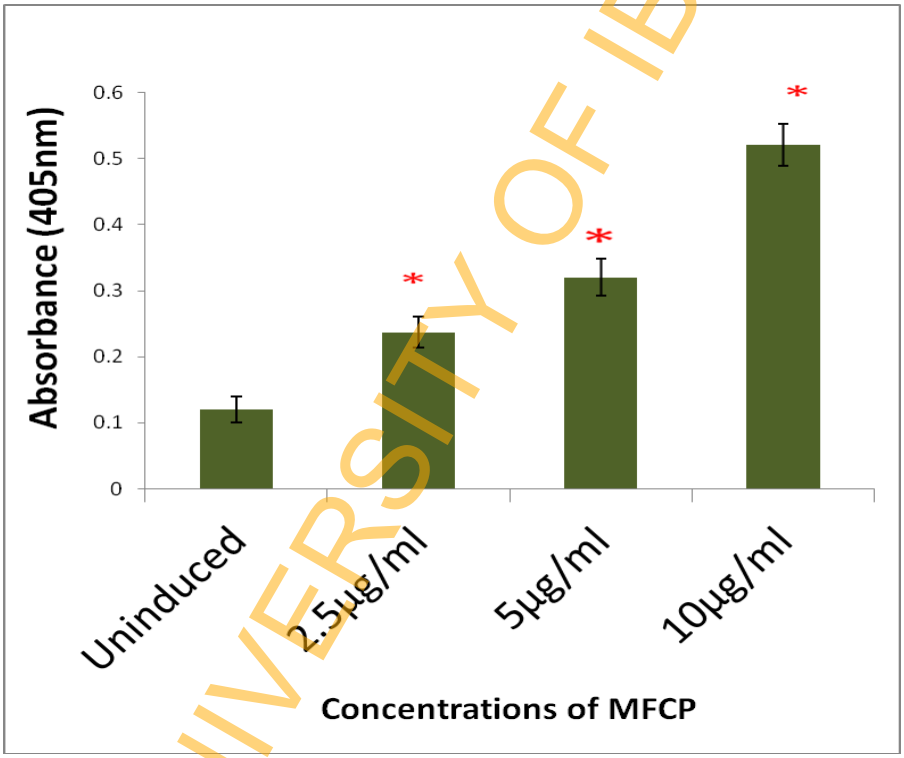


Fig 46: Induction of Caspases -3 activity in prostate cancer cell lysates following 24 hour exposure to varying concentrations of MFCP (2.5, 5, &10µg/ml)

EXPERIMENT 16: FLOW CYTOMETRIC DETERMINATION OF INDUCTION OF APOPTOSIS BY METHANOL AND PARTIALLY PURIFIED FRACTIONS OF *C. PORTORICENSIS IN* PROSTATE LNCaP CELLS

INTRODUCTION

A characteristic feature of a healthy cell is the asymmetric distribution of plasma membrane phospholipids between the inner and outer leaflets. Under physiological conditions, choline-containing phospholipids (phosphatidylcholine, sphingomyelin) are exposed on the external leaflet while aminophospholipids (phosphatidylserine, phosphatidylethanolamine) are exclusively located on the cytoplasmic surface of the lipid bilayer (Wlodkowic *et al.*, 2011)**.** This asymmetry is scrambled during apoptosis when phosphatidyl serine constituting less than 10% of the total membrane phospholipids, becomes exposed on the outside leaflet of the membrane (Fadok *et al.*, 2000) Exposure of PS on cell surface provides signaling to macrophages, which then become attracted and initiate to phagocytize apoptotic cells and apoptotic bodies. The detection of exposed PS allows for a precise estimation of apoptotic incidence. Annexin V, a fluorochrome-tagged 36-kDa anticoagulant protein. reversibly binds to PS residues in the presence of divalent calcium ions (Tait and Gibson, 1994;Gerke and Moss, 2002). Annexin V conjugated to fluorochromes of different absorption and emission wavelength has found many applications as a marker of apoptotic cells, in particular for their detection by flow cytometry and fluorescence microscopy (van Genderen et al., 2006). Propidium Iodide (PI) is a nucleic acid binding fluorophore commonly used a counter-stain in multicolour fluorescent techniques (Moore *et al.*, 1998). It intercalates with DNA by binding to its base pairs (and to some extend also RNA) and is membrane impermeable. Therefore, it is usually excluded from viable cells and

can be used to identify dead or damaged cells (Rieger *et al.*, 2011). Excitation of the propidium–nucleic acid complex at 485nm gives rise to maximum emission at 650nm. Dual staining with AnnexinV/PI allows identification of three major populations within the cell. Viable cells (lower left Quadrant) stain negative to both annexin V and PI, early apoptotic cells (lower right Quadrant) are Annexin V^{+}/PI^{-} while cells in their late apoptotic phase(upper right quadrant) are $Annexin^{-}PI^{+}$. Dead/damaged cells (upper right quadrant) are both $Annexin^{+}/PI^{+}$. This experiment was carried out in order to quantify methanol-fraction-induced apoptosis.

PROCEDURE

Prostrate LNCaP cells were exposed to 2.5, 5 and 10 MFCP and pMFCP for 24 hours. They were harvested by trypsinization, washed twice with PBS (4°C) and re-suspended in 1X binding buffer (component of Annexin V-FITC assay kit). AnnexinV-FITC and propidium iodide (PI) solution (5µL) were then added to stain the cells. Apoptosis quantification by flow cytometry was done by flow cytometry as earlier described on page 107.

RESULTS

Determination of apoptosis by flow cytometry in LNCaP cells following treatment with different concentrations of MFCP are shown in Figure 47. Treatment of prostate LNCaP cells with 2.5, 5 and 10 µg/mL MFCP for 24 hrs resulted in significant increases in the ratios of early and late apoptotic cells, while percentage of viable cells reduced compared with untreated control.

In this regard, percentage of viable cells decreased significantly from 97% to 19.4% at 0.1% DMSO (control) and 10 µg/mL, respectively while, percentage of early apoptotic cells increased from 0.08% in the control cells to 35.1% at 10 µg/mL. Likewise, percentages of dead cells increased significantly from 2.91 % (control cells) to 41.3% at 10 µg/mL.

Similarly, Figure 48 shows that treatment of LNCaP cells with different concentrations (2.5, 5 and 10 µg/mL) of PPF 3 resulted in decrease in number of viable cells from 97% in untreated cells to 23.6 % in cells exposed to 10 µg/mL. Again, percentage of early apoptotic cells increased significantly from 0.08% in DMSO-treated cells to 62.9% following treatment with 10 µg/mL MFCP. An increase in the percentage of dead/damaged cells also increased from 2.91% (control cells) to 8.71% (10 µg/mL).

CONCLUSION

These experimental data provides evidence for MFCP-induced apoptosis in prostate LNCaP cells.

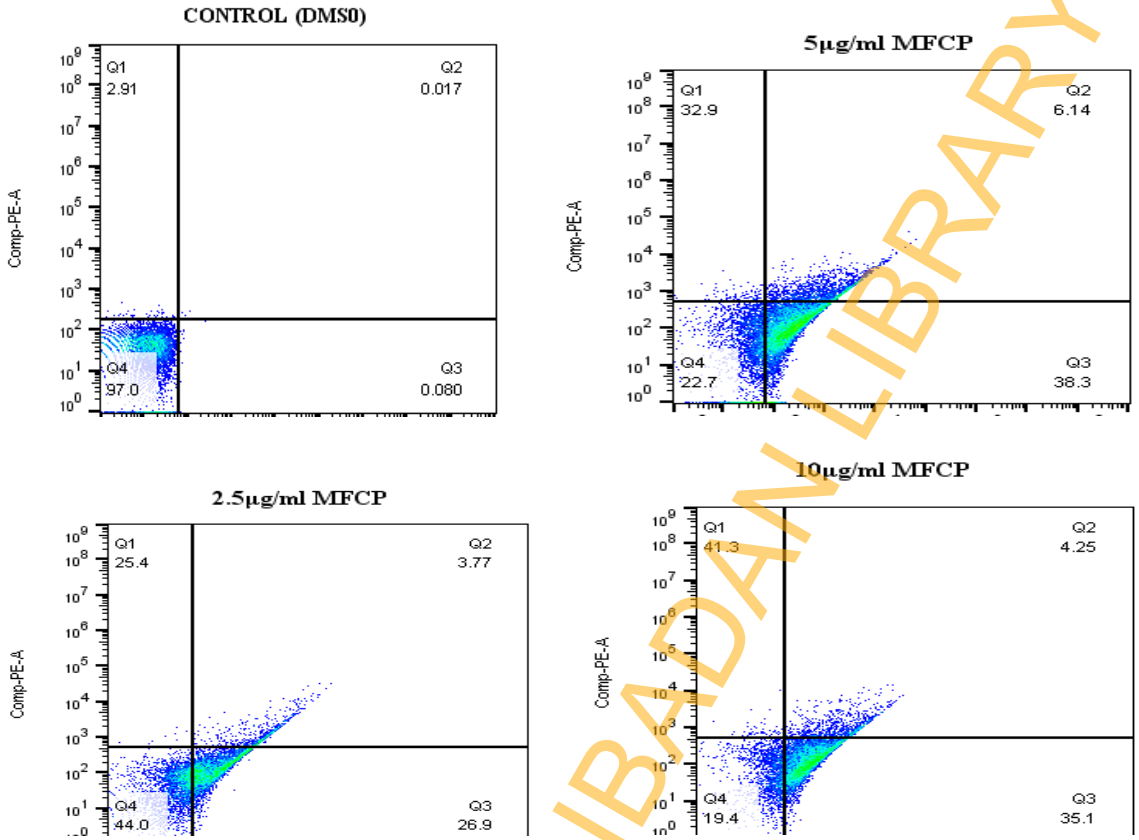


Fig 47: Flow cytometric determination of apoptosis induction in prostate cancer cells by MFCP with Annexin-V/propidium iodide (*PI) staining was done after* cells were treated with or without MFCP

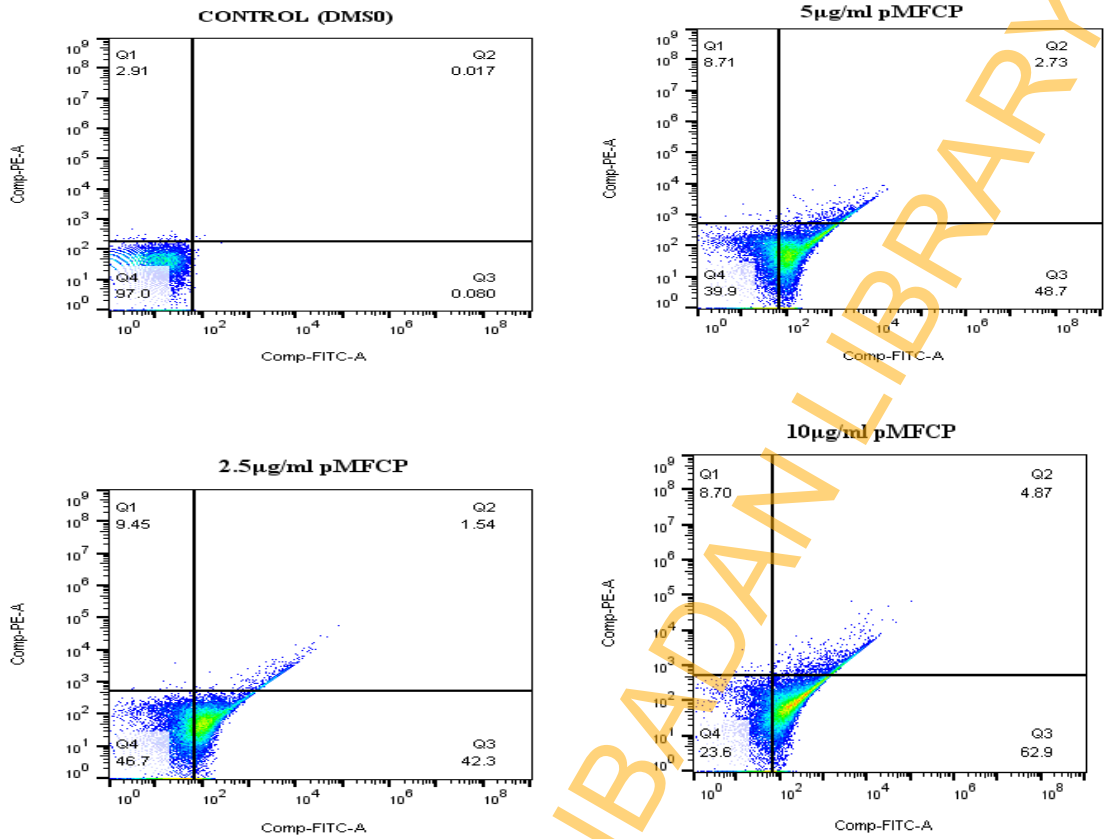


Fig 48: PPF 3 promoted cell apoptosis in LNCaP cells. Flow cytometric analysis of apoptosis with Annexin-V/propidium iodide (*PI) staining was done after* cells were treated with or without MFCP

EXPERIMENT 17: EFFECTS OF METHANOL FRACTION OF *CALLIANDRA PORTORICENSIS* ON REPRESENTATIVE BCL-2 FAMILY MEMBERS IN PROSTATE LNCaP CELLS

INTRODUCTION

Bax (Bcl-2 Associated X protein) is a pro–apoptotic factor of Bcl-2 family. It promotes apoptosis by binding to and antagonizing the apoptosis repressor Bcl-2. In inactive state, Bax resides in the cytosol, but under conditions of cellular stress, it undergoes a conformational change that causes translocation to the mitochondrial membrane where Bcl-2 anti-apoptotic proteins reside **(**Youle and Strasser, 2008; Luna-vargas and Chipuk, 2016) . The interaction of Bax with Bcl-2 proteins promotes Bax oligomerization and association with mitochondrial membranes with subsequent release of cytochrome c which triggers apoptosis. Studies have shown that over expression of Bcl-2, an apoptosis suppressor have been critically linked in all forms of cancers. (Roy *et al.*, 2014; Delbridge *et al.*, 2016). Given the emergence of Bcl-2 family members as critical regulators of mitochondrial-mediated apoptosis, this experiment was aimed at testing whether MFCP-induced apoptosis is regulated by pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins by assessing their levels in both cytosolic and mitochondrial lysates of cells exposed to different concentrations of the methanol fraction.

PROCEDURE

Mitochondria of human prostatic LNCaP cell line were isolated by conventional differential centrifugation as described by Clayton and Shadel (2014**)**. Mitochondrial and cytosolic lysates were prepared according to the method described on page 103.

Samples/standards (50µL) was added to appropriate wells followed by addition of 50µL of antibody cocktail (antibodies specific to Bax & Bcl-2) to each well. The plates were sealed and incubated for an hour at 25^0C on a plate shaker set to 400rpm. The wells are washed thrice to remove unbound material. Complete removal of liquid at each step was also ensured for good performance. After the last wash the microplate was inverted and blotted against a clean paper towel to remove excess liquid.100µL of TMB substrate was added and incubated for 10minutes in the dark during which it is catalyzed by HRP generating a blue colour. This reaction is then stopped by the addition of 100µL stop solution completing any colour change from blue to yellow. The intensity of developed colour is measured at 450nm.

RESULTS

Effects of MFCP on levels of Bax in cytosol and mitochondrial lysates of prostatic LNCaP cells are depicted in Figures 49 and 50, respectively. Cytosolic levels of Bax reduced significantly up to 3 folds at 10 µg/mL relative to control while a concomitant 2.5 fold increase in levels of Bax in the mitochondria was observed at the same concentration (10 µg/mL) when compared to control.

Figure 51 and 52 show the effects of MFCP on levels of Bcl-2 in cytosol and mitochondrial lysates of prostatic LNCaP cells. Interestingly, levels of anti-apoptotic Bcl-2 proteins increased significantly in the cytosol up to 4.2 folds at 10 µg/mL while a 5-fold decrease was observed in the mitochondrial lysate at highest concentration of 10 µg/mL when compared with control.

CONCLUSION

Methanol fraction-induced apoptosis is regulated by Bax and Bcl-2 proteins.

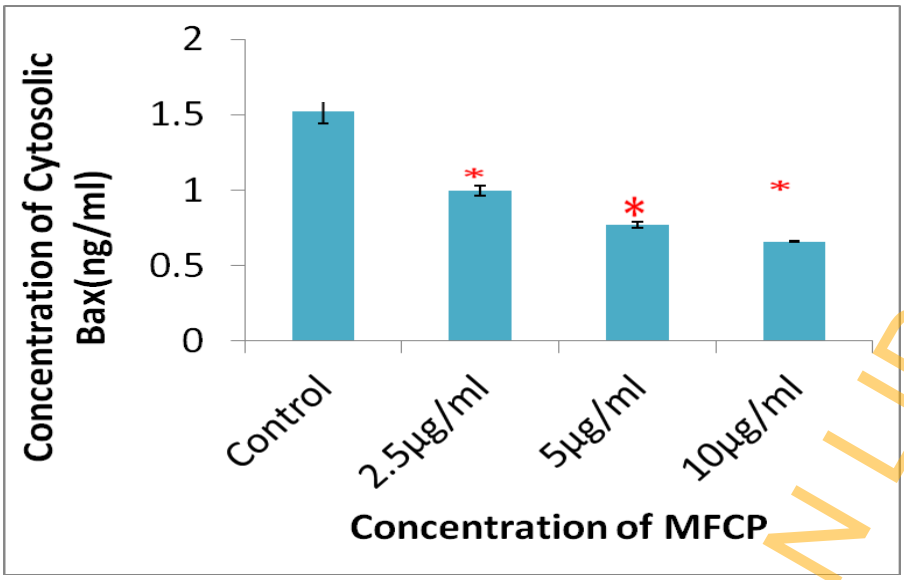


Fig 49: Effect of MFCP on levels of Bax Activity in cytosol of prostate cancer cells. LNCaP cells were treated with DMSO, 2.5, 5.0, 10µg/mL.

