MODULATION OF MITOCHONDRIAL – MEDIATED APOPTOSIS BY SOLVENT FRACTIONS OF *Daniellia oliveri* (ROLFE) STEM BARK

BY

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ABSTRACT

The Mitochondrial Permeability Transition (mPT) pore opening is a calcium-dependent process resulting from an increased permeability of the mitochondrial membrane. This mPT is a major factor which promotes apoptosis. Increasing mPT has evolved as a target for the treatment of tumours. *Daniellia oliveri* (DO) is a medicinal plant used in folkloric management of tumours in Africa. Compared to other tissues, liver cells have the highest concentration of mitochondria. Therefore, this study was carried out to investigate the effects of DO on mPT in rat livers.

The DO stem bark was collected, authenticated at Department of Botany, University of Ibadan (UIH: 22383), air dried, pulverized and extracted with ethanol for 72 hours. The filtrate gave ethanol extract (EEDO) that was partitioned successively with chloroform (CFDO), ethyl acetate (EAFDO) and ethanol (EFDO) to obtain three fractions of DO. An *in vitro* experiment was performed on liver mitochondria from fifteen male rats (110.0±1.8 g) using 60-300 µg/mL of EEDO and its fractions. Twenty-four male rats (90.0±0.5 g) were divided into four groups (n=6) and treated intraperitoneally for fourteen days thus; control (distilled water), 25, 50 and 100 mg/kg EFDO. After overnight fasting, rats were sacrificed and liver mitochondria were isolated by differential centrifugation (same as for *in vitro*). The mPT, mitochondrial ATPase (mATPase) and nuclear DNA (nDNA) fragmentation were assessed using spectrophotometry. Expressions of Bcl-2, Bax, p53 proteins and Cytochrome C Release (CCR) were determined using immunohistochemistry. Caspase 3 (C3) and Caspase 9 (C9) activities were determined by standard method using ELISA technique. The GC-MS was used to identify the compounds in EFDO. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The mPT was increased by 60, 120, 180, 240 and 300 µg/mL EEDO (0.03, 0.09, 0.20, 0.40, and 0.65 folds) and EFDO (0.04, 0.10, 0.35, 0.68, 0.75 fold), respectively. Graded concentrations (0.75, 2.25, 3.75, 4.25 and 6.75 µg/mL) of EFDO (3.2±0.2, 4.0±0.1, 4.5±0.3, 5.1±0.2, 5.9±0.1 µmpi/mg protein), respectively, CFDO (2.7±0.4, 3.0±0.2, 3.7±0.3, 4.1±0.3, 4.5±0.4 µmpi/mg protein) and EAFDO (2.9±0.1, 3.5±0.3, 4.3±0.2, 4.8±0.4, 5.1±0.1 µmpi/mg protein) enhanced mATPase activities relative to control (2.4±0.2 µmpi/mg protein), respectively. These five concentrations also increased CCR; EEDO (0.7±0.04, 0.8±0.03, 0.9±0.04, 1.1±0.06, 1.5±0.04 nmol/mg protein), EFDO (0.6±0.03, 0.7±0.03, 0.8±0.06, 0.9±0.02, 1.0±0.04 nmol/mg protein)

and CFDO (0.6±0.05, 0.7±0.02, 0.8±0.04, 0.9±0.03, 1.1±0.02 nmol/mg protein) relative to control (0.5±0.01 nmol/mg protein). The EFDO at 25, 50 and 100 mg/kg increased mPT (0.5, 0.6 and 0.7 folds) and mATPase activity (5.9±0.2, 7.0±0.4, 7.7±0.2 against 3.5±0.3 µmpi/mg protein). The Bax (35.6, 200.2 and 330.8%), p53 expression (91.3, 100.2 and 204.4%), CCR (50.2, 100.7 and 300.3%) and nDNA fragmentation (20.8, 55.2 and 115.6%) increased, while Bcl-2 (5.5, 34.5 and 56.4%) decreased compared to control. The C3 (15.8, 68.4 and 88.2%) and C9 (87.5, 150.5 and 180.4%) activities were increased compared to control. The GC-MS revealed the presence of oleic (54%) and palmitic acids (39%) in EFDO.

Daniellia oliveri increased apoptosis *via* mitochondrial membrane permeability transition pore opening. It also enhanced mitochondrial ATPase activity.

Keywords: Mitochondrial membrane permeability transition pore, *Daniellia oliveri*, Apoptosis, Cytochrome C release

Word count: 496

CERTIFICATION

I certify that this research work was carried out by Achem, Jonah in the Biomembrane Research and Biotechnology Laboratories of the Department of Biochemistry under my supervision.

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DEDICATION

This research work is dedicated to our LORD and SAVIOUR, JESUS CHRIST, who was delivered over to death for our sins and was raised to life for our justification. And to my late father, Mr. Achem, Ogili, who gave priority to academy of his children and this gave me a sound foundation.

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ABBREVIATIONS

ADP: Adenosine diphosphate

AIF: Apoptosis Inducing Factor

AMC: Aminomethylcoumarin

AMPK: Adenosine Monophosphate Kinase

ANOVA: One Way Analysis of Variance

ANT: Adenine Nucleotide Translocase

Apaf-1: Apoptosis Protease-Activating factor

ATP: Adenosine Triphosphate

ATPase: Adenosine Triphosphatase

Bak: BCL-2 Associated Killer protein

BAX: BCL-2 Associated X- protein

BCL-2: B - Cell Lymphoma 2

BH: BCl-2 Homology

BSA: Bovine Serum Albumin

CAD: Caspase-activated DNase

CARD: Caspases Recruitment Domain

CCR: Cytochrome c Release

CD: Clusters of differentiation

CFDO: Chloroform Fraction of *Daniellia Oliveri*

CPT: Camptothecin

CsA: Cyclosporin A

Cyp D: Cyclophilin D

DFF40: DNA Fragmentation Factor

DIABLO: Direct IAP Binding Protein with Low Pi

DISC: Death Inducing Signaling Complex

DMRT: Duncan's Multiple Range Test

DNA: Deoxyribonucleic Acid

DO: *Daniellia oliveri*

DNP: Dinitrophenol

DPA: Diphyenylamine

EAFDO: Ethyl Acetate Fraction of *Daniellia oliveri*

EDTA: Ethylene Diamine tetraacetic Acid

EEDO: Ethanol Extract of *Daniellia oliveri*

EFDO: Ethanol Fraction of *Daniellia oliveri*

EGCG: Epigallocatechin-3- Gallate

ELISA: Enzyme – Linked Immunosorbent Assay

EndoG: Endonuclease G

ER: Endoplasmic Reticulum

ETC: Electron Transport Chain

FFA: Free Fatty Acids

GC-MS: Gas Chromatography Mass Spectrometry

HK: Hexokinase

HNE: 4-hydroxynonenal

IAP: Inhibitor of Apoptosis Proteins

IIM: Intact Isolated Mitochondria

IMM: Inner Mitochondrial Membrane

LPO: Lipid Peroxidation

MAC: Mitochondrial Apoptosis-induced Channel

MAM: Mitochondrial ER Associated Membrane

MDA: Malondialdehyde

MIM: Mitochondrial Interior Membrane

MMPT: Mitochondrial Membrane Permeability Transition

MOMP: Mitochondrial Outer Membrane Permeabilization

MPT: Mitochondrial Permeability Transition

MPTP: Mitochondrial Permeability Transition Pore

MSH: Mannitol, Sucrose, HEPES-KOH

NADH: Nicotinamide Adenine Dinuleotide

NADPH: Nicotinamide Adenine Dinucleotide phosphate

NO: Nitric Oxide

NOS: NO Synthase

eNOS: endothelial NO Synthase

iNOS: stimulatory NO Synthase

nNOS: neuronal NO Synthase

NTA: Non Triggering Agent

OA: Oleic Acid

OMM: Outer Mitochondrial Membrane

Omi/HtrA2: Omi/high Temperature Requirement Protein A

PBS: Phosphate Buffer Saline

PS: Phosphatidylserine

PT: Permeability Transition

PTP: Permeability Transition Pore

PTPC: Permeability Transition Pore Complex

PUFA: Polyunsaturated fatty acids

RNA: Ribonucleic acid

ROO: Peroxy radical

ROS: Reactive Oxygen Species

SD: standard deviation

SDS: Sodium Dodecyl Sulphate

TA: Triggering Agent

TBA: Thiobarbituric acid

TBARS: Thiobarbituric acid reactive species

TET: Tris EDTA Triton x

TP 53: Tumour Protein 53

TopI: Topoisomerase I

TRAF2: TNF Receptor Associated Factor 2

TNFR: Tumour Necrosis Factor Receptor

Smac/Diablo: Second Mitochondrial – Derived Activator of Caspase

UIH: University of Ibadan Herbarium

UV: Ultra Violent

VDAC: Voltage Dependent Anion channel

VLC: Vacuum Liquid Chromatography

CHAPTER ONE

1.0 INTRODUCTION

The Mitochondrial Permeability Transition (mPT) means permeation of MitochondrialInteriorMembrane (MIM) that could be attributed to effects of some harmful stimulus includingcytotoxic drugs,oxidative stress, hypoxia andCa^{2+} accumulation (McCommis and Baines, 2012). The mPT is always brought about as a consequence ofmPT pore (mPTP) gap, in spite of the fact that what constitute this pore are still to be fully confirmed (Nakagawa *et al*., 2005). The Mitochondrial permeability Transition Pore (mPTP) is a macro-protein with diameter measuring between 1.0 – 1.3 nm andit is non-distinctly water-permeable to solid moleculeshaving weightsbelow 1.5KDa. This complex protein is suggestively constitutedprimarily ofCyclophilin D,Voltage Dependent Anion Channel (VDAC), Adenine Nucleotide Translocase (ANT) and BAX-BCL-2. Opening of mPTP always result in reduction of unpolarized condition of MIM and enlargement of the matrix gaps, with subsequent irregular burst of the OuterMitochondrialMembrane (OMM) resulting from increased MIM surface area compared to that of theOMM (Kinnally and Antonsson, 2007). In spite of severalthoughts and debates on the role play bymPTP in cell death through mitochondrial channel, there are many proofs for essential components and regulation of mPTP in apoptosis. Several investigations have attributed mPT as an integral and a major agent in injury toneurocytes caused by excitotoxicity (Ichas and Mazat,1989; White and Reynolds,1996).

The stimulation of mPT, which raises permeabilityof mitochondrial membrane, causes mitochondrionto become more depolarized, indicating abolition of membrane potential. This decrease of membrane potential will causeproton and some molecules to pass throughOMM without restriction(White and Reynolds,1996).During harsh stresses and disease state, mPTP opening could initiateharmed cell death primarily through necrotic process (Haworth and Hunter, 2001). An increasing number of researches on several animal tumour structures and cancer cellshave shown that initiation of mPTP opening by pharmacologic applications induces cell death by apoptosis and precludes gradual cell formation during tumour development, which is an extreme harmfulseries of events with specific tissue metabolic reprogramming (Fantin and Leder, 2006; Brenner and Grimm, 2006; Armstrong, 2007).Application of chemotherapy is an effective strategy to elevateReactive Oxygen Species (ROS) generation, ultimately inciting

opening of mPTP. The ROS upregulation and/or interruption of antioxidant activities in cancer always give rise to ROS accumulation (Fang *et al*., 2009).

Mitochondria are cell components that carry out many functions in cell by controlling survival and death signalling processes.Theyserve as the powerhouse of the cell generating more thanninety percent (90%) of Adenosine Triphosphate (ATP) through oxidative phosphorylation for cell metabolism. They are also responsible for regulatingvarious cell death processes including apoptosisand necrosis. Extreme pressure andcalcium retention in the mitochondria causes rise in permeation of the mitochondrial membrane and subsequently formation of pathologic and non-distinctiveburst of mPT pores (Halestrap and Pasdois, 2009). The mPT pore opening with enormous passage of molecules enhances depolarization of Mitochondrial Inner Membrane (MIM) resulting to ATP exhaustion withmore ROS formation. The abrupt rise in MIM permeationincreasesmitochondrial matrixcolloidal osmotic pressure and subsequently matrix enlargement and bursting of OMM. Bursting of OMM triggers discharge of pro-apoptotic proteins thus, starting both caspase – dependent and caspase – independent apoptotic processes. Mitochondrial Outer Membrane Permeabilization(MOMP) could equally be caused by assembling of non-selective channels triggered by transferring pro-apoptotic proteins to mitochondria (Armstrong *et al*., 2009; Baines, 2009). The regulationof mPT pore by numerous signalling agents, cellular metabolic products and ions is an intricate process. The formation of mPT pore isdependent on difference between agents promoting and impeding pore opening.The mPT pore opening causes mitochondria to be uncoupled and this will cause the F_0F_1-ATPase to operate in reverse mode, i.e., breaking downATP instead of synthesizing it. Intense ATP exhaustion destroys the structure and function of cells, causing damage to the cell and subsequently causingcell death.

The primary focus of using drug to treat mitochondrial – relatedailments is promotion of extrinsic and intrinsic pro-apoptotic processes in orderto incitedeath of cancerous cells thereby, preventingtheir proliferation (Fulda *et al.,* 2010). Therapeutic applications can directly or indirectly affect mitochondria and reducing their ATP synthesizing capability andthus, interfering with structure of OMM to enhance discharge of pro-apoptotic proteins to the cytosol. It has been proven that poor response ofcancerous cells to pro-apoptotic signals and decreased occurrence of programmed cell death are major difficulties in cancer management and

cure(Hanahan and Weinberg, 2000). Numerous bioactive compounds are known to incitemPT poreopening resulting incelldeath because of their straight interference with Voltage Dependent Anion Channel (VDAC) and Adenine Nucleotide Translocase (ANT). Pharmacologicsuccess of anti-tumour drugs is commonly known to be facilitated by mPTP-targeted agents. Also, it has been proved that compounds that evoke calcium accumulation in the mitochondria and ROS formation, coupled with exhaustion of elevatedphosphate energy (ATP and creatine phosphate) can in an indirect manner incitemPT pore formation (Fulda and Debatin, 2006; Kroemer *et al*., 2007).

The anti-cancer impacts of various pharmacological agents on cancerous cells, resulting from interruption ofaccumulation of ROS and anti-oxidant system seems to be influenced by mitochondrial – controlled cell death where the role of mPT pore is evident (Tonissen and Di Trapani, 2009; Palmeira and Wallace, 1997). Different conditions, such as prevalence of Ca^{2+} along with phosphate ions could cause isolatedmitochondria to pass throughmPT. This series of events is characterized by a Ca^{2+}-depending upon rise in permeation of MIM, bringing about decrease ofmembrane potential,mitochondrialswelling andOMMbursting. The mPT is considered to result from opening of a purported track complex, which is usually known asPermeability Transition Pore (PTP), and it is thought to be constituted of ANT andVDAC (ininternalandexternal membrane channel, respectively)and cyclophilin D (Cyp D) including other substance(s) (Crompton, 2003).

Apoptosisand description of its molecular hypothesis is presently fully comprehended. Mammalian cells exhibit two prominent cell death channels, theextrinsic and intrinsic route(Green and Evan, 2002). Mitochondria carry out majorfunction in intrinsic route: a rise of outer membrane permeation enhancing discharge of apoptogenicproteins from mitochondria to cytosol, like cytochrome c, Smac/Diablo, DNaseG and Apoptosis Inducing Factor (AIF)(Wang, 2001).The possible function of mPT in death of cell is equally corroborated by results of research that it is occasionally restrainedby bonkrekic acid (Zamzami and Kroemer, 2001).The CsA-sensitive mPT isinvolved in appearance changing of cristaeand cytochrome cstores assemblingin the course of apoptosis, thus promoting absolute exudation of cytochrome c (Scorrano *et al*., 2002).

The mPT pore opening is usually connected tomitochondrial abnormality becauseits formation causesdepolarization of mitochondria, discontinuance ofATP production, release of calcium ions, respirationinhibition and swelling of matrix,which thencauses assemblage of cytochrome c with apoptosis activating factor 1 (Apaf 1), MOM rupture and eventually discharge of pro-apoptotic proteins (Rasola and Bernardi,2011). It is worthmentioning that these deleterious impacts on energy preservation andviability of cell are only seen for prolong mPT pore openings(Petronilli*et al*.,2001),while temporary openings – which have been recorded inbothisolated mitochondria and in situ (Petronilli *et al*., 1989)– could be implicated in calciumphysiologicmodulation and homeostasis of ROS(Zorov *et al*., 2014), and offer mitochondria with quick mechanism for release of Ca^{2+}(Barsukova*etal.*,2011).

Mitochondria are organelles producing energy for the cell and they also participate in several essential activities including ROS formation and detoxification, fatty acid oxidation, stress awareness, Ca^{2+} signalling, cross-talk with other organelles, and orchestration of some cell death modalities (Wallace, 1999; Kroemer*et al*., 2007). When there isalteration of mitochondrial functionand its dysfunctions are extravagant, evacuation by autophagy or death of cell is stimulated.Consequently, mitochondrial homeostasis is an intricate and tautly manipulated process, and inability to sustain this process is often connected with serious abnormalities (Wallace, 1999; Kroemer*et al*., 2007).

The mechanism called Permeability Transition (PT) facilitated bypore opening, the PTP, leading to a change in permeability properties of MIM has been discovered (Hunter *et al.,* 1976). From the time of description of PT, important endeavours have been attained to decode its physiologic function and, more recently, its role in pathology and death of cell (Griffiths and Halestrap, 1995). This has permitted its awareness as effective pharmacologic focus in ailments connected with dysfunction of mitochondria and excessive death of cell (e.g., heart failure, cancer and neurodegeneration(Elrod*et al*., 2010).

It has been suggested that mPT pore is preferentially found at the contact site between MIM and MOM and this correspond with its physiological functions in energy transpose and cell death by enhancing certain protein–protein engagement and structural modifications (Brdiczka, 1991; Vyssokikh and Brdiczka, 2003). ThePT is known to be associated with rise in ROS (e.g,

hydrogen peroxideand superoxide anion), ROS-incited destruction to lipids, proteins and DNA and cytochrome c discharge, thus involving PT in cell death.The ROSoverproduction, Ca^{2+}accumulation in the mitochondrial matrix, ATP reduction and phosphate accumulation are major metabolic alterations that favour mPTporeformation(Vander*et al*., 1999).

The mPT pore is connected withseveral disease conditions includingneurodegenerative,hepatotoxicity, cardiac necrosis among other harmful occurrences triggering cell injury and subsequently cell demise (Lemasters*et al*., 2009; Bernardi and Bonaldo, 2008; Baines, 2010).The mPT is the major source of cell death under differentsituations. For instance, it is a major factor in death of neuron cells in excitotoxicity, in which hyperactivation of receptors of glutamate initiate excess movement of calcium into the cell(Ichas and Mazat,1989). The mPT pore also seems to carry out major function in injury, disturbance in blood circulation being the source, as it happens duringstroke and heart assault (Honda and Ping2006). Nevertheless, investigation has demonstrated that mPT pore is kept sealed for all the time ofischemia, however,it becomes accessible as soon as the tissues are suffused with blood following the period of ischemia, exhibiting tremendous part in reperfusion injury (Bopassa *et al*., 2005).

Epidemiological researches have illustrated correlation between plant antioxidants and reduction of chronic diseases (Sasikumar*et al*., 2010; Lieu, 2003). These boons are believed to be attributed to antioxidant components of plant origin (Rice-Evans, 2001).Several epidemiological studies have also proven that plants with rich antioxidants as constituent serve in keeping health and protect against diseases (Milner, 1999), and eating them was found to ameliorate risk of neurodegenerative and heart diseases, cancer, disorder of abnormally high blood pressure and loss of brain function (Vinsion *et al*., 2001; Wolfe and Liu, 2003). The main composites bioactive classesthat enhance total capacity of antioxidant of plant arevitamins (C and E) and polyphenols. Medicinal plants are applied as panaceae for human sicknesses from time immemorial and the rationale for using them is attributed to the fact that they contain bioactive constituents that exhibit curative value (Nostro *et al*., 2000).Recent studies have illustrated that phenolic substances in plants removeROS and inhibit oxidative cell damage (Divya and Mini, 2011). Application of herbal outputs could be a better choice to attain the goal of discovering a required cure for illness and for mitigating free radicals generation.

Daniellia oliveri (*Caesalpiniacea*) is a plant that is majorly found in the Amazon land and parts of South America and Africa (Langenhein, 1973; Gentry, 1973).This plant is traditionally used in treating several human diseases including breast tumours,swellings, vestibule vaginal tube and abscesses (Survey Report,1998). The bark of stem can be prepared intoconcoction which can be used to treatsickle cell malfunctions and diabetes (NCAC Policy and Operational Guidelines, 1992). It produces liquid called oleoresin which is used as medicine by indigenous people of Mali, Ghana and Nigeria for over four centuries (Gilbert, 2000). It is also traditionally applied as agent of anti-inflammation and management of several genito-urinary tract diseases and skin ailments (Raffauf, 1992; Duke and Vasquez, 1994). In a traditional manner, all parts of *D. oliveri* are useful incure for sickness of various illnesses in Nigeria and some West African Countries (Fleury, 1997).

Since chemotherapy is the major aim to instigate apoptosis in cancer treatment, mPT poreevocation maybe suggestedas a fascinating endeavour for developing new therapeutics for cancer to evokemediation ofmitochondria in death of cell and impedeproliferation of cancerous cell. This research is aimed at elucidating the function of mPT pore in death of cell, and pharmacologic effects of certain solvent fractions of *Daniellia oliveri* stem bark to inducemPT-mediated apoptosis.

1.1 JUSTIFICATION

Mitochondrial permeability transition plays physiological role and contributes significantly to abnormality and demise of cell. This has permitted its awareness as an effective pharmacologic focus in diseases connected to mitochondrial dysfunction and where apoptosis has been impeded.The restrained achievement of clinical treatment of ailments including radiation, chemotherapy, immunomodulation and surgery in managing cancer, as illustrated by high morbidity and mortality rates shows that there is an imperative need of new cancer treatment (Akbar *et al*., 2011).Certain bioactive compounds of medicinal plants have been demonstrated to trigger cell death via mPT pore. For instance, resveratrol gotten from wine and grapes, is shown to hinder synthesis of ATP and evoke MOMP;betulinic acid of the lupane class, is reported to evoke death of cells in cancerand also regulates proteins of Bcl-2 class (Selzer *et al*., 2002) and berberine from the family of*Berberidaceae*plants apply constant impacts on mitochondria, such as engagement with ANT, variations inBcl-2/Bax ratio, generation of ROS, reduction inmembrane potentialand discharge of cytochrome c(Fulda *et al*., 2010).

Danthron (1,8-dihydroxyanthraquinone), a commonly existing constituent, separated from rhizome and root of *Rheumpalmatum* L. has been illustrated to depolarize membrane potential and stimulate opening of mPT pores incancer cellsof human gastric (Jo-Hua *et al*., 2011). In malignant cells, natural product, such asanthraquinone and its derivatives have been found to cause membrane potential loss as one of their proapoptotic mechanisms (Ismail *et al*.,2013).Recent results from our laboratory have proved that crude extracts of *Brysocarpus coccinues* and *Cnestis ferruginea* can induce mPT pore opening (Adedosu *et al.,* 2012). Recent research using cancer cell lines showed that extracts of *Daniellia oliveri* in combination with *Capsicum frutescens* had cytotoxic effect on breast cancer, prostate cancer and colon cancer cell lines (Howard, 2011).It is in view of these claims that we investigated the effects of certain fractions of *Daniellia oliveri* stem barkon mitochondrial – mediated apoptosis.

1.2 AIM OF STUDY

This study was carried out to ascertain if any fractions of *D. oliveri* stem bark would have effects onmitochondrial–mediated cell death and thus could serve as a potential drug candidate in inducing apoptosis using animal model.

1.3:SPECIFIC OBJECTIVES

The specific objectives of this research are:

- To carry out preliminary phytochemical screening of *D. oliveri*stem bark in order to ascertain its constituent bioactive compounds.
- To ascertain the *in vitro*effects of solvent fractions of *D. oliveri*stem bark on mPT pore opening.
- To determine the effect of solvent fractions of*D. oliveri*stem bark on mitochondrial lipid peroxidation, ATPase activity and cytochrome c release *in vitro*.
- To investigate the effects of ethanol fraction of *D. oliveri*stem bark on mPT pore, mitochondrial lipid peroxidation and ATPase activity *in vivo.*
- To ascertain the effects of ethanol fraction of *D. oliveri* stem bark on the histology of visceral organs of male albino rats *in vivo*.
- To determine the modulatory effects of EFDO stem bark on apoptotic biomarker proteins, caspases and DNA fragmentation

- To identify compounds present in EFDO stem bark responsible for mPT poreopeningand effects of these compounds on some markers of mitochondrial–mediated cell death.

CHAPTER TWO
REVIEW OF LITERATURE

2.0: *DANIELIA OLIVERI*

Daniellia oliveri (*caesalpiniacea*) is a plant that grows mainly in the Amazon land and parts of South America and Africa (Langenhein, 1973; Gentry, 1973). Theheight of this tree is approximately 100 feet and trunk diameter of 4 feet (Record and Mell, 2000). This plant grows majorly in the forest and grass land region, and can withstand any weather. In Nigeria, it is renowned differently in different ethnic tribes such as, “iya” in Yoruba, “maje” in Hausa “ubakwa” in Idoma, “oda” in Igala, “chiha” in Tiv “abwa” in Igbo (Dalziel, 1937).

This plant is traditionallyused for the treatment of breast tumours, swellings,vestibule vaginal fistula and cavities caused by tissue destruction (Survey Report,1998). Preparation of *D. oliveri*stem bark with other substance is used in the treatment of diabetes and sickle cell malfunctions (NCAC Policy and Operational Guidelines, 1992). It produces a liquid called oleoresin which has been used as medicine by indigenous people of Mali, Ghana, Ivory Coast and Nigeria for more than 400 years (Gilbert, 2000). The oleoresin is traditionally used as an agent against inflammation and in the treatment of different genito-urinary tract diseases and skin ailments (Raffauf, 1992; Duke and Vasquez, 1994). The oleoresin is also used as an anti-rheumatic, antibacterial, diuretic, hypotensive agent, laxative, purgative, expectorant, vermifuge and vulnerary (Fleury, 1997). The leaves are also used by native inhabitants as medicine for the treatment of diabetes. Someof these medicinal uses of oleoresin have been authenticated by modern scientific studies such as its effectiveness as antibacterial, anti-inflammatory and anti-oxidant agent (Verpoorate and Dahl, 1987; Basile *et al,* 1988).

