MODULATORY EFFECTS OF *Cajanus cajan* LINN. LEAVES ON MITOCHONDRIAL-MEDIATED APOPTOSIS IN EXPERIMENTAL MURINE MODELS

BY

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ABSTRACT

The opening of the Mitochondrial Membrane Permeability Transition (MMPT) pore causes cytochrome C release (CCR) which is a point of no return for apoptosis to take place. Some medicinal plants are known to induce MMPT pore opening and may be useful in the management of cancer. *Cajanus cajan* (CC) is used traditionally to treat breast cancer, however there is no scientific basis for this claim. This study was designed to assess the effects of CC on MMPT, mitochondrial ATPase (mATPase) activity and CCR in experimental murine models.

The leaves of CC were obtained from Bode market in Ibadan and authenticated at the Department of Botany, University of Ibadan (UIH22500). They were extracted with methanol to obtain methanol extract of CC (MECC). Fractionation of MECC by Vacuum Liquid Chromatography yielded chloroform fraction (CFCC) and ethyl acetate fraction (EACC). The EACC was purified on silica gel and sephadex LH 20 column chromatography and structure of compounds determined using Fourier transform infrared (FTIR), Ultraviolet (UV) spectroscopy and Gas chromatography mass spectrometry (GC-MS). The MECC, CFCC and EACC at different concentrations: 10, 30, 50 and 70 µg/mL were used for *in vitro* assays. In the *in vivo* studies, animals were treated with MECC and EACC.

Eighteen male mice (25±0.04 g) were assigned into three groups of six animals each and orally treated with corn oil (control), EACC (100 mg/kg) and EACC (200 mg/kg) separately. The mice were treated daily for fourteen consecutive days. Similar treatment was repeated in male Wistar rats (150±2.00 g) and animals were sacrificed by cervical dislocation.

The MMPT pore opening, mATPase activity and CCR (Rat and Mice) were determined spectrophotometrically. Caspase 3(C3), caspase 9(C9) and CCR were determined immunohistochemically. Formalin-fixed, H&E stained tissues were examined under light microscope. Data were subjected to descriptive statistics using ANOVA at $\alpha_{0.05}$

In vitro, the MECC at 10, 30 µg/mL had no effect on MMPT pore, while 50 and 70 µg/mL induced pore opening by 7 and 13 folds. Similarly, enhancement of ATPase activities was by 3, 3, 4 and 4 folds at 10, 30, 50 and 70 µg/mL, respectively. The EACC induced MMPT pore opening at the same concentrations by 19, 20, 21 and 23 folds, and enhanced ATPase activities by 9, 11, 13 and 15 folds respectively. In the *in vivo* studies; the MECC had no effect on the MMPT pore at 100 mg/kg but induced opening at 200 mg/kg by 8 folds and activated C3, C9 and CCR significantly.

The EACC induced MMPT pore opening *in vivo* by 0.136±0.03 and 0.229±0.03nm relative to control 0.023±0.002 nm and stimulated CCR at 100 and 200 mg/kg by 15.19±0.3 and 19.14±0.3 ng/mL relative to control 10.15±0.1 ng/mL, respectively. Histological changes revealed perivascular infiltration by inflammatory cells in hepatic tissue. The column fractionation yielded a partially pure compound; 2-Methyl-z, z, 3, 13 Octadecadienol.

The ethyl acetate fraction of *Cajanus cajan* induced mitochondrial-mediated apoptosis via the induction of mitochondrial membrane permeability transition and therefore could be useful in conditions of downregulated apoptosis such as cancer.

Keywords: Mitochondria membrane permeability transition pore, Mitochondrial ATPase, Apoptosis, *Cajanus cajan*

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Olajumoke .O. Alao

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CERTIFICATION

I certify that this work was carried out by Olajumoke Olufunlayo Alao under my supervision in the Laboratories for Biomembrane Research and Biotechnology in the Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria.

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DEDICATION

This thesis is dedicated to the Lord Almighty

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ABBREVIATIONS

ΔΨm: mitochondrial membrane potential

ΔpH: pH gradient

Δμ_: electrochemical gradient

ANT: adenine nucleotide translocator

ADP: adenosine diphosphate

AIF: apoptosis inducing factor

Apaf-1: apoptotic protease-activating factor-1

ATP: adenosine triphosphate

Bcl-x : Bcl-2 related protein

BH3: Bcl-2 homology 3

BAX: Bcl-2 associated X protein

BAK : Bcl-2 antagonist killer 1

BID : BH3 interacting domain death agonist

BAD : Bcl-2 antagonist of cell death

BIM : Bcl-2 interacting mediator of cell death

PUMA: p53-upregulated modulator of apoptosis

NOXA: Phorbol-12-myristate-13-acetate-induced protein 1

BMF: Bcl-2-modifying factor

Hrk: Harakiri

Bik: Bcl-2-interacting killer

A1: Bcl-2-related gene A1

Bcl-2: B-cell lymphoma 2,

Bcl-xL: Bcl-2-related gene, long isoform,

Bcl-w: Bcl-2-like protein 2 baculoviral IAP

Mcl-1: 23 yeloid cell leukemia 1

TOM : mitochondrial protein import

TSPO: mitochondrial lipid transport,

CsA: Cyclosporin A

Cyp-D: Cyclophilin D

Diablo: direct inhibitor of apoptosis binding protein with low pI

DISC: death-inducing signaling complex

DNP: 2,4-Dinitrophenol

EACC: Ethylacetate fraction of *Cajanus cajan*

EGTA: ethylene glycol tetraacetic acid

EGCG: EpiGalloCatechinGallate

FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

HEPES: N-(2-idroxyethyl)-piperazin-N'-2-ethansolfonic acid

IAP: inhibitor of apoptotic proteins

IF1: inhibitor protein 1

IMS intermembrane space

IMM: inner mitochondrial membrane

MECC methanol extract of *Cajanus cajan*

MPT: Mitochondrial Permeability Transition

MMPT: Mitochondrial Permeability Membrane Transition

MOMP: mitochondrial outer membrane permeabilization

mtDNA: mitochondrial DNA

CFCC: chloroform fraction of *Cajanus cajan*

NADH: reduced form of nicotinamide adenine dinucleotide

OMM: outer mitochondrial membrane

OSCP: oligomycin sensitivity conferring protein

PBR: peripheral benzodiazepine receptor

Pi: inorganic phosphate

PiC: phosphate carrier

PMCA: plasma membrane Ca^{2+}-ATPase

PT: permeability transition

PTP: permeability transition pore

OXPHOS: Oxidative phosphorylation.

ROS: reactive oxygen species

SDS: sodium dodecyl sulfate

Smac: second mitochondrial-derived activator of caspases

TLC: Thin layer chromatography

TMB: 3,3,5,5-tetramethyl benzidine,

TNF: tumor necrosis factor

Ub0: ubiquinone 0

UCP 2: uncoupling protein 2

VDAC: voltage-dependent anion channel

XIAP: X-linked inhibitor of apoptosis proteins

CHAPTER ONE

INTRODUCTION

Apoptosis is a signaling pathway involved in the regulation of tissue homeostasis and the elimination of transformed and mutated cell (Kuranaga, 2011). Apoptosis is initiated by signals inside and outside the cell via two main pathways, the intrinsic and the extrinsic pathways (Kuranaga, 2011, Circu and Aw, 2010). It is characterised by changes in cell morphology that ultimately culminates in eukaryotic cell death. Common features in the intrinsic pathway include the activation of specific cysteine aspartic-proteases (caspases) and externalisation of phosphatidylserine. The extrinsic pathway is mediated by the binding of several death receptors to appropriate ligand causing the recruitment of adaptor protein which associates with procaspase 8, resulting in its conformational changes. This leads to its binding to a supramolecular complex; Death Inducing Signaling Complex (DISC) which activates caspase 8 which in turn activates caspase 3 resulting in cell death. In the intrinsic pathway, internal damage to the cell causes a group of proteins, the Bcl-2 proteins, to interact and cause the release of some proapoptotic protein into the intermembrane space. This leads to a series of reactions which eventually causes cell death (Halestrap *et al.,* 1999).

Mitochondria are organelles involved in the health and survival of an organism. They are frequently introduced as adenosine triphosphate (ATP) producing powerhouses of eukaryotic cells. Paradoxically, mitochondria play an integral part in apoptotic cell death through the opening of a non-specific membrane channel, the Mitochondria Membrane Permeability Transition (MMPT) pore. Groundbreaking studies of Hunter and Harworth in the late 1970s showcased MMPT pore universally and it has since become a research topic in several laboratories. Though, initially considered an *in vitro* artifact induced by

calcium, several drugs have been directed to act on the MMPT pore in order to cause cell death with a view to treating several pathological conditions such as cancer. The molecular composition of the MMPT remains uncertain, but the classical model comprises of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), adenine nucleotide translocase (ANT), in the inner mitochondrial membrane (IMM) and cyclophilin D (CypD) in the matrix. Under physiological conditions the MMPT is responsible for cellular homeostasis: regulating movement of ions and fluid (Lemasters *et al*., 2009). However, perturbations to the environment of the cell leads to intracellular apoptotic signals in the mitochondria (Cotran *et al.,* 1999). Dysregulation of apoptosis results in malformations and diseases. These diseases include Acquired immune deficiency syndrome (AIDS), heart failure, neurodegeneration, rheumatoid arthritis, diabetes and cancer. Cancerous cells are abnormal cells, which have the capacity to multiply indefinitely, as a result of evasion of apoptosis (Hanahan and Weinberg, 2000). In almost all instances, deregulated cell proliferation and suppressed cell death synergestically cause neoplastic transformations.

Cancer, a genetic disorder, is the most widespread killer disease in the world; economically developed and developing countries across have expressed concerns about this menace. Cancer can be treated with surgery, ionizing radiation and chemotherapy or a combination of these. Research has shown these treatments cause DNA damage and could be genotoxic. Prolonged treatment also leads to drug resistance as well as severe side effects such as: hair loss, gastrointestinal effects such as nausea and vomiting, lethargy and depression. Hair loss and nausea/vomiting are very common because hair follicles and intestinal stem cells are the most rapidly dividing normal cells of the human body. These side effects cause harm to the patient if not managed well and could be a burden to caregivers as well.

Many of the therapeutic agents used for cancer treatment are quite costly and are not easily available is some developing nations. Chemotherapy is currently being undertaken with caution since mortality arises from this form of treatment, rather than from the cancer itself. It is therefore necessary to conduct research on alternative treatment options. Chemoprevention involves defensive mechanisms that prevent the onset of carcinogenesis. Most anticancer drugs of plant origin showed fewer side effects and the likelihood of drug resistance as compared to the conventional chemotherapy drugs (Belayachi *et al.,* 2013). Medicinal plants have been shown to induce apoptosis and are now used in the management of cancers. Accumulating evidence from scientific research has shown that chemopreventive agents could exert their therapeutic effects by inducing apoptosis in malignant cells. Modulation of apoptosis through the induction of permeability transition could therefore be a promising strategy in the management of conditions associated with irregular apoptosis such as cancer. Hence, the development of drug from natural products would provide innovative and alternative avenues for chemoprevention and management of cancer.

1.1 JUSTIFICATION OF THE STUDY

An increase in the demand for low-cost drugs has lead to an increase in medicinal plant usage, especially in rural areas where in most cases the health care system is nothing to write home about. Patronage of medicinal plants as an alternative to synthetic drugs in both rural and urban areas is due to poverty and high cost of treatment in hospitals (Efferth *et al*., 2007). Medicinal plants contain bioactive agents that can treat a diverse symptoms, ranging from minor ailments such as gastrointestinal issues to chronic problems such as infections. In addition, they can modulate immune function and prevent chronic diseases (Zhang *et al*., 2015). Medicinal plants contain complex mixtures of bioactive compounds that can target multiple pathways of a disease, they may also have additive or synergistic effect which can improve their potency.

Although the pharmacological activities of most of these medicinal plants have not been determined scientifically, their therapeutic uses have been based on their folkloric prescriptions, and they are generally well-tolerated in the body (Aggarwal and Shishodia, 2006). Medicinal plants serve as a major source of drugs since most of the pharmaceuticals in use today are derived from natural products. The presence of phytochemicals such as flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, tannins, anthocyanins, chalcones, xanthones, liganans, depsidones, terpenes, alkaloids and organic sulfur compounds confer chemopreventive properties on them. (Haque *et al.,* 2016). Researchers are highly interested in exploring medicinal plants to discover and manufacture several novel compounds, since they are rich in bioactive compounds (Poe, 2017). Dietary phytochemicals, have been demonstrated to inhibit carcinogenesis i*n vitro* and substantial evidence indicates that they can also do so *in vivo*. Flavonoids seem to be a promising approach to cancer chemoprevention and their mechanism of action has been identified to include carcinogen inactivation,

antiproliferation, cell cycle arrest, inhibition of angiogenesis, antioxidation reversal of multidrug resistance and induction of apoptosis and differentiation or a combination of these mechanisms (Akinmoladun *et al*., 2007). The discovery of new natural products for cancer treatment is of great urgency to improve prospects of people with the disease. Many naturally occuring compounds that have demonstrated anticancer activity are potent inducers the MMPT pore. Some of these compounds are curcumin, lycopene, capsaicin and Epigallocatechingallate (EGCG) (Martin, 2006). Berberine, α bisabol, betunilic acid, cucurmin and resveratol have been tested in tumor cell line and preclinical animal. Curcumin and resveratrol are promising and are currently undergoing further clinical and preclinical trials.

Similarly, (Burkhill, 1978) emphasized that *Cajanus cajan* is a leguminous plant cultivated in East and West Africa. It is administered in Folk medicine for the treatment of liver and stomach disorders, anemia, dysentery, measles, jaundice and breast cancer. Some biological activities reported by Scientists include antioxidant, hepatoprotective, antidiabetic and antisickling properties (Ashidi *et al.*, 2010). The popularity of *Cajanus cajan* in traditional medicine suggests the presense of bioactive components that would be useful in drug development. To the best of our knowledge, it is not known if bioactive agents of *Cajanus cajan* would have modulatory effects on the mitochondrial membrane permeability transition MMPT pore.

1.2 OBJECTIVE

The main objective of the research is to determine if the extract of *Cajanus cajan* contains bioactive components that can modulate mitochondrial-mediated apoptosis and therefore can be used in drug development where apoptosis is dysregulated

.Specific Objectives:

1. To determine the phytochemicals present in *Cajanus cajan* leaves.
2. To fractionate the methanol extract of *Cajanus cajan* and to determine the potency of fractions on the MMPT pore.
3. To assess the effects of the fractions of *Cajanus cajan* on mitochondrial ATPase activity.
4. To investigate the effects the fractions of the *Cajanus cajan* leaves on Fe-induced lipid peroxidation.
5. To investigate the effects the fractions of the *Cajanus cajan* leaves on cytochrome c release.
6. To investigate the effects of the most potent fraction of *Cajanus cajan* leaves on caspase 9, 3 and cytochrome c, *in vivo*.
7. To assess the effects of methanol extract of *Cajanus cajan* on immunohistochemical expression of caspase 9, 3 and cytochrome c.
8. Purification and structural elucidation of the most potent fraction of *Cajanus cajan* using chromatographic and spectroscopic techniques

CHAPTER TWO

LITERATURE REVIEW

2.1 MITOCHONDRIA

The word mitochondrion is from two words: "mitos" and "chondros" which means thread and granule respectively in Greek (Scheffler, 2008). The symbiotic relationship between a primordial eukaryotic cell which was unable to use oxygen and an engulfed aerobic bacteria has been reported in several scientific reviews to culminate in the formation of a special organelle in eukaryotic cell. The aerobic bacteria evaded digestion and acquired nutrition and protection from the engulfing cell which in return supplied energy to the host (Prasai, 2017). This engulfed bacteria evolved to form the mitochondrion. Mitochondria contain their own DNA and can synthesise their own protein (Gray, 2012). Mitochondria are commonly refered to as powerhouses of cells because they synthesis ATP and supply the cell with chemical energy required for normal cellular functions. Apart from the generation of ATP, other biological functions of mitochondria involved include numerous cell processes such as cell growth, cell messaging, cell signaling, aging, replication, cell cycle, cell differentiation and cell death. Several characteristics make mitochondria unique. The number of mitochondria in a cell varies widely with cell type. In the heart, mitochondria occupy approximately 30% of cardiomyocyte volume and provide more than 90% ATP necessary for cardiac function. Human spermatozoa contain a fixed number of 16 mitochondria and oocytes up to 100,000. Organs that are very active metabolically, such as muscles, liver, brain, and cardiac and skeletal muscles, contain the largest number of mitochondria and are most susceptible to drugs acting on mitochondria and to mitochondrial pathologies (Fischer *et al.*, 2012).

2.2 THE STRUCTURE OF MITOCHONDRIA

Mitochondria is compartmentalized into outer and inner membrane, critae matix, intermembrane space, inner boundary membrane, cristae junction, cristae membrane and mitochondrial matrix, respectively. The outer mitochondrial membrane (OMM) envelopes the organelle and facilitates exchange of solutes and other molecules with its immediate environment (van der Laan *et al*., 2016). It is fairly homogeneous, consisting about 1.0-1.5 µg proteins per µg phospholipids (Walther *et al*., 2009).

Phospatidylcholine and phosphatidylethanolamine are the most abundant lipid of the OMM and phospholipid cardiolipin is only present sparingly (Tatsuta *et al*., 2014). The outer mitochondrial membrane is readily permeable to small molecules as a result of the transmembrane channels formed by a family of integral membrane proteins called porins, also known as, the voltage-dependent anion channel (VDAC), which forms relatively large internal channels (about 2–3 nm) and allows ions, metabolites, and certain small molecules to move between the cytoplasm and mitochondria.

These pore-forming channels render the membrane freely permeable to all ions and molecules of 5000 Da or less (Lodish, 2013). Larger molecules, however, pass through the MOM via special translocases (Kühlbrandt, 2015). Apart from VDAC, other major protein components of MOM include those involved in mitochondrial protein import (TOM complex), mitochondrial lipid transport (TSPO), intrinsic apoptotic pathway (Bak), and mitochondrial fission (Mff) and fusion (Mfn1). The IMM contains an inward fold called cristae which is highly impermeable to most small molecules and ions, including protons ; and they transport ions, molecules, and metabolites across with specialized membrane transporters and exchangers; it is also rich in cardiolipin, an unusual phospholipid which is typically found in bacterial plasma membranes. The inner trans

membrane potential is highly negative (-180 mV) and is being maintained by pumping protons into the intermembrane space (Fischer, 2012). The inner membrane bears the components of the respiratory chain and the ATP synthase.

Mitochondria are membranous organelles that perform several biological processes which includes energy production, heme biosynthesis and porphyrin metabolism, Ca^{2+} homeostasis and apoptosis. Apart from their role in energy generation, they also control cell death (Nunnari and Suomalainen, 2012).They provide energy for most cellular activities including, synthesis of lipids and folding, as well as force generation (Duchen, 2004). They also contain enzymes involved in the synthesis of lipids and ketone bodies.

2.3 OXIDATIVE PHOSPHORYLATION

The production of energy is achieved by transporting electrons from NADH or $FADH_2$ which are generated from substrates in the TCA cycle to the final acceptor, molecular oxygen, to produce water. The five macromolecular protein complexes of the mitochondrial ETC are embedded in the mitochondrial inner membrane (MIM), and, by passing electrons from CI and CII via CIII to CIV with the help of electron carriers, UbQ and cytochrome c, generate energy that is maintained as an electrochemical proton gradient across the MIM. Complex V, the $F_o F_1$ ATP synthase, then uses the energy from the proton gradient to generate ATP from ADP and inorganic phosphate, which provides the cell with its fundamental energy substrate. The ETC is the major source of mitochondrial reactive oxygen species (ROS) due to the large electron flows, with CI and CIII identified as prime superoxide-generating sites.

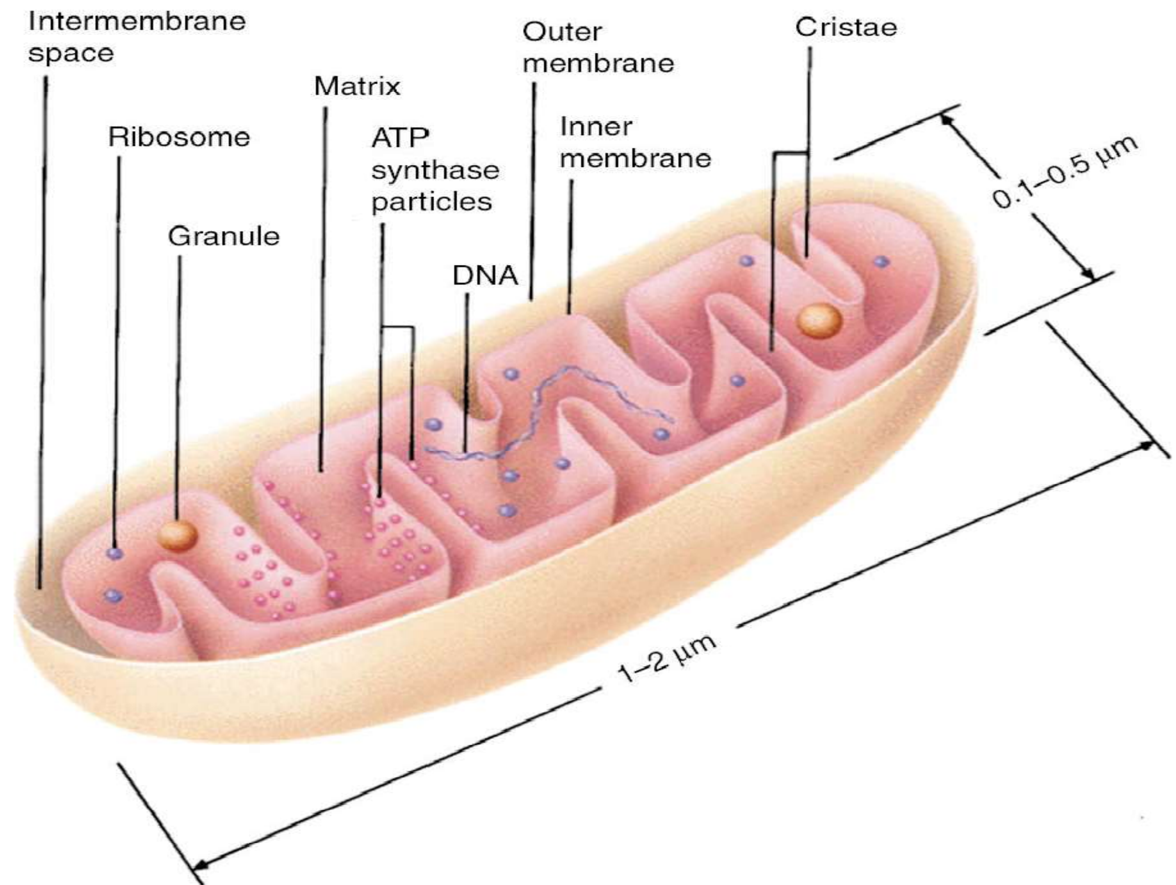


Figure 1: Structure of Mitochondrion (Frey and Mannella, 2000)

2.4 MITOCHONDRIAL F_OF_1 ATP synthase

FoF_1 ATP synthase is a large oligomeric complex whose general structure is highly conserved among bacteria, chloroplasts, and mitochondria. The mitochondrial F_OF_1 ATP synthase creates the energy storage molecule adenosine triphosphate ATP, from adenosine triphosphate (ADP) and inorganic (P_i). Accumulating evidence have shown that the F_OF_1 ATP synthase also contributes to the formation of the mitochondrial membrane permeability transition pore. The monomeric mammalian F_OF_1 ATP synthase is a multiprotein assembly made of 15 different protein subunits with an overall molecular weight of about 550 kDa (Walker, 2013). It has enzymatic activity localized in the inner membrane of eukaryotic mitochondria. The enzyme consists of two major domains, a water soluble catalytic F_1 domain and a membrane-bound F_O domain, joined together by central and peripheral stalks (Pedersen and Amzel, 1993, Boyer, 1997).

The F_1 region comprises the subunits α, β (3 of each), γ, δ and ε whereas the F_O region comprises subunits a, b, c (multiple subunits, 8 in mammals), d, e, f, g, A6L and F6 (Abrahams *et al*., 1994, Wittig and Schagger, 2008). The central stalk formed by the subunits γ, δ, and ε as and the peripheral stalk, comprised of the oligomycin sensitivity conferring protein (OSCP) and subunits b, d, and F6 (Collinson *et al*., 1996). In mammals, all the ATP synthase subunits, except two mitochondrial encoded subunits (ATP6 and A6L), are nuclear encoded. The main role of the peripheral stalk, which is a predominantly α-helical structure made from single copies of the oligomycin sensitivity conferral protein (OSCP), subunits b and d, and factor 6 (F6) is to link the α3β3 domain to subunit ATP6, which interacts with the two predicted transmembrane α-helices of the b-subunit, so that together the α3β3 domain, the peripheral stalk, and subunit a form the stator of the enzyme. The functioning of ATP synthase is dependent on a rotary catalytic mechanism. The unique property of the $F_O F_1$ ATP synthase is that it produces ATP at the

expense of expense of an ion (H^+)-motive force by this rotary catalytic mechanism (Boyer, 1997). During ATP synthesis, the c-ring, which is organized like a barrel turns with estimated speeds of up to 300 Hz.The generation of rotation involves the translocation of protons across the membrane domain of the enzyme via a pathway at the interface between the surface of the c-ring and subunit A6L. The movement of the c-ring in a circular manner induces the rotation of the central stalk which is attached to the c-ring inside the α3β3 hexamer of the F_1 part (Abrahams, 1994; Noji *et al*., 1997). At three of the six α-β interfaces are found the catalytic sites of the enzyme, where ATP is formed from ADP and inorganic phosphate, using energy supplied (Abrahams, 1994).

During rotation, the F_1 region is held stationary relative to the Fo region by the peripheral stalk, a connection which is essential for the proper function of the enzyme (Walker and Dickson, 2006). Without a proton gradient the ATP synthase can function in reverse, pumping protons into the intermembrane space and hydrolyzing ATP in order to create a mitochondrial membrane potential (ΔΨm). Furthermore ATP synthase can associate with the inhibitor protein 1(IF1) which inhibits hydrolysis of the enzyme. IF1 acts as a dimer and binds to the α-β interfaces of two F1 ATPases via its N-terminal inhibitory sequence (Cabezon *et al*., 2003). Binding of IF1 requires ATP and is favored by low pH and ΔΨm. The restoration of ΔΨm favoring ATP synthesis displaces IF1 from the F_O F_1 ATP synthase (Green and Grover, 2000). In eukaryotic mitochondria oligomycin can inhibit F_O F_1 ATP synthase, by binding to subunit c (Symersky *et al*., 2012). It thereby blocks synthesis as well as hydrolysis of ATP and abolishes ADP stimulated respiration in intact mitochondria. To date, considerable progress has been made in elucidating details of the ATP synthase structure and the mechanisms of rotary catalysis, both synthesis and, to a greater extent, hydrolysis and catalysis.

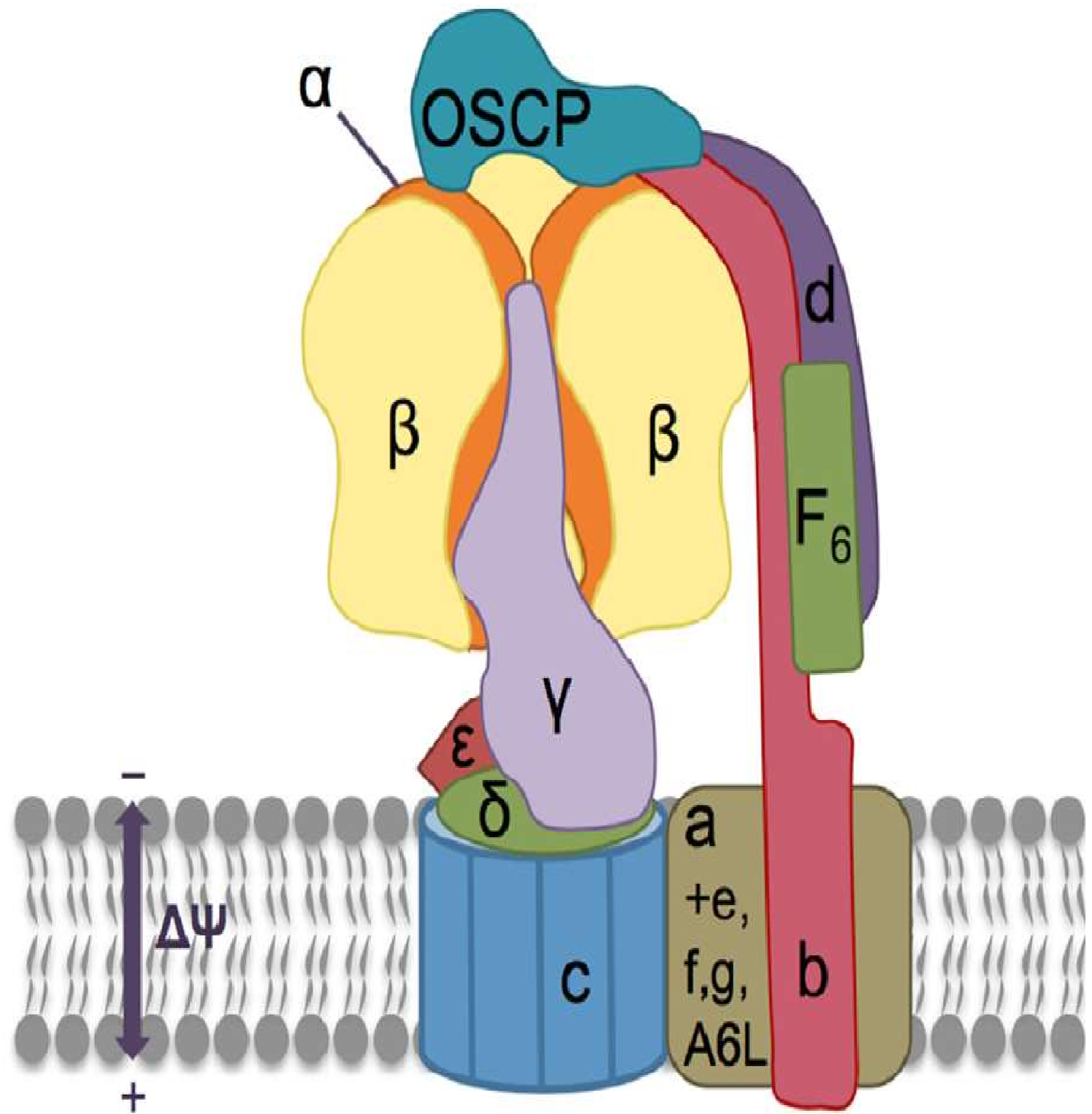


Figure 2. Mitochondrial $F_O F_1$ ATP synthase (Jonckheere *et al*,. 2012)

2.5 MITOCHONDRIA AND ENERGY PRODUCTION

Oxidation phosphorylation (OXPHOS) is the oxidation of substrates by cytosolic and mitochondrial enzymes to release energy that is transferred to ATP (the energy currency of the cell.) The main metabolic substrates are the carbohydrate, fatty acids and protein. Carbohydrates are converted into glucose via the glycolytic pathway, pyruvate which enters the mitochondrion is converted to Acetyl-Co A. Fatty acids are also converted to Acetyl Co A through beta oxidation. Proteins are hydrolysed to peptides and amino acids, which are the deaminated and converted into pyruvate or acetyl-Co A. In the mitochondria matrix, acetyl Co A enters the TCA cycle, which generates NADH and $FADH_2$, the substrates of the mitochondrial ETC, which is located in the mitochondria cristae membrane. The mitochondrial electron transport chain (ETC) consists of five large, multi-protein complexes I, II, III, IV and V. Energy released from the flow of electrons is used by complexes to pump protons against their electrochemical gradient into the intermembrane space. This gradient is called electrochemical gradient and is proportional to the proton motive force that is the product of the electrochemical potential (m) and proton gradient (p) across the IMM. The proximal four complexes of the ETC accept and transfer electrons from NADH and the TCA cycle and subsequent proton tranlocation by complexes I,III and IV from the mitochondrial matrix into the intermembrane space creates the proton motive force (p) to drive $F_O F_1$ ATP synthase (Jonckheere *et al*,. 2012, Watt *et al*. 2010).

2.6 MITOCHONDRIA AND CALCIUM CYCLING

It is well documented that Ca^{2+} ions are among the most important second messengers and they play a key role in cell signaling. The regulation of Ca^{2+} in cells is a complex process, based on several transport systems located in the plasma membrane and in the

intracellular organelles. The plasma membrane contains a channel for the downhill penetration of Ca^{2+} into the cell from the environment. In excitable cells, the channel is controlled by electrical currents. It also contains 2 systems which are responsible for the pumping of Ca^{2+} out of the cell, a specific ATPase, and a Na^{+}/Ca^{2+} exchanger. Inside cells, Ca^{2+} is transported reversibly by mitochondria and sarcoplasmic reticulum. The former organelle accumulates Ca^{2+} electrophoretically, and releases it through a separate pathway. The latter accumulates Ca^{2+} through a specific ATPase, and releases it through another route (Carafoli , 2012, Lehninger et al., 1978).

The ability of mitochondria to take up and release calcium is well established. The response of the mitochondria to addition of calcium depends on the amount of calcium added and the level of mitochondrial calcium (Nicholls, 1978). Mitochondrial inner membrane depolarization occurs not only through proton movement but also via the flux of other ions including Ca^{2+} across mitochondrial membranes. Movement of Ca^{2+} into the mitochondrial matrix is a physiological event that takes place in response to increased cytosolic Ca^{2+} levels.

Buffering of Ca^{2+} is frequently employed by mitochondria in cells that experience rapidly changing cytosolic Ca^{2+} levels such as those of excitable tissues. Mitochondria regulate cytosolic levels of Ca^{2+} and the release of Ca^{2+} and metabolites using several ion channels and exchangers. The Ca^{2+} uniporter ion channel (MCU) located at the mitochondrial inner membrane participates in mitochondrial Ca^{2+} uptake within the cell body of many types of cells and also in the presynaptic terminals of neurons (Chouhan *et al*., 2012; De Stefani and Rizzuto, 2014). Mitochondrial calcium overload profoundly influence cell survival, as excess matrix Ca^{2+} can initiate cell death through opening of the MMPT.

2.7 MITOCHONDRIA AND CELL DEATH

Mitochondria orchestrate the life and death of a cell. Apart from acting as the power houses of animal cells they also coordinate signaling pathways associated with cell death. The mitochondria act as powerhouses by suppling the energy from oxidation of energy-rich compounds in foodstuffs to generate a chemical potential difference for hydrogen ions, called the proton motive force (pmf), across the inner mitochondrial membrane. Disruption of this membrane dissipates the proton motive force, and the cells die for lack of fuel. This event happens, for example, when the concentration of calcium ions inside human mitochondria is increased. The mitochondria respond by opening a pore, water enters, and the mitochondria swell and burst.The involvement of mitochondria in cell death has been subject of intense research for the past three decades.

Mitochondria are actively involved in apoptotic cell death (Dalla Via, 2014). In apoptosis cells retain theirplasma membrane integrity and it occurs during homeostasis, metamorphosis, immune response, cellular response to growth factors and hormones. The series of morphological changes in dying cells include rounding up of the cell, pseudopod retraction, cell shrinkage, chromatin condensation and nuclear fragmentation (Kerr *et al*., 1972). Mammalian cell death is most widely classified into two processes, apoptosis or necrosis. Cells undergoing a sudden stress situation such as infection or exposure to toxins die by necrosis resulting in loss of plasma membrane intergrity, uncontrolled release of cellular components and inflammation.

On the contrary, apoptosis is a highly regulated form of progtammed cell death that governs normal body sculpture, defense against pathogen invasion and developmental processes. Exposure of the cell to an apoptotic stimulus leads to a series of morphological changes such as rounding up of the cell, pseudopod retraction, cell shrinkage, chromatin

condensation and nuclear fragmentation (Kerr *et al*., 1972). Many human pathologies such as degenerative diseases (too much apoptosis) or cancer (too little apoptosis) are associated to the abnormal regulation of apoptosis. Until recently, necrosis and apoptosis were considered to be two distinct phenomena. However, there is now evidence that both processes can be interconnected and that necrosis can be initiated by death receptor involved in apoptotic cell death in a process called necroptosis (Wu *et al*., 2012).

2.8 APOPTOSIS

Apoptosis is employed by multicellular organisms for immune surveillance and for maintenance of tissue homeostasis during development and differentiation. It is evolutionarily conserved and genetically regulated (Kuranaga, 2011). It also plays a significant role in tumour cell biology by preventing the growth of cells mutated due to DNA damage. Glùcksmann (1951), in his study of cells undergoing embryogenesis, discovered a common conserved pattern of morphological features. In his classic description of cell death, one can recognize almost all of the morphological alterations that have since been found to be characteristic features of apoptosis. Saunders (1966) observed this phenomenon in the chick limb during embryogenesis.

The term apoptosis was used for the first time by Kerr (1972) to describe a morphologically distinct form of cell death. Kerr, in an experiment caused ischaemic liver injury in rats by the ligation of a branch of the portal vein; and Wyllie *et al.,* (1980) observed that the morphological characteristics of cell death in the adrenal gland following withdrawal of adrenocorticotropic hormone matched those seen in the ischaemic liver. Later, Wyllie *et al*., (1980) observed that the morphological characteristics of cell death in the adrenal gland following withdrawal of adrenocorticotropic hormone matched those seen in the ischaemic liver. In their seminal

paper Kerr and co-workers described this phenomenom as apoptosis. They discovered morphological changes in cell death that led to membrane blebbing and the digestion of the resulting small vesicles by macrophages. The blebbing of the cells reminded researchers of leaves, which were scattered around a tree in autumn. From that time on, the Greek word 'apoptosis' not only described the falling of leaves but also the process of programmed cell death (Kerr *et al.,* 1972).

The components of apoptosis have recently been described in detail after an explosion in the study of apoptosis. Critical discoveries included the caspases, a family of highly conserved proteases that executes the program of cell death; receptor (tumor necrosis factor (TNF) superfamily, bone morphogenetic protein (BMP) and nonreceptor (hypoxia, genotoxic stress, and growth factor withdrawal) means of activation of the caspase cascade; the Bcl-2 family of proteins, which reside within the mitochondria and regulate cytochrome *c* release and caspase activation; and a family of inhibitor of apoptosis proteins (IAPs) that inhibit caspase activity and thus prevent the fortuitous activation of the pathway. The significance of apoptotic research has been recognized in many ways, including the awarding of the 2002 Nobel prize in physiology and medicine to three scientists: Sydney Brenner, John Sulston and Robert Horvitz.

2.9 MORPHOLOGICAL CHARACTERISTICS OF APOPTOSIS

Apoptotic cells show morphological changes such as cell shrinkage, pyknosis and extensive plasma membrane blebbing followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding leading to the formation of apoptotic bodies, apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment) which are subsequently phagocytosed by macrophages, an inflammatory response, since apoptotic cells do not release their

constituents into the surrounding interstitial tissue, apoptotic cells are quickly phagocytosed by cells in the surrounding tissue, preventing secondary necrosis, and the engulfing cells do not produce inflammatory cytokines (Kurosaka *et al*., 2003)

2.10 BIOCHEMICAL CHARACTERISTICS OF APOPTOSIS

Apoptotic cells show several biochemical changes such as cleavage and cross-linking of protein, DNA degradation and phagocytic recognition (Hengartner, 2000). Caspases are important group of proteases that proteolytically cleave proteins at specific sites containing asparate during the apoptotic scenario with different caspases having specificities and functioning at different points during the apoptosis. Studies have shown that once caspases are activated, cell death must surely take place (Nemes *et al*., 1996).

Deoxyribonucleic acid degradation by Ca^{2+}-and Mg^{2+}-dependent endonucleases also occurs, resulting in DNA fragments of about 200 base pairs, observed as "DNA ladder" can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination. Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue (Bortner *et al*., 1995). This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to 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 are also be exposed on the cell surface during apoptotic cell clearance.

2.11 NECROSIS

Necrosis originated from a Greek word for death. It is a pathological form of cell death that is highly disorganized. Several scientists have associated necrosis with inflammation (Kelly *et al*., 2001). Necrotic cells do not usually send the same chemical signals to the immune system that cells undergoing apoptosis do. This prevents nearby phagocytes from locating and engulfing the dead cells, leading to a build up of dead tissue and cell debris at or near the site of the cell death. Necrotic cells typically show cytoplasmic swelling and vacuolation, rupture of the plasma membrane, dilation of organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation. When cells swell and burst, they spill their contents over their neighbours and elicit a damaging inflammatory response (Festjens *et al*., 2006).

Necrosis unlike apoptosis, is not energy-dependent. Biochemically, most prominent features include massive energy depletion, the formation of reactive oxygen species and the activation of non-apoptotic proteases. All these events result in a loss of function of homeostatic ion pumps and damage to membrane lipids with cell membrane swelling and rupture. Furthermore, during necrosis, a substantial rise in intracellular calcium is observed. The elevated calcium levels in the cytosol trigger mitochondrial calcium overload, leading to depolarization of the inner mitochondrial membrane and a shut-down of ATP production (Orrenius, 2003).

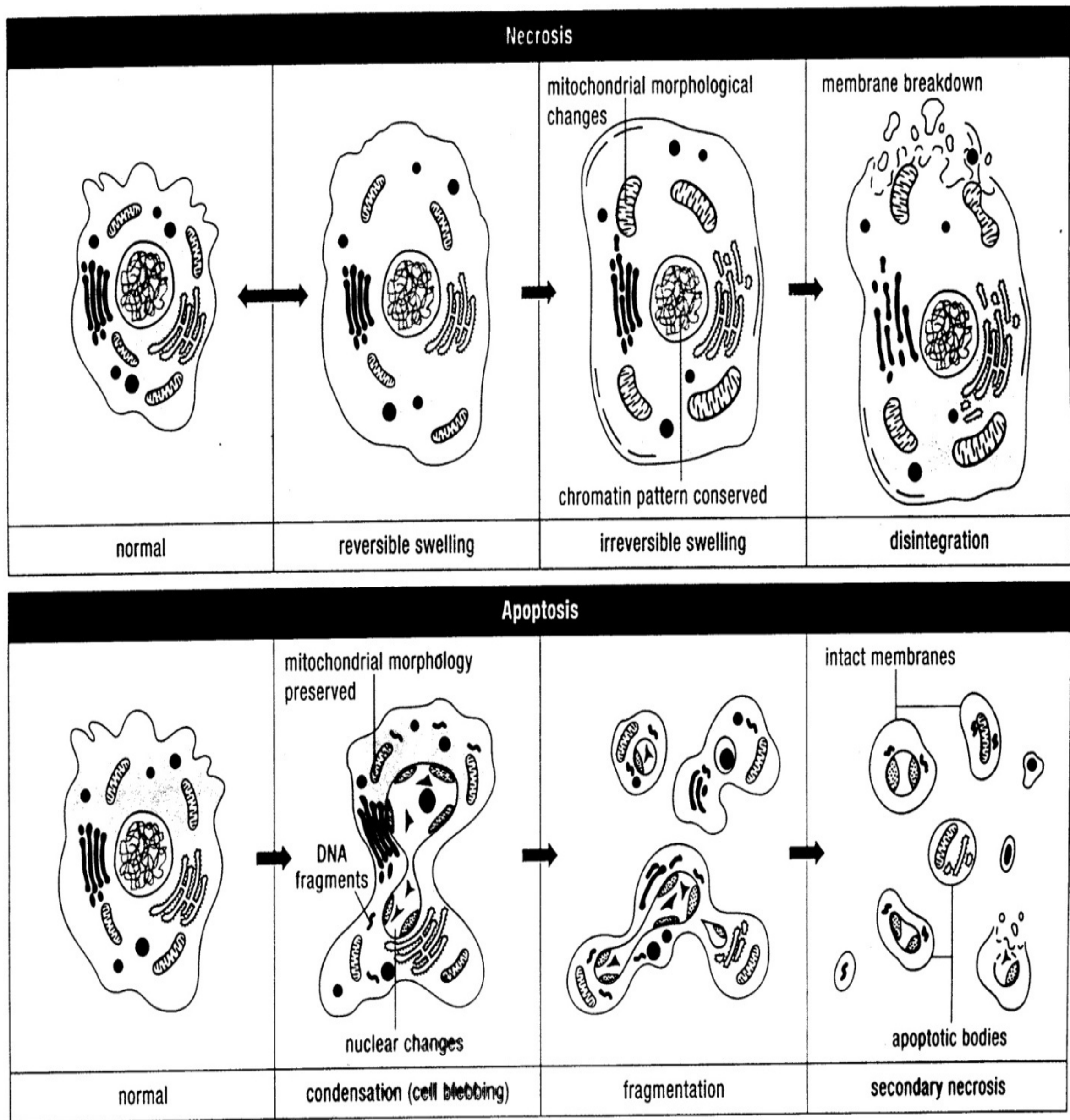


Figure 3: Apoptosis and Necrosis (Rode, 2008)

2.12 THE PATHWAY OF APOPTOSIS

2.12.1 EXTRINSIC PATHWAY OF APOPTOSIS

Extrinsic pathway initiates apoptosis through transmembrane receptor-mediated interactions. The binding of specific ligands activates death receptors located on cell surface leading to the transmission of apoptotic signals. This pathway is useful in the elimination of infected cells by the immune system. Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, which comprises almost 20 members of cytokine receptors, such as TNFR-1, Fas/ CD95, and the TNF-related apoptosis inducing ligand (TRAIL) receptors These receptor recruit adapter proteins, to their cytoplasmic domain called the "death domain" This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways (Ashkenazi, 2002). TheFasL/FasR and TNF-α/TNFR1 are the commonest ligand receptor model in the extrinsic phase of apoptosis. In these models, these ligand receptor interactions leads to recruitment of adaptor proteins. For example, the binding of Fas ligand to Fas receptor results in the recruitment of the adapter protein FADD, Subsequently, dimerization of the death effector domain results from the association of procaspase-8 with FADD.