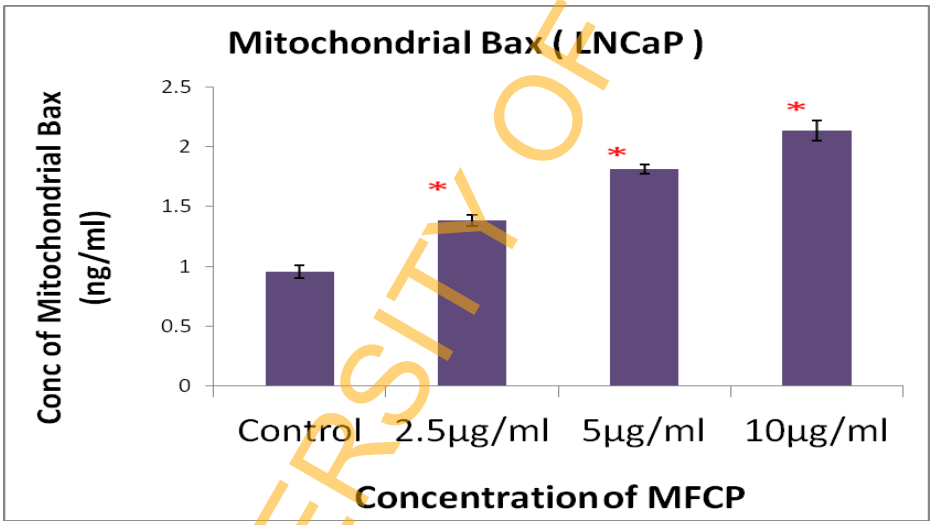


Fig 50: Effect of MFCP on levels of Bax Activity in mitochondria of prostate cancer cells. LNCaP cells were treated with DMSO, 2.5, 5.0, 10µg/mL

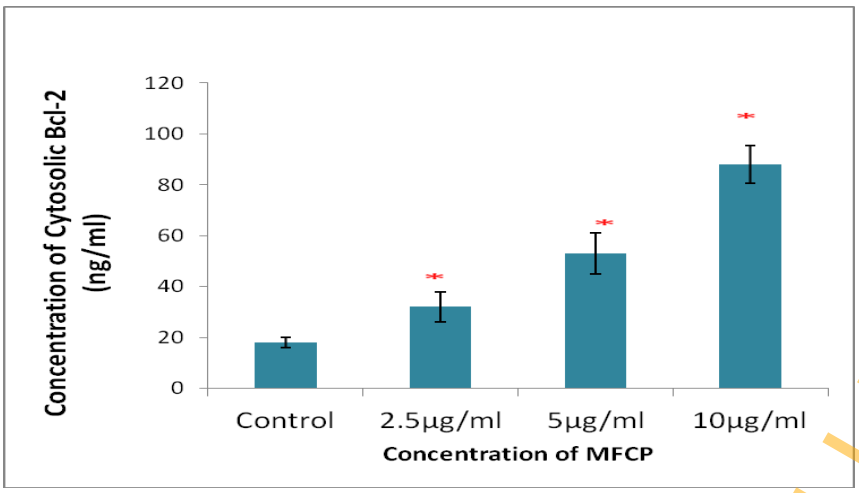


Fig 51: Effect of MFCP on levels of antiapoptotic Bcl-2 Activity in cytosol in prostate cancer cells. LNCaP cells were treated with DMSO, 2.5, 5.0, 10µg/ml MFCP

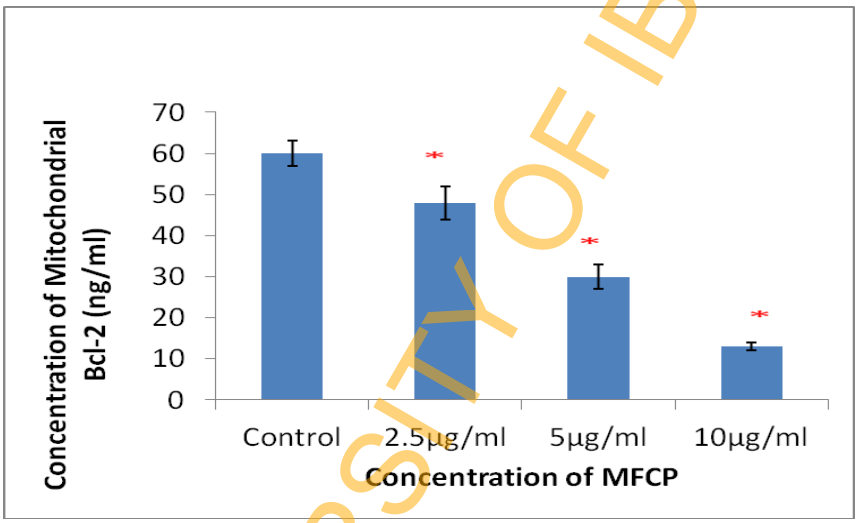


Fig 52: Effect of MFCP on levels of antiapoptotic Bcl-2 Activity in mitochondria in prostate cancer cells. LNCaP cells were treated with DMSO, 2.5, 5.0, 10µg/ml MFCP

EXPERIMENT 18: ASSESSMENT OF THE EFFECTS OF METHANOL AND PARTIALLY PURIFIED FRACTIONS OF *C. PORTORICENSIS* ON CELL CYCLE DISTRIBUTION IN DIFFERENT CANCER CELL LINES

INTRODUCTION

Normal mammalian cellular proliferation is tightly regulated at each phase of the cell cycle by the activation and deactivation of a series of proteins that constitute the cell cycle machinery (Vermeulen *et al.,* 2003). The 'life cycle' of a dividing eukaryotic non-embryonic cell starts with the cell triggered to enter the cell cycle and ends with the equal partitioning of the genetic material and cleavage of the cell during cytokinesis. The whole process is called the cell cycle and consists of four main phases. Entry to the cycle is made in Gap 1 (G_1) phase and this is followed in sequence by a DNA synthesis (S) phase, Gap 2 (G_2) phase, and Mitosis (M). After mitosis (M) some cells enter the G1 phase of a new cell cycle whilst others may diverge at the start of G1 into a phase called Gap O (zero). Phases G_1, S and G_2 are often grouped and called 'interphase' (Bertoli *et al.,* 2013). Cells in G_0 (zero) are quiescent and not dividing (hence zero), this may be permanent or temporary. Loss of cell cycle regulation has been directly linked to cancer incidence (Strzyz, 2016). This may occur through mutation of tumour suppressor genes responsible for inhibiting uncontrolled cellular growth. This experiment was carried out to examine whether methanol fraction- growth inhibition is mediated by cell cycle arrest.

PROCEDURE

Prostate LNCaP cells (1×10^6) were treated with MFCP at its maximum inhibitory concentration (IC_{50}), five times its maximum inhibitory concentration ($5IC_{50}$) and 0.1% DMSO. Adherent cells were collected by centrifugation and were fixed in 70% ethanol at 4°C overnight and cell cycle progression was determined by flow cytometry following staining with propidium iodide using a BD C6 Accuri flow cytometer.

RESULTS

Effect of MFCP on DNA distribution in prostate LNCaP cells is shown in figure 53. The effect of LNCaP on cell cycle progression as measured by flow cytometry showed that a 24 hr exposure of MFCP increased significantly the percentage of cells in S- phase from 37.4 % in uninduced cells to 40.1% at 2.5 µg/mL (IC_{50}) and 64.3% at 12.5 µg/mL ($5IC_{50}$). In addition, the percentage of LNCaP cells in G_1 phase decreased in a concentration-dependent manner in control cells (57%) relative to cells treated with 2.5 µg/mL(56.0%) and 12.5 µg/mL (27.7%) MFCP.

Figure 54 depicts the effect of PPF 3 on DNA distribution in prostate LNCaP cells. The results show that LNCaP cells underwent a concentration-dependent inhibition of proliferation when treated with PPF 3 with significant increase in the proportion of cells arrested in S-phase from 37.4% (control) to 53.7% (12.5 µg/mL).

Figures 55 and 56 depict the effects of MFCP on Cell cycle distribution in prostate DU-145 and lung adenocarcinoma cells respectively. According to the results, DU-145 cells exposed to MFCP displayed an increase in S phase- cell population from 32.8% in control cells to 41% (3.5 µg/mL) and 42.8% (17.5 µg/mL).

As shown in Figure 56, treatment of lung adenocarcinoma cells for 24 hrs also caused 45.3 % (19.1 µg/mL) of the cell population to arrest at S- phase from 21.5% in control cells. A significant decrease in cells distribution in G_1 phase was observed for all cell lines. This shows that methanol fraction could inhibit the growth of prostate LNCaP, DU-145 and lung adenorcarcinima (A549) cells.

CONCLUSION

Methanol-fraction-induced growth inhibition is mediated by cell cycle arrest in Prostate LNCaP, DU-145 and Lung adenocarcinoma cells.

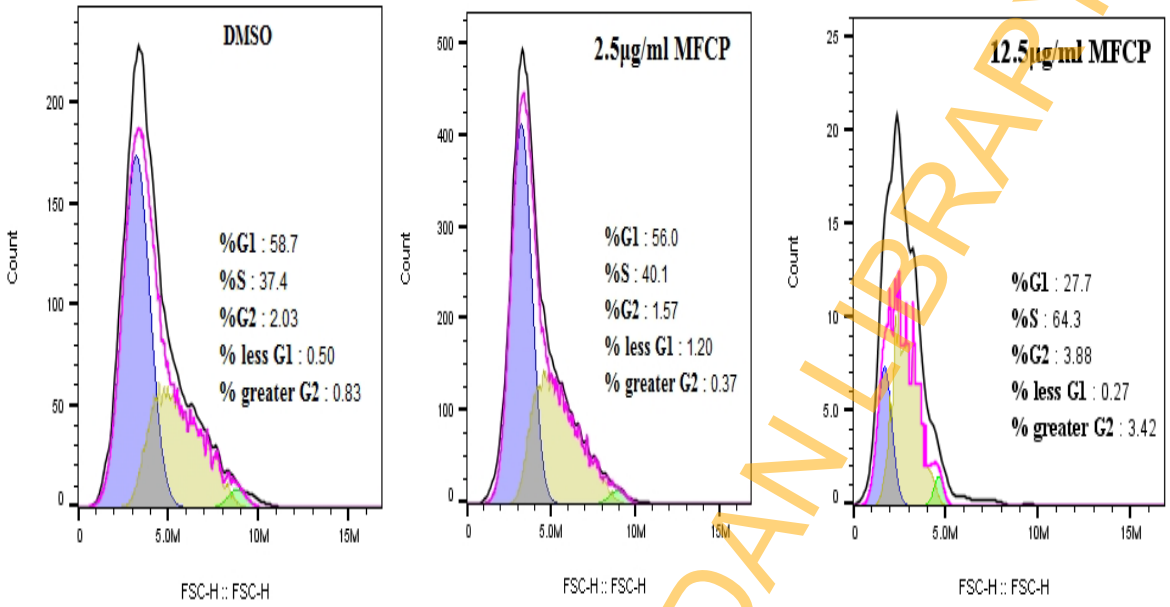


Fig 53: Effect of MFCP on DNA distribution in prostate cancer cells. LNCaP cells (1 X 10^6/5ml) were treated with 2.5µg/ml & 12.5 µg/ml methanol fraction of *Calliandra portoricensis (MFCP)* for 24h after which cells were detached and aliquots of cell suspensions were incubated with a propidium iodide (PI) solution for 30 mins

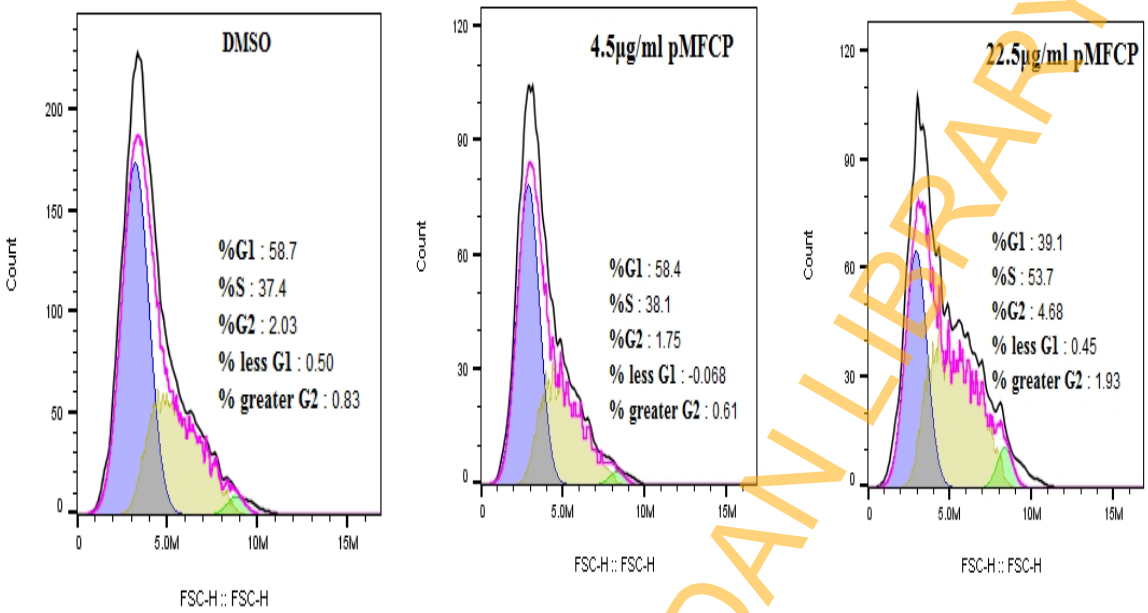


Fig 54: Effect of PPF 3 on DNA distribution in prostate cancer cells. LNCaP cells (1 X 10^6/5ml) were treated with partially purified fractions (4.5 µg/ml & 22.5 µg/ml) of the crude MFCP for 24h after which cells were detached and aliquots of cell suspensions were incubated with a propidium iodide (PI) solution for 30 mins

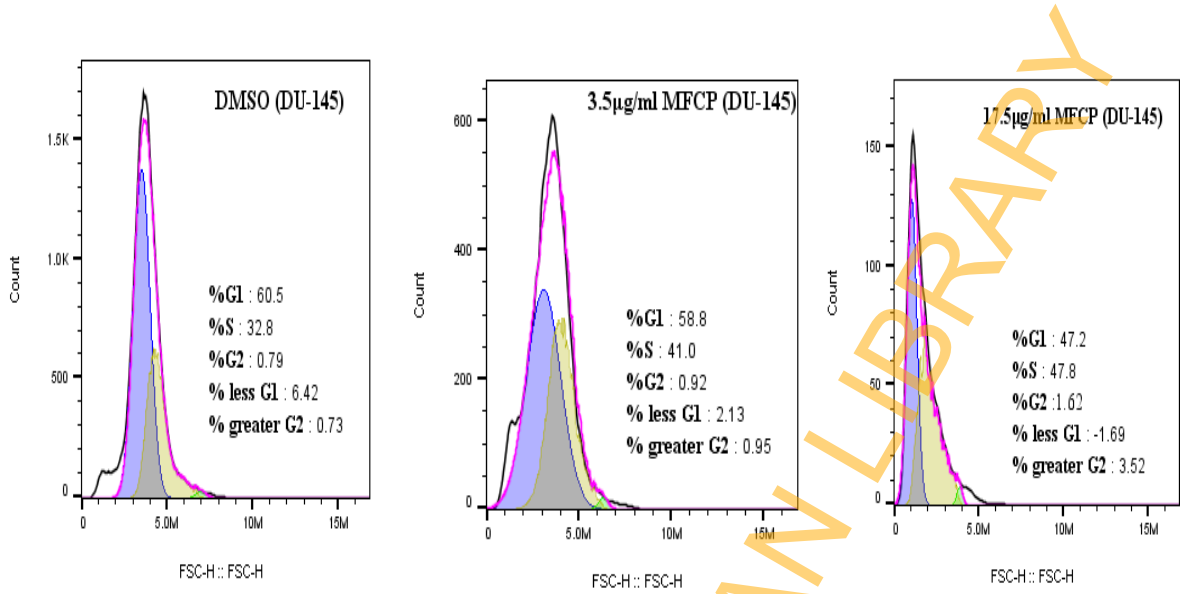


Fig 55: Effect of MFCP on DNA distribution in prostate DU-145 cells. DU-145 cancer cells (1 X 10^6/5ml) were treated with partially purified fractions (3.5 µg/ml & 17.5 µg/ml) of the crude MFCP for 24h after which cells were detached and aliquots of cell suspensions were incubated with a propidium iodide (PI) solution for 30 mins

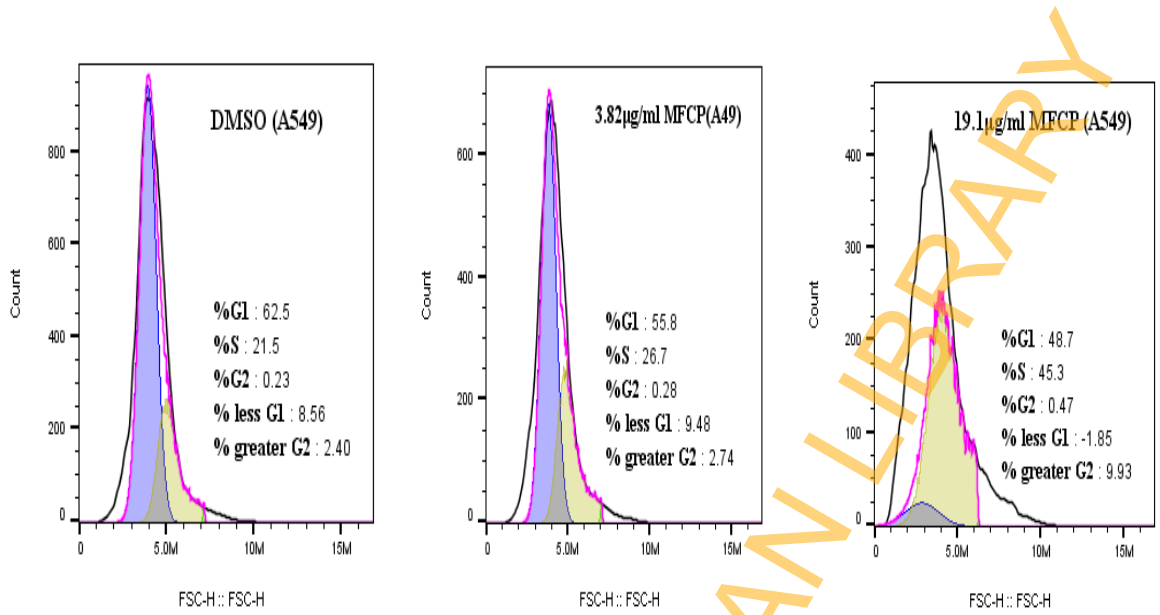


Fig 56: Effect of MFCP on DNA distribution in A549 cells. Lung Adenocarcinoma cells (1 X 10^6/5ml) were treated with partially purified fractions (3.82 µg/ml & 19.1 µg/ml) of the crude MFCP for 24h after which cells were detached and aliquots of cell suspensions were incubated with PI

EXPERIMENT 19: ISOLATION OF POTENT FRACTIONS FROM MODIFIED METHANOL FRACTION OF *CALLIANDRA PORTORICENSIS* ROOT BARK USING THIN LAYER CHROMATOGRAPHY (TLC), PREPARATORY TLC, HPLC-MS AND NUCLEAR MAGNETIC RADIATION (NMR) SPECTROSCOPY

INTRODUCTION

In recent years, a substantial body of evidence has demonstrated a wide range of pharmacological activities for a number of medicinal herbs. In addition, the investigation of the crude plant extracts through ethno-pharmacological evaluation of plants with folk medicinal value showed that a number of plants exhibit medicinal properties which may include antioxidant, anti-inflammatory and antitumour activities (Borgia *et al*., 1981). The presence of various compounds like flavonoids, polyphenolics, tannins and steroids has been implicated in a number of medicinal properties of the plants (McClure, 1975). In its traditional form, thin layer chromatography (TLC) is frequently used for the analysis of botanical raw materials. Thin layer chromatography has a long record in almost all pharmacopeias for its use in the identification of herbal medicines**.** High Performance/Pressure Liquid Chromatography is an analytical technique used for identification, quantitation and mass analysis of materials (Sasidharan *et al.*, 2011). It utilizes a compounds intrinsic ability for both mobile mobile phase (typically a buffered solvent) and a stationary phase (porous solid support with specialized coating). Mass Spectroscopy works by ionizing chemical compounds to generate molecular fragments and measuring their mass to charge ratios (m/z) which is unique for each compound (Glish and Vachet, 2003).