In a traditional manner, all parts of *D. oliveri* are usedto cure and manage manifold sicknesses in some West African Countries including Nigeria. The leaves are beneficial in healing diabetes, gastrointestinal imbalance, yellow fever, as diuretic and aphrodisiac (Ahmadu *et al.,* 2003) and as well for wounds dressing, precisely circumcision (Igoli, 2005). The barks of the root are frequently applied for treating disorder of the muscles, tendons, joints, nerves and lameness, and condition of any part of the body consisting of congestion of blood vessels (Mac Donald and

Olorunfemi, 2000). InIvory Coast,the root and stem barks have been of benefit as chewing stickwhen dried (Bhat *et al*., 1990; Delaveau *et al*., 1979).

Daniellia oliveri(Rolfe) Hutch and Dalz is widely recognized as Ilorin balsam or African copaiba balsam (Adaku and Okewesili, 2000 and Adegoke *et al*., 1968). This plant is found to be in the family fabacae and is very useful both as timber and forest enrichment tree. *Daniellia oliveri* is important in agro forestry systems, soil and water conservational (Agunu *et al*., 2005). It belongs to fire resistance savanna species (Ahmadu *et al*., 2007). This planthas been known to possess high medicinal benefit being active in healing gastro intestinal disorders (Adegoke *et al*., 1968), as antiaborifacients in pregnancy, skin mucosa and as anxiolytic (Ainstie, 1990), for healingpains of rheumatism (Akhtar *et al.,* 2000) and effective as antimicrobial agent (Adegoke *et al.,* 1968).

Fig. 1: Picture of *Daniellia oliveri*(Ibadan, June,2016)

Many bioactive compounds are demonstrated to exhibitbiological activity and they undergo interactions to guard against cancer.Over 4000 distinct flavonoids have been identified in different plantsand have been linked to reduction of cancer risk and other chronic ailments.

Chemo-preventive agents show their effects by retarding the process of carcinogenesis at different stages (Ritesh *et al.,* 2010).

Bioactivecompounds obtained from plants having medicinal value exhibit noticeable applications in potential practice of managing manifold clinical circumstances such as diabetes, stroke, neurodegenerative diseases and cancer (Desai *et al.*, 2008; Guilford *et al*., 2008). Much inquiry has been projected in the direction of assessment of extracts of plant as preventive factors, which proffer enormous endowment to restrain tendency to cause cancer. The down regulating mechanisms of tumour promotion by bioactive agentsof natural originrange from inhibition of toxicity to genes, elevation of substances that act to prevent or slow the oxidation of other chemicals and anti-inflammatory operation, retardation of proteases and cell proliferation (Soobrattee *et al*., 2006). Researches have pointed out that varieties of new chemo-preventive bioactive agents from medicinal plants have been sorted out based on their capacity to adjust one or more definite molecular occurrences. Discovery of effective leafy plant and exposition of their fundamental processes of action could proffer solution to directed change of an alternative and supporting process for cancer management.

In the 1950s, the search for anticancer factors from plant sources commenced and culminated in finding and development ofvinca alkaloids, vincristine, and podophyllotoxinsisolationwhich are cytotoxic (Reddy, 2003; Pezzuto, 1997). Bioactive agents from plants are significant drug candidates that could possibly pave way to fresh and improve treatments for different human sicknesses, such as diabetes and autoimmune diseases. Chemotherapeutic application is known to destroy healthy cells together with cancer cells and at some points they can build up resistance to medical care through heritable change of the base-pair sequence of genetic (Wiseman and Spencer, 1998). Medicinal plants have been used for prevention and medication of differenthuman sicknesses (Adebajo *et al*., 1983). It has been reported that plants contain a wealth of agents which could be applied in the management of diseases (Cowmann, 1999; Banso and Olutimayin, 2001). Accordingly, checking for wealth of biodiverse constituents is essential for study before vegetations are completely destroyed.

2.1PHYTOCHEMICALS

Phytochemicals are bioactive compounds produced in plants during their metabolic activities. These compounds are known as “secondary metabolities” such as alkaloids, flavonoids,

glycosides, polysaccharides etc (Harborne, 1973; Okwu, 2004).These bioactive agents are non-nutritive compounds that possess disease protective properties. The production of these bioactive agents in plants is well known to protect the plants but recent researcheshave also demonstrated that they protect humans against diseases.

Many medicinalplants produce their helpful phytochemicals by means of synergistic action of multiple bioactive factor performing at a sole or multiple mark points in connection with a physiologic system, whilesynthetic pharmaceuticals is based upon single chemical. As pointed out by Tyler (1999), these combined actions of pharmacologic activities can be of tremendous benefit by excluding the danger associated with domination of a single xenobiotic agent. Collective interactionsof different Phytomedicinesform basis for their efficacy (Kaufman *et al*., 1999). Majority of bioactive agents are very powerful substances which actas harbingers for production of several drugs (Sofowora, 1993).

Bioactive agents from plants for a very long time have been used as drugs, for instance, Salicin, which possessespain-relieving properties and protect inflammationwas initially isolated from white willow tree and subsequently synthetized to becalled Aspirin,a staple over counter drug. Facts from laboratory investigations demonstratedthat bioactive agents contained in plantcould ameliorate danger of several human diseases including diabetes, cancer, etc, property which could be attributed to dietary fibres, such as polyphenol. Explicit phytochemicals, for instance fermentable dietary fibres were also very good examples (Amos *et al*., 1998).

2.2APOPTOSIS

Apoptosisis the usual physiologic pathway of event which regulatesgrowth and health of multicellular organisms (Dash *et al*. 2005). It is a physiological activity that serves a crucial function in growth and homeostasis of tissue (Robby, 2010). Apoptosis is an arranged and controlled cellular procedure that transpires in physiologic and pathologic states (Mohan, 2010; Merkle, 2009; Rebecca, 2011). Problems associatedalongside the control of apoptosis have been included in several maladies, such asdiabetes, autoimmune, uncontrolled proliferation of tissuesand neurodegenerative ailments. Cancer is a diseasethat is frequently demonstrated by little occurrence of apoptosis, thus giving rise to malignant cells. Cancer is always associated with imbalance between cell proliferation and cell death (Rebecca, 2011).

2.3 MORPHOLOGICAL MODIFICATIONS IN APOPTOSIS

These refer totransformations in apoptotic cell beingconcerned with the nucleus and it is similarin all type of cells (Hacker, 2000; Saraste and Pulkki, 2000). Final cellular fragmentation usually require several hours from the stimulation of cell death process. Not withstanding, the time involved is relying on the category of cell, the stimulus and the approach of apoptosis(Ziegler and Groscurth, 2004).

Concentration of chromatin and nuclear DNA breakdown are remarkablestructural hallmarks of apoptosis, which is attended by mopping up of the cell and decrease involume of cell (pyknosis). Chromatin concentration begins at nuclear membrane periphery, creating a curved morphology and moreover concentrates till it fragmentswithincell with an uninjured boundary, a form explained as karyorrhexis (Manjo and Joris, 1995). Plasma membrane is not damaged during the entire event and atthe end phase of cell death, some of the structural shapes include blisteringof membrane, organellesdetailed structuretransformation in thecytoplasm and destruction of membrane integrity (Kroemer *et al*., 2005).

2.4 BIOCHEMICAL CHANGES IN APOPTOSIS

Three major kinds of transformation involving chemical processes in living organismsare seen in death of cell includingcaspases actuation, protein and DNA decomposition,membrane cytoplasm alteration and identification by cells that undergo phagocytosis (Kumar *et al*., 2010). Phosphatidylserine (PS) appears on outside stratum of cell membrane at the onset of apoptosis, being “flipped out” from the interior stratum. This bestows first awareness ofcells that aredead by macrophages, bringing aboutphagocytosis of cells that lack the discharge of pro-inflammatory components (Hengartner, 2000). This is ensued by a distinguished hydrolysis of deoxyribonucleic acid into huge fifty to three hundred kilobase units (Vaux and Silke, 2003). Later, there is inter-nucleosomal DNAdegradationby endonucleases into oligonucleosomes of 180 to 200 manifolds pairof bases. Another definite characteristic of death of cell is the actuation of caspases, a group of cysteine protease. The “c” in “caspase” alludes to a protease called cysteine, whereas the “aspase” means splitting after aspartic acid remains, which is the enzyme’s explicit attribute (Kumar *et al*., 2010). Caspases that are stimulatedsever large number of functional proteins within cell and disintegrate nucleus of the cell platform and cellular structure

resembling skeleton contained within the cytoplasm. Furthermore, they instigate DNAase, which subsequently breakdown DNA in the nucleus (Lavrik *et al*., 2005). These biochemical modifications expound some of the morphologic and physiologic alterations in cell death, but it is essential to observe that biochemical study of severance of DNA or incitation of caspase ought not to be applied to determine death of the cell, as it could take place in the absence of oligonucleosomal disintegration of DNAand can be caspase independent(Galluzi *et al*., 2007).

2.5 APOPTOSIS AND MECHANISMS INVOLVED

The knowledge of mechanisms of cell death is essential and serves a necessary role in comprehending the origin and developmentof ailments as a consequence of disarrayed apoptosis. This can subsequently assist in development of substances for medical purposes that focus on certain apoptotic genes or routes. Caspases are important in executing apoptosis as they are both the initiators and executioners. There are three routes through which apoptosis occur. Death receptorand mitochondrialare thetwo common initiation channels (Green and Evan, 2002). Both channels finally converge to a usual route or the final phase of cell death. Endoplasmic reticulum intrinsic route is the third less familiar instigation channel(O'Brien and Kirby, 2008).

INTRINSIC MITOCHONDRIAL APPROACH

The intrinsic route of apoptosis is initiated within the cell resulting from influence of certain stimuli on cell. Interior stimuli like genetic destruction, anoxia,cytosolic calciumoverload and oxidative pressure are some factors that evoke intrinsic channel through the mitochondria (Karp, 2008). Irrespective of stimuli, this routeresults in increased mitochondrial permeation and evacuation of pro-apoptotic proteins. This track is in close manner controlled byproteins found in BCl-2 group (Danial and Korsmeyer, 2004).

Two primary classes of proteins of BCl-2 exist, specifically the anti- and pro-apoptotic (Reed, 1997). Anti-apoptotic proteinsinhibition of cell deathis carried out by preventingcytochrome cdischarge to the cytosol, whereas pro-apoptotic counterparts instigate apoptosis through enhancing such discharge. The fate of apoptosis initiation is figured out by fairness between anti- and pro-apoptotic proteins and not necessarily on quantity of these proteins (Reed, 1997). Discharge ofcytochrome c instigates caspase 3 stimulation bythe assemblage of an intricate, apoptosome that consist of caspase 9, cytochrome cand Apaf-1. Other pro-apoptotic proteins,

Smac/DIABLO and Omi/HtrA2 enhancecaspase stimulation through attachment to IAPs andsubsequent interference incaspase-3 or -9engagementwith IAPs (LaCaasse *et al*., 2008).

2.6 MITOCHONDRIA, CENTRAL REGULATORS OF INTRINSIC APOPTOSIS PATHWAYS

Mitochondria serve an essential function in differentiation and spreading of death signals which always originate within cells, likedamage to DNA, pressure of oxidation, deprivation and those provoked due to cytotoxicsubstance. They also serve in mediating and amplifying the extrinsic pathways of apoptosis (Kaufmann and Earnshaw, 2000). Mitochondrial swellingcaused by osmosis has been noticed during instreaming of water to the matrix with gradual bursting of Mitochondrial Outer Membrane (MOM), which always brings about liberation of pro-apoptotic proteinsto cytosol from mitochondria(Loeffler and Kroemer, 2000). Released proteins include AIF, endoG,cytochrome c, Htr/Omi and Smac/Diablo (Verhagen*et al*., 2002).

The mPT is always a consequence of the abolition of membrane potential, but membrane potential loss is not often brought about by mPT, and discharge of cytochrome c has been noticed without membrane potential (Bernardi*et al*., 1999). Furtherance to mitochondrial proteins liberation, the wastage of membranepotentialand mPT as well, enhance a loss of biochemicalcellhomeostasis: ATP production is halted, redox substances like NADH and NADPH oxidized, and ROSare enormously produced (Kroemer *et al*., 1997). The increase in concentrations of these species subsequently results in oxidation of biomolecules thereby facilitating the interruption of membrane potentialof the mitochondria as part of positive feedback (Marchetti*et al*., 1997). There are many possible mechanisms proposed for mPT, but there seems to be a common notion that a so-called Permeability Transition Pore (PTP) is constituted ofVoltage Dependent Anion Channel (VDAC)andAdenine Nucleotide Translocator (ANT)as its central contents. The ANTfound inthe interiormembrane of the mitochondria serves for exporting ATP andreplaced with ADP (antiport). In cancer cell lines extreme expression of ANT-1 prevalently evokes death of cell with all its characteristics, whileANT-2 does not, showing anexplicit physical or biological function of ANT-1 in occurrence of mitochondrial apoptosis(Bauer*et al*., 1999). The VDAC, also referred to as porin, is situated in mitochondrial exteriormembrane and creates indistinct pore through the external membrane. It has been pointed out that straight protein-protein engagement, VDAC-ANT complex is believed to linked interior

and exteriormembrane of mitochondria to so-called ‘contact sites’, equivalence to a tight connection and perhaps forming the PTP (Beutner *et al*., 1998).

2.7ANT and VDAC

The ANTand VDACare the most teemingproteins of MIM and MOM, respectively.They have been illustrated to react with family of Bcl-2 proteins and instigateinjuryto mitochondriaduring apoptosis (Shimuzu *et al*. 2001). Recommendations have indicated that engagement of Bax with VDAC bringsabout alteration of VDAC permeation to enhancepassage of proteins such as cytochrome c. Furthermore, since these two proteins carry out essential function in promoting movement of little metabolites and nucleotides across membraneof the mitochondria, binding of Bax could as well add to noticed obstruction of exchange of ATP/ADP and creatine phosphate export during cell death mediated by cytokine removal(Vander Heiden *et al.* 1999).

2.8 EXTRINSICDEATH RECEPTOR CHANNEL

The death receptor (extrinsic)route usually begins withdeath ligands binding to death receptor. Signalling extrinsic apoptosis is influenced by so-called triggering of “death receptors” that are cell periphery receptors which relaysigns of apoptosis after definite ligands bind to them. All family of TNFRmembersare madeof subdomains rich in cysteine outside the cellthat enable them know their ligands with explicity, bringing about trimerization and instigation of corresponding death receptor. Assemblage of numerous procaspase-8 at the DISC results in their autocatalytic activation and liberation of caspase 8being activated. Stimulated caspase-8 thus actson downstream effector caspases that eventually sever explicit substrates bringing aboutdeath of cell. Cells harbouring the ability to stimulate such direct and majorlydependent on caspase cell death routes were grouped to belong to type I cells (Scaffidi *et al*., 1998).

In some cells, stimulus from incited receptor does not produce a sufficient caspase signalling sequence sufficientfor carrying out apoptosis directly. Under this condition, the signal requiresenlargement through apoptotic pathways that isdependenton mitochondria. The connection betweensignalling of caspase chain and mitochondria is supplied by Bcl-2 group member, Bid. This is splitted and moves to the mitochondria for interaction with Bax and Bak to incitedischargeto the cytosol of apoptogenic factors(Luo*et al*., 1998).

THE COMMON PATHWAY

The carrying out (execution) stage of apoptosis is performed by actuation of streams of caspases. The intrinsic upstream caspase is caspase 9 whereas extrinsic route is caspase 8. These two pathways always converge at caspase 3, which splits inhibitor of caspase-incited deoxyribonuclease, which is the primary cause of nuclear apoptosis. Subsequently, caspases for downstream mediate breakdown of protein kinases, repair of DNA proteins and inhibitory small units of endonucleases(Ghobrial *et al*., 2005).

2.9 THE ENDOPLASMIC RETICULUM (ER) INTRINSIC ROUTE

TheER intrinsic approach is another but less prominent pathway, which is thought tobecaspase 12 – dependentand independent on the mitochondria (Szegezdi *et al*., 2003). When ER is disfigured by pressures on the cells, there will be proteins exposition and formation of protein in the celldecreased, and an adaptor protein, TNF receptor associated factor 2 (TRAF2) disengages from procaspase 12, bringing about evocation of the latter (O'Brien and Kirby 2008).The lumen of ER is the major storage of Ca^{2+} within cell and Ca^{2+}-binding chaperones mediate the proper enclosing of proteins in the lumen of ER. It is well known fact that Ca^{2+} movement in and out of ER controls numerous responses of the cell and signalling transduction routes that are connected to pressure response, regulation of transcriptional processes and development. For instance, large release of Ca^{2+} from the ER can activate several signalling mechanisms that enhance death of cell majorly by Ca^{2+}-mediated mitochondrial apoptosis *(*Rizzuto *et al*., 1998).

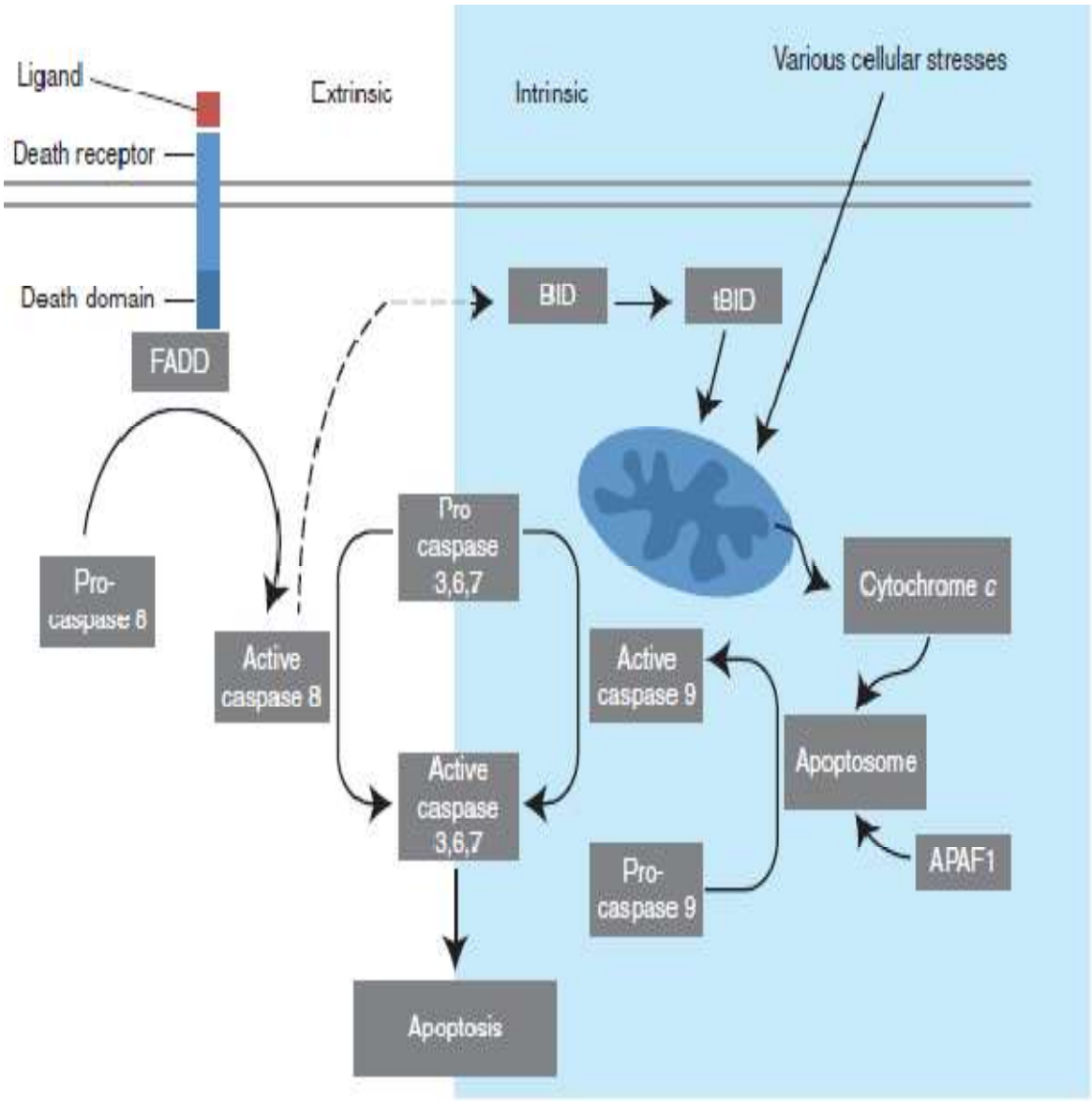


Fig. 2: Extrinsic and intrinsic channels of apoptosis (David *et al*., 2013)

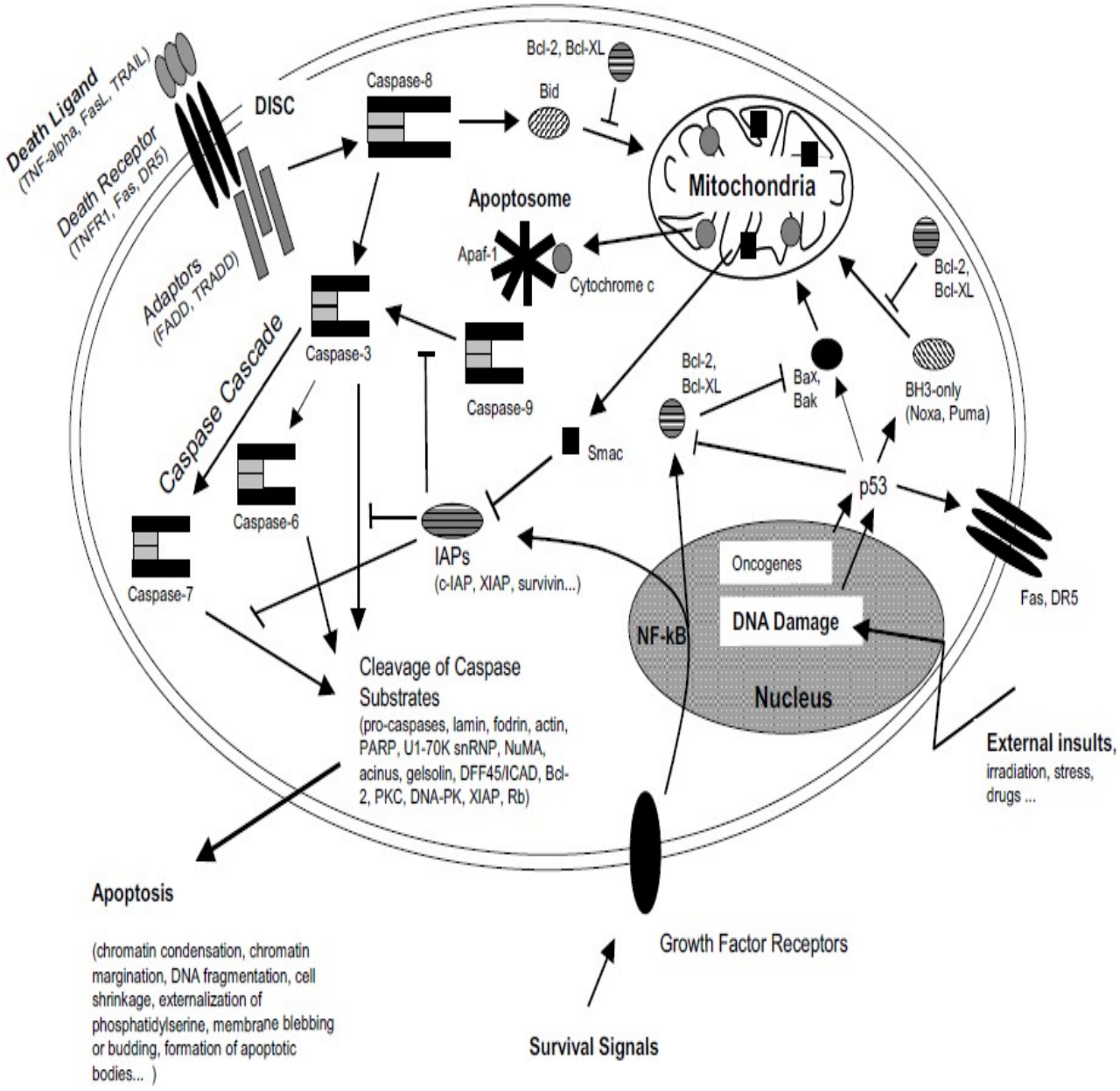


Fig. 3: Schematic representation of some major apoptotic signaling pathways (Andreas, 2003)

2.10APOPTOTIC SIGNALS EXECUTION

Liberation of Cytochrome c

Cytochrome c, a smaller unit of ETCof the mitochondria,participates actively in initiation of caspaseswhen set free from mitochondria to the cytosol at the onset of cell death (Liu *et al*. 1996). The attachment of cytochrome *c*, which is not a function ofnucleotide being present, raises Apaf-1 fascination for dATP/ATP, probably by exposing the nucleotide attachment domain or making firm the attached nucleotide to Apaf-1. The uniting of nucleotide to Apaf-1/cytochrome cintricate facilitates its oligomerization to produceapoptosome. In this complex the CARD folded sections of Apaf-1 become unfolded, which eventually mobilizes numerous procaspase9 to the intricate and enhance their autoactuation. Caspase 9 bound to apoptosome is the only caspase capable of efficiently cleaving and stimulating downstream execution caspases like caspase 3. These executioner caspases afterwards split manifold relevantsubstrates within the cell, resulting in certainstructural alterations in apoptotic cell such as condensationof chromatin, disintegration of nucleosomal DNA, breakdown of nuclear membrane, bringing phosphatidylserine to the external surface, and generation of apoptotic bodies (Hengartner, 2000).

Release of Smac

The Smac/Diablo, a 25-kD protein located in the mitochondria, is as well liberated with cytochrome c to the cytosol during death of cell. This protein is a natural nuclei-encoded protein of the mitochondria that contains55-amino acid-mitochondrial focusing series at its N- terminus and this chain is always eliminated on transferto the mitochondria. The revealed N-terminal Ala of matured Smac is perfectly needed for uniting with IAPs. Due to the fact that this Ala begins to be opened only when the signal peptide become severed after entranceto the mitochondria, mitochondrial targeting grows to be an important stage for functioningof Smac. This putting in order also guarantees that Smac and other apoptosis proteins of mitochondriadon’t prompt premature cell death before their movementto the mitochondria (Wu *et al.,* 2000).