Procaspases-8 and -10 binds to a supramolecular complex called death-inducing signaling complex (DISC). Caspase 3 is activated by caspase 8. Studies have shown that caspase-8 activation can be blocked by cellular FADD-like interleukin- 1β-converting enzyme inhibitory protein (c-FLIP). Unlike type I cell, the signalling cascade from type II cells is not sufficient to result in cell death. In this regard, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Caspase 8 cleaves Bid to its truncated form (tBID) which translocates to the mitochondria where it

acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol which eventually results in cell death (Luo *et al*., 1998; Chinnaiyan, 1999).

2.12.2 INTRINSIC PATHWAY

This is an apoptotic pathway that is initiated in response to signals within the cell. It does not require ligand receptor-mediated interactions but relies solely on mitochondrial-mediated events within the cell. This pathway is caused by perturbations in the microenvironment of the cell due to intracellular signals that may either be stimulatory or inhibitory in nature. Inhibitory signals causing apoptosis include the absence of certain cytokines, growth factors and hormones. Other stimuli that are stimulatory include, oxidative stress, radiation, toxins, hypoxia, hyperthermia, viral infections, and chemotherapeutic drugs. These stress signals arise as a result of internal damage to the cell thereby causing the release of Bcl-2 family of proteins, Bax and Bak. These proteins is regulated by p53 a tumor suppressor gene which activates transcription of proapoptotic proteins from the Bcl-2 family.

Bax and Bak oligomerize once activated, then translocate to the mitochondria and oligomerize forming pores causing proapototicproteins to move from the intermembrane space to the cytosol. Cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi, the first group of proapoptotic proteins released and they activate the caspase-dependent mitochondrial pathway (Chinnaiyan, 1999). Cytochrome c binds Apaf 1 (apoptotic proteinase activating factor 1) is a CARD containing adaptor protein, which recruits procaspase 9 in an ATP (Adenosine triphosphate)-dependent manner in order to form a heptamer of cytochrome c, Apaf1 and procascapse 9 known as apoptosome .This provides the platform for the clustering andactivation of the initiator caspase 9. Consecutive

recruitment of the executioner procaspase 3 to the apoptosome, leads to its maturation into active caspase 3 by the resident caspase 9.

2.12.3 PERFORIN/GRANZYME PATHWAY

This is a relatively novel pathway utilised by immune cells in addition to Fas-Fas ligand interactions. Tumor cells and virus-infected cells undergo apoptosis by signals sent to them by cytotoxic T lymphocytes (CTLs) through the Perforin/granzyme Pathway. They are exert their their cytotoxic effects via the binding and subsequent release of granules which contain transmembrane pore-forming molecule perforin and granzymes which are serine proteases. The perforins interact with intracellular secretory granules which release granule associated enzymes (granzymes) A and B (Bots and Medema, 2006).

A specific DNAase, NM23-H1, is responsible for destroying nuclear material. This DNAase is normally bound to the SET complex, which is cleaved by granzyme A causing the release and activation of the said DNAase. Proteins are cleaved at aspartate residues by Granzyme B and will activate procaspase-10 which can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse). Amplification of the death signal by specific cleavage of Bid, helps granzyme B to utilize the mitochondrial pathway. However, granzyme B can also directly activate caspase-3. Direct induction of the execution phase of apoptosis occurs after bypassing the upstream signaling pathways. (Bots and Medema, 2006)

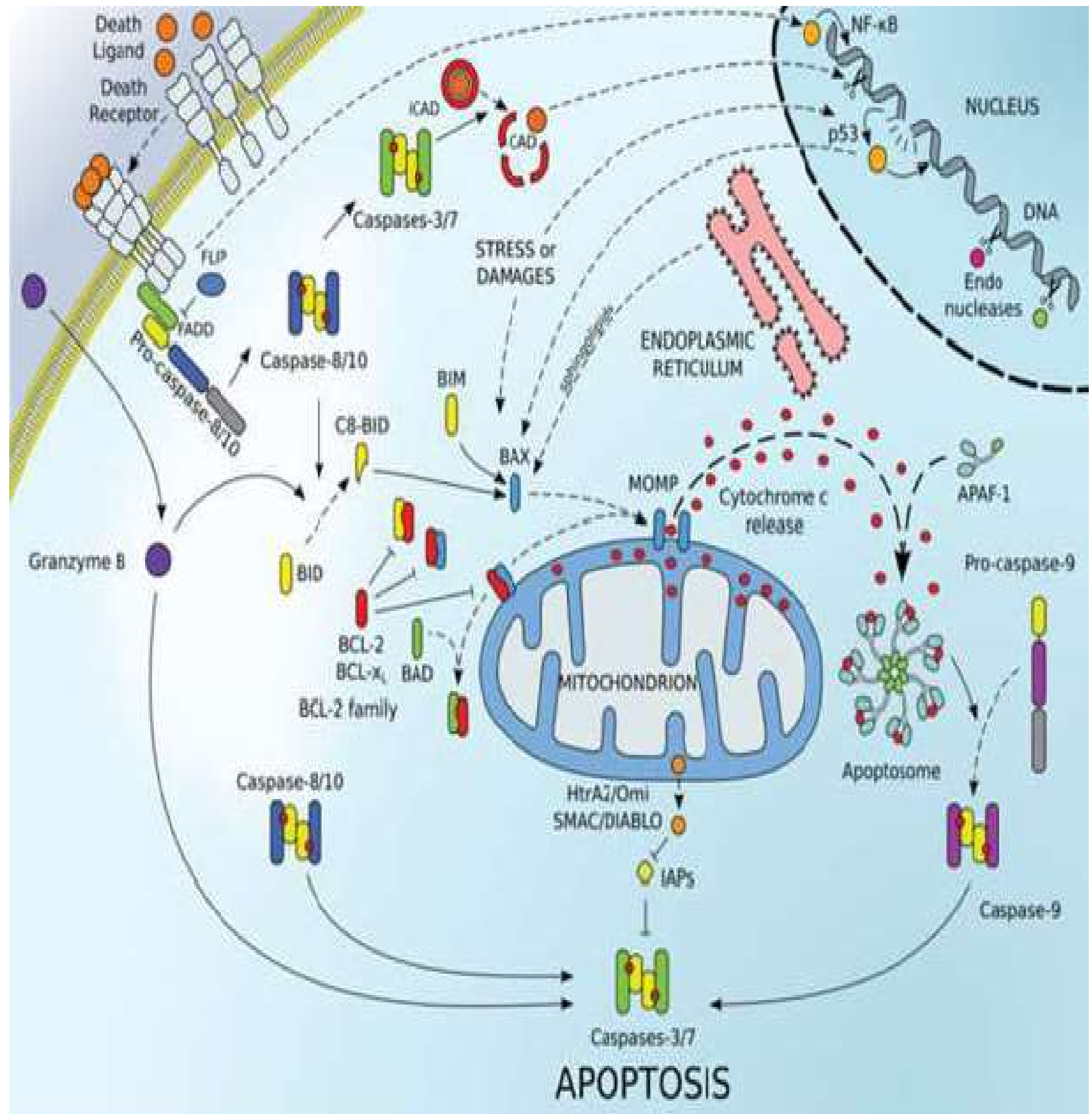


Figure 4: The pathways of apoptosis (Renault and Chipuk, 2013)

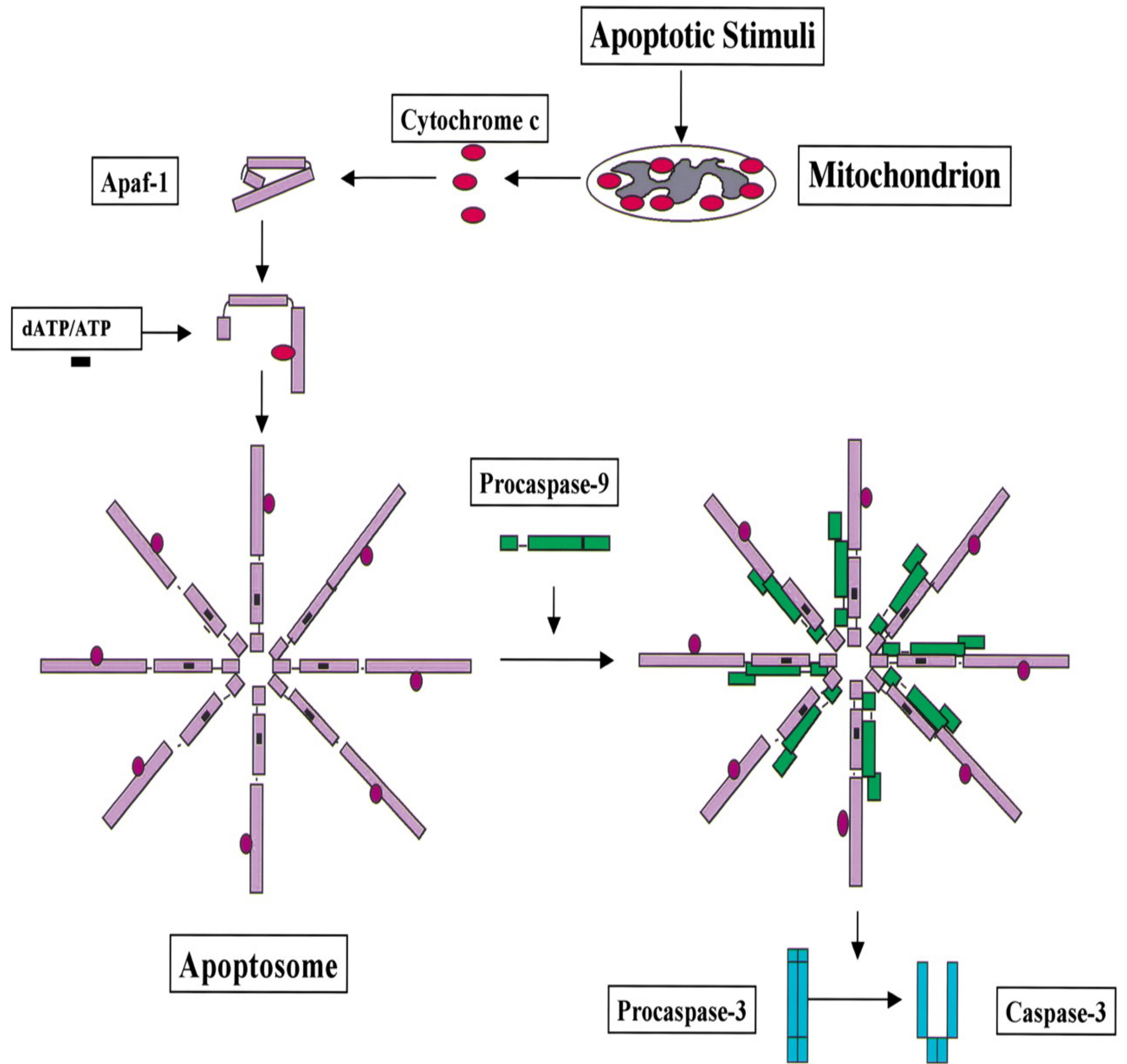


Figure 5: Formation of Apoptosome (Zou *et al.,* 1999*)*

2.13 PROTEINS REGULATING APOPTOSIS

The extrinsic and intrinsic apoptotic pathways are regulated by proteins such as the p53 NF-κ B, the ubiquitin proteosome system and the PI3K pathway

2.13.1 p53

Several papers have reported p53 as a tumour suppressor genethat elicits apoptosis by upregulating expression of Bax in response to sensing DNA damage, Bax stimulates cytochome C release and cell cycle arrest. It is well established that, p53 functions as a transcription factor regulating downstream genes important in cell cycle arrest, DNA repair and apoptosis. The critical role that p53 plays is evident by the large number of tumors that bear a mutation in this gene. Loss of p53 in many cancers leads to genomic instability, impaired cell cycle regulation, and inhibition of apoptosis. After DNA damage, p53 holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. The mechanism by which p53 promotes apoptosis is still not fully understood (Yu *et al.,* 2003, Cantley, 2002)

2.13.2 NF-κ B

NF-κB is a nuclear transcription factor that regulates expression of a large number of genes involved in the regulation of apoptosis, viral replication tumorigenesis, iniflammation, and many autoimmune diseases (Maldonado *et al*., 2003). NF-κB is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, radiation, pharmacological agents, and stress. In its inactive form, NF-κB is sequestered in the cytoplasm, bound inhibitor proteins of the IκB family.

The various stimuli that activate NF-κB cause phosphorylation of IκB, which is followed by its degradation. This results in exposure of the nuclear localization signals on NF-κB subunits and the subsequent translocation of the molecule to the nucleus.

In the nucleus, NF-κB binds with the consensus sequence of various genes and thus activates their transcription.NF-κB has been shown to have both anti- and proapoptotic functions that may be determined by the nature of the death stimulus rather than by the origin of the tissue. (Kuhnel *et al.*, 2000). Under physiologic conditions, the activation of NF-κB induces resistance to apoptotic stimuli through the activation of many complex proteins including TNF receptor-associated factor, IAP, and X-linked IAP. However, in response to certain stimuli, NF-κB activation may lead to induction of apoptosis. (Kuhnel *et al.*, 2000).This may be explained by the activation of some proapoptotic proteins such as interferon-regulated factor-1, c- myc, p53, and caspases such as caspase 1. In some viral infections, induction of apoptosis by he virus is dependent on NF-κB activation (Kuhnel *et al.*, 2000).

2.13.3 THE UBIQUITIN/PROTEOSOME SYSTEM

The ubiquitin/proteosome system is composed of a large proteinase complex that is responsible for the turnover of most intracellular proteins and consequently regulates cell growth and apoptosis (Myung *et al*., 2000). Protein degradation is a highly coordinated process that involves recognition of the protein by attaching it to multiple ubiquitin molecules and then its digestion by the 26S proteosome. Many cell cycle regulators and transcription factors such as p53, cyclins and cyclin-dependent kinase inhibitors, and NF-κB are regulated by the ubiquitin/pro-teosome system (Myung *et al*., 2001).

2.13.4 PI3K

PI3K is a kinase that plays a central role in signaling pathways important to cell survival, pro- liferation, motility, and tissue neovascularization. PI3K is upregulated in many cancers (Hidalgo and Rowinsky, 2000, Cantley, 2000). Cell-surface receptors induce the production of second messengers such as phosphatidylinositol 4, 5-bisphosphate 3 and phosphatidylinositol 3, 4, 5-trisphosphate, which convey signals to the cytoplasm from the cell surface. Phosphatidylinositol 4, 5-bisphosphate 3 signals activate the kinase 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt. Akt activation leads to phosphorylation of certain pro-teins that lead to cell survival. For example, phosphorylation of IκB by Akt leads to activationof NF-κB that promotes survival. Phosphorylation of Bad by Akt leads to its inactivation and block-ing of the apoptotic signal. In addition, phosphorylation of caspase 9 blocks the induction of apoptosis. Akt also phosphorylates the protenForkhead-related transcription factor 1. Several proteins important in human cancers can be dysregulated in the PI3K pathway. These are also important because of Food and Drug Administration (FDA) approved agents targeting them.For example, epidermal growth factor stimulation activates Akt via PI3K, as does HER-2/ neuactivation. 3 Loss of phosphatase and tensin homolog tumor suppressor gene also augments the activity of this pathway.

2.14 APOPTOSIS IN HEALTH AND DISEASE

For the formation of body cavities, shaping of tissues and organs, morphogenesis of fingers and toes, apoptosis is essential (Lawen *et al*., 2003). Apoptosis is pivotal to the pathogenesis of disease since an upregulation can lead to tissue damage and atrophy, whereas down-regulation can lead to cellular overgrowth. In acquired immunodeficiency

syndrome (AIDS), the HIV virus causes death of T-helper cells which are required for proper immune function. Excess apoptotic cell death is also implicated in cardiovascular diseases (Quadrilatero and Bloemberg, 2010) and in neuronal disease (Kermer *et al*., 2004). Insufficient apoptosis in a number of tissues is the basis of cancerous tumour formation (Lawen, 2003). Due to its involvement in numerous disease states, a great deal of research is examining apoptotic regulators as potential therapeutic treatments.

2.15 APOPTOSIS AND CARCINOGENESIS

Common characteristics of cancer cells include uncontrolled growth, angiogenesis and apoptosis evasion. A normal cell is transformed into a malignant cell due to genetic changes in the cancerous cell. Evasion of cell death may cause this malignant transformation (Hanahan and Weinberg, 2000). Kerr *et al,* as early as the 1970's, had linked apoptosis to the elimination of potentially malignant cells, hyperplasia and tumour progression (Kerr *et al*, 1972). Hence, reduced apoptosis or its resistance plays a vital role in carcinogenesis. Reduction in apoptosis by malignant cells could be due disrupted balance of pro-apoptotic and anti-apoptotic proteins, reduced caspase function and impaired death receptor signalling

2.16 CASPASES

Caspases are central initiators and executioners of apoptosis. Cell death can be classical apoptosis if execution of cell death is dependent on caspase activity, hence caspases are involved in the regulation of apoptosis. Caspases are members of a family of cysteine proteases synthesized as inactive zymogens (Bratton and Cohen**,** 2001). The term caspases is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity involves cleavage of substrates after certain aspartic acid residues within specific

pentapeptide QACRG regions (Los *et al*., 1999). Till date, 7 different caspases have been identified in *Drosophila*.

In mammals, caspases are subdivided into three functional groups, initiator caspase 2, 8, 9, and 10, executioner caspases such as caspase 3, 6, and 7 and inflammatory caspases 4, 5, 11 and 12 (Bratton and Cohen, 2001). In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. The prodomain is also frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase.

The proapoptotic caspases can be divided into the group of initiator caspases including procaspases 2, 8, 9 and 10, and into the group of executioner caspases including procaspases 3, 6, and 7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing Death Effector Domains (DED) in the case of procaspases 8 and 10 or Caspase Recruitment Domains (CARD) as in the case of procaspase 9 and procaspase 2. Via their prodomains, the initiator caspases are recruited to and activated at death inducing signalling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways).

2.17 INHIBITORS OF APOPTOSIS PROTEINS (IAPS)

A family of proteins that inhibit cell death are known as IAPs. The presence of BIR region of 70 to 80 amino acid zinc binding domain required to provide inhibition of

apoptosis characterise these proteins (Liston *et al*., 2003). Scientist during their phase I studies in some cancers, designed AEG35156 an antisense oligonucleotide to selectively target XIAP, the only IAP family member that directly inhibits caspase 3, 7 and 9and apoptosis was observed (Dean *et al*., 2009). Antimicrobial responses, tumorigenesis, metastasis and cellular migration are other signaling pathways modulated by IAPS.

2.18 MEDIATORS OF CASPASE-DEPENDENT APOPTOSIS

2.18.1 CYTOCHROME C

Cytochrome c is a 12.3 kDa nuclear encoded protein, that shuttles electron from complex III to complex IV in the electron transport chain. However, its' movement out of the mitochondrion stimulates apoptosis. Its' dissociation from a particular hemoprotein initiates it's release from the IMM (Ott *et al*., 2002). Accordingly, deficiency of cytochrome c abrogates activation of caspase upon mitochondrial apoptosis induction (Li and Li, 2000). Upon release to the cytosol, cytochrome c binds Apaf 1 and several other initiators of apoptosis other proteins in mitochondrial-mediated apoptosis.

2.18.2 SMAC/DIABLO AND HTRA2/OMI

Smac/DIABLO is a 25-kD nuclear encoded mitochondrial protein mitochondrial protein that is released along with cytochrome *c* during apoptosis. As cytochrome c is released into cytosol Smac/Diablo and HtrA2/Omi are also released. Caspase activation is mediated after these proapoptotic molecules neutralize a protein family of endogenous inhibitors of caspases (IAPs) (Du *et al*., 2000; Suzuki *et al*., 2001). In the cytosol interacts and antagonizes IAPs thus allowing activation of caspase thus facilitating apoptosis. Smac/Diablo can be a pharmacological target in the design of anticancer drugs.

2.18.3 CAD

Caspase-activated DNase is activated in later stages of apoptosis via caspase-dependent cleavage of its inhibitor (ICAD). It translocates to the nucleus resulting in pronounced chromatin condensation and DNA fragmentation (Enari *et al*., 1998).

2.19 CASPASE-INDEPENDENT EXECUTION OF CELL DEATH

Classic apoptosis requires the activation of initiator and executioner caspases. Mitochondrial mediators of caspase-independent apoptosis; AIF and Endonuclease G both translocate to the nucleus upon release from the mitochondrial IMS to contribute to nuclear DNA condensation and fragmentation. Granzymes (granule enzymes) belong to a family of highly homologous serine proteases contained in the granules of innate and adaptive immune killer cells (cytotoxic T lymphocytes and natural killer cells). These cytotoxic granules are I released via exocytosis and are used to eliminate virally infected cells or transformed target cells.

There are five human granzymes, including Granzyme A and Granzyme B, the most abundant and extensively studied granzymes. Granzyme B has been shown to cleave substrates after aspartic acid residues and shares many substrates with effector caspases. The cytotoxic granules also contain perforin, a pore-forming protein that is needed to deliver the granzymes into the target cell. Cathepsins are proteases, which normally reside within the lysosomal compartments. Different mechanisms and certain stimuli induce the release of these lysosomal enzymes into the cytoplasm where they can induce apoptosis by releasing apoptogenic factors from mitochondria. Calpains are Ca^{2+} activated cysteine proteases localized to the cytoplasm and the mitochondria. Calpains generally promote apoptosis (as well as necrosis) by cleavage of cytoskeletal proteins, induction of

mitochondrial dysfunction (e.g. cleavage of electron transport chain proteins) and cleavage and activation of caspases However, it remains vastly unclear, how calpain activity is regulated physiologically in a cell.

Also some physiologic and artificial scenarios of apoptosis-like cell death that do not require the activation of caspases. Artificially, the cell-permeable irreversible synthetic inhibitor of caspases zVAD-fmk can be used to examine whether certain stimuli depend on caspase activation for the execution of cell death. zVAD-fmk binds the catalytic site of all caspases by mimicking the cleavage site and fluoromethyl ketone forms a covalent bond, thus resulting in an inactive inhibitor-enzyme complex (Slee *et al*., 1996).

While for some cell death stimuli, apoptosis induction can be potently blocked by zVAD-fmk, other stimuli still induce cell death with apoptotic characteristics. The first reported example is the overexpression of Bax, which triggers cell death via the breakdown of mitochondrial function even in the presence of zVAD-fmk .Similarly, other proteases such as granzymeA/B, cathepsins and calpains can trigger caspase-independent apoptosis. Hence, although some caspases are substrates of these proteases and caspase activation can still be observed, it is not necessarily required for cell death execution.

2.20. MEDIATORS OF CASPASE-INDEPENDENT APOPTOSIS

2.20.1 APOPTOSIS INDUCING FACTOR

The AIF translocates to the nucleus and induces condensation of peripheral nuclear chromatin and DNA fragmentation although it lacks intrinsic DNase activity (Cande *et al*., 2004). Compared to cytochrome c and Smac, AIF is released relatively late and slow

during mitochondria-mediated apoptotic cell death, although the timing has been controversially discussed (Arnoult *et al.*, 2002).

2.20.2 ENDONUCLEASE G

Nucleosomal DNA fragments are formed after endonuclease G migrates to the nucleus and cleaves nuclear chromatin, after their release from the mitochondrial IMS. However, knock-out models of endonuclease G proved that deficiency of endonuclease G does not abrogate apoptotic DNA fragmentation (Irvine *et al*., 2005).

2.21 MITOCHONDRIAL MEMBRANE PERMABILITY TRANSITION PORE

A non-specific pore that allows the passage of virtually any molecule with a molecular mass inferior to 1500 Da, is the MMPT pore. It occurs as a result of conformational changes in a multiprotein assembly at the junction of the IMM and OMM. The widespread and irreversible permeabilization of the OMM is a crucial step in intrinsic apoptosis. The permeabilization of the inner mitochondrial membrane due to the opening of a wide pore has progressed from being considered a curious artifact induced in isolated mitochondria by Ca^{2+} and phosphate to a key cell-death-inducing process in several major pathologies.

Its relevance is now universally acknowledged and pharmacology targeting the phenomenon is being developed. Accumulating evidence from scientific studies have been based on mitochondrial swelling assays, calcium retention capacity, patch clamp analysis and genetic loss of function analysis. The molecular composition of the MMPT pore is still unsolved, although it is generally believed to be a multiprotein assembly made up of several components. Years of persistent biochemical and pharmacological studies

have shown that the pore consists VDAC in the OMM (Szabo and Zoratti, 1993), the ANT in the IMM, and CyP-D in the matrix (Griffiths and Halestrap, 1995) but genetic studies have ruled out this possibility.

Other proteins been proposed to participate in the MMPT assembly and regulation include hexokinase, the translocator protein TSPO (a.k.a. peripheral benzodiazepine (Bz) receptor) and Bcl-2 family members. With these proposed components, the model predicted that the MMPT pore was generated as one contiguous pore spanning the intermembrane space by apposition of VDAC within the outer membrane and ANT within the inner membrane, regulated by CypD within the mitochondrial matrix. Progress has recently been made with the identification of the F_O F_1 ATP synthase as the probable proteic substrate.

2.22 FEATURES AND REGULATION OF THE MITOCHONDRIAL MEMBRANE PERMABILITY TRANSITION PORE

Most classical studies of the MMPT pore were carried out in mitochondria obtained from mammalian cells or tissues. These studies allowed a thorough characterization of the functional properties and regulation of the putative channel, but its molecular nature remains still elusive. The transition between the “open” and “closed” state of the MMPT pore can be modulated by many different compounds, ions or conditions. These pore effectors can be subdivided into inducers, that increase the probability of the pore to open; and inhibitors, that increase the probability of the pore to close. Perhaps the single most important factor, which is essential for opening of the pore therefore is called a “permissive factor”, is matrix $Ca^{2+.}$ Importantly, all the other divalent metals, such as $Mg^{2+,}$ Mn^{2+} and Sr^{2+} instead decrease the pore open probability.

Another important modulator of the pore is matrix pH, with an optimum for MMPT pore opening at pH 7.4. More basic as well as more acidic pH desensitizes the pore to opening (Nicolli *et al*., 1993).The pore is a voltage-dependent channel, in the sense that a decrease in ΔΨm favors it's opening, whereas a high inside-negative ΔΨm tends to stabilize the pore in the closed conformation (Bernardi, 1992). Many PTP modulators appear to modify the threshold potential, bringing it closer (inducers) or moving it further away (inhibitors) from the resting potential. Thus, an inducer brings the threshold potential needed for pore opening closer to the resting potential, and makes mitochondria more susceptible to pore opening even after slight depolarizations. Inhibition of PTP can also be observed with bongkrekate (Le Quoc and Le Quoc, 1998), whereas atractylate opens the pore .

Since atractylate and bongkrekate are selective inhibitors of the adenine nucleotide translocase (ANT), these results led to the suggestion that the MMPT may be entirely or partially formed by the ANT, a debatable issue. The MMPT can be directly regulated by electron flux within complex I of the respiratory chain, with an increased open probability when flux increases (Fontaine *et al*., 1998). This led to the discovery that quinonescan modulate the MMPT, with some of them acting as inhibitors (such as ubiquinone 0 (Ub0) or decylubiquinone) and others as inducers (such as 2,5- dihydroxy-6-undecyl-1,4-benzoquinone). A third class of MMPT-inactive quinones (such as ubiquinone are able to counteract the effects of both inhibitors and inducers.

However, despite a large number of studies, the exact relationship between quinine structure and effect on the pore remains unsolved. Opening of the pore is strongly favored by an oxidized state of the pyridine nucleotides NADH and NADPH as well as of critical thiol groupsat distinct matrix or membrane sites. Both of these effects can be reversed by

reducing agents (Costantini, *et al*, 1996; Chernyak *et al*., 2004). The interconversion between dithiol and disulfide groups correlates with the redox state of glutathione, suggesting that there is a redox equilibrium between the dithiols and matrix glutathione. This finding explains the effect of thiol reacting agents such as *N*ethylmaleimide (NEM) (Kowaltowski *et al*., 1997) and monobromobimane (Costantini *et al*., 1995) on the pore

2.23 PHYSIOLOGICAL CONSEQUENCE OF THE PORE

The opening of the pore causes reversal of the ATP synthase to consume ATP this also plays a role in cell death. Over the years, the pore has been extensively studied for its role in ischemic injury in brain, heart, and other organs as well as in neurodegenerative conditions (Bonora *et al*., 2015). In the heart, data suggest that opening of the pore during early reperfusion after ischemia is a harmful event that precipitates further damage to the myocardium (Griffiths and Halestrap, 1993). Additional data suggest that cell death pathways may regulate or be regulated by the MMPT pore, and uncontrolled opening of the MMPT pore leads to cellular necrosis and, perhaps, apoptosis (Baines, 2011; Bernardi, 2013).

However, more recent data suggest that transient pore opening can serve a physiologic purpose. Transient opening was described in the late 1990s (Huser and Blatter, 1999; Petronilli *et al*. 1999). In the heart, transient opening of the pore during preconditioning can be protective, thus serving a physiological role even during injury. This transient pore opening in striated muscle mitochondria may be associated with transient increases in ROS (so-called superoxide flashes) which are proposed to serve as a signaling mechanism (Wang *et al.*, 2012).

Furthermore, it has been hypothesized that transient opening of the pore releases mitochondrial matrix Ca^{2+} to maintain mitochondrial homeostasis, although this function of the pore has recently been questioned (De Marchi *et al*., 2014). In the embryonic heart, the pore appears to be open at early stages of myocyte differentiation (Beutner *et al*., 2014; Hom *et al*., 2011).

The early heart derives most of its energy from anaerobic glycolysis (Porter *et al.*, 2011), and the activity of the ETC is low (Beutner *et al*., 2014). At an early stage of development (mouse embryonic day 9.5), myocytes have an open pore, low $\Delta \psi m$, and high levels of ROS, and closure of the pore using CsA increases $\Delta \psi m$ and decreases ROS, leading to further myocyte differentiation (Hom *et al*., 2011). These changes in MMPT activity are associated with increased assembly and activation of the ETC at or after embryonic day 11.5 (Beutner *et al*., 2014).

2.24 COMPONENTS OF THE PORE

In the first model, PT pores form from the adenine nucleotide translocator (ANT) in the inner membrane, the voltage- dependent anion channel (VDAC) in the outer membrane, and cyclophilin D (CypD), a CsA binding protein from the matrix. Lately the Fo F_1 ATP synthase has received commendable attention as a candidate for the formation of the pore for several reasons.

2.24.1 VDAC AS A COMPONENT OF THE PORE

Voltage dependent anion channel (VDACs) are a family of ion channels localized in the outer membrane of mitochondria. There are three distinct *VDAC* genes, *VDAC* 1, VDAC 2 and VDAC 3, in mammals that exhibit high degree of redundancy as far as their

metabolic functions are concerned. While VDAC 1 and VDAC 3 deletion mutants in mice are viable with minor defects in mitochondrial respiration and abnormalities in mitochondrial ultrastructure, VDAC 2 deletions are embryonic lethal and show enhanced activation of intrinsic apoptotic pathway (Baines *et al*., 2007). Experiments on the electrophysiological properties of VDACs incorporated into planar phospholipid membranes showed their similarities to MMPT, they were thought to be integral component of the pore forming complex.

It was also shown that VDACs copurified with ANT on a GST-Cyp-D affinity column, though this results have been contested by other groups (Woodfield *et al*., 1998). It has also been speculated that the ATP/ADP mediated regulation of MPTP is through the enzyme Hexokinase that interacts with VDACs at the outer membrane of mitochondria. Scientific reports have shown that factors which modulate VDAC channel properties, like addition of Ca^{2+}, NADH and glutamate also affect pore activity. However, recent literature has clearly ruled out a role for VDACs in constituting the pore. It has been shown that deletion of the mammalian VDAC genes (VDAC1 and VDAC3), either individually or in combination, has no effect on the formation of MMPT (Baines *et al*., 2007).

To address for possible compensatory role played by VDAC 2, the authors knocked down VDAC 2 by siRNA. It was shown unambiguously that the induction of pore formation in either Ca^{2+}-induced or stress-induced mitochondria was identical between the wild-type and various VDAC null mutants ruling out a role for VDAC in MPTP formation. PiC, a member of the family of hexatransmembrane proteins that facilitate the transport of metabolites across the IMM, is a protein recently speculated to constitute MMPT. A CsA-sensitive binding of PiC to Cyp-D and a CsA-insensitive binding of ANT to PiC was seen

in pull-down and co-immunoprecipitation studies with GST-Cyp-D and ANT, respectively. The above associations, given the regulatory role of ANT and Cyp-D, implicate PiC as an essential component of MMPT pore.

It should also be noted that inorganic phosphate, an activator of MMPT pore, binds PiC with high affinity (Leung *et al*., 2008). Other circumstantial evidence implicating PiC as an essential component of MMPT are that a genome-wide cDNA screen has identified that overexpressed PiC can efficiently trigger the intrinsic apoptotic pathway (Alcala *et al*., 2008). It is also shown that cytomegalovirus-encoded protein vMIA, an inhibitor of apoptosis, may interact with PiC as shown by the reduced uptake of 33 p phosphate into isolated mitochondria (Poncet *et al*., 2006) The other proteins that are tentatively speculated as components of MPTP are peripheral benzodiazeine receptors (PDBR), hexokinase, creatine kinase, Bcl-2 (Halestrap, 2009) The last word on what constitutes the pore is yet to be pronounced.

2.24.2 ANT AS A COMPONENT OF THE PORE

The movement of adenosine triphosphate (ATP) to the cytosol is carried out by ANT. It is functionally bound to the voltage dependent anion channel, (VDAC) on the OMM, while in the IMM; ANT is bound to cyclophilinD (CyD) and, the $F_o F_1$ ATP synthase (Boekema and Braun, 2007). The involvement of ANT as a key component of the MPT was first proposed by Hunter and Haworth (Hunter and Haworth, 1979). Later, stronger evidence was provided by other scientists (LeQuoc and LeQuoc, 1988; Halestrap and Davidson, 1990). A role for ANT in the formation of MMPT is suggested that carboxyatractyloside (CAT) activates MMPT and Bongkrekic acid inhibits it.

Further, it was shown that oxidative stress alters a cysteine residue in ANT leading to abolishment of binding of adenine nucleotides to the protein, in turn accounting for sensitization of MMPT to calcium by oxidative stress. It was also demonstrated using patch clamp that isolated and reconstituted ANT could be reversibly converted into an unusually large cation selective channel that requires Ca^{2+}. It was shown that the channel was inhibited by low pH and ANT inhibitors (Brustovetsky and Klingenberg, 1996). However, recent experiments done on liver mitochondria from mice lacking both isoforms of ANT (ANT1 and ANT2) showed the formation of MMPT, although the amount of calcium required to open the pore was more than 2 times that required for the wild type mouse(Kokoszka *et al*., 2004). It was also shown that MMPT formation becomes insensitive to the ligands of ANT in mitochondria from these mice. These data ruled out the possibility of the ANT as a pore component as described in the first model. Therefore suggesting a regulatory role for ANT in MMPT formation (Leung and Halestrap, 2008).

Mitocans are a group of compounds with anti-cancer activity involved in mitochondrial destabilization. Deregulation of the VDAC/ANT complex results in apoptosis induction in cancer cells. Compounds that modulate the PTPC include lonidamine, arsenites and steroid analogues (represented by CD437) (Belzacq *et al.,* 2001). Interestingly, an arsenite analogue 4 - [N-(S-glutathionylacetyl)amino] phenylarsineoxide (GSAO) was shown to inhibit the function of ANT by crosslinking its cysteine residues. This was followed by the generation of oxidative stress and induction of apoptosis, which was selective for proliferating, angiogenic endothelial cells (ECs) while being non -toxic to growth arrested ECs (Don *et al*., 2003).

2.24.3 CYP-D: A COMPONENT OF THE PORE

Cyclophilin-D (Cyp-D) is an 18 KDa protein encoded by a nuclear gene, *Ppif*, and subsequently transported to the mitochondria by a mitochondrial signal sequence. It is a peptidyl prolyl cis-trans isomerase (PPIase) and plays an important role in protein folding. It is a member of cyclophilin family is implicated as an important regulator of MMPT pore because cyclosporine A (CsA), a specific inhibitor of cyclophilin family, inhibits MMPT.

Three studies reported the creation of knockout strain of mice lacking Cyp-D to test the indispensability of this protein in MMPT pore formation (Baines *et al*., 2005; Nakagawa *et al*., 2005). It was shown that mitochondria from mice lacking Cyp-D were impervious to Ca^{2+}- induced permeability transition and were insensitive to CsA inhibition of MMPT. They were also resistant to Ca^{2+}-overload and oxidative-stress induced cell death characteristic of necrosis. However, beyond a particular Ca^{2+} threshold, MMPT formation ensues in mitochondria from Cyp-D knockout mice as seen in the wild-type. This supports a model whereby Cyp-D is involved in bringing about a Ca^{2+}-triggered conformational change in a membrane protein that constitutes the actual pore. The studies also point out that a Cyp-D-independent conformational change can happen in the presence of high Ca^{2+} overload. These findings make the role of Cyp-D appear regulatory in facilitating channel formation rather than being a component of the pore.

2.24.4 OTHER PREDICTED COMPONENTS OF THE PORE

2.24.4 PHOSPHATE CARRIER

The phosphate carrier (PiC) is a member of the mitochondrial carrier family and promotes the transport of Pi across the IMM. Uptake of Pi into the mitochondria is essential for the

production of ATP from ADP. Different studies hypothesized a role for PiC in the MMPT composition due to the fact that some inhibitors of the PTP such as NEM, Ub0 and Ro 68-3400, are also PiC inhibitors (Leung *et al*,.2008, Varanyuwatana & Halestrap, 2012). The PiC of the IMM is encoded by a single gene in mammals and is critical for ATP synthesis by serving as the primary means for mitochondrial Pi transport across the IMM.

While long suspected as playing a role in MMPT formation, interest in the PiC was renewed following the realization that antibodies critical for the earlier studies on the ANT outlined above were, in fact, primarily directed toward the PiC Additional biochemical studies demonstrated the CsA-sensitive bind-ing of CyPD to the PiC and its modification by chemical reagents (e.g., by phenylarsine oxide and NEM) correlated with PTP opening and inhibition. These and a suite of ad-ditional biochemical studies led to the idea that PiC may be a key component of the MMPT that undergoes a Ca^{2+} induced change in conformation to induce pore formation in cooperation with the ANT Consequently, in addition to the ANT, recent models of the MMPT also include PiC Genetic tests of the role of PiC in MMPT formation initially involved the application of siRNA techniques in either mammalian cells or transgenic animals. In both situations, mitochondria lacking up to 60% PiC expression showed no alteration in MMPT function, and no effects on MMPT activity were observed after overexpression of PiC .Furthermore, in mitochondria prepared from cells where the gene encoding PiC had been genetically inactivated (resulting in over 90% reduction in PiC levels, the MMPT could still form and displayed marginally reduced sensitivity to activators. Finally, patch clamp experiments with the reconstituted, functionally active PiC revealed an anion channel function with a mean conductance as low as 40 pS which was further decreased to 25 pS by Ca^{2+} and Mg^{2+} , inhibited by phosphate, and unaffected by ADP. Taken together, these

experiments indicate that PiC, like the ANT, cannot constitute a key structural element of the PTP.

2.24.5 PERIPHERAL BENZOODIAPINE RECEPTOR (TSPO)

Peripheral peripheral benzoodiapine receptor (TSPO) was initially identified as an OMM protein that bound a series of Bz analogs that do not target benzoodiapine receptors in the central nervous system. Hence, it was initially referred to as the peripheral benzoodiapine receptor. Early studies appeared to demonstrate that TSPO is intimately involved in two separate functions, mitochondrial transport of cholesterol and protoporphyrin IX, and PTP regulation. In cells that produce steroid hormones (e.g., in the adrenal cortex), TSPO was thought to promote the transport of cholesterol into the mitochondrial matrix, the rate-limiting step in steroid synthesis However, the ubiquitous expression of TSPO suggested that it could serve more general functions, one of which was long thought to be the regulation of MMPT activity. TSPO was initially linked to PT function based on its association with other proteins thought to be required in traditional models of the PT (i.e., VDAC and ANT) In addition, experiments with Bz specifically targeting TSPO suggested that its ligands promoted the opening of the pore.

2.24.6 MMPT PORE FORMATION OF BY DIMERS OF THE F_OF_1 ATP SYNTHASE

$F_O F_1$ ATP synthase monomers are enzymatically active, but recent studies show that in vivo the ATP synthase forms dimers (Wittig and Schagger, 2008). Electrophysiological studies of the pore supports the dimeric structure believed to be formed by two cooperative channels (Zoratti *et al*., 2009). The assumption that the pore is made up of

dimers of the $F_O F_1$ ATP synthase was presented in a recent review by Bernardi *et al*. (Giorgio *et al*., 2009, Giorgio *et al.,* 2013). It has also been proposed that Ca^{2+} binding induces a conformational change that leads to channel formation in their inner membrane sector.The fact that CypD interacts with the OSCP subunit of the peripheral stalk on ATP synthase structure makes it a good candidate on ATP synthase pharmacology.

In eukaryotes the mitochondrial ATPase complexes are organized in dimers and higher order oligomers. The monomomer units interact via their Fo sectors; subunits a, e, and g are the major ones involved in mammals. The ATP synthase also forms supercomplexes with the mitochondrial creatine kinase (mtCK), adenine nucleotide translocase (ANT), and phosphate carrier (PiC) These synthasomes connect the machinery required for ATP generation (ATP synthase) to that of ADP/ATP and phosphate exchange (ANT, PiC) and energy transfer to the cytoplasm (ANT, mtCK), therefore creating a regulatory unit where energy production and exchange pathways meet (Saks *et al*., 2012).