Di-*tert*-butyl dicarbonate ($C_9H_{18}O_3$) is a reagent widely used in organic synthesis . The protection of amines with *tert*-butyloxycarbonyl (BOC) group is a widely used reaction in organic synthesis because of its inertness toward catalytic hydrogenolysis and resistance toward hydrolysis under most basic conditions and nucleophilic reagents (Agami and Couty, 2002; Ragnarsson and Leif, 2013) *N*-Boc deprotection is generally achieved under mild acidic conditions such as trifluroacetic acid (TFA), aqueous phosphoric acid in THF (Li *et al.,* 2003; Bose *et al.,* 2003) acid. The *tert*-butyloxycarbonyl (Boc) is easily introduced using commercially available di-*tert*-butyldicarbonate under standard basic condition. Amines are converted to *N*-*tert*-Boc derivatives by reaction with di-*tert*-butyldicarbonate $(BOC)_2O$ in the presence of a suitable base (Le Corre *et al.,* 1978) thus rendering them inert/ inactive.

PROCEDURE

The crude and VLC fractions were obtained as earlier described. These were spotted on the TLC plates. The best solvent system was chosen via variations of the different solvents. The dissolved extracts were spotted on the plates and placed in the chromatographic tank containing the appropriate solvent system as the eluent. As the solvent ascended the plates, resolution of the constituents was made and the elution was terminated at about 1cm from the end of the plate. The plates were air-dried and activated where necessary. After this, the plates were labeled and then sprayed with the appropriate chromogenic agents.

Di-tert-butyl bicarbonate (BOC anhydride) (20g) was added to MFCP (10g) and dissolved in 1M NaOH (480mls) and acetonitrile (120mls) in a 1 lL round bottom flask. The resulting solution was constantly stirred at 25^0C for 48 hours. Work up, isolation of compounds from BOC-protected MFCP using column chromatography was carried out as described on pages 111 and 112.

RESULTS

Figure 57 shows the TLC pattern of the potent MFCP and its BOC- protected derivative under UV light at 254nm. From the figure, there was no movement of MFCP from the origin while two UV active compounds were identified in its BOC-protected derivative using the same solvent system.

Isolation of the UV active fraction present in the MFCP-BOC-derivative was achieved by preparatory TLC. Figure 61 showed the TLC pattern of sub fraction-BOC-MFCP (100mg) isolated from BOC-MFCP. The figure showed that the fraction contains two compounds which have very close retardation factors (R_f) using two different solvent systems (Figures 58a and b).

The sub fraction-BOC-MFCP was subjected to column chromatography on silica gel F. Gradient solvent elution was achieved by using dichloromethane and ethanol which gave 33 eluents which were monitored and pooled according to TLC behaviour. They were pooled into 6 sub-fractions which initially gave single bands on TLC as depicted in Figure 59a and b.

Further concentration of the pooled single band fractions produced an additional band on TLC (Figure 59c). Figure 59d shows the TLC pattern of subfraction-MFCP after deprotection by removal of the BOC-group.

The anti-proliferative activity of the deprotected polar compound designated partially purified fraction 4 (PPF 4) was carried out on prostatic LNCaP and DU-145 cells. The data obtained were compared with the inhibitory effect of the parent methanol fraction on prostatic cancer cells (Table 8). Treatment of LNCaP and DU-145 cells with PPF-4 showed that it decreased cell viability with IC_{50} value of 2.5µg/mL and 2.43µg/mL, respectively while the parent MFCP initially gave IC_{50} values of 2.37 and 3.34µg/mL, respectively.

Figures 60 and 61show the proton NMR spectra of BOC-MFCP derivative and its subfraction-BOC-MFCP. According to the figure, the signature of BOC- anhydride shows up at 2 in both spectra.

Figure 62 shows the proton NMR profile of the deprotected subfraction-MFCP from which BOC-anhydride has been removed. The NMR spectra show the disappearance of the BOC-anhydride signature at 2. It also reveals the presence of two compounds present in the fraction.

The total-ion chromatogram of PPF 3 is shown in Figure 63. Figures 64 and 65 also reveal the total-ion chromatogram of the PPF 3 in its positive and negative-ion mode. A phenolic compound identified as Afzelechin, showed up in both modes of ionization. Besides, gallic acid was also identified to be present.

CONCLUSION

MFCP- protected amine subfraction (PPF 4) exhibited higher growth- rate inhibition in prostate cancer cells compared with unprotected MFCP (PPF-3).

Amine Protection of Methanol Fraction of *C. portoricensis* (MFCP)

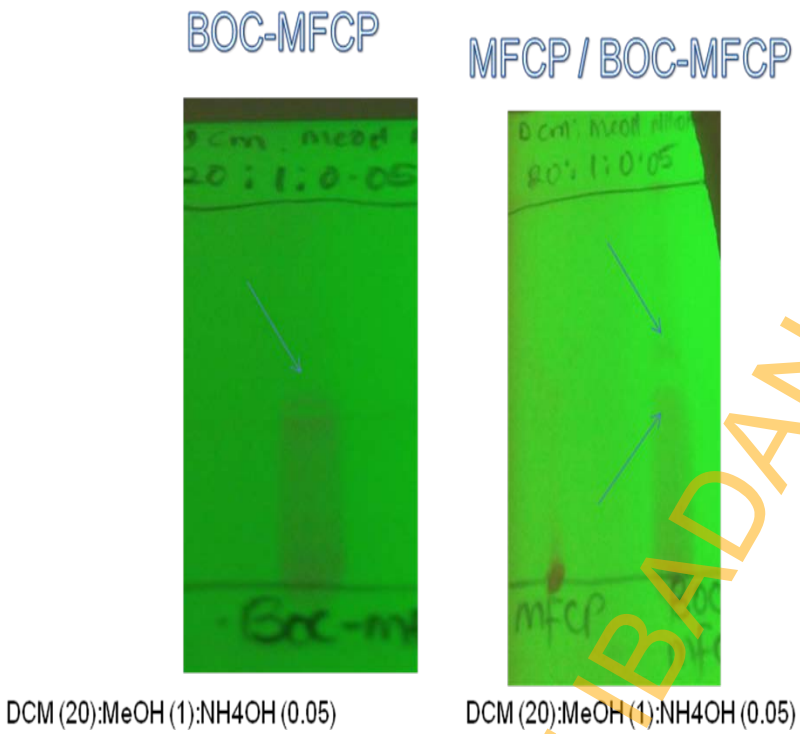


Fig 57: Comparison of TLC patterns of MFCP and its derived BOC-MFCP fractions

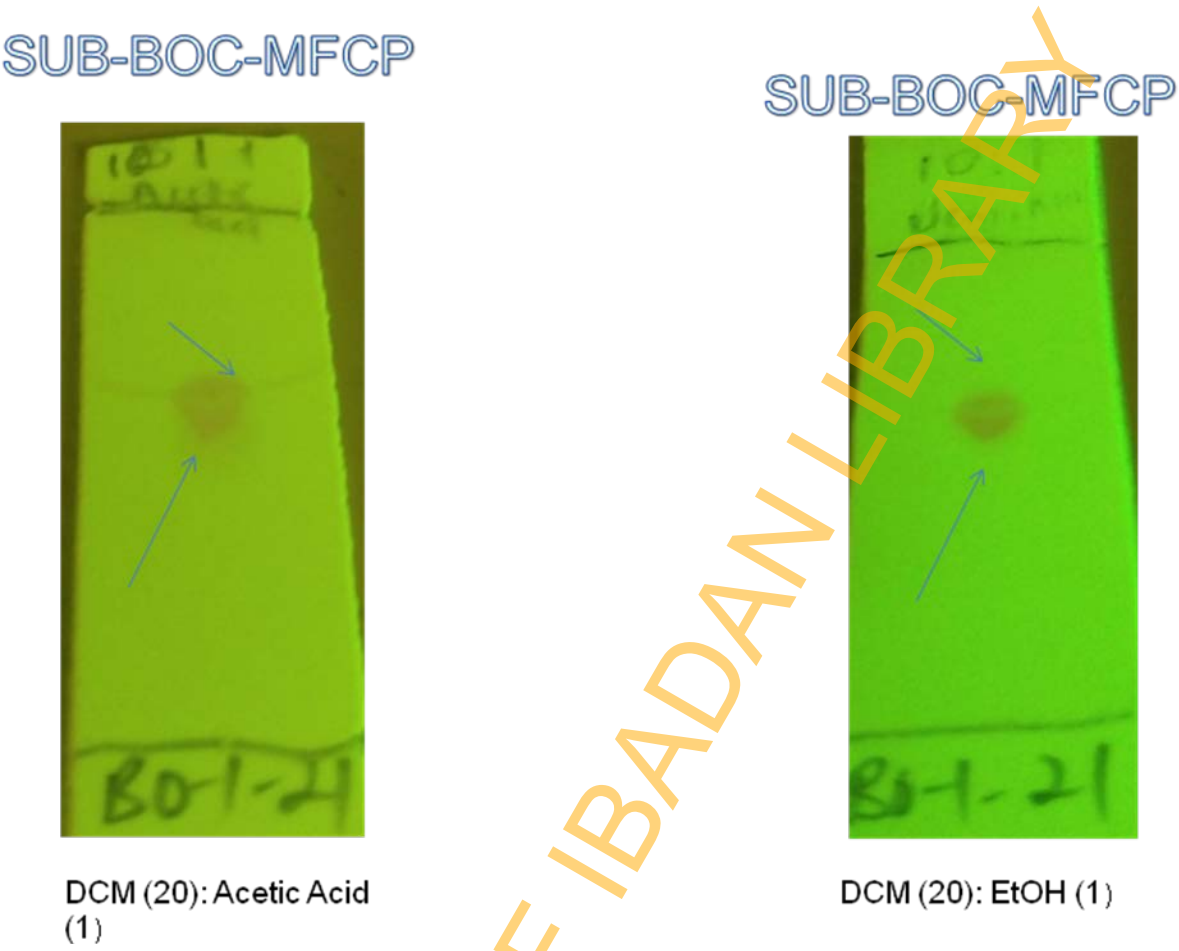


Fig 58: TLC patterns of fractions obtained from Prep-TLC of BOC-MFCP and its subsequent deprotection (removal of the amine protecting group)

Column chromatography of Sub-BOC-MFCP fraction

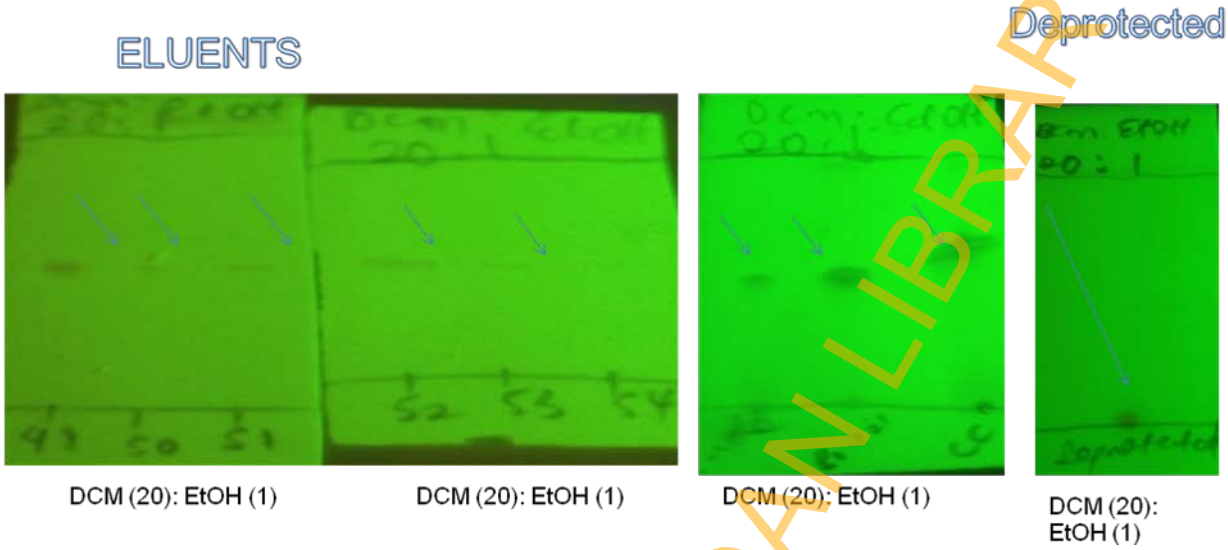


Fig 59: TLC patterns of 6 pooled eluents and their concentrated forms obtained from gradient elution of sub-BOC-MFCP fraction using DCM and methanol in order of increasing polarity

Table 8: Comparison of the cytotoxic activity of partially purified fraction on LNCaP and DU-145 from Sub-BOCMFCP with crude fraction

Purified Fraction	CRUDE MFCP	PPF-3	PPF-4
IC_{50} (µg/ml) (LNCaP	2.37 ± 0.16	4.16 ± 0.34	2.5 ± 0.9
IC_{50} (µg/mL) (DU-145)	3.34 ± 0.19	6.77 ± 0.26	2.43 ± 0.4