Liberation of Apoptosis Inducing Factor (AIF)

The AIF, a 57-kD flavoproteinlooks like oxidoreductase of bacterial and is located in space between themembranesofmitochondria. Translocation of AIF from mitochondria to nucleus results during instigation of cell death and this tiggerschromatin condensation and mass DNA

cleavage. These outcomes are not dependent on oxidoreductase activity of AIFandcaspases. Insufficient AIF is reported to have severe imparts in animal growth. For instance, interference of AIF in mice hinders necessary death of cells responsible for making cavity of embryoid bodies in the embryo. Besides, embryonic stem cells deficient of AIF show opposition to death of cell after administrationof vitamin K3 and serumdeprivation (Joza *et al*., 2001).

Discharge of Endonuclease G

The EndoG is a 30-kD nuclease found in the mitochondria. The EndoG is converted into another form by a gene in the nucleus, transcribed in cytosol and transfered subsequently to the mitochondria. It is being suggested that it takes part in duplication of mitochondria by voting off RNA primers for provoking mitochondrial DNA synthesis. The purpose of EndoGsuggested in DNA of mitochondrial repetition was founded on its position and explicity of substrate (Cote *et al*., 1993).

EndoG possesses the ability to prompt nucleosomal DNA disintegration once released from mitochondria. The isolation of AIF and EndoG proves that death of cells can take placewithout caspase action duringdestruction of mitochondria. During this condition, liberation of AIF and EndoG triggers a process of cell deathin the same direction as caspase triggering. Pro-death members of this protein elevate discharge of these apoptogenic agents while anti-death members cause their retardation (Korsmeyer *et al*., 2000).

2.11Function of Nitric Oxide in Apoptosis

Among relevant biochemical signalling molecule is Nitric Oxide (NO) which functions in several tissues to control various physiologic processes such as vasodilation, inflammation and immune function. This compound (NO)as well, has been illustrated to participate in regulating apoptosis. The outcomes of death of cell are found to be relyingon the quantity of NO and category of basic unit of life used, and has been indicated to play dual role, both inducing and protecting from death of cell in variety of cells. Hinderance to apoptosis byNO has been demonstrated in a manifold sort of cells, for example, leukocytes, hepatocytes and trophoblasts. As a whole, opposition to apoptosis outcomes of NO can be influenced viavarious mechanisms like nitrosylation and rendering inactive a considerable number of caspases likecaspases 3, 1 and 8.

ROLE OF NO (*S*-NITROSYLATION) IN RESISTANCE TO APOPTOSIS AND CARCINOGENESIS

The NO is an unstable gaseous free radical thatparticipates majorly as a message carrier and muscles or organs that respond to stimulus. The NO is generated by different cells of mammals and is essential for various biologic mechanisms, for example, cell death (Mocellin *et al*., 2007). The roleplay by NO in biological system made it a useful agent in neoplasia management since apoptosis is always impeded at time given room for creation of tumour cells (Chang*et al*., 1997; Morin*et al*., 1996). A group of NO Synthases (NOS)that is expressed in different isoformsin mammals helps to produce NO. Examples of these include neuronal (class I or nNOS), stimulatory (category II or iNOS), and endothelial (category III or eNOS). The group I and III isoforms are known essentially to display NO at reduced concentrations in a calcium-relying upon manner (Knowles and Moncada, 1994), whereas the category II isoform, activated majorly through inflammatory cytokines and gram-negative toxins secreted by microorganisms, generates NO in higher quantities and this is not dependent on intracellular calcium concentrations.Irrespective of differences in NO isoforms, several studies have demonstrated that all three isoforms are necessary in enhancing or retarding the origin of malignancy(Xu *et al*., 2002).

The operation of NOS is often linked with grade of tumour, rate of proliferation and occurrence of significant signalling components that are traced to cancer development (Fukumura *et al*., 2006). The NOS has been expressed in numerous tumours such as brain, breast, lung, prostate, bladder e.t.c. (Fukumura *et al*., 2006; Thomsen and Miles, 1998; Lala and Orucevic, 1998; Lancaster and Xie, 2006). Large number of researches have illustrated that NO is an essential constituent of tumour immediate environment, where it is generatedby either tumour and endothelial cells in the tumour tiny vasculature, or stromal cells in tumours (Lala and Chakraborty, 2001). However, the relevance of NO in tumour is complicated and remains to be fully understood.

It has been proved by several studies during the past few decades that NO can exhibit opposite effects and this is dependent on factors such as concentration of NO instigation, temporal-spatial style of NO way of functioning, targets of NO inside cell, and diverse surroundingsand pathophysiological fettles. The inhibition and management of cancer by NO is still ambiguous

due to discrepancies of reports from different researchers. While sundry studies reveal that presence of NO in tumour is injurious to it, other manifold clinical studies present an enhancing action of NO in tumour development and metastasis (Mocellin *et al*., 2007). Hence, a difference in impacts of NO is noticed, prompting investigators to insinuate that NO possibly exhibit a biphasic answer in a manner that when NO quantities go above a severe levels that would be conducive for neoplasmto grow and survive, growth inhibitionapproach is triggered (Ridnour *et al.,* 2006).

Researches have indicated that NO has straight effect on secondary metastasis and could increase creation of cancer cells, subsequently resulting in the formation of tumours as a result of structural changes of healthy cells (Sawa and Ohshima, 2006). This could be attributed to the fact that NO has been revealed to exhibit pleitropic effects such as, cell escalation, smooth-muscle looseness, neurotransmission, toxicity and cell death. These are important conditions which when dysregulated might result in formation of tumourigenesis (Hirst and Robson, 2007). The chemistryand redox condition of NO make easy for its reaction with motley proteins hence, controlling diverse intra- and inter-cellular signalling occurrences (Stamler *et al*., 1992). Results from recent studies present evidence that NO can provoke an intricate mechanism of responses, hence causing death of cell through mitochondrial death receptor (Kuzushima *et al*., 2006). The NO could stimulate its apoptosis-opposing effect through various mechanisms including rendering caspase inactive, instigation of tumour cellantigen p53 gene expression, promotion of cellular Flice Inhibitory Protein (FLIP), and overproduction of BCl-2 and BCl-X_L with resultant prevention of liberationof cytochrome *c*(Chanvorachote *et al*., 2006). S-nitrosylation is one of the main processes by which NO controls the action of sundryproteins targeted(Stamler *et al*., 1992).

2.12FLIP and NO (death receptor route)

The FLIP is one of the major proteins that serve a regulatory part in the death receptor pathway of cell death. This protein makes cells to exhibit resistant to apoptosis mediated through death receptor in varioustype ofcells(Lee *et al*., 2003), and a riseof this proteinexpression has been linkedto neoplastic cells that could avoid immune continuous monitoring *in vivo*. In addition, suppression of FLIP by compounds that are toxic to cells has been demonstrated to trigger cells to apoptosis via death receptor route. Thus, FLIP could stand as potential necessary phase in

tumourigenesis and an encouraging objective for manufacturing drug and treatment against cancer(Kinoshita *et al*., 2000).

2.13NO and Bcl-2 (mitochondrial channel)

Bcl-2 is an essentialprotein that controls deathof cell through mitochondrial death approach (Green and Reed, 1998). The oncogenic tendency of Bcl-2 protein is highly expressed with its overproduction explained in different kind of malignancies like breast, prostate, and colorectal neoplasia (Osford *et al*., 2004). Evidences from up-to-date studies have illustrated a rise in theconcentration of Bcl-2 and NO in various malignancies(Haendeler *et al*., 2002).For instance, encounter with death ligand and glutathione exhaustion (Azad *et al*., 2006).

2.14 S-nitrosylation and carcinogenesis

The primary feature of cancer cells is their resistance to death of cell and this is responsible forthe origin and cancer progression. It could also be attributed to failure of manifold drug treatment against neoplasia. Molecular transformations which render cellsresistant to death can be induced by *S*-nitrosylation by proteins which are against apoptosis such as Bcl-2 and FLIP. This mechanism is brought about by NO and could lead to promotion of proteins seen in various types of tumours. Nonetheless, direct proofs connecting *S*-nitrosylation and carcinogenesis are insufficient. Opposition to cell death is a distinguishing feature of cancer development, thusmarking of cells that evade apoptosis by S-nitrosylation could be a key determinant in tumour sequence.

2.15 APOPTOSIS IN HEALTH AND DISEASE

Apoptosis is a usual occurrence during growing period of organisms with multiple cells and progresses during the entire period of adult stage. The combined effectsof cell death and cell enlargement form the basis for giving shape to tissues and organs during the early stages of embryos. For instant, death of cells foundin-between toes facilitates the formation of gaps between them (Dash *et al*. 2005).Apoptosis also serve essentiallyto maintain order of the immune system. T lymphocytes are cells that participte in destruction of infected or injured cells in organisms. They get matured in the thymus, and preceding their release into the flow of blood, they are examined to found out that they can destroy foreign antigens and are also able to damage healthy cells. All ineffectual T-cells are eliminated by the process of

apoptosis.Difficulties associated with maintenance of order of apoptosis are responsible for cause of several sicknesses. Cancer is an ailment which is always associated withinsignificant apoptosis. Cancer cells commonly exhibitseveral alterations that have permitted them to turn a blind eye to usual cellular signals controlling their expansion hence, become more propagated than ordinary(Dash *et al*. 2005).

CARCINOGENESIS AND APOPTOSIS

Cancer is often seen to be the consequence of chronological order of hereditary alterations which cause transformation ofordinary cell into abnormal kind whereas equivocation of death of cell is one of the necessary modifications that lead to this injurious transmogrification (Hanahan and Weinberg, 2000). For the past four decades, apoptosis has been linked to excretion of strong cancerous cells and tumour advancement. Consequently, decreased apoptosis or its opposition serves a crucial part in creation of cancer cells. The generalmechanism by which cell deathprevarication takes place can be widely clssified into three, namely interruption in balance of pro- and anti-apoptotic proteins;declined activity of caspase and less effectivesignallingdeath receptor (Kerr, 1972).

2.16MITOCHONDRIAL PERMEABILITY TRANSITION (mPT) PORE

The mPT refers tosnell permeation of the Mitochondrial Interior Membrane (MIM) caused by noxious stimuli, for example, hypoxia, cytotoxic drugs e.t.c. (McCommis and Baines, 2012). The mPT is observed to take place sequel tomPTP opening,in spite of the fact that what make up mPTP is yet to be comprehended (Nakagawa *et al.,*2005). Depolarization of the MIM withenlargement of the matrix space isbrought about by opening of mPTP, resulting in randomburst of MOM due to higher surface area of MIM than the MOM (Kinnally and Antonsson, 2007). There are different lines of proof for the conformation and controlling of mPTP in cell death in spite of several debates on functionof mPTP in mitochondrial channel of cell death. The mPT pore is associated withdepolarizationof mitochondria, enlargement of matrix, liberation of cytochrome *c*, stimulation of caspase sequences, splitting of downstream death effector proteins and finally death of cell (Gerl and Vaux, 2005; Zhao *et al.,* 2009).

Liberation of apoptogenic proteins, cytochrome c especially, is deemed “a commitment step,” which will eventually end in programmed death of cell. This committal phase of cell death seems to be majorly dependent on MOMP. Although the processes that are fundamental to MOMP

stand to be ascertained, it seems to be obvious that diverse protein complexes onmembranes of mitochondria, such as Bcl-2 proteins, arrange the lastresponses in cell death like DNA disintegration andplasma membraneblebbing(Dejean *et al.*, 2010).

2.17 ROLES OF mPT IN PATHOLOGY

Originally,mPTP was revealed by Haworth and Hunter (1979) and was known to participate in neurodegeneration, hepatotoxicity from Reye-related factors, cardiac necrosis and other harmful occurrences inciting damage to cell (Lemasters *et al*., 2009; Bernardi and Bonaldo, 2008 and Baines, 2010). The mPT is among event responsible for cell death in diverse circumstances. The mPT as well seems to serve a major function in injury brought about by ischemia, as is the case in heart injury and loss of brain function (Honda and Ping, 2006). Still and all, inquiry has demonstrated that mPT pore stands sealed during disturbance in blood circulation, but becomes unimpeded during reperfusion of tissues with blood sequel to ischemic period, serving a crucial function in reperfusion damage(Bopassa *et al.*, 2005).The mPT is equally believed to underline death of cell instigated by Reye's syndrome, since drugs that could induce the syndrome, for example,valproateandsalicylate, enhancemPT. The mPT could as well participate in mitochondrial autophagy(Lemasters *et al*., 1998). Cells subjected to toxic concentrations of Ca^{2+}ionophores as well pass throughmPT and death by necrosis (Lemasters*et al.*, 1998).

2.18 mPTPORE REGULATION

VDAC-HK II Interaction

Hexokinase (HK) is anenzyme of glycolysis that catalyses the initial stage of this pathway where phosphorylation of glucose results to formation of glucose-6-phosphate. They are four main types of this enzyme and among theseisoforms,HK II is reported to be over produced in many tumour cells where it assists in enlargement and continued life of tumour cells by improving aerobic degradation of glucose(Mathupala *et al*., 2006). Besides furnishing precursor for glucose degradation and biosynthesis of major metabolites, HK II of the mitochondria is also thought to act an essential function in sustaining MOM intactness by means of engagement with VDAC and subsequently inhibition of mitochondrial-influenced cell death.

Fastening of HK II to VDAC hindersmPT pore formation in two different ways. First, HK II interaction would alterstructural arrangement of VDACthat can subsequently changeorganisation

of ANT which is not suitable formPT pore arrangement. The option of HK II in attaching to VDAC during synthesis of ATP by mitochondria recommends that HK II uniting with VDAC perhaps trigger alteration of VDAC for the in- and outflow of ATP. Second, VDAC occupied by HK II has been demonstrated to preclude pro-apoptotic proteins interaction with it, which could subsequently inhibits their oligomerization require for actuation ofmPT pore.The regulatory function of HK II-VDAC engagement as part of cellular apoptotic signalling is more corroborated by swot of Shulga *et al*. (2009).

2.19 THE BCL-2 FAMILY

The group of proteins commonly called BCl-2 is consisted of two groups namely, pro- and anti-apoptotic proteins. These proteins serve a central purpose in regulating the processes of cell death, particularly through intrinsic route as they are located upstream of permanent injury to cell and exert its action majorly on the mitochondria (Gross *et al*., 1999). Traditionally, these proteins are categorised into one of three subfamilies; anti-apoptotic, BH3-only (pro-apoptotic), and pore-forming or 'executioner' (pro-apoptotic) proteins.

The first class of Bcl-2 group is the anti-apoptotic proteins which contains domains of BH 1, 2, 3 and 4, eg Bcl-2, Bcl-xL and Mcl-2. The Bcl-2 is the first to be isolated in this family of proteins which was discovered more than 20 years ago. The name BCl-2 is derivedfrom B– cell lymphoma 2 (Kroemer *et al*., 2005).This group of proteins is actively engagedin cell death processes. Cells will be more susceptible to apoptosis whenever the pro-apoptotic proteins are in excess, whereas when the anti-apoptotic proteins are present in excess, the cells will exhibit resistant to apoptosis. Presence of more pro-apoptotic Bcl-2 at the periphery of the mitochondria brings about permeability transition poreformation.Cellular apoptosis is mostly regulated via the mPTP and this is dependent on Bcl-2 proteins (Gogvadze *et al*., 2009b).

The second class of Bcl-2 groupis the pro-apoptotic proteins which contains domains of BH 1, 2 and 3, e.g.Bax and Bak. The Bax arelocated in the cytosol while Bak are located on the mitochondria during physiological conditions. However, cell death stimuli could result to Bax transfer to the mitochondria and subsequently insert into the OMM. When found at the mitochondria, Bax could undergo homodimerization or heterodimerization with Bak or truncated Bid, with subsequent interruption ofintegrity ofOMM by pore formation on the membraneof the

mitochondriaand enhancing its permeability. Some reports have as well proposed that Bax reacts with proteins from the PTPC to triggerpermeabilization of mitochondrial membrane(Marzo*et al*., 1998). The Bax protein is frequently found in the cytosol, while Bak is often located on the OMM under physiological conditions. Although the mechanism through which Bax and Bak incite MOMP remains poorly understood, it appears to be obvious that Bax come together and homodimerize by means of movement to the OMM and Bak actuation stimulates disintegration of mitochondria andliberation of cytochrome c (Youle and Strasser, 2008).

The third group is composed of the BH-3 only proteins, e.g.,Bid, Bim, Puma, Noxa, Bad, Bmf, Hrk, and Bik. Theyare limited to the BH3 only domain and thus their name arise from this.During cellular pressures such as damage to DNA, deprivation of growth factor and pressures on the ER, the BH3-only proteinsare stimulated, hence they are pro-apoptotic. They act by impeding anti-apoptotic proteins. For instance, Bim, Puma, Bad and Bmf undergo heterodimerization with Bcl-2 and Bcl-XL and segregatethem, resulting in the blockage of their anti-apoptotic activity (Reed, 1998).

2.20 CASPASES

Caspases meaning cysteine-aspartic proteases, cysteine aspartasesbelong to a group of protease enzymes which participate actively in cell death processes. The name Caspases originates from definitecysteine protease activity of these enzymes - a cysteine amino acid in their active site that attacks nucleophilicallyand splits a protein only at aspartic acid C-terminal. They are family of genes that play an essential role in sustaining homeostasis by means of controlling apoptosis and inflammation (David *et al*., 2016). Caspases possess other known functions in cell death such as Pyroptosis and Necroptosis. These forms of cell death are very crucial in keeping an organism from pressure signals and harmful incursion. Other known functions of caspases include cell progression, suppression of tumour and cell enlargement etc (Shalini *et al*., 2015).

The cause of neoplasm development in organism has been attributed to caspase deficiency. Abnormal growth can take place due to several factors, for example, mutation in a cell cycle gene which eliminates cell growth restrains, along side mutations inproteins of apoptosis likecaspases that would react by triggering apoptosis in anomalous proliferating cells (Goodsell and David, 2000). On the other hand, over stimulation of some caspases likecaspase-3 can result

inextreme occurrence ofcell death. This has been observed in manyneurodegenerative disorders,such as Alzheimers diseasewhere there is loss of neural cells (Goodsell and David, 2000). Caspases that participate in processing inflammatory signals have equallybeen involved in diseases. Inadequatetriggering of these enzymes can result in the organism's vulnerability to infection as the right immune response may not be triggered (Goodsell and David, 2000). Furthermore, scientists have applied caspases as cancer treatment to destroy unnecessary cells in neoplasms (McIlwain*et al*., 2013).

2.21 CASPASES IN PROLIFERATION

Caspases are always connected with cell death and there are also frequent evidences that some groups of these enzymes are actively involved in cell proliferation. It has been observed from earlier remarks that treatment of T cells along side inhibitors ofcaspase resulted in an amazing decrease of CD3-induced T-cell increase. This promotion of abnormal growth by caspasewas subsequently connected to caspase-8, because c-FLIP, a known inhibitor of caspase-8, was demonstrated to regulate T-cell progression. It has also beenshownthat caspase-8 and -6 can actively promote proliferation of B-cell. Nevertheless, caspase-3 perhaps exhibit a reverse effect, as B cells devoid of caspase-3 revealed an elevated enlargement*in vivo* and over proliferation subsequent to mitogenic provocation *in vitro*(Woo *et al*. 2003).

2.22MITOCHONDRION

The mitochondrion (plural mitochondria) is anorganelle with double membrane and ispresent in almost alleukaryotes(Henze*et al*., 2003). Mitochondria can measure between 0.5 and 1.0 μm in diameter. Hugedifferences can be observed in the formation and size of these organelles andthey are not visible unless explicitly stained. They synthesize largersupply of ATPthat serves as a source of chemical energyfor the cell, hence they are regarded as "the powerhouse of the cell"(Campbell*et al*., 2006).They arecellorganelles crucial for energy production and as well for diverse types of cell death via MOMP (Martel *et al*., 2012).Furtherance to providingenergy for the cell, mitochondria also participate in other tasks, likesignalling, cell enlargement and deathof cell, and equally maintaining manipulation of cell cycle and growth (McBride*et al*., 2006). These organelles have been involved in variousailments of human, like disorders of the mitochondria(Gardner and Boles, 2005),cardiac malfunction and heart failure (Lesnefsky*et al*., 2001). Many features make mitochondria one of a kind. Depending onorganism, tissue andtype

of cell,the number of mitochondria can widely vary. Red blood cells, for instance, are devoid of mitochondria, whereas liver cells possess more than 2000 (Alberts *et al*., 1994; Voet*et al.*, 2006).

2.23 STRUCTURE OF MITOCHONDRION

This organelle is composed of five prominentparts namely:

.exterior membrane,

.intermembranespace (between the exterior and interior membranes),

.interior membrane,

.cristae, and

.matrix (spacesin the interior membrane).

EXTERIOR MEMBRANE

This is the part of the mitochondria which surrounds the entire organelle having a thickness that ranges between 60 and 75 angstroms (Å). The exterior membrane as well contains enzymes that participate in numerousactivities like oxidation of epinephrine, degradation of tryptophan and fatty acidselongation. Mitochondria which outer membranes are stripped are called mitoplasts (Hayashi*etal*., 2009).

INTERMEMBRANE SPACE

Thisis equally referred to as the space between the exterior and interior membrane.The exterior membrane is easily permeable to little molecules and because of this the quantities of small molecules including, sugarsand ions, in theperimitochondrial space is the same as the cytosol(Alberts*et al*., 1994). Cytochrome c is one major molecule that is localized in thespace between the membranesin this manner(Chipuk*et al*., 2006).

INNER MEMBRANE

This partof the mitochondria contains proteins with five kinds of functions, including ATP synthase, synthesizingATP in the matrix (Alberts*et al*., 1994). Furthermore, this part of the mitochondria has a rich content of an uncommon phospholipid, cardiolipin. Originally, cardiolipin was identified in cow hearts in 1942, and is often associated with bacterial and mitochondrial plasma membranes (McMillin and Dowhan, 2002). Cardiolipin possesses four fatty acids instead of two, and may contribute to the impermeability of the interior membrane

(Albertset *al*., 1994). The internal membrane in contrast to the external membrane, is devoid of porins, and is extremely impermeable to all molecules. The internal membrane of the mitochondria is compartmentalized into severalcristae, which improve the surface area of the interior membrane, promoting its capacity to generate ATP. For instance, an average liver mitochondrial inner membrane surface area is approximately five times larger than the exterior. This proportion is not constant and mitochondria from cells that possess larger requirement for ATP, e.g., muscle, have even greater cristae (Mannella, 2006).

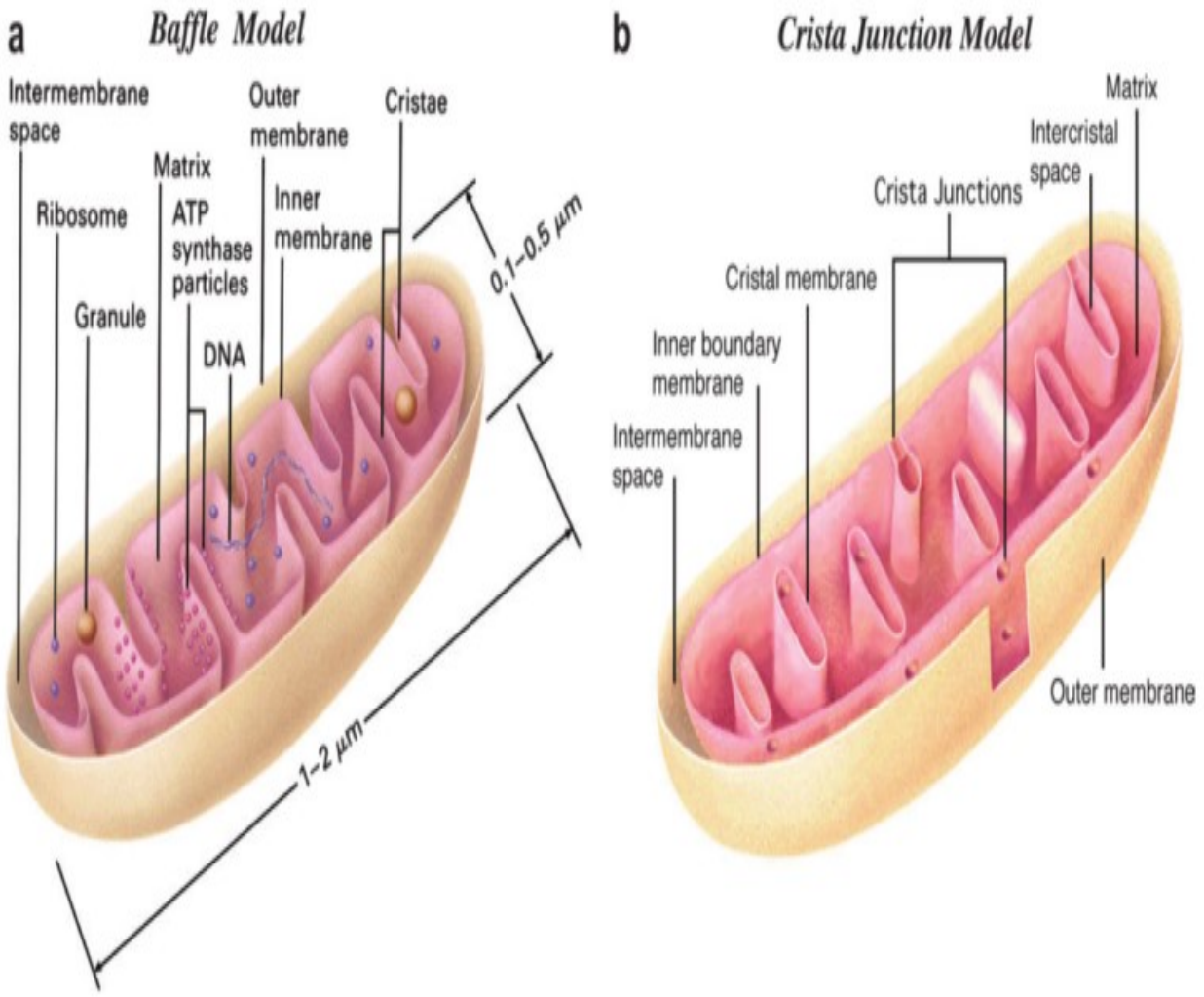


Fig.4:Structure of mitochondrion (a) The palade model of mitochondrion, often called the "baffle" model. It is the most commonly depicted model in textbooks. (b) An alternative model. This supplemented Palade model especially for mitochondria found in higher animals. It provided an explanation for structures observed using electron tomography. The topologies of membrane predicted by these two models are quite different (Lapajne J. 2015).