2.24.7 MMPT PORE FORMATION BY THE C-RING OF F_OF_1 ATP SYNTHASE

The ATP synthase complex comprises of a membrane-spanning channel, namely the membrane embedded ring of c subunits detached from the F1 sector. This portion of the complex thus became a candidate. Alavian *et al*., present data that the c-subunit creates the regulated pore. They go on to conclude that that the F_1β-subunit plays an inhibitory role on the c-subunit pore. Studies by Paolo Pinton's group also surpotted this hypothesis. Therefore, the authors suggest the channel of the MMPT forms within the c-ring itself after calcium dependent extrusion of F_1, i.e., of the γ, δ and ε subunits..; Indeed, Ca^{2+}-induced swelling released c-subunit oligomers from F_1, whereas MMPT pore inhibitors (CsA and ADP) blocked this release. Although these exciting results support a novel model for the MMPT, the F_O F_1ATP synthase cannot explain regulation by all known

MMPT effectors, such as the ANT binding agents bongkrekic acid or atractyloside, which can dramatically effect MMPT activity through altering the conformational states of ANT (Alavian *et al.,* 2014).

When all three mammalian isoforms of the c-subunit (ATP5G1, ATP5G2, ATP5G3) were deleted in HeLa cells using siRNA, there was no effect on ATP production. However, depletion of c-subunits prevented ionomycin-induced opening of the MMPT, mitochondrial fragmentation, oxidative stress-induced mitochondrial depolarization, and release of cytochrome c. Overexpression of ATP5G1 had the opposite effect on MMPT. Under conditions of Ca^{2+} overload (ionomycin) or oxidative stress, c-subunit depletion prevented cell death in HeLa cells and attenuated glutamate-induced cell death in primary cultures of cortical neurons

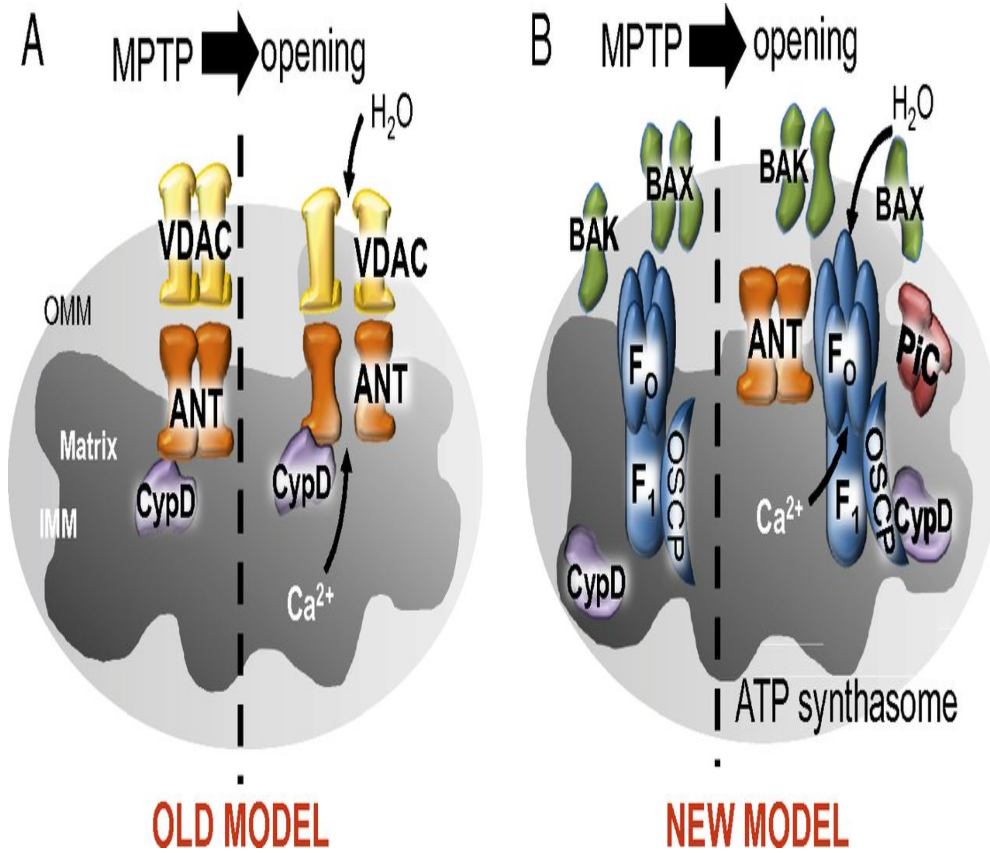


Figure 6a and 6b: Original model (A) and proposed model (B) of the MMPT pore. (Karch and Molkentin, 2014).

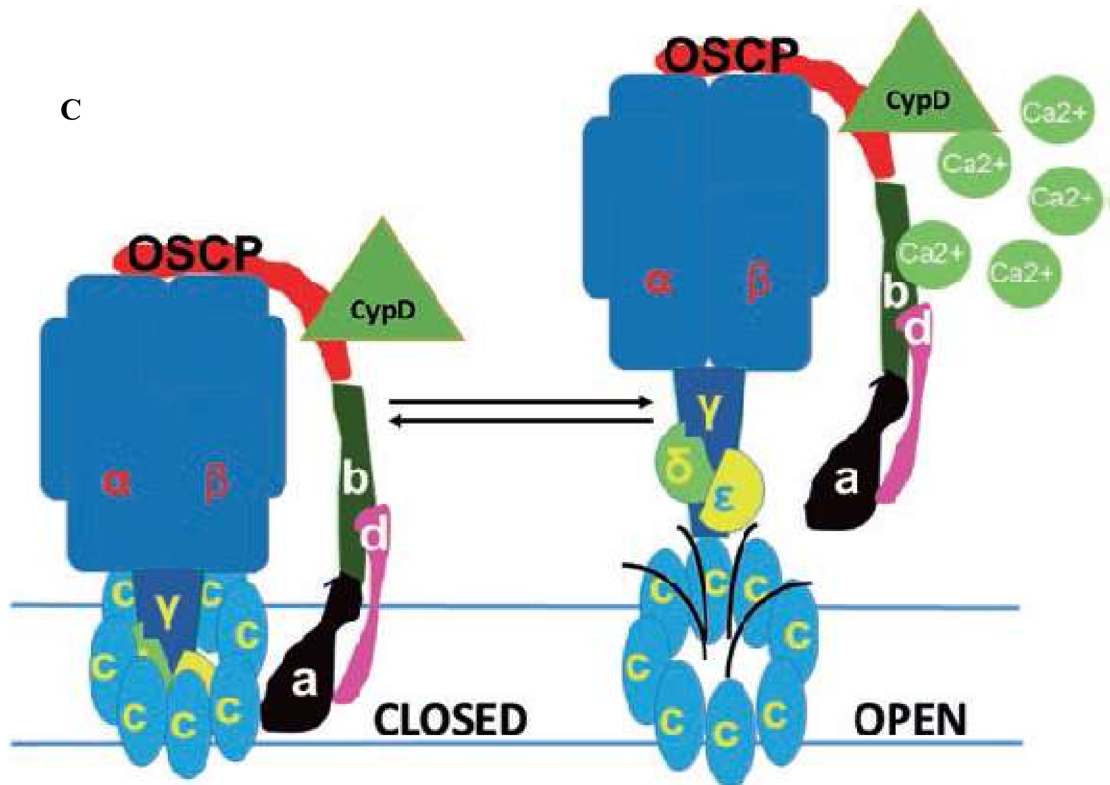


Fig 6 C.: Proposed model of the mPTP pore as the C ring . The F1 or enzymatic portion of the FoF1ATP synthase serves as the gate of the channel, which resides in the Fo (within the c-subunit ring). Upon Ca^{2+} influx into the matrix, CypD assists the F1 to lift off of the Fo, exposing the mouth of the pore which then conducts ions.(Rostovseva T. 2017)

2.24.8 MMPT PORE OPENING DURING ISCHEMIA/REPERFUSION INJURY

The effects of ROS and Ca^{2+} on MMPT have been widely reported as key players during ischemia and reperfusion damage. During ischemia, the accumulation of factors, including Ca^{2+}, long-chain fatty acids and ROS, progressively increases the susceptibility to MMPT, thus MMPT opening increasing the likelihood that MMPT will occur upon reperfusion. Indeed, the conditions that occur during ischemia and reperfusion are identical to those that induce MMPT opening. During ischemia, increased glycolysis causes the accumulation of lactic acid and the reduction of pH. To restore the pH, the Na+/H+ antiporter is activated, but it acts inefficiently because Na^{+} cannot be pumped out of the cell, as the Na^{+}/K^{+} ATPase is inhibited by the absence of intracellular ATP.

Consequently, the cytosolic Ca^{2+} concentration increases because the activity of the Na^{+}/Ca^{2+} antiporter is reduced or reversed. In addition, during ischemia, there is a decrease in the adenine nucleotide concentration, which is associated with an increased phosphate concentration, thereby sensitizing in response to Ca^{2+}; however, low pH inhibits MMPT opening. If the period of ischemia is prolonged, the heart becomes irreversibly damaged due to the activity of degradative enzymes, such as phospholipases and proteases, which also compromise mitochondrial function. Upon reperfusion, the mitochondria recover their ability to respire and rescue the sustained mitochondrial membrane potential, which is required for ATP synthesis. However, the mitochondrial membrane potential is the driving force for mitochondrial Ca^{2+} uptake, thus leading to Ca^{2+} overload. In addition, rapid and extensive production of ROS occurs when the inhibited respiratory chain is re-exposed to oxygen. Thus, the following resulting

conditions are nearly optimal for MMPT opening: high Ca^{2+} levels within the mitochondrial matrix, increased levels of phosphate and oxidative stress, depletion of adenine nucleotide concentration, and rapid return of the pH to a physiological value.

2.25 BCL-2 PROTEIN FAMILY

The proteins belonging to the Bcl-2 family exert the greatest control over the mitochondrial pathway of apoptosis by regulating the permeabilization of the OMM. (Hardwick *et al*., 2013; Du *et al*., 2000; Verhagen *et al*., 2000). Based on structure and function they are subdivided into :(1) BH3 proteins with eight members: Bcl-2-interacting domain death agonist (Bid), Bcl-2-interacting mediator of cell death (Bim), p53-upregulated modulator of apoptosis (Puma), Noxa (latin word for damage, also known as Phorbol-12-myristate-13-acetate-induced protein 1), Bcl-2 antagonist of cell death (Bad), Bcl-2-modifying factor (Bmf), Harakiri (Hrk), and Bcl-2-interacting killer (Bik), whose sequence homology with other members is restricted to the BH3 domain, BH3-only proteins Bad, Hrk and Noxa, Bid, Bim and Puma (2) the executioner proteins Bax or Bak (Chipuk *et al*., 2008; Hsu *et al*., 1997) (3) the antiapoptotic members ; Bcl-2-related gene A1 (A1), B-cell lymphoma 2 (Bcl-2), Bcl-2-related gene, long isoform (Bcl-xL), Bcl-2-like protein 2 (Bcl-w), and 23 yeloid cell leukemia 1 (Mcl-1) are the major members of this group and are responsible for protecting cells from apoptotic stimuli by preservation of OMM integrity (Chipuk *et al*., 2010)

Class 2 mitocans are compounds targeting Bcl 2 family proteins. This class of mitocans includes compounds acting as mimetics of the Bcl 2 homology-3 (BH3) domains, integral parts of Bcl -2 family of proteins.These agents have been shown to interact with BH3 binding domains, thereby interfering with the interaction between Bcl -2, Bcl-xL or Mcl

1 and the pro-Apoptotic proteins, Bax or Bak. The result is the oligomerisation of Bax or Bak to form channels and activation of the post -mitochondrial poptotic signalling (Oliver *et al.*, 2005). Gossypol has served as a lead compound for developing more efficient BH3 mimetics, such as the highly intriguing agent ABT -737 (van Delft *et al.*, 2006). ABT-737 as well as its orally applicable version, ABT -263 (Tse *et al.*, 2008) are now being tested in clinical trials. ABT-263 (Navitoclax) has been successfully tested in phase 1 clinical trial of lymphoid tumour and chronic lymphocytic leukemia, resulting in the design of phase 2 study (Wilson *et al.*, 2010; Roberts *et al.*, 2012). It has also been tested on solid tumours. Phase 1 clinical trial with mall -cell lung cancer (SCLC) or pulmonary carcinoid, patients resulted in good outcome, prompting a phase 2 study (Roberts *et al.*, 2012).

2.26 ACTIVATION OF BAK AND BAX

During apoptosis, Bax and Bak undergo conformational changes and oligomerize at discrete sites on the mitochondria (Nechushtan *et al.*, 2001). The oligomerization of Bax and Bak is proposed to result in the permeablization of the mitochondrial membrane and the release of pro-apoptotic molecules involved in the execution of apoptosis (Du *et al.*, 2000; Verhagen *et al.*, 2000). Wei and colleagues in 2001, described that cells lacking both Bax and Bak, but not cells lacking only one of these components, are completely resistant to tBID-induced cytochrome c release and apoptosis. Moreover, double deficient cells are resistant to multiple intrinsic apoptotic stimuli (Wei *et al.*, 2001). Given their critical role in mediating mitochondrial apoptosis, Bax and Bak have to be strictly regulated by other members of the Bcl-2 family.

Bax is a monomeric protein that is predominantly localized in the cytoplasm with minor population loosely associated with mitochondria and endoplasmic reticulum (Hsu *et al.*,

1997). Unlike Bax, Bak is constitutively associated with the mitochondrial membrane and the endoplasmic reticulum. Early during apoptosis, activated Bax translocates to the mitochondria, inserts into the OMM and oligomerizes (Nechushtan *et al*., 2001; Lovell *et al*., 2008). Bax and Bak contain nine α-helices with a hydrophobic α5 helix at the protein core, surrounded by amphipathic helices (Suzuki et al., 2000). When Bax and Bak become activated, they undergo major conformation changes involving the C terminus (of Bax only), N terminus, the BH3 domain and the α5 and α6 helices, resulting in formation of membrane oligomers. The locally concentrated lipophilic residues could then penetrate the membrane bilayer, provoke positive membrane curvature and eventually membrane rupture (Westphal *et al*., 2014). Bax and Bad double knockout mouse embryonic fibroblasta are resistant (MEFS) are resistant to apoptotic, necrotic and autophagic forms of cell death. Specifically, loss of inhibition of Bax and Bak rendes MEFs resistant to calcium overload as well as protecting mice from ischemia-reperfusion injury in the heart and brain when specifically deleted from cardiomyoctes and neurons respectively.

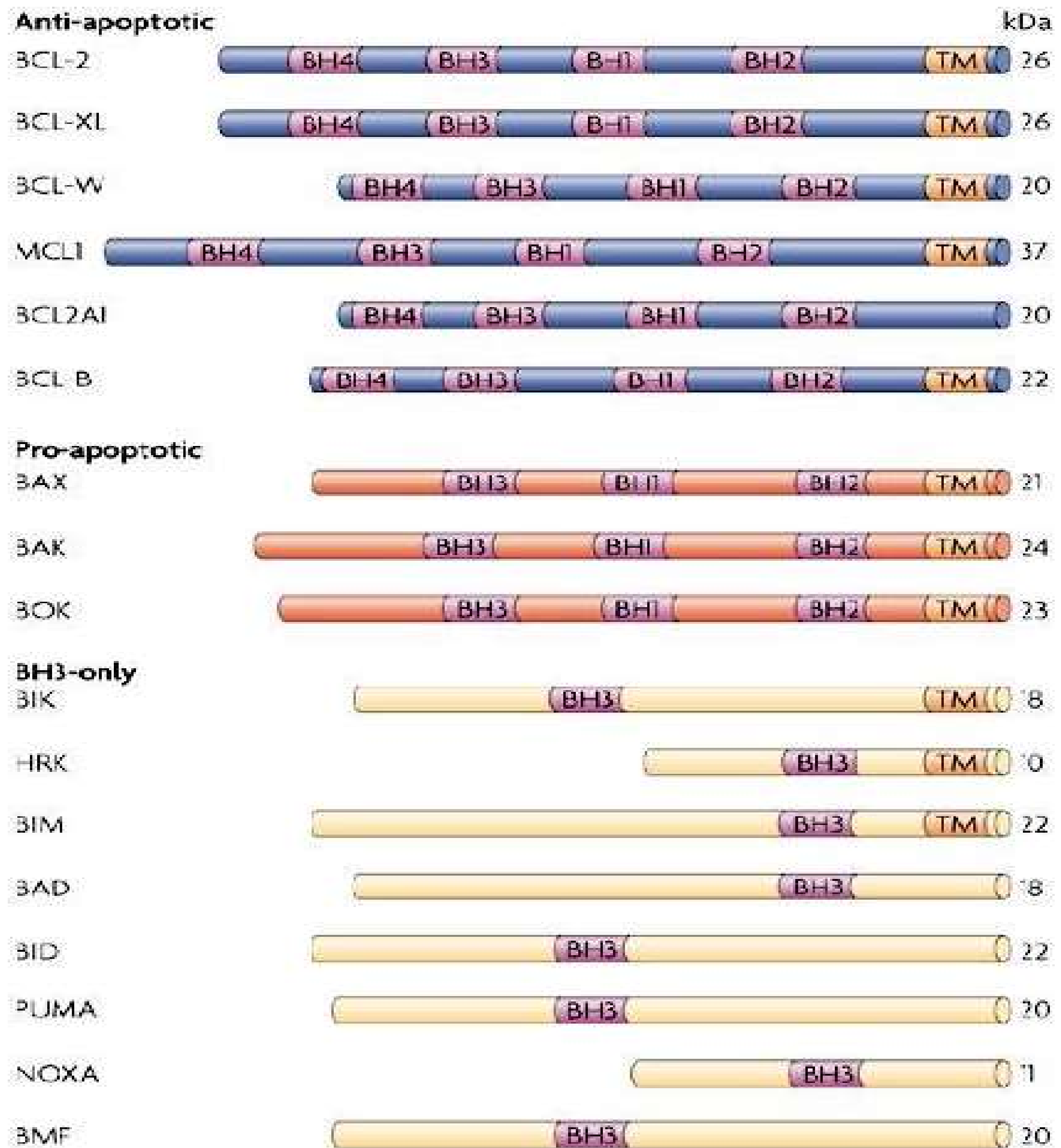


Figure 7: The schematic structure of Bcl-2 family proteins (Taylor *et al.*, 2008).

2.27 CASPASE ACTIVATION

Caspases are proteases which degrade intracellular proteins during apoptosis.Their enzymatic process takes place in a controlled manner with minimal effect on surrounding tissue. There is increased interest in the levels of caspases in biological tissue since this has been linked with the pathogenesis of different neurodegenerative diseases and cancer. In cancer, there is downregulation of caspase or insufficient caspase activation. Hence caspase antibodies play a clinical role in prognosis and progression of cancer in addition and also to identify apoptotic cells (Xu *et al.,* 2018).

2.28 MEMBRANE ALTERATIONS

The processes that affect cell membrane dynamics during the apoptotic program have attracted considerable interest over the years. Alterations in the plasma membrane has been shown by several studies to be a characteristic feature of apoptosis.The anionic phospholipid phosphatidylserine (PS) normally confined to the inner plasma membrane in healthy cells is translocated to the outer membrane leaflet during apoptosis (Julian and Olson, 2015). It is believed that caspases are responsible for this flip-flop mechanism, although how caspases promote PS externalization remains a mystery.

2.29 DNA FRAGMENTATION

The fragmentation of DNA into nucleosomal units is caused by an enzyme known as CAD, or caspase activated DNase,which cut the internucleosomal regions into double-stranded DNA fragments of 180–200 base pairs (bp) and it is the major biochemical hallmark of apoptosis (Wyllie, 1980). Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD). During apoptosis, ICAD is cleaved by caspases, such as caspase 3, to release CAD. The inability to degrade DNA can give rise to autoantibodies

that can cause lupus erythematosus-like autoimmune disease. Since apoptosis and DNA cleavage go hand in hand, detection of DNA fragmentation is currently one of the most frequently used techniques for highlighting apoptotic cells in tissues. A traditional method for demonstrating internucleosomal DNA degradation is gel electrophoresis of genomic DNA. In tissue sections, the TUNEL (terminal dUTP nick end-labeling) method is widely used to measure DNA fragmentation (Agarwal, 2017). The principle of the assay is that endonuclease-generated DNA breaks are enzymatically labeled by terminal transferase with UTP derivatives coupled to fluorochromes or biotin that can be detected in an immunoperoxidase reaction. This assay is very sensitive, and allows DNA fragmentation to be assessed quantitatively by light and fluorescence microscopy or by flow cytometry.

2.30 MITOCHONDRIAL CHANGES

The permeabilization of the mitochondrial outer membrane i this subsequently leads to the release of some proteins.Western blotting or immunocytochemistry are the techniques used to quantify these proteins particularly in the cytosolic, mitochondrial and nuclear fractions. Loss of the inner mitochondrial transmembrane potential the electrochemical proton gradient generated by the respiratory chain also takes place along with the opening of the MMPT pore (Kroemer *et al*., 2007). The MMPT pore may be detected by measuring the mitochondrial transmembrane potential $\Delta\Psi m$), which is disrupted once the outer membrane is permeabilized. The loss of $\Delta\Psi m$ can be observed using positively charged (cationic) dyes such as TMRE and JC-1 that accumulate in the mitochondrial matrix when $\Delta\Psi m$ is maintained in healthy cells, in apoptotic cells, a shift in mitochondrial transmembrane potential prevents the dye from accumulating in the mitochondria.

2.31 MODULATION OF APOPTOSIS BY BIOACTIVE AGENTS IN MEDICINAL PLANTS

Bioactive agents from medicinal plants have been demonstrated to modulate apoptosis in diverse cell model and their mechanism of action been identified to include upregulation of Bax;,downregulation of Bcl-2, downregulation of BAD, upregulation of p53 , activation of caspases, induction of FasL, increasing TRAIL receptors, Inhibition of p38 and NF-kB, activation of MAPK, JNK, AP-1, ERK, PI3K, JNK and Akt, disruption of mitochondrial membrane potential and cytochrome c release or a combination of these mechanisms. carotenoids, curcumin, genistein, Indole-3-carbinol (I3C), 3, 39-diindolylmethane (DIM) the garlic compounds diallyl sulfide (DAS) and diallyl disulfide (DADS) and plumbagin, can regulate proapototic/antiapoptotic protein ratios. Numerous examples demonstrate that dietary bioactive agents can induce mitochondrial release of cytochrome c. Lycopene, EGCG, curcumin, 6-gingerol, grapeseed extract has been shown to induce apoptosis in human hepatoma cells by cytochrome c release. Bioactive agents in medicinal plants can induce apoptosis through caspase activation. Resveratrol increases caspase activity (caspases 6, 3, and 9) in numerous models, including normal and hematopoietic cells. Indole-3-carbinol upregulates Bax which induces the release of cytochrome c, and activates caspases 3 and 9. The organosulfur compounds found in garlic have shown potent pro-apoptotic activity in numerous models. Accumulating evidence indicates that dietary bioactive agents can trigger specific aspects of the extrinsic apoptosis pathway. Resveratrol has been shown to trigger CD95 signaling dependent apoptosis in human tumor cells. Dietary diterpenoids induce apoptosis in human prostate cells by upregulation of Fas ligand.

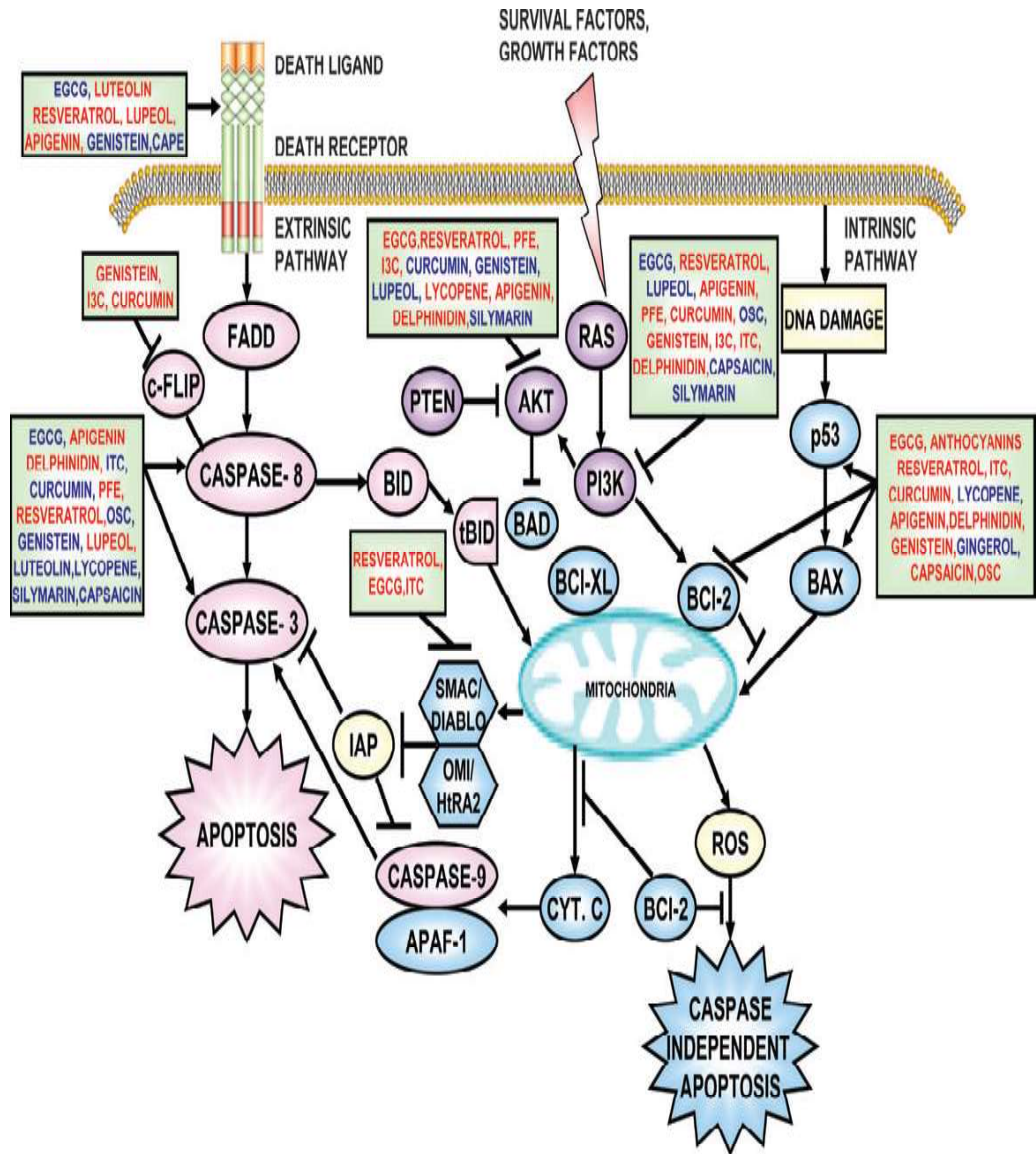


Figure 8: Induction of apoptosis by dietary chemopreventive agents

2.32 MEDICINAL PLANTS

Medicinal plants are defined as plants that can be used for medicinal or therapeutic purposes (Rekha *et al.,* 2013). Secondary metabolites are structurally diverse group of compounds found in plants. The functions of secondary metabolites are varied. Some secondary metabolites are toxins used to deter predation, and others are pheremones used to attract insects for pollination. Phytoalexins protect against bacterial and fungal attacks while allelochemicals inhibit rival plants that are competing for soil and light. Paradoxically these compounds present in medicinal plants are useful to human for the treatment of diseases. Medicinal plants have been used in traditional healthcare systems for many centuries. Traditional knowledge of indigenous communities about plant diversity has made them major contributors to primary health care for majority of the population around the world particularly those in rural areas. Decoctions from the leaves, bark, seeds, roots, fruits and other parts of plants have been the first source of therapeutic relief to various ailments for many communities (Iwu, 1993). The World Health Organization has estimated that 80% of the earth's inhabitants rely on traditional medicine for their health care needs, and most of this therapy involves the use of plants extracts or their active components.

Tropical countries have a wide variety of medicinal plants, some species are found in the wild while some are domesticated by farmers. The medicinal power of these plants lies in phytochemical constituents that cause definite pharmacological actions on the human body. Phytochemical are natural compounds which occur in plants such as medicinal plants, vegetables and fruits that work with nutrients and fibers to act against diseases or more specifically to protect against diseases (Akinmoladun *et al*., 2007). Many antioxidants such as vitamin C, vitamin E and carotenoids, occur as dietary constituents.

Antioxidant are found in fruits, vegetables and in different beverages. For example, antioxidants have been found in berries, apples, citrus fruits and in fruit juices . Highly active antioxidants were found in olives and olive oil. Red wines contain a variety of polyphenolic compounds, the most abundant being anthocyanins, and they have been shown to have high antioxidant activity. Green and black teas have been extensively studied for antioxidant properties. The main compounds responsible for antioxidant activity were found to be catechins. Epigallocatechin 3-gallate, epigallocatechin, epicatechin 3-gallate, epicatechin, gallocatechin and catechin were identified and their antioxidant activities have been studied. Herbs and spices are also good sources of antioxidants.

Plants have also been used in the production of stimulant beverages: tea, coffee, cocoa and cola or intoxicants :Wine, and Beer. The active components of these stimulants are methylated xanthine derivatives namely theophylline and theobromine, which are the main constituents of coffee, tea, and cocoa, respectively. The most important intoxicant in society today are wine, beer, and liquor made from fermentation of fruits and cereals. Higher plants have been used as a source of drugs by mankind for several thousands of years. In fact ancient man was totally dependent on green plants for day to day needs of medicament.

Despite the development of modern medicine, synthetic drugs and antibiotics, there has been increased emphasis on the use of plant materials as source of medicine as a result of the severe side effects associated with frequent use of synthetic drugs. Researchers select indigenous plants based on their use by traditional healer throughout the world. Scientific proof of the use of these traditional medicinal plants has been considered and about 20% of medicinal plants have been studied for their biological activities (Coopoosamy and

Naidoo, 2012) Several researches conducted showed that indigenous plants have several biological activities such as antioxidant, anticarcinogenic, antimutagenic, antitumor, antibacterial, antiviral, anti-inflammatory antifungal, insecticidal, hepatoprotective, antirheumatic, and antivenin (Akrout *et al.,* 2011; Iqbal *et al.*, 2012). These biological activities emanate from the presence of different types of compounds that constitute the various plant parts known as secondary metabolites. Secondary metabolites include flavonoids, tannins, polyphenols, sapnosides, chromones and tophenones (Akrout *et al.,* 2011). Furthermore plants are used as a basis of some of the most important drugs and it has been documented that extract of some plants useful in crude form. Plant-derived compounds have been an important source of several clinically useful anti-cancer agents. These include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and paclitaxel (taxol).

The search for novel anticancer agents currently targets chemical entities that selectively induce apoptosis and/or reverse MDR. Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural sources. Plant extracts can be regarded as chemical libraries of structurally diverse compounds, their investigation therefore constituting a promising approach in drug discovery. The discovery of natural products for cancer treatment is of great urgency to improve prospects of people with the disease. Phytochemicals in food, such as flavonoids have been demonstrated to inhibit carcinogenesis in vitro and substantial evidence indicates that they can also do so in vivo. Flavonoids seem to be a promising approach to cancer chemoprevention and their mechanism of action been identified to include carcinogen inactivation, antiproliferation, cell cycle arrest, induction

of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms

2.33 *CAJANUS CAJAN*

Cajanus cajans is a perennial legume of the family Fabaceae, it is a shrub that is common in the South Western part of Nigeria. It is commonly known as Pigeon pea in English, Otili in Yoruba, Fio-fioin in Igbo, Aduwa in Hausa and Olele in Edo (Wu *et al*., 2009). Traditionally, several medicinal uses have been documented for this plant. It is used for treatment of diabetes in India for febrifuge, stabilization of the menstrual period and dysentery in South America, and for treatment of hepatitis and measles in Africa. The leaves of *Cajanus cajan* are used to arrest blood, relieve pain and kill worms. The young leaves of the pcan be chewed for treating aphtha, and the decoction of the leaves has been proved to treat cough and diarrhea effectively It is used as a strong diuretic, the fresh seeds are said to help incontinence of urine in males, while immature fruits are used in liver and kidney ailments. The seeds (pigeon peas) are also a popular food in developing countries such as Nigeria (Burkhill, 1978). It has been used for many years traditionally for treating sores, skin irritations, diabetes, hepatitis, measles, jaundice, dysentery and stabilizing the menstrual period (Grover *et al*., 2002, Yuan-guang Zu, 2010).

2.34 PHARMACOLOGICAL PROPERTIES OF *CAJANUS CAJAN*

In recent years, it has also been explored for the treatment of ischemic necrosis of the caput femoris, aptha, bedsore and wound healing. Chemical investigation has revealed the presence of two globulins, cajanin and concajanin (Ambasta, 2004). Chemical studies reveal 2’-2’ methylcajonone, 2’- hydroxygenistein, isoflavones, cajanin, cahonones which impart antioxidant properties.The roots are also found to posses genistein and genisten. It

also contains hexadecanoic acid, α amyrin, β Sitosterol, pinostrobin, longistylin A, longistylin C which impart anticancer activity. Presence of Cajanuslactone, a coumarin imparts antibacterial activity. Presence of cajanin, stilbene acid, pinostrobin, vitexin and orientin is responsible for antiplasmodic activity.

A new natural coumarin cajanuslactone has been isolated from the leaves of *Cajanus cajans* which is a potential antibacterial agent against Gram-positive micro-organisms. The three stilbenes, cajanin, longistylin C and longistylin A from leaves have been found to possess hypocholesterolemic effects. Anti-plasmodial activities have also been confirmed in betulinic acid isolated from roots and longistylin A and C obtained from leaves (Burkhill, 1978).

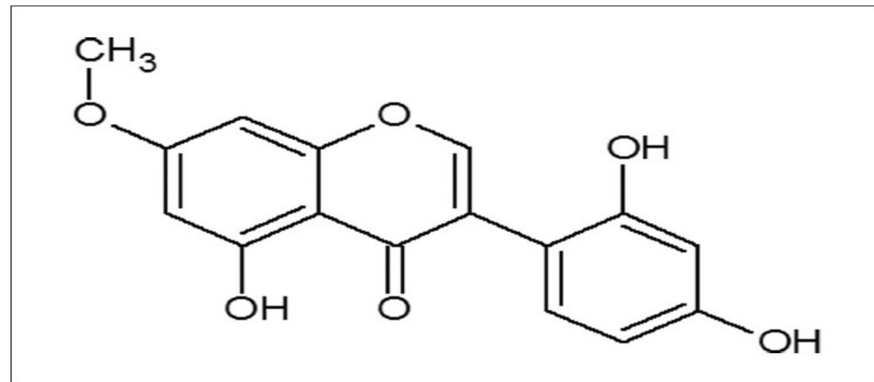
Two isoflavanoids genistein and genistin isolated from the roots were found to possess antioxidant activity. Four important compounds, pinostrobin, cajaninstilbene acid, vitexin and orientin isolated from ethanolic extracts of leaves were found to possess significant antioxidant properties (Burkhill, 1978). Particularly, Pinostrobin, a substituted flavanone isolated from leaves possesses anti-inflammatory activity and inhibits sodium channel-activated depolarization of mouse brain synaptoneurosomes (Nicholson *et al*., 2010). Previous studies on the evaluation of the in vitro activities of isoflavanoids isolated from ethanolic extract of on some microbial strains showed marked inhibitory effects against several strains Some protein fraction isolated from leaves also showed hepatoprotective effects and the presence of phenolics (flavanoids and tannins) impart anthelmintic activity. *In vivo* antimicrobial activity was studied in mice that had been inoculated with *S. aureus* and the potential mechanism of antimicrobial activity was studied by histopathology (Yuan-gang *et al*., 2010). Methanol extract of *Cajanus cajan* significantly reduced the fasting bold sugar of alloxan-induced diabetic rats in a dose dependent

manner with maximum hypoglycemic effects at 4 to 6 hours (Ezike *et al*., 2010). Negative pressure cavitation extraction of *Cajanus cajan* roots showed notable concentration dependent antioxidant activity (Pal *et al*., 2008).

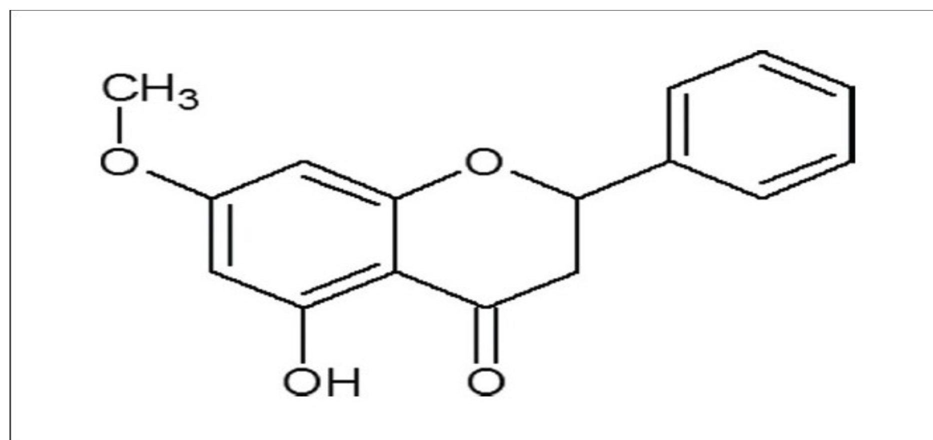
Methanol aqueous fraction of the leaf reversed liver damage by decreasing the activities of liver enzymes and augmenting antioxidant enzyme activities showing a promise in alcohol-induced liver dysfunction (Ashlan and Islam, 2009) Cajanol an isoflovanone found in the roots is found to possess anticancer activity. The three stilbenes, cajanin, longistylin C and longistylin A from leaves have been found to possess hypocholesterolemic effects. Anti-plasmodial activities have also been confirmed in betulinic acid isolated from roots and longistylin A and C obtained from leaves. Some protein fraction isolated from leaves also showed hepato-protective effects and the presence of phenolics (flavanoids and tannins) impart anthelmintic activity. The aqueous methanolic extract of the seeds of *Cajan* (pigeon pea- a major constituent/base of a herbal formula, CiklaviteR, used in the Management of Sickle Cell disease in Nigeria)has been found to posses significant anti-sickling activity in concentration dependent doses. This was achieved based on previous studies by Ekeke and Shode, (1990).

Plate 1: ***CAJANUS CAJAN*** **LEAVES**

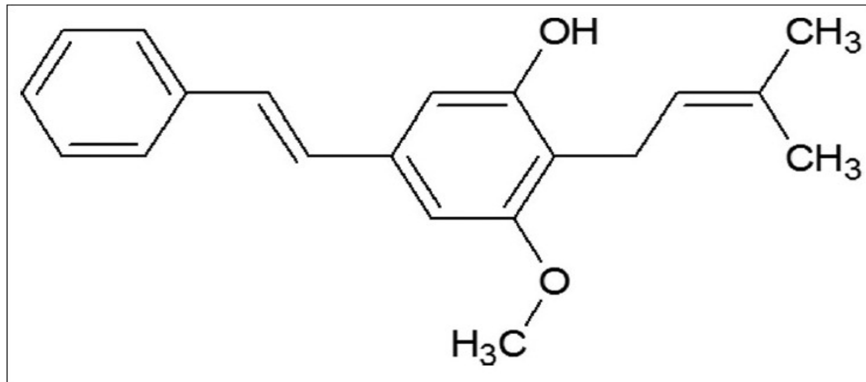
Cajanin



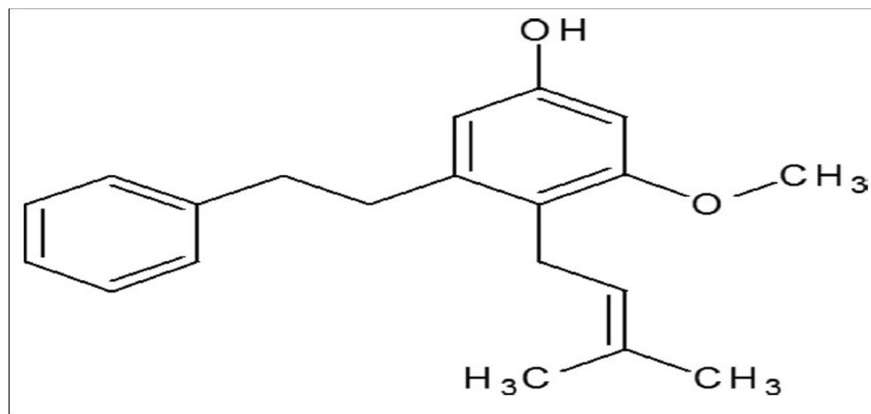
Pinostrobin



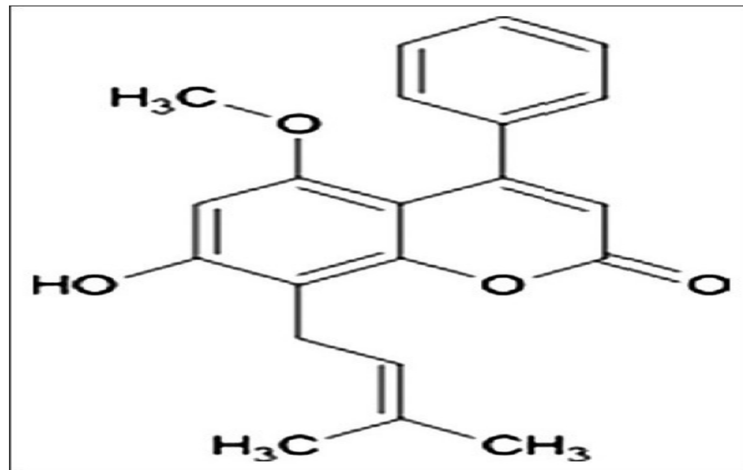
Longistylin A



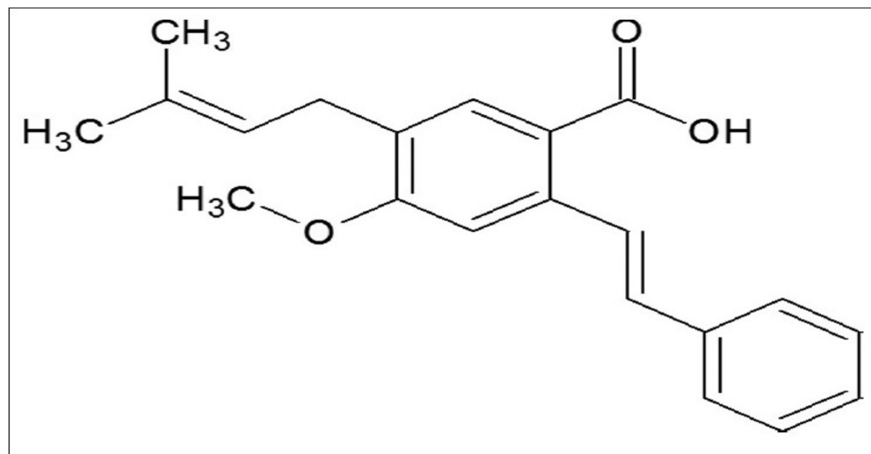
Longistylin C



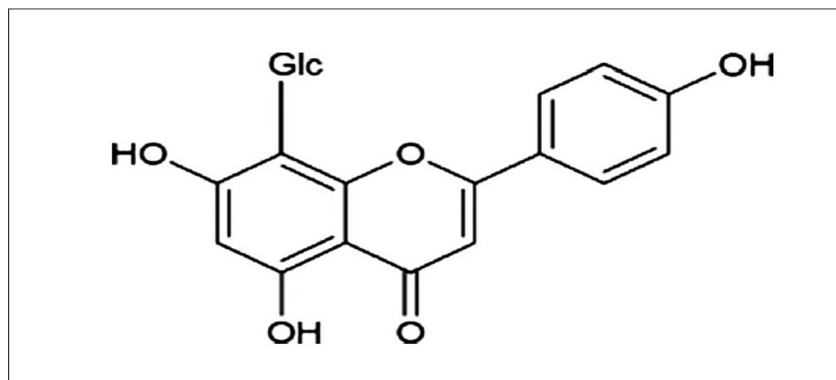
Cajanuslactone



Cajaninstilbene acid



Vitexin



Orientin

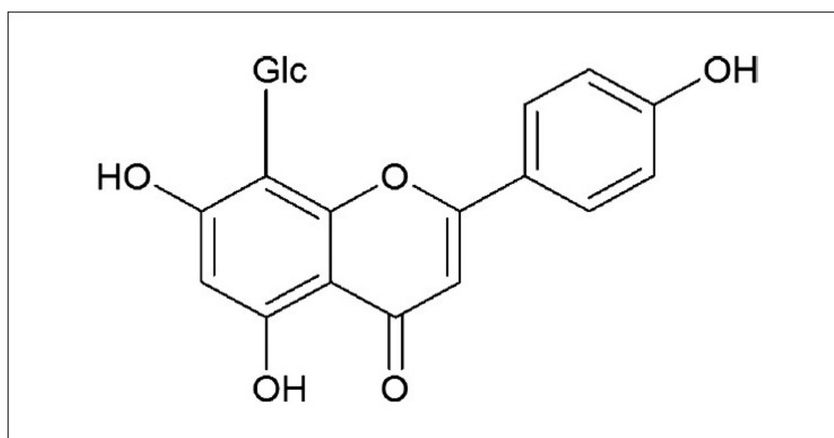


Figure 9: **FLAVONOIDS IN *CAJANUS CAJAN* LEAVES** (Pal *et al*., 2011)

CHAPTER THREE

MATERIALS AND METHODS

3.1 COLLECTION AND IDENTIFICATION OF THE PLANT MATERIAL

The fresh leaves of *Cajanus cajan* were obtained from Bode market in Ibadan, Nigeria and authenticated by the Department of Botany, University of Ibadan and a specimen Voucher No.UIH22500 was deposited in the Herbarium. The leaves were air dried for 2 weeks, pulverised and soaked in methanol for 72 hours and filtered. The filterate was concentrated using a rotary evaporator under reduced pressure at 40°C and the concentrate was made solvent-free by further concentrating in a water bath until a constant weight was obtained. The MECC was kept in a glass stoppered bottle in a refridgerator.