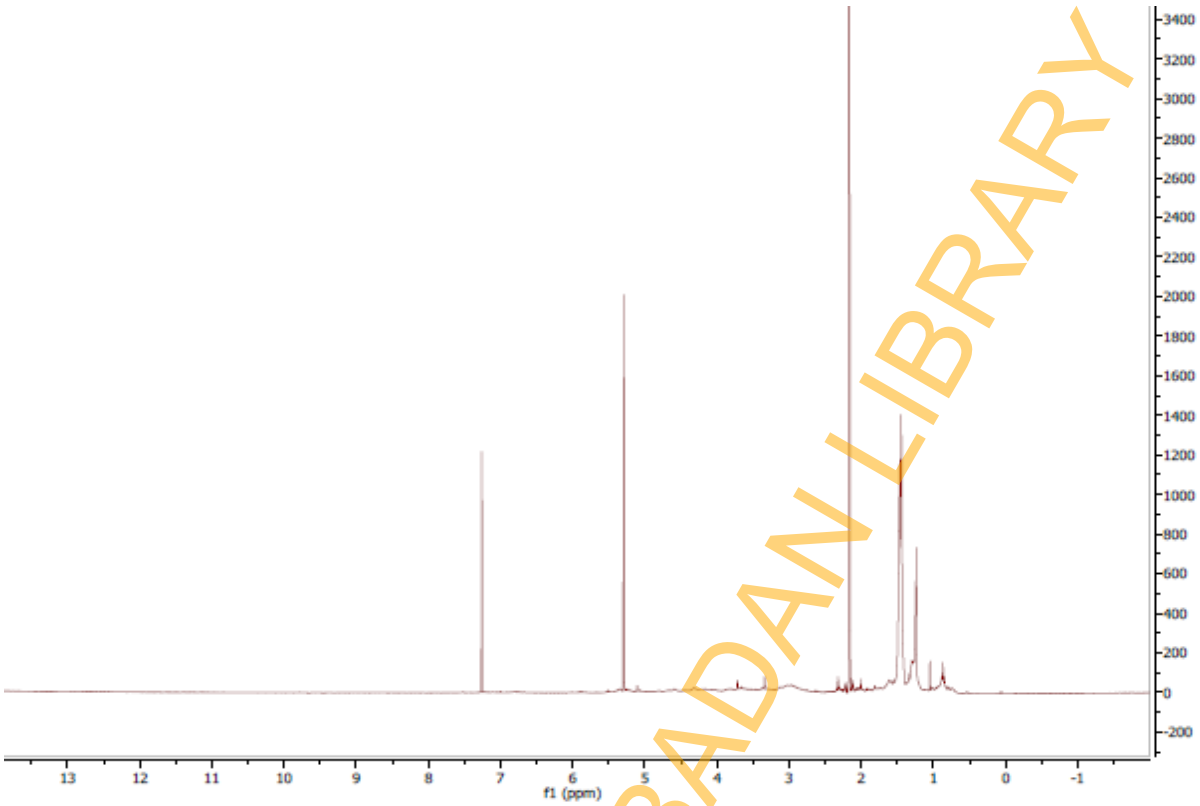


Fig 60: ^{1}H NMR Spectrum of BOC-MFCP

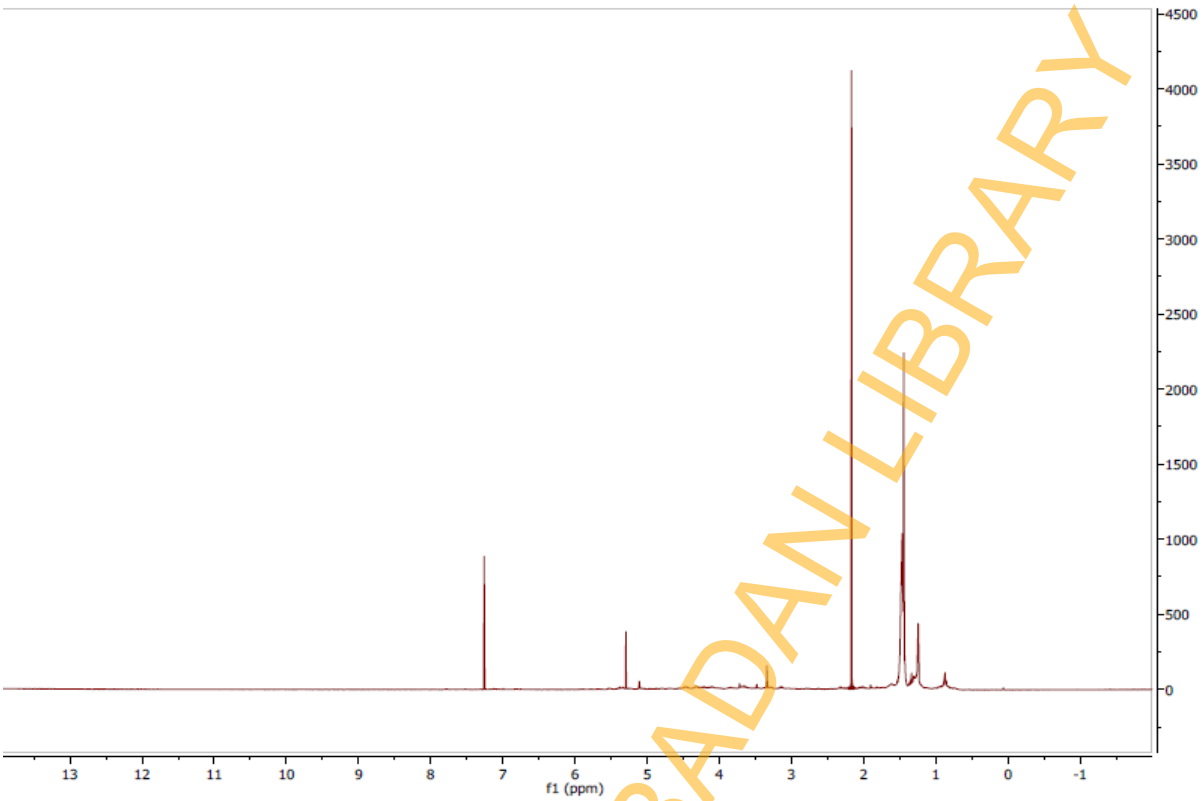


Fig 61: ^{1}H NMR Spectrum of Sub-BOC MFCP

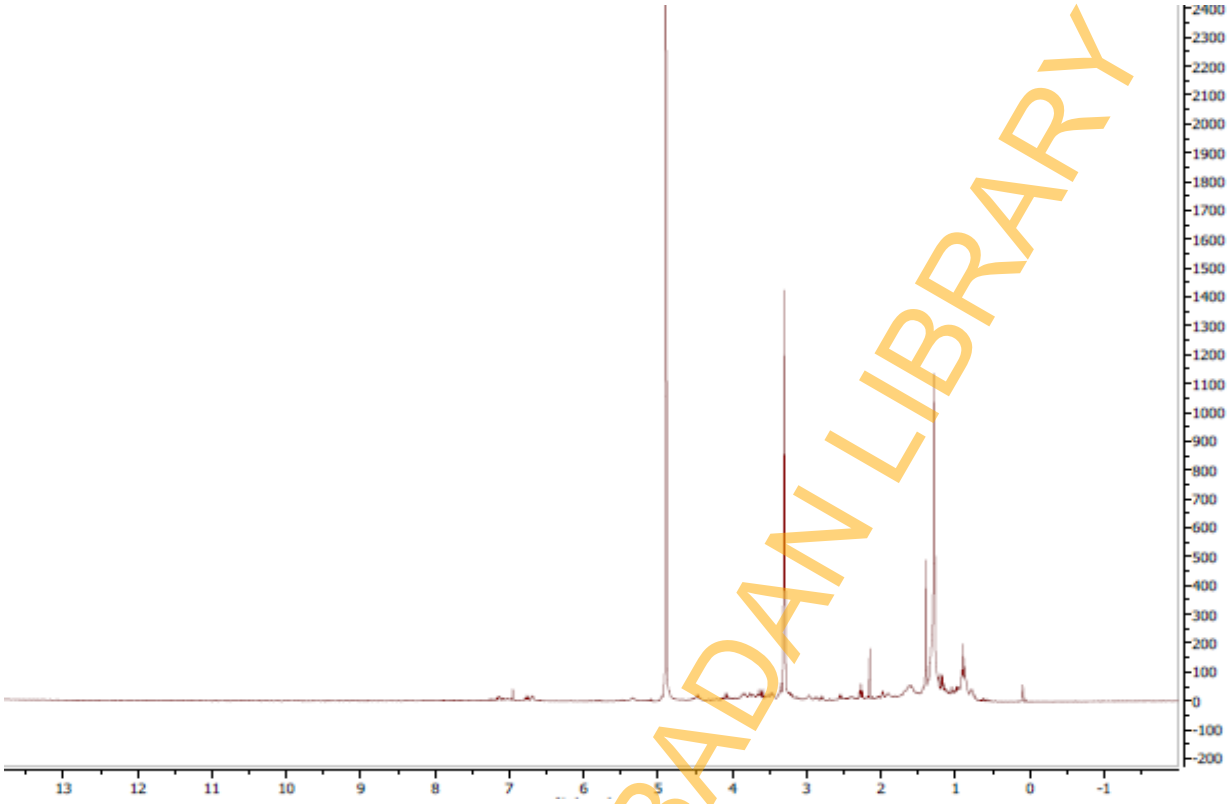


Fig 62: ^{1}H NMR Spectrum of Sub- MFCP (PPF-4) (Deprotected)

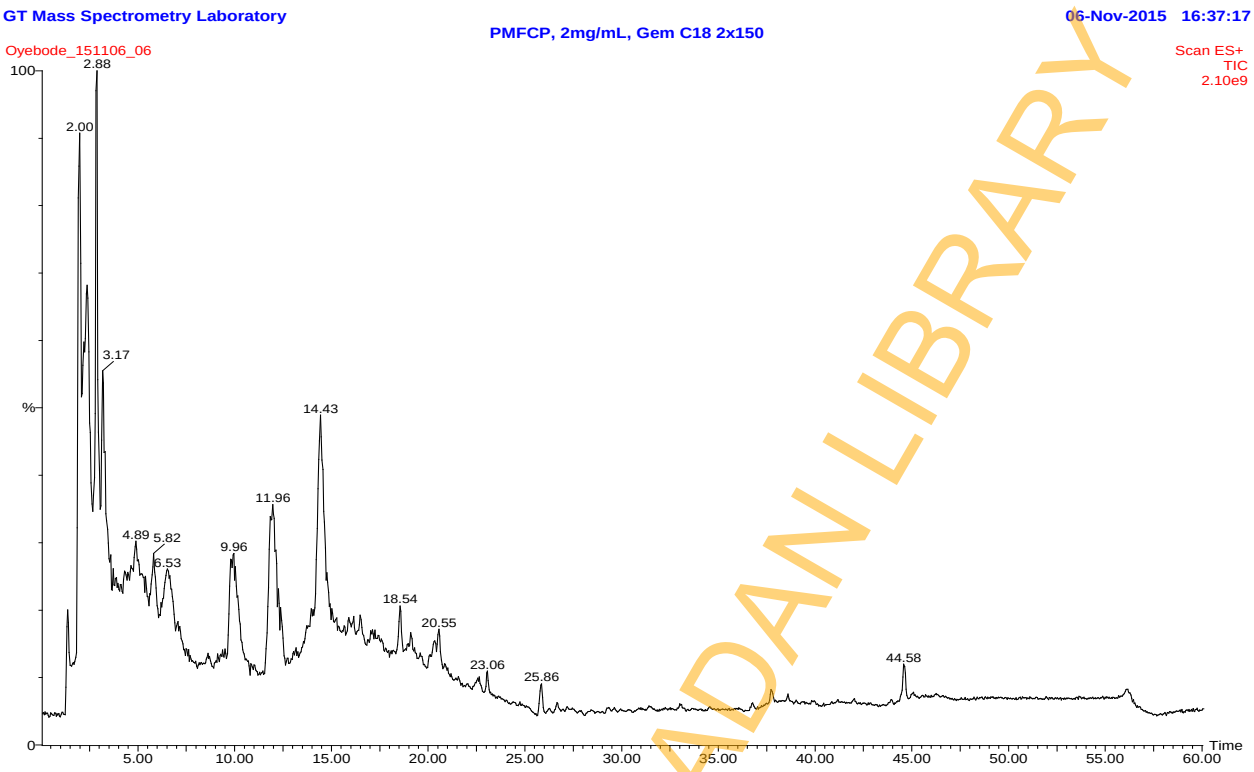


Fig 63: Total ion chromatogram (XIC) of PPF-3

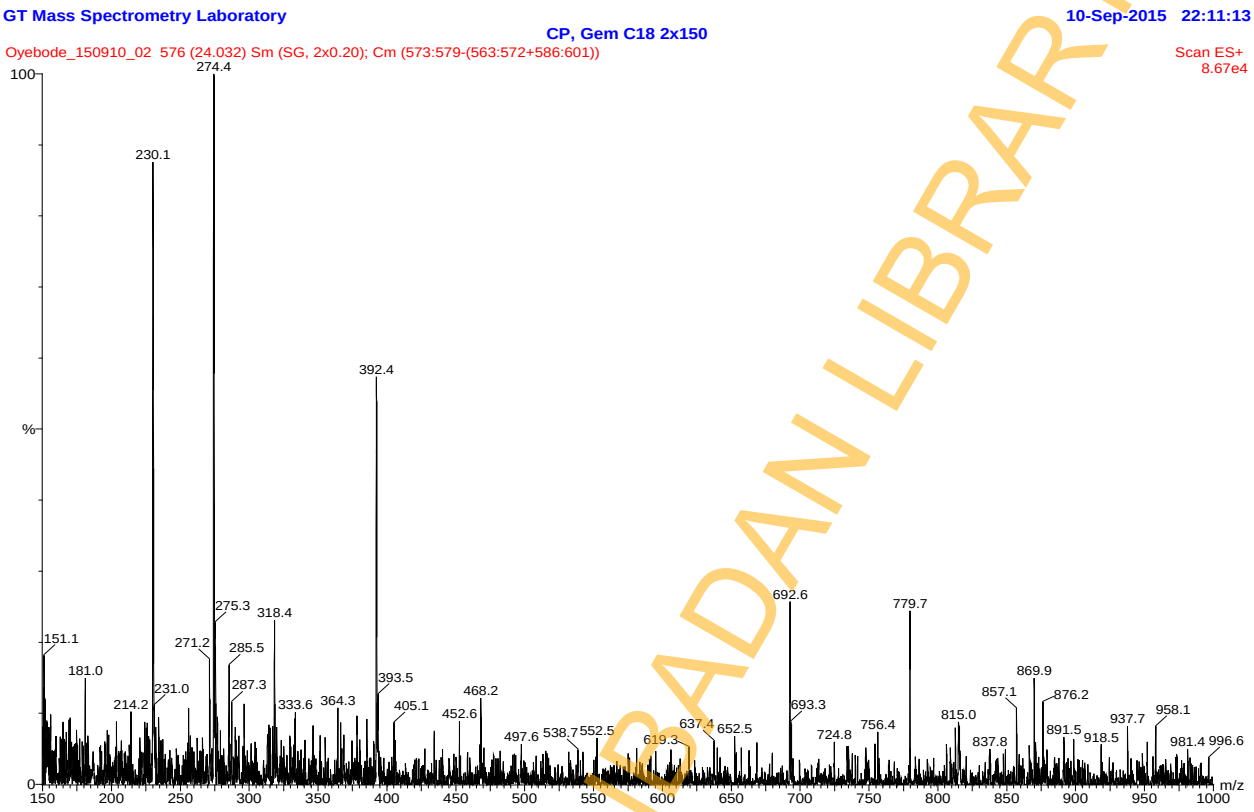


Fig 64: Extracted chromatogram from PPF-3 (positive-ion mode)

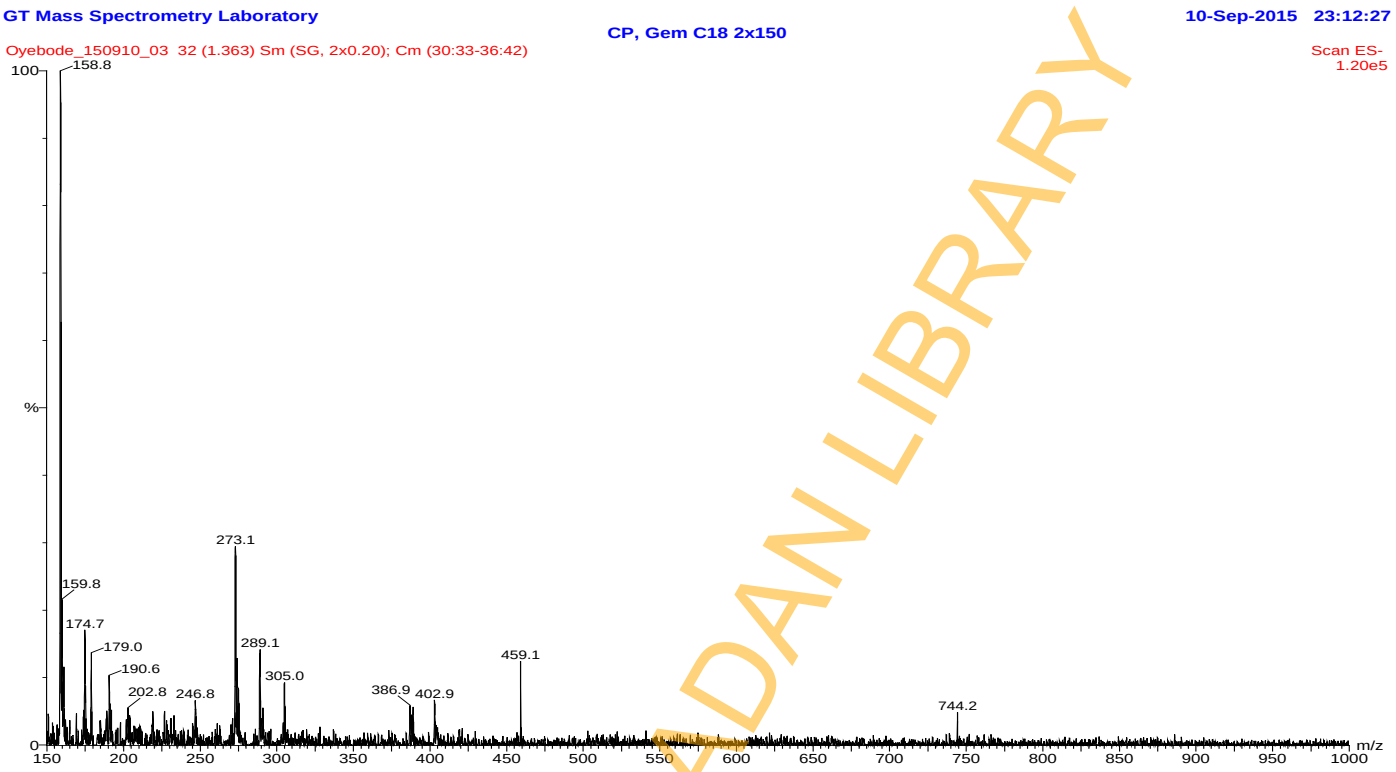


Fig 65: Extracted chromatogram from PPF-3 (Negative–ion mode)

EXPERIMENT 20: STRUCTURAL ELUCIDATION AND ANTI-PROLIFERATIVE POTENTIAL OF PURIFIED COMPOUND FROM CHLOROFORM FRACTION OF *C.PORTORICENSIS* (CFCP)

INTRODUCTION

Bioactive compounds in plants are secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes for compounds associated with plant growth and development, and are regarded as products of biochemical "side tracks" in the plant cells and not needed for the daily functioning of the plant. Several of them are found to hold various types of important functions in the living plants such as protection, attraction or signalling (Chikezie *et al.,* 2015). Given the ease of isolation of hydrophobic compounds, isolation of bioactive compounds from chloroform fraction of C. *portoricensis* was explored.

PROCEDURE

The chloroform fraction was obtained as earlier described. These were spotted on the TLC plates. The best solvent system was chosen via variations of the various constituents. The dissolved extracts were spotted on the plates and placed in the chromatographic tank containing the appropriate solvent system as the eluent. As the solvent ascended the plates, resolution of the constituents was made and the elution was terminated at about 1cm from the end of the plate. The plates were air-dried and activated where necessary. After this, the plates were labeled and then sprayed with the appropriate chromogenic agents.

Prostatic LNCaP cells were incubated with CFCP /vehicle (0.1% DMSO) for 24 h. Caspase-9 and 3 activities were assayed using a colorimetric substrate, Ac-LEHD-pNA and Ac-DEVD-pNA, respectively using Biovision Caspase kit (Biovision Incorporated, CA, USA). Assay was carried out according to manufacturer's instruction as described on page 99-101. Cleavage of the C-terminal peptide bond by the enzyme-released p-nitroaniline, the concentration of which was measured at 405 nm.