2.24 PHOSPHOLIPID TRANSFER

The Mitochondrial Associated ER Membrane (MAM) is endowed with enzymes that play an essential role in lipid biosynthesis, likephosphatidylserine decarboxylase is found on the mitochondrial side and phosphatidylserine synthase on the ER side(Vance and Shiao, 1996; Lebiedzinska*et al.*, 2009). Mitochondriaare powerful organelles that frequently undergo splitting and fusion events, thus, they need a steady and well-controlled provision of phospholipids forstability of the membrane (Twig*et al*., 2008; Osman*et al*., 2011; Lebiedzinska*et al.*, 2009). When compared to the standard vesicular process of lipid transfer, there is proof indicating that physical closeness of the ER and mitochondrial membranes at the MAM permits lipid flipping between opposed bilayers (Osman*et al*., 2011). In spite of this uncommon and apparently energetically unfavorable phenomenon, this type of transport does not require ATP (Osman*et al*., 2011).

2.25 FUNCTIONS OF MITOCHONDRIA

The major significant role of mitochondria is production ofATP (i.e.,ADP phosphorylation), by means of respiration, and regulationof metabolism of cells(Voet*et al*., 2006). The central groups of reactions that resulted in ATP generation are generally known as the citric acid cycle. Nonetheless, mitochondrion performs several other functions besidesynthesis of ATP, and these include the following:

Apoptosis: Mitochondria are known to carry out an essential function in the phenomenon of apoptosis.Excess and unwanted cells are trimmed during the developmental stage of an organism. Aberrant cell death that results from mitochondrial dysfunction can adversely interferes with the function of the affected organ (Green, 1998).

Heat generation:Under specificcircumstances, protons can return into the matrix without enhancingsynthesis of ATP. This process is referred to as mitochondrial uncoupling or proton leak and is often attributed to enhance spreading of protons into the matrix. The process brings aboutunused stored energy being given out as heat(Mozo*et al.*, 2005).

Control of cell proliferation:The correlation betweenmitochondria andmultiplication of cellhas been carried out using cervical cancerHeLa cells. Tumour cells need a large quantity of ATP

(Adenosine triphosphate) to enable them produce bioactive compounds includingproteins, lipids and nucleotides for fast cell proliferation (Weinberg and Chandel, 2009).

Detoxification of ammonia:Some functions of the mitochondria are carriedout only in some specified kinds of cells. For instance, mitochondria that are present in cellsof theliverpossess enzymes that permit them to detoxify ammonia, a protein metabolism by – product.

Production of ROS: It generates ROS, which have beeninvolved in controlling different physiological processes, but which could equally be detrimental if generated more than necssary (Li *et al*., 2013).

Blood and hormones building: It helps in synthesizing certain components of blood and hormones like testosterone and estrogen.Other functions of these organelles are:

- Membrane potentialregulation (Voet*et al*., 2006)
- Calcium signalling (including calcium-evoked apoptosis) (Hajnóczky *et al*., 2006)
- Cellular metabolismregulation (McBride*et al*., 2006)
- Steroid synthesis (Rossier, 2006).

2.26P53 PROTEIN

The p53 protein, as well referred to as tumour protein 53 (or TP 53), is a prominent tumour suppressor known and it is encoded by the tumour suppressor gene TP53 sited at the short arm of chromosome 17 (17p13.1). This protein derived its named from itsmass, 53 kDa (Levine *et al*., 1991). Since p53 was identified, many studies have investigated to ascertain its influence and the role it plays in neoplasia. It does not solely participate in triggering cell death, but as wellplay a crucial part in modulation of cell cycle, gradual formation of organs,DNA recombination, gene enlargement, separation of chromosome and ageing of cell (Oren and Rotter, 1999) and is referred as “guardian of the genome” (Lane, 1992).

Over 50% of human cancers have been connected to malfunctions in p53 tumour suppressor gene (Bai and Zhu, 2006). It has also been reported recently that some target genes of p53 that are engaged in regulation of cell cycle and cell death are exceptionally present in melanoma cells, resulting in the aberrant action of p53 and promotingenlargement and propagation of these cells. Furthermore, it has been discovered that when p53 mutant was made inactive or

suppressed, such suppression of mutant p53 expression caused a decreased cell group proliferation in human malignancies, which was ascertained to be caused by stimulation of cell death (Vikhanskaya *et al*., 2007).

The notions for the main function of cell death checkpoints in defence in opposition to malignant transfiguration presents the tumour suppressor p53, which is presumptively the very widely examined cell death factor, as promoting neoplasia because it is rendered inactivate in approximately over fifty percent of allneoplasia in human. The p53 is a tumour suppressor protein that is actuated as a transcription factor in reaction to stimuli, such ashypoxia,activation of oncogene and especially,injury to DNA, resulting toarrest of growth and/or cell death by triggering different p53 target genesexpression,e.g., p21, Bcl-2 associated x-protein, Puma, Noxa, Apaf-1, First Apoptosis Signal (Fas), and Death Receptor 5 or by repressing antiapoptotic proteinsexpression, e.g. Bcl-2, Bcl-XL or survivine. Up-to-date proof recommends transcription-independent p53 cell death routes in which p53 transfers to the mitochondria, interacts with Bcl-X_L, induce PT and discharge of cytochrome c (Hainaut and Hollstein, 2000).

The p53 protein is a transcription agent playing an important functionas abnormal growth suppressor. The p53 quantities are sustained at low concentrations in normal cells by disintegration brought about by MDM2 (Liang *et al*., 2001). Whenever stresses likedamage to DNA and hypoxia occur, p53wild-type will respond by instigating genes that bring aboutarrest of cell cycle and, if the stress is highly extreme, apoptosis will result (Alarcon-Vargas *et al*., 2002). The relevance of p53 is depicted by mutant p53 presence in 50% neoplasia in human. When there isinactivation of p53 either by mutation or deletion, cellsbecome more and more susceptible to injurious transfiguration. The lack of functioning p53 proteinenhances accumulation ofmutations that could bring about tumourigenesis (Hainaut and Hollstein, 2000). The p53, as well referred toas the “guardian of the genome” is being illustrated to serve a crucial role in intrinsic tumour retardation by two processes, and these include arrest of cell cycle and instigation of apoptosis. Diverse triggers like injury to DNA, stimulation of oncogene, and erosion of telomere could lead to provocation of p53. Injury to DNA can be as a result ofdrugs or radiation exposure (Lane, 1992).

2.27CYTOCHROME C

Thiscomplex is a minutehemeprotein loosely found connected with the interiormitochondrialmembrane. It is an important constituent of the ETC, where it transfers a single electron. It has the ability of participating inreductionand oxidationreactions, but does not interact withoxygen. It is encoded by *CYCS*gene in human (Schneider and Kroneck, 2014).

FUNCTIONS

Cytochrome c is a known constituent of ETC in the mitochondria. The heme portion of cytochrome c receives electrons from complexbc_1 and relays them tocomplex IV. This protien is as wellfound to be actively implicated in triggeringapoptosis. Whenever it is liberated, it attaches to Apaf-1 (Tafani *et al*., 2002). It catalyses various reactions likearomaticoxidation andhydroxylation, and exhibits peroxidase activity by oxidation of severaldonors of electron, for example,4-aminoantipyrine and 2-keto-4-thiomethyl butyric acid(McPherson and Delucas, 2015).

Cytochrome c is as well an intermediary in apoptosis, a regulated type of cell death applied to destroy cells in the process of growth or as a consequence of infection or damage to DNA. Cytochrome c is fastened to cardiolipin,which is found in the interior membrane of the mitochondria, thus stabilizing its presence in the internal mitochondrial membrane. This binding of cytochrome c to cardiolipin prevents its liberation from mitochondria and triggering death of cell(Orrenius and Zhivotovsky, 2005).

At the initial stage of apoptosis, production ofROS in the mitochondria is prompted, and cardiolipin undergoes oxidation through a peroxidase working of the cardiolipin–cytochrome c intricate. This thus, enhances detachment ofhemoprotein from mitochondrial internal membrane and could cause its extrusion into the soluble cytosol by means of pores present in the exterior membrane (Boehning *et al*., 2003). When cytochrome c is released, it prompts caspase 9, andcaspase 9 can subsequnetly go on to triggercaspases 3 and 7, which are the primary cause for ravaging the cell from inside(Neupert, 1997).

2.28 EXTRAMITOCHONDRIAL LOCALIZATION

Under normal physiological conditions, cytochrome c is commonly believed to be fixed mainly in the space between membranes of the mitochondria(Kroemer *et al*., 1998). The actuation

ofcaspases, which is primarily known asthe evocation, bringing about the initial phase of cell death is preceded by the liberation of cytochrome c to the cytosol(Loo *et al.,* 2013). The degree of occurrence of apoptosis could be monitored byquantifying the concentration of cytochrome c leaking into cytosol and culture medium (Waterhouse and Trapani, 2003; Soltys *et al*., 2001). There are compelling evidencefrom thorough immunoelectron microscopic studies with tissues sections of rat making use cytochrome c-definite antibodies that cytochrome c under usual physiological states is as well present at places outside the mitochondria (Gupta *et al*., 2008). Sturdy and explicit presence of cytochrome c has been identified in granules of zymogen and in growth hormone granules,inanterior pituitary andpancreatic acinar cells,respectively. Cytochrome c as well, was identified in the pancreas condensing vacuoles and in the acinar lumen (Gupta *et al.*, 2008).

2.29SPERMINE

This is apolyaminethatoccurs naturallyin all eukaryotes, but not very common in prokaryotes. This compound serves an essential role in cell formationboth in healthy and neoplastic tissue. It is produced by an enzyme known as spermine synthaseby means of adding an aminopropyl group to spermidine. Spermine is characteristically a strong base, and in water solution, all of its amino groups will be positively chargedat physiologic pH (Totowa, 1998).The function of spermine including other polyamines in affecting RNA structure and protein function has been published ina review (Igarashi and Kashiwagi, 2000). This compound is widely applied in molecular biology and biochemistry studies. Utilization of spermine is being employed in chromosome separation and in the gathering together of chromatin (Cram, *et al.*1990; Marquet, *et al*., 1986). Spermine may also serves as micromolecules for the preparation of gene transfer agents (Ronsin*et al*, 2001; Azzam *et al*. 2002). The formation of an intricate between spermine and DNA to form particles with diameter <100 nm has been investigated (Trubetskoy*et al*., 2003). Spermine has been used in the crystallization of DNA (Shui, *et al*. 1998; Saminathan, *et al*. 2002).

2.30ROTENONE

The compound rotenoneis described as an odourless, colourless, crystallineisoflavone and it is important as a wide-rangeinsect killer,pesticide and piscicide. It is naturallyavailable instems andseeds of numerous plants, like theroots of many members of Fabaceae and thejicama vine

plant. It is anoutput naturallyobtained from definitetropical and subtropical family members of the pea found in Southeast Asia and South America. The application of this compound byhuman could be for a long time ago, as early explorers found Peruvian natives applying crude extracts of the Cubé plant to incapacitate fish for consumption. Its benefits include as insecticide, pesticide, and as a nondiscriminate piscicide (fish killer). It has been recorded that rotenone is being used by native peoples to catch fish(Peter, 2007).

Toxicity of Rotenone

The World Health Organizationclassifiedrotenone as mildly harmful. It is softly harmful to other mammals and humans, but severely harmful to insects and life in water, such as fish. This severeharm ininsects and fish is because the lipophilic rotenone is freely breathed in by means oftrachea or gills, but not as freely viagastrointestinal tractor the skin. Rotenone is known to be harmful to erythrocytes *in vitro* (Lupescu*et al*., 2012). The lowest fatal dose for an infant is 143 mg/kg, but deaths in human resultingfrom poisoning by rotenone are uncommon because its irritating effect prompts puking. It has been revealed that deliberate ingestion of rotenone can be deadly (Wood *et al*., 2005).Rotenone is toxic due to its efficacity in interfering mitochondrial electron transfer which impedes the utilization of oxygen in respiratory organisms, resulting todeath of cell and subsequently death of the entire living thing if the measured portion taken is elevated enough.

Rotenone breaks down as soon as it is subjected to sunlight and it normally possesses an activity that can last for about six days in the surrounding (Vitax, 1998). It undergoes oxidation to rotenolone, which is approximately a sequence of measurenot as toxic as rotenone. The extent of its decomposition in wateris dependenton diverse conditions, such aspH, temperature, sunlight and hardness of water.

Mechanism of action of Rotenone

Rotenone works through its interruption with mitochondrialelectron transfer sequence. Itspecifically impedes transport of electrons to ubiquinonefrom centers of iron-sulfur in complex I. This interrupts NADH in the process of generation of cellusable energy (ATP) (Hayes, 1991). Complex I isnot able to transportto CoQits electron, generating accumulation of electrons inside the matrix of the mitochondria. Molecular oxygen is then diminished to the radical, generating

ROS, which could cause injury toDNA and other mitochondrialcomponents (Mehta, 2014).The mechanism of how rotenone interrupts electron transport is identical with other well-studiedinhibitors of respiration.Themechanism ofrespirationof fish is exactly connected to water by means of gills, thus rotenone may pass straight into the bloodstream of fish, resulting in death. It exhibits lowerharmful effect toavesand mammals as their route of ingestion is via the gut where a lot of the compound is decomposed to lower toxic components before toxic quantities can pass into the bloodstream.

2.31LIPID PEROXIDATION

Lipid peroxidation is a sequence of linkedprocess of reaction that is characterized by repeatedlyremoval of hydrogenby groups, such as HO. and RO., and addition of O_2 to alkyl radicals (R.) bringing about production of ROO., and in the oxidative damage offatty acids that are polyunsaturated, in which the methylene group (=RH-) is the major focus (Halliwell and Gutteridge, 1984).Lipid peroxidation refers to the effect of accumulation of ROS, which brings about degeneration of biological systems. It could be triggered mostly byROS, which act by removing allylic atom of hydrogen from a methylene group of polyunsaturated fatty acid side series. This is subsequently followed by rearrangementof bondsthat lead to stabilization throughformation of diene conjugate. The lipid radicals that are formed then pick up oxygen to generate peroxy species.Human beings live in an environmentthat is highly characterized with oxidative activities and many processes which are involved in metabolism could lead to the generation of greater oxidants (Rui and Boyer, 2004).

Lipid peroxidation generally brings about a reduction in fluidity of membrane and also interfers in the membranesbarrier functions. During pathological conditions, the nitrogen species and reactive oxygen are generated at rates that are higher than normal, and consequently, lipid peroxidation results with α-tocopherol deficiency. Moreover, cells and organelles membranesare frequently being exposed to several kinds of injuries and possessing elevated quantities of transition metals and polyunsaturated fatty acids (Halliwell &Gutteridge, 1984).

Peroxidationof lipid brings about the formation of intricate variety of outputs and majority of these products are reactive electrophiles. Some of these products interact withDNA and protein and thus, are harmful and capable of causing mutation (Porter, 1986). The malondialdehyde (MDA) seems to be the greatest mutagenic output resulting from peroxidation of lipid, whereas

4-hydroxynonenal (HNE) is the highest harmful (Esterbauer, *et al*.,1990). The MDA has been applied for several years as an easyperoxidation of lipidbiomarker due to its ready reaction with thiobarbituric acid to generate an extremely coloured chromogen. Furthermore, the reactionof thiobarbituric acid is widely knownto be non-selective and has contributed to significant arguments upon its application for measurement of MDA from *in vivo* samples. Isoprostanes haverecentlyemerged as prominent biomarkers of peroxidation of lipid and their benefit has become effective in many*in vivo* studies (Morrow and Roberts, 1996). Analysis of greatly isolated MDA prepared by three independent methods, however, indicated that this compound iscapable of causing mutation (Basu and Marnett, 1983).

Oxygen radicals and some other reactive species are formed in living systems either as wasteoutputs of oxygen reduction or through xenobiotic decomposition (Pradeep and Ajudhin, 2011). These ROS, for example,nitric oxide (NO),hydroxyl radicals (OH.),superoxide anion (O^{2-})and peroxy radical (ROO.) are not constant and can interact with the major macromolecules likeproteins, lipids and nucleic acids (Amarowicz*et al*., 2010). The repercussions of oxidation of these biomolecules have been connected to manydisorders inhuman such ascancer,atherosclerosisand ailment of the nervous system (Supardy *et al*. 2011). Free radicals likeROS which are synthesized from metabolism and or by environmental conditions attack biological systemsdirectly and the substantial production of these radicals have been linked to different chronic diseases including atherosclerosis, cancer,diabetes, arthritise.t.c. (Dzingiral *et al.*, 2007; Chance *et al.*, 1979).

Cells possess an all-encompassing order of antioxidant defence processes to ameliorate free radical generation or restrict their injurious outcomes (Sato *et al*., 1996). These processes are inadequate when the balance moves in favour of free radicals production (Gulcin *et al*., 2002), thus body requires antioxidant supplements to decrease oxidative damage and slow down lipid peroxidation. Nowadays, the application of synthetic antioxidants is restricted because of harmful effects associated with them when used at optimum concentration.Antioxidants are compounds commonly known to suppress or completely impede the oxidation of fats and oil, or other molecules by restraining the dissemination of sequence of reactions involved in oxidation (Halliwell *et al*., 1999). The antioxidant present in the body's defence system functions to preserve the cells from over production ofROS and is composed of both endogenous including

uric acid,bilirubin, superoxide dismutases, glutathione peroxidase, catalase e.t.c., and exogenous such as tocopherols,bioflavonoids, ascorbatee.t.c. (Cross*et al*., 1987).

The natural antioxidant mechanisms is often inadequate and inefficient, therefore intake of dietary antioxidant compounds becomes imperative. Plants contain wealth of phytochemicals such as flavanoids,saponin, tannin, alkaloids and phenolic, which exhibit several biological activities including antioxidant potential. Damagesthat are often caused in living organisms by immoderate ROS generation and concurrentperoxidation of lipid, damage to protein and breaking of DNA strand can be prevented by antioxidants. The applications of antioxidants which occur naturallyare in high demand in bio-pharmaceuticals, food additives and nutraceuticals. Thus, great attention is giving tonatural antioxidants present in herbs and plant. Phytochemical constituents of plants are known to serve as lipid peroxidation inhibitorsand free radicalsscavengers (Beutner *et al*., 2001).

2.32 BIOLOGICAL IMPLICATIONS OF PEROXIDATION OF LIPID

There are interesting proofs in experimental models ofratliver, of elevated lipid peroxidation secondary to enhanced mitochondrial generation of O^{2-} and H_2O_2 (Navarro & Boveris, 2007; Navarro *et al*., 2009).Peroxidation of lipid in living organisms has two repercussions namely,damage to membrane structure and secondary products generation. This outcome is harsh for biological systems, cause destruction to membrane function, inactivation of enzymatic and toxic effects on cell division and function(Catala, 2006).

Oxidative stress is a common mechanism, which always leads toinjury to cellsthat takes place with elevated lipid peroxidation of phospholipids of cell and has been involved in diverse cell abnormalities (Sies, 1991a; Catala, 2006). Aldehydes are known to highlyintereact with macromolecules, such as DNA,phospholipids and proteins producing intra- and intermolecular adducts. Under normal physiologicstates, thequantities of these outputs are low; nonetheless,pathological situations will lead to higher concentrations of these products. Therefore, damage to DNA resulting fromperoxidation of lipid end products could profferencouraging markers for risk prognostication and focus for preventive measures.The HNE and MDA which are outcome of lipid peroxidation cause destruction toproteinthrough their

addition reactions withcysteine sulfhydryl groups, lysine amino groups and histidine imidazole groups (Esterbauer, 1996).

Structural alterations of protein by aldehyde products obtained fromperoxidation of lipid are responsible for neurodegenerative abnormalities and actuation of kinases and nuclear transcription factor inhibition.The correlation betweenplant antioxidants and decrease of chronic diseases has been indicated by several epidemiological studies(Sasikumar *et al*., 2010; Lieu, 2003). These beneficial effects are believed to be attributedto antioxidant components present in plants including vitamins, carotenoids and flavonoids (Rice-Evans, 2001). Recentstudies have demonstrated that phenolic substances obtained from plants can scavenge ROS and efficiently inhibit oxidative cell destruction. A better choice to satisfy the purpose of finding a suitable management for ameliorating free radicals generation could be achieved through the use of herbal products (Divya and Mini, 2011).

2.33ATPases

ATPasesare a group of enzymes that catalyze the breaking down of ATP into ADP and a free phosphate ion (Geider and Hofmann-Berling, 1981; Njus *et al*. 1981; Kiley and Peters, 1981). This reaction which involves the removal of phosphate group from ATP(dephosphorylation) is accompanied with the release ofenergy, which the enzyme always uses to carry out other chemical reactions that would not anyway occur. This process is generally useful in all forms of lives.

In the interior membrane of eukaryotic mitochondria are present F0F1-ATPase/ATP synthase (F-type ATPase, complex V) which serves as powerhouse by generating ATP for the cell. This enzyme can equally work in the reverse modeby decomposing ATP and pumping protons during adverse conditions such as cytotoxic drugs, ROS etc. This enzyme can be differentiated into two main intricacies namely, F1 and F0 (Pedersen & Amzel, 1993; Boyer, 1997).

2.34 TRANSMEMBRANEATPase

These are integral proteins of the membrane which are anchored within biological membranes, and they function to transport solutes across the membrane, specifically against their concentration gradient. Transmembrane ATPases play significant role in importing many of the

metabolites essential for cellmetabolism and exporting by-products,solutes andtoxins that can inhibitprocesses of cell.

CHAPTER THREE
MATERIALS AND METHODS

3.0**Collection of *D. oliveri*Stem Bark**

Stem bark of *Daniellia oliveri*(DO) was purchased from Bode market, Ibadan and authenticated by Department of Botany, University of Ibadan Herbarium (UIH - 22383). The stem bark was dried at room temperature (28 – 30^{o}c) for four (4) weeks (30 days) and pulverized to minute particles by means of a mortar and pestle. The powdered stem bark was stored at room temperature in a clean bottle.

3.1 EXTRACTION

Ethanol (500 ml) was poured unto 50g of the pulverized stem bark in a glass container. The mixture was stirred, covered, and left standing for 72 hours and filtered applying sterile whatmann No 1 filter paper. With the use of rotory evaporator the bright yellow filtrate (extract) referred to as ethanol extract of *D. oliveri*(EEDO) was concentrated and the crude extract obtained was evaporated to dryness using water bath at 40^{o}c. n-hexane was used to defat the crude ethanol extract which was then separated successively between, chloroform, ethyl acetate and ethanol using Vacuum Liquid Chromatography (VLC) method to obtain the various fractions of chloroform, ethyl acetate and ethanol fraction of *D. oliveri*(CFDO, EAFDO and EFDO) respectively. The fractions were then concentrated using rotary evaporator, evaporated to dryness using water bathat 40^{o}c and stored in clean glass bottles.

3.2 Vacuum Liquid Chromatography of Ethanol Fraction of *Daniellia oliveri*

3.2.1 Packing of the Chromatographic Column

The prewashed sintered Buchner glass was further washed using concentrated H_2SO_4 to remove impurities from the sieve. The column was then packed with silica gel (0.04 – 0.063 mm MERCK) to three quarter full. The column was then packed on a conical Buchner flask and

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

3.2.2 Preparation of the sample slurry

Silica gel 60(0.04 – 0.063 mm MERCK) 12 g was added to 20 g of the ethanol fraction of *D. oliveri* sample. The gel – sample mixture was stirred until a homogenous mixture was obtained. The mixture was air-dried to obtain a powdered form.

3.2.3 Loading of sample on the column

The sample was applied to the top of the column with the pump switched on. The first solvent system – 100% n-hexane was added to the column. This was done with 700 ml of the n-hexane solvent. The column was eluted again with n-hexane: chloroform (1:1), made by mixing 50 ml of n-hexane with 50 ml chloroform. This was done until there was a complete exhaustion of the fraction in the column. The column was further eluted with chloroform only (100%); chloroform: ethyl acetate (1:1); ethyl acetate (100%); ethyl acetate: ethanol (1:1) and lastly with ethanol (100%). The fractions were concentrated using rotary evaporator at 40^{o}c and then transferred into pre-weighed all-glass sample bottles and labelled. Thin layer chromatography (TLC) of the fractions were carried out in order to ascertain the purity and also to identify the phytochemicals present in each of the fractions gotten from the solvent systems.

3.2.4 Thin Layer chromatography

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

3.3 EXPERIMENTAL ANIMALS

Albino rats (male)each weighing 80 -100 g were purchased from Veterinary MedicineDepartment animal house, University of Ibadan, Ibadan, Nigeria. The rats were kept in ventilated cage with 12 hours light/dark cycling and were allowed access to food and water

freely. The rats were kept for a fortnight for acclimatization and standardization of their body weight before experimental work.

3.4.0 METHODOLOGY/ PROCEDURES

Phytochemical Screening

Screening forthe secondary metabolites present in *D. oliveri* was performed using standard methods (Sofowora, 1993; Trease and Evans, 1989).

Reducing Sugars test(Fehling's Test): Ethanol fractions (0.5 g) were dissolved in 5 ml of water and boiling Fehling's solution (A and B) were added to test tubes.The changes in colour were viewed for the various fractions, which show thatreducing sugar is present.

Flavonoids:Three methods were carried out to prove presence of flavonoids in *D. oliveri*fractions. First, 5 mlof solution of ammonia was introduced to a portion of fractions of *D. oliveri* in different test tubes followed by 1 ml of concentrated H_2S0_4. The appearance of yellow colour which vanishes on standing confirms thatflavonoids are present. Second, to a portion of fractions of *D. oliveri*a few drops of 1% solution of aluminium were added. Flavonoidspresence is confirmed by the formation of yellow colour. Third, a portion of the fractions of *D. oliveri* were heated in separate test tubes with 10 ml of ethyl acetate over a steam bath for three minutes. The mixtures were filtered and 1 ml of ammonium solution is added to 4 ml of the filtrate and shaken. Flavonoids presence is confirmed by the formation of yellow colour.