3.2 PARTITIONING OF CRUDE METHANOL EXTRACT

A Buchner funnel filled with silica gel 60 (0.043-0.063, MERCK) was fitted into a flat bottomed flask which was attached to a vacuum pump. A slurry of the sample was prepared by mixing the crude extract with silica gel (1:1) in a petri dish. Then, the sample was placed in the funnel and the surface of the sample was leveled using a spatula. Partitioning of the extract was done by eluting the column with n- hexane, chloroform and ethyl acetate (in increasing polarity). The resulting fractions were collected and concentrated using a rotary evaporator. Fractionation of methanol extract by vacuum liquid chromatography yielded the n-hexane fraction, chloroform fraction and ethylacetate fraction.

Weight of the fraction= weight of sample and bottle- weight of bottle only:

Percentage yield of extract = $\frac{X-Y}{Z}\ x\ 100$

Where X, Y and Z are weight of fraction and bottle, weight of bottle only and weight of fraction only respectively.

3.3 PHYTOCHEMICAL SCREENING OF THE LEAVES OF *CAJANUS CAJAN*

Preliminary qualitative phytochemical screening of each fraction was carried out using the following methods

3.3.1 Test for Flavonoids:

Flavonoids are polyphenolic compounds composed of two benzene rings. They react with ammonia or base to give an intense yellow colour. To a small amount of fraction 5 mL of dilute ammonia solution were added, followed by addition of concentrated H_2SO_4. Yellow coloration in each fraction indicated the presence of flavonoids (Edeoga *et al*., 2005). Flavonoid was further confirmed by adding few drops of dilute NaOH solution to 1 mL of stock solution. An intense yellow colour appeared which turned colourless on addition of a few drop of dilute acid.

3.3.2 Test for Tannins

Tannins are phenolic compounds defined as substances present in plant which are able to combine with protein forming stable water- insoluble copolymers**.** Tannins react with ferric chloride forming, a blue black, green or blue-green precipitate. To 2 mL extract a

few drops of 1% lead acetate were added. A yellowish precipitate indicating the presence of tannins was observed (Savithramma *et al*., 2011).

3.3.3 Test for Saponins

Saponins are a diverse group of compounds. Saponins consist of aglycone unit linked to one or more carbohydrate chains. The frothing test is based on soap-like effect produced by saponins because of their surfactant properties which enable them to lower surface tension when dissolved in water. To 5 mL fraction 20 mL of distilled water was added for 15 minutes. Formation of foam indicates the presence of saponins.

3.3.4 Test for Anthocyanins

Anthocyanins are a group of coloured compounds found in plants. To 2 mL of extract, 2 mL of 2N HCl and ammonia was added. The pink-red colouration turned blue-violet showed the presence of anthocyanins (Savithramma *et al*., 2011).

3.3.5 Test for cardenolides

Cardenolides are cardiac glycosides with a five member lactone ring and a deoxy sugar as constituents. They are a special group of steroids that have effect on the cardiac muscle. They can be detected by analyzing for the presence of deoxy sugars using Keller-killiani‘s procedure. The deoxy sugars in cardenolides react with ferric chloride in an acidic medium forming reddish-brown complex that gradually becomes blue.

3.3.6 Test for Alkaloids

Alkaloids are organic nitrogenous compounds that are weak bases. Alkaloids react with potassium bismuth iodide in Dragendroff's reagent to form yellowish brown coloured complex. About 0.5 g fraction was stirred with 5 mL of 1% dilute hydrochloric acid on a water bath. The resulting solution was then filtered and 1 mL of the filtrate was then treated with a few drops of Mayer's reagent and a second 1 mL portion with Dragendorff's reagent. Turbidity or precipitation with either of these reagents was taken as evidence for the presence of alkaloids in the extract (Harborne, 1984; Trease and Evans, 1989). Alkaloid was further confirmed by Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate (Harborne, 1984; Trease and Evans, 1989).

3.3.7 Test for terpenoids

Terpenes are are hydrocarbons built from isoprene units consisting of 5 carbon atoms attached to eight hydrogen atoms. Salkowski test confirms the presence of terpenes. About 5mLs of each fraction was mixed in 2mLs of chloroform and concentrated H_2SO_4 (3mLs) was carefully added to form a layer. A reddish brown coloration on the interface was formed to show positive results for the presence of terpenoids.

3.4 ANIMALS AND EXPERIMENTAL DESIGN

Male wistar albino rats (150±2.00 g) and male wistar albino mice (17±0.04 g), were purchased from the Central Animal House of the College of Medicine, University of Ibadan, Nigeria. They were acclimatized for two weeks in well ventilated cages under standard environmental conditions of 12 hr light /dark cycle and given Ladokun animal

feed and water *ad libitum*. The rats were divided into three groups with 5 animals each; group 1 animals were given corn oil (vehicle), groups 2 and 3.They were orally administered 100 and 200 mg/kg body weight of MECC. The extract was orally administered to rats once daily for 30 days. The mice were divided into three groups with 5 each.; mice in group 1 were given corn oil (vehicle), groups 2 and 3, and were orally administered 100 and 200 mg/kg body weight of EACC.The extract was administered to rats once daily for 14 days.

3.5 COLLECTION OF BLOOD

The animals were sacrificed by cervical dislocation and blood was quickly collected from retro-orbital plexus into plain and EDTA bottles, placed in a table centrifuge and spun at 3,000 rpm for 10 minutes, then the serum was separated from the clot. The serum obtained was used to assay for alanine amino transferase, aspartate amino transferase alkaline phosphatase, caspase 3, 9 .and cytochrome C.

3.6 HISTOLOGY OF THE ORGANS

After sacrificing the animals, they were dissected and the organs were harvested and processed. Then the sections were observed under microscope and their photomicrographs were taken.

3.7 IMMUNOHISTOCHEMISTRY OF ORGANS

Liver sections were collected in 10% phosphate buffered formalin. Liver specimen were processed and embedded in parafin using standard procedures. The formalin fixed tissue sections were deparafinised in xylene, and the primary antibody was added followed by the addition of the secondary antibody. This was followed by the addition of horse radish

peroxidase for 10 minutes, then immunoperoxidase diaminobenzidine (DAB) colour (Dako, Glostrup, Denmark). Finally counterstaining was performed using Mayer's hematoxylin.

3.8 ASSESSMENT OF MITOCHONDRIAL MEMBRANE TRANSITION PORE IN RAT AND MOUSE LIVER MITOCHONDRIA

Reagents

Homogenising Buffer: **(Buffer C)**: **(210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH, pH 7.4 and 1mM EGTA).**

About 0.12g HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]
(Sigma Aldrich, USA), 3.83 g of Mannitol (Sigma Aldrich, Inc., USA), 2.4 g of sucrose and 0.038 g EGTA were dissolved in 70 mL of distilled water and adjusted to pH 7.4 using KOH (Sigma Aldrich, Inc., USA)) and the solution made up to 100mL with distilled water. The solution was swirled gently in order for the BSA to dissolve and prevent foaming. The buffer was also stored at 4˚C.

Washing buffer (Buffer D): (210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH, pH 7.4 and 0.5% BSA)

About 0.12 g of (HEPES) 4-(2-hydroxyethyl)-piperazin-N’-2-ethansolfonic acid, 2.4g of sucrose 3.83g of mannitol and 0.5g of Bovine Serum Aluminium (BSA) were dissolved in 70 mL and made up to 100 mL with distilled water. The pH adjusted to 7.4 using KOH .

Suspension buffer (MSH buffer): (210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH, pH 7.4)

About 0.12 g of (HEPES) 4-(2-hydroxyethyl)-piperazin-N'-2-ethansolfonic acid, 19.15 g of mannitol, 12 g of sucrose were dissolved in 480 mL of distilled water, standardized with 1M KOH pH (7.4) and then made up to 500 mL. Reagents were purchased from Sigma and Co, USA.

3.9 ISOLATION OF LOW IONIC STRENGTH LIVER MITOCHONDRIA

Low ionic strength mitochondria were isolated according to the method of Johnson and Lardy (1967) as modified by Olorunsogo *et al*., (1979). The animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in buffer C then weighed and minced with a pair of scissors. Liver suspension (10 %) was prepared in ice cold buffer C by homogenizing the liver in a potter-Elvehjem glass homogenizer. The homogenate was sedimented twice at 2500 rpm for 5 minutes to remove the nuclear fraction and cellular debris. The supernatant obtained was centrifuged at 13000 rpm for 10 minutes and the mitochondrial fraction obtained was washed three times at 12000 rpm for 10 minutes with buffer D.

The mitochondria were immediately suspended in a solution of ice cold MSH buffer then dispensed in precooled Eppendorf tubes as aliquots and placed on ice for immediate use. Mitochondria used to assay for mitochondrial ATPase activity was prepared using the procedure above except that the ice-cold sucrose buffer was used for the isolation.

3.10 ASSAY ON MITOCHONDRIAL PERMEABILITY TRANSITION IN RAT AND MOUSE LIVER MITOCHONDRIA

Principle

Mitochondria undergoing calcium induced permeability transition (PT) show colloidosmotic, large amplitude swelling which results in a decrease in photometric absorption at 540 nm. Permeability transition was assessed by measuring the swelling of mitochondria based on the principle that as mitochondria swells with concomitant release of mitochondria proteins, the refractive index changes and less light is scattered (Lapidus and Sokolove, 1993). This is detected as a decrease light absorbance spectrophotometrically. To avoid any complications that changes in the redox state of respiratory chain components might cause, the wavelength of the incident light should be at the isobestic point for the cytochromes (520 nm or 540 nm) as used in several studies on isolated mitochondria.

Reagents

0.8µM Rotenone

About 0.00079 g Rotenone was dissolved in 50 % Ethanol (5 mL ethanol was dissolved with 5 mL water). The rotenone was also stored in a dark container because of its photosensitivity and stored at 4^0C.

5mM Succinate

Sodium succinate (Sigma-Aldrich, Inc., USA) (0.6754 g) was dissolved in 10 mL of distilled water.

300µM $CaCl_2$

About 0.01764 g of $CaCl_2$ (May and Baker Lab., Products) was dissolved in 10 mL of distilled water and stored in a container.

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

About 0.12 g of HEPES (Sigma-Aldrich, Inc., USA) was dissolved in 80 mL of distilled water and pH adjusted to 7.4 using KOH, mannitol (3.83g) and 2.4g of Sucrose were dissolved in the solution containing HEPES-KOH (7.4) and made up to 100 mL with distilled water in a standard volumetric flask and stored in the refrigerator.

1mM Spermine

Spermine (Sigma-Aldrich, Inc., USA) (0.00809 g) was dissolved in 10 mL of distilled water. The Spermine was stored in a dark container due to its photosensitivity and stored at 4^0C.

Assay of mitochondrial membrane transition pore in rat and mouse liver mitochondria

Mitochondrial swelling is measured by continuous time scan of the difference in absorbance at 540nm at 30 seconds interval, over 12 minutes.

NTA: Buffer → Rotenone → water →Mit 3½minutes → Succinate

TA: Buffer → Rotenone → water → Mit 3minutes →$CaCl_2$ ½minutes→Succinate

I: Buffer → Rotenone → water → Spermine → Mit 3minutes → $CaCl_2$ ½ min → Succinate

CC: Buffer → Rotenone → water → Mit →CC 3minutes → $CaCl_2$→ ½ min → Succinate→*Cajanus cajan*

Procedure

Mitochondria (0.4 mg/mL) were incubated in the presence of of 0.8 µM Rotenone and MSH buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH pH 7.4 for 3.5 minutes prior to the additon of 5 mM Succinate. For the assay containing calcium as triggering agent, 120 µM calcium was added as triggering agent 3 minutes after mitochondrial incubation in rotenone and 30 seconds after 5 mM succinate was added .When inhibition by spermine was assayed spermine was added prior to mitochondria. Temperature was maintained at 30°C and swelling rate was quantified as decrease in absorbance at 540nm wavelength at interval of 30 seconds. In order to monitor the MMPT in the presence of extract, mitochondria samples are incubated with varying concentrations of extract.

NTA: No Triggering Agent; **TA**: Triggering Agent; **Mit-** The volume of intact mitochondria. *CC* : *Cajanus cajan* .The volume of the reagents is in µL .The volume of the mitochondria depends on the value derived from protein determination. The spectrophotometer reading was taken at 540 nm

Table 1: Protocol for Mitochondria Membrane Permeability Transition Assay

Samples	Buffer	Distilled Water	Rotenone(0. 8μM)	Spermine(1 mM)	Mit(0.4 mg/mL)	$CaCl_2$ (120 μM)	Succinate (5mM)	CC
Blank	2200	300	-	-	-	-	-	-
NTA	2200	-	10	-	-	-	50	-
TA	2200	-	10	-	-	25	50	-
S.I	2200	-	10	63	-	25	50	-
CC	2200	-	10	-	-	-	50	10
CC	200	-	10	-	-	-	50	30
CC	2200	-	10	-	-	-	50	50

3.11 PROTEIN DETERMINATION BY LOWRY'S PROCEDURE

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

Principle

The colour reagent used is phospho-18-molybdictungstic complex (a mixture of several molecular forms such as $3H_20.P_2O_5.9MoO_3$ and $3H_2O.P_2O_5.10WO_3.8MoO3$), which can be reduced by phenol groups giving a blue colour at alkaline pH. Tyrosine and tryptophan residues presenting the protein are responsible for the reduction of this phospho-18-molybdictungstic complex. This method is very sensitive.The colour reagent which is called 'phenol' reagent is very unstable and decomposes easily in alkaline solutions since it reacts with tyrosine only at alkaline pH, an excess of the reagent need to be added for complete reaction.However, a high concentration of this phospho-18-molybdictungtic acid can maintain the phospho-molybdictungstic reagent in the oxidized stage during storage.Lowry *et al* (1951) found that pretreatment of the protein sample with alkali copper markedly increased the colour developed during the reduction reaction of phospho-18- molybdictungstic reagent .In their assay medium, they also added a mixture of Na_2CO_3 and NaOH to buffer the pH around 10 to neutralize the phosphoric acid produced by the degradation of the phosphormolybdictungstic complex at alkaline pH. The Folin-Ciocalteau test is sensitive, samples containing as little as 5mg of protein can be readily analyzed

Reagents

Reagent A: 2% Na_2CO_3 in 0.1M NaOH

About 2 g Na_2CO_3 (BDH Chemicals Ltd, England) and 0.4g NaOH (Sigma Chemical Co, USA) were dissolved in 100mL of distilled water and stored at room temperature.

Reagent B: 2% Na-K-tartarate

About 2 g Sodium potassium tartarate (Hopkins and Williams England) was dissolved in 100mL of distilled water and stored.in a standard volumetric flask.

Reagent C: **1% $CuSO_4.5H_2O$**

About 1 g of $CuSO_4.5H_2O$ (Sigma Chemical Co, USA) was dissolved in 100mL of distilled water.

Reagent D: **Alkaline Copper Sulphate solution**

Alkaline Copper Sulphate solution was prepared just before use by mixing Reagents A, B and C together in the ratio 100:1:1. Reagent B was added first to prevent cloudiness of the solution.

Reagent E: **Folin-Ciocalteau reagent**

The reagent is commercially available in 2N, it is diluted with distilled water to 1N just before use. The reagent is kept in a black container because it is photolytic.

Procedure

Reagent D (3 mL) was added to samples((100 µL to 800 µL of the prepared standard BSA solution) mixed and allowed to stand at room temperature for 10 minutes. 0.3mL of Reagent E was added after 10 minutes. After 30 minutes the absorbance was measured at

750 nm wavelength in a Camspec M105 spectrophotometer. The results obtained were used to plot a standard protein curve.

3.12 CALIBRATION OF BSA STANDARD CURVE

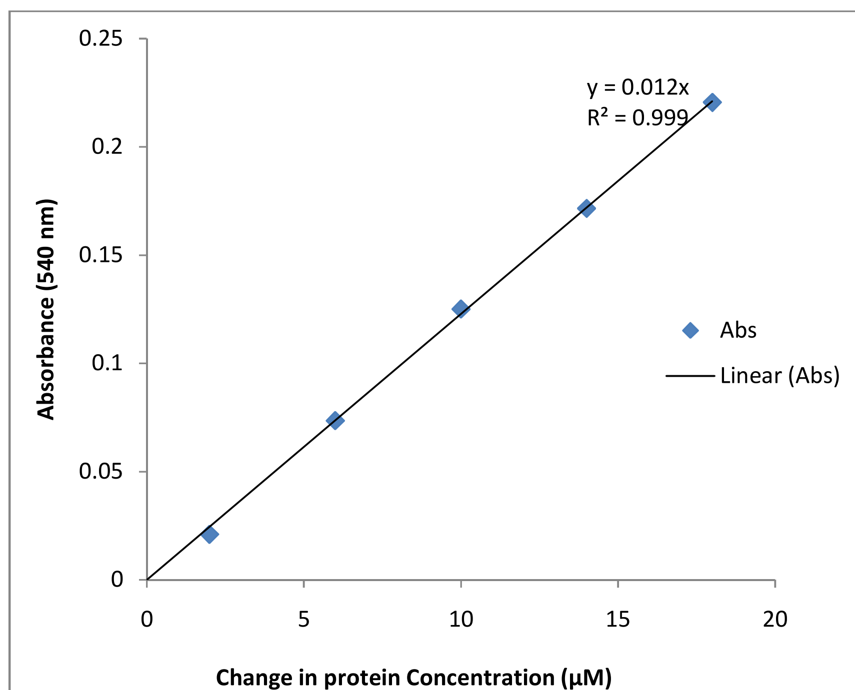
Bovine Serum Albumin (BSA) 1 mg/mL was prepared by dissolving 5 mg BSA in 5 mL distilled water. from The stock solution (1 mL) was taken and mixed with 19mL distilled water to prepare a 200 µg/mL for the solution. For blank preparation, 1000 µL of distilled water was put in a test tube, 3.0 mL (3000 µL) of Reagent D was added to the test and allowed to stand for 10 minutes. After this, 0.3 mL (300 µL) of Reagent E (Folin-C) was added to all the test tubes and allowed to stand for 30 minutes after which readings were taken at 750 nm wavelength on a Camspec M105 spectrophotometer. This process was repeated using serial dilutions of BSA stock solution.

3.13 DETERMINATION OF PROTEIN CONCENTRATION IN MITOCHONDRIA

About 10 µL of the mitochondria was dissolved in 990 µL of distilled water in a test tube, 3.0 mL (3000 µL) of Reagent D was added to the test and allowed to stand for 10 minutes. After this, 0.3 mL (300 µL) of Reagent E (Folin-C) was added to all the test tubes and allowed to stand for 30 minutes after which readings were taken at 750 nm wavelength on a Camspec M105 spectrophotometer. Protein concentration in mitochondria was extrapolated from the standard curve calibrated with BSA, and multiplied by the dilution factor (x100) for the actual concentration of protein.

Table 2: Protocol for Protein Estimation according to the method of Lowry *et al*., 1951

Test tubes in duplicates	1	2	3	4	5	6	7	8	9
Standard BSA (1mg/mL)	-	100	200	300	400	500	600	700	800
Water	1000	900	800	700	600	500	400	300	200
Reagent D	3000	3000	3000	3000	3000	3000	3000	3000	3000
Reagent E (Folin C)	300	300	300	300	300	300	300	300	300

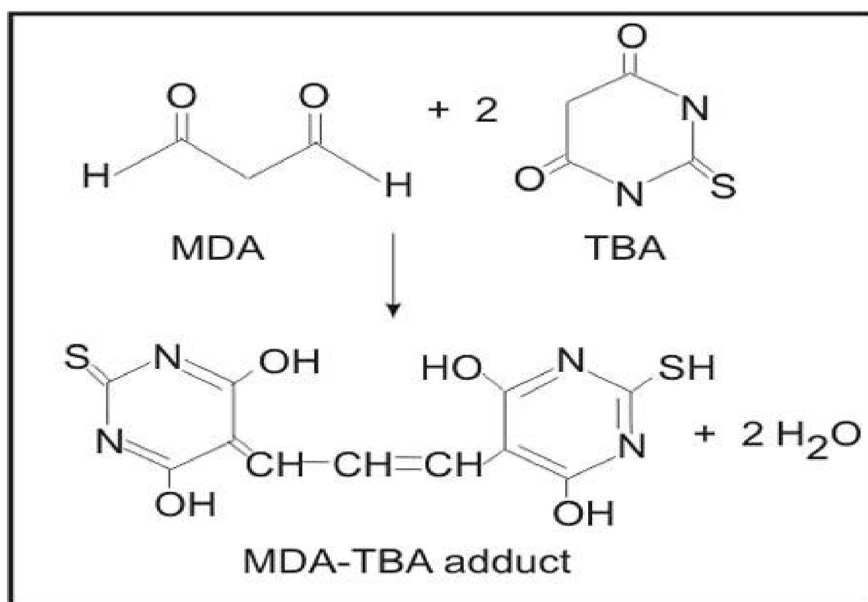


0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
y = 0.012x
R² = 0.999
Abs
Linear (Abs)
Absorbance (540 nm)
Change in protein Concentration (µM)

3.14 LIPID PEROXIDATION DETERMINATION

Principle

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using mitochondrial suspension as lipid-rich media, as described by Ruberto *et al*., (2000). This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde (MDA) an end product of lipid peroxides during lipid peroxidation (LPO). On heating a pink coloured complex is generated which absorbs maximally at 532nm. It is readily extractable into organic solvents such as butanol. This test is often caliberated using MDA as the standard and thus the results are expressed as the amount of free MDA produced.



Reagents

0.07 M Ferrous sulphate

0.106 g of ferrous sulphate was dissolved in 10 mL distilled water and stored in a container.

20% Acetic acid (pH 3.5)

20 mL of acetic acid with 80 mL of distilled water.

0.8% TBA in 1.1% SDS

0.8 g TBA and 1.1 g SDS dissolved in distilled water and made up to 100 mL.

Procedure

Mitochondrial suspension (0.5 mL) was added to 0.1 mL of the extract. The volume was then made up to 1.0 mL with distilled water. About 0.05 mL of $FeSO_4$ (0.07 M) was added and the mixture was incubated at 37°C for 30 min. Then, 1.5 mL of acetic acid was added, followed by 1.5 mL of TBA in SDS (0.8% TBA in 1.1% SDS).

The resulting mixture was vortexed and heated at 95°C for 1 hour. After cooling, 5 mL of butanol was added and the mixture was centrifuged at 3000 rpm for 10 min. 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 =

% inhibition of lipid peroxidation = $1 - \frac{A(sample)}{A(control} x\ 100$

Table 3: Protocol for lipid peroxidation

	Mit.	Extract	Water	$FeSO_4$ (0.07M)	Acetic acid (20%)	TBA:SDS	Butanol (mL)
Mit. Only	35	-	965	50	1500	1500	5
Extract only	-	800	200	50	1500	1500	5
Without Mit	-	-	1000	50	1500	1500	5
50µL	35	50	915	50	1500	1500	5
100µL	35	100	865	50	1500	1500	5
200µL	35	200	765	50	1500	1500	5
400µL	35	400	565	50	1500	1500	5

3.14 DETERMINATION OF MITOCHONDRIAL ATPase ACTIVITY

PRINCIPLE

Determination of mitochondrial ATPase activity was according to the method of Lardy and Wellman (1953) as modified by Olorunsogo *et al.,* (1979). The concentration of inorganic phosphate released was determined as described by Bassir (1963).

REAGENTS

0.1M Tris-HCl pH 7.4

About 1.2 g of Tris (hydroxymethyl) aminomethane was dissolved in a little quantity of distilled water. After adjusting the pH to 7.4 with HCl, the solution was made up to 100 mL in a standard volumetric flask. This was stored in the refrigerator at 4^oC.

0.25 M Sucrose

This was prepared by dissolving 8.56 g of sucrose in distilled water and the solution was made up to 100 mL in a standard volumetric flask and kept in the refrigerator at 4^0C.

0.5mM KCl

This was prepared by dissolving 37.25mg of KCl in a little quantity of distilled water and made up to 100 mL in a standard volumetric flask.

0.01M ATP (pH 7.4)

This was prepared by dissolving 0.2757 g of disodium salt of ATP in a little quantity of distilled water and the pH adjusted to 7.4. This was then made up to 100mL in a standard volumetric flask.

9% Ascorbate

About 9 g of ascorbic acid was dissolved in 100 mL distilled water and it was stored in brown reagent bottle at 4°C. This reagent is usually prepared fresh.

1.25% Ammonium molybdate

Ammonium molybdate (Hopkins and Williams Ltd) England, 1.25 g was dissolved in 100 mL of 6.5% H_2SO_4. This reagent is usually stored in plastic container.

10% Trichloroacetic acid

About 10 g of trichloroacetic acid was dissolved in 100 mL distilled water in standard volumetric flask. This was transferred into a reagent bottle and stored at 4°C.

Procedure

To each test tube, 0.25 M sucrose (200µL), 0.5mM KCl (200µL), 0.1M Tris-HCL buffer pH 7.4 (1300µL) were added. Distilled water or 10, 30, 50 and 70 µL of extract were added to the designated tubes respectively in a final reaction volume of 2000µL accordingly. The uncoupler (DNP) was added followed by 0.01M ATP. 10% SDS was added to the zero time tube before the addition of mitochondria. Mitochondria were added to each test tube, after the addition of ATP, every 30 seconds while continuously shaking in the water bath at 27°C for 30 minutes. While still shaking, 1mL SDS was added to each test tube (except zero time) every 30 seconds. 1mL of ammonium molybdate is added to each test tube followed by 1mL ascorbate. The solutions were allowed to stand for 20 minutes and absorbance was measured at 660nm.

Table 4: Protocol for mitochondrial ATPase activity

	Sucrose (0.25 M)	KCl (5mM)	Tris (0.1M)	Ext (µL)	UCP (µL)	ATP (0.01M)	Mit (µL)	SDS (µL)	NH_4Mb (µL)	Ascorbate (9%)
Blank	200	200	1300	-	-	-	-	1000	1000	1000
1	200	200	1300	-	-		35	1000	1000	1000
2	200	200	1300	-	-	40	-	1000	1000	1000
3	200	200	1300	-	-	40	35	1000	1000	1000
4	200	200	1300	10	-	40	35	1000	1000	1000
5	200	200	1300	30	-	40	35	1000	1000	1000
6	200	200	1300	50	-	40	35	1000	1000	1000
7	200	200	1300	70	-	40	35	1000	1000	1000
DNP	200	200	1300	-	50	40	35	1000	1000	1000
Zero Time	200	200	1300	-	-	40	35	1000	1000	1000

3.16 STANDARD PHOSPHATE CURVE DETERMINATION

PRINCIPLE

Ammonium Molybdate (1.25%) and 9% L-Ascorbic acid react with inorganic phosphate to form a blue colour. Serial dilutions of 1mM Na_2HPO_4 were used to calibrate the standard curve for the determination of concentration inorganic phosphate liberated. Absorbance was measured at 660 nm after incubation at room temperature for 30 minutes.

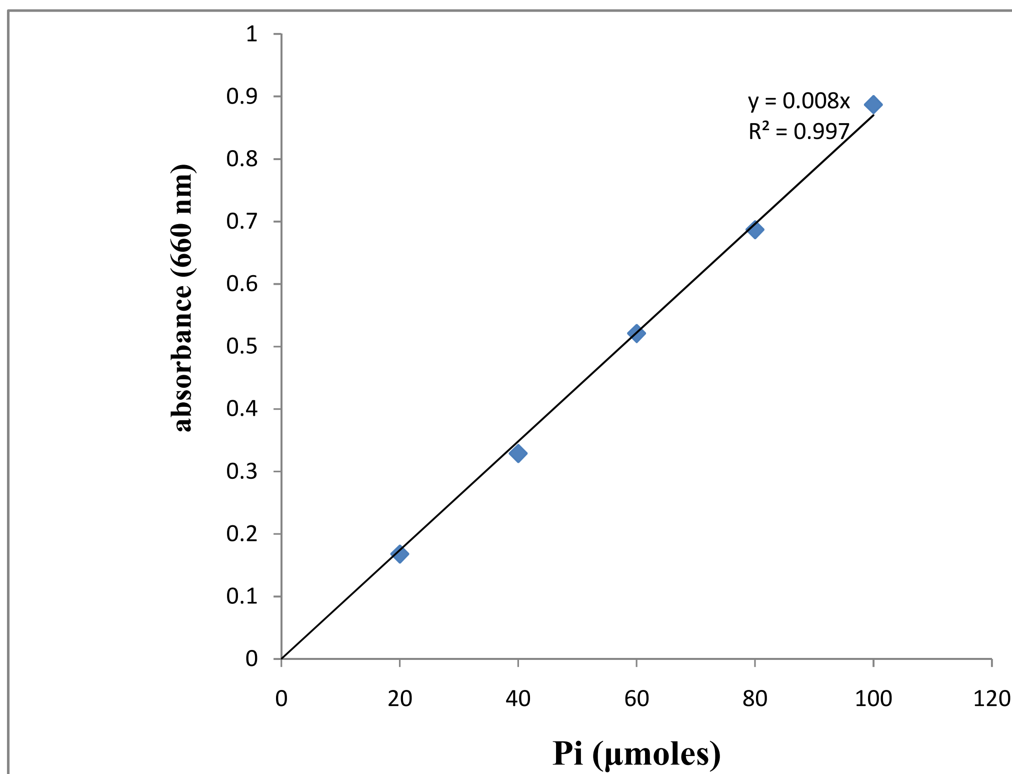


Figure 3.2: Standard phosphate curve

3.17 DETERMINATION OF INORGANIC PHOSPHATE

Principle

Concentration of inorganic phosphate released was determined as described by Bassir (1963). This method is based on the fact that molybdic acid in the presence of inorganic phosphate gives a yellow coloured compound, which is reduced to give a blue coloured compound in the presence of ascorbate as reducing agent. The intensity of the colour thus formed is directly proportional to the concentration of inorganic phosphate.

Reagents

1mM Na_2HPO_4

To 100mL distilled water, 0.0143g of Na_2HPO_4 was added.

9% Ascorbic acid:

To 100mL distilled water, 9g ascorbate was added.

1.25% Ammonium molybdate

Ammonium molybdate, 1.25 g was dissolved in 100mL of 6.5% H_2SO_4. This reagent is usually stored in plastic container.

Procedure

To 5 mL of deproteinized supernatant in a test tube, 400 µL of 5% solution of ammonium molybdate was added and 0.2 mL of 2% freshly prepared ascorbate solution was also added. After thorough mixing by gentle shaking, the tube was allowed to stand for 20 minutes. A standard solution of potassium dihydrogen phosphate a (0.2 mg inorganic

phosphate per 5mL) was similarly treated. The intensity of the blue colour, which developed was read at 660nm in a spectrophotometer.

3.18 DETERMINATION OF CYTOCHROME C RELEASE

Principle

Cytochrome c was determined according to the method of Appaix *et al.,* 2000. The method utilizes a very intensive (γ) or Soret peak at 414 nm in the spectrum of cytochrome c (ε =100 mM^{-1} cm^{-1}). This value of the extinction coefficient, which is a molecular property of cytochrome c, is then used to calculate the concentration of cytochrome c in a medium from any measured value of optical density. The results of spectrophotometric assay of cytochrome c release are in accord with those of oxygraphic determination of cytochrome c-dependent respiration of isolated mitochondria.

Reagents

The reagents are the same as that of assessment of permeability transition as indicated in Materials and methods in Section 3.11..of Materials and Methods.

Procedure

Cytochrome c release was determined in the cause of MMPT pore opening assay for thirty minutes for appreciable amount of cytochrome c to be released. Briefly, mitochondria were incubated in MSH buffer in the presence of 0.8µM rotenone for three minutes after which 300µM $CaCl_2$ was added after three minutes and 5mM succinate was added thirty seconds after calcium. Thirty minutes after, the samples in the test tubes were centrifuged at 13,000 rpm for ten minutes. A similar set up containing the methanol

extract and chloroform fractions and buffer only was treated the same way and also centrifuged at 13,000 rpm for ten minutes. Absorbance of the supernatant was read at 414 nm (the soretic peak of cytochrome c) and the absorbance of the samples containing only the extract was subtracted from the one containing the released cytochrome c. The concentration of cytochrome c released was determined by extrapolation from a standard curve. Cytochrome c release was expressed in picomole per milligram protein.

3.19 DETERMINATION OF SERUM CASPASE 3

Principle

Caspase 3 was determined by enzyme linked adsorbent immunoassay. The kit is an enzyme immunoassay for *in vitro* quantitative measurement of Caspase 3 in mouse serum.

Reagents

Biotin-conjugated antibody

Avidin conjugated to Horseradish Peroxidase (HRP)

3,3,5,5-tetramethyl benzidine,TMB substrate solution.

Stop solution(sulphuric acid)

Procedure

Caspase 3 activity was determined according to the manufacturers instructions as shown below. 100µL of prepared standards (20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.312ng/mL, 0.312ng/mL) and samples were added to the

appropriate microtiter plate wells containing. a Biotin-conjugated antibody(the wells are pre-coated with an antibody specific to CASP3). Avidin conjugated to Horseradish Peroxidase was added to each microplate well resulting in a yellow colour. This was followed by incubation for thirty minutes. Then TMB subsrate was added giving a blue colour (only those wells that contain CASP3, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in colour). This was followed by another incubation for thirty minutes then 50 μL of sulphuric acid was added to terminate the reaction . Then the absorbance was measured in a microplate reader at 450 nm.

The concentration of CASP3 in the samples is then determined by comparing the O.D. of the samples to the standard curve

Calculation of results

The result was calculated by finding the average of the duplicate readings for each standard, control, and samples and subtracting the average zero standard optical density. A standard curve of the mean O.D was plotted against the concentration for each standard and a best fit curve was drawn through the points on the graph or create a standard curve on log-log graph paper with CASP3 concentration on the y-axis and absorbance on the x-axis.

Calculation: The relative O. D. 450 = the O. D. 450 of each well- O. D. 450 of control well

3.20 DETERMINATION OF SERUM CASPASE 9

The procedure for the determination of serum caspase 9 is similar to the protocol for determination of serum caspase 3 as indicated in Materials and methods under Section 3.21.

3.21 DETERMINATION OF SERUM CYTOCHROME C

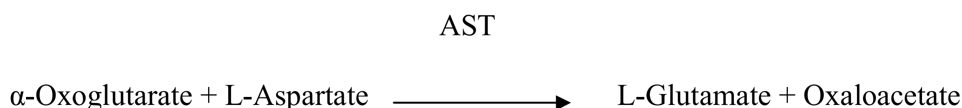
The procedure for the determination of serum cytochrome c is is similar to the protocol for determination of serum caspase 3 as indicated in Materials and methods under Section 3.21

3.22 ASSESSMENT OF LIVER FUNCTION

3.22.1 DETERMINATION OF SERUM ASPARTATE AMINO TRANSFERASE (AST) ACTIVITY

AST activity was determined according to the method of Reitman and Frankel (1957).

Principle



AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

Reagents

1. **Reagent 1 and 2**

Reagent 1consistsof phosphate buffer (100 mmol/L pH 7.4), L-aspartate (100 mmol/L) and α-Oxoglutarate(2 mmol/L). Reagent 2containing 2, 4-dinitrophenylhydrazine (2 mmol/L)

2. Sodium hydroxide(0.4mol/L) 16g of NaOH was dissolve in one liter of distilled water

Protocol

The activity of AST was determined according to manufacturers instruction

	Reagent Blank	Sample
Sample	-	0.1 mL
Reagent 1	0.5 mL	0.5 mL
Distilled water	0.1 mL	-

Procedure

Reagent 1 (0.5mL) and 0.1mL of sample were incubated for exactly 30 minutes at 37°C. Thereafter 0.5 mL of Reagent 2 was added and the mixture was vortexed and allowed to stand for exactly 20 minutes at 20-25°C. Then 5.0 mL of 0.4mL Sodium Hydroxide was added and the absorbance of the sample was measured against the reagent blank after 5 minutes at a wavelength of 546mm.

3.22.2 DETERMINATION OF SERUM ALANINE AMINO TRANSFERASE (ALT) ACTIVITY

ALT activity was determined following the principle described by Reitman and Frankel (1957).

Principle

ALT

α-Oxoglutarate + L-Alanine L-Glutamate + Pyruvate

ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

Reagents

R1.	**Buffer**	
	Phosphate buffer	100 mmol/L pH 7.4
	L-alanine	200 mmol/L
	α-Oxoglutarate	2 mmol/L
R2.	**2, 4-dinitrophenylhydrazine**	2 mmol/L

Wavelenght	Hg 546mm
Cuvette	1 cm light path
Temperature	37°C
Measurement	Reagent blank

The following were pipetted into test tubes

	Reagent Blank	Sample
Sample	-	0.1 mL
Reagent 1	0.5 mL	0.5 mL
Distilled water	0.1 mL	-

Procedure

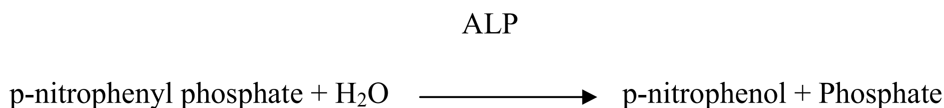
Reagent 1 (0.5mL) and 0.1mL of sample were incubated for exactly 30 minutes at 37^oC. Thereafter 0.5 mL of Reagent 2 was added and the mixture was vortexed and incubated for exactly 30 minutes at 37^oC. Thereafter 0.5 mL of Reagent 2 was added to both Reagent Blank and the sample and the mixture was mixed and allowed to stand for exactly 20 minutes at 20-25^oC. Then 5.0 mL of Sodium Hydroxide was added and the absorbance of the sample was measured against the reagent blank after 5 minutes.

3.22.3 DETERMINATION OF SERUM ALKALINE PHOSPHATASE (ALP) ACTIVITY

The method of Wright and Vandenberg, 2007 was employed in this assay.

Principle

P-Nitrophenyl phosphate (PNPP) is hydrolysed to p-nitrophenol and phosphoric acid at pH 10.1 by alkaline phosphatase (ALP). The p-nitrophenol confers yellowish colour on the reaction mixture and its intensity can be monitored by the spectrophotometer at 405 nm to give a measure of the enzyme activity.



$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow{ALP} \text{p-nitrophenol} + \text{Phosphate}$$

Reagents

R1a.contains Diethanolamine buffer, (1 mol/L, pH 10) and $MgCl_2$ (0.5 mmol/L)

R1b. contains the substrate P-nitrophenylphosphate (10 mmol/L)

Procedure

Wavelenght	Hg 405mm
Cuvette	1 cm light path
Temperature	25°C, 30°C, 37°C
Measurement	against air

The following was pipetted into cuvette

Sample	0.05 mL
Reagent	3.00 mL

The mixture was mixed and initial absorbance read and timer started simultaneously. The absorbance was read again at 1 minute interval for 3 minutes.

Calculation

ALP activity was calculated using the following formulae

U/I = 3300 × ΔA 405 nm/min

3.23 CHEMICAL ANALYSES

3.23.1 DISTILLATION OF SOLVENTS

Methanol, n-hexane and ethyl acetate were distilled. In each case, the solvent to distill was poured into a two litre quick fit round bottom flask , adouble surface condenser v was then connected through a quick fit adaptor to the flask. The round bottom flask was then heated on a heating and the distilled solvent was collected and transferred to a Winchester bottle.

3.23.2 PARTITIONING OF EXTRACT BY VACUUM LIQUID CHROMATOGRAPHY

The Vacuum liquid chromatography (VLC) was the form of column chromatography that was used to separate crude extract . The Buchner flask was filled with Silica gel and was attached to a vacuum pump with a T piece glass adaptor.. The extract was prepared by mixing the sample with silica gel (1:1). The silica gel and sample mixture was preadsorbed properly, and filter paper was placed on the silica after which the preadsobed sample was poured and then covered with another filter paper. The vacuum was applied to to make the adsorbent layer level. After the vacuum was released a solvent of was poured quickly onto the surface of the adsorbent and the the vacuum was re-applied. The following solvents systems representing the mobile phase were used to elute bioactive fractions from the column: n-hexane, chloroform and ethylacetate respectively. The three fractions were concentrated in a rotary evaporator at 40^oC.

3.23.3 Column chromatography

Column chromatography was used for the separation of fractions into partially pure compounds. The stationary phase (silica gel) was placed in a vertical glass column and the mobile phase was added at the top and made to flow down through the column by gravity. This is generally used as a purification technique to isolate desired compounds from a mixture

3.23.4 Thin layer chromatography

Thin layer chromatography was used to monitor the eluted fractions. The eluted compound was first spotted by using a capillary tube to transfer a small amount of eluent to one end of a TLC plate. The solvent evaporated leaving behind a small spot on the material. which was developed in closed jar by placing the bottom of the TLC plate in a beaker containing a small pool of development solvent and the spot traveled up the plate by capillary action until it is 1cm from the top. Then the plate was removed immediately from the beaker and the solvent front was marked with a pencil .Visualisation was done in an iodine tank.. TLC plate was placed in iodine chamber for a few minutes and the organic compounds present in the plate formed a dark spot with iodine .The spots on a TLC plate were circled showing how far the compound travelled on the plate. The component visible as separated spots were identified by comparing the distances they travelled with known reference material. The Rf value was used to quantify the movement of the materials along the plate. Rf is equal to the distance travelled by substance divided by the distance moved by solvent front.