In order to determine the mass of the isolated compound, mass pectroscopywas employed to produce chemical compounds that would generate molecular fragments while measuring their mass to charge ratios (m/z). This property is unique for each compound (Glish and Vachet, 2003)

RESULTS

Figure 66a shows the TLC behaviuor of CFCP under UV light at 254nm, as seen fron the figure, a single compound spot was identified from the chromatogram. The isolaton of the single spot was achieved by preparatory TLC and it was designated pCFCP.

The TLC pattern of pCFCP is depicted in Figure 66b. As seen from the plate, the isolation of the single compound spot was achieved. The antiproliferative activity of pCFCP on prostate LNcaP cells is shown in Figure 66c. According to the results, pCFCP decreased cell viability with a lower IC_{50} of 74.6 µg/mL relative to 95.0 µg/mL obtained for in cells treated with crude CFCP.

Assessment of potency of pCFCP on induction of apoptosis is shown in Figure 67. Evaluation of apoptotic effects of pCFCP showed that it enhances caspase -3 activity at 80 µg/mL (IC_{50}) and 160 µg/mL IC_{50} by 1.2 and 1.6 folds, respectively.

Figures 68 and 69 are the proton MNR spectra and total ion chromatogram of pCFCP.

The mass and ^{13}C spectra of pCFCP are depicted in Figures 70 and 71. From the figure, ^{13}C spectra reveals the presence of 32 carbons present in the compound while the mass spectroscopy shows that the compound has a molecular weight of 409.3g/mol. Elemental analysis also revealed the presence of carbon, hydrogen and oxygen.

CONCLUSION

The CFCP contains potent chemicals that could elicit apoptotic effects.

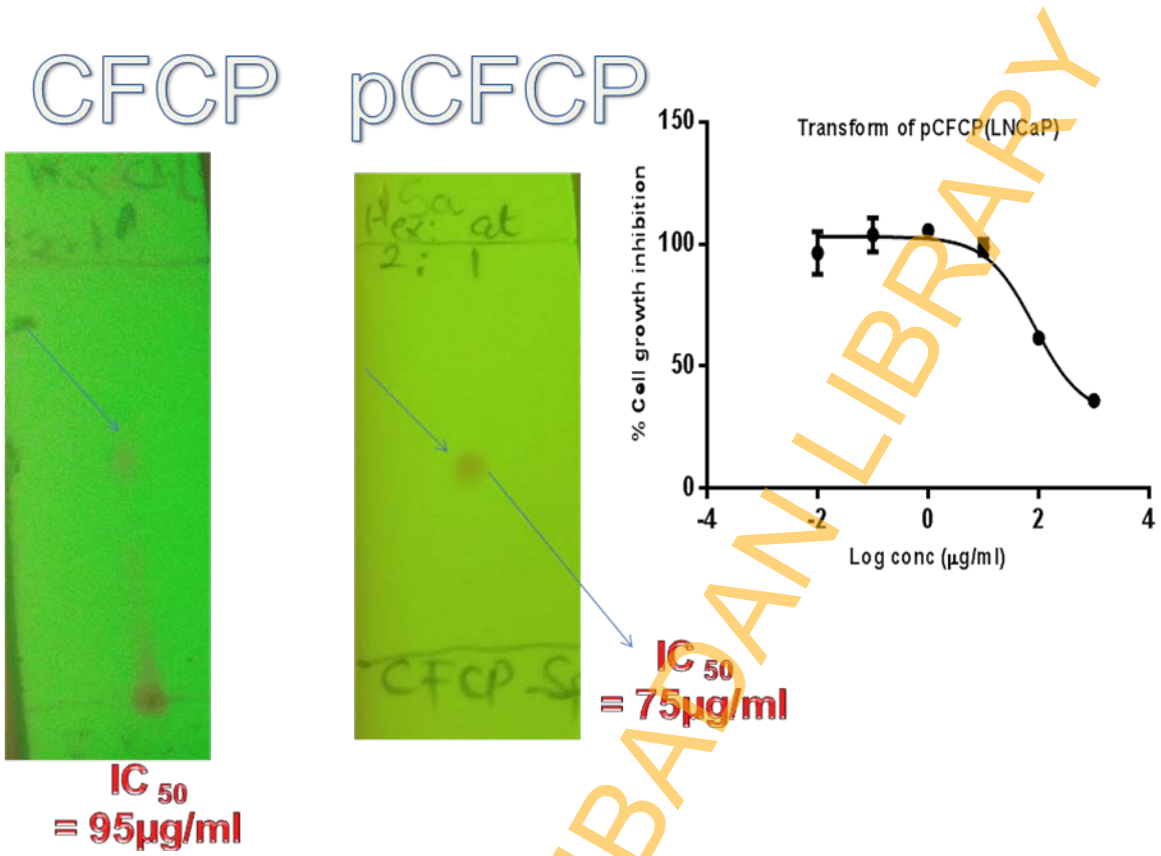


Fig 66: TLC patterns of fractions obtained from TLC and Prep-TLC of CFCP

Assessment of potency of the purified fraction of chloroform extract of *Calliandra portoricensis* on induction of apoptosis

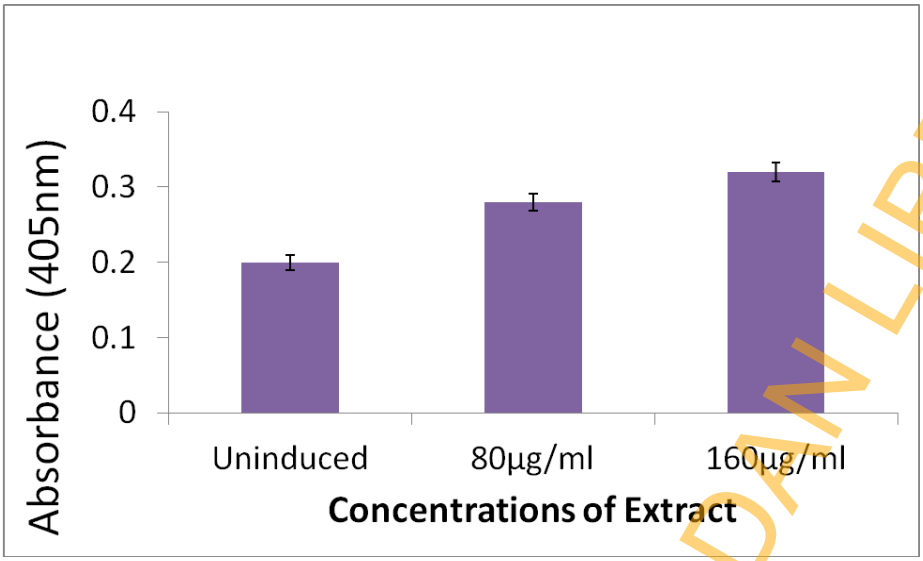


Figure 67: Effect of pCFCP on Caspase -3 activation.

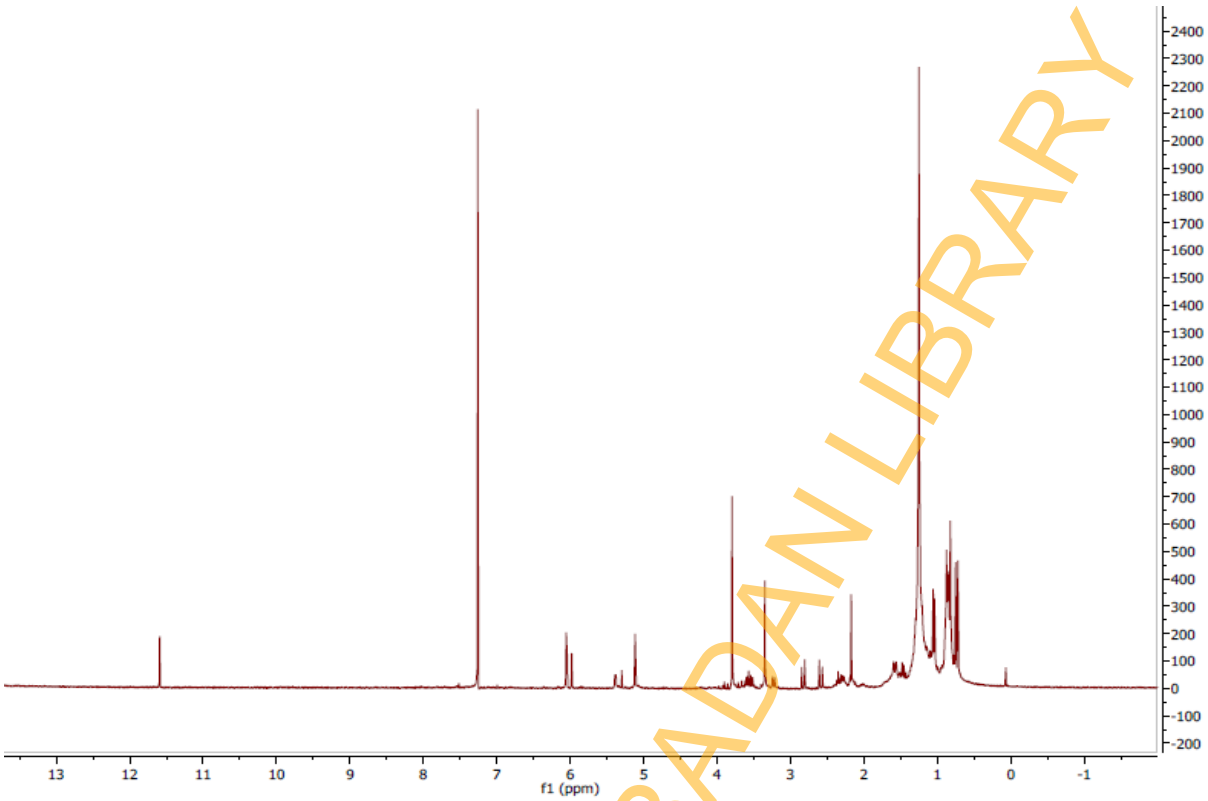


Fig 68: ^{1} I H NMR spectrum of pCFCP (Hydrophobic Compound)

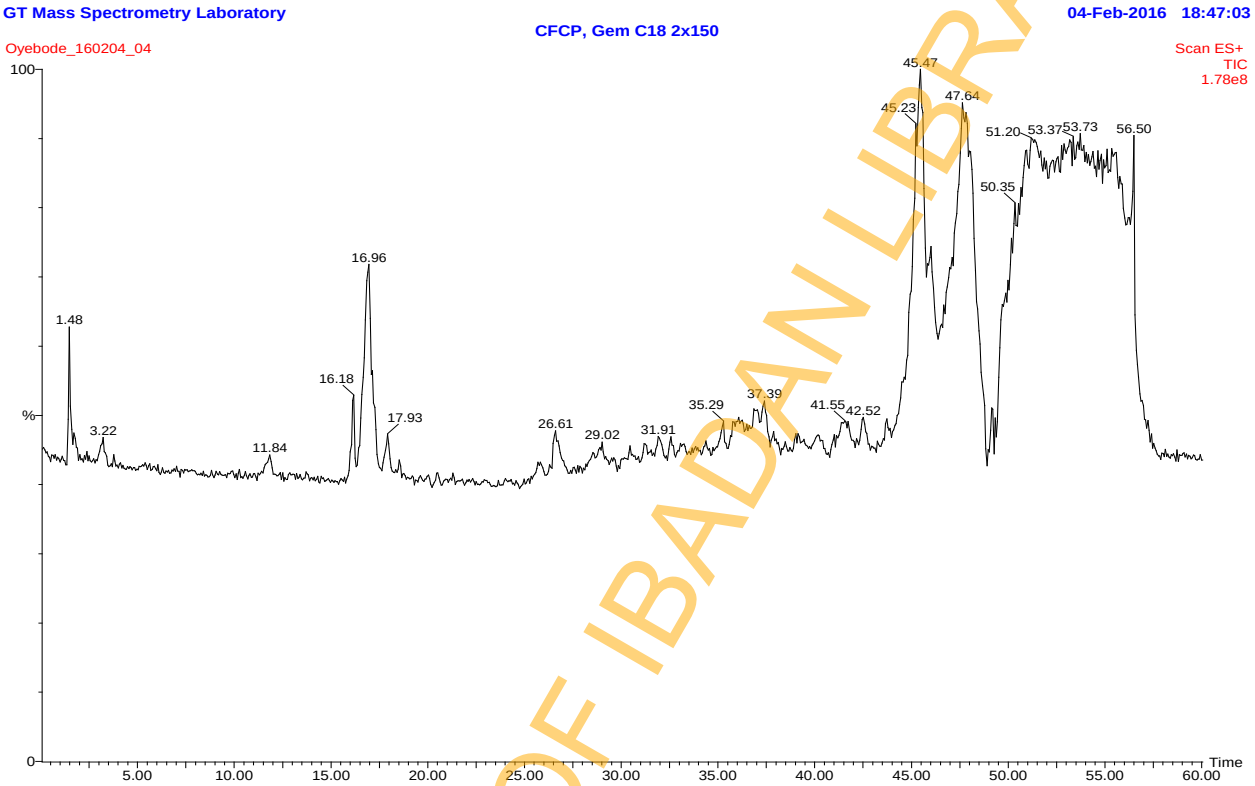


Fig 69: Total ion chromatogram of pCFCP (Hydrophobic Compound)

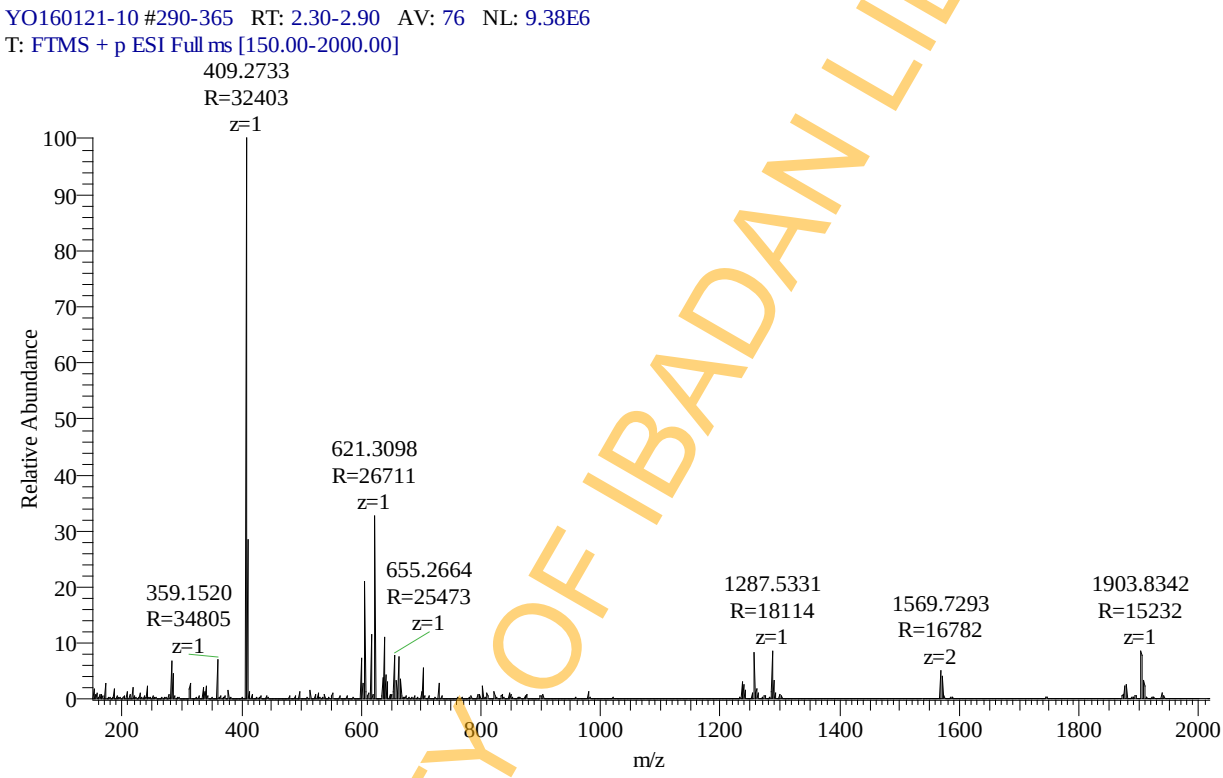


Fig 70: Mass spectrum of pCFCP (Hydrophobic Compound)

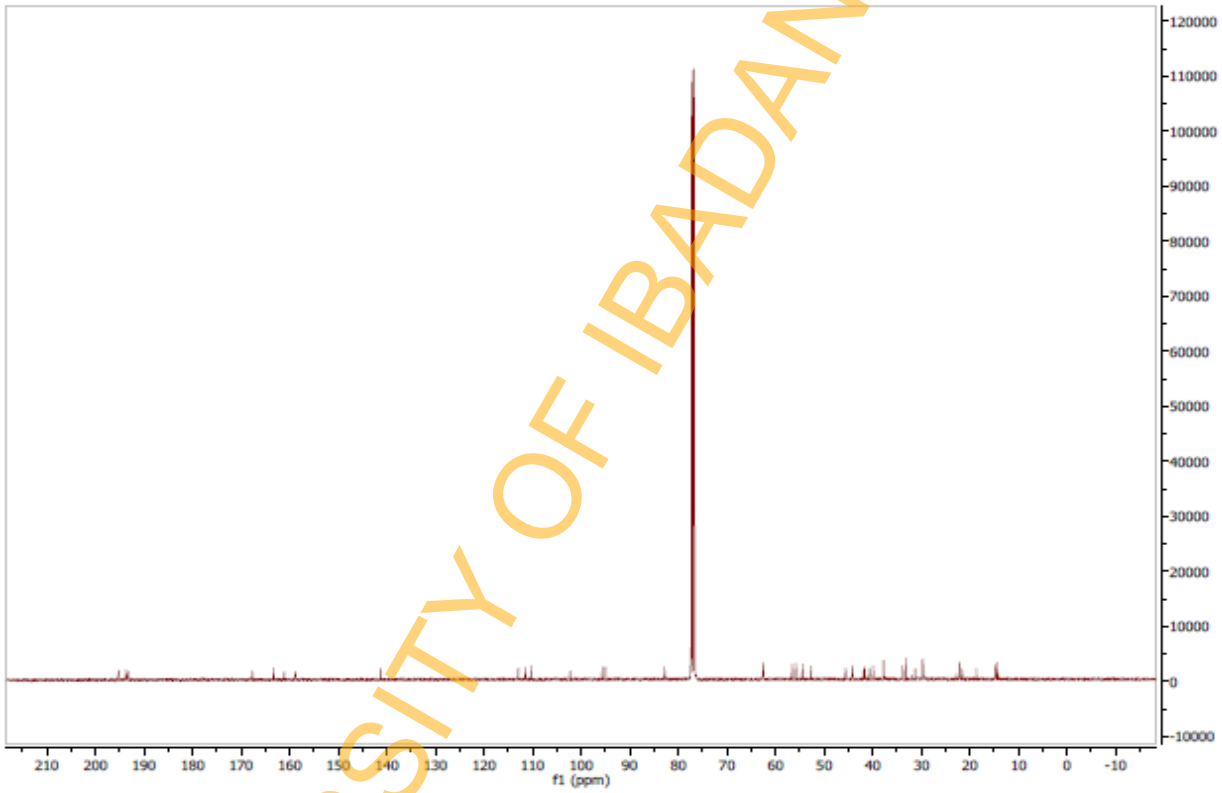


Fig 71: ^{13} C Spectrum of pCFCP (Hydrophobic Compound)

CHAPTER FIVE

5.0 DISCUSSION

Research has indicated that cancer remains the world second leading cause of death with prostate cancer rated the second most common cancer and sixth leading cause of cancer deaths among men globally (IARC, 2015). Sadly, there is evidence that African men suffer disproportionately from prostate cancer compared to men from other parts of the world (Adeloye *et al.*, 2016). Consequently, a dramatic change in the approach of cancer treatment has evolved over the years with the emergence of disease treatment via targeting of the molecular features of cancer. This has put an end to the age when surgery and radiotherapy were the only effective way to fight tumour growth (Urruticoech *et al.*, 2010).