Saponins:To 0.5 g of fractions of *D. oliveri* in separate test tubeswas added 5 ml of distilled water. The mixture were vigourously mixed by shaken rapidlyand viewed for constant continuous foam.3 drops of olive oil were mixed with the frothings and were shaken vigourously after which it was viewed foremulsion formation, which confirms saponins presence.

Coumarins:Distilled water (5 ml) was addedto 0.5 g offractions of *D. oliveri*and then heated to evaporate.The residue is then dissolved in 1-2 ml of hot distilled water and shared into two portions. To each one portion was added 0.5 ml 10% NH_4OH, while the second part served as

control. Two spots of each is then put on filter paper and viewed under UV light. Intense fluorescence confirms that coumarin is present.

3.4.1. Isolation of rat liver mitochondria

Isolationof low ionic strength mitochondria was carried out by the method of Lapidus andsokolove (1993). The animals for the assay were sacrificed viadislocationat the cervical region, dissected and the livers were excised and washed with isolation bufferuntil a neat liver was obtained.It was then weighed and chopped with a pair of scissors. The minced liver was then homogenized in a 10% prepared suspension in a Teflon-glass cup homogenizer. The above steps were all carried out on ice in order to maintain mitochondrialintegrity. The suspended tissue (liver) in isolation buffer was then poured into a centrifuge tube and spinned in a refrigerated MSE centrifuge at 2,300 rpm twicefor 5 minutes in order to sediment nuclear fraction and cell debris.The supernatant was decanted and spinned for 10 minutes at 13,000rpm to sediment mitochondria. The brownish mitochondrial pellet gotten after discarding the supernatant was then washed twice by re-suspending in washing buffer and spinned for 10 minutes at 12,000rpm. The washed mitochondria were at once suspended in a solution of ice-cold suspension buffer, then poured in Eppendorf tubes in aliquot and kept on ice for use.

3.4.2.ASSESSMENT OF MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE

Calcium overloadcan cause mitochondrial membrane permeability transition. The interior membrane under this condition becomes non-discriminately permeable to small molecules (1500 Da). Isolated mitochondria undergoing Permeability Transition (PT) exhibit colloid property, that is, large amplitude swelling which brings about a decrease in photometric absorption at 540 nm. Many experimental PT were performed by measuring the enlargement of mitochondria through overseeing the associated reduction in light scattering.

PRINCIPLE

The tenetof the assay is established on the fact that during the swelling of the mitochondria, their refractive index varies and hence low light is allowed to pass through the cuvette bringing about a reduction in absorbance of light measured with a spectrophotometer. In a means to evade any

intricacies that variations in the redox condition of respiratory sequence components may result, the wavelength of the striking light should be at the isobetic point from the cytochromes (540nm) as applied in manifolds investigation on separated mitochondria.

Reagents

- Rotenone (0.8µM): Rotenone (0.00079 g) (Sigma-Aldrich, Germany) was dissolved in 50% ethanol (5ml ethanol with 5ml water). The rotenone was preserved in a dark container because of its photosensitivity at 4^0C.
- Sodium Succinate (5mM): Sodium succinate (0.6754g) was dissolved in 10ml of distilled water(Sigma-Aldrich, Germany).
- $CaCl_2$(3µM): $CaCl_2$(0.01764g) (May and Baker Lab., Products) was dissolved in 10ml of distilled water and kept in a container.

PROCEDURE

Mitochondria (0.4 mg/mL protein)were pre-incubated in the presence of 0.8µMrotenone for 3.5 minutes.Then 25 µL of 5mM Sodium succinatewas added and absorbances were taken for 12 minutes at 30 seconds interval.Assay for mitochondrial swelling without a stimulating agent (Ca^{2+}) was carried out by preincubation of the mitochondria in a suspension buffer, 0.8µM rotenone for $3^1/_2$minutes after which 25 µl of 5mM succinate was introduced and absorbances were read after every 30 second for 12 minutes. Assay for mitochondrial swelling in the presence of an inducing agent (Ca^{2+}) was carried out by preincubation of the mitochondria in a suspension buffer, 0.8mM rotenone for 3minutes after which Ca^{2+} was added. Thirty (30) seconds later 25 µL of 5mM Succinate was added and absorbances were read after every 30 second for a period of 12 minutes. Assay for mitochondrial swelling inhibition in the presence of Spermine involved preincubation of mitochondria in suspension buffer, 0.8mM rotenone and 1mM Spermine for 3minutes after which Ca^{2+}, the triggering agent was added then 30 seconds after, 25 µL of 5mM Succinate was introduced to energise the reaction. Absorbances were taken at 540nm wavelength in a Camspec M105 spectrophotometerfor 12 minutesat 30 seconds interval.

Table 1: Protocol for Mitochondrial swelling

Samples	Swelling Buffer(µl)	Rotenone (µl)	Spermine (µl)	Mitoch-ondria (µl)	$CaCl_2$ (µl)	Extract(µl)	Succinate (µl)
Blank	2500	-	-	-	-	-	-
No triggering agent	2200	10	-	30	-	-	50
+ triggering agent	2200	10	-	30	25	-	50
+ Spermine	2200	10	62.5	30	25	-	50
Daniellia oliveri	2200	10	-	30	-	10	50
Daniellia oliveri	200	10	-	30	-	30	50
Daniellia oliveri	2200	10	-	30	-	50	50
Daniellia oliveri	2200	10	-	30	-	70	50

Note: Readings were taken at 540nm

3.4.3.DETERMINATION OF PROTEIN

Mitochondrial protein was determined as describedby Lowry *et al.*, 1951.

DETERMINATION OF PROTEIN BY LOWRY'S PROCEDURE

Protein level of the mitochondria was quantified by the procedure of applying Bovine Serum Albumin (BSA) as level of quality.

PRINCIPLE

The colour reagent applied in this assay is a phosphor-18-molybdictungstic intricate, which is adifferent mixture of molecular forms like $3H_2O.P_2O_5.9MoO_3$ and $3H_2O.P_2O_5.10WO_3.8MoO_3$, which can be reduced by phenol groups giving a blue colour at pH of alkaline. Tyrosine and/or tryptophan presence in the protein accounts for this phosphor-18-molybdictungstic intricatereduction. This procedure is very sensitive andthe colour reagent which is as well referredto as "phenol reagent", is very fickle and disintegrates freely insolutions that are alkaline because it reacts with tyrosine only atpH that is alkaline; an excess of the reagent need to be introduced to it for absolute reaction. Furthermore, an increased concentration of this phosphor-18-molybdictungstic acid can give turbidnessbecause of theinsoluble salt formation. In order to surmount this difficulty, the procedure was re-examined and it was illustrated that lithium salts addition to the reagent would prevent turbidness. In their mixture called 'Folin-Ciocalteau reagent', they also added some bromine water to maintain the phosphor-18- molybdictungstic reagent in the oxidized condition during storage.

Lowry *et al.*(1951) ascertained that pre-treatment of sample of protein with copper in alkaline mediumconspicuously raised the formed colour during reduction reaction of phosphor-18-molybdictungstic reagent. In their assay medium, they equally introduced NaOH and

Na_2CO_3mixtureto stabilize the pH around 10 and to make the Phosphoric acid formed neutral by decomposition ofphosphormolybdictungstic intricate at pH that is alkaline.

The Folin-Ciocalteau test is sensitive: as small as 5mg samples of proteincan easily be assessed. The protein reaction in solution with Folin reagent takes place in two stages, which lead to the ultimate protein colour.

1) Reaction with Cu in alkaline medium: Cu^{2+} + Protein $\xrightarrow{Cu2+}$ Protein

2) Reduction of the phosphomolybdictungstic reagent by the Cu-treated protein

Reagents

- Reagent A: 2% Na_2CO_3 in 0.1M NaOH. 2g Na_2CO_3(BDH Chemicals Ltd, England) and 0.4g NaOH (Sigma Chemical Co, USA) were dissolved in 80ml of distilled water, made up to 100 mL in a volumetric flask and kept at room temperature.
- Reagent B: 2% Na-K-tartrate. 2 g Na-K-tatarate (Hopkins and Williams England) was dissolved in 80mL of distilled water, made up to 100 mL in a volumetric flask and stored.
- Reagent C: 1% $CuSO_4.5H_2O$. 1g $CuSO_4.5H_2O$ (Sigma Chemical Co, USA) was dissolved in 80 mL of distilled water, made up to 100 mL in a volumetric flask.
- Reagent D: Copper Sulphate in alkaline solution was prepared just before use- 50ml,0.5ml and0.5mLof Reagent A, B and C, respectively were mixed.
- Reagent E: Folin-Ciocalteau reagent- a solution of Reagent E was prepared by diluting 2ml of 2N Folin's reagent (Sigma Chemical Co, USA) with 2mL of distilled water.

PREPARATION OF FOLIN-CIOCALTEAU REAGENT

100g of Sodium Tungstic ($Na_2WO_4.2H_2O$) and then 25g of Sodium Molybdate ($Na_2MoO_4.2H_2O$) were dissolved in about 700ml of H_2O. 100ml of concentrated HCl and 50ml of 85% phosphoric acid and a few drops of bromine was introduced to the mixture and refluxed for 10hours in an all-glass apparatus. The mixture was later cooled, filtered after diluted to 1 litre, and stored in a refrigerator at 4^0C. The reagent is prepared in a fume cupboard and stored in a black container because it is photolytic. The colour of the reagent is golden-yellow and if it acquires a green colour it is unsatisfactory for use and may be regenerated by boiling with a few drops of bromine. The 2Nsolution was usually diluted to 1N using distilled water just before use in the experiment.

3.4.4.PROTEIN STANDARD SOLUTION

1mg/ml Bovine Serum Albumin (BSA) (Sigma Chemical, USA) was prepared by dissolving BSA (5mg) in distilled water (5 mls). Out of this stock solution 1ml was taken and mixed with distilled water (19 ml)to get a solution with an absorbance of 1.140 at 279nm. This is established on the basis that the molecular extinction coefficient (E) of BSA is 45,000 and its molecular weight is 65,000. This absorbance will give a concentration of 200µg/ml for the solution. The assay was performed in duplicates.

PROCEDURE

Reagent D (3mL) was added to protein sample,mixed together and standat room temperature for 10 minutes.Reagent E (0.3ml)was subsequently addedand the mixture was quickly and vigorously shaken and left standing at room temperature for 30 minutes.Theabsorbance was then read at 750nm wavelength usingCamspec M105 spectrophotometer and the absorbances were plotted against protein concentration to obtain theprotein standard curve.

SAMPLE PREPARATIONS

Sample: 990 µl distilled water and10µl of mitochondriawere mixed in test tubes in duplicate.Blank Preparation: 1000µl of distilled water was put in a test tube. 3.0ml (3000µl) of Reagent D was introduced to the test tubes (sample and blank) and left for 10 minutes. After this, 0.3ml (300µl) of Folin-C was introduced and left to stand for 30 minutes after which absorbance readings were taken at 750nm wavelength using a Camspec M105 spectrophotometer.

Table 2: Protocol for Protein Estimation (Lowry *et al*., 1951)

Test tubes in duplicates	1	2	3	4	5	6	7	8	9
Standard BSA (µl) solution	-	100	200	300	400	500	600	700	800
Distilled H_2O (µl)	1000	900	800	700	600	500	400	300	200
Reagent D (µl)	3000	3000	3000	3000	3000	3000	3000	3000	3000
Folin C (µl)	300	300	300	300	300	300	300	300	300

BSA: Bovine Serum Albumin

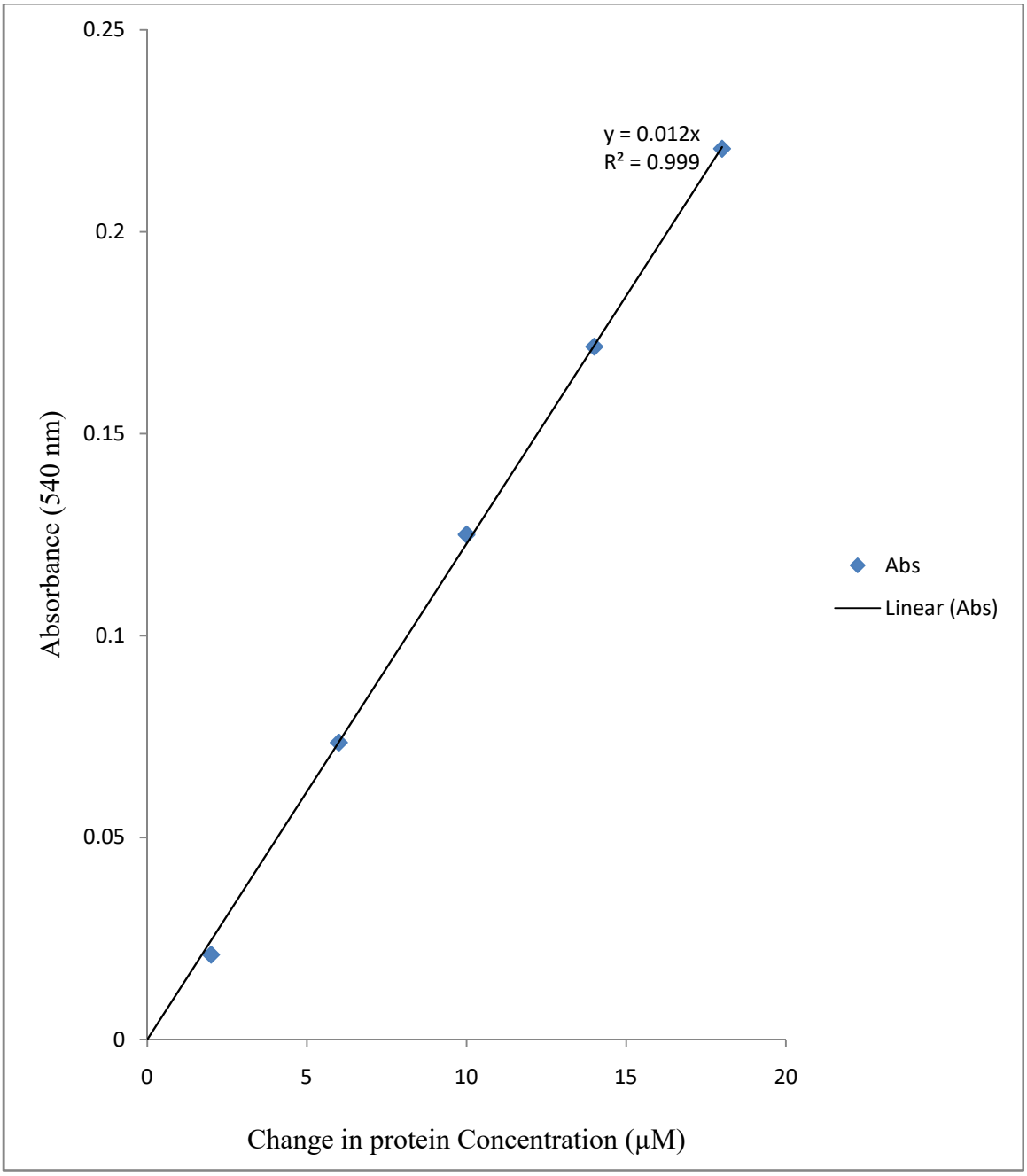


Figure 5: Standard protein curve

3.4.5LIPID PEROXIDATION (LPO) DETERMINATION:Thiobarbituric acid reactive species (TBARS)modified assay method was employed to investigateformation of lipid peroxide,using mitochondria homogenates as media rich in lipid, as portrayed by Ruberto *et al*., (2000).

PRINCIPLE

The principle for this assay is established on the reaction between Thiobarbituric acid (TBA) and malondialdehyde (MDA), an end product of lipid peroxides during LPO. A pink coloured intricate is generated on heating in acid solution, which maximally absorbs at 532nm and fluoresces at 533. It is easily extractable into organic solvents like butanol. This assay is always caliberated using MDA as the standard hence, the results are represented as quantity of free MDA generated.

REAGENTS

A. 20% Acetic acid

   This was prepared by measuring 20 ml of acetic acid into 80 ml distilled water and make up to 100 ml in a standard volumetric flask.

B. 0.8% (w/v) TBA in 1.1% SDS

   This was prepared by weighing 0.8 g TBA and 1.1 g Sodium Dodecyl Sulphate (SDS) into a beaker and dissolved with distilled water and make up to 100 ml in a standard volumetric flask.

C. $FeSO_4$ (0.07 M)

   This was freshly prepared by weighing 0.001064 g of $FeSO_4$, dissolved in 1 ml of distilled water.

PROCEDURE

To each test tube in duplicates, 35 µl of mitochondria was added followed by plant extract (50, 100, 200, 400 and 800 µl) to designated tubes and these were made up to 1000 µl, respectively with distilled water. Freshly prepared 0.07M $FeSO_4$ (50 µl) was then added to each test tube and incubated for 30 minutes at room temperature. Acetic acid (1500 µl) was then added followed by TBA in SDS (1500 µl). The solutions were then incubated in a water bath at 95^0c for 1 hour. The solutions were removed, allowed to cool and butanol (5 ml) was added and vigorously shaken. These were then centrifuged at 3000 rpm for 10 minutes using table centrifuge. The absorbances of the clear supernatant were then read at 532 nm using spectrophotometer.

Table 3: Protocol for lipid peroxidation determination

	Mit. (µl)	Extract (µl)	H_2O (µl)	$FeSO_4$ (µl)	Acetic acid (µl)	TBA:SDS (µl)	Butanol (mls)
Mit. only	35	-	965	50	1500	1500	5
Extact only	-	800	200	50	1500	1500	5
NME	-	-	1000	50	1500	1500	5
50µl extract	35	50	915	50	1500	1500	5
100µl extract	35	100	865	50	1500	1500	5
200µl extract	35	200	765	50	1500	1500	5
400µl extract	35	400	565	50	1500	1500	5
800µl extract	35	800	165	50	1500	1500	5

TBA:Thiobarbituric acid

SDS: Sodium Dodecyl Sulphate

NME: No Mitochondria and Extract

3.4.6 ATPase ACTIVITY DETERMINATION

ATPase activity was determined as described previously(Lardy and Wellman, 1953) as modified by Olorunsogo and Bababunmi (1979).Concentration of inorganic phosphate released was determined as described by Bassir (1963).

REAGENTS

A. 0.25M sucrose

This was prepared by adding distilled water to 8.56g of sucrose, dissolved and make up to 100ml in a standard volumetric flask with distilled water and kept in the refrigerator at 4^0C.

B. **KCl (5mM KCl)**

Distilled water was added to 37.25mg KCl, dissolved and make up to 100mlin a standard volumetric flaskwith distilled water.

C 0.01M ATP (pH 7.4)

This was prepared by addinglittle quantity of distilled water to 0.2757 g of disodium salt of ATP, dissolved and the pH adjusted to 7.4. More distilled water was then added to make up to 100ml in a standard volumetric flask and stored in appendoff tubes at very low temperature.

D **9% Ascorbate**

100ml distilled water was added to 9g of ascorbic acid,dissolved by shaking and stored in brown reagent bottle and stored at 4^oC. This reagent is usually prepared fresh.

E. Ammonium molybdate

1.25g NH_4Mo (Hopkins and Williams Ltd) England was dissolved in 100ml of 6.5% H_2SO_4. This reagent is usually stored in plastic container.

G. **10% Trichloroacetic acid**

In 100 ml standard volumetric flask 10g of trichloroacetic acidwas dissolved and make up with distilled water to 100 ml, transferred into a reagent bottle and stored at 4^oc.

PROCEDURE

To each test tube in duplicate, sucrose (200µl), KCl (200µl), Tris (1300µl) were added to all test tubes. This was followed by the addition of 10, 30, 50 70 and 90µl of extract to the designated tubes respectively and the solutions were made up to 2000µl accordingly. Uncoupler was added followed by ATP. Thento the zero time tube SDS was added before the addition of mitochondria.

(SDS was added to zero time tube to stop the reaction after adding ATP.) After adding ATP, mitochondria were added to each test tube every 30seconds or 1minute while continuously shaking for 30 minutes in the water bath at 27^{o}C. While still shaking, 1mL SDS was added to each test tube (except zero time) every 30seconds or 1minute. Then 1mL of ammonium molybdate was added to each test tube followed by 1mL ascorbate. The solutions were then allowed to stand for 30minutes to develop the blue colouration and absorbance was read at 680nm. Absorbance is read by taking 1200mL of water and 200µL of sample (this was done so as to reduce turbidity which may affect spectrophotometer reading) into the cuvette.

Table 4: Protocol for mitochondrial ATPase activity

	Sucrose (µl)	KCl (µl)	Tris (µl)	Ext (µl)	H_2O (µl)	DNP (µl)	ATP (µl)	Mit (µl)	SDS (µl)	NH_4Mo (µl)	Ascorbate (µl)
Blank	200	200	1300	-	300	-	-	-	1000	1000	1000
1	200	200	1300	-	265	-		35	1000	1000	1000
2	200	200	1300	-	260	-	40	-	1000	1000	1000
3	200	200	1300	-	225	-	40	35	1000	1000	1000
4	200	200	1300	10	215	-	40	35	1000	1000	1000
5	200	200	1300	30	195	-	40	35	1000	1000	1000
6	200	200	1300	50	175	-	40	35	1000	1000	1000
7	200	200	1300	70	155	-	40	35	1000	1000	1000
8	200	200	1300	90	135	-	40	35	1000	1000	1000
Ucp	200	200	1300	-	175	50	40	35	1000	1000	1000
0 Time	200	200	1300	-	225	-	40	35	1000	1000	1000

DNP: Dinnitrophenol (Standard uncoupler)

SDS: Sodium Dodecyl sulphate

3.4.7 DETERMINATION OF INORGANIC PHOSPHATE

Concentration of inorganic phosphate released was determined as described by Bassir (1963).

REAGENTS

A. 1mM Na_2HPO_4: Dissolve0.143g in 1000ml or, 0.00143g in 10ml

B. 9% ascorbic acid: Dissolved 9g ascorbate in 100ml of distilled water.

PRINCIPLE

Thisassayprinciple is established based on the fact that in the presence of inorganic phosphate molybdic acid generates a yellow coloured compound, which undergoes reduction to give a blue coloured compound. Ascorbate is used as the reducing agent and thecolour intensitythus formed is exactly proportional to the level of inorganic phosphate released.

PROCEDURE

400 µl of 5% solution of ammonium molybdate and 5ml of deproteinized supernatant were mixed in test tube, and 0.2ml of 2% freshly preparedsolution of ascorbate was added. The tube was kept for 20 minutes after thorough mixing by gentle shaking. A standard solution of potassium dihydrogen phosphate (0.2mg inorganic phosphate per 5ml) was equally treated. Distilled water was used to blank and the blue colour intensity formed was read in a spectrophotometer at 680nm.

CALCULATION

$$\text{Mg inorganic phosphate} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \frac{1}{1000}$$

$$\text{Mole of inorganic phosphate released} = \frac{\text{mg of inorganic} \times 1000}{\text{Molecular mass of pi}}$$

Therefore, mole of inorganic phosphate (pi) released per minute per milligram of mitochondrial protein is given by the expression: mole/min/mg protein.

$$\frac{\text{Mg pi released per ml}}{\text{Molecular mass of pi}} \times \frac{1000}{1000} \times \frac{1000}{\text{Mg protein}} \times 30$$

Table 5: Protocol for inorganic phosphate determination

1 mM Na_2HPO_4 (µl)	H_2O (µl)	NH_4Mo (ml)	Ascorbate (ml)
-	1000	1	1
20	980	1	1
60	940	1	1
100	900	1	1
140	860	1	1
180	820	1	1

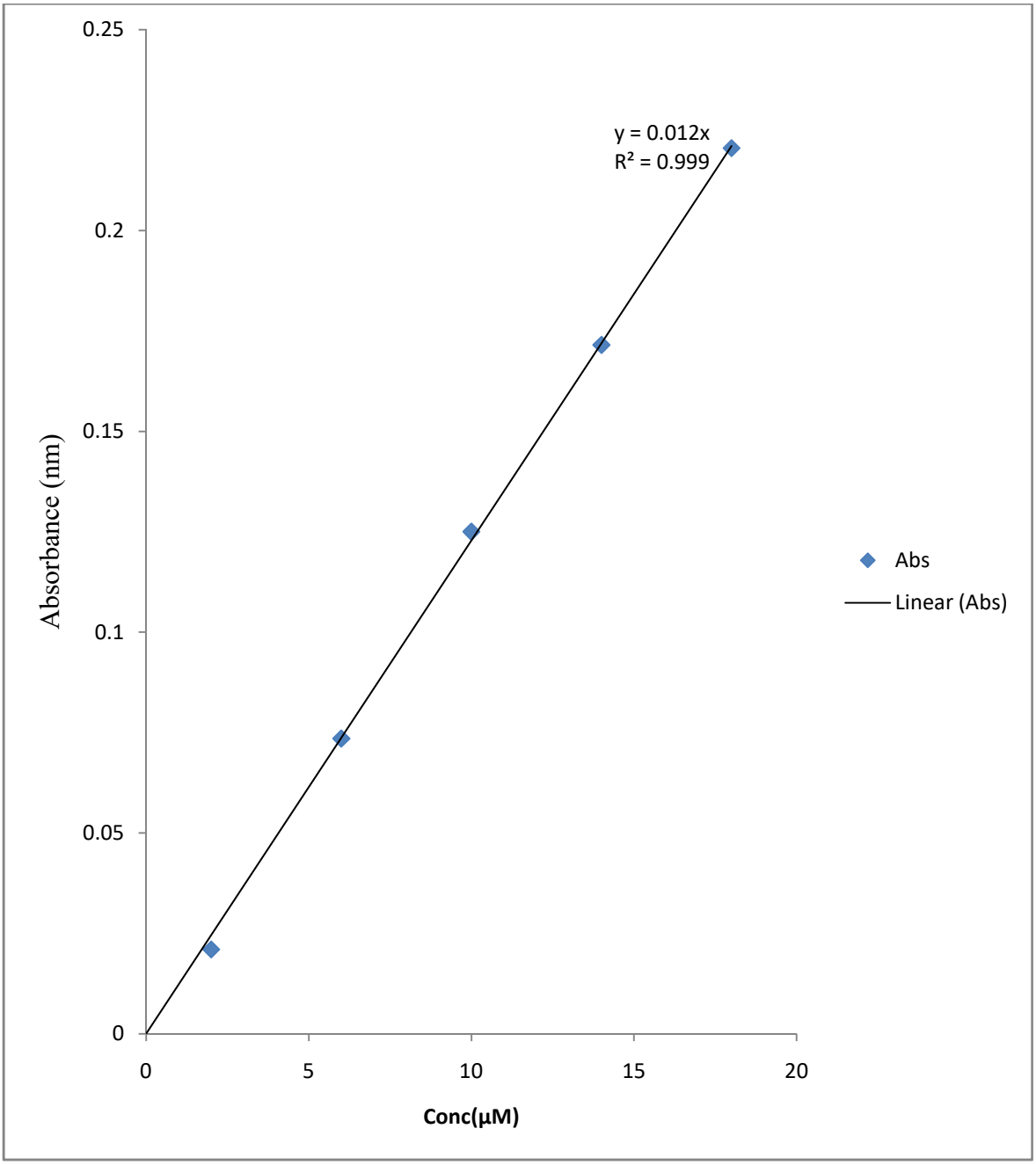


Fig. 6: Standard phosphate curve

3.4.8Quantification of Released Cytochrome C

Quantification of cytochrome c was assayedas described by Appaix *et al*. (2000), in a quick and plain spectrophotometric process for assessment of quantitative liberation of cytochrome c following the permeabilization of mitochondrial membranes.