3.23.5 Spectroscopic Method

3.23.5.1 Ultra -Violet and Visible Spectroscopy

Ultra -violet and visible spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample in the ultraviolet-visible spectral region. It is useful in the detection of functional groups. It gives information about the chromophores and conjugations present in the structure of the compound.

3.23.5.2 Fourier Transform Infra-Red Spectroscopy.

Fourier transform Infra-red spectroscopy is a spectroscopic technique that specifically characterizes different functional groups present in a compound based on individual absorption in the infra-red region of the electromagnetic spectrum (Williams and Fleming, 1989). It provides information on function al groups of various types. The infrared spectra of a sample is collected by passing a beam of infrared light through the sample. Examination of the transmitted light reveals how much energy was absorbed at each wavelength. This can be done with a monochromatic beam, which changes in wavelength over time, or by using a Fourier transform instrument to measure all wavelengths at once. transmittance or absorbance spectrum can be produced, showing at which IR wavelengths the sample absorbs. Analysis of these absorption characteristics reveals details about the molecular structure of the sample.

3.23.5.3 Gas Chromatography Mass Spectrometry Analysis

This chromatographic technique is used to identify the phytocomponents present in a sample It can analyse pure compound at less than 1 gram. While the GC resolves the complex mixture into single compound, the MS provides their mass spectra which gives information about their molecular weight and structural details. system Gas Chromatograph interfaced to a Mass Spectrometer. The mass spectra of the compounds found is usually matched with the data in the library of National Institute of Standards and Technology (NIST).The result is presented in a spectrogram showing the peak identities of the compound is presented. Identification of the resulting compound is done using spectroscopic techniques.

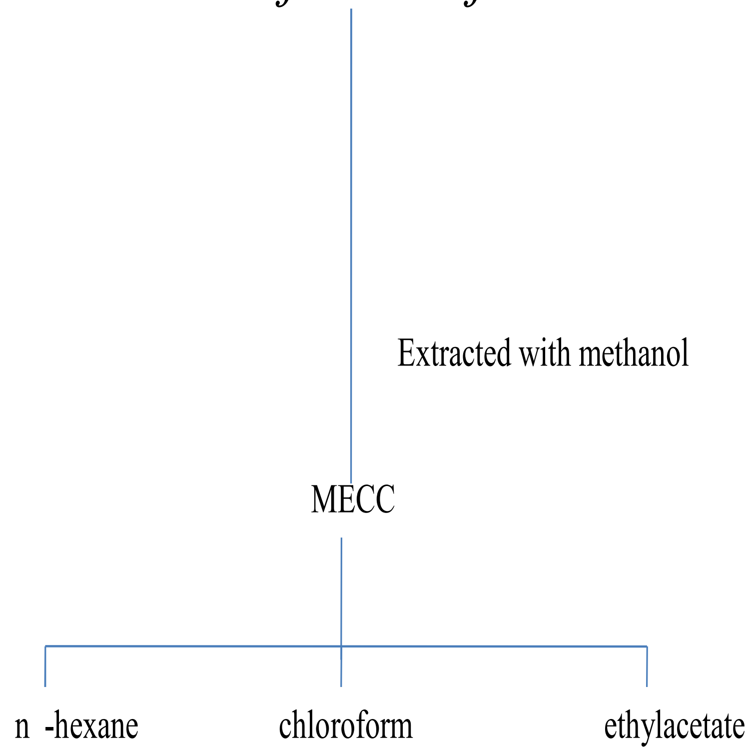


Figure 10 Fractionation flow chart of *Cajanus cajan* leaves

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT 1: PHYTOCHEMICAL SCREENING OF THE LEAVES OF *CAJANUS CAJAN*

INTRODUCTION

Medicinal properties of different parts of plants such as the bark, seed, fruits and leaves have been exploited by man. Contemporary science has acknowledged their action, and quite a number of drugs have their active ingredient derived from plants. The study of medicinal plants starts with extraction, which is the processing of the bioactive constituents from plant prior to phytochemical screening assays and other biological assays. Traditional healers commonly use water to extract plant compounds, but it cannot extract nonpolar compounds, for this reason they also use nonpolar solvents like alcohol.

Phytochemical evaluation of most natural products has shown the presence of alkaloids, cardenolides, saponins, tannins, anthraquinones and flavonoids. *Cajanus cajan* leaves is a common medicinal plant among the "Yorubas", South west of Nigeria administered for treating liver and stomach disorders, anemia, dysentery, measles and jaundice (Burkhill, 1978). In this study, phytochemical evaluation was done on the leaves in order to know the possible chemical substances relevant for drug development.

PROCEDURE

The phytochemical screening was done by standard procedures as described in Section 3.3 of Materials and Methods.

RESULTS

The fractions from *Cajanus cajan* leaves were evaluated by phytochemical qualitative reactions for plant secondary metabolites. The screening was performed for alkaloids, cardenolides and flavonoids, anthraquinones, saponins, tannin and terpenes.

CONCLUSION

Phytochemical screening showed that the fractions of *Cajanus cajan* tested positive for all the secondary metabolites.

Table 5 : PHYTOCHEMICAL SCREENING OF VARIOUS FRACTIONS OF *CAJANUS CAJAN* LEAVES

Phytochemicals	MECC	CFCC	EACC
alkaloids	+	+	+
cardenolides	+	+	+
Anthraquinones	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Flavonoids	+	+	+
Terpenes	+	+	+

+ : PRESENT

- : ABSENT

EXPERIMENT 2: EFFECTS OF METHANOL EXTRACT OF *CAJANUS CAJAN* (MECC) ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

INTRODUCTION

The mitochondrial membrane permeability transition (MMPT) pore is a non-specific channel 3nm in diameter. An increase in the permeability of this pore allows the passage of molecules of less than 1500 Daltons in molecular weight. Experimental evidence based on Kroemer' studies on cell death in different tissues showed that, pore opening is a key step before apoptosis can take place.

The pore is believed to extend through the outer and inner mitochondrial membranes and the molecular constituentsis still uncertain. Initially, the widely accepted structure included the the voltage dependent anion channel (VDAC), adenine nucleotide transporter (ANT) and Cyclophilin D (Adams *et al* 1989; Bucheler *et al.,* 1991). It is well established that calcium is essential for cell survival, however intracellular calcium overload causes the opening of the mitochondrial membrane permeability transition (MMPT) pore. In the original study by Haworth and Hunter they found that MMPT pore opening resulted in a conformational change in isolated mitochondria which was accompanied by swelling that can be followed by measuring changes in light scattering. Thus a common and convenient assay for the permeability transition is to measure changes in light scattering .Modulation of pore opening constitutes a useful molecular target or novel drug design as strategies for killing unwanted cells. This study was designed to assess the modulatory effects of MECC on mitochondrial membrane permeability transition pore, *in vitro.*

PROCEDURE

The procedure for the assessment of MMPT is described in Section 3.6.in Materials and Methods.

RESULTS

The results obtained showed that there was no significant change in absorbance obtained in the absence of calcium, indicating intactness of the mitochondria. On addition of calcium there was a large amplitude change in absorbance when compared with the intact state, (NTA) and this was almost completely reversed by spermine, the synthetic inhibitor. At 10 and 30 µg/mL, no significant changes on the MMPT was observed, but at 50 and 70 µg/mL induction of the opening of the pore by 7 and 13 folds, without calcium. With calcium there was induction by 16, 19, 20 and 23 folds respectively at the same concentrations.

CONCLUSION

Methanol extract of *Cajanus cajan* had non significant effects on the MMPT pore at 10 and 30 µg/mL but there was induction of opening of the pore by 7 and 13 folds at 50 and 70 µg/mL, without calcium. On addition of calcium, there was induction by 16, 19, 20 and 23 folds respectively at the same concentrations. The observed results might be due to the presence of phytochemicals in the leaves of *Cajanus cajan* which might be responsible for the opening of the pore.

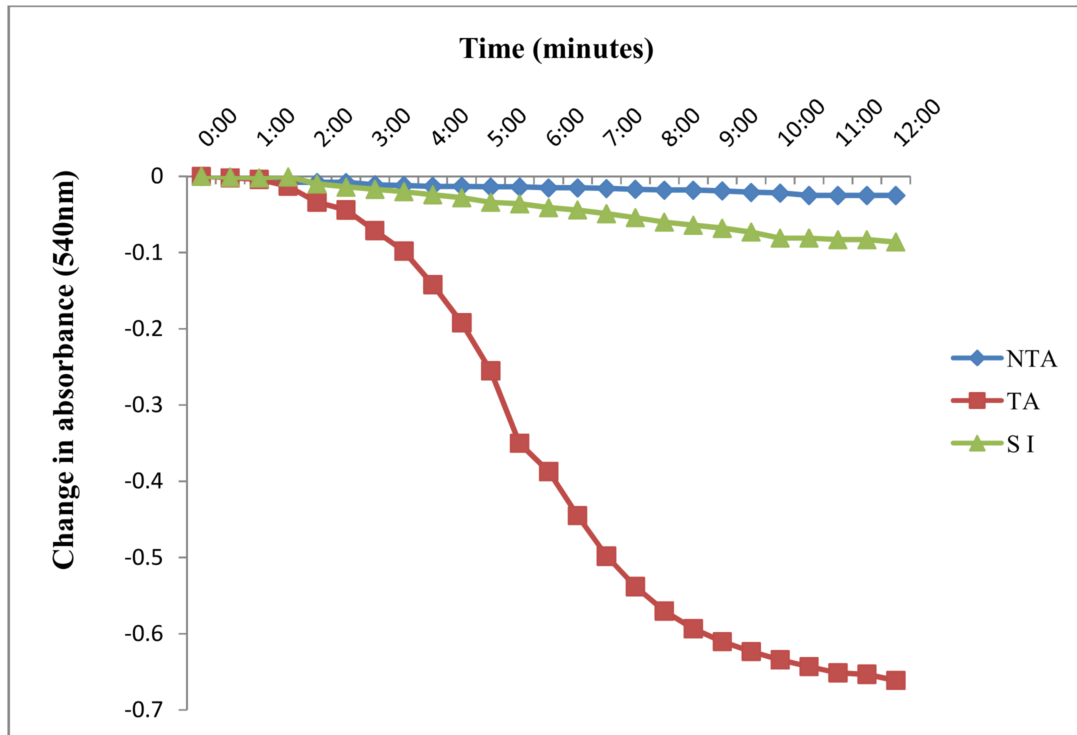


Figure 11**:** Induction of the opening of mitochondrial membrane permeability transition pore in isolated rat liver mitochondria by calcium and inhibition by spermine. NTA-No triggering agent (without calcium), TA -Triggering agent (with calcium), SI-spermine inhibition.

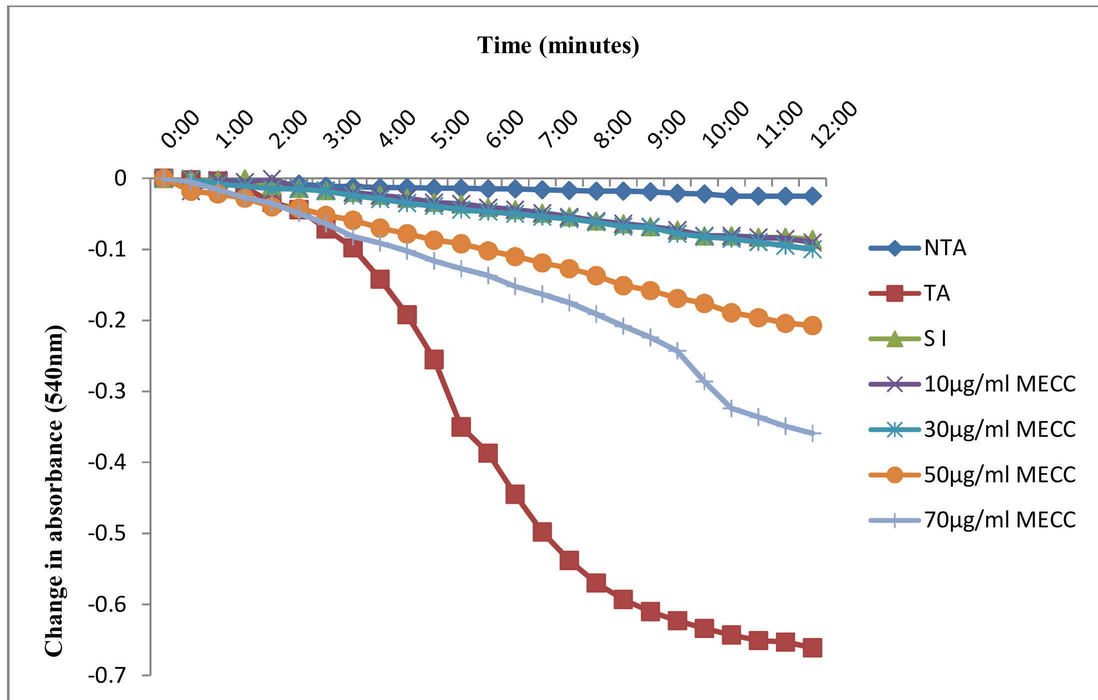


Figure 12: Effects of MECC on MMPT pore in the absence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition. MECC - methanol extract of *Cajanus cajan*

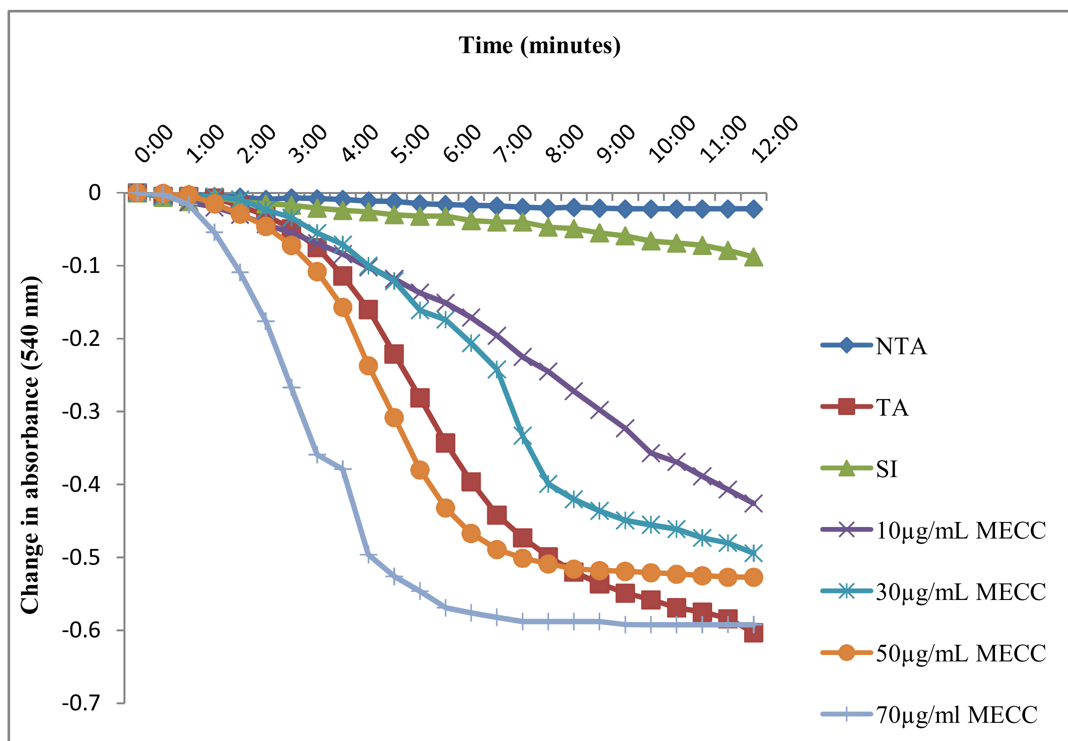


Figure 13: Effects of MECC on MMPT pore in the presence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition, MECC - methanol extracts of *Cajanus cajan*

EXPERIMENT 3: EFFECTS OF CHLOROFORM FRACTION OF *CAJANUS CAJAN* (CFCC) ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

INTRODUCTION

Apoptosis enables an organism to destroy unwanted cells during development and it is also responsible for cellular homeostasis (Thompson, 1995). The interference in apoptotic pathway has been linked to the neoplastic transformations that occur in cancers according to several authors. The MMPT is well documented to be triggered by certain pathway that leads to apoptosis and certain phytochemicals and plant extracts have been demonstrated to modulate apoptosis in certain pathological conditions (Kokoszka *et al.,* 2004; Baines *et al.,* 2007). Eventhough transient opening of the pore is important for release of ions and metabolites to maintain mitochondrial homeostasis (Leung and Halestrap, 2008; Leung *et al*., 2008). The irreversible opening of the MMPT can be triggered by a variety of events including calcium overload and reactive oxygen species production. In this study we assessed the effect of chloroform fraction of *Cajanus cajan* (CFCC) on the MMPT pore.

PROCEDURE

The procedure for the assessment of MMPT is described in Section 3.6 in Materials and Methods.

RESULTS

The assessment of the modulatory effects of chloroform fraction of *Cajanus cajan* (CFCC) on mitochondria membrane permeability transition pore shows that change in absorbance obtained in the absence of calcium was 0.02nm indicating intactness of the mitochondria. On addition of calcium there was a large amplitude change in absorbance

when compared with the intact state, and this was almost completely reversed by spermine. Chloroform fraction of *Cajanus cajan* in the absence of calcium at 10, 30, 50 and 70 µg/mL did not have effect on the pore. In the presence of calcium there was induction of the opening of the MMPT pore by 20, 21, 23 and 24 folds respectively, in a concentration dependent manner.

CONCLUSION

Chloroform fraction of *Cajanus cajan* had no effect on the mitochondrial membrane permeability transition pore in the absence of calcium.

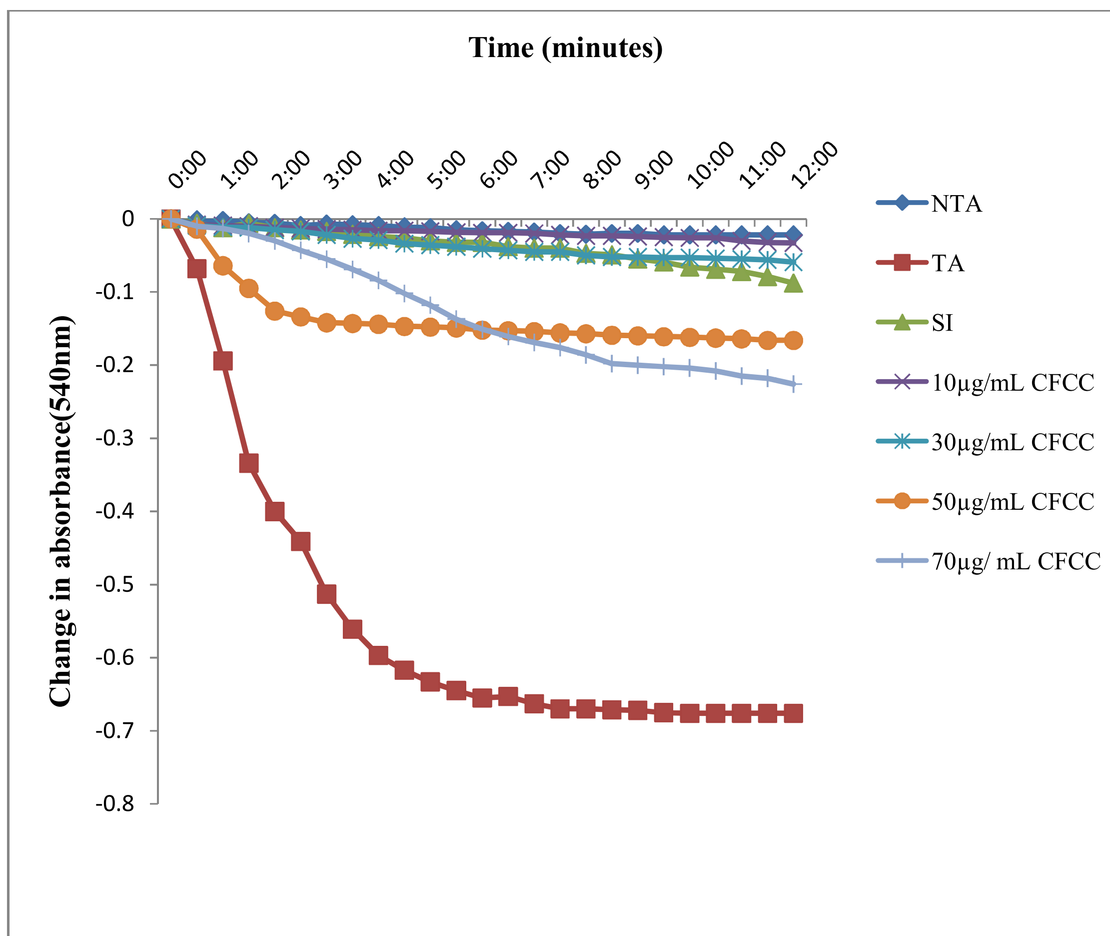


Figure 14: Effects of Chloroform fraction of *Cajanus cajan* on MMPT pore in the absence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition. CFCC – Chloroform fraction of *Cajanus cajan*

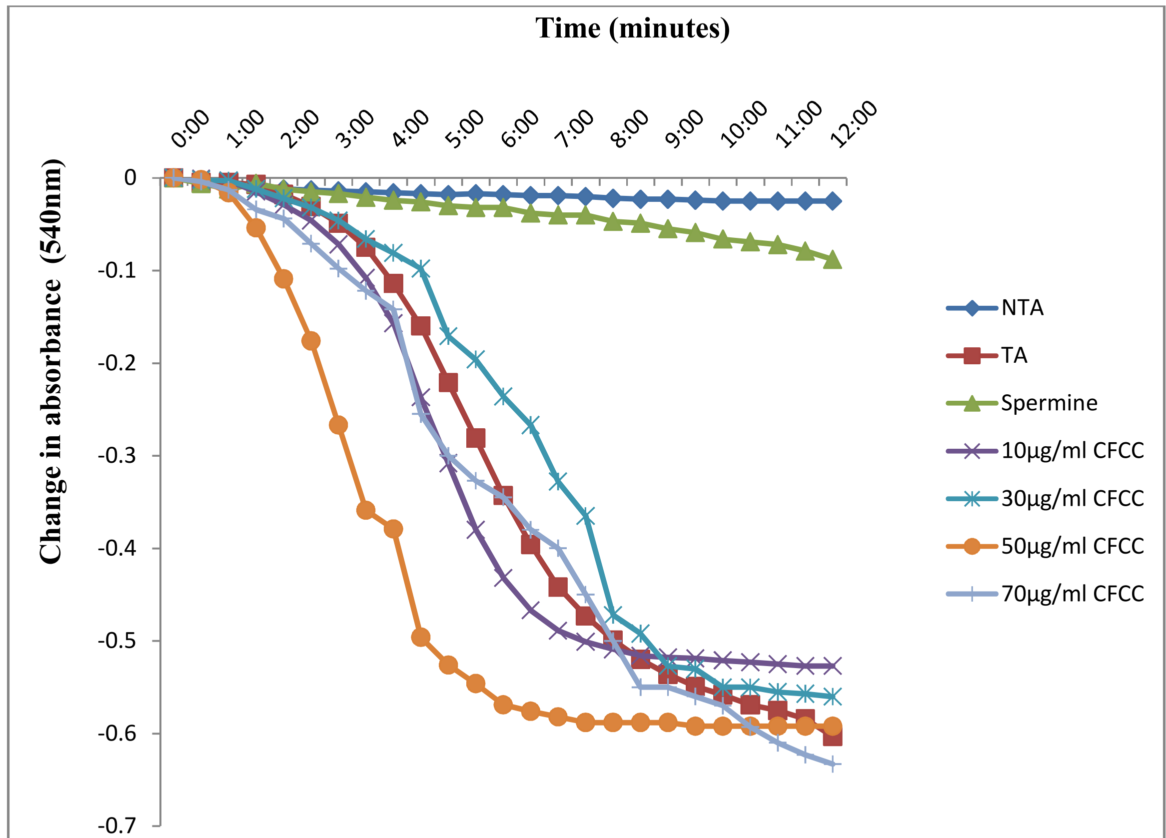


Figure 15: Effects of CFCC on MMPT pore opening in the absence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition. CFCC – Chloroform fraction of *Cajanus cajan.*

EXPERIMENT 4: EFFECTS OF ETHYLACETATE FRACTION OF *CAJANUS CAJAN* (EACC) ON THE MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

INTRODUCTION

Apoptosis is form of programmed cell death responsible for the removal of harmful and mutated cells by organisms (Kuranaga, 2011). During this process, cells exhibit cytosplasmic shrinkage, plasma membrane blebbing, nuclear condensation, and later fragmentation of both the cytoplasm and nucleus into membrane-enclosed apoptotic bodies. The extrinsic pathway utilizes cell surface receptors, whereas the intrinsic pathway involves the mitochondria. These two pathways lead to caspase activation, and have been shown to be critical in carcinogenesis. Dysregulation of apoptosis is implicated in the pathogenesis of many diseases for example insufficient apoptosis is linked to cancer.

The widespread and irreversible opening of the MMPT pore is a crucial step in intrinsic apoptosis. The MMPT is the major mechanism that mediates calcium-induced injury (Leysens *et al*., 1996). The molecular mechanisms underlying this transition that is calcium dependent and cyclosporine A-sensitive is still undergoing scientific investigations. The MMPT is well documented to be triggered by certain pathway that leads to apoptosis and certain phytochemicals and plant extracts have been demonstrated to modulate apoptosis in certain pathological conditions. It is also considered to be an early marker of mitochondrial alterations, which is thought to precede cytochrome c release and apoptosome formation.

PROCEDURE

The procedure for the assessment of MMPT is described in Section 3.6.of Materials and Methods.

RESULTS

The results of the assessment of the modulatory effect of EACC on mitochondria membrane permeability transition pore shows that the change in absorbance obtained in the absence of calcium was (0.02± 0.01) indicating intactness of the mitochondria. On addition of calcium there was a large amplitude change in absorbance when compared with the intact state, (NTA) and this was almost completely reversed by spermine in the absence of calcium. The EACC at 10µg/mL, 30µg/mL, 50 and 70 µg/mL opened the pore by 19, 20, 21 and 23 folds and 22, 26, 27 and 29 folds with and without calcium respectively.

CONCLUSION

The EACC at 10µg/mL, 30µg/mL, 50 and 70 µg/mL induced the opening the MMPT pore by 19, 20, 21 and 23 folds and 22, 26, 27 and 29 folds with and without calcium respectively. The EACC is the most potent fraction.

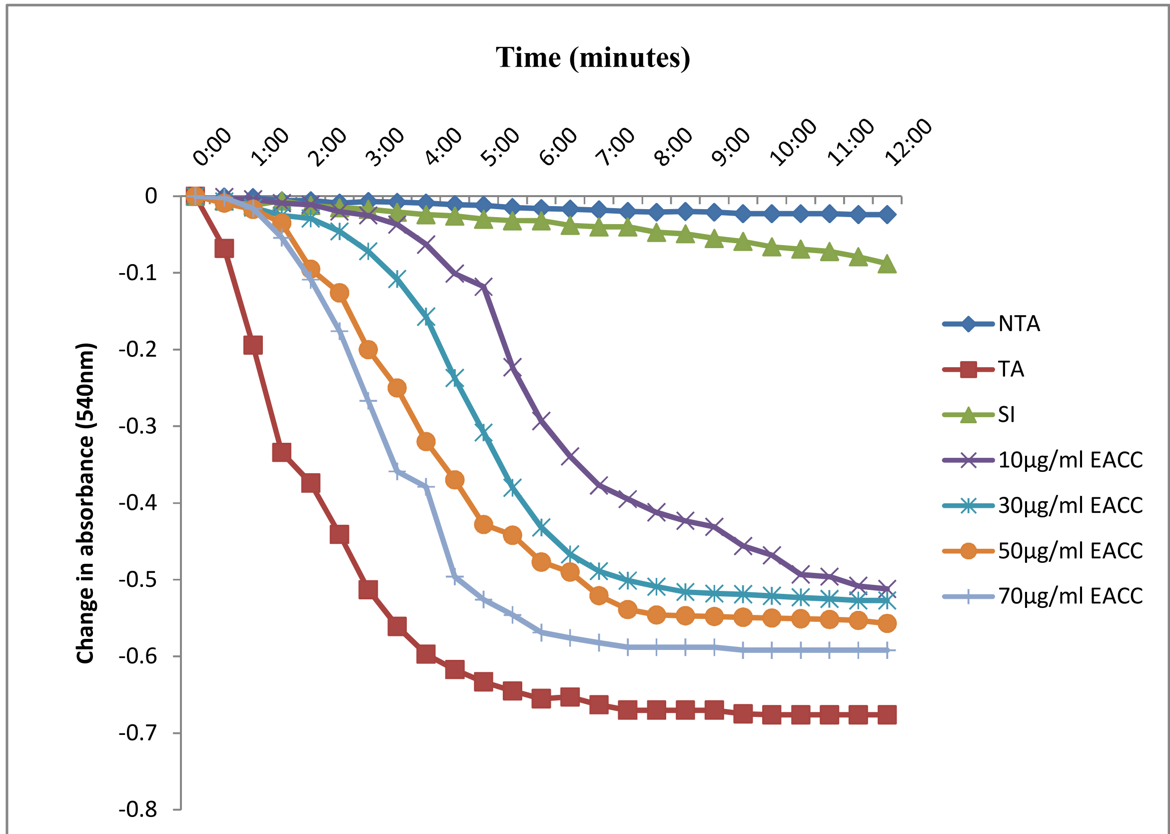


Figure 16: Effect of EACC on MMPT pore in the absence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition, EACC - ethylacetate fraction of *Cajanus cajan*

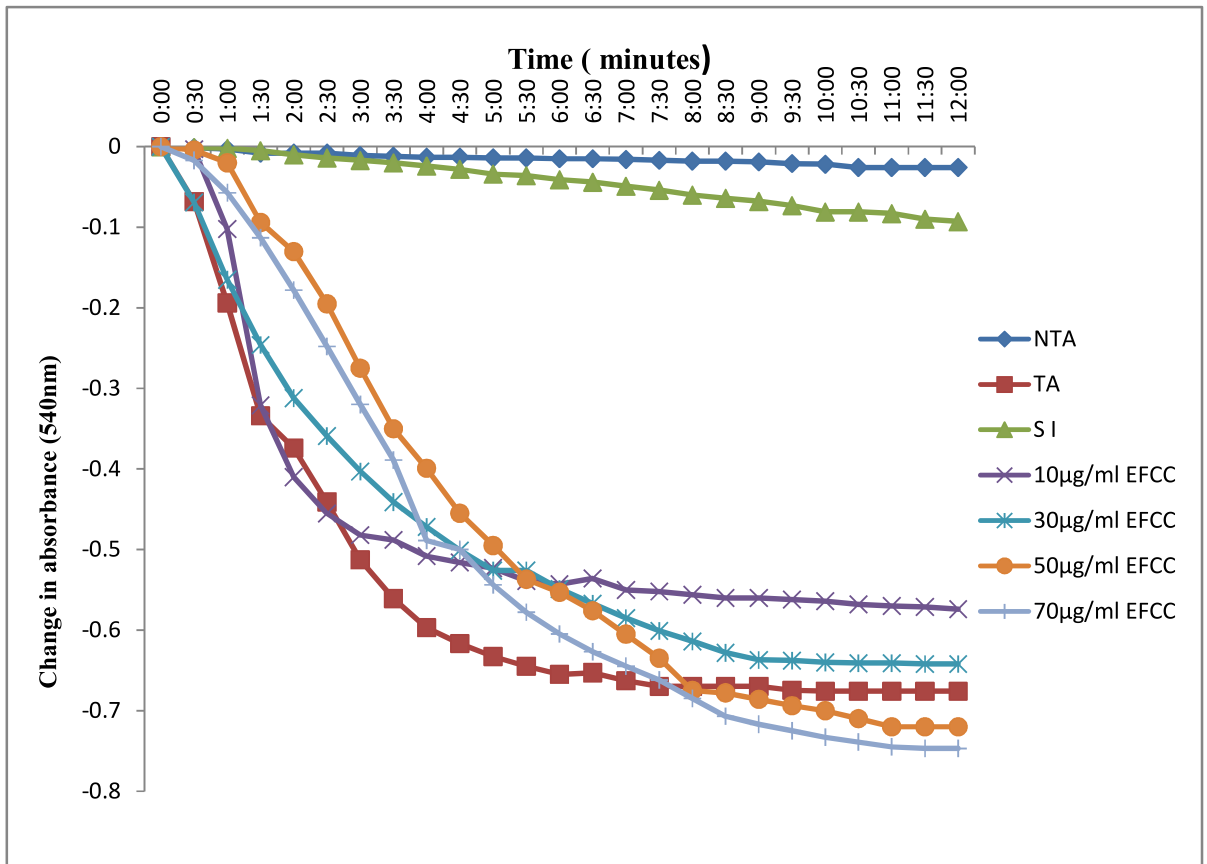


Figure 17: Effect of EACC on MMPT pore in the presence of calcium. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition, EACC- ethylacetate fraction of *Cajanus cajan*

EXPERIMENT 5: DETERMINATION OF CYTOCHROME C RELEASED IN MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION (MMPT) PORE OPENING

INTRODUCTION

Most apoptosis-inducing conditions associated with the intrinsic pathway involve the disruption of the mitochondrial inner transmembrane potential ($\Delta\psi$), resulting in the entry of solutes and water into the mitochondrial matrix, driven by the colloid osmotic pressure of the matrix that is tightly packed with enzymatic complexes. The resulting increase in mitochondrial matrix volume known as large-amplitude swelling. This is accompanied by the distension and disorganization of the cristae as well as by a reduction of the electron density of the matrix leading to the release of some proteins including cytochrome c, which activates the apoptosome and therefore the caspase cascade (Hunter *et al*.,1976).

Cytochrome c is a highly water soluble protein, unlike other cytochromes, with a solubility of about 100 g/L and is an essential component of the electron transport chain, where it carries one electron. It is capable of undergoing oxidation and reduction, but does not bind oxygen. It transfers electrons between Complexes III (Coenzyme Q – Cyt C reductase) and IV (Cyt C oxidase). In humans, cytochrome c is encoded by the CYCS gene. When released from the mitochondria under stress conditions it is able to activate the apoptotic signaling pathway condemning the cell to death.

PROCEDURE

The procedure for determination of cytochrome C release is described in Section 3.6 of Materials and Methods.

RESULTS

The data in figure shows that Cytochrome c released at 10µg/mL, 30µg/mL, 50 and 70 µg/mL was 0.03, 0.05, 0.14, 0.15 and 0.03, 0.08, 0.09, 0.13 and 0.04, 0.05, 0.08 and 0.15 ng/mg mitochondrial protein respectively for the methanol extract, chloroform fraction and ethylacetate fraction.

CONCLUSION

Cytochrome c released for the methanol extract, chloroform fraction and ethylacetate fraction as a result of permeability transition.

Table 6 **:** The release of cytochrome c by various fractions of *Cajanus cajan*

Concentration (µg/mL)	MECC **(**ng/mg protein**)**	CFCC **(**ng/mg protein**)**	EACC **(**ng/mg protein**)**
10	0.03±0.003	0.03±0.001	0.04±0.004
30	0.05±0.003	0.08±0.003	0.05±0.003
50	0.14±0.002	0.09±0.004	0.08±0.006
70	0.15±0.004	0.13±0.004	0.15±0.004

Each value represents the mean of five determinations ± SD

EXPERIMENT 6: EFFECT OF VARIOUS FRACTIONS OF *CAJANUS CAJAN* ON MITOCHONDRIAL ATPase ACTIVITY

INTRODUCTION

The opening of a nonspecific pore in the inner mitochondrial membrane, the mitochondrial membrane permeability transition pore causes a loss in the inner mitochondrial transmembrane potential which subsequently leads to cell death. The mitochondria are powerhouses that provide the energy necessary for cellular activities through the enzymatic action of the FoF1 ATP synthase. The FoF1 ATP synthase is a rotary enzyme which is localized in the inner mitochondrial membrane. The enzyme which is reversible and consists of two parts; the membrane bound Fo which is a proton pore, and the F1 part which has catalytic activity (Stock *et al.,* 1999). However when the concentration of calcium ions inside human mitochondria increases, the MMPT pore opens.

Opening of the MMPT pore is accompanied by loss of the mitochondrial membrane potential and proton gradient across the IMM. At low electrochemical potential, FoF1 synthase begins to hydrolyse ATP in an attempt to maintain the mitochondrial membrane potential (Leysens *et al.,* 1996). The mechanism of oxidative phosphorylation requires the permeability of the inner mitochondrial membrane is maintained, hence dissipation of membrane potential and hydrolysis of ATP are hallmarks of permeability transition.

These three phosphate groups of ATP are linked to one another by two high-energy bonds called phosphoanhydride bonds. When one phosphate group is removed by breaking a phosphoanhydride bond during hydrolysis, energy is released, and ATP is converted to adenosine diphosphate (ADP). The amount of ADP is equal to the amount of inorganic phosphate

In view of the fact that *Cajanus cajan* induced opening of the pore it was necessary to assess mitochondrial ATPase activity in this study.

PROCEDURE

The procedure for determination of Adenosine triphosphate activity was described in section 3.8 of Materials and Methods.

RESULTS

Mitochondrial ATPase activity at 10, 30, 50 and 70 µg/mL was stimulated by 2.6, 3.0, 3.8 and 4. 2 folds respectively, for the MECC. Similarly, stimulatory effects of 1.8, 2.3, 7.2, 10.2 and 9, 11, 13 and 15 folds were observed for the chloroform fraction and and ethylacetate fraction. Dinitrophenol, the classical uncoupler, had the highest ATPase of activity of 23 folds

CONCLUSION

The various fractions of *Cajanus cajan* stimulated ATPase activities with the ethyl acetate having the highest stimulatory activity.

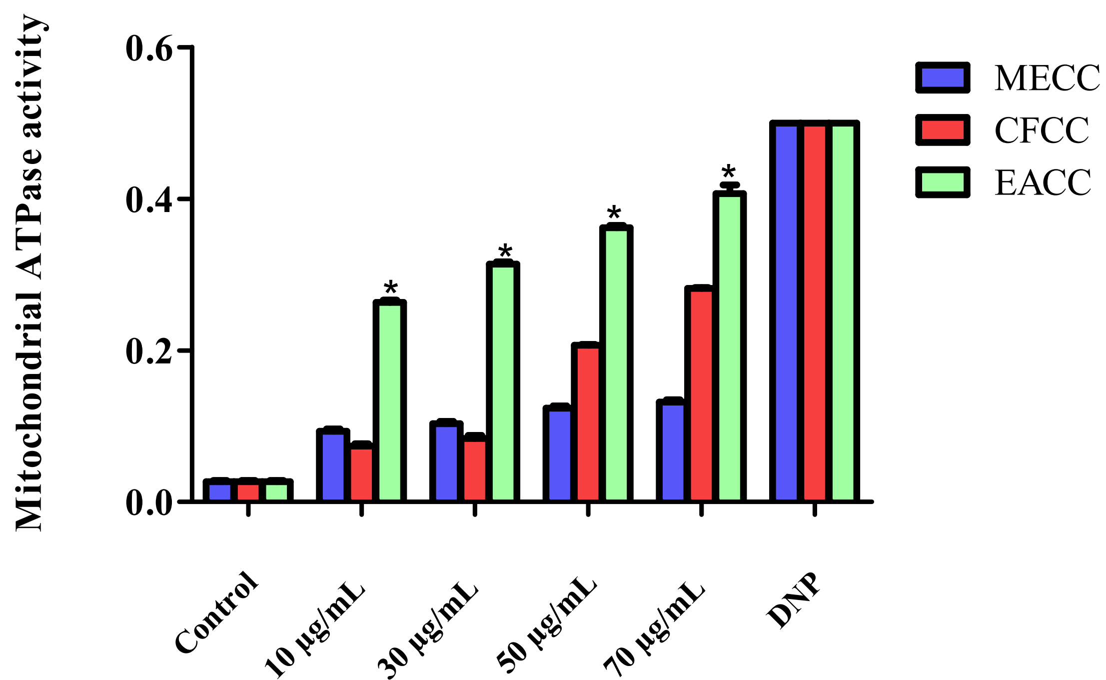


Figure 18: Effects of various fractions of *Cajanus cajan* on mitochondrial ATPase activities. MECC - methanol extracts of *Cajanus cajan*, CFCC – chloroform fraction of *Cajanus cajan* and EACC - ethylacetate fraction of *Cajanus cajan.* Each value represents the mean of five determinations ± SD.

EXPERIMENT 7: EFFECTS OF VARIOUS FRACTIONS OF *CAJANUS CAJAN* ON THE INHIBITION OF LIPID PEROXIDATION

INTRODUCTION

Cellular injury can be quantified by lipid peroxidation, a well-established indicator of oxidative stress in cells and tissues. A complex series of compounds such as reactive carbonyl compounds are formed when lipid peroxides decompose. Also when polyunsaturated fatty acid peroxides decompose malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) are formed. Measurement of malondialdehyde and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation (Esterbauer *et al*; 1991).

PRINCIPLE

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using liver homogenates as lipid-rich media, as described by Ruberto *et al*., (2000).

PROCEDURE

Mitochondrial suspension were added to varying concentrations of the test sample in test-tubes, along with various fractions of *Cajanus cajan* leaves. Then 0.05 mL of 0.07M $FeSO_4$ was added to induce lipid peroxidation and the mixture incubated for 30 minutes at 37^0C. Then 1.5 mL of 20 % acetic acid (pH 3.5) and 1.5 mL of 0.8 % (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 mL of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm.

Inhibition of lipid peroxidation percent by the extract was calculated as [1-(E-C)/C] x 100, where C is the absorbance value of the fully oxidized control and E is the absorbance in presence of the extract.

RESULTS

The percentage inhibition of lipid peroxidation by fractions of *Cajanus cajan* in figure 3.6, showed that at varying concentrations (50, 100, 200 and 400 µg/mL) of the extract, there was an increase in percentage inhibition of Fe^{2+}- induced lipid peroxidation. There was an inhibition of 6, 8, 11 and 15% for the MECC. Similarly, there was an inhibition of lipid peroxidation by 31, 49, 54 and 62% for the CFCC and 6, 13, 42 and 53% for the EACC, respectively. The CFCC had the highest inhibitory effect

CONCLUSION

The various fractions of *Cajanus cajan* inhibited of lipid peroxidation.