At the molecular level, one of the hall marks of cancer is dysregulated apoptosis. Apoptosis plays a unique physiological role in tissue homeostaisis, embroyonic development and removal of deleterious cells from the whole organism. It is a process by which a cell is involved in it's own death, it affords the cell the privilege to put its house in order before its demise (Elmoore, 2007). On the other hand, dysregulation of the apoptotic process plays a pivotal role in all forms of diseases. In this regard, excessive apoptosis is one of the hall marks of tissue wastage while downregulated apoptosis is a major feature of all cancer cells. It is now well established that the mitochondrion is a central regulator of mammalian cell apoptosis (Clavier *et al.*, 2016) in addition to its vital function of promoting cell survivial via oxidative phosphorylation (Alam *et al.*, 2016) . Mitochondria therefore play a dual role in life and death.

The permeabilization of the mitochondrial outer membrane and release of apoptogenic proteins notably cytochrome *c* are undoubtedly central to the events in apoptosis. Mitochondrial permeabilization is due to the opening of the mitochondrial Permeability Transition (mPT) pore formed by a protein complex, a pore whose enduring interest is due to the role it plays in virtually all disease conditions (Brenner and Moulin, 2012).

Given the fact that chemotherapy results in drug resistance and loss of normal cells in addition to cancer cells, attention is being shifted to bioactive agents that specifically target cancer cells while sparing the normal ones. Compelling evidence indicate that dietary bioactive agents may trigger apoptosis through the mitochondrial-mediated pathway and other numerous molecular targets (Chen and King, 2005). Examples of promising dietary chemopreventive compounds include plumbagin, a plant-derived naphthoquinone, isolated from *Plumbago zeylanica*, which is used concurrently with *Calliandra portoricensis* in the traditional treatment of prostate emlargement. Besides, honokiol and oridnonin isolated from *Magnolia officinalis* and *Isodon rubescens* respectively, have been shown to restrain cancer by modulating targets for the apoptotic signalling pathways including caspases 3 and 8, Bcl-2/Bax ratio and cyclins (Millimouno *et al.*, 2014).

However, lack of scientific information to validate the rationale for the use of the herbal medication stimulated further investigation on the potential of *C.portoricensis* as a possible anticancer agent with particular emphasis on evasion of the apoptotic signal which is a hallmark in all cancer forms.

Phytochemical screening of *C. portoricensis* in our laboratory revealed that the root bark of *C.portoricensis* contains tannins, saponins, flavonoids and cardiac glycosides (Page 119). Although, certain flavonoids have been shown to induce mPT pore opening (Martin, 2006), the nature of the substances responsible for the effects of the plant extract on mPT pore are still unknown.

One commonly used method for the measurement of the opening of the mPT pore in isolated mitochondria involves the addition of extra-mitochondrial calcium that must first enter the mitochondria. This task is performed by monitoring the calcium–induced decrease in light scattering that reflects mitochondrial swelling accompanying calcium-induced opening of the mPT pore (Johnson and Lardy, 1967; Javadov *et al.*, 2009) . Using this method, we have shown that the mPT pore of the preparations employed in this study was susceptible to induction by the addition of extra-mitochondrial calcium and that like that of normal intact mitochondria, the induction of pore opening was reversible by spermine, a standard inhibitor of the openingof the mPT pore (Fig 15). This observation confirmed that the mitochondria were intact and not uncoupled, hence they were sutiable for further use.

The observations that crude MECP, in the absence of calcium had an inductive effect (13.8 folds) that was higher than that of calcium suggests that *C. portoricensis* contains bioactive agents that may interact with the pore or mitochondrial membrane and cause the release of cytochrome c and caspase co-factors that will subsequently activate signalling events leading to programmed cell death. Moreover, the finding that the inductive effect was enhanced in the presence of calcium suggests that calcium potentiated the effect of MECP.

Furthermore, the observation that the inductive effect of *C.portoricensis* was concentration-dependent suggests that the active component increased with increasing quantity of the fractions used and that the components of the plants may be interacting with specific components of the pore possibly cyclophilin D, (Baines *et al.*, 2005) which has been shown to be a major regulator of the pore, until saturation was obtained.

Although it is yet to be determined which of the active component(s) exerts this effect, it seems likely that the opening of the pore elicited by exposure to C. *portoricensis* to isolated mitochondria will subsequently lead to the release of cytochrome c and activation of executional caspases. This view is in consonance with previous observations that bioactive agents that alter mitochondrial membrane permeability and /or dissipate the mitochondrial potential could induce apoptosis. (Martin, 2000; Millimouno *et al.*, 2014). In this regard, the vanilloids found in turmeric and capsaicin found in chili peppers open the mPT pore and collapse mitochondrial potential leading to induction of apoptosis (Suh *et al.*, 2013). Also, the flavonoid baicilin induces apoptosis in T-lymphocytes by inducing cytochrome c release and disrupting mPT before activation of caspase 3 (Ueda *et al.*, 2001).

We have also shown in this study that methanol fraction of *C.portoricensis* (MFCP) in the absence of calcium had an inductive effect on the opening of the pore which was greater than that of calcium at higher concentrations (Fig 19). The observations that the inductive effects were enhanced even in the presence of calcium, and could be reversed almost totally by spermine, a standard mPT pore inhibitior, indicate that there was no disruption of mitochondrial membrane integrity while the induction of mPT pore occurred. These findings indicate clearly that the bioactive agents in these fractions are not interacting directly with the membrane but

rather acting in a manner to facilitate the opening of the mPT pore possibly by binding to contact sites on the pore where calcium occupies to facilitate induction.

The observation in this study that mitochondrial permeability transition was not affected by ethylacetate (EFCP) and chloroform fractions (CFCP) of *C. portoricensis* in the presence and absence of calcium is clearly due to the absence of the active principle in chloroform and ethylacetate fractions in comparison to methanol fraction. This suggests that the bioactive agents responsible for the opening of the pore are somehow highly polar constituents and hence could not dissolve in non and less-polar solvents such as chloroform and ethylacetate.

It has been established that opening of the pore results in reduction in efficiency of oxidative phosphorylation which impacts on decrease in energy levels in addition to dissipation of mitochondrial transmembrane electrochemical proton gradient, cessation of ATP synthesis and loss of respiratory substrates as well as nucleotides from the mitochondrial matrix (Kroemer *et al.*, 2007; Rasola and Bernardi, 2011).

Currently, researchers are proposing that mPT pore is the mitochondrial ATPase. A group of thought hypothesizes that the mPT pore forms at the interface between ATP synthase dimers or perhaps tetramers, (Alavian *et al.*, 2011; Bonora *et al.*, 2013; Alavian *et al.*, 2014; Morciano *et al.*, 2015) and there is another hypothesis that the mPT pore arises from the c-ring of the F_0 sector of the mitochondrial ATPase (Giorgio *et al.*, 2013; Carro *et al.*, 2014; Bernadi and Di Lisa, 2015). Although, both points of view are supported by experimental results and still have some weak points, these studies provide compelling evidence that the ATP synthase is required for proper mPT pore function. Therefore, in order to ascertain the involvement of mPT pore in mitochondrial ATP synthase, effects of fractions of *C.portoricensis* on mitochondrial ATPase

activity were investigated. The data obtained in this study showed that crude methanol extract (MECP) and methanol fraction (MFCP) of *C. portoricensis* enhanced mitochondrial ATPase activity to varying extents in male Wistar rats. The enhancement observed by MECP and MFCP were close to that of 2,4 dinitrophenol (DNP), a classical uncoupler of oxidative phosphorylation. Studies have shown that 2,4 DNP, a proton ionophore induces mitochondrial dysfunction and its toxicity causes death in humans (Miranda *et al.*, 2006). Moreover, Issekatz (1984) had stated that uncoupling of oxidative phosphorylation by 2,4 DNP may be due to its interference with the final energy production pathway by importing protons into the mitochondria and thus preventing the uptake of inorganic phosphate molecules into the mitochondria (Issekatz, 1984).

These results lay credence to our previous findings that these fractions (MECP and MFCP) induced opening of the mPT pore in rat liver mitochondria. It is also consistent with reports that mPT pore opening causes uncoupling of oxidative phosphorylation by inhibiting uptake of inorganic phosphate. This converts mitochondria to ATP consumers instead of producers, thus, leading to elevated levels of inorganic phosphate (Bernardi and Di Lisa, 2015). Of note is the key discovery that the mPT pore forms from ATP synthase (Giorgio *et al.*, 2009; Giorgio *et al.*, 2013; Alavian *et al.*, 2014) and this opens entirely new perspectives to the molecular definition of its role in pathophysiology. Our results further provide evidence that the status of the mPT pore is involved in the proper functioning of the mitochondrial ATP synthase.

It has been revealed that the signalling pathways that affect the transition of the ATP synthase from an energy-conserving to an energy-dissipating device are of great relevance to cell death and survival, and are exploited by cancer cells to boost their resistance to apoptosis (Bonora *et al.*, 2013)

The finding that EFCP and CFCP did not show significant enhancement of mitochondrial ATPase activity at all concentrations tested is in consonance with our previous reports on their insignificant effect on the opening of the mPT pore in rat liver mitochondria. It seems likely that the bioactive agents responsible for induction of pore opening are the same candidates interacting with the mitochondrial ATP synthase. Thus, this may be another pointer that attests to the view of Alavian and his collegues (Alavian *et al.,* 2011) that the ATP synthase may be an essential part of the mPT pore.

It is well established that the mitochondrion produces reactive oxygen species as "side products" of respiration (Zorov *et al.*, 2014). A decline in energy is common in aging, and the restoration of mitochondrial bioenergetics may offer a common approach for the treatment of numerous age-associated diseases (Szeto, 2014). Phospholipid bilayers of cellular and subcellular membranes are undoubtedly major targets for free radicals. Mitochondrial lipid, cardiolipin makes up 18% of the total phospholids and 90% of the fatty acyl chains are unsaturated. Oxidation of cardiolipin may be one of the critical factors in initiating apoptosis by liberating cytocochrome c from the mitochondrial inner membrane and facilitating Mitochondrial Outer Membrane Permeabilization (MOMP) (Shi, 2010). Changes in cardiolipin not only alter fluidity and folding of the IMM, but can profoundly alter the organization and function of respiratory complexes (Szeto, 2014).

Given the role of reactive oxygen species as mediators of aging and age-related diseases, it became important to determine if fractions of *C.portoricensis* utilize mitochondrial membrane peroxidation as a probable mechanism of promoting mitochondrial dysfunction.

Incubation of mitochondria in the presence of $FeSO_4$ causes a significant increase in lipid peroxidation due to the fact that some metals have a strong catalytic power to generate highly reactive hydroxyl radicals via the fenton reaction mechanism. The effects of fractions of *C. portoricensis* on iron-induced lipid peroxides were tested using the method of Ruberto *et al.*, (2000) using mitochondria as lipid rich source.

Our results revealed that varying concentrations (100-800µg/mL) of both MECP and MFCP strongly inhibited generation of mitochondrial lipid peroxides in a concentration–dependent manner. The CFCP and EFCP displayed moderate and minimal inhibitory effects respectively, in this regard.

Inhibition of mitochondrial lipid peroxidation by fractions of *C.portoricensis* could be related to the presence of phenolic compounds, which accounts for the antioxidant activity of natural plant products and which have been shown to be free radical terminators (Nijveldt, 2001).

Interestingly, the finding that fractions of *C. portoricensis* inhibited generation of mitochondrial lipid peroxides indicate their protective effect on membrane bilayers by shielding the mitochondrial membranes from free radical induced severe cellular dysfunction. Moreover, it suggests that the mechanism of induction of pore opening is not via generation of reactive oxygen species that could lead to peroxidation of mitochondrial membrane lipids but rather via interaction with components of the pore. Hence, our results rules out mitochondrial membrane peroxidation as a possible mechanism for mitochondrial membrane permeabilization by fractions of *C.portoricensis.*

Phytochemicals have been shown to intercept or terminate lipid peroxidation by chelating metal ions, such as copper or iron (Armida *et al.*, 2005). The phytochemicals present in fractions of *C.*

portoricensis may also be acting by complexing iron to prevent the generation of ROS aside fron being antioxidants. This is in consonance with the findings of Armida and other colleagues (2005) who stated that one of the ways phytochemicals elicit their antioxidant activity is by complexing metal ions.

The inductive effect of the potent MFCP on mPT pore in *vitro* in rat liver mitochondria has been well established in this study. Given that the plant decoction is normally ingested, it is necessary to determine whether MFCP on its own can induce pore opening *invivo* and if the signalling pathways of apoptosis are involved in the real mechanism of interaction of MFCP.

Owing to the relevance of bioavailability of the compound of interest at the target site and to determine the safety dose regimen for the potent methanol fraction of *C. portoricensis (MFCP),* male abino rats were orally admistered different doses of MFCP for 21 and 30 days while control animals received distilled water accordingly.

Mitochondrial permemability transition was assessed spectrophotometrically in the animal subjects at 540nm. The results obtained showed that the intergrity of the mitochondria isolated from control animals were not compromised thus, they were suitable for further use.

After 21 days of oral administration of MFCP, the methanol fraction showed no ability whatsoever in inducing mitochondrial permeability transition (mPT) at all doses tested. This might be due to poor oral bioavailability of the active principle of the fraction which is a potential drawback associated with dietary compounds after ingestion (Bansal *et al.*, 2011) . However, after 30 days of administration (Figure 29), MFCP induced mPT pore opening in the absence of calcium at doses 100 and 200 mg/kg with induction folds of 2.6 and 3.3, respectively while there was no induction of mPT pore opening at doses lower than 50 mg/kg. This inductive

effect, though at higher doses, is in consonance with the reported *in vitro* data. These results suggest that at higher doses of methanol fraction, the active compound was available at the target site to interact with the components of the mPT pore in order to ellicit inductive effect.

Furthermore, the data revealed that oral administration of higher doses of MFCP for 21 (200 and 400mg/kg) and 30 days (100 and 200 mg/kg) potentiated calcium-induced opening of the pore. This suggests that methanol fraction ellicited synergistic effect with calcium in inducing the opening of the mPT pore.

In order to determine the risk of toxicity associated with exposure to high treatment concentrations of MFCP, enzymatic, haematological and histological profiles of animal subjects were screened for hepatotoxicity. The hematocrit or packed cell volume (PCV) is a part of the complete blood count (CBC) and denotes the percent of the whole blood that is composed of red blood cells. A reduced value denotes anemia (of any cause) while a high hematocrit value may indicate dehydration, increased red cell production (erythrocytosis due to any cause) or malignant proliferation of red cells (polycythemia). White blood cells (WBC) play a vital role in the body's immune defense against disease. The number of WBC may be reduced or increased depending on the disease condition or reaction occurring in the body, such reactions could be normal or abnormal (McKenzie *et al.*, 2005).

The effects of methanol fraction on haematological parameters after oral administration were investigated. It was seen that the animals tolerated the fraction up to 50mg/kg after which decrease in levels of all haematological parameters (WBC, PCV, Hb, and RBC) under investigation was observed, after 21 days of oral administration. Moreover, cell counts were adversely affected at the highest dose (200mg/kg) compared with control and other treated

groups (25, 50 and 100mg/kg) after 30 days of administration. The decrease in haematological parameters observed may be associated with toxicity of the fraction at these doses. These findings, suggests that MFCP could have hematotoxic effects which are also highly dependent on the dose and duration of administration.

Meyer and Harvey (2004) had stated that serum enzyme levels could be indicative of liver fuction. Our results showed that MFCP caused significant increases in the serum ALT and AST activities at 200 and 400 mg/kgbw dosage groups after twenty one days of administration. However, serum AST and ALT levels were significantly elevated after thirty days of administration at all the test groups used for this study. These results suggest that MFCP may result in hepatic damage. However, the extent of damage is highly dependent on the dose and duration of administration. Hence, use of doses within therapeutic limits is essential especially in the traditional treatment and indiscriminate use of plant decoctions and extracts should be discouraged.

Histopahtological examination of liver, kidney and prostrate of animals orally admistered MFCP for 30days was determined after fixing in 10% formalin. Severe diffuse vacuolar degeneration of hepatocytes was observed in livers of animals that received the highest dose (200mg/kg) of MFCP. Similarly, protein casts were also abundant in kidney of animals that received the highest dose of 200mg/kg, which suggests that the kidney tubules may be leaky to proteins, while no visibe lesions were observed in kidney of animals that received doses 25, 50 and 100mg/kg MFCP. Furthermore, distortion of the entire prostate gland as well as necrotic debris was observed in the prostates of animals that received the highest dose of 200mg/kg. Taken together,

these observations suggest that methanol fraction of *C. portoricensis* could be toxic at higher concentrations and hence should be taken with caution.