Reagents

10 µM HEPES (pH 7.4)

HEPES (2-[4-(2-hydroxymethyl) piperazin-1-yl]ethanesulfonic acid (Sigma Aldrich, St.Louis, USA). 2.38 g was dissolvedin 80 ml of distilled water and the pH adjustedto 7.4 using 5M NaOH. The final volume was made up to 100 ml with distilled water.

10 µM $CaCl_2.2H_2O$

Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Sigma Aldrich St. Louis) was dissolved in 200 mlof distilled water and the volume was made up to250 ml mark of standard volumetric flask with distilled water.

Suspension Buffer(210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH pH7.4)

Distilled water (60 ml) was added to HEPES (0.12 g) (Sigma-Aldrich, Germany),dissolvedand pH regulated to 7.4 using KOH. Mannitol (3.83 g) and Sucrose (2.4 g) were dissolved in the HEPES-KOH (7.4) solution and make up to 100ml and then stored in the refrigerator.

PROCEDURE

Mitochondrial permeability transition was induced as previously described. 1 mg/ml of intact mitochondria isolated from liver, at a final concentration of 2.5 ml ofthe reaction medium, were incubated for 30 min at 25°C in the presence of different buffers as illustrated in the protocol table below. After incubation with the different concentrations for 30 minutes, mitochondria were spinnedfor 10 minutes at 13000 rpm. The supernatant was then filtered via membrane of 0.2µM Millipore. The clear supernatants absorbance was recorded against the medium as a reference at 414nm.

Table 6: Protocol for Cytochrome c Quantification

Sample	Buffer (µl)	Rotenone (µl)	Mitochondria (mg/ml)	Extract (µg/ml)	$CaCl_2$ (µl)	Succinate (µl)	Dil. H_20 (µl)
Blank	2200	10	_		_	50	240
IIM	2200	10	1		_	50	VARIABLE
TA	2200	10	1		25	50	
Test	2200	10	1	10-90 µg/ml	_	50	

IIM: Isolated Intact Mitochondria

TA: Triggering Agent (Ca^{2+})

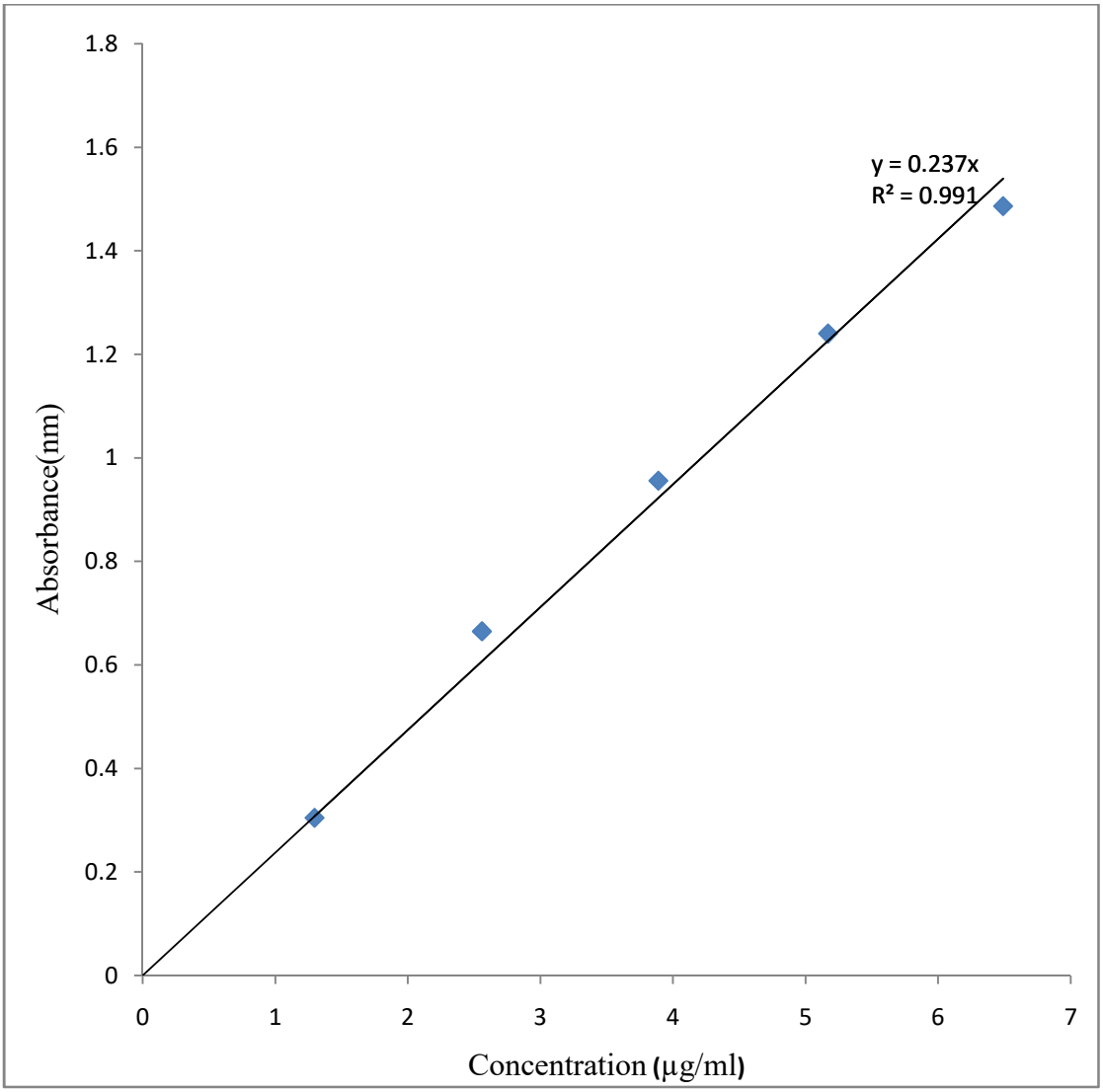


Fig. 7: Standard cytochrome c curve

3.4.9 HISTOLOGICAL ASSESSMENT OF VISCERAL ORGANS OF MALE ALBINO RATS

Tissues were excise, mopped and then preserved in 10% formalin. The tissues were then subsequently cut into sections for histological assessment.Histopathological investigation of tissues begins with surgeryor autopsy followed by cutting the tissue and placed in a fixative which makes the tissues stable to avoiddecay. The most frequently used fixative is formalin (10% formaldehyde in water).

PROCEDURE

The tissue section is deparaffinized by by attaching it to the slide and dipping in fresh xylene in a coplar jar. This stepis repeated.The tissue section is then hydrated by passing via reducing alcohol concentration baths and water (100%, 90%, 80% and 70%). This is then stained with hematoxylin for 3-5 minutes andwashed in running tap water till sections turns “blue”.This is then mounted in mounting media andview under microscope.

3.4.10 IMMUNOHISTOCHEMICALDETERMINATION OF APOPTOTIC BIOMAKERS

PROCEDURE

Immunostain formalin-fixed, paraffin-embedded tissue sections:The tissue was deparaffinizedby attaching on the slidesand deep the slides in xylene twice, 5 min each and thentransfer slides to 100% alcohol twice, 3 min each. This was followed by passing through 95% twice, 70% once alcohols respectively for 3 min each.The section was rinsed with Wash Buffer twice, 5 min each time and thenperformed antigen regaining to reveal the antigenic epitope. Other steps were sequentially carried out as specified and the staining colour of the antibody in the tissue sections was thenobserved under microscope.

3.4.11 DETERMINATION OF DNA FRAGMENTATION (Tunel assay method)

The TUNEL assay is a system planned for the explicit identification and quantitationof apoptotic cells in a cell population. This system makes possible for simple, exact and quick identification of celldeath. The system could be applied to determine apoptotic cell death in many systems, such as formalin-fixed, paraffin-embedded tissue and cultured cells sections. This process

assesses nuclear DNA disintegration, an essential biochemical distinguishing characteristic of apoptosis in varioustypes of cells.

Principles of TUNEL staining / the TUNEL assay

The TUNEL staining / TUNEL assay method relies on the enzyme terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks. TdT is expressed in certain immune cells and acts during recombination – the process that generates antibody diversity.

PROCEDURE

Tissue sections were deparaffinized (i.e., attached to microscope slides) by dipping slides in fresh xylene in a Coplin jarat room temperature for 5 minutes. The rest processes were sequentially carried out in accordance to the manufacturer's instructions.

3.4.12 DETERMINATION OF DNA FRAGMENTATION (Assay via Dipheylamine)

PRINCIPLE

A hallmark of late apoptosis is extensive genomic DNA fragmentation that generates a multitude of DNA double-strand breaks (DSBs) with accessible 3'-hydroxyl (3'-OH) groups. This characteristic forms the basis for a well-established apoptosis detection method: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL)

BUFFERS

TET: Tris HCl +EDTA +Tritonx -100

DPA: Diphyenylamine (1.5 g) + Acetic acid (100 ml)+ Conc. H_2SO_4 (1.5 ml)

TET: Tris HCl + EDTA

PROCEDURE

1 g of the liver wasweighedinto 20 ml TET buffer and pureed. Thehomogenate was then spinnedfor 20 min at 27,000 rpm. The supernatant was separatedfrom the pellet, and 2 ml of TET was added to the pellet and 3 ml of DPA to the supernatant. Incubate at 37^{o}c for 16 – 24 hrs. The absorbances of the supernatant and the pellet were read at 620 nm.

$$\% \text{ DNA fragmentation} = \frac{\text{Absorbance of supernatant}}{\text{Absorb.of supernatant + pellet}} \times 100$$

3.4.13DETERMINATION OF CASPASES 3 AND 9 ACTIVITIES

Cells that are suspected to or have been induced to undergo apoptosis are first lysed to collect their intracellular contents. The cell lysate can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the colour reporter molecule p nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the colour reaction.

PROCEDURE

The number of microwell strips that were needed to carry out the assay test, the desired samples number and the exactwellnumber required for assaying standards and blanks. Each sample:blank,standard and optional control were carried out in duplicate. Excessstrips of microwell were removed from holder and kept along side the desiccant provided at 2°-8°C and tightly sealed.

The strips of microwell were washed with approximately 400 µl Wash Buffer per welltwice with complete removal by means of suctionbetween microwell contents. The Wash Buffer was then allowedto stay in the wells for about 10 – 15 seconds before removal by means of suction and being meticulous to avoid scraping the periphery of the microwells. Excess Wash Buffer were removed after the final wash stage by emptying wells and tappingstrips of microwellon absorbent pad orpaper towel. The microwell strips were used at once after washing. Wells were not allowed to dry. Sample Diluent (100 µl)were added to all standard wellsin duplicate. Prepared standard (100 µl of 200 ng/ml) was pipetted into well A1 and A2in duplicate. From this stage the rest procedures were carefully carried out in accordance with the instructions of the manufacturer.

3.4.14 CHARACTERIZATIONOF *Daniellia oliveri* STEM BARK

PRINCIPLE

Column chromatography separates mixtures based on varying solubilities of components in solvent systems and adsorbent to the stationary phase. Introduction of mobile phase and sample of mixturesto be separated from top of the column cause each componentto travel with varying speed.

MATERIALS/ REAGENTS

- Glass column with a knob at the lower end
- Silica gel (stationary phase) with a uniform size and shape
- Mixture of solvents – ethanol, acetone and ethyl acetate (mobile phase)
- Cotton wool

PROCEDURE

Two methods are commonly applied to set up a column and these are the dry method and the wet method. The dry method was used in this study.For setting up the dry method, the column was first packed with dry silica gel of mesh size 60 – 200, which served as the stationary phase, followed by the addition of mobile phaseto the column.The sample to be separated was then preadsorbed with the silica gel in ratio 1:1 before loading on the stationary phase. The preadsorbed sample in the column was washed with varying solvent systems prepared in order of increasing polarity. The eluted fractions were pooled based on similarity in $R_f$value on thin layer chromatographic plate. Samples/fractions with two or more components were further separated using micro-column. Eluents with high degree of purity were then subjected to GC-MS analysis.

3.4.15 STATISTICAL ANALYSIS OF DATA

Three independent measurements (assays) at leastwere expressed as mean ± standard deviation (SD)for all the experimentdataused. One Way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were performed andvalues of p below 0.05 were taken as statistically significant.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT 1

Phytochemical Screening of Crude Extract and Fractions of *Daniellia oliveri* Stem Bark

INTRODUCTION

Phytochemicalsare chemical compounds produced in plants during their normal metabolic processes, which are sometimes referred to as "secondary metabolities". The use of plants for traditional medicine could be due to theability of these secondary metabolites in plants to treat, cure or prevent diseases.Plants withmedicinal properties for time immemorial have been used as remedies for human diseases and the reason for using them as medicine is attributed to the fact that they contain chemical substances of therapeutic importance (Nostro *et al*., 2000).

Plants having medicinal value contain biochemically active principles that traditional medicine practitioners have taken advantage of over the past years for the treatment of several diseases (Adebanjo *et al*., 1983). The drug candidates presentin medicinal plants are referred to as active principles andit has been documented that plants contain numerous active principles (Cowmann, 1999; Banso and Olutimayin, 2001). There is a reasonable possibility that these plants which have been appliedby human for time immemorial will ultimately result to novel drug prototypes (Eshrat and Hussain, 2002).

PROCEDURES

Phytochemical Screening**:** Phytochemical investigation was perfomed accordingto the standard methods stated under materials and methods (Section 3.4.0).

RESULTS

Results of the phytochemical screening ofsolventfractions of*Daniellia oliveri* stem bark is depicted in table 7. The results showed that flavonoids, saponins, tannins, glycosides, coumarin, phlobatannins, terpenoids and steroids arepresent (table 1). The crude extract and ethanol fraction contain several of the phytochemicals screened, while chloroform and ethyl acetate fractions contain only few of these phytochemicals. The absence of some phytochemicals in the various fractions of *D. oliveri* stem bark could be due to insolubility of these compounds in some solvents used.

Table 7: Crude ethanol extract and solvent fractions of *D. oliveri*stem barrk phytochemical screening

Phytochemical	Crude extract	Ethanol	Ethyl acetate	Chloroform
Saponins	+	+	-	-
Flavonoids	+	+	-	-
Alkaloids	+	+	-	-
Terpenoids	+	+	+	+
Cardiac glycosides	+	+	+	+
Anthraquinones	+	+	-	-
Tannins	+	+	+	-
Steroids	+	+	+	+
Reducing sugar	-	-	-	-
Coumarin	+	-	+	-
Phlobatannins	+	+	+	-

+ = present

- = absent

EXPERIMENT 2a

ASSESSMENT OF EFFECTS OF Ca^{2+}AND SPERMINE ON INTACT RAT LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE.

INTRODUCTION

Permeability Transition (PT) is the sudden rise of the IMM permeability to solutes of approximately 1500 Da. The study of mitochondrial route has tremendously added to thecomprehension of mitochondrial physiology (Szabò and Zoratti 2014). The PTP opening is usually associated with dysfunction of the mitochondria because its occurrence results to depolarization of mitochondria,Ca^{2+} discharge,inhibition of respiration, cessation of synthesis of ATP, pyridine nucleotide depletion, andswelling of matrix.Swelling, in turn, causes cytochrome c mobilization, OMM burst and finally liberationof cytochrome c and AIF (Bernardi *et al*.,2006).

PROCEDURE

Isolation of mitochondria and assessment of mitochondrial membrane permeability transition pore opening followed the procedure described under materials and methods (pages 44 and 45 respectively).

RESULTS

Figure 8 illustrates the results of effects of Ca^{2+} and spermine on intact rat liver mitochondria. Treatment of the mitochondria in the absence of Ca^{2+} shows no significant change in absorbance implying that the mitochondrial membrane was intact, whereas in the presence of Ca^{2+}, the MMPT pore significantly opened (large amplitude swelling) and this was reversed upon treatment with spermine. Spermine inhibited exogenous calcium- induced mitochondrial swelling in intact rat liver. Spermine reversed calcium–induced pore opening by approximately 75%.

SUMMARY

Ca^{2+} significantly induced mPT pore opening while spermine inhibited mPT pore opening.This indicated that the mitochondrial membrane permeation transition pore was intact and not uncoupled and therefore suitable for use.

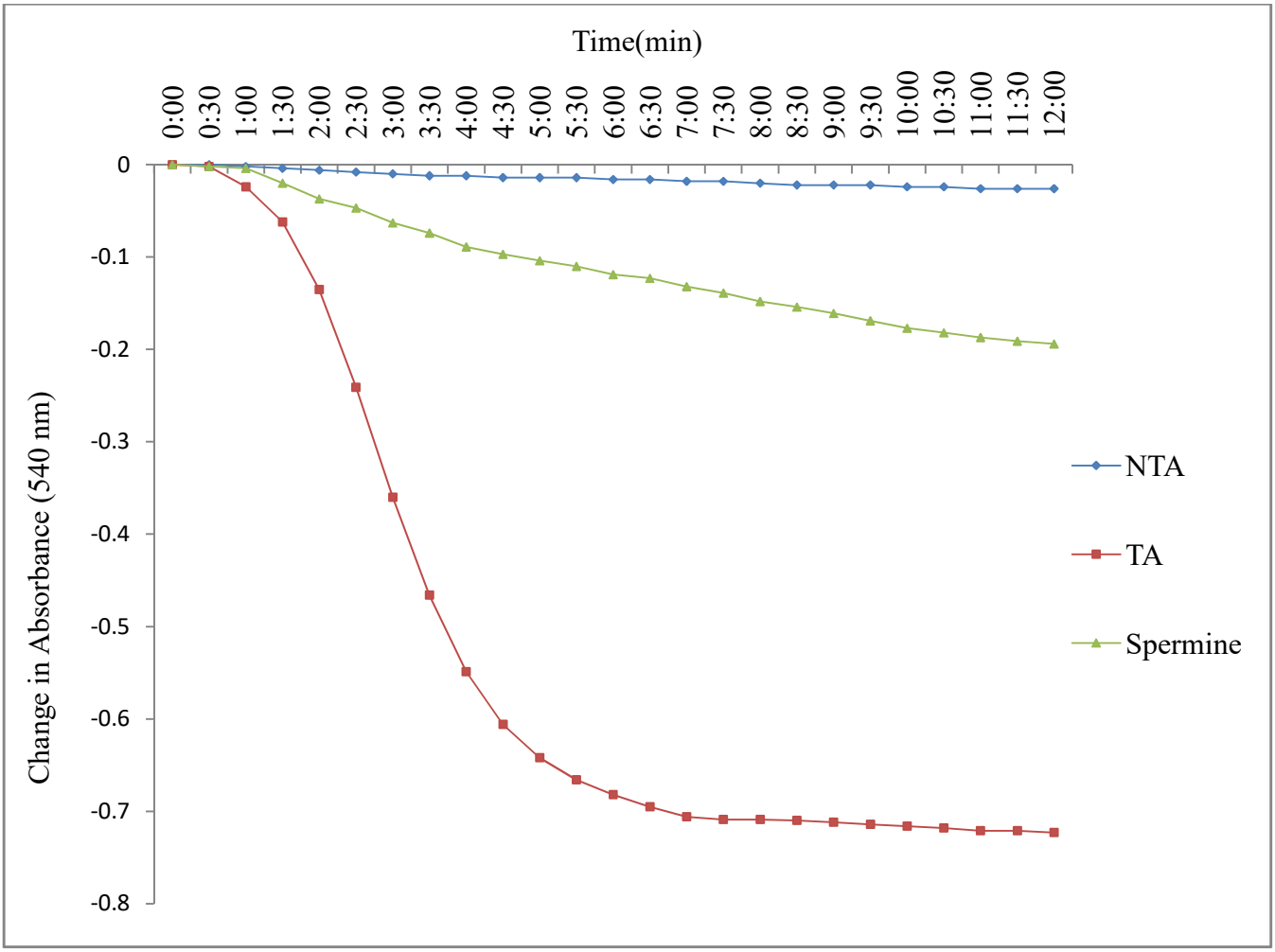


Fig.8: Effects of TA and spermine onmPT pore on intact rat liver *in vitro*

NTA: Non Triggering Agent

TA: Triggering Agent (Ca^{2+})

EXPERIMENT 2b

ASSESSMENT OF EFFECTS OF VARYING CONCENTRATIONS OFSOLVENT FRACTIONS OF *Daniellia oliveri* STEM BARK ON INTACT RAT LIVER MITOCHINDRIAL MEMBRANE PERMEABILITY TRANSITION PORE IN THE ABSENCE AND PRESENCE OF Ca^{2+} *IN VITRO*.

INTRODUCTION

Mitochondria are known to play crucial function in the life of cell by controlling bothsignalling routesof survival and death.The mPT pore represents a potential therapeutic target for both cell survival and death strategies and this is attributed to its central role in lethal functions.Severe oxidative pressure followed by accumulation of calcium in the matrix of the mitochondria enhances an elevation in the permeation of the mitochondrial membranes with creation of the pathological and non-explicit mPTPs (Chipuk *et al*., 2000). The MOMP may as well take place as result of the formation of random routes triggered by movement of pro-apoptotic BCl-2 proteins to the mitochondria (Armstrong *et al*., 2009; Baines, 2009). The mPT pore is well documented to be triggered by certain pathway that leads to apoptosis and certain phytochemicals and plant extracts/fractions have been illustrated to evoke death of cell in certain cells of cancer. It is inrespect to this that this research was planned to ascertain effects of certain solvent fractions of *Daniellia oliveri* stem bark on mPT pore in both absence and presence of calcium.

PROCEDURE

Isolation of mitochondria and assessment of mitochondrial membrane permeability transition pore opening followed the procedure described under materials and methods (section 3.4.1 and 3.4.2 respectively).

RESULTS

Figures 9 and 10 depict inductive effects of crude Ethanol Extract of *Daniellia oliveri* (EEDO) on intact rat liver mPTP*in vitro* in both absence and presence of triggering agent, respectively.Treatment of intact mitochondria with non triggering agent showed that the mitochondria were intact. Treatment with a triggering agent (Ca^{2+}) significantly ($p < 0.05$) induced mPTP opening with induction fold of 26.81 and treatment with spermine significantly (p

< 0.05) reversed Ca^{2+} - induced opening with inhibition fold of 1.28. This shows that the mitochondria were intact, uncouple and therefore suitable for use. Treatment of the mitochondria with 60, 180, 300and 420 µg/ml of EEDO stem bark in the absence of Ca^{2+} exhibited an induction fold of 0.03, 0.09, 0.20 and 0.65, respectively. This shows a concentration – dependent induction of mPTP opening by the EEDO stem bark. Treatment of the mitochondria with 60, 180, 300 and420 µg/ml of the EEDO stem bark in the presence of Ca^{2+} exhibited induction folds of 0.58, 0.54, 0.48 and 0.42, respectively.Figure 11 depicts inductive effects of EFDO stem bark on intact rat liver mPT pore *in vitro*. Treatment of mitochondria with 60, 180, 300and 420 µg/ml ofEFDO stem bark in the absence of Ca^{2+} exhibited an induction fold of 0.04, 0.10, 0.35 and 0.68, respectively. This shows a concentration – dependent in induction of mPT pore opening by EFDO stem bark. Treatment of the mitochondria with 60, 180, 300 and 420 and µg/ml of the EFDO stem bark in the presence of Ca^{2+} exhibited induction folds of 0.64, 0.62, 0.56 and 0.52, respectively(Figure 12). There were significant induction at the varying concentrations of EFDO used in the presence of Ca^{2+} but these decreased with increase in concentration. This shows that there was no synergy in mPT pore induction between Ca^{2+} and EFDO stem bark. EFDO stem could thus be inhibiting Ca^{2+} - induced mPT pore opening as the concentration of the fraction increases.

Figure 13 illustrates inductive effects of varying concentrations of Chloroform Fraction of *Daniellia Oliveri* (CFDO) stem bark on intact rat liver mitochondrial membrane permeability transition pore. Assessment of pore opening in the absence of calcium showed that the mitochondria were intact. Treatment of the intact mitochondria with calciumshowed induction of 13.82 folds and spermine inhibited calcium - induced opening by 2.44 folds. Treatment of the intact mitochondria with varying concentrations - 60, 180, 300 and 420 µg/ml of the CFDO stem bark in the absence of Ca^{2+} exhibited induction folds of 0.01, 0.02, 0.04 and 0.05, respectively. These showed there was no remarkable induction in mPTPcompare to inductions in the presence of calcium at varying concentrations of CFDO used. Treatment of the intact mitochondria with varying concentrations (60, 180, 300 and 420 µg/ml) of the CFDO stem bark in the presence of Ca^{2+} exhibited induction folds of 0.56, 0.47, 0.21 and 0.14, respectively (Figure14). This showed a decrease in induction of mPT pore as the concentration increased.Figure 15presents results of the treatment of intact mitochondria with Ethyl Acetate Fraction of *Daniellia oliveri*(EAFDO)

stem bark in the absence of Ca^{2+}. Varying concentrations - 60, 180, 300 and 420 μg/ml of the EAFDO stem bark treated with intact rat liver mitochondria in the absence of Ca^{2+} exhibited induction folds of 0.32, 0.50, 0.35 and 3.4 respectively. In the presence of Ca^{2+} EAFDO stem bark exhibited induction folds of 21.53, 20.38, 20.18, 18.21 and 13.32, respectively (Figure 16).