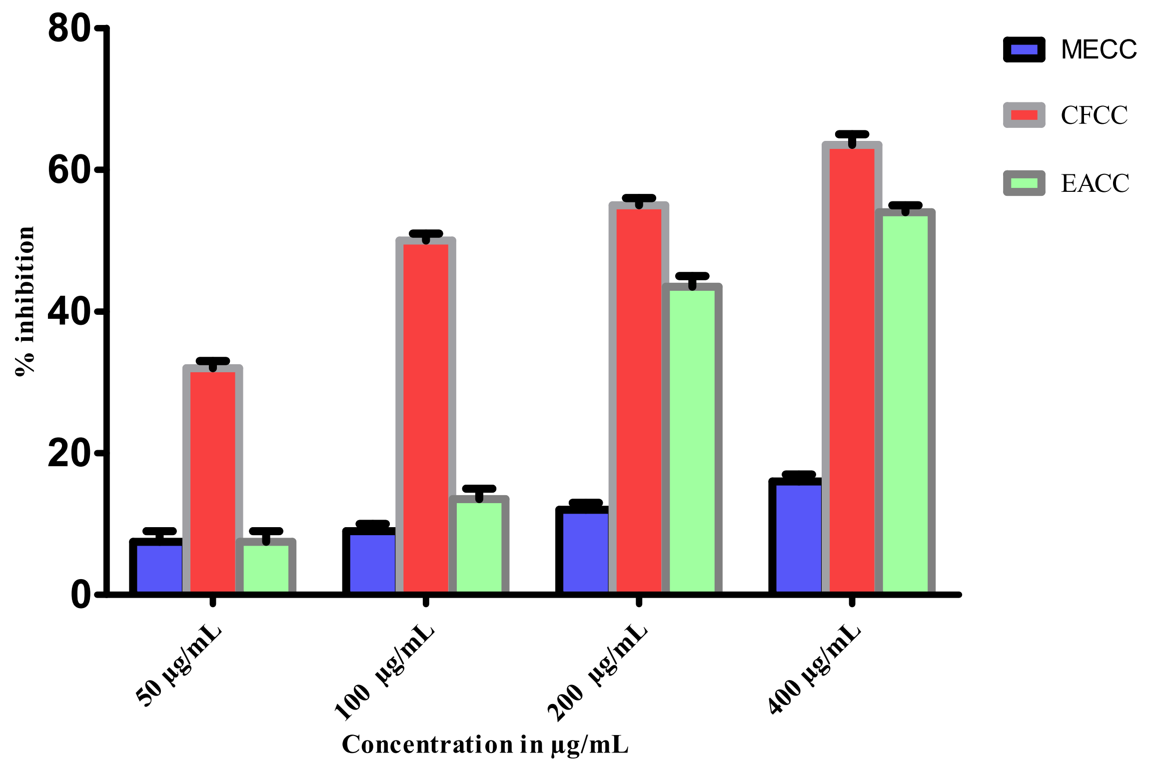


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Figure 19: Effects of various fractions of *Cajanus cajan* on the inhibition of lipid peroxidation. MECC - methanol extracts of *Cajanus cajan*, CFCC – chloroform fraction of *Cajanus cajan* and EACC - ethylacetate fraction of *Cajanus cajan.* Each value represents the mean of five determinations ± SD.

EXPERIMENT 8: EFFECT OF ETHYLACETATE FRACTION OF *CAJANUS CAJAN* ON MMPT PORE OPENING, RELEASE OF CYTOCHROME C AND ACTIVATION OF CASPASES, *IN VIVO*

INTRODUCTION

Caspase activation is initiated by two major signaling routes: namely the death receptor pathway and the mitochondrial mitochondrial pathway. Death receptor-mediated apoptosis plays a fundamental role in the maintenance of tissue homeostasis, especially in the immune system, whereas the mitochondrial pathway is used extensively in response to extracellular and internal insults such as DNA damage. This present study investigated the effects of EACC on MMPT pore, the release of cytochrome c, activation of caspases and histological changes in mouse liver after fourteen days oral administration.

PROCEDURE

The procedure for the assessment of MMPT is described in Section 3.6. of Materials and Methods.

RESULTS

The EACC induced MMPT pore opening invivo by 5 and 8 folds at 100 and 200mg/kg bwt doses, respectively. Also there was significant increase in the serum levels of caspase 9, 3 and cytochrome C in the treated mice when compared to the control mice. The histopathology of the liver showed periportal and perivascular infiltration by inflammatory cells in the mice treated with EACC

Conclusion

Methanol extract of *Cajanus cajan* (MECC) increased the liver enzyme activities due to the damage caused to the liver which resulted in increases in cytochrome C and caspases in the treated animals.

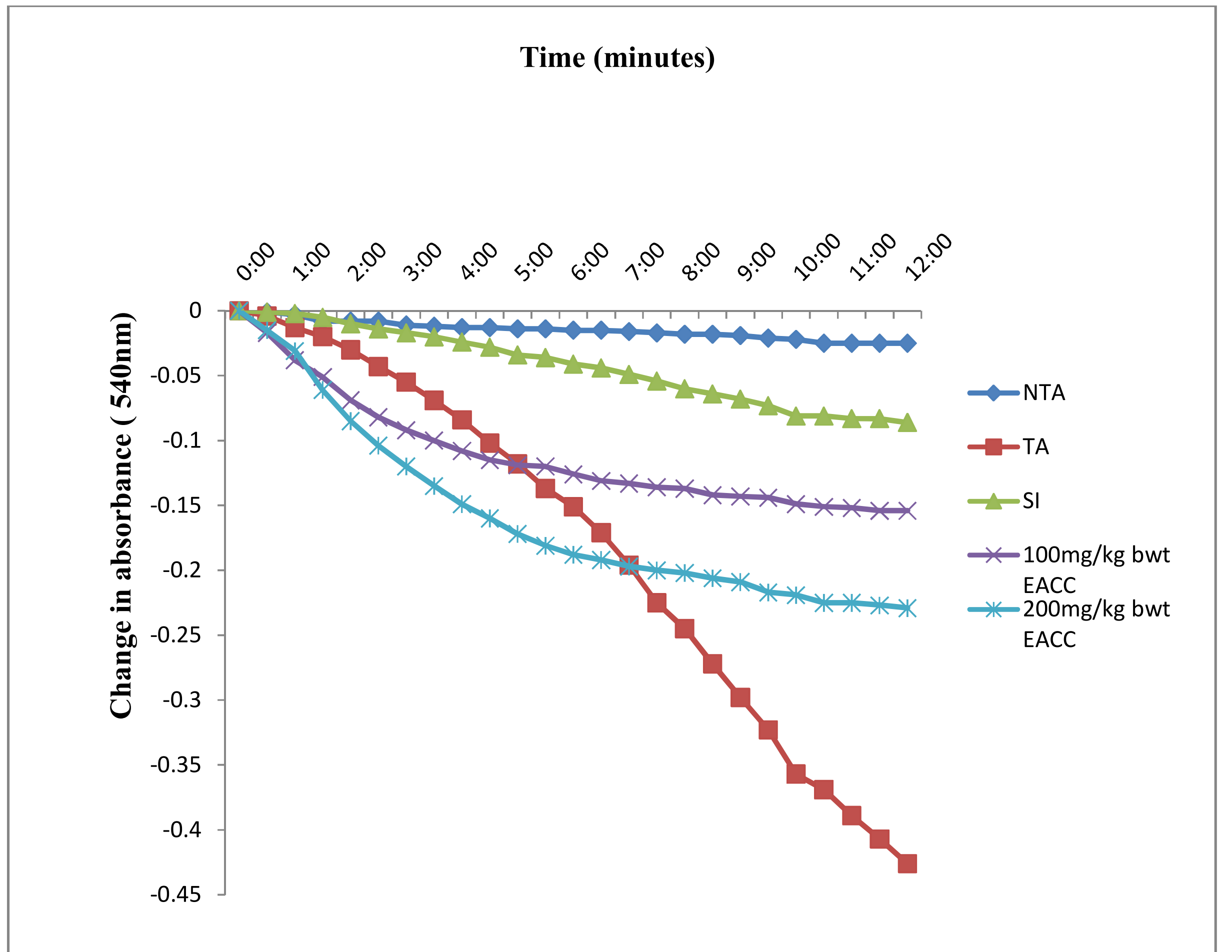


Figure 20: Effects of EACC on the opening of the MMPT pore after 14 days oral administration. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition, EACC - ethylacetate fraction of *Cajanus cajan*

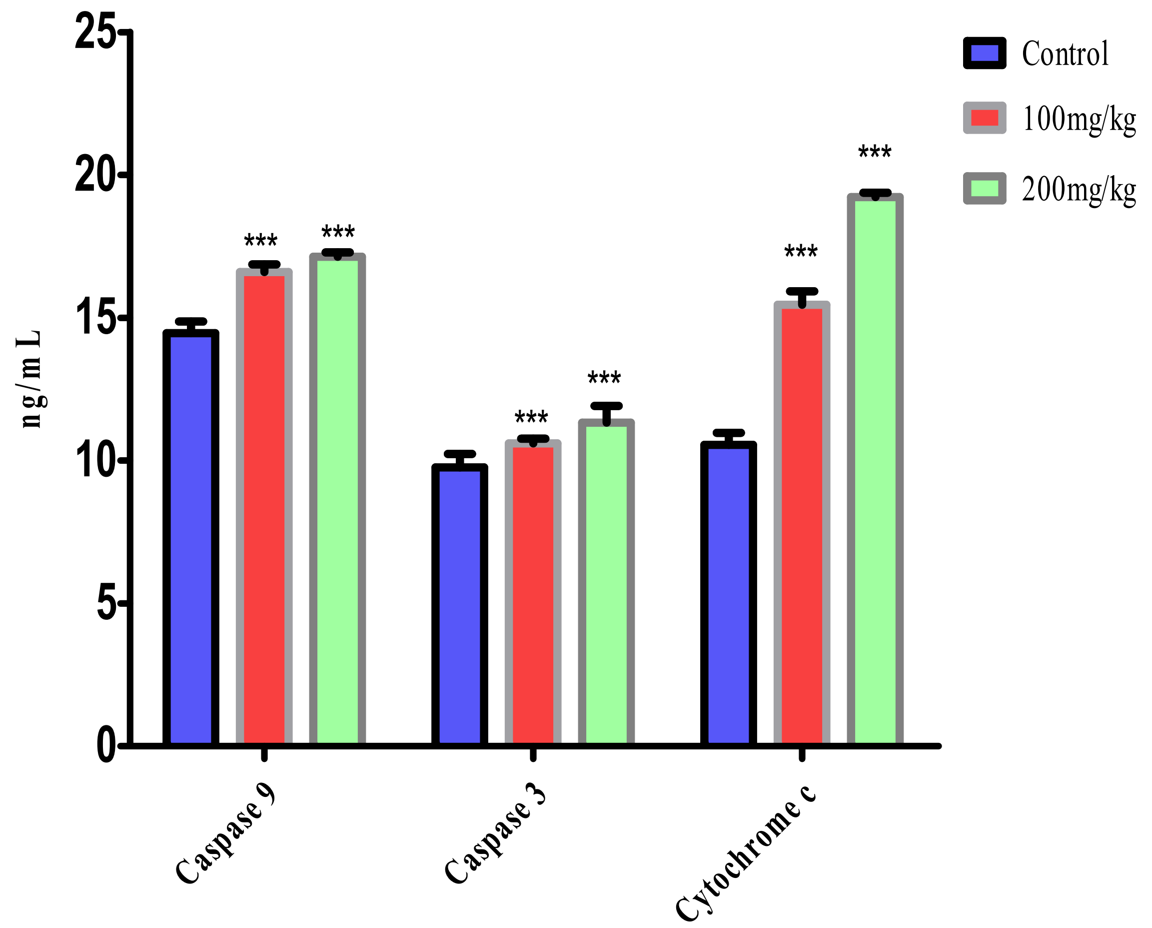


Figure 21: Effects of Ethylacetate fraction of *Cajanus cajan* on the release of cytochrome c and activation of caspase 9 and 3. Each value represents the mean ± SD for five rats per group. * Significantly different from control at $p < 0.05$

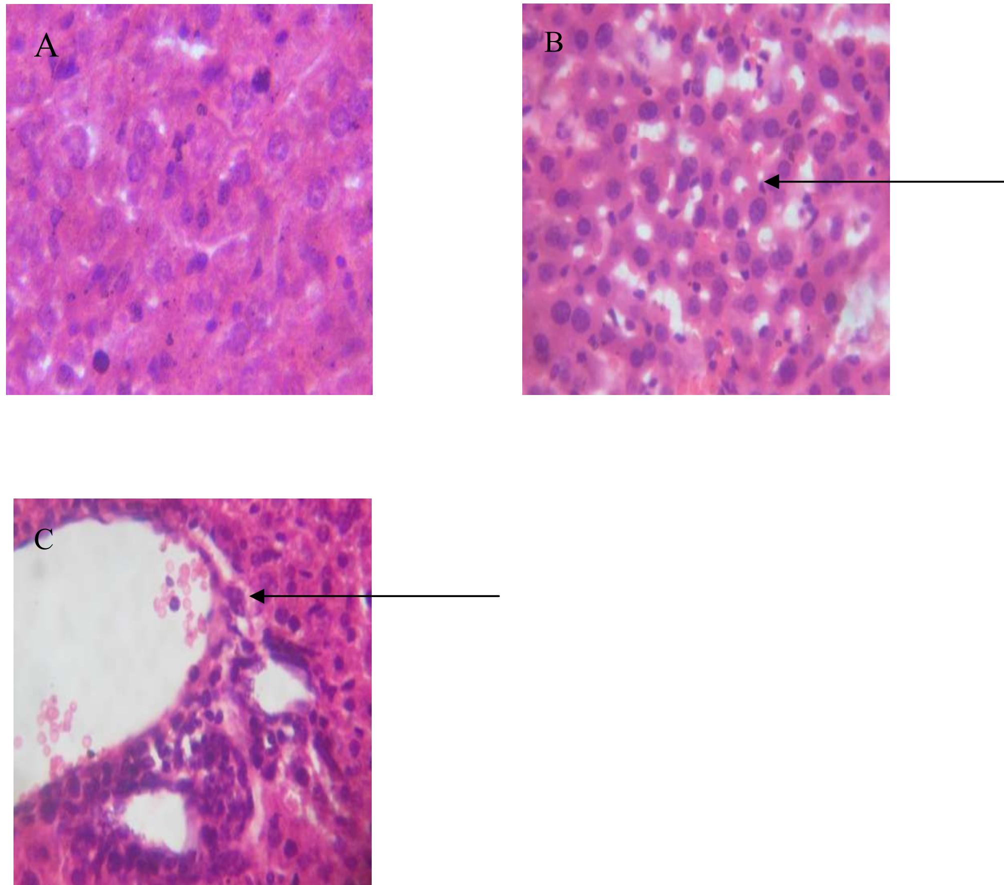


Figure 22: Histopathology of the liver of controland animals treated with ethylacetate fraction of *Cajanus cajan.* The control A shows no visible lesion while the treated animals B (100mg/kg bwt) and C (200 mg/kg bwt) shows periportal and perivascular infiltration by inflammatory cells. The arrows indicate inflammatory cells. (Magnification, X400)

EXPERIMENT 9: EFFECTS OF METHANOL EXTRACT OF *CAJANUS CAJAN* ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE OPENING AND LIVER ENZYME ACTIVITIES

INTRODUCTION

Drugs that are currently used in the treatment of cancer have exhibited side effects, hence researchers are on the search for alternative cancer drugs from natural products. Many studies have been carried out to attain this goal. The last decade has witnessed an unprecedented focus and discovery of novel agents that target mitochondria to induce cancer cell death. The paramount importance of discovering novel and efficient anticancer agents is even more accentuated by the fact that neoplastic diseases are now the greatest threat of the Western society and are likely to increase in frequency (Jemal *et al*., 2011; Siegel *et al*., 2012).

Evasion of apoptosis is considered to be one of the hallmarks of human cancer and one of the mechanism is through disruption of the mitochondrial-mediated pathway. The MMPT pore regulates the movement of molecules across the inner mitochondria membrane. .Induction of mitochondrial-mediated apoptosis by compounds of natural origin could provide an avenue for drug development in the management of cancer.

This study investigated the effects of MECC on MMPT pore opening, liver enzyme activities and immunohistochemical expression of cytochrome c, caspase 9 and 3 and histologically changes after thirty days administration of MECC.

Procedure

The procedure for the assessment of MMPT is described in Section 3.6.

Results

There were significant increases in the activities of AST, ALT and ALP in the treated animals when compared to the animals in the control group. Histological changes observed showed infilteration by inflammmatory cells and significant immunohistochemical expression of cytochrome C and caspases 9 and 3 were observedin the liver of the treated animals when compared with the control.

Conclusion

The MECC induced mitochondrial mediated apoptosis.

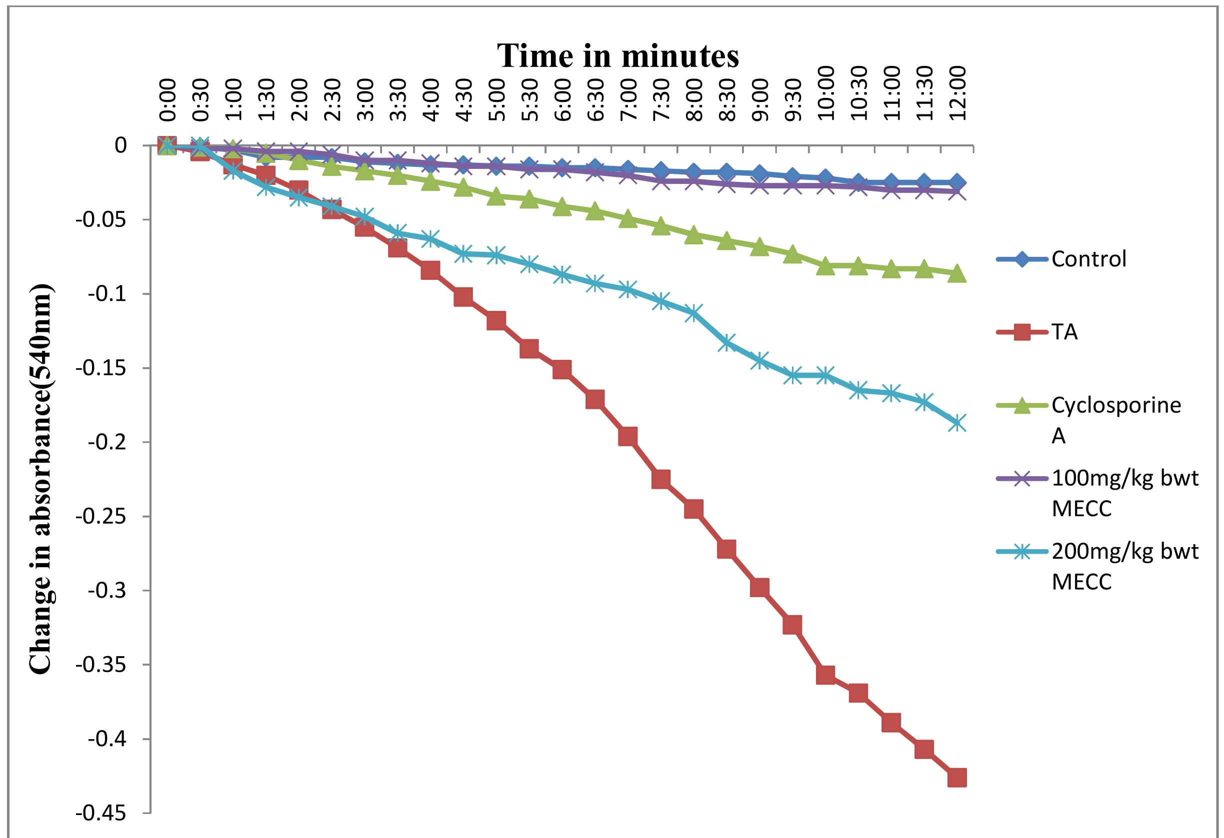


Figure 23: Effects of on the opening of the MMPT pore after 30 days oral administration. NTA- No triggering agent (without calcium), TA- Triggering agent (with calcium), SI-spermine inhibition, CI-cyclosporin inhibition, MECC - methanol extracts of *Cajanus cajan.*

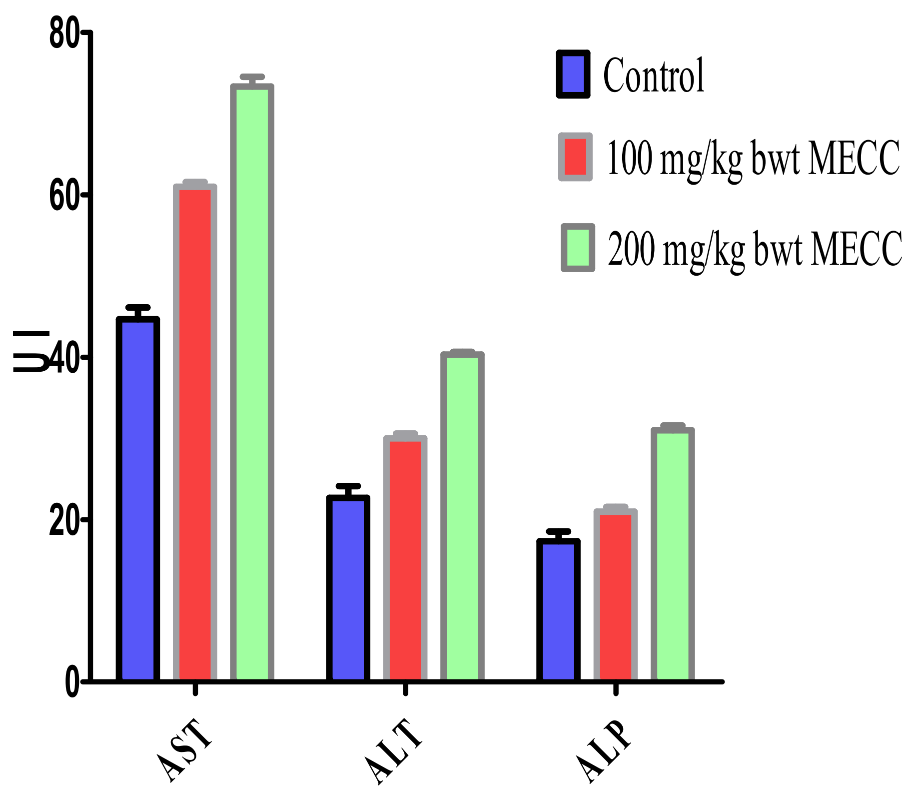


Figure 24 : Effect of methanol extract of *Cajanus cajan* on liver enzyme activities after 30 days oral administration. MECC - methanol extract of *Cajanus cajan*. Each value represents the mean of five determinations ± SD.* Significantly different from control at $p < 0.05$

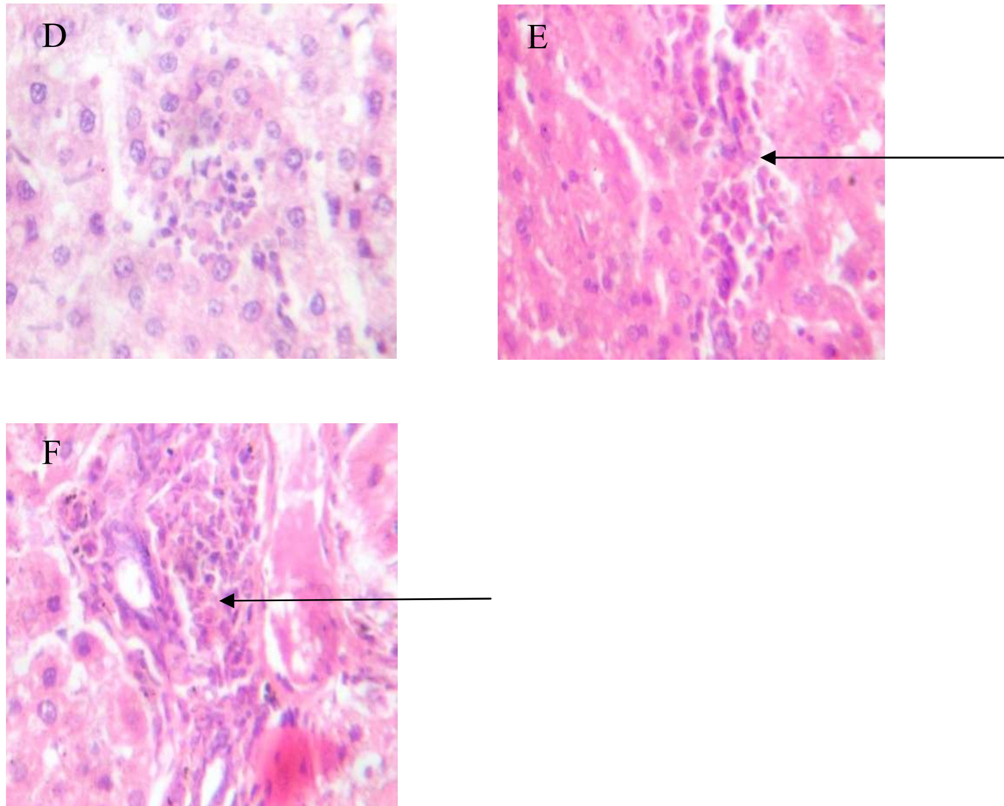
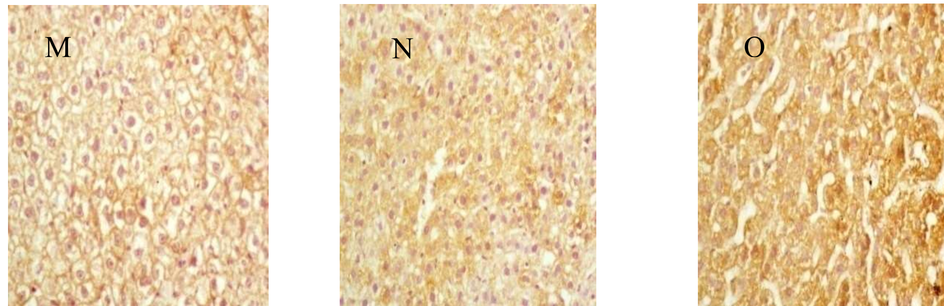


Figure 25: Histopathology of the liver showing the control and animals treated with methanol fraction of *Cajanus cajan.* . No lesion was observed in control D but there was infilteration by inflammmatory cells in the extract-treated animals E (100mg/kg bwt) and F(200mg/kg bwt) . The arrows indicate inflammatory cells. (Magnification., X400)



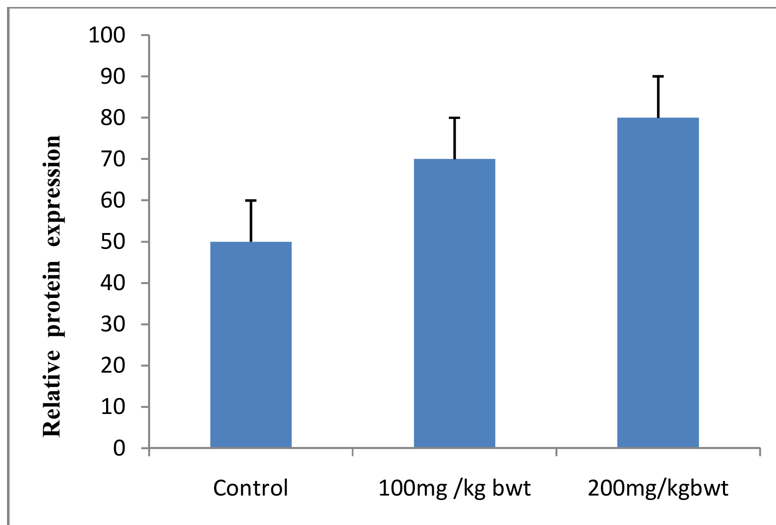
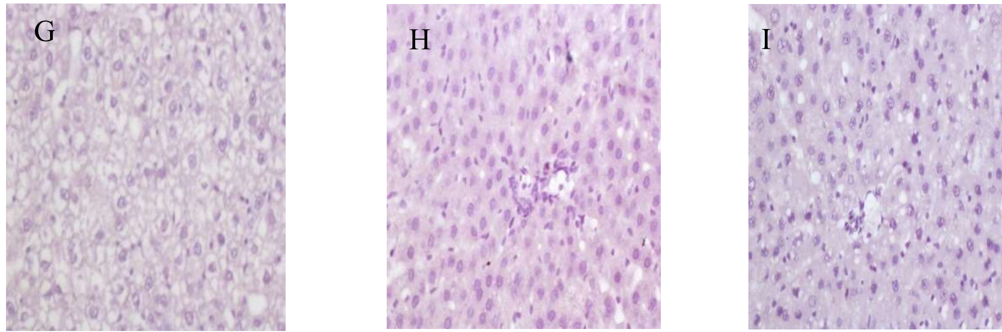


Figure 26; Immunohistochemistry showing the effects of MECC on cytochrome c expression in the liver of control(M) 100mg treated rat (N), 200mg treated rats (O) * Significantly different from control at $p < 0.05$



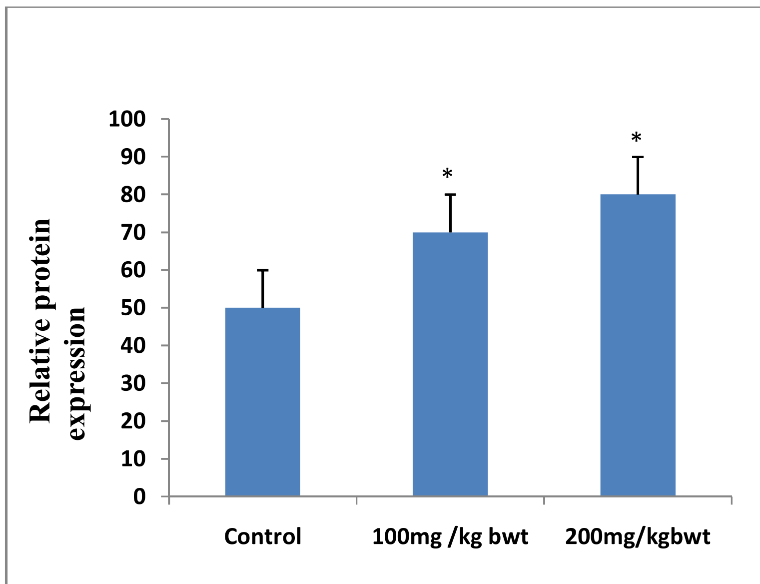
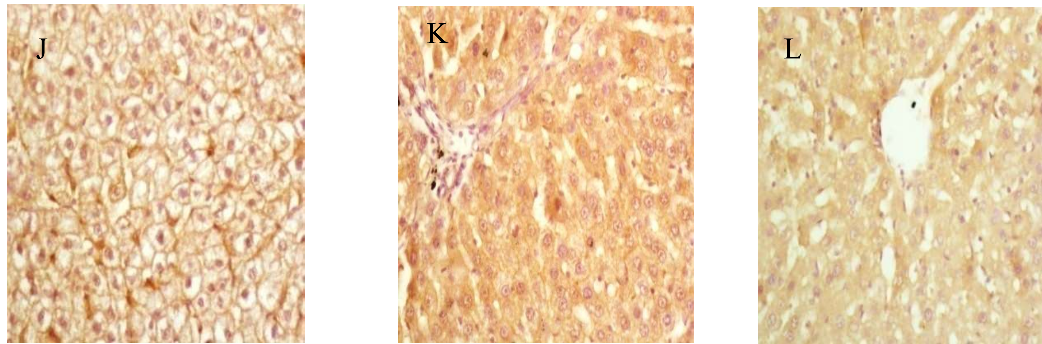


Figure 27: Immunohistochemistry showing the effects of MECC on caspase 9 in the liver of control(G) 100mg/kg bwt treated rat (H), 200mg/ kg bwt treated rats (I) *Significantly different from control at p< 0.05



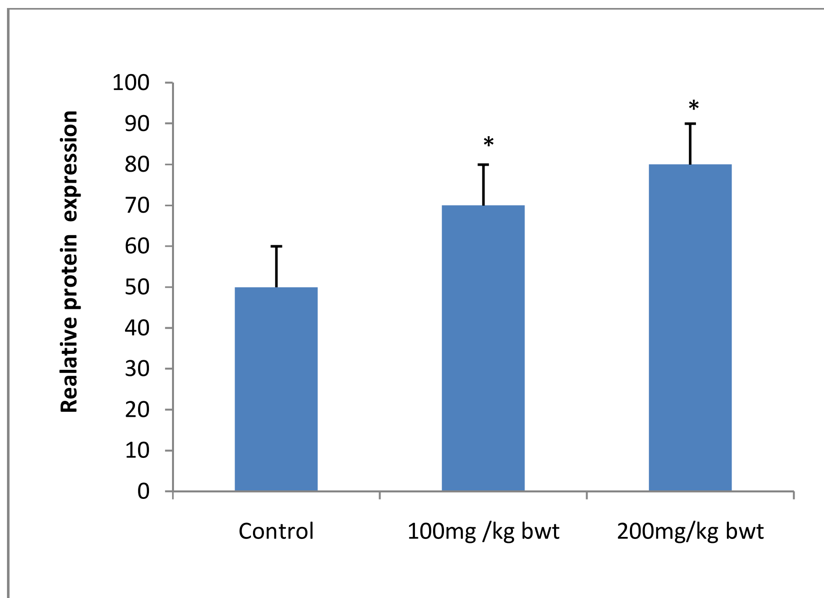


Figure 28: Immunohistochemistry showing the effects of MECC on caspase 3 in the liver of control(J) 100mg/kg bwt treated rat (K), 200mg/ kg bwt treated rats (L)

EXPERIMENT 10: PURIFICATION, ISOLATION AND CHARACTERISATION OF COMPOUND FROM EACC USING SPECTROSCOPIC TECHNIQUES

INTRODUCTION

Several techniques are employed to identify the compounds present in medicinal plants. The Vacuum liquid chromatography (VLC) is a chromatographic separation method which uses vacuum to speed up effluent flow rates. It has s several advantages such as simple equipment, shorter separation time, better resolution and large separation capacity. Column chromatography separates mixtures of organic compounds into pure compounds. It is generally used as a purification technique to isolate desired compounds from a mixture (Kenkel, 2003). Thin Layer Chromatography (TLC) is a used to monitor the purity compounds in a mixture, particularly to confirm purity of isolated compound (Visht and Chaturvedi, 2012). TLC is considered to be one of the simplest of the chromatographic techniques. It is a sensitive technique and (0.000001 g) microgram quantities can be analysed by TLC. It takes little time for an analysis (about 5-10 minutes), and is a relatively inexpensive procedure. It consists of four steps spotting, development, visualization and analysis. First, the sample to be analysed is dissolved in a volatile solvent to produce a very dilute solution (about 1 %).

PROCEDURE

The most active fraction (EACC) was subjected to column chromatography (40 x 6 cm) under gravity. Silica gel (60-230 mesh) was used to load a glass column. The sample to load the column was prepared by weighing 1 g of EACC and preadsorbing in silica gel ratio 1:1. Then, the dried silica fraction mixture was layered on the column layer bed. The column was first eluted with n-hexane 100% hexane, the polarity of the solvent was

increased gradually by 10% increments of ethyl acetate in increasing polarities. compounds . This yielded 170 fractions, fraction 154 appeared as a mixture of crystalline compounds. Fraction 54 was purified by another column (37 x 3 cm) packed with sephadex LH- 20 mesh and eluted with methanol. The subfractions eluted were developed on developed TLC plates plates precoated with silica gel 60 H $F_{254}$and fraction 54 appeared as a single spot

RESULTS

Fraction 154 that was eluted from the column appeared as a crystalline compound was subjected to GCMS and yielded several compounds. This fraction was further purified and subfraction 54 was subjected to spectroscopic techniques. FTIR of sub fraction 54 (0.13g) gave the characteristic absorption peak represented by figure .Absorption bands at 3636 cm-1 for O-H stretching vibration of an alcohol and 1633 cm-1 for C=C stretching vibration of an alkane .Its' UV spectrum revealed absorption maxima, λ max at 303 and 259 nm which indicated carbonyl groups and conjugated double bonds. Subfraction 54 was employed for GCMS analysis. The GC-MS studies revealed that subfraction 54 as a partially pure compound with a sharp peak with m/z value of 280, which corresponds to the molecular weight of 2 methyl z, z, 3, 13 Octadecadienol.

Table 7: Different percentages of solvents used for elution of column chromatography

Elution	Ratio of solvents (%)
Hexane	100
Hexane; ethylacetate	90; 10
Hexane; ethylacetate	80:20
Hexane; ethylacetate	70:30
Hexane; ethylacetate	60:40
Hexane; ethylacetate	50:50
Hexane; ethylacetate	40:60
Hexane; ethylacetate	30:70
Hexane; ethylacetate	20:80
Hexane; ethylacetate	10:90
Ethylacetate	100

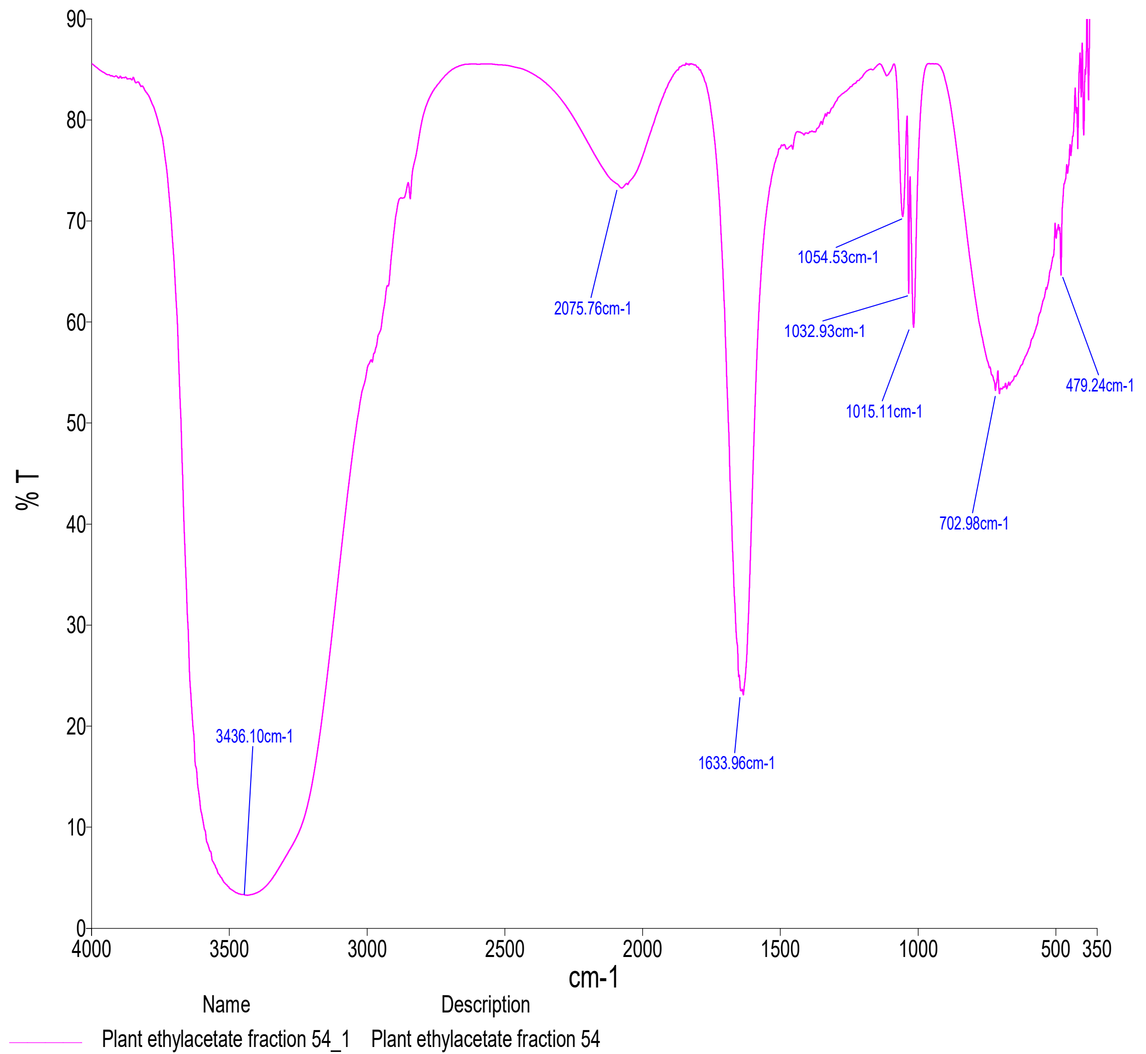


Figure 28; IR spectrum of subfraction 54

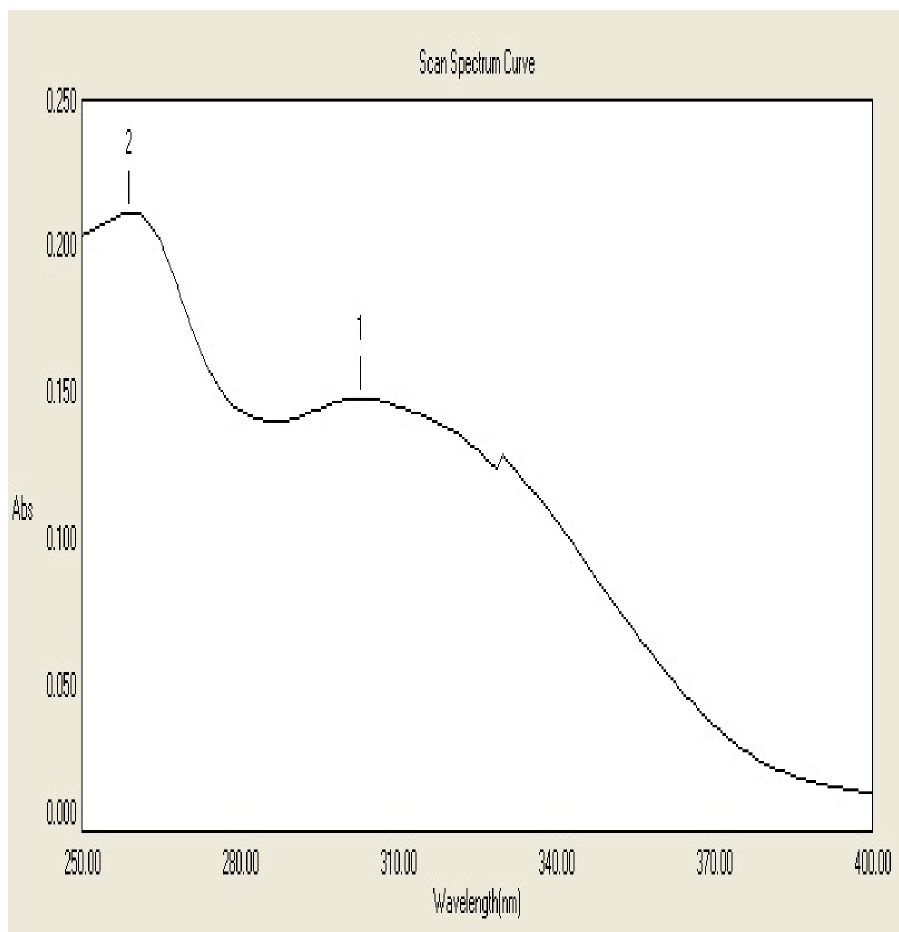


Figure 29 ; UV spectrum of subfraction 54

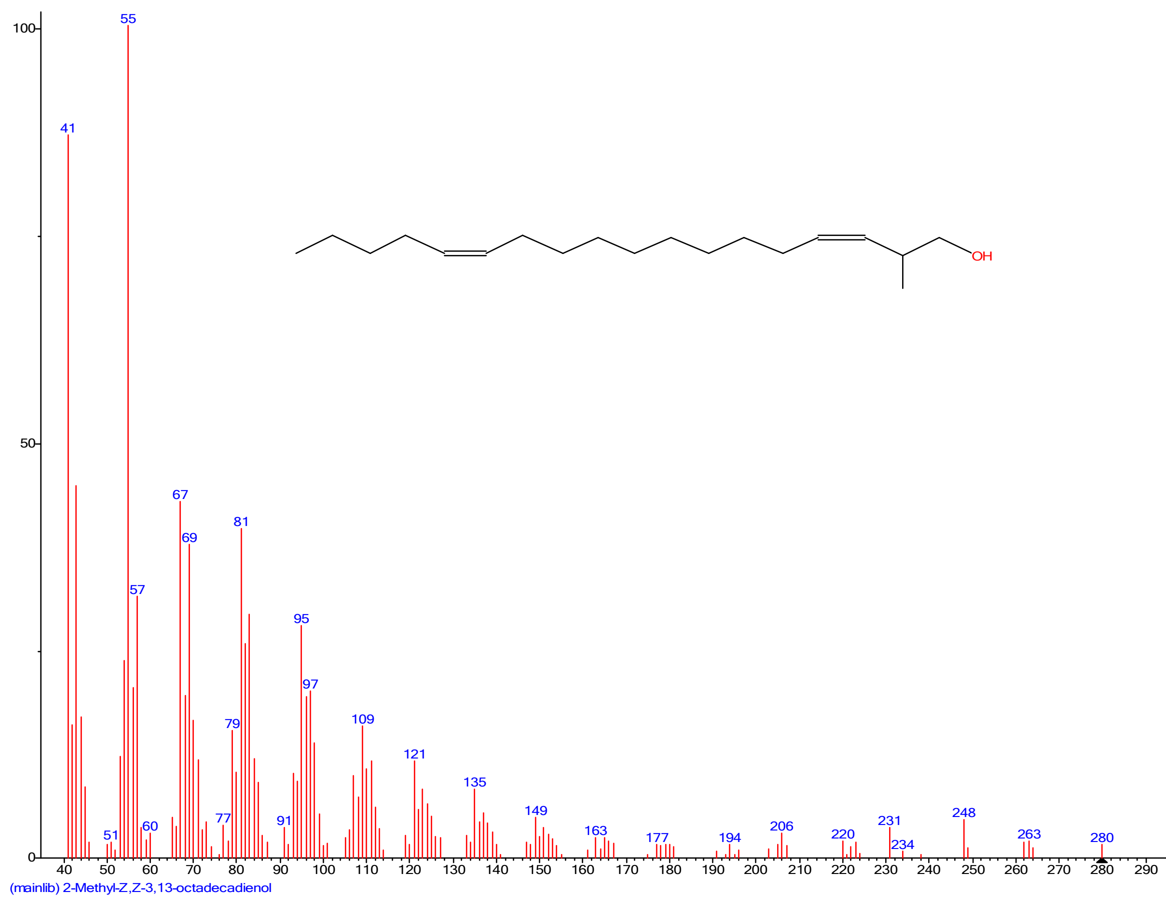


Figure 30: GCMS of subfraction 54 (2 methyl z, z, 3, 13 Octadecadienol)

CHAPTER FIVE

DISCUSSION, CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE

5.1 DISCUSSION

Diverse mechanisms have been proposed for the chemopreventive effects of dietary phytochemicals on human health with respect to combating disease conditions emanating from dysregulation of apoptosis such as cancer. Attention has recently focused on mitochondrial mediated apoptosis as a signaling pathway that is disrupted during carcinogenesis (Martin, 2006). Since MMPT pore opening results in apoptosis and the neoplastic transformations that occur in cancer have been linked to insufficient apoptosis, targeted induction of the opening of MMPT pore is now considered as an indispensable step in the development of anticancer chemopreventive agents (Xu *et al*., 2018). Hence, modulation of mitochondrial-mediated apoptosis through the induction of MMPT pore opening and expression of caspases is now considered a new prototype in the management of conditions associated with dysregulation of apoptosis such as cancer.