Evidence abound that sustained mPTP opening causes cell death via a cytochrome c-Apaf-1 (Apoptotic Protein Acivating Factor-1)-dependent conformational change leading to the fornation of apoptosome, which becomes a platform on which a caspase-cascade reaction is triggered (Rasola and Bernadi, 2011). This is accompanied by loss of ionic homeostasis, matrix swelling, outer membrane rupture, and the loss of proteins from the inter-membrane space, the so-called pro-apoptotic factors. These events result in the triggering of the pro-apoptotic pathway mediated by the cascade of the caspases whose activity together with the mitochondrial bioenergetic collapse and redox catastrophe finally leads to cell death. The mPT is therefore a decisive event which defines the line between survival and death and the mitochondrial membranes constitute the battleground on which opposing signals combat to seal the fate of the cell (Dalla Via *et al.*, 2014). Hence, mPT pore activation stimulates apoptosis and prevents the differentiation of many tumor cells. Given that methanol fraction after being ingested (*in vivo)* ellicits inductive effects on the mPT pore, it would be instructive to determine the involvement/ modulation of apoptotic proteins. Levels of pro- and anti- apoptotic proteins, Bax and Bcl-2 were assessed in cancer cell lines because these proteins are major players in the regulation of mitochondrial-mediated apoptosis. First, we determined the optimum concentration of MFCP that will inhibit the proliferation of prostatic tumour cells, LNCaP (androgen-sensitive) and DU-145 (androgen-insensitive).

Metabolically active cells reduce colourless or weakly coloured tetrazolium salts to coloured derivatives known as formazans to distinguish live cells from their dead counterparts (Riss *et al.*, 2013). In order to study the effect of fractions of *C.portoricensis* on cell growth, cell viability was measured by addition of MTS, a tetrazolium compound and the development of a purple-coloured formazan product was monitored at 490nm. Our results showed that prostate LNCaP cells (2.4µg/mL) were more susceptible to inhibition by methanol fraction than prostate DU-145 cells (3.3µg/mL) as indicated by its lower IC_{50} which translates to a higher potency.

Prostate cancer is dependent on circulating testosterone in its early stages and is treatable with radiation and surgery. However, recurrent prostate tumors advance to an androgen-independent state in which progression occurs in the absence of circulating testosterone, leading to metastasis and death (Heinlein and Chang, 2004). During the development of androgen independence, prostate cancer cells are known to increase intracellular testosterone synthesis, which maintains cancer cell growth in the absence of significant amounts of circulating testosterone. Overexpression of the androgen receptor (AR) occurs in androgen-independent prostate cancer and has been proposed as another mechanism promoting the development of androgen independence (Hong *et al.*, 2008).

The observation that androgen–expressing (LNCaP) prostate cancer cells are more responsive to MFCP than androgen refractory cells (DU-145) suggests that methanol fraction of *C.portoricensis* may disrupt androgen receptor signalling. Although, the mechanism through which MFCP does this is not known, however, studies have shown certain compounds could downregulate androgen receptor expression or may disrupt pathways dependent on AR signalling (Xing *et al.*, 2001). In this regard, Hong *et al.*, (2008) reported that pomegranate

polyphenols down-regulate expression of androgen-synthesizing genes in human prostate cancer cells overexpressing the androgen receptor. Inhibition of growth of LNCaP prostatic cells by MFCP may be of particular importance in androgen-independent prostate cancer cells and the subset of human prostate cancers where AR is up-regulated.

One of the biggest mysteries in cancer research remains why mutations in certain genes cause cancer only at specific sites in the human body. The poor correlation between the expression level of a cancer gene and the tissues in which it causes malignant transformations raises the question of which factors determine the tissue-specific effects of a mutation (Schaefer and Serrano, 2016).

Some genes are associated with tumorigenesis in a broad variety of tissues (general-cancer genes) while others are mutated only in very few cancer types (specific-cancer genes). The lack of agreement between expression and pathology location suggests the presence of other factors contributing to the tissue specificity of cancer genes. Different physiological scenarios likely contribute to the tissue specificity of cancer genes (Sieber *et al.*, 2005). Factors responsible for such include oncogenic transformations which might be driven directly by the inactivation or constitutive over-activation of the specific function in the disease tissue.Cellular characteristics (e.g., sensitivity to apoptosis or rate of cell division) might also affect the impact of a mutation on a cell. Besides, in unaffected tissues, another gene might compensate for the loss or activation of a cancer gene, while the compensating gene might be not expressed or inactive in the affected disease tissue (functional redundancy and compensation) (Schaefer and Serrano, 2016).

In agreement with the idea that cell-intrinsic differences could explain the cell type-specificity of cancer genes. An example of a general-cancer gene is p53, which has been found mutated in the

majority of cancer types (Lawrence *et al.*, 2014). In contrast, BRCA1 is an example for a specific-cancer gene: germline loss-of-function mutations in this important double-strand DNA repair protein increase the risk by up to 80% for breast and ovarian cancers but to a much lower rate for other cancer types (Friedenson, 2005).

In order to investigate cell-type specificity and potency, lung adenorcarcinoma cells (A549) and healthy kidney VERO cells were treated with varying concentrations of the methanol fraction of *C. portoricensis.*

Our results revealed that inhibition of A549 cells was achieved with IC50 value of 3.64µg/mL when treated with varying concentrations of methanol fraction. The observation that methanol fraction clearly inhibited the growth of lung adenocarcinoma cells (A549) indicated that MFCP is not cell-type specific as it could also inhibit the growth / proliferation of other cancer cells in particular, A549 cells. Moreover, the findings that MFCP was about 6-fold less toxic in healthy kidney VERO cells (IC_{50} = 17.86 µg/mL) than in cancer cells suggest that MFCP contains anticancer agents that may be cancer-cell selective.

Cytotoxic effects of chloroform (CFCP) and ethylacetate fractions (EFCP) on prostate LNCaP cells were also investigated to correlate our data on mitochondrial permeability transition with inhibition of grwith of cancer cells. The cell growth inhibition data showed that CFCP and EFCP decreased cell viability with IC_{50} of 95 and 221µg/mL, respectively. These suggest that potent anticancer agents are abundant in methanol fraction (MFCP) than in EFCP and CFCP. This is in agreement with our previous data that showed that these fractions (CFCP and EFCP) had no effect with respect to opening of the pore. This points to the fact that opening of the mPT pore, a consequence of mitochondrial matrix swelling and permeabilization of the outer

mitochondrial membrane is a reliable assay for assessment of cell death. This is particularly in order since a cross talk exists between extrinsic and intrinsic pathway of apoptosis (Roy and Nicholson, 2000) with the mitochondria amplifying signals from the cell surface through members of the Bcl-2 family. These data provide evidence to support the fact that induction of pore opening (mitochondrial-mediated pathway) is a valid mechanism that leads to cell death.

Methanol fraction having demonstrated positive responses is considered an active lead for bio-assay guided fractionation. In order to isolate a potent compound from MFCP, partially purified fractions were obtained by thin layer chromatography and their ability to inhibit cancer cell growth was assessed. Our results show that partially purified fractions 1 and 2 decreased cell viability with IC_{50} of 6.32 and 5.74µg/mL respectively in prostate LNCaP cells. The potency of these fractions was equally found to be lower in DU-145 cells compared to that of the parent methanol fraction with IC_{50} value of 2.37µg/mL. However, our results showed that addition of equal concentrations of partially purified fractions 1 and 2 (PPF 3) gave a lower IC_{50} of 4.16µg/mL in prostate LNCaP cells and DU-145 cells. These translate to a better potency compared with their individual inhibitory activities; hence PPF3 may be employing synergistic effect in decreasing number of viable cells in androgen -sensitive and refractory cancer cells.

According to the Mitchell's chemiosmotic theory of oxidative phosphorylation, the respiratory chain converts redox energy into an electrochemical gradient of protons (proton-motive force) (Mitchell, 1961) , which subsequently drives ATP formation from ADP and inorganic phosphate by the ATP synthase. The proton motive force is a thermodynamic potential that develops across the inner mitochondrial membrane is composed of two components: electrical membrane potential ($\Delta\Psi$) and the difference between the cytosolic and matrix pH (ΔpH).The membrane potential is the main driving force for ATP production and depicts the status of the cell's health

(Dzbek and Korzeniewski, 2008). In view of the fact that methanol fraction prevented uptake of inorganic phosphate for ATP production, a process that is dependent on the cells mitochondrial membrane potential, the effecs of MFCP on ΔΨ was investigated via the ability of the cells to take up the mitochondrial dye JC-1 into the mitochondria or remain in the cytosol. When the transmembrane potential is low, as in many cells undergoing apoptosis, JC-1 exists as a monomer and produces green fluorescence while healthy cells exhibit red aggregate forms of the dye (Perry *et al.*, 2011).

Carboxy cyanide 4-fluoro trimethoxy phenylhydrazone (FCCP) is an uncoupler of oxidative phosphorylation and was used as a depolarization control in this study. FCCP transports protons into the mitochondrial matrix via a pathway other than the proton pore of the ATP synthase thereby uncoupling nutrient oxidation from ADP phosphorylation(Kenwood *et al.*, 2014). Our results showed that percentage of LNCaP cells exhibiting monomeric forms of the dye (cytosol) was high in the FCCP-treated cells indicating that the cells had ungergone apoptosis. Prostate LNCaP cells treated with the vehicle (0.1% DMSO) displayed higher percentage of cells exhibiting JC-1 aggregates (mitochondria). This indicates that the cells' had intact mitochondria membrane hence they emitted red JC-1 fluorescence. However, when mitochondria were preincubated with parent methanol (MFCP) and its partially purified fraction 3 (PPF3) for 72 hours, a significant reduction in mitochondrial membrane potential was observed as indicated by the JC-1 dye fluoresecent intensity ratio (aggregates: monomers). The observation that methanol fraction caused a significant decrease in mitochondrial membrane potential in prostate LNCaP cells reveals that methanol fraction dissipates mitochondrial membrane potential in these cells.

This agrees with the fact that a decrease in the $\Delta\psi_m$ is considered as one of the earliest events in apoptosis and is mediated by the opening of the mPT pore.

It is now well established that the release of cytochrome c to the cytosol is a point of no return in the apoptotic process, owing to the fact that cytosolic cytochrome *c* combines with monomeric apoptotosis activating factor -1(APAF-1) in order to assemble the apoptosome which becomes a platform for the activation of caspase 9 which subsequently initiates a caspase cascade involving caspases 3 , 6 and 7 (Elmore, 2007; Kroemer *et al.*, 2007; Tait and Green, 2010). Furthermore, determination of cytochrome c release was investigated to ascertain if collapse in mitochondrial membrane potential resulted in the elevated levels of cytochrome c. In this study, cytosolic cytochrome c levels were measured by subjecting cell lysates to Enzyme Linked Immunosorbent Assay (ELISA) for cytochrome c following exposure of prostate LNCaP cells to varying concentrations of MFCP and PPF 3 (2.5, 5 and 10 µg/mL).

According to the results, increase in cytochrome c levels was observed in cell lysates of cancer cell lines treated with parent methanol and its partially purified methanol fraction 3 (PPF 3). In this regard, MFCP and its partially purified fraction (PPF 3) caused significant ($p < 0.05$) increases in cytchrome c release at 10µg/mL with about 4.2 and 3.6 folds, respectively. However, no significant increase was observed at 2.5µg/mL in cells exposed to the partially purified fraction (PPF 3).

The fact that levels of cytochrome c increased with increase in concentrations of the methanol fraction at 2.5, 5 and 10 µg/mL suggested that the collapse of mitochondrial membrane potential elicited by MFCP is accompanied by release of the apoptogenic protein, cytochrome c. Further more, the finding that there was no significant increase in levels of cytochrome c release in prostate LNCaP cells treated with PPF 3 at 2.5 µg/mL could be associated with its IC_{50} value of

4.16µg/mL in prostate LNCaP cells. Hence, it could be inferred that its half maximum inhibitory concentration (IC_{50}).has not been reached.

One of the hallmarks of classical apoptosis is the activation of unique cysteine-dependent aspartate-specific proteases, otherwise called CASPASES ,a family of protease enzymes that have critical roles in controlling homeostasis in apoptosis and inflammation processes (Shalini *et al.,* 2015), hence plays an important role in determining the cells fate. It is believed that caspases are present as inactive monomeric precursor enzymes that must be dimerized for full activation before mediate most of their activity by cleaving their target proteins (McIlwain *et al., 2*013). Caspases promote cell death and it would be expected therefore that loss of caspases activity promotes tumor development.

In order to define a downstream event in MFCP-induced apoptosis since methanol fraction induced the release of cytochrome c in prostrate LNCaP cells, investigation of its effect on the activation of initiator caspase 9 and executioner caspase -3 were determined using colorimetric substrate, Ac-LEHD-pNA and Ac-DEVD-pNA, respectively.

The data obtained demonstrate that methanol fraction induced the activiation of caspases 9 and 3 in a concentration –dependent manner. The findings that methanol and its partially purified fractions (PPF 3) activated caspase 9 and 3 activites in prostate LNCaP cells after a 24-hour treatment confirms caspases 3 and 9 are activated in response to exogenous cytochrome c and that cytochrome c is a point of no return for the apoptotic process. The release of cytochrome c is therefore an important event in methanol fraction caspase –mediated apoptosis. Further more, increase in caspase activity correlated very well with increase in concentration of the fraction.

This is in agreement with the findings of McIIwain *et al.,(* 2013) which confirm that caspases are central to the execution of the apoptotic process.

Detection, analysis and separation of phytochemicals are usually achieved via thin layer chromatography, a common and efficient technique for separation and isolation of plant bioactive constituents (Lade *et al.,* 2014). It is estimated that 60% of analyses are performed based on TLC worldwide (Maitland and Maitland, 2010).

Fractions of *C.portoricensis* were sprayed with chromogenic stains anisaldehyde and ninhydrin after developemtn on thin layer chromatography in order to monitor bioactive compounds present. The resuls shows that MFCP developed a purple coloration to the ninhydrin stain which suggests the presence of primary amines while all fractions (MFCP, CFCP, and EFCP) of *C. portoricensis* tested positive to the anisaldehyde stain which reveals the presence of terpernes.

A cell undergoing apoptosis demonstrates multitude characteristic morphological and biochemical features, which vary depending on the inducer of apoptosis, cell type and the "time window" at which the process of apoptosis is observed. In view of the fact that, the gross majority of apoptotic hallmarks can be revealed by flow and image cytometry, cytometric methods have become a technology of choice in diverse studies of cellular demise (Wlodkowic *et al.,* 2011). A universal phenomenum accompanying apoptotic cell death is a change in plasma membrane structure via surface exposure of phosphatidylserine (PS) independent of the species, the cell type or the apoptosis induction system used, while the membrane integrity remains unchallenged (Arur *et al.,* 2003). PS can easily be detected by staining with a fluorescent conjugate of Annexin V, a 35-36 kDa Ca^{2+} - dependent protein that has a high affinity for PS.

Annexin V may be conjugated to fluorochromes such as FITC and Phycoerythrin (PE), or to biotin or tagged with EGFP (Enhanced Green Fluorescent Protein). These formats retain their high affinity for PS and thus serve as sensitive probes for flow cytometric analysis of cells undergoing apoptosis. A distinction can be made between live, early apoptotic and late apoptotic cells when annexin V is used in conjuction with plasma membrane permeability markers such as propidium iodide, (PI) (a membrane impermeable DNA stain). Live cells stained with fluorochrome-tagged Annexin V and PI, have minimal Annexin V fluorescence and minimal PI fluorescence. At the early stages of apoptosis, cells stain brightly with Annexin V but still exclude PI. Finally, when apoptotic cascade advances to later stages, the secondary/late apoptotic cells stain intensely with both probes.

Apoptosis was quantified in prostate LNCaP cells by staining the cells with Annexin V/PI following analysis by flow cytometry.Treatment of prostate LNCaP cells with different concentrations (2.5, 5 and 10 µg/mL) of MFCP and PPF 3 for 24 hrs resulted in significant increases in the ratios of early and late apoptotic cells, while percentage of viable cells reduced compared with untreated control. In this regard, percentage of viable cells decreased significantly from 97% to 19.4% at 0.1% DMSO (control) and 10 µg/mL, respectively upon MFCP treatment while exposure to PPF 3 caused decrease in viable cells from 97% in untreated cells to 23.6 % in cells exposed to 10 µg/mL. The distribution of prostate LNCaP apoptotic cells upon PPF3 treatment belonged predominantly to those of early apoptosis (0.08% in DMSO-treated cells to 62.9% at 10 µg/mL) while percentages of dead/damaged cells upon (2.91 % in control cells) to 41.3% at 10 µg/mL) MFCP treatment were significantly higher than those in their early apoptotic phase. This may be attributed to the fact that the parent methanol fraction is more potent (higher

IC_{50}) than the PPF 3. In addition, it was observed that decreases in viable cells and increases in apoptotic cells were dependent on MFCP and PPF 3 concentrations. These findings are in line with the observations made by Castedo *et al.,* (1996) that early during apoptosis, cells undergo a disruption of the mitochondrial transmembrane potential prior to exposing PS at the outer membrane leaflet. These data provide a strong correlation between inhibition of cell proliferation and apoptosis (Alenzi, 2004) and indicate that PS externalization is a downstream event of early caspase activation and possibly an early phenomenon of the so-called execution phase.