SUMMARY

Ethanol fraction exhibited more induction of mPT pore when compared to other fractions of *D. oliveri* stem bark used in this assay.

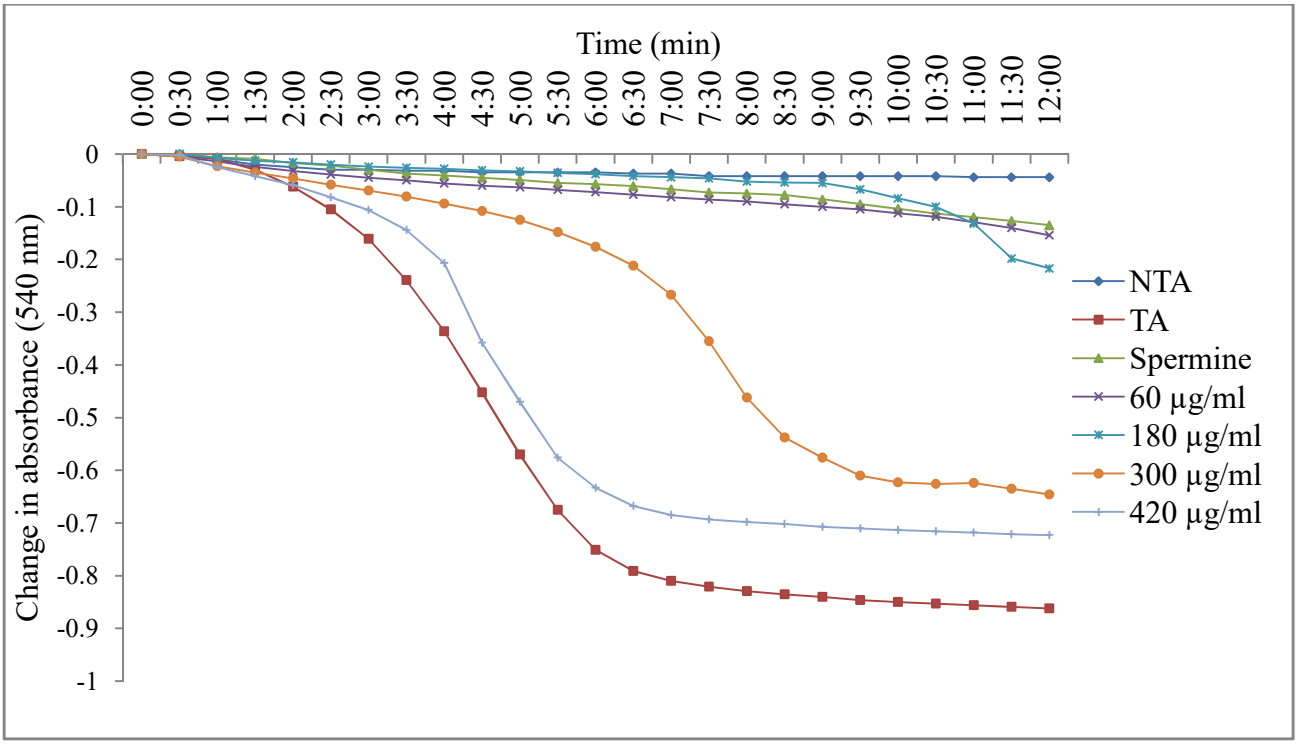


Fig. 9: Effects of crude ethanol extract of *D. oliveri* on intact rat liver mPT pore *in vitro* in the absence of TA

NTA: Non Triggering Agent

TA: Triggering Agent (Ca^{2+})

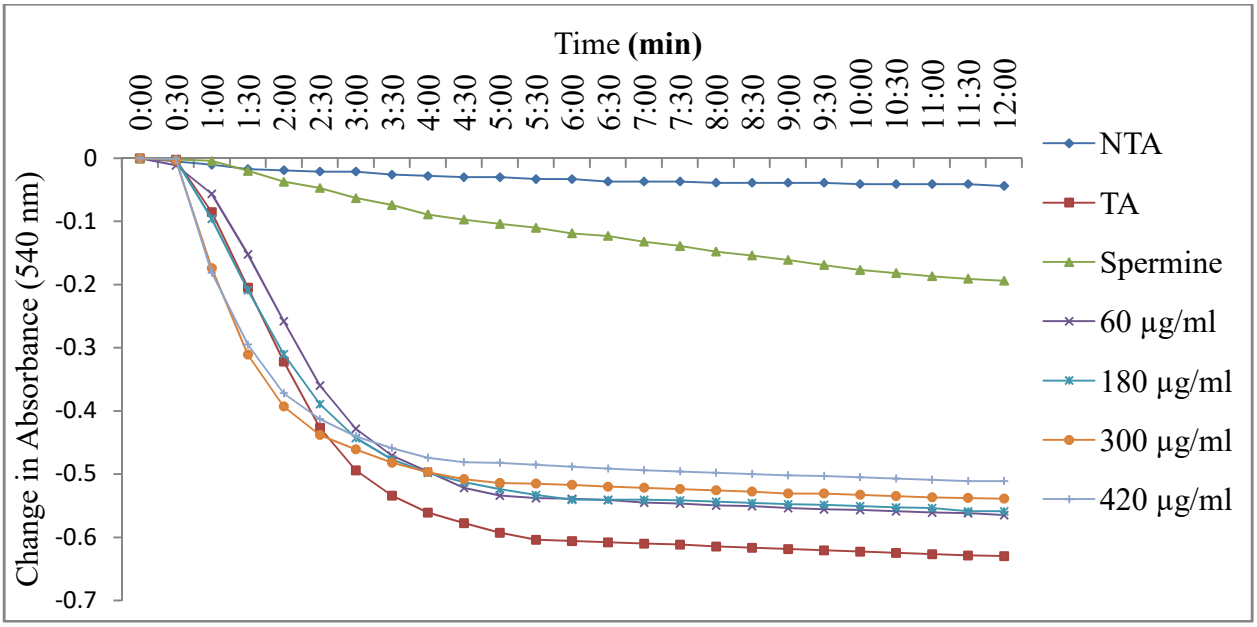


Fig. 10: Effects of crude ethanol of*D. oliveri* on intact rat liver mPT pore *in vitro* in the presence of TA

NTA: Non Triggering Agent

TA: Triggering Agent (Ca^{2+})

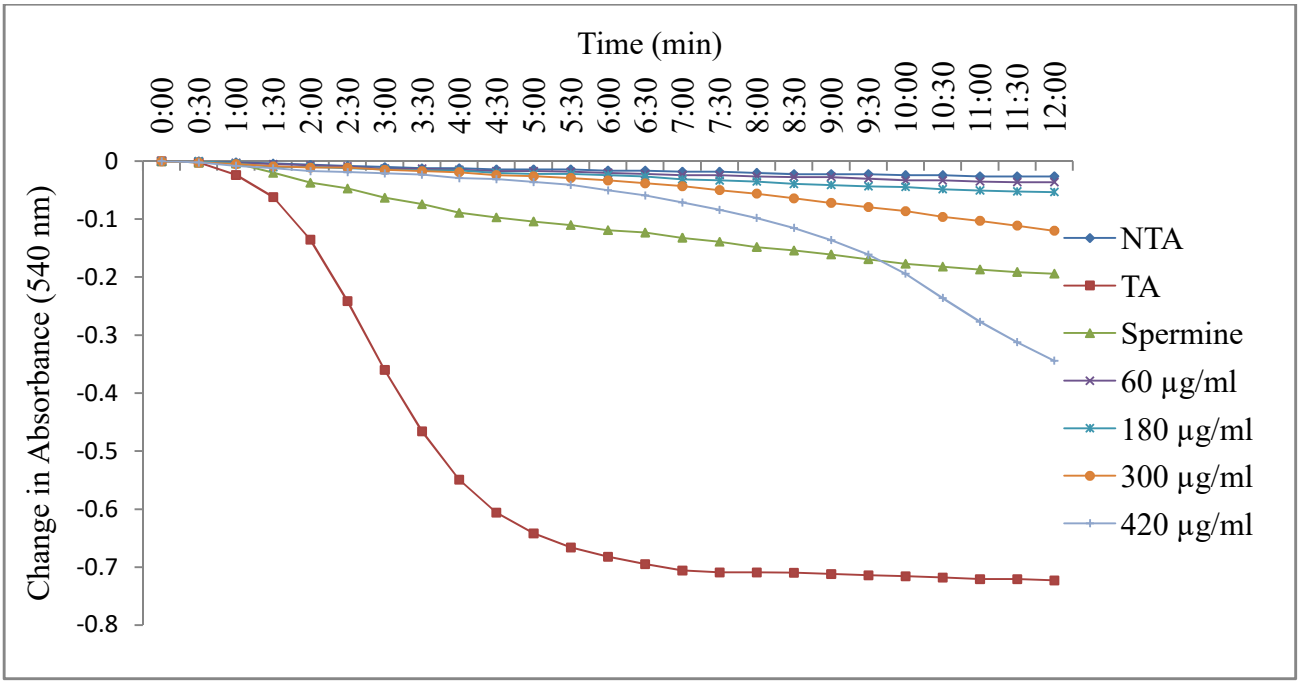


Fig. 11: Effects of ethanol fraction of *D. oliveri* stem bark on intact rat liver mPT pore *in vitro* in the absence of TA

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

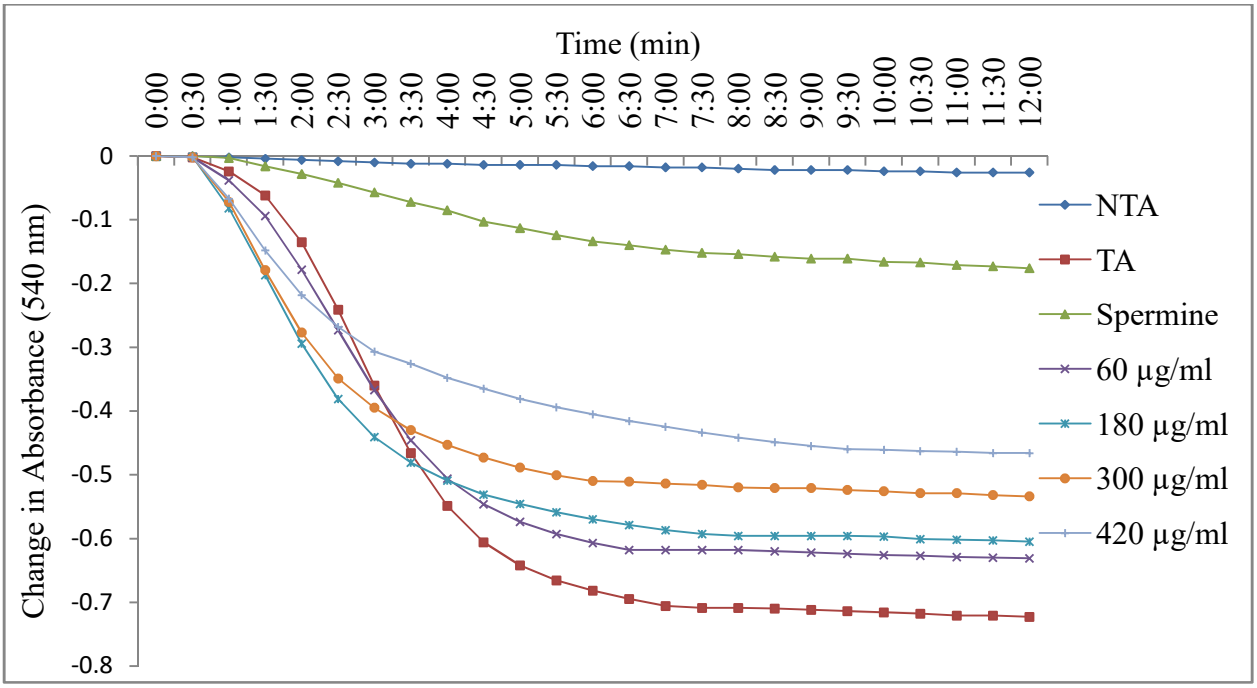


Fig. 12: Effects of Ethanol fraction of *D. oliveri* stem bark on rat liver mPT pore *in vitro* in the presence of TA

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

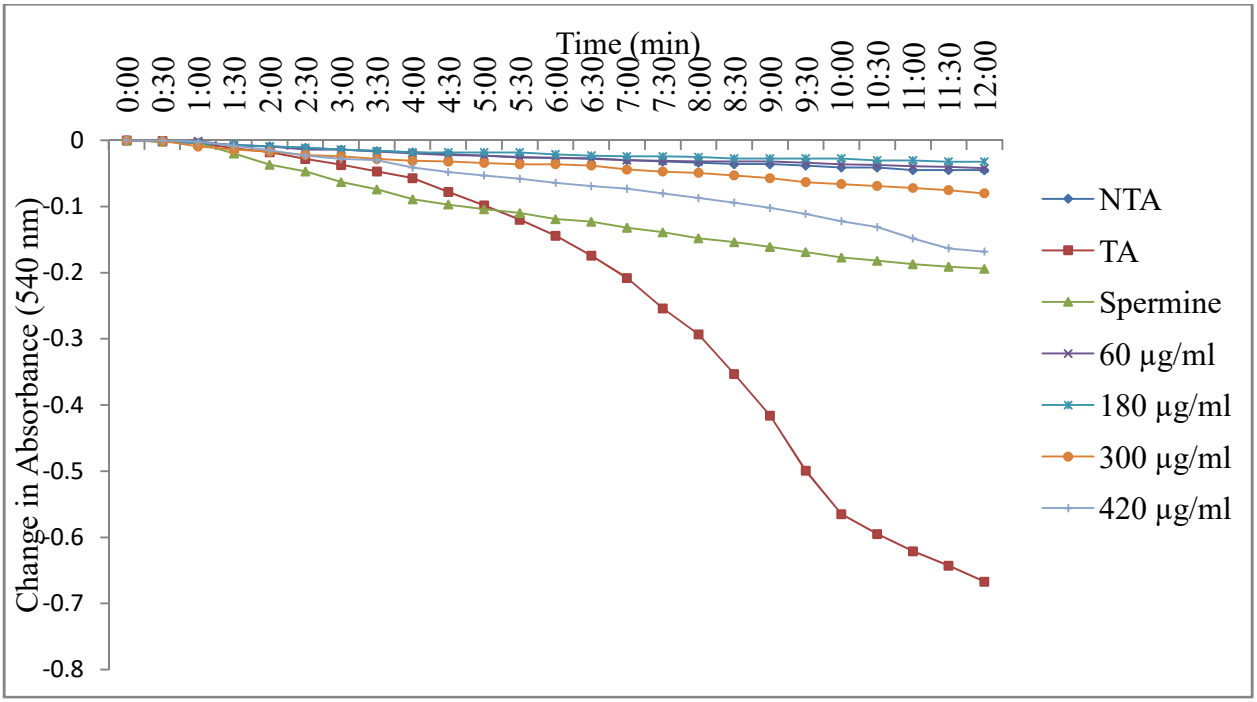


Fig. 13: Effects of Chloroform fraction of *D. oliveri* stem bark on rat liver mPT pore *in vitro* in the absence of TA

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

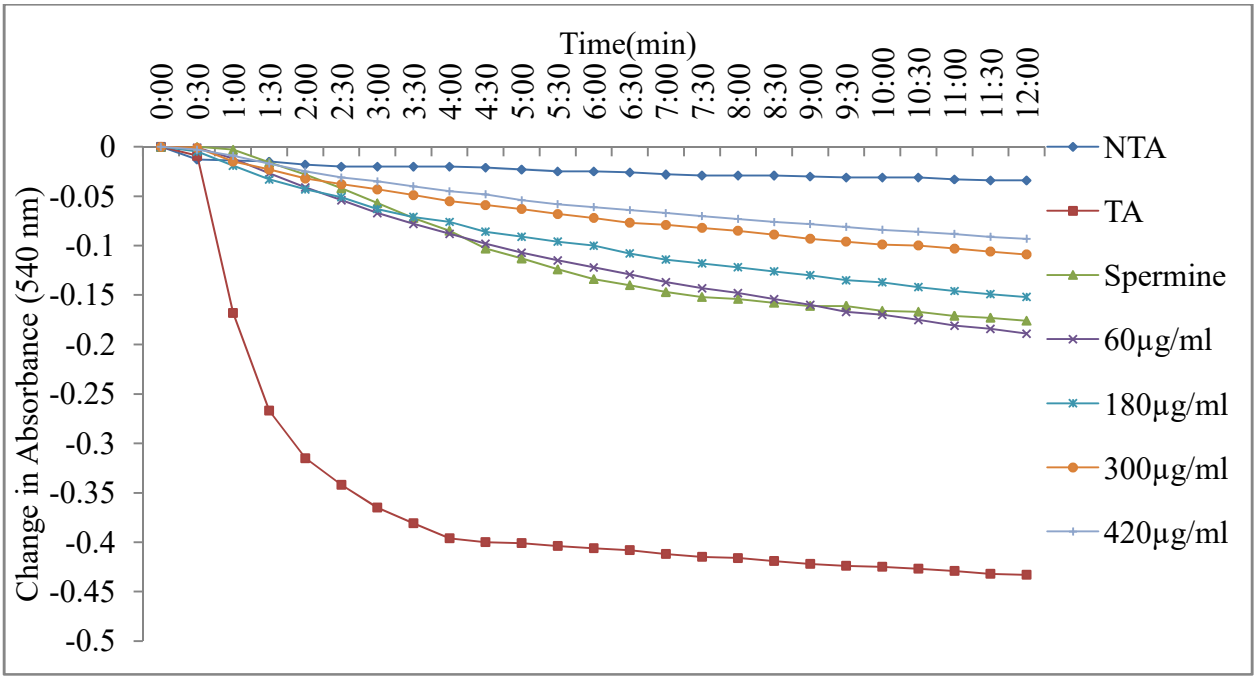


Fig. 14: Effects of Chloroform fraction of *D. oliveri* stem bark on rat liver mPT pore *in vitro* in the presence of TA

NTA: Non Triggering Agent

TA: Triggering Agent

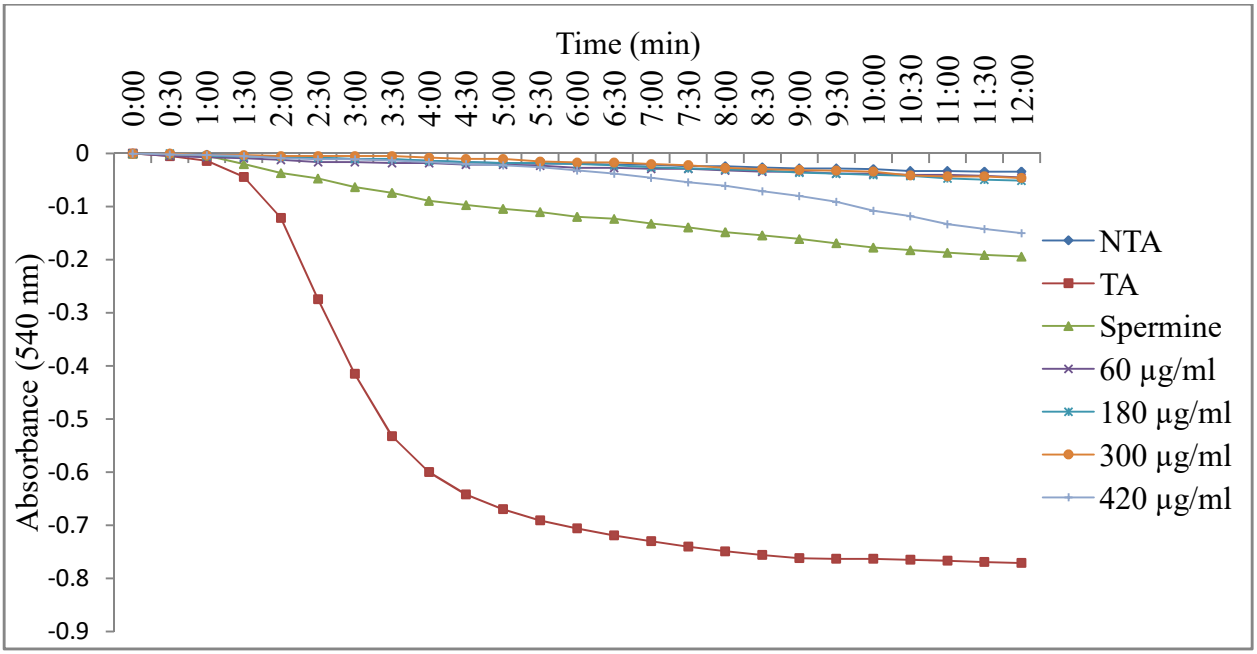


Fig. 15: Effects Ethylacetate fraction of *D. oliveri* stem bark on hat liver mPT pore *in vitro* in the absence of TA

NTA: Non Triggering Agent

TA: Triggering Agent (Ca^{2+})

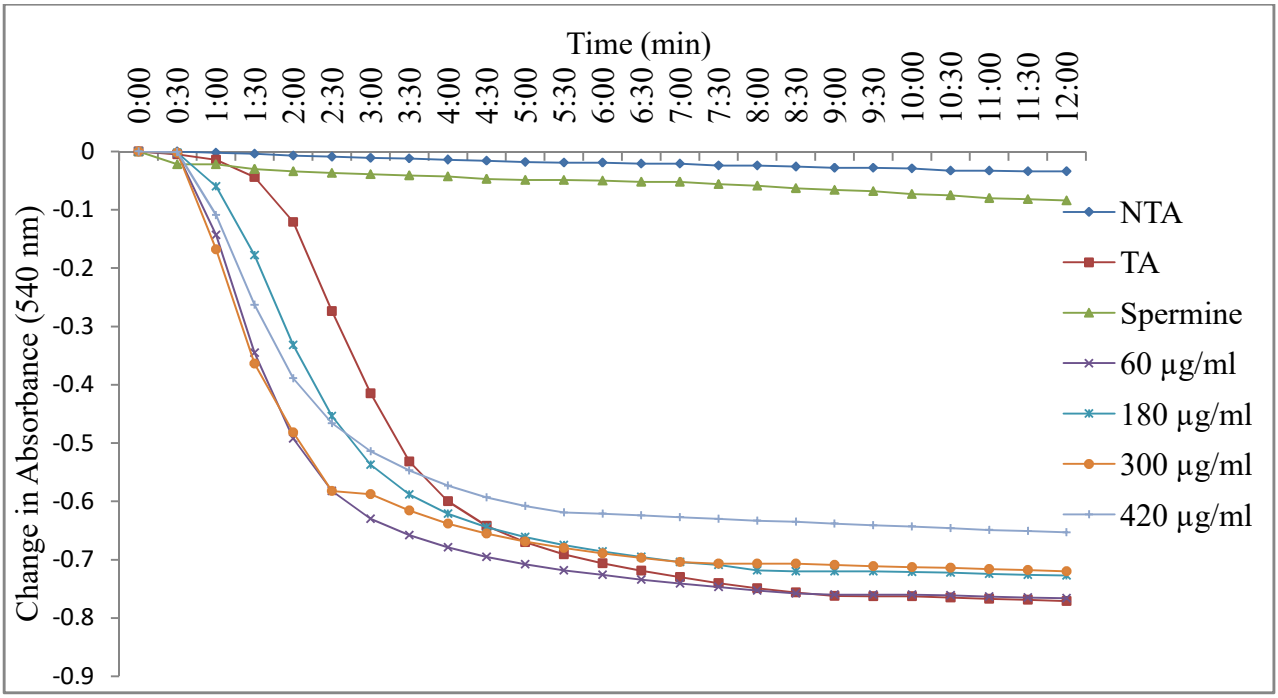


Fig. 16: Effects of Ethylacetate fraction of *D. oliveri* stem bark on rat liver mPT pore *in vitro* in the presence of TA

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

EXPERIMENT 3a

ASSESSMENT OF EFFECTS OF VARYING CONCENTRATIONS OF SOLVENT FRACTIONS OF *Danielliaoliveri* STEM BARK ON Fe^{2+}-INDUCED LIPID PEROXIDATION AND MITOCHONDRIAL ATPase ACTIVITY.

INTRODUCTION

Lipid peroxidation refers to an accumulated effect of ROS, which results in worsening and destruction of biological systems. The process of lipid peroxidation could be caused by ROS, which act by removing an allylic atom of hydrogen from a methylene group of polyunsaturated fatty acids. Several phytochemicals which are constituents of plants are knownto scavenge free radicals and inhibitperoxidation of lipid (Beutner *et al*., 2001). The defensive impacts on living cellsof most plants have been connected to their non-nutrient components including flavonoids, alkaloids, phenolic acid, terpenoids etc. These investigations have resulted to elevated attention on cancer prevention techniques in which these dietary substances are employed (Keith, 2000). Studies haveshown that diverse phytochemicals exhibit a range of activities, which may aid in prevention against chronic diseases like cancerand guard against peroxidation of lipid (Hollman and Katan 1997; Liu, 2003). The use of inherent antioxidants of plant origin for the management and treatment of diseases is receiving great attention. We therefore investigate the effects of solvent fractions of *D. oliveri* stem bark on Fe^{2+} - induced lipid peroxidation.

PROCEDURE

Isolation of mitochondria and assessment of Fe^{2+}- induced lipid peroxidation followed the procedure described under materials and methods (section 3.4.1 and 3.4.5, respectively).

RESULTS

Figure 17illustrates the effects of solvent fractions of *D. oliveri*stem bark on Fe^{2+}-induced lipid peroxidation using rat liver mitochondria as lipid – rich media. All fractions of *D. oliveri* stem bark used in this assay significantly ($p < 0.05$) inhibited Fe^{2+} - instigated lipid peroxidation in a concentration-dependent pattern. 0.75, 1.5, 3, 6 and 12mg/ml of ethanol fraction used inhibited Fe^{2+}-induced lipid peroxidation by 13.2, 33.1, 52.5, 79.3 and 133.5%, respectively. Chloroform fraction inhibited by 3.4, 14.4, 39.4, 51.1 and 57.8% and ethyl acetate fraction by 3.9, 25.5, 31.9, 85.8 and 114.4%, respectively.