Medicinal plants have received commendable attention because of their usefulness in managing life threatening conditions because they contain composite of phytochemicals. For disease manifestation and progression involving multifactorial and complex signaling pathways, the cocktail of phytochemicals will target multiple sites for their mode of action. Plants produce a variety of compounds known as secondary metabolites which are the non-nutrient chemicals. They help to boost the immune system, kill pathogenic microorganisms and also to scavenge free radical molecules (Poe, 2017). Carotenoids, phytosterols, saponins, tannins, steroids, and alkaloids are among the phytochemicals present in medicinal plants (Karuppusamy, 2009). Phenolic compounds and flavonoids are also extensively dispersed in plants and have several biological activities (Zhang *et*

al., 2015). These secondary metabolites that help to protect the body against various illnesses (Haque *et al.,* 2016). The indigenous knowledge of chemical constituents of medicinal plants helps to disclose the importance of traditional remedies.

It has been reported that these compounds may act individually or synergistically to elicit their therapeutic effects making them lead compounds for drug development. In this study, phytochemical screening shows the presence of alkaloids, cardenolides, flavonoids, anthraquinones, saponins and tannins in the fractions of *Cajanus cajan,* this is also confirmed from literature (Oyewole *et al.,* 2008). Alkaloids have also been reported to have antitumor, antimalarial, antihypertensive, antipyretic, antiparasistic, anti-inflammatory activity and antimicrobial activities (Obumselu *et al.,* 2013). Some alkaloids have exhibited significant anticancer activity in preclinical and clinical trial (Kruczynski *et al.,* 1998). Cardiac glycosides are used in treatment of cardiac failure and congestive heart failure. Tannins are well known to posssess antioxidant, antihypertensive, antipyretic, anti-inflammatory activities. Park and co-workers published the ability of flavonoids to induce G2/M cell cycle arrest through regulation of proteins such as cyclin B1, cdc2, cdc25c and p21.

There is evidence from several studies that various classes of phytochemicals present in vanilloids, curcumin, turmeric and capscacin can alter permeability transition and favour the opening of the MMPT pore, a critical event needed to commit mutated cell death (Martin, 2006).

In this study, the opening of the MMPT pore was assessed by measuring the rate of decrease in light absorbance that reflects the mitochondrial swelling. It was necessary to ascertain the integrity of the mitochondria at the start of the experiment.

We observed that there were no significant changes in the volume of intact mitochondria, addition of calcium caused large amplitude swelling which was almost completely reversed by spermine, a standard inhibitor of opening of mitochondrial membrane permeability transition pore (Figure 11). In this study *Cajanus cajan* leaves was extracted and crude methanol extract was partitioned using n-hexane, chloroform and ethyl acetate in order of increasing polarities. Assessment of various fractions on permeability transition was determined. Figure 12 shows that MECC at 50 and 70 µg/mL induced of MMPT pore opening by 7 and 13 folds, respectively when compared with the non triggering agent. The chloroform fraction (figure 14) had no significant effects on the pore, while the EACC (figure 16) induced pore opening at 10, 30, 50 and 70 µg/mL by 19, 20, 21 and 23 folds, in a concentration-dependent manner.

In this study we observed the induction of permeability transition by fractions of *Cajanus cajan*. The order of potency in inducing MMPT pore opening was CFCC< MECC< EACC. The differences in the observed inductive effects observed is ascribable to the active ingredient present which appears to be different as a result of the various extracting solvents.From our results this induction of the MMPT pore by calcium was reversed by Spermine which confirms that the mitochondrial swelling observed resulted from MMPT induction. Spermine the standard inhibitor of the MMPT pore is a polyamide that has been shown to inhibit both in isolated rat heart and liver mitochondria .Several alternatives for the site of spermine action have been proposed, an external binding site which is occupied by divalent cations such as calcium decrease the probability of pore

opening. Lapidus and Sokolove, 1993 have demonstrated that spermine competes for calcium binding site at low affinity. Our results in figure 11 shows that calcium, the triggering agent induced pore opening by 30 folds. It could be that these fractions had as similar mechanistic effect on the pore.

Anticancer drugs such as Etoposide and some naturally occuring compounds that have demonstrated anticancer activity have been shown to elicit their action directly by inducing MMPT pore (Martin, 2000). Induction of permeability transition by medicinal plants is well documented by previous scientific studies (Adisa *et al*., 2012; Salako *et al*., 2010; Olanlokun, *et al*., 2017 and Oyedeji *et al*., 2018).

The MMPT acts as a sensor of calcium ions, protons and thiol groups contributing to mitochondrial homeostasis. However exposure of mitochondria to calcium overload, high inorganic phosphate, alkaline pH, reactive oxygen species and low membrane potential results in pore opening. Several reports have shown that abnormally elevated cytosolic Ca^{2+}can injure cells by stimulation of mitochondrial Ca^{2+} uptake which eventually trigger pore opening or other forms of mitochondrial injury (Nicholls, 2009, Xiong *et al*, 2002).

Inorganic phosphate is a long known inducer of pore opening, a process that more recently has been proposed to involve the inorganic phosphate carrier as the pore's CypD binding component (Leung *et al*., 2007). The electron transport chain and oxidative phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane (Mitchel, 1979).

Complete loss of the ATP synthase enzyme has been reported incompatible with life. However, partial loss of the complex has been associated with human diseases (Houstek *et al*., 2004). Uncoupling of oxidative phosphorylation is a key event in the opening of the

MMPT pore.When mitochondria becomes uncoupled the permeability barrier in the IMM is lost and they begin to hydrolyse instead of synthesizing ATP. It is well established that ATP synthase which is localized in the IMM is highly sensitive to the intergrity of the membrane. Opening of the MMPT pore is accompanied by loss of the membrane potential across the IMM. F_OF_1ATP synthase hydrolyse ATP in an attempt to maintain the mitochondrial membrane potential (Leysens *et al.,* 1996). Hence, dissipation of membrane potential and hydrolysis of ATP are hallmarks of permeability transition.

Our results have shown that methanol extract of *Cajanus cajan* stimulated mitochondrial ATPase activity at 10µg/mL, 30µg/mL, 50 and 70 µg/mL was by 2.6, 3.0, 3.8 and 4. 2 folds respectively, by methanol extract. Similarly stimulatory effects of 1.8, 2.3, 7.2, 10.2 and 9, 11, 13 and 15 folds were observed for the chloroform and ethylacetate fraction respectively in a concentration dependent manner. It was observed that the Dinitrophenol, a classical uncoupler had the highest ATPase stimulatory activity, of 23 folds, this was followed by EACC.

A well-established mechanism of biomembrane injury is lipid peroxidation, which is a biomarker of oxidative stress in tissues. It is well documented that some medicinal plants elicit their chemopreventive activity through the generation of reactive oxygen species. Reactive oxygen species causes the peroxidation of unsaturated fatty acids leading to a decrease in membrane fluidity and disruption of membrane intergrity and function. Cellular damage associated with lipid peroxidation is implicated in serious pathological conditions such as heart diseases, artherosclerosis, carcinogenesis as well as aging process. Lipid peroxidation is initiated by ROS and strongly affected by transition metals like iron which catalyses the generation of hydroxyl radicals according to Fenton reactions (Halliwell and Gutteridge, 1998). This peroxidation reaction causes the decomposition of lipid hydroperoxides into alkoxy radical. Damage to biological

molecules occurs when the highly reactive hydroxyl radical reacts with polyunsaturated fatty acids in membrane phospholipid yielding carbonyl products like MDA. Mitochondria are highly dynamic structures made up membranes, each composed of phospholipid bilayers making them highly susceptible to free radical attack. Under normal physiological conditions, the body has a pool of scavengers such as reduced glutathione, α-tocopherol and ascorbate which are usually obtained from the diet (Bababunmi and Bewaji, 2002). Also the mitochondrial antioxidant systems; enzymatic such as mitochondrial enzyme superoxide dismutase (SOD) and non-enzymatic such as mitochondrial glutathione scavenge free radicals and preserve mitochondrial integrity; however on exposure to free radicals or reactive oxygen species induces extensive lipid peroxidation of mitochondrial membranes leading to membrane dysfunction and disruption of integrity of mitochondria.

Documented evidence shows that reactive oxygen species is one of the main mechanism for the induction of MMPT pore opening. In this study we assessed the effects of various fractions of *Cajanus cajan* on the inhibition of lipid peroxidation in order to find out if the induction of MMPT was as a result of reactive oxygen species. We observed that *Cajanus cajan* prevented lipid peroxidation in a concentration-dependent manner. This is in consonance with findings on antioxidant activities of Xylopia Aethopica were reported by Adaramoye *et al*., (2011) and by Farombi *et al*., (2013) respectively. Screening of the phytochemicals in *Cajanus cajan* fractions in the previous study revealed the presence of flavonoids which can preserve mitochondrial integrity. This findings rule out oxidative damage as the mechanism of the opening of the MMPT pore .

The MMPT pore opening has long been associated with many disease conditions and can be seen as a double-edged sword. For example, in cardiac ischemia the pore is an

open state and inhibitors of the MMPT pore (cyclosporin A) employed in the treatment of this condition have been shown to reduce cardiac ischemia-reperfusion injury.reperfusion by decreasing the size of tissues with infart. Hence, inhibition of MMPT with has been shown to reduce cell dysfunction and death following cardiac ischemia-reperfusion (Griffiths and Halestrap, 1995). On the other hand induction of pore opening is a pharmacological tool in the treatment of cancers. Therefore compounds that favouring opening of the pore can be administered as anticancer agents. More importantly, a great body of evidence has lent support for the concept that pharmacological induction of the mitochondrial membrane transition pore is an effective and promising strategy for the management of cancer.

Cancer is among the leading causes of death worldwide. In 2012, there were 14.1 million new cases and 8.2 million cancer-related deaths worldwide. The incidence of cancer is on the rise in United States is in 2018, an estimated 1,735,350 new cases of cancer will be diagnosed in the United States and 609,640 people will die from the disease. Studies have shown that the commonest cancers in Nigeria in 2009 to 2010 were breast and cervical cancer among women and prostate cancer among men.

Apoptosis is highly beneficial to living systems for the elimination of deleterious and unwanted cells from an organism. Apoptosis research is increasing by the day in order to provide management stratergies for differerent forms of cancer. It is well established that several anticancer drugs elicit their action by targeting mitochondria via different mechanisms. Accumulating evidence has shown that cancer cells may block apoptosis by upregulating anti-apoptotic Bcl-2 proteins thereby preventing MMPT pore opening or by inhibiting caspase function following permeability transition. Hence medicinal plants that

can modulate mitochondrial-mediated apoptosis might be useful in targeting and subsequently killing cancer cells.

The MMPT pore is a vulnerable target for experimental and pharmacological intervention in several experimental models. Since the discovery of the mitochondrial permeability transition, many proposals have been made about the protein constituents, yet till date the molecular nature of the pore is unknown. Also the mode of action is still an ongoing research. The long standing idea that the pore forms at contact sites of the inner and outer membranes through voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) and CypD paled into insignificance since genetic studies shows that VDAC- and ANT-null mitochondria still display a CsA-sensitive permeability transition leaving CypD as the only component standing.

Lately the FoF1 ATP synthase is now considered a reasonable candidate for the formation of the pore. Recent evidence from the Bernardi group suggests that the suggests that the pore was formed in the membrane surrounding dimers of ATP synthase (Giorgio *et al*., 2013).Their work showed that CypD binds to the stator of ATP synthase (Giorgio *et al*., 2009), also that this binding is specifically at the OSCP subunit and that decreasing the association of CypD with ATP synthase (Giorgio *et al*., 2013). Furthermore, they demonstrated that a novel inhibitor of ATP synthase that binds to OSCP, benzodiazipine-423, inhibits the interaction of OSCP and CypD and, like CypD, opens the PTP in response to $Ca^{2+.}$

The argument that the C-ring of the FoF_1 ATP synthase constitute the pore was put forward by Alavian. His hypothesis that the main membrane embedded portion of mammalian FoF1 ATP synthase, i.e., the c-subunit ring, forms the pore was substantiated (Alavian *et al*., 2014; Azarashvili *et al*. 2014). Indeed, electrophysiological recordings of

the purified mitochondrial c-subunit yield a multi-conductance, voltage-dependent channel with prominent subconductance states (Alavian *et al*., 2014). Alavian *et al*., 2014 in their present data explained that the c-subunit of the FoF_1 ATP synthase creates the regulated pore with convincing evidence. Both these models can be challenged. According to some school of thought, FoF_1 ATP synthase dimers exhibit no obvious pore-forming features, dimerization has been reproducibly associated with improved bioenergetic metabolism and cell survival (rather than cell death (Daum *et al*., 2013) The FoF_1 ATP synthase is mostly monomeric and responds normally to MMPT induction (Masgras *et al*., 2012). It seems unlikely for C-rings to lose their lipid plugs in relatively physiological conditions (Gerle *et al*., 2016). So far, a direct interaction between C-rings and CypD has not been identified. Despite all these findings there are still some unanswered questions about the precise nature of the pore. Thus, additional investigation is still on going to obtain precise structural and functional insights into the molecular machinery that mediate pore opening.

In this study, we evaluated the effects of various fractions of *Cajanus cajan* on calcium-induced MMPT in rat liver mitochondria .We observed that oral administration of the MECC for 30 days resulted in mitochondrial dysfunction and hepatotoxicity. Opening of MMPT pore was observed as shown in Figure 4 MECC induction pore opening invivo at 200mg/kg body weight by 8 folds when compared with the control. Mitochondrial dysfunction is a major mechanism of liver injury by some toxic agents and the MMPT pore plays a mitochondrial- mediated hepatocyte injury. The MMPT pore opening has been reported to be involved in the toxicity process of different xenobiotics and in different pathologies ultimately resulting in cell damage and death (Nakagawa and Moore 1999, Rao *et al*., 2014).

The biotransformation of foreign compounds and drugs takes place in the liver and involves their conversion into simple and harmless substances by making them soluble enough to be excreted from the body make the liver becomes susceptible to injury. Abnormal liver enzyme levels signal liver damage. AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of ketoglutaric acid to generate oxalacetic and pyruvic acids respectively. AST is also diffusely represented in the heart, skeletal muscle, kidneys, brain and red blood cells, and ALT has low concentrations in skeletal muscle and kidney (Rej, 1989). ALP is an enzyme that transports metabolites across cell membranes. Liver and bone diseases are the most common causes of pathological elevation of ALP levels.

The increases in the activities of AST, ALT and ALP in the treated animals suggesting that leakage of liver enzymes into the serum was as a result of damage to the hepatocytes leading to the breakdown of cell integrity (Figure 23).

These biochemical changes observed was further confirmed by the histopathological changes in which there was periportal and perivascular infiltration by cells. Immunohistochemical examination of the liver showed a significant increase in the expression of caspase 3, 9 and cytochrome c in the liver of the animals administered extract when compared with the control. Taken together this results demonstrates that the plant has the ability to increase the levels of transaminases and induce mitochondrial-mediated apoptosis, several studies have that cell death is the outcome of liver toxicity . This present result is consistent with the findings in which curcumin-induced apoptosis through mitochondrial pathway involving caspase-9 and 3 activation (Jana *et al* ., 2004)

In order to validate the results of the invitro data on the most potent fraction, an in vivo study assessed the effect of EACC on the induction of MMPT in animals after oral

administration was conducted. Along with this, the serum levels of biomarkers of apoptosis (caspase 3, 9 and cytochrome C) were also determined. The signaling pathway of apoptosis pathway involve the release of apoptogenic factors such as AIF and cytochrome C. Apoptosis inducing factor is released in caspase independent apoptotic pathway and this is specific for neuronal cells.The mitochondrial-mediated pathway involves the release of cytochrome c from the mitochondria from the mitochondria and this activates a caspases cascade.

Caspases are intracellular serine proteases that play a pivotal role in apoptosis. They are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Caspase-3 activity first becomes detectable early in apoptosis, continues to increase as cells undergo apoptosis, and rapidly declines in late stages of apoptosis. Its activity is an early marker of cells undergoing apoptosis. Caspase 3, is considered to be the most important of the executioner caspases and is activated by Caspases 9. Caspases 9 cleaves procaspase 3 thereby activating it to caspase 3, which then cleave and inactivate proteins crucial for the maintenance of cellular cytoskeleton, DNA repair, signal transduction and cell cycle control (Hengartner, 2000). There are over 300 in vivo caspase substrate: among them are poly (ADP- ribose) polymerase (PARP-1) and ICAD/DFF45 which is cleaved to CAD which degrades chromosomal DNA reslting in DNA fragmentation which is characteristic of apoptotic cells (Fischer *et al*., 2003).

Cytochrome c is a nuclear encoded molecule that plays an indispensable role in apoptosis. (Kalkavan and Green, 2018). It interacts with various cytosolic and nuclear partners during the onset of apoptosis. It has been documented that, cytochrome c is normally attached to the outer surface of the IMM by an association with the anionic phospholipid,

cardiolipin which is particularly rich in unsaturated fatty acid and occupies almost the entire inner mitochondrial membrane. The molecular interaction between cardiolipin and cytochrome c involves electrostatic as well as hydrophobic interactions and hydrogen bonding. It has been suggested that one of the acyl chains of cardiolipin may be inserted into a hydrophobic pore in cytochrome c, whereas the others extend into the phospholipid bilayer (Luo, 1998). The release of cytochrome *c* during apoptosis signaling might be a two-step process, involving detachment of the hemoprotein from oxidized cardiolipin followed by its release into the cytoplasm through Bax/Bak-induced pores in the outer mitochondrial membrane (OMM).

Oxidation of cardiolipin reduces cytochrome c binding and this is occurs by pore formation mediated by pro-apoptotic Bcl-2 family such as Bax (Luo, 1998). Once released to the cytoplasm, cytochrome c interacts with WD40 domains of the apoptotic protease activating factor-1 (Apaf-1) in the cytosol, enabling the assembly of the apoptosome (Riedl *et al*., 2005). This protein platform activates caspase-9 and later caspase-3, initiating the caspase cascade, which executes cell death in an orchestrated way. Apart from Apaf 1 activation and apoptosome assembly recent findings have shown that cytochrome c interacts with other proteins that regulate apoptosis such as 14-3-3ε (a direct inhibitor of Apaf-1) prevents it from binding Apaf 1 and favoring apoptosis.

This results in figure 5 shows an induction of the opening of the MMPT pore which corrobrates our in vitro findings. It also shows an increase in serum levels of cytochrome c, and caspases 9 and 3 in animals treated with EACC when compared with control, thereby confirming the occurrence of apoptosis. Furthermore, histopathology of the liver of the animals treated with extract showed infilteration by inflammatory cells. This observation supports our serum biochemical findings. It is also consistent with the

findings of Subbarayan *et al*., (2012), who reported that Masilinic acid isolated from Olea europaea produced mitochondria disruption, cytochrome c release leading finally to activation of caspase 3 and 9. The EACC had the most potent biological activity invivo, and was further purified using column chromatography. The possible mechanism by which fractions of *Cajanus cajan* might cause mitochondrial-mediated apoptosis involves the induction of permeability transition is due to the opening of the MMPT pore which is followed by osmotic swelling of the mitochondrial matrix and rupture of the outer mitochondrial membrane and the release of inter membrane protein cytochrome C and subsequent uncoupling of the mitochondria would lead to hydrolysis of ATP.

The fraction may act on the pore components directly favouring the open conformation. The major role of the Bcl-2 family of proteins is to regulate apoptosis through the interactions between the proapototic and the antiapoptotic proteins. Furthermore, alterations in the expression of Bax and Bcl-2 leads to high Bax/Bcl-2 ratio which is an important factor in determining the cell's vulnerability to apoptosis. In response to apoptotic signals the proapoptotic Bax or Bak, dimerise thereby and form a lipidic pore in the OMM.

Previously literature by several scientists revealed the presence of structurally diverse compounds in *Cajanus cajan* leaves. Pal *et al*., (2011) reported the presence of cajaninstilbene acid (3-hydroxy-4-prenylmethoxystilbene-2-carboxylic acid), pinostrobin, vitexin and orientin Also Duker-Eshun et al. (2004) reported the presence of cajanin, longistylin C and longistylin A and Luo *et al*., (2010) reported the presence of Cajanol. Of these compounds, Cajanol was reported to have anticancer activity.

Given that the EACC the most potent fraction we proceeded to purify this fraction and fraction 154 appeared as whitish crystals. GC-MS analysis was done on the crystal and

the compounds identified were Tridecane, Pentadecane, β Bisabolene, Methoxyacetic acid 3 pentadecylester, hexadecanoic methyl ester, 9- Octadecenoic acid methyl ester,E 15 heptadecenal. Fraction 154 was subjected it to further purification which yielded subfraction 54 and using spectroscopic techniques this partially pure compound was found to be 2 Methyl z, z, 3,13 Octadecadienol.This compound was isolated for the first time in *Cajanus cajan* leaves.

Antioxidants act as pro-oxidants in a diversity of systems based on its structure, concentration, and cellular re-dox context (Bouayed and Bohn, 2010) .There is evidence from several studies that various classes of phytochemicals present in vanilloids, curcumin, turmeric and capscacin can alter permeability transition and favour the opening of the MMPT pore (Martin, 2006). Interestingly this partially pure compound 2 Methyl z, z, 3, 13 Octadecadienol induced the opening of the MMPT pore.. This compound is a terpene alcohol with an O-H group and previous studies by Yan *et al.,* 2007 showed that O-H containing compounds have the ability to induce pore opening. This result is also consistent with findings of Park *et al*., (2005) who reported that Genistein from Soy bean induced MMPT pore opening. It is therefore plausible to suggest that this compound modulates the MMPT pore thus favouring pore opening.

CONCLUSION

This study shows that *Cajanus cajan* induced apoptosis in normal rat liver through the induction of MMPT, stimulation of mitochondrial ATPase activity and increased activities of caspase 3 and 9. These findings suggest that certain bioactive components of *Cajanus cajan* may be involved in the induction of the opening of the pore with the eventual release of cytochrome C which is a prelude to the progression of mitochondrial-mediated apoptosis. Hence, *Cajanus cajan* may be useful in the management of conditions associated with dysregulated apoptosis such as cancer. cancer.

CONTRIBUTIONS TO KNOWLEDGE

- This study provides scientific validation on the use of *Cajanus cajan* leaves in the management of conditions associated with dysregulated apoptosis.

- The fractions of *Cajanus cajan* leaves was able to cause mitochondrial mediated apoptosis by

  1. inducing mitochondrial membrane permeability transition pore, in rat liver mitochondria
  2. stimulated the release of cytochrome c and activation of caspase 3 and 9
  3. stimulated ATPase activities in a concentration dependent manner
  4. increasing liver enzyme activities in the liver of animals

- Purification of *Cajanus cajan* yielded a partially pure compound, which induced permeability transititon in rat liver mitochondria.
- Partially pure compound was 2 Methyl z, z, 3,13 Octadecadienol, this was reported for the first time in *Cajanus cajan* leaves

REFERENCES

Abrahams, J. P., Leslie, A. G. W., Lutter, R. and Walker, J. E. 1994. Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria *Nature* 370: 621-628

Adams, J, Palombella, V.J. Akrout A., Gonzalez L. A., Jani H. E., and Madrid P.C. 2011. Antioxidant and antitumor activities of *Artemisia campestris* and *Thymelaea hirsuta* from southern Tunisia. *Food Chem Toxicol* 49: 342–347.

Adams, V., Bosch, W., Schlegel, J., Wallimann, T.and Brdiczka, D. 1989. Further characterization of contact sites from mitochondria of different tissues: topology of peripheral kinases. *Biochim Biophys Acta* 81: 213–216

Adaramoye, O. A., Okiti, O. O., and Farombi, E. O. 2011. Dried fruit extract from *Xylopia aethiopica* (Annonaceae) protects Wistar albino rats from adverse effects of whole body radiation. *Exp Toxicol Pathol* 63: 635–643.

Adisa, R.A., Alabi, T.D. and Olorunsogo, O.O. 2012. Modulation of opening of rat liver mitochondrial membrane permeability transition pore *by* different fractions *of the* leaves *of Cnestis ferruginea. D.C. Afr J Med Med Sci* 41: 157–69.

Agarwal, A., Cho, C.L., Esteves, S.C. and Majzoub, A. 2017. Current limitation and future perspective of sperm DNA fragmentation tests. *Transl Androl Urol* 6 (Suppl 4): S549–S552

Aggarwal, B.B. and Shishodia S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer *Biochem Pharmacol* 71: 1397–1421

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Obuotor, E.M. and Farombi, E.O. 2007. Phytochemical constituents and antioxidant activity of extract from the leaves of the Ocimum graticcimum. *Sci Res Essay* 2: 163–166.

Akrout, A., Gonzalez, L. A., Jani, H. E. and Madrid, P.C. 2011. Antioxidant and antitumor activities of *Artemisia campestris* and *Thymelaea hirsuta* from southern Tunisia. *Food Chem Toxicol* 49: 342–347.

Alavian, K.N., Beutner, G., Lazrove, E., Sacchetti S., Park, H.A., ***et al.*** 2014. An uncoupling channel within the c-subunit ring of the F_1F_O ATP synthase is the mitochondrial permeability transition pore. *Proc Natl Acad Sci U S* A 111:10580–5 A.

Alcala, S. Klee, M., Fernandez, J., Fleischer, A. and Pimentel-Muinos, F.X. 2008. A high-throughput screening for mammalian cell death effectors identifies the mitochondrial phosphate carrier as a regulator of cytochrome *c* release. *Oncogene* 27: 44–54.

Ambasta, S.P., 4thed. New Delhi. National institute of Science Communication. The useful plants of India, 2004, 94–95.

Appaix, F., Minatchy, M., Riva-Lavieille C., Olivaires, J., Antonnson, B. and Saks, V.A. 2000. Rapid spectrophotometric method for quantitation of cyctochrome c release from isolated mitochondria or permealized cells revisited. *Biochim. et Biophys. Acta* 1457: 175–181.

Arnoult, D., Parone, P., Martinou, J.C., Antonsson, B., Estaquier, J. and Ameisen, J. C. 2002. Mitochondrial release of apoptosis-inducing factor occurs downstream

of cytochrome c release in response to several proapoptotic stimuli. *J Cell Biol.* 159: 923–929.

Ashidi, J.S., Houghton, P.J., Hylands P.J.and Efferth T. 2010. Ethnobotanical survey a nd cytotoxicity testing of plants of South-western Nigeria used to treat cancer, with
isolation of cytotoxic constituents from *Cajanus cajan* Millsp. leaves. *J Ethnopharmacol* 128: 501–512.

Ashkenazi, A .and Dixit, V.M. 1998. Death receptors: signaling and modulation. *Scienc e* 81:1305–1308

Ashkenazi, A. 2002. Targeting death and decoy receptors of the tumour-necrosis factor superfamily *Nat Rev Cancer* 26: 420–30.

Ahsan, R. and Islam, M. 2009. Hepatoprotective activity of methanol extract of some medicinal plants against carbon tetrachloride-
induced hepatotoxicity in rats. *Euro J Sci Res* 37: 302–310.

Azarashvili, T., Odinokova, I., Bakunts, A., Ternovsky, V., Krestinina, O., Tyynela, J. and Saris, N.E. 2014. Potential role of subunit c of Fo F_1-ATPase and subunit c of storage body in the mitochondrial permeability transition. Effect of the phosphorylation status of subunit c on pore opening. *Cell Calcium* 55:69–77.

Bababunmi E.A. and Bewaji C.O. 2002. Oxidative stress and antioxidant therapy in wasting conditions associated with malnutrition, HIV/AIDS, tuberculosis and malaria. *Biokemistri*12: 135–139.

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H. *et al.* 2005. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434: 658–662.

Baines, C.P., Kaiser, R.A., Sheiko, T., Craigen, W.J, and Molkentin, J.D. 2007.Voltag e -dependent anion channels are dispensable for mitochondrial dependent cell death. *Nat Cell Biol* 9: 550–555.

Baines, C.P. 2011. The mitochondrial permeability transition pore and the cardiac necrotic program. *Pediatr Cardiol* 32: 258–262.

Bassir, O. 1963**.** Handbook of practical Biochemistry Bassir O. Ibadan University Press, Ibadan, Nigeria; 13.

Belayachi, L., Aceves-Luquero C., Merghoub N., Bakri Y., de Mattos S.F. and Amzazi S. 2013.Screening of north African medicinal plant extracts for cytotoxic activity against tumor cell lines. *Eur J Med Plant* 3: 310-332.

Belzacq, A.S., El Hamel, C., Vieira, H.L., Cohen, I., Haouzi, D., Metivier, D., Marche tti, P., Brenner, C.and Kroemer, G. 2001.Adenine nucleotide translocator media tes the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. *Oncogene* 20: 7579-7587.

Bernardi, P. 1992. Modulation of the mitochondrial cyclosporin A-sensitive permeability .*Biochim Biophys Acta* 1777: 592-560

Bernardi P. 2013.The mitochondrial permeability transition pore: a mystery solved? *Front Physiol* 4: 95-101

Bernardi, P., Rasola, A., Forte, M.and Lippe, G. 2015.The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. *Physiol Rev* 95: 1111–11155

Beutner, G., Eliseev, R.A. and Porter, G.A. Jr. 2014. Initiation of electron transport chain activity in the embryonic heart coincides with the activation of mitochondrial complex 1 and the formation of supercomplexes. *PLoS One* .9: e113330

Boekema, E. J. and Braun, H.P. 2007. Supramolecular structure of the mitochondrial oxidative phosphorylation system. *J Biol Chem* 282: 1–4

Bonora, M., Bononi, A., De Marchi, E., Giorgi, C., Lebiedzinska, M., Marchi, S., Patergnani, S., Rimessi, A., Suski, J.M., Wojtala, A., Wieckowski, M.R., Kroemer, G., Galluzzi, L., Pinton, P. 2013. Role of the c subunit of the Fo ATP synthase in mitochondrial permeability transition. *Cell Cycle* 12: 674–683

Bonora, M., Wieckowski, M.R., Chinopoulos, C., Kepp, O., Kroemer, G., Galluzzi, L . and Pinton, P. 2015. Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition. *Oncogene* 34: 1475–1486

Bortner, C.D., Oldenburg, N.B.and Cidlowski, J.A. 1995. The role of DNA fragmentati on in apoptosis. *Trends Cell Biol* 5: 21–26

Bouayed, J. and Bohn, T. 2010. Exogenous antioxidants–double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev*. 3:228–237

Boyer, P. D. 1997. The ATP synthase a splendid molecular machine. *Ann. Rev. Biochem* 66: 717-749

.**Bots, M and Medema, J.P.** 2006.Granzymes at a glance. *Cell Science at a Glance*. 119 (Pt24): 5011-5014

Bratton, S.B. and Cohen, G.M. 2001 Apoptotic death sensor: an organelle‘s alter ego? *Trends Pharmacol Sci* 22: 306–315

Bratton, D.L., Fadok, V.A., Richter, D.A., Kailey, J.M., Guthrie, L.A, and Henson, P .M. 1997. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem* 272: 26159–26165

Brustovetsky, N. and Klingenberg, M.1996. Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by $Ca^{2+.}$ *Biochemistry* 35: 8483–848

Bucheler K., Adams V. and Brdiczka D. 1991. Localization of the ATP/ADP translocator in the inner membrance and regulation of contact sites between mitochondrial envelope membranes by ADP. A study on freeze fracture isolated liver mitochondria. *Biochim. Biophys. Acta* 1053: 233–42

Burkhill, H. M. 1978. Tropical Africa, 2nd Ed Vol 1 families A-D. Royal Botanic Gardens, Kew, Richmond, UK

Cabezon, E., Montgomery, M. G., Leslie, A. G. W. and Walker, J. E. 2003. The structure of bovine F_1-ATPase in complex with its regulatory protein IF1. *Nat. Struct. Biol* 10: 744–750

Cande, C. N., Vahsen, Vahsen N., Kouranti,I.,Schmitt, E., Daugas E. *et al.* 2004. AIF and cyclophilin. A cooperate in apoptosis-associated chromatinolysis. *Oncogene* 23: 1514-1521

Cantley, L.C., 2002. The phosphoinositide 3-kinase pathway. *Science*., 296:1655–1657

Carafoli, E., 2012. The interplay of mitochondria with calcium: an historical appraisal. *Cell Calcium* **52**, 1–8.

Chernyak, B.V. and Bernardi, P. 2004.The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites. *European Journal of Biochemistry* 238: 623–630

Chinnaiyan A.M. 1999. The apoptosome: heart and soul of the cell death machine. *Neoplasia*. 1:5-15..

Chipuk, J.E. and Green, D.R. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* 18, 157-164

Chipuk, J.E., Moldoveanu, T., Llambi, F., Parsons, M.J., and Green, D.R. 2010. The BCL-2 family reunion. *Mol Cell* 37: 299–310

Chouhan A.K, Ivannikov M.V, Lu Z., Sugimori M., Llinas R.R. and Macleod G.T. 2012. Cytosolic calcium coordinates mitochondrial energy metabolism with presynaptic activity. *J. Neurosci* 32:1233–1243

Circu, M. L. and Aw, T. Y. 2010. Reactive oxygen species, cellular redox systems, and apoptosis, *Free Radical Biology and Medicine* 48: 749–762

Collinson, I. R., Skehel, J. M., Fearnley, I. M., Runswick, M. J.and Walker, J. E. 1996. The F_OF_1 ATPase complex from bovine heart mitochondria: the molar ratio of the subunits in the stalk region linking the F1 and FO domains. *Biochemistry* 35: 12640–12646

Coopoosamy A.and Naidoo A. 2012. An ethnobotanical study of medicinal plants used by traditional healers in Durban, South Africa, *Journal of pharmacy and pharmacology* 6: 818–823.

Costantini, P., Chernyak, B. V., Petronilli, V. and Bernardi, P. 1995. Selective inhibition of the mitochondrial permeability transition pore at the oxidation-reduction sensitive dithiol by monobromobimane. *FEBS Lett* 362; 239–240.

Costantini, P., Chernyak, B. V., Petronilli ,V. and Bernardi, P. . 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J. Biol. Chem* 271: 6746–6751

Cotran, R.S., Kumar, V., Collins, T., Cortan, R.S., Kumar, V. and Collins, T. 1999. Cellular pathology I: cell injury and cell death., *Robbins Pathologic Basis of Disease Philadelphia*, PAW.B. Saunders Co129.

Dalla Via, L., García-Argáez Aída, N., Martínez-Vázquez M., Grancara S., Martinis P. and Toninello A. 2014. Mitochondrial Permeability Transition as Target of Anticancer Drugs, *Current Pharmaceutical Design* 20: 223–244.

Daum, B., Walter, A., Horst A., Osiewacz, H.D.and Kuhlbrandt, W. 2013.Age dependent dissociation of ATP synthase dimers and loss of inner membrane cristae in mitochondria. *Proc Natl Acad Sci* USA,110: 15301 –15306.

Dean, E., Jodrell D., Connolly, K., Danson, S., Jolivet, J., Durkin, J., Morris, S. *et al.* 2009. Phase I trial of AEG35156 administered as a 7-day and 3-day continuous intravenous infusion in patients with advanced refractory cancer. *J Clin Oncol* 27: 1660–1666.

De Marchi E., Bonora M., Giorgi C. and Pinton P. 2014.The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux. *Cell Calcium* 56:1–13

De Stefani, D. and Rizzuto R. 2014. Molecular control of mitochondrial calcium uptake. *Biochem Biophys Res Commun* 449: 373–376

Don, A.S., Kisker, O., Dilda, P., Donoghue, N., Zhao, X., Decollogne, S., Creighton, B., Flynn, E., Folkman, J. and Hogg, P.J. 2003. A peptide trivalent arsenical inhibits tumor angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. *Cancer Cell* 3: 497–509

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102: 33–42

Duchen, M.R. 2004. Roles of mitochondria in health and disease. *Diabetes* 53: 96–102

Duker-Eshun, G., Jaroszewski, J.W., Asomaning, W.A., Oppong-Boachie, F. and Chistensen, S.B. 2004. Antiplasmodial constituents of *Cajanus cajan*. *Phytotherapy Research* 18: 128–130

Edeoga, H.O., Okwu, D.E. and Mbaebie, B.O. 2005. Phytochemical constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol* 4: 685–688

Efferth T., Li P.C., Konkimalla, V.S.and Kaina, B. 2007. From traditional Chinese me dicine to rational cancer therapy. *Trends Mol Med* 13:353–361

Elena-Real C.A., Díaz-Quintana A., González-Arzola K., Velázquez-Campoy A., Orzáez M., *et al.,* 2018. Cytochrome c speeds up caspase cascade activation by blocking 14-3-3ε-dependent Apaf-1 inhibition. *Cell Death Dis* 6: 365–375

Elliott, P.J. 2000.Proteaome inhibition: a new strategy in cancer treatment. *Invest New Drugs* 18: 109–121

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. A. 1998. Caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391:43–50.

Ekeke, G.I. and Shode, F.O. 1990. Phenylalanine is the predominant antisickling agent in *Cajanus cajan* Seed Extract, *Planta Medica* 56: 41–45

Ezike, A.C., Akah, P.A., Okoli, C.C. and Okpala C.B. 2010. Experimental evidence for the antidiabetic activity of *Cajanus cajan* leaves in rats. *J Basic and Clinical Pharm*1:25–30

Farombi, E.O. 2011. Dried fruit extract from *Xylopia ethiopica* (Annonaceae) protects Wistar albino rats from adverse effects of whole body radiation. *Exp Toxicol Pathol* 63: 635–43

Festjens, N., Vanden Berghe, T. and Vandenabeele P. 2006 Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta* 1757: 1371–1387.

Fischer, U., Janicke, R. and Schulze-Osthoff, K. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death & Differentiation* 10: 76–100

Fischer, F., Andrea H., and Heinz D. and Osiewacz K. 2012. Mitochondrial quality control: An integrated network of pathways. *Trends Biochem Sci*. 37: 284–292

Fontaine, E., Eriksson, O., Ichas, F. and Bernardi, P. 1998. Regulation of the permeability transition pore in skeletal muscle mitochondria. *J. Biol. Chem* 273 12662-12668.

Frey, T. G. And Mannella C.A. 2000**.** The internal structure of mitochondria. *Trends Biochem Sci* 25: 319-324.

Gerle C. 2016. On the structural possibility of pore forming mitochondrial F_OF_1 ATP synthase. *Biochim Biophys Acta* 1857: 1191–1196

Giorgio, V., Bisetto, E., Soriano, M.E., Dabbeni-Sala, F., Basso, E., Petronilli, V., Forte, A. M., Bernardi, P. and Lippe, V. 2009. Cyclophilin D modulates mitochondrial FoF_1-ATP synthase by interacting with the lateral stalk of the complex. *J Biol Chem* 283, 33982±8

Giorgio, V., von Stockum S., Antoniel, M., Fabbro, A., Fogolari, F. and Forte, M. 2013. Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc Natl Acad Sci* 110: 5887–5892

GlÜCksmann A.1951.Cell deaths in normal vertebrate ontogeny., *Biol Rev*. 26:59–86

Green, D. W. and Grover, G. J. 2000. The IF(1) inhibitor protein of the mitochondrial F(1)F(0)-ATPase. *Biochim Biophys Acta* 1458: 343-355.

Gray, M.W. 2012 Mitochondrial evolution, Cold Spring Harbor Perspect. Biol. 4.

Griffiths, E.J. and Halestrap, A.P. 1993. Protection by Cyclosporin A of ischemia/reper fusion-induced damage in isolated rat hearts. *J Mol Cell Cardiol*, 25:1461–1469

Griffiths, **E. J. and Halestrap, A.P.** 1995. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion, *Biochem J* 307 (Pt 1) 93–98.

Grover, J. K., Yadav, S. and Vats, V.J. 2002. *Ethnopharmacol* 81: 81–100

Halestrap, A. P. and Davidson A.M. 1990. Inhibition of Ca^{2+-}induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem J* 268: 153–160.

Halestrap, A. P. 1999. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem Soc Symp 66: 181–203.

Halestrap, A. P. 2009.What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol* 96 : 821–831.

Halestrap, A.P. 2010. A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. *Biochem Soc Trans* 38:841–846

Halliwell, B. and Gutteridge, J.M.C. 1998. The Antioxidants of Human Extracellular Fluids. *Arch Biochem Biophys* 280:1–8

Hanahan, D. and Weinberg, R.A. 2000.The Hallmarks of cancer. *Cell* 100: 57–70

Haque, M. U., Ferdiousi, N. and Sajon, S. R. 2016. Anti-cancer agents derived from plant and dietary sources: a review. *International Journal of Pharmacognosy* 32: 55–66.

Harborne, J.B. 1988. Phytochemical methods., 3rd Edition, Chapman and Hill.

Hardwick, J.M. and Soane, L. 2013. Multiple functions of Bcl-2 family proteins. *Cold Spring Harb Perspect Biol* 10: 4–10.

Hengartner, M.O. 2000. The biochemistry of apoptosis. *Nature* 407: 770–776

Hidalgo, M. and Rowinsky, E.K. 2000.The rapamycin sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* 19: 6680–6686.