Escape from apoptosis is a key attribute of tumour cells and facilitates chemo-resistance. The 'Bcl-2-regulated' or 'intrinsic' apoptotic pathway integrates stress and survival signalling to govern whether a cancer cell will live or die (Delbridge and Strasser, 2015). Permeability of the outer mitochondrial membrane can be influenced by Bcl-2 protein family members which may be anti or pro –apoptotic. The delicate balance of these (pro/anti apoptotic) proteins determine the susceptibility of cancer cells to undergo apoptosis (Vaux and Korsmeyer, 1999 ; Fulda *et al.*,2010). Mitochondrial permeabilization transition is often altered in cancer cells possibly as a result of overexpression of mPT pore components (Rasola and Bernardi, 2014), upregulation of anti-apoptotic members of the Bcl-2 family and/or downregulation of Bax (Bonora and Pinton, 2014). Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane in healthy cells. This distribution contrasts to the restricted localization of Bcl-2 to cellular membrane compartments, including the endoplasmic reticulum, outer mitochondrial membrane, and nuclear envelope (Gross *et al.,* 1998). Upon the induction of apoptosis, the cytosolic Bax translocates to the outer mitochondrial membrane (Hsu *et al.,*1997 ; Wolter *et al.,* 1997) and deeply inserts into the membrane (Goping *et al.,* 1998). In contrast,

antiapoptotic Bcl-2 members surround the outer mitochondrial membrane and are saddled with the responsibility of guarding the integrity of the mitochondria. They are critical for their ability to bind to the pro-apoptotic Bcl-2 family members and thereby exert their antiapoptotic function (Delbridge and Strasser, 2015). Overexpression of Bcl-2 or Bcl-XL potently inhibits apoptosis in response to many cytotoxic insults, among others by suppressing the generation of ROS, stabilizing $\Delta\psi$, preventing mPT and consequently blocking the release of cytochrome c (Elmore, 2007)

Levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 were assessed in mitochondrial and cytosilic lysates of LNcaP cells exposed to varying concentrations of MFCP. Our results showed that levels of Bax reduced significantly up to 3 folds at 10 µg/mL in the cytosol relative to control while a 2.5-fold increase in levels of Bax in the mitochondria relative to control. This suggests that MFCP caused translocation of Bax from the cytosol to the mitochondria and possibly facilitated Bax oligomerization which is considered crucial for mitochondrial membrane permeabilization (Sharpe *et al.,* 2004). This strongly supports the view that the Bax rings surround an opening required for mitochondrial outer membrane permeabilization (MOMP) (Große *et al., 2*016). Also, the fact that levels of Bcl-2 proteins increased significantly in the cytosol while a decrease was observed in the mitochondria attests to the fact that the antiapoptotic Bcl-2 protein is downregulated by MFCP in prostate LNCaP cells. These data is in line with the finding Magnolol, a flavonoid isolated from *Magnolia officinalis* induced apoptosis in human breast-cancer cells via the intrinsic pathway with release of AIF from mitochondria accompanied by down- regulation of antiapoptotic protein Bcl-2 and upregulation of proapoptotic protein p53 and Bax (Zhou *et al., 2*013).

This suggests that the downregulation of Bcl-2 by MFCP may be as a result of displacement of Bcl-2 from the Bax membrane insertion site or posibly by influencing an upstream event necessary for Bax insertion. These data further corroborate our findings on the downstream signalling events that have been earlier observed. Besides, it reveals that Bax and Bcl-2 proteins play an important role in the regulation of MFCP-induced cellular commitment to death via apoptosis.

Apoptosis and proliferation are tightly coupled because it is essential to identify and eliminate cells proliferating inappropriately.Consequently, cell cycle regulators can influence both cell division and cell death (Meikrantz and Schlegel, 1995). The susceptibility of cells to apoptosis frequently depends on the differentiation state of the cell. Additionally, deregulation of the cell cycle components has been shown to induce mitotic catastrophe and also may be involved in triggering apoptosis.Compounds that can induce cell cycle arrest and apoptotic cell death are generally considered to be potential anticancer drugs (Vermeulen *et al.*, 2003).

To measure the cellular DNA content after treatment with MFCP, the cells were stained with a Propidium Iodide (PI), a fluorescent dye that binds to DNA with a red fluorescence and can be excited at 488nm in a manner that reflects accurately the amount of DNA present (Alenzi, 2004). PI is only weakly fluorescent in aqueous solution but fluoresces strongly when bound to DNA in a stoichiometric manner. However, it stains all double stranded nucleic acids so the cells were incubated with RNase to remove any double stranded RNA. Given that PI is excluded by

the plasma membrane, the cells were fixed with 70% ethanol before adding the dye(Bertoli *et al.*, 2013).

The results showed that in prostate LNCaP cells, treatment with MFCP and PPF increased significantly the percentage of cells in S-phase. In this regard, the percentages of cells in S-phase from 37.4 % in uninduced cells to 40.1% at 2.5 µg/mL (IC_{50}) and 64.3% at 12.5 µg/mL ($5IC_{50}$) on exposure to MFCP. Similarly, treatment with PPF 3 led to significant increase in the proportion of cells arrested in S-phase from 37.4% (control) to 53.7% (12.5 µg/mL). Analysis of DNA content in different cell cycle phases was also conducted in lung adenorcarcinoma cells (A549) and prostate DU-145 cells exposed to MFCP in order to inquire if the pattern of cell arrest is the same.Our results show that in both cell lines, there was a progressive increase in the percentages of cells in the S-phase in a concentration-dependent manner. These results suggest that cancer cells exhibited similar patterns of inhibition of cell proliferation in all cell lines tested hence, methanol fraction prompts cancer cells to arrest at S-phase of the cell cycle.

In this study, several attempts to purify bioactive compounds from methanol fraction (MFCP) proved abortive owing to degradation as indicated in changing proton NMR profiles; therefore modification of MFCP was carried out. Given the fact that MFCP tested positive to ninhydrin indicating the presence of primary amines, we took advantage of this information and protected the amines present in the methanol fraction with Ditert-butyl dicarbonate (BOC-anhydride). This pyrocarbonate reacts with amines to give *N-tert*-butoxycarbonyl or BOC-derivatives. These carbamate derivatives exhibit reduced polarity as a result enhances separation on TLC.

The proton NMR spectrum of the BOC-MFCP shows that the reaction of MFCP with the BOC –anhydride was successful.The BOC –anhydride signature showed up at 2 in the observed spectra (Figure 65). This revealed that BOC-anhydride has been incorporated into the methanol fraction. In addition, two UV active compounds were identified in its BOC-protected MFCP derivative while there was no movement of MFCP from the origin, this demonstates that protection of amines in MFCP enhanced hydrophobicity and increased separation on silica gel TLC plate.

Isolation of the two UV active compounds present in the MFCP-BOC-derivative was done using preparatory TLC. The TLC chromatogram of the two isolated compounds (sub fraction BOC-MFCP 1 and 2) showed that they had very close retardation factors (R_f) using two different solvent systems (DCM: Acetic Acid; DCM Ethanol) (20:1). Because further preparatory TLC did not separate these compounds; column chromatography was therefore employed to separate PPF 4 into cpmpounds 1and 2. Gradient elution was achieved by using dichloromethane and ethanol which gave 33 eluents and were monitored and pooled into 6 sub-fractions according to observed TLC behaviour.

After subjecting the subfractions (1-6) obtained from column chromatography to TLC in a DCM: Ethanol (20:1) solvent system, the developed chromatogram initially produced single bands for each of the sub-fractions having the same Rf on TLC as depicted in Figure 62. Having the same R_f, the fractions were pooled together and further concentrated. This produced an additional band on developed TLC in the same solvent system DCM: Ethanol (20:1) (Figure 62b). This observation demonstrates that subfraction MFCP-BOC 1 and 2 with close retardation factors may be isomers of each other, hence could not be separated in the same solvent system.

The resulting subfractions 1-6 designated partially purified fraction 4 (PPF4) was then deprotected via treatement wth trifluoroacetic acid solution i.e removal of the BOC-anhydride

group to produce a polar PPF-4. The success of the deprotection procedure was validated via TLC showing no movement of the fraction (PPF 4) from the origin (Figure 62c). This was further confirmed in the proton NMR spectrum of the deprotected PPF4 with the disapearance of the BOC signature at two (2) (Figure 66).

Determination of the efficacy of the BOC-protected technique and potency of the partially purified fraction 4 obtained from tthe MFCP BOC-anhydride protection was done and compared our results with partially purified fraction 3 isolated from unprotected MFCP. This was carried out by treatment of varying concentrations of PPF-4 on LNCaP and DU-145 cells. According to the results, PPF 4 decreased cell viabilities in on LNCaP and DU-145 cells with IC_{50} values of 2.5 and 2.43µg/mL, respectively. An important observation is that this cytotoxic effect observed is greater compared with that obtained with the PPF3 in LNCaP and DU-145 cells (4.16 and 6.77 µg/mL). PPF-4 also gave IC_{50} values that were close to the effect seen with the crude methanol extract, 2.37 and 3.34 µg/mL in LNCaP and DU-145 cells, respectively.

These points to the fact that the BOC-anhydride- (amine protected) MFCP led to isolation of a purified fraction with higher potency compared to that obtained in PPF 3 (unprotected MFCP sub-fraction). Extracted chromatogram of PPF 4 revealed the presence of Afzelechin (2-(4-hydroxyphenyl)-3, 4-dihydro-2H-chromene-3, 5, 7-triol) (flavan-3-ol), a type of flavonoid, in both modes of ionization (positive and negative) (m/z = 275/274), while gallic acid showed up as a protonated ion in its positive mode of ionization at m/z 171. Studies have shown that gallic acid possesses cytotoxic effects by reducing cell viability, proliferation, invasion and angiogenesis in human cervical cancer cells (Zhao and Hu, 2013) while characterization and antimicrobial evaluation of epiafzelechin from the stem bark of *Calliandra surinamensis* Benth had initially been reported by Iftikhar *et al.*, (2013).

Plant biologists' fall into two categories: those who believe polyamines play an important role in plant growth and development, and those who are skeptical. The latter group by far exceeds the former. Over the years polyamines have been implicated in being involved in a wide array of processes in plants, ranging from triggering organogenesis to protecting against stress (Walden *et al.*, 1997). A large body of data indicates that polyamine pathway can be a molecular target for therapeutic intervention in several types cancer since polyamines are absolutely required for cellular proliferation and also have a role in facilitating cell death (Thomas and Thomas, 2001; Hyvönen *et al.*, 2016). However, the basis of these diverse cellular responses is currently not known. Our results from this study reveal that amines may be contributing to the enhanced cytotoxic activity observed in BOC-MFCP compared with unprotected MFCP. This is in consonance with the report of Hyvonen and colleagues concluded that triethylenetetramine attacks multiple proven anticancer drug targets (Hyvonen *et al.*, 2016).

Put together, a schematic model of mechanism of action of *Calliandra portoricensis* is depicted in Figure 72.

Given the ease of isolation of compounds from hydrophobic plant extracts, the choloroform fraction (CFCP) was subjected to TLC in a solvent system hexane: chloroform (2:1) in order to monitor and isolate the bioactive compounds present.

The developed chromatogram produced a single spot which was designated pCFCP (Figure 70b). In order to obtain more of this compound, preparatory TLC was employed and the ability of pCFCP to inhibit proliferation of cancer cell was examined and compared with its crude form (CFCP).

Interestingly, the isolated hydrophobic compound (pCFCP) decreased viability of prostate LNCaP cells with a lower IC_{50} (80µg/ml) compared with the parent crude CFCP (95µg/ml) (Figure 70c). This suggests that purification enhances activity with respect to the hydrophobic compound present in the fraction. Furthermore, evaluation of the ability of the growth-inhibition mediated by pCFCP to elicit activation of executioner caspase- 3 was carried out. using spectrophotometric technique. As seen from the results (Figure 71), pCFCP displayed apoptotic effects by enhancing caspase-3 activity at its IC_{50} concentration (80µg/mL) and at twice the IC_{50} concentration (160µg/mL). This implies that the hydrophobic compound is capable of mediating an apoptotic response.

The mass spectra of the pCFCP shows that the compound has a molecular weight of 490g while the carbon- 13 NMR reveals that 32 carbon atoms are present in pCFCP. Furthermore, elemental analysis reveals that the hydrophobic compound contains carbon (69.63%), hydrogen (7.46%) and oxygen (22.91%).

The isolated hydrophobic compound could be subjected to structural modification to produce enhance anticancer activity while minimizing its toxicity as previously reported for taxol, a antimitotic antitumor agent (Kingston, 1991). Minimal structural modifications of the C-13 side chain and C-10 substitution were made to develop antitumour taxoids with more potent activity or lower critical concentrations than taxol. Synthesis of taxotere with slight modifications produced improved drug resistance, water solubility problems and more potent activity in promotion of tubulin polymerization (Guenard *et al.*, 1993). Hence, the C-13 side chain became a target for structural modification.

Put together, our observations provide an insight into the mechanistic relationship between changes in mitochondrial organization and function with specific emphasis on mitochondrial permeability alterations. Based on these results and in conjunction with data obtained in the animal study, we propose the followed model (Fig 72). Upon treatment with MFCP, certain interactions with the membrane occur which leads to the aggregation and eventual opening of the mPT pore as a result of relocalization and upregulation of Bax from the cytosol to the mitochondria. Increased pro-apoptotic Bax levels permeabilize the outer mitochondrial membrane with concomittiant downregulation in anti-apoptotic Bcl-2 levels which results in the release of cytochrome c into the cytosol. This is accompanied by depolarization of the mitochondrial membrane potential and an enhancement of mitochondrial F_0F_1 ATPase activity. Accordingly, opening of the mPT pore results in the release of cytochrome c which forms an apoptosome complex with Apaf-1 and procaspase-9 in the presence of residual ATP. The complex then becomes a platform for activation of initiator caspase-9. Subsequently, caspase-9 activates executioner procaspase-3 which ultimately results in programmed cell death. In keeping

pace with the ability of MFCP to block cell proliferation, MFCP halted the proliferation of the cancer-cell cycle at S-phase boundary before obeying the final death command.

These facts confirm that the folkoric use of *C.portoricensis* in combination with *P. zeylanica* for the treatment of prostate tumour is successful because the two plants contain chemical components whch are anticancer agents. Particular emphasis is placed on *C.portoricensis in* this thesis which has clearly demonstrated that *C.portoricensis* contains chemical constituents such as xanthone and gallic acid which can induce mitochondrial-mediated apoptosis in cancer cell line. These findings will be of invaluable use in chemotherapy of cancer cells especially if the compounds are subjected of structural modification to enhance potency.

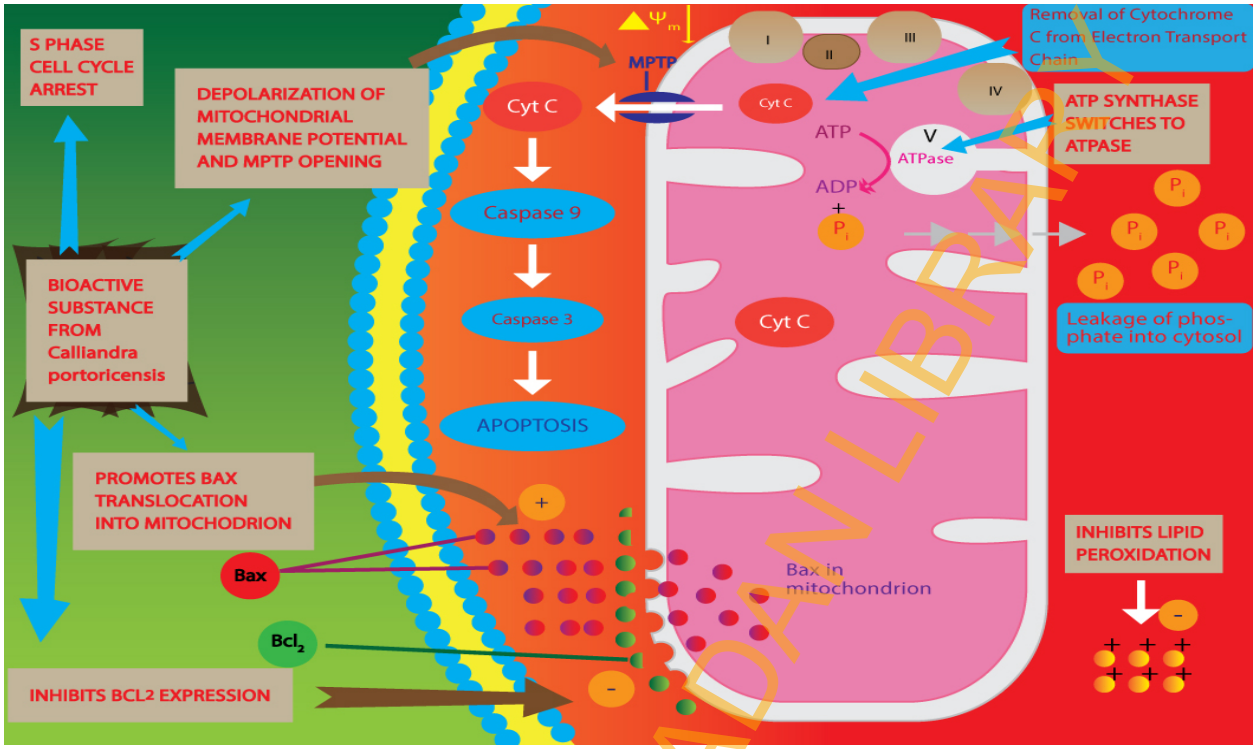


Figure 72: Schematic model of mechanism of action of *Calliandra portoricensis*

CONTRIBUTIONS TO KNOWLEDGE

- *C.portoricensis* contains bioactive agents that induce mpT pore opening with the release of cytochrome c and enhancement of mitochondrial ATPase activity
- Bioactivity guided assay reveals that methanol fraction is the most potent with respect to opening of the mPT pore.
- Oral administration of methanol fraction caused tissue damage and alters heamatological parameters at high doses.
- Molecular mechanism of MFCP-induced apoptosis involves strong inhibition of cell growth not only in prostate cancer cell lines (LNCaP and DU-145) but also in lung adenocarcimona cells (A549).
- Inhibition of cell proliferation correlated with collapse of mitochondrial membrane potential, decreased levels of Bcl-2 with increased translocation of Bax into mitochondria .
- The MFCP-mediated growth inhibition is also associated with S-phase arrest, activation of procaspases 9 and 3 and apoptosis induction with concomittant reduction in viable cell numbers.
- Though, the active principle of *Calliandra portoricensis* is unknown till now, we propose that there are two major bioactive compounds present in MFCP, which have anticancer and antiproliferative activity and may be isomers of each other.
- Methanol fraction also contains a xanthone derivative which may contribute to the cytotoxic and apoptotic effects observed.

- In addition, *C.portoricensis also* possesses a potent hydrophobic compound capable of structural modification to enhance potency.

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