Hom, J.R., Quintanilla, R.A., Hoffman, D.L., de Mesy Bentley, K.L., Molkentin, J.D., Sheu S.S. and Porter G.A. Jr. 2011.The permeability transition pore controls cardiac mitochondrial maturation and myocyte differentiation. *Dev Cell* 21: 469–478

Houstek J, Mracek T, Vojtiskova A. and Zeman J. 2004 . Mitochondrial diseases and ATPase defects of nuclear origin. *Biochim Biophys Acta* 1658: 115–121.

Hsu, H., Xiong, J. and Goeddel, D.V. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 81: 495–504.

Hunter, D. R. and Haworth, R. A. 1979. The Ca^{2+}-induced membrane transition in mitochondria. II Nature of the Ca^{2+} trigger site, *Arch Biochem Biophys* 7: 460-467

Hunter, D.R. and Haworth, R.A., Southard, J.H. 1976. Relationship between configur ation, function, and permeability in calcium-treated mitochondria. *J Biol Chem* 251: 5069–5077

Huser, J. and Blatter, L.A.,1999. Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem J* 343:311–317

Iqbal, S., U. Younas, Chan, K.W., Zia-Ul- Haq, M. and Ismail, M. 2012. Chemical Composition of *Artemisia annua* L. Leaves and Antioxidant Potential of Extracts as a Function of Extraction Solvents. *Molecules* 17: 6020–6032

Irvine, R. A., Adachi N., Shibata D.K., Cassell G.D., Yu K., Karanjawala Z.E., Hsieh C.L.and Lieber M.R. 2005. Generation and characterization of endonuclease G null mice. *Mol. and Cell. Biol* 25: 294–302.

Iwu, M, Handbook of African Medicinal plants, CRC press.

Jana, N. R., Dikshit, P., Goswami, A. and Nukina, N. 2004. Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway. *J .Biol Chem*, 279: 11680–11685

Jedy-Agba, E., Curado, M.P., Ogunbiyi, O., Oga, E., Fabowalem T., Igbinoba, F., Osubor, G., Otu T., Kumai H., Koechlin, A., Osinubi, P., Dakum, P., Blattner, W., and Adebamowo G. 2012. CA.Cancer incidence in Nigeria: a report from population-based cancer registries.*Cancer Epidemiol.*, 5: 271–278

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. and Forman, D. 2011.Global cancer statistics. *CA Cancer J Clin*; 61: 69–90

Johnson D and Lardy H. 1967. Isolation of liver or kidney mitochondria. *Methods Enzymol* 10: 94-96.

Jonckheere, A.I., Smeitink, J.A.M.and Rodenburg, R.J.T. 2012. Mitochondrial ATP s ynthase: architecture, function and pathology, *J. Inherit. Metab. Dis*. 25: 35211–35225

Julian, L. and Olson, M. 2015. Apoptotic membrane dynamics in health and disease, *Cell Health and Cytoskeleton* 7:133–142

Kagan, V. E., Tyurin, V.A., Jiang, J., Tyurina, Y.Y. and Ritov, V.B. 2005. Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat. Chem. Biol* 1:223-232

Kalkavan H. and Green D.R. 2018. MOMP,cell suicide as a BCL-2 family business *Cell Death and Differentiation* ,25: 46–55

Karch, J. and Molkentin, J. D. 2014. Identifying the components of the elusive mitocho ndrial permeability transition pore, *PNAS* 111: 10396-10397

Karuppusamy S. 2009. A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *J Med Plants Res* 3:1222-1239

Kelly, K.J., Plotkin, Z. and Dagher, P.C. 2001.Guanosine supplementation reduces apoptosis and protects renal function in the setting of ischemic injury. *J Clin Invest* 108: 12911298

Kenkel, J. 2003. Analytical Chemistry for Technicians. CRC Press, 3rd edn., 205-237

Kermer, P., Liman J., Weishaupt, J.H. and Bähr, M. 2004.Neuronal apoptosis in neurodegenerative diseases: from basic research to clinical application.*Neuro-degenerative diseases* 1: 9-19

Kerr, J. F., Wyllie, A. H. and Currie, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26: 239-257

Kokoszka, J.E., Waymire, K.G., Levy, S.E., Sligh, J.E., Cai, J., Jones, D.P., MacGregor, G.R. and Wallace, D.C., 2001. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* X; 427: 461-465.

Kowaltowski, A. J., Vercesi, A. E. and Castilho, R. F. 1997. Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca^{2+} : correlation with mitochondrial permeability transition. *Biochim. Biophys. Acta* 1318: 395- 402.

Kroemer, G., Galluzzi, L. and Brenner, C., 2007. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 87:99–163

Kuhnel, F., Zender, L. and Paul, Y. 2000. NF kappa B mediates apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis. *J. Biol Chem* 275: 6421–6427.

Kundu, R., Dasgupta, S., Biswas, A., Bhattacharya, A., Pal, B.C., Bandyopadhyay, D .,Bhattacharya, S. and Bhattacharya, S. 2008. *Cajanus cajan* Linn. (Leguminosae) prevents alcohol-induced rats liver damage and augument cytoprotective function. *J. Ethnopharmacol* 118: 440–447.

Kuranaga, E., Matsunuma,T., Kanuka, H., Takemoto, K., Koto, A., Kimura, K.and Miura, M. 2011. Apoptosis controls the speed of looping morphogenesis in *Drosophila* male terminalia *Development* 138: 1493–1499

Kurosaka, K, Takahashi, M, Watanabe, Nand Kobayashi, Y. 2003. Silent cleanup of very early apoptotic cells by macrophages. *J Immunol* 171: 4672–4679

Kühlbrandt, W. 2015. Structure and function of mitochondrial membrane protein complexes. *BMC Biol*; 13: 1–11

Kruczynski, A., Barret, J.M., Etie ́ vant C., Colpaert F., Fahy, J. and Hill, B.T. 1998. Antimitotic and tubulin-interacting properties of vinflunine, a novel Fluorinated Vinca alkaloid. *Biochem Pharmacol.* 55: 635–648

Lapidus, R.G.and Sokolove P.M. 1993. Spermine Inhibition of the permeability transition of isolated rat liver mitochondria: An investigation of mechanisms. *Biochem. Biophys. J* 64: 246–253

Lardy, H.A. and Wellman, H. 1952. Oxidative phosphorylation: role of inorganic phosphate and acceptor system in control of metabolic rates. *J.Biol. Chem* 195: 215–221

Lawen, A. 2003.Apoptosis-an introduction., *BioEssays: news and reviews in molecular, cellular and developmental biology* 25: 888–896.

Lehninger, A.L., Vercesi, A.E., and Bababunmi, E.A. 1978. Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides .*Proc. Natl. Acad. Sci. USA* 75: 1690–1694.

Lemasters, J. J., Theruvath T. P., Zhong, Z. and Nieminen A. L. 2009. Mitochondrial calcium and permeability transition in cell death. *Biochim Biophys Acta* 1787: 1395–1401.

LeQuoc, K. and LeQuoc, D.1988. Involvement of ADP/ATP carrier in calcium-induced perturbations of the mitochondrial inner membrane permeability: importance of the orientation of the nucleotide binding site, *Arch. Biochem. Biophys* 265: 249-257.

Leung A.W. and Halestrap A.P. 2008. Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. *Biochim Biophys Acta* 1777: 946–952.

Leung, A.W.C., Varanyuwatana, P., and Halestrap, A.P. 2008. The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. *J Biol Chem* 283: 26312–26323

Leyssens, A., Nowicky, A.V., Patterson L.,Crompton M., and Duchen M.R. 1996. The relationship
between mitochondrial state, ATP hydrolysis, $Mg^{2+}$and Ca^{2+} studied in isolated rat cardiomyocytes. *Journal of Physiology* 496: 111–128

Li, K.and Li Y.2000. Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 101: 389–399

Liston, P., Fong, W.G., Korneluk, and R.G. 2003. The inhibitors of apoptosis: there is more to life than Bcl-2. *Oncogene* 22: 8568–8580.

Lodish, H. F. 2013. Molecular Cell Biology., 7th ed., W.H. Freeman and Co., NewYork.

Los, M., Wesselborg, S. and Schulze-Osthoff, K. 1999. Cell death and differentiation *Immunity* 10 : 629–634

Lovell, J.F., Billen, L.P., Bindner, S., Shamas-Din, A., Fradin, C., Leber, B., and Andrews, D.W. 2008. Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell* 135: 1074–1084.

Lowry, O.H., Rosebrough, N.J., Farr A.L. and Randall R.J. 1951.Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193: 262−275.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 944 : 481–90.

Luo M., Liu X., Zu Y., Fu Y., Zhang S., Yao L., Efferth T. 2010. Cajanol, a novel anticancer agent from Pigeonpea *Cajanus cajan* (L.) Millsp. roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway. *Chemico-Biological Interactions*, doi:10.1016/j.cbi.2010.07.009.

Maldonado, V., Melendez-Zajgla J., and Ortega A., Modulation of NF-kappa B, and Bcl-2 in apoptosis induced by cisplatin in HeLa cells. *Mut Res.* 1997.381:67–75.

Martin, K.R., 2006**.** Targeting apoptosis with dietary bioactive agents. *Exp Biol Med.* 231: 117-129

Masgras, I., Rasola, A. and Bernardi, P., 2012. Induction of the permeability transition pore in cells depleted of mitochondrial DNA. *Biochim Biophys Acta*.;1817: 1860 – 1866.

Mazzeo, A.T., Beat, A., Singh, A., and Bullock, M.R., 2009.The role of mitochondrial transition pore, and its modulation, in traumatic brain injury and delayed neurodegeneration after TBI. *Exp Neurol*, 218:363 -370

Mitchell, P.,1979**.** Keilin's respiratory chain concept and its chemiosmotic consequences. *Science.* 206: 1148-1159

Myung J., Kim K.B., and Crews C.M. 2001, The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev* 21:245-273.

Nakagawa, Y.and Moore, G. 1999**,** Role of mitochondrial membrane permeability transition
in phydroxybenzoate ester induced cytotoxicity in rat hepatocytes. *Biochem Pharm acol* 58: 811-816.

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., Inohara, H., Kubo, T., and Tsujimoto, Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005:*434*, 652–658.

Nechushtan, A., Smith, C.L., Lamensdorf, I., Yoon, S.H. and Youle, R.J., 2001. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* 153, 1265-1276.

Nemes, Z., Jr, Friis, R.R., Aeschlimann, D., Saurer, S., Paulsson, M. and Fesus, L. 1996. Expression and activation of tissue transglutaminase in apoptotic cells of involuting rodent mammary tissue. *Eur J Cell Biol*. 70:125-133

Nicholls, D.G.1978. Calcium transport and porton electrochemical potential gradient in mitochondria from guinea-pig cerebral cortex and rat heart. *Biochem J.* 170: 511-522

Nicholls, D.G. 2009, Mitochondrial calcium function and dysfunction in the central nervous system. *Biochim Biophys Acta* 1787: 1416-1420

Nicholson, R.A., David L.S., Pan R.L. and Xin M. L. 2010. Pinostrobin from *Cajanus cajan* (L.) Millsp. inhibits sodium channel-activated depolarization of mouse brain synaptoneurosomes. *Fitoterapia* 81: 826-829

Nicolli, A., Petronilli, V. and Bernardi, P. 1993. Modulation of the mitochondrial cyclosporine A-sensitive permeability transition pore by matrix pH. Evidence that the pore open closed probability is regulated by reversible histidine protonation. *Biochemistry* 32: 4461-4465

Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K.1997. Direct observation of the rotation of F_1-ATPase opened by membrane depolarization. *J Biol Chem.*, 267, 8834-8839.

Nunnari, J. and Suomalainen A. 2012. Mitochondria: In sickness and in health, *Cell* 148: 1145-1159.

Obumselu, F. O., Okerulu, I. O., Onwukeme, V. I., Onuegbu T. U. and Eze, R. C. 2013. Phytochemical and antibacterial analysis of the leaf extracts of *Ricinus communis. Journal of Basic Physical Research,* 2: 68–72.

Olanlokun, O.J., Oyebode, T.O.and Olorunsogo, O.O. 2017. Effects of Vacuum Liqui d Chromatography (Chloroform) Fraction of the Stem Bark of *Alstonia boonei* on Mitochondrial Membrane Permeability Transition Pore. *J Basic Clin Pharma*.,8: 2212–25

Oliver, C.L., Miranda, M.B., Shangary, S., Land, S., Wang, S.and Johnson, D.E. 2005. Gossypol acts directly on the mitochondria to overcome Bcl-2 and Bcl-xL-mediated apoptosis resistance. *Mol Cancer Ther* 4: 23–31.

Olorunsogo, O.O., Bababunmi E.A., and Bassir, O.1979. Uncoupling effect of Nphosphonomethylglycine on at liver mitochondria. *Biochem. Pharm.*. ; 27: 925–927.

Orrenius, S., Zhivotovsky, B. and Nicotera P. 2003. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 4: 552–565.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsk.y, B., and Orrenius, S. 2002 Cytochrome *c* release from mitochondria proceeds by a two-step process. *PNAS*. 99: 1259–1263

Oyewole, O.I., Malomo, S.O. and Adebayo, J.O. 2008. Comparative studies on antisickling properties of thiocyanate, tellurite and hydroxyurea. *Pak. J. Med. Sci*, 24:18–22

Pal, D., Mishra, P., Sachan, N. and Ghosh, A. K. 2011. Biological activities and medicinal properties of *Cajanus cajan* (L) Millsp. *J Adv Pharm Tech Res* 2 : 207–213

Park, S. S., Kim, Y. N., Jeon, Y. K., Kim, Y. A., Kim, J. E., Kim, H. and Kim, C.W .2005. Genistein-induced apoptosis via Akt signaling pathway in anaplastic large-cell lymphoma, *Cancer Chemother. Pharmacol* 56: 271–278.

Pederson, L. and Amzel, L.M. 1993**.** ATP synthase: structure, reaction center, mechanism and regulation of one of nature's most unique machines. *J. Biol. Chem* 268: 9937–9940.

Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P., D.I. and Lisa, F. 1999. Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J* 76: 725–734.

Poe, K., 2017. Plant-Based Diets and Phytonutrients: Potential Health Benefits and Disease Prevention, *Arch Med*. 9:6-7.

Porter, G.A. Jr, Hom, J., Hoffman, D., Quintanilla, R., de Mesy, Bentley, K. and Sheu S.S., 2011.Bioenergetics, mitochondria, and cardiac myocyte differentiation. *Prog Pediatr Cardiol*. 31:75–81

Poncet, D., Pauleau, A-L, Szabadkai, G., Vozza, A.and Scholz, S.R. 2006. Cytopathic effects of the cytomegalovirus-encoded apoptosis inhibitory protein vMIA. *J Cell Biol* 174: 985–996.

Prasai, K. 2017. Regulation of mitochondrial structure and function by protein import: A current review**.** *Pathophysiology* 24: 107–122.

Quadrilatero, J. and Bloemberg, D. 2010. Apoptosis repressor with caspase recruitment domain is dramatically reduced in cardiac, skeletal, and vascular smooth muscle during hypertension. *Biochem. Biophys. Res. Commun.* 391: 1437–1442.

Rao, V.K., Carlson, E. A. and Yan, S. S. 2014. Mitochondrial permeability transition pore is a potential drug target for neurodegeneration. *Biochim Biophys Acta* 1842: 1267–1272

Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R. and Shi, Y. 2005. Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* 434: 926–933.

Reitman, S. and Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Amer. J. Clin. Path.* 28 : 56.

Rej, R. 1989.Aminotransferases in disease. *Clin Lab Med* 9: 667–687.

Rekha, D., Tamil Selvi, S., Bharathidasan, R., Panneerselvam, A., Ilakkiya, R. and Jayapal, R. 2013. Study of Medicinal Plants used from Koothanallur and Marakkadai, Thiruvarur district of Tamil nadu, India. *Hygeia J D Med* 5: 164 – 170.

Renault T.T. & Chipuk J.E. 2014. Death upon a kiss: mitochondrial outer membrane composition and organelle communication govern sensitivity to BAK/BAX-dependent apoptosis. Chemistry & Biology 21; S1074-5521: 383-389

Rizzuto, R. and Pozzan T. 2006. Microdomains of intracellular Ca^{2+}: molecular determinants and functional consequences. *Physiol Rev* 86: 369–408.

Roberts, A.W., Seymour, J.F., Brown, J.R., Wierda, W.G., Kipps, T.J., Khaw, S.L., Carney *et al*., 2012. Substantial susceptibility of chronic lymphocytic leukemia to Bcl-2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol* 30: 488–496.

Rode H. 2008 Apoptosis, Cytotoxicity and cell proliferation manual.Roche Diagnostics

Rostovseva T. 2017 Molecular basis for mitochondrial signaling, Biological and medical physics, Biomedical engineering Springer: 69

Ruberto, G., Baratta, M., Deans, S. and Dorman, H. 2000. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Med* 66:687–693.

Saikumar, P. Dong, Z. Mikhailov V., Denton M, Weinberg J.M., and Venkatachalam M.A. 1999. Apoptosis: definition, mechanisms, and relevance to disease. *The American journal of medicine* 107:489–506.

Salako, T.A., Adisa, R.A., Alao, O.O., Adeniran, O.O., Atanu, F.O. and Olorunsogo, O. O. 2010. Effects of methanolic and chloroform extracts of leaves of *Alstonia boonei* on rat liver mitochondrial membrane permeability transition pore. *Afr J Med Med Sci*. 39 (Suppl 1): 109–116.

Saks, V., Kuznetsov, A.V., Gonzalez-Granillo, M., Tepp, K., Timohhinam, N., Karu-Varikmaa M., Kaambre, T., D.O.S. Santos, P., Boucher, F.and Guzun, R. 2012. Intracellular energetic units regulate metabolism in cardiac cells. *J Mol Cell Cardiol* 52: 419–436

Saunders J.W. 1966. Death in embryonic systems. *Science*.154: 604–612

Savithramma, N., Linga Rao, M. and Suhrulatha, D. 2011.Screening of medicinal plants for secondary metabolites. *Middle-East J. Sci. Res* 8: 579–584.

Scheffler, I.E. 2008. Mitochondria, second ed., Wiley-Liss, Alberts, Molecular Biology of the Cell, 5th ed., Garland Science, New York.

Schon, E.A., DiMauro, S. and Hiran,o M., 2012. Human mitochondrial DNA: roles of inherited and somatic mutations, *Nat. Rev. Genet*., 13: 878–890.

Siegel, R., Naishadham, D., and Jemal, A. 2012. Cancer statistics, *CA Cancer J. Clin* . 62,10–29.

Slee, E. A., Zhu, H., Chow, S. C., Mac Farlane, M., Nicholson, D.W. and Cohen, G.M., 1996. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP 32. *Biochem. J.* 319: 21–24.

Sullivan, P.G., Rabchevsky, A.G., Waldmeier, P.C., and Springer J.E. 2005.Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death? *J Neurosci Res* 79:231–239.

Suzuki, M., Youle, R.J., and Tjandra, N. 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 103: 645–654

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi R., A. 2001.Serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell.* 8: 613–621.

Symersky, J., Osowski, D., Walters, D. E. and Mueller, D. M. 2012. Oligomycin frames a common drug binding site in the ATP synthase. *Proc. Natl. Acad. Sci*, USA, 109: 13961–13965

Szabo, I. and Zoratti, M. 1993. The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore. *FEBS Lett* 330: 201–205

Tatsuta, T., Scharwey, M. and Langer, T. 2014. Mitochondrial lipid trafficking, *Trends Cell Biol* 24: 44–52

Taylor, R.C., Cullen, S.P., and Martin, S.J. 2008.Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 9:231–241.

Thompson, C. B.1995. Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456–1462.

Trease, G.E. and Evans, **W.C.** 1989. A Textbook of Pharmacognosy. 13th Edn., Bailliere Tindall Ltd., London.

Tse, C., Shoemaker, A.R., Adickes, J., Anderson, M.G., Chen, J., Jin, S., Johnson, E.F.*et al*. 2008. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res.* 68, 3421-3428.

van der Laan, M., Horvath, S.E. and Pfanner, N. 2016. Mitochondrial contact site and cristae organizing system. *Curr. Opin. Cell Biol* 41: 33-42.

van Delft, M.F., Wei, A.H., Mason, K.D., Vandenberg, C.J., Chen, L., Czabotar, P.E *et al*. 2006.The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and

efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 10: 389-399.

Varanyuwatana, P. and Halestrap, A. P., 2012 The roles of phosphate and the phosphate carrier in the mitochondrial permeability transition pore. *Mitochondrion* 12: 120-125.

Verhagen, A.M., Ekert, P.G., Pakusch, M. and Silke J., 2012. Identification of DIABLO, a Mammalian Protein that Promotes Apoptosis by Binding to and Antagonizing IAP Proteins 102: 45-53.

Visht, S. and Chaturvedi, S. 2000., Isolation of Natural Products. *Curr. Pharm. Res.* 2: 584 -599.

Walther, D.M. and Rapaport, D. 2009., Biogenesis of mitochondrial outer membrane proteins, *Biochim. Biophys. Acta (BBA) – Mol. Cell Res*.1793: 42–51.

Walker, J. E. and Dickson, V. K. 2006. The peripheral stalk of the mitochondrial ATP synthase. *Biochim. Biophys. Acta*, 1757:286–96

Walker, J.E. 2013. The ATP synthase: The understood, the uncertain and the unknown. *Biochem Soc Trans*, 41:1–16.

Wang W., Fang H., Groom L., Cheng A., Zhang W., Liu J., Wang X., Li K., Han P., *et al.* 2011. Navitoclax, a targeted high-affinity inhibitor of Bcl-2, in lymphoid malignancies: a phase 1 dosees calation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. *Lancet Oncol.* 1149-59.

Wang X., Jian C., Zhang X., Huang Z., Xu J., Hou T., Shang W., Ding Y., Zhang W., Ouyang, M.,Wang Y, Yang Z., Zheng M. and Cheng H. 2012**.** Superoxide

flashes: elemental events of mitochondrial ROS signaling in the heart. *J Mol Cell Cardiol*.52: 940–948.

Watt, I.N., Montgomery M.G., Runswick M.J., Leslie A.G. and Walker J.E. 2010. Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. *Proc Natl Acad Sci U S A*107:16823–16827.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. 2001. Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. *Science* 292, 727–730.

Williams, D.H. and Fleming, I.,1989. Spectroscopic methods in Organic Chemistry, Berkshire-England: McGraw-Hill.

Westphal, D., Kluck, R.M., and Dewson, G. 2014. Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. *Cell Death Differ* 21, 196–205.

Wittig, I. and Schagger, H. 2008. Structural organization of mitochondrial ATP synthase. *Biochim. Biophys. Acta* 1777: 592–598

Woodfield K., Rück A., Brdiczka D. and Halestrap A.P. 1998. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. *Biochem J* 336: 287–290.

Wright, T.M. and Vandenberg, A.M., 2007. Risperidone- and quetiapine-induced cholestasis. *Ann. Pharmacother* 41:1518–1523

Wu, N., Fu, K., Fu, Y.J., Chang,, F.R., Chen, Y.H., Liu, X.L., Kong, Y., Liu, W. and Gu, C.B., 2009.Antioxidant activities of extracts and main components of Pigeon pea [*Cajanus cajan (L.)* Millsp.] leaves. *Molecules* 14: 1032–1043.

Wu, W., Liu, P. and Li, J., 2012. Necroptosis: An emerging form of programmed cell death. *Crit. Rev. Oncol.* 82: 249–258.

Wyllie, A. H., 1980**.,** Glucocorticoid-induced thymocyte apoptosis is associated with endogenous nuclease activity. *Nature*. 284: 555–556

Wyllie A.H., Kerr J.F.R. and Currie A.R., 1980. Cell death the significance of apoptosis. *Int. Rev. Cytol.* 68: 251–306

Xiong J., Verkhratsky A., and Toescu E.C. 2002.Changes in mitochondrial status associated with altered calcium homeostasis in aged cerebellar granule neurons in brain slices. *J Neurosci.22*: 10761–0771

Xu D.C., Arthurton L., and Baena-Lopez L.A. 2018.Learning on the Fly: The Interplay between Caspases and Cancer, BioMed Research International, Article ID 5473180, 18 pages,

Yu J., Wang Z., Kinzler K.W. 2003. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. *Proc Natl Acad Sci* USA.;100: 1931–1936

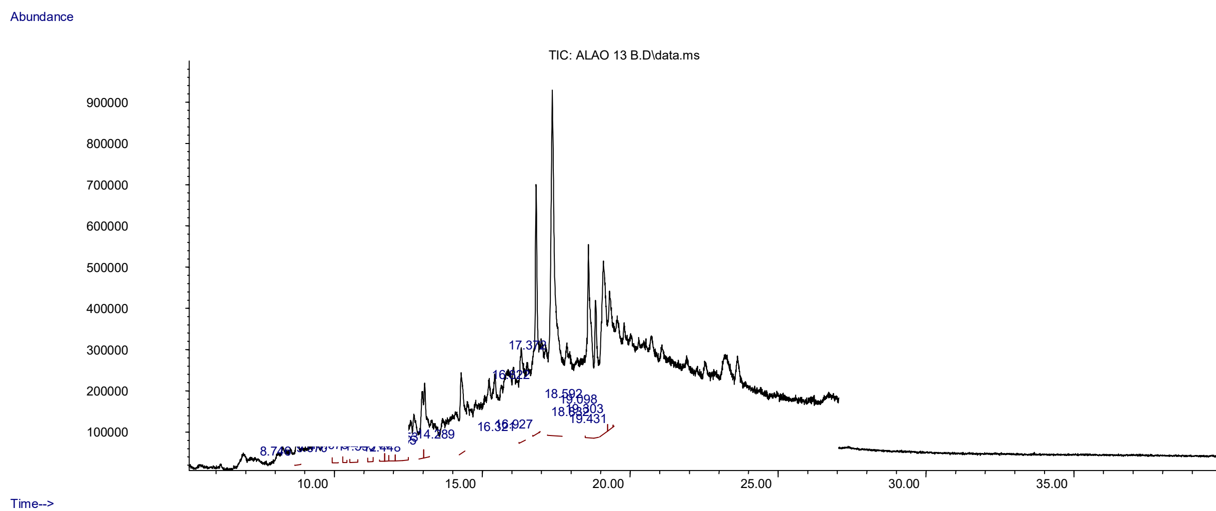
Yuan-gang Z., Xiao-lei, Y. F., Nan Wu, Y. and Michael W. 2010. Chemical composition of the SFE-CO2 extracts from *Cajanus cajan* (L.) Huth and their antimicrobial activity *in vitro* and *in vivo*. *Phytomed* 17:1095–1101

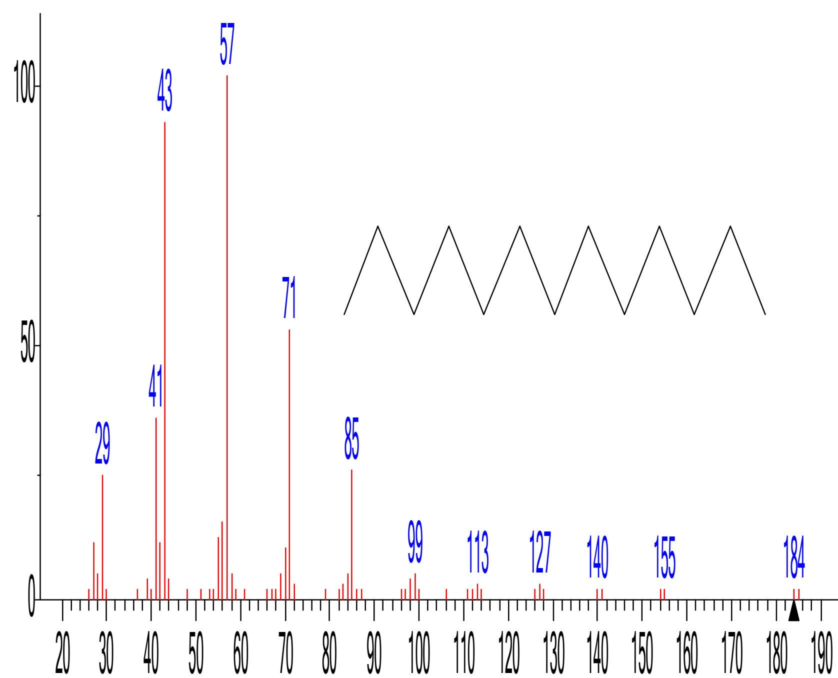
Zhang Y. J, Gan R.Y, Li S., Zhou Y., Li A.N., Xu D.P., and Li H.B. 2015. Antioxidant Phytochemicals for the Prevention and Treatment of Chronic Diseases. Molecules. 20: 21138-21156.

Zoratti, M., Marchi, U.D., Gulbins, E., and Szabo, I. 2009. Novel channels of the inner ochondrial membrane. *Biochim Biophys Acta*. 1787: 351-363.

Zou H., Li Y., X Liu, and Wang X .1999. *J. Biol. Chem.* 274: 11549-11556

APPENDIX

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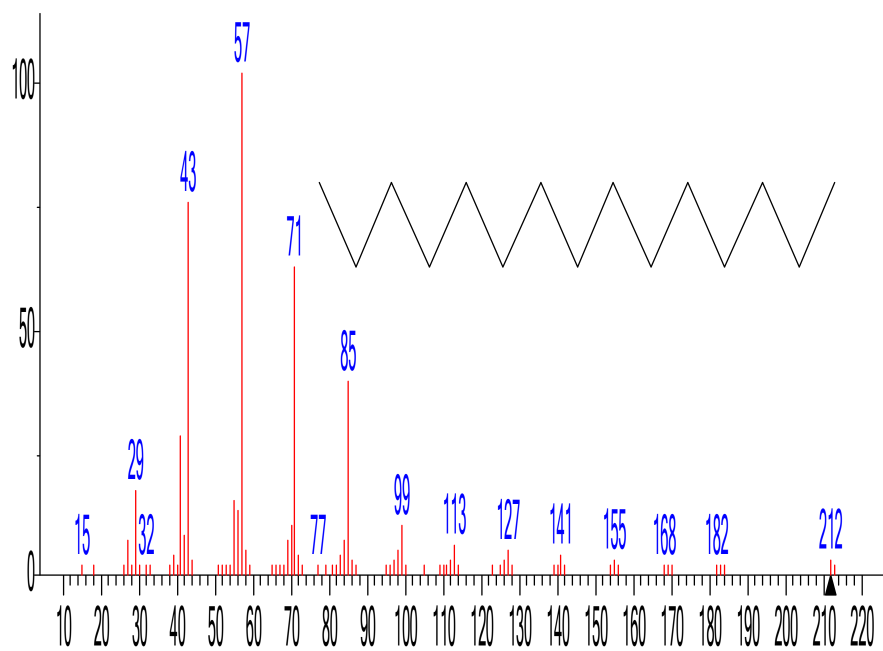
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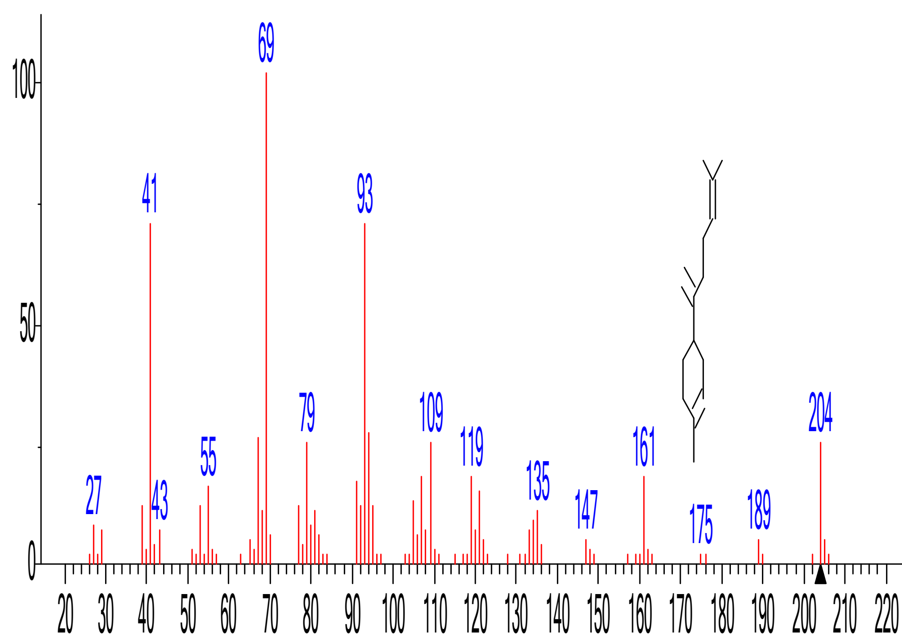
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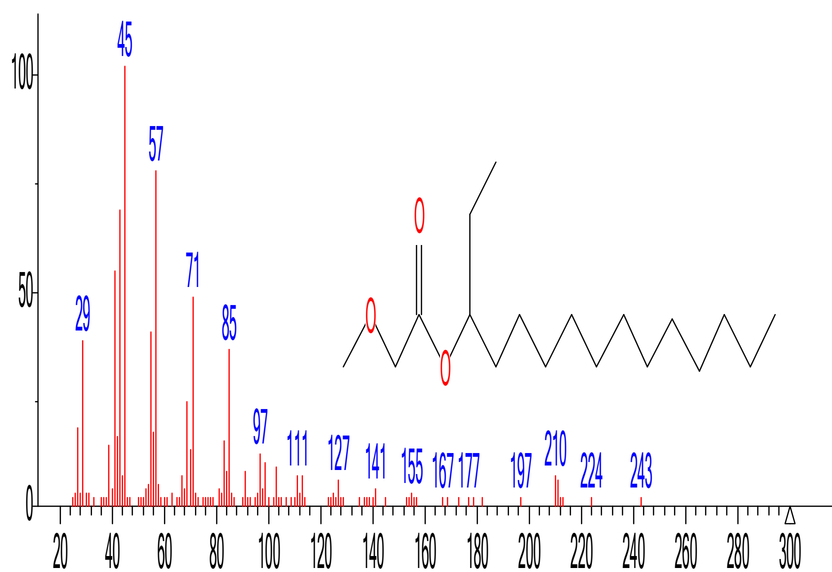

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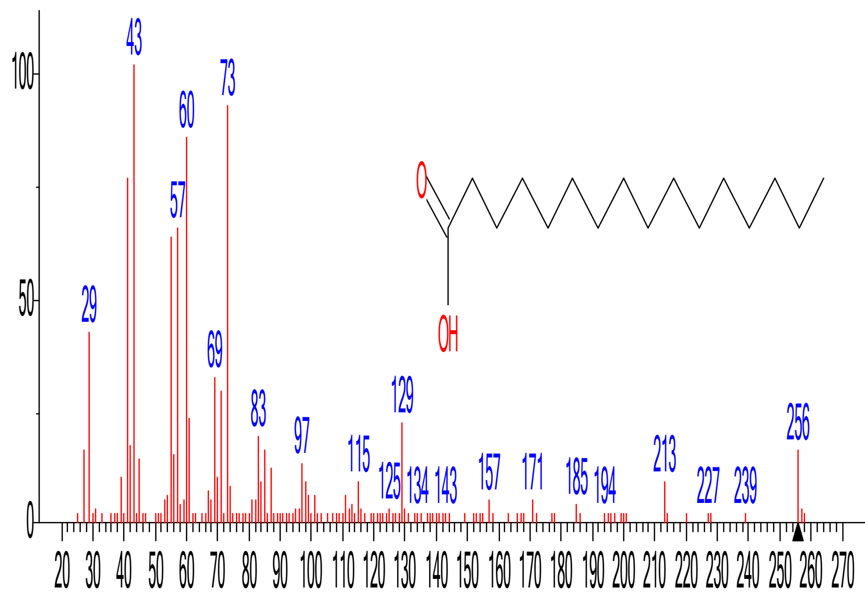

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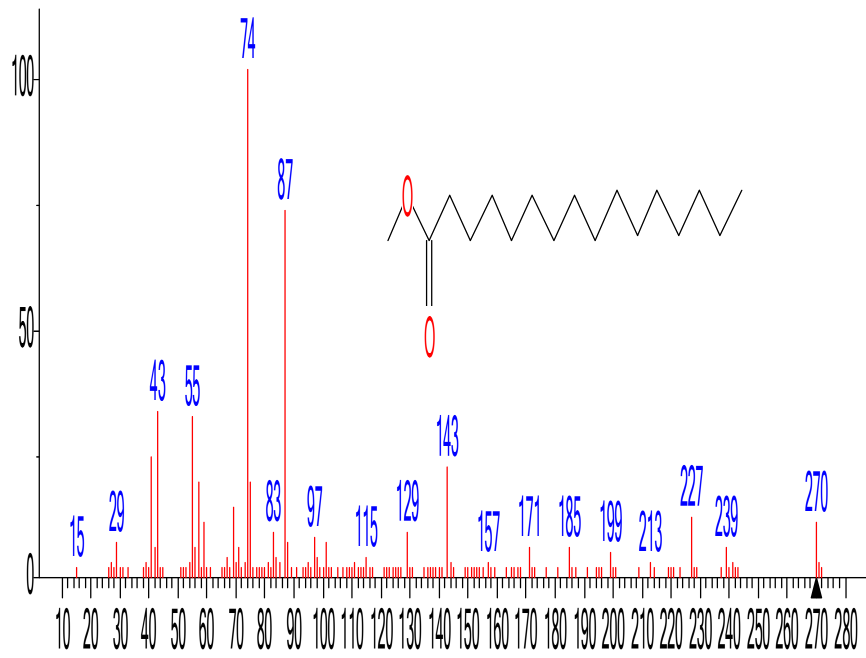

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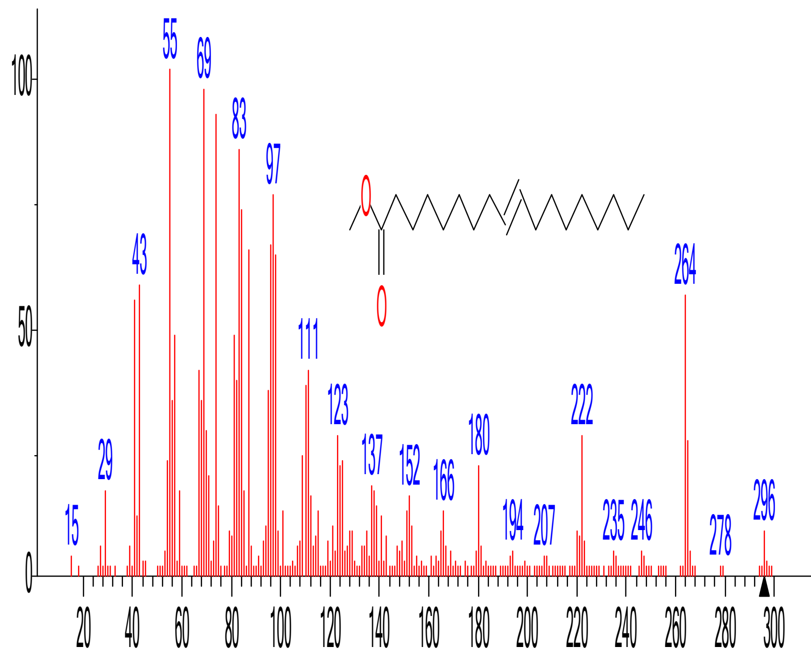

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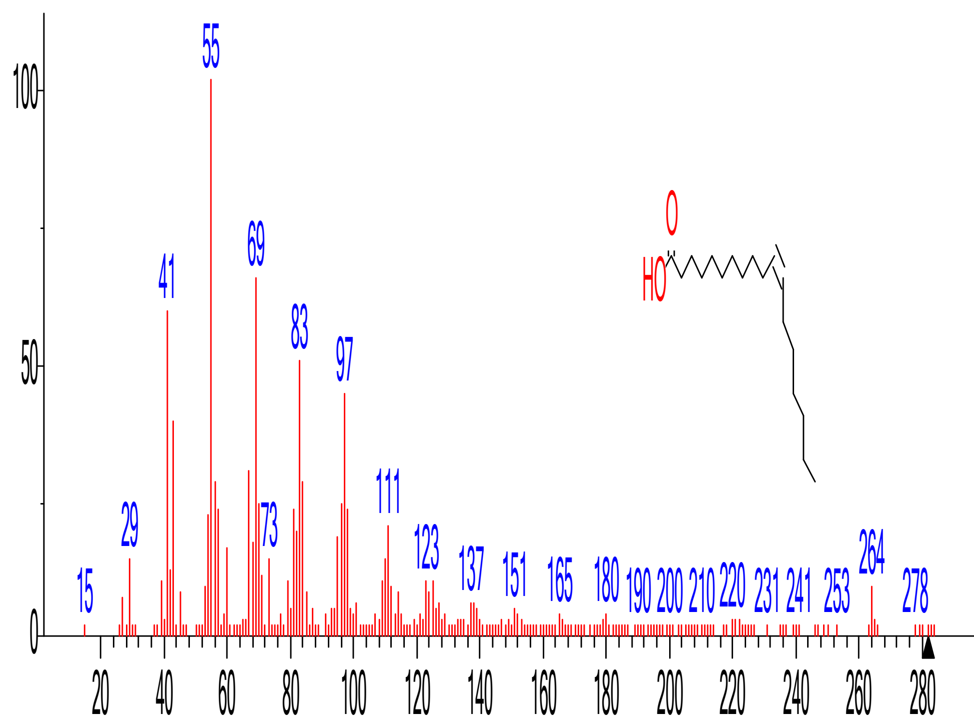

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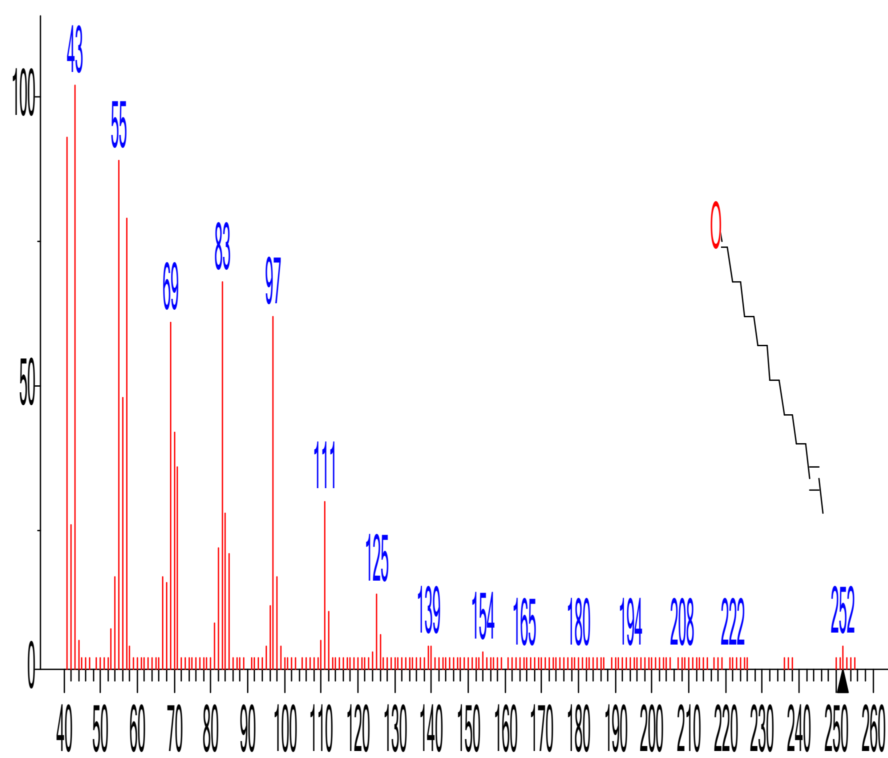

Fig 31 GCMS of ethylacetate subfraction of *Cajanus cajan* leaves