SUMMARY

Fractions of *D. oliveri* stem bark contain bioactive components which scavaged ROSand could protect biological membranes from damage caused by lipid peroxidation. Inhibition of Fe^{2+}-induced lipid peroxidation exhibited by fractions of *D. oliveri* shows that induction of mPT pore by EFDO demonstrated earlier in this work could not be attributed to ROS generation, which are known to induce mPT pore opening.

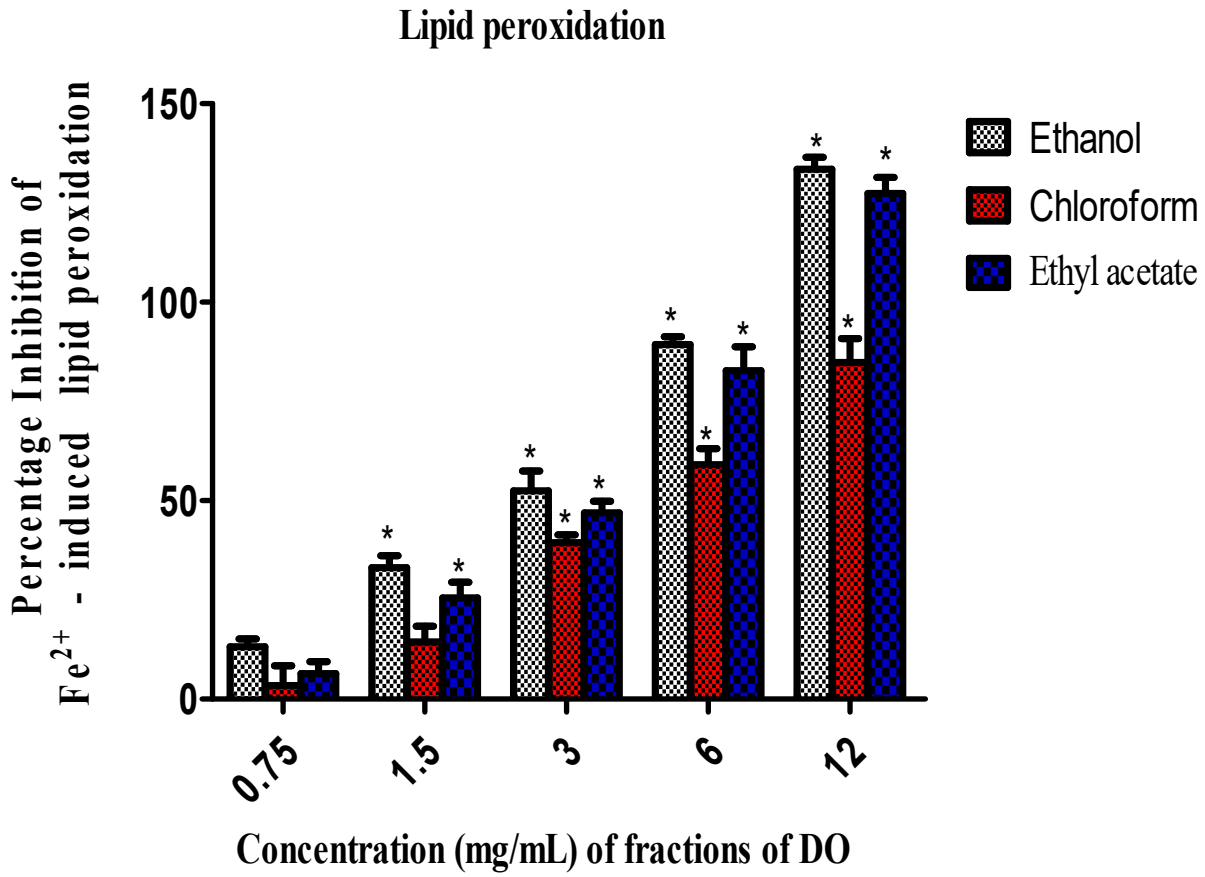


Fig. 17: Effects of solvent fractions of *D. oliveri* stem bark on Fe^{2+}-induced lipid peroxidation of rat liver mitochondria

DO: *Daniellia oliveri*

(*: statistical significance, p< 0.05)

EXPERIMENT 3b

ENHANCEMENTS OF ATPASE ACTIVITY OF RAT LIVER MITOCHONDRIA BY SOLVENT FRACTIONS OF *Danielliaoliveri* STEM BARK*in vitro*.

INTRODUCTION

ATPasesare a class of enzymes that catalyze the splitting of ATP into ADP and a free phosphate ion (Geider and Hofmann-Berling, 1981; Kielley, 1961; Martin and Senior, 1980; Njus*et al*., 1981). Energy isliberated as a result of this dephosphorylation reaction, which the enzyme harnesses to perform other chemical reactions. Some of these enzymes are integral membrane proteins and they function to transportsolutes across the membrane. ATP exhaustion and accumulation of phosphate are the primary metabolic changes that favour mPT pore formation. Thus, this assay was performed to ascertain the modulatory effects of fractions of *D. oliveri* on the activities of mitochondrial ATPase.

PROCEDURE

Isolation of rat liver mitochondria and assessment of ATPase activities followed the procedure described under materials and methods (section 3.4.1 and 3.4.6, respectively).

RESULTS

Figure 18depicts the effects of certain fractions of *D. oliveri* stem bark on ATPase activities in rat liver mitochondria. All fractions of *D. oliveri* stem bark used in this assay significantly ($p < 0.05$) enhanced ATPase activities relative to control and the enhancement isconcentration – dependent. The60, 180, 300, 420 and 540µg of ethanol fraction used enhanced ATPase activities by 42.0, 53.8, 55.3, 58.9 and 63.1% respectively, while Dinitrophenol (DNP) enhanced by 68.6%. The same concentrations used for CFDO and EAFDO enhanced it by 42.1, 45.0, 46.3,

+++47.9 and 47.6%; 34.4, 35.7, 37.0, 38.2 and 45.7%, respectively.

SUMMARY

Fractions of *D. oliveri* stem bark enhanced decomposition of ATP to ADP and inorganic phosphate (p_i), which are among factors known to induce mPT pore opening, thus substantiating the induction of mPT pore opening in the earlier experiment since ATP exhaustion and accumulation of phosphate are the major metabolic factors that favour mPT pore formation.Enhancement of ATPase activity by *D. oliveri* could remarkably influencemitochondrial function and change ATP concentration, mitochondrial transmembrane potential and synthesisof ROS, which have been involved in various cellular processes like cellular protection, apoptosis, O_2 sensing and ageing.Enhancement in the activity of ATPase by *D. oliveri* stem bark fractions could thus enhance its anti-tumour activity and could be potential therapeutic targets for mitochondrial – dependent cell death.

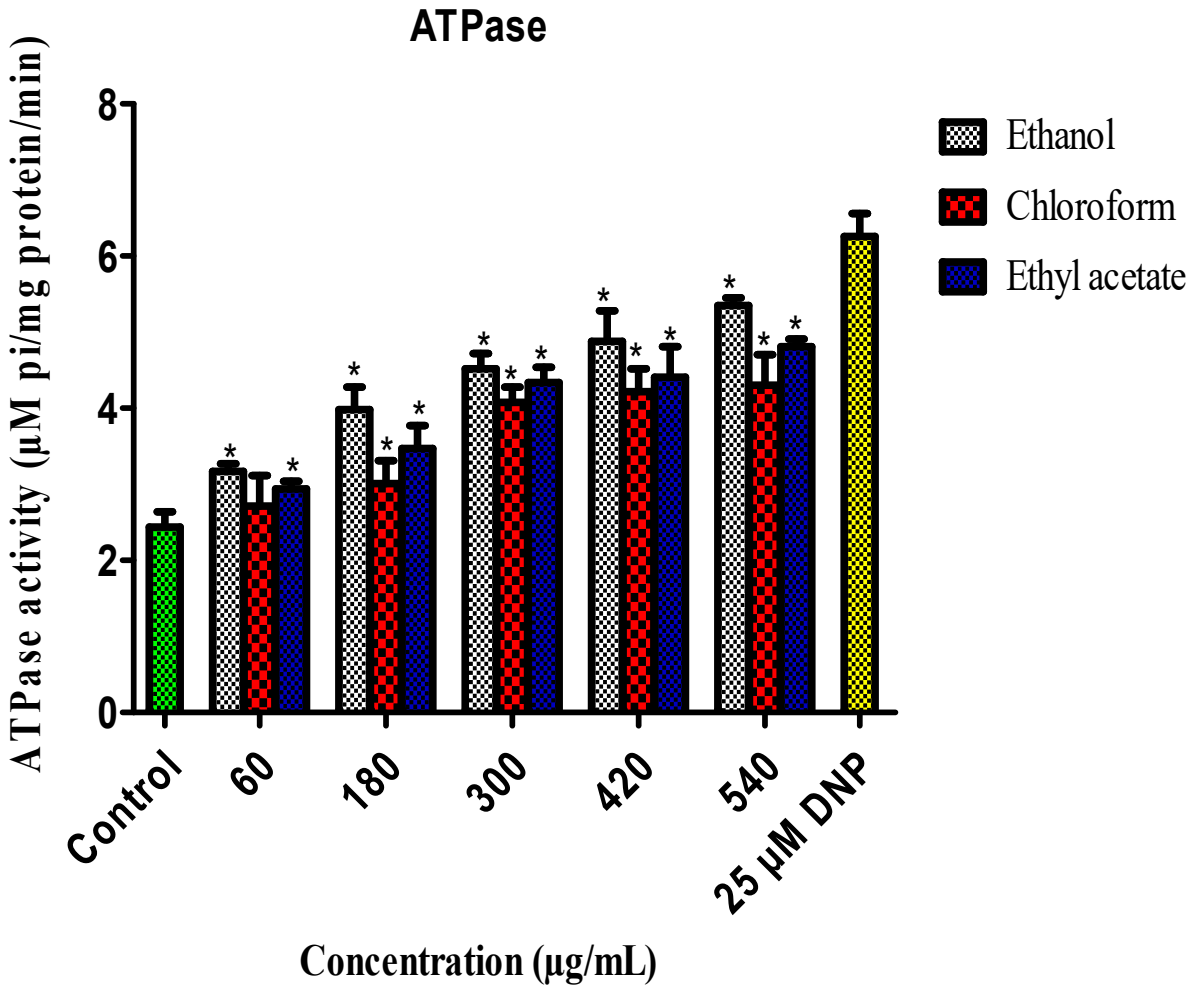


Fig. 18: Effects ofsolvent fractions of *Daniellia oliveri* stem bark on ATPase activity of rat liver mitochondria*in vitro*

(*: statistical significance, p< 0.05)

EXPERIMENT 3c

IN VITRO ASSESSMENT OF THE EFFECTS OF SOLVENT FRACTIONS OF *Danielliaoliveri*STEM BARK ON RELEASE OF CYTOCHROME C

INTRODUCTION

Cytochrome c complex is a small hemeprotein that is located and loosely bound to the interior membrane of the mitochondrionand is an important component of ETC. Cytochrome c as well, plays an important role in starting the processes of apoptosis. Following liberationof cytochrome c to the cytosol, it binds Apaf-1 (Dipiro, 1999). Hence, this experiment was carried out to assess the effects of solvent fractions of *D. oliveri* on activities of cytochrome c.

PROCEDURE

Isolation of rat liver mitochondria and assessment of cytochrome c release followed the procedure described under materials and methods (section 3.4.1 and 3.4.8, respectively).

PRINCIPLE

The method makes use of a very intensive (γ) or Soret peak at 414 nm incytochrome c the spectrum (ε =100 mM^{-1} cm^{-1}). This value is referred to as the extinction coefficient, which is a molecular property of cytochrome c, is then used to determine the level of cytochrome c in a medium from any quantified value of absorbance.

RESULT

Figure 19 depicts the results of the cytochrome c release by certain fractions of *D. oliveri*. The varying concentrations – 60, 180, 300, 420 and 540µg/ml of EFDO treated with intact isolated mitochondria (IIM) caused the release of 0.279, 0.388, 0.521, 1.084 and 1.544nmol/mg mitochondrial protein of cytochrome c, respectively. This shows a remarkable ($p < 0.05$) release of cytochrome c in a concentration–dependent pattern when compared with the Intact Isolated Mitochondria(IIM) and Ca^{2+} which caused the release of 0.245 and 1.636nmol/mg mitochondrial protein of cytochrome c, respectively. The same concentration used for CFDO and EAFDO induced the release of 0.605, 0.620, 0.640, 0.655 and 0.690; 0.69, 0.74, 0.77, 0.86 and 0.92, respectively. All the fractions used in this assay induced the liberation of cytochrome c in a concentration–dependent pattern and EFDO is seen to have instigated the highest liberation of cytochrome c.

SUMMARY

The ability of solvent fractions of *D. oliveri* to cause the liberation of cytochrome c in this assay gave credence to stimulation of mPT pore opening in the previous experiment and the application of *D. oliveri* in cell death since discharge of cytochrome c is often referred to as the commitment step in the processes of apoptosis.

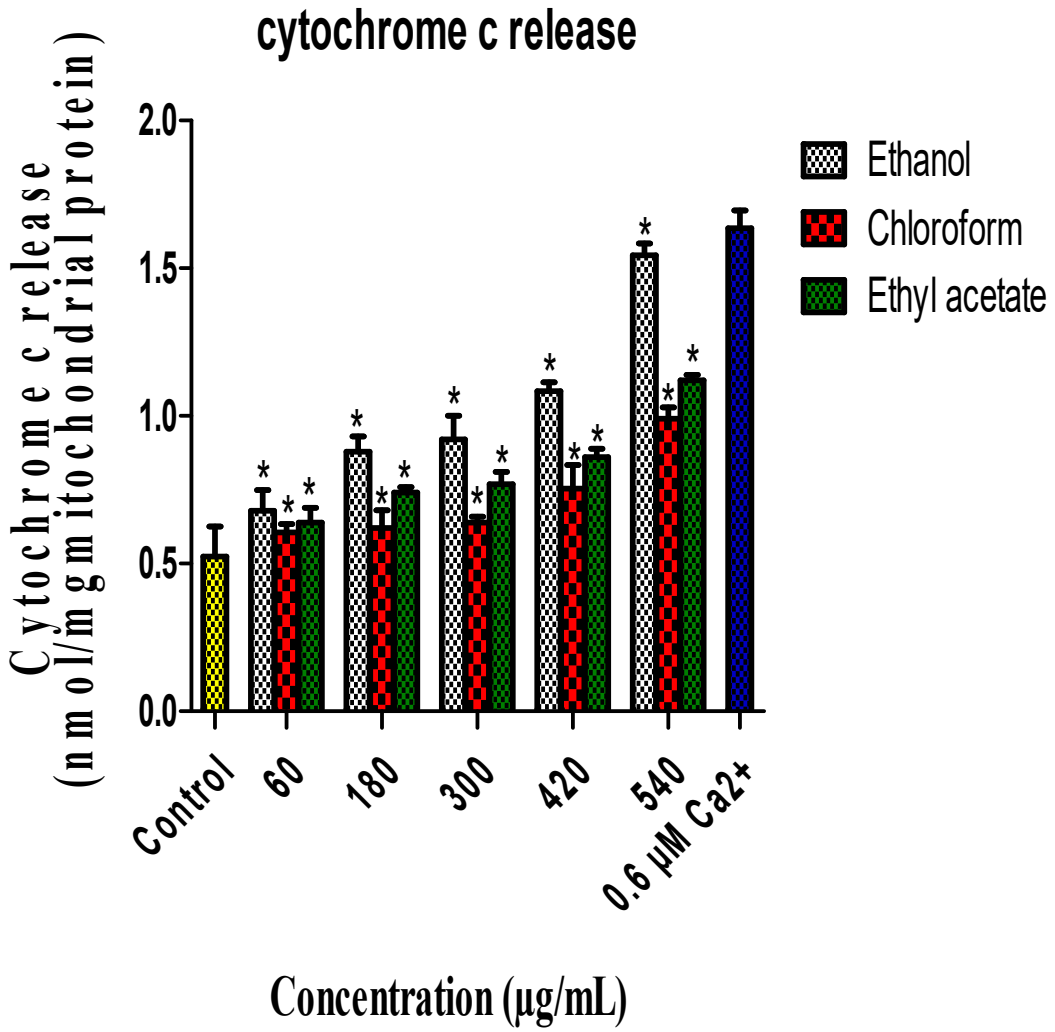


Fig. 19: Effects of solvent fractions of *D. oliveri* stem bark on cytochrome c release *in vitro*

(*: statistical significance, $p < 0.05$)

Experiment 3d

EFFECTS OF SUBFRACTIONS OF ETHANOL FRACTION ON mPTP*IN VITRO*

The Ethanol Fraction of *Daniellia oliveri* (EFDO) was subfractionated using n-hexane (100%), n-hexane: chloroform; chloroform (100%); chloroform: ethyl acetate (50:50); ethyl acetate (100%); ethyl acetae: ethanol (50:50), ethanol (100%); ethanol: methanol (50:50) and methanol (100%). The various subfractions were tested on mPT pore using different concentrations (120, 200 and 280 µg/ml). Ethyl acetate (100%) exhibited induction folds of 0.05, 0.06 and 0.08, respectively (Figure 20); ethyl acetate: ethanol (50:50), 0.07, 0.09 and 0.15 (Figure.21), ethanol (100%), 0.18, 0.20 and 0.28 (Figure.22),ethanol: methanol (50:50), 0.09, 0.15 and 0.21 (Figure 23) and methanol (100%), 0.06, 0.08 and 0.09 (Figure 24).

SUMMARY

Ethanol (100%) subfraction of the ethanol fraction was found to be the most potent in inducing mPT pore opening and was thus subjected to further assays.

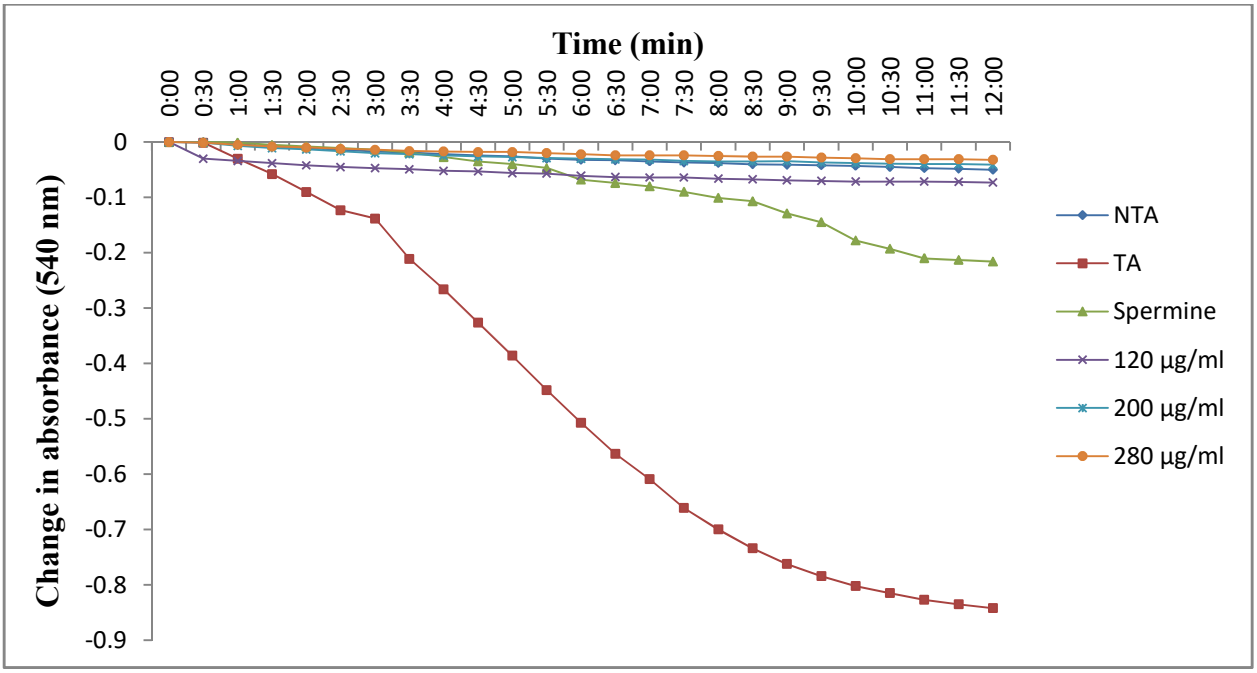


Fig.20: Effects of 100% ethyl acetate subfraction of EFDO on mPTP

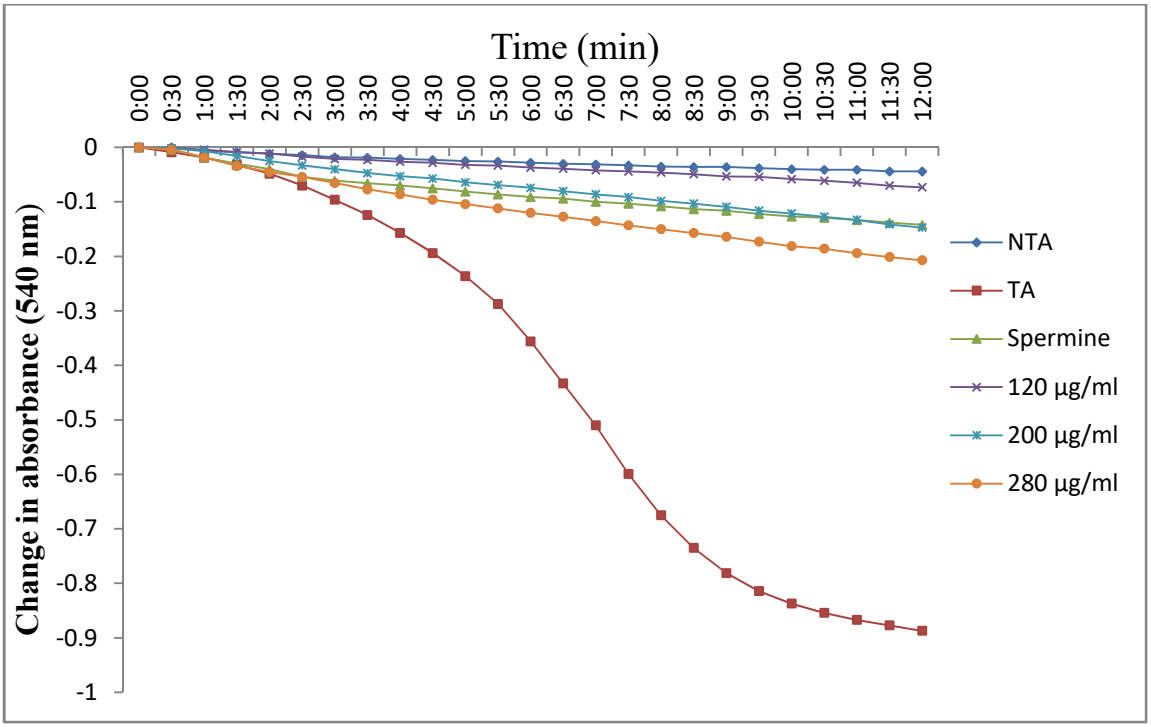


Fig.21: Effect of ethyl acetate: ethanol (1:1) subfraction of EFDO on mPTP

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

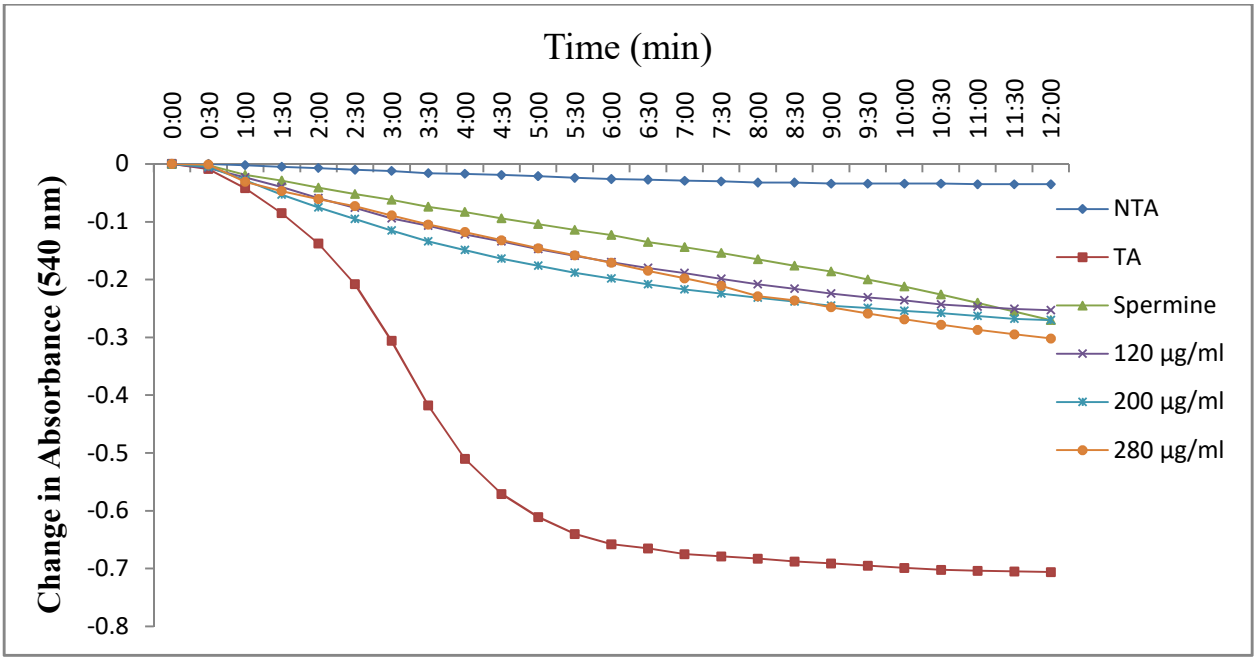


Fig.22: Effect of ethanol (100%) subfraction of EFDO on mPTP*in vitro*

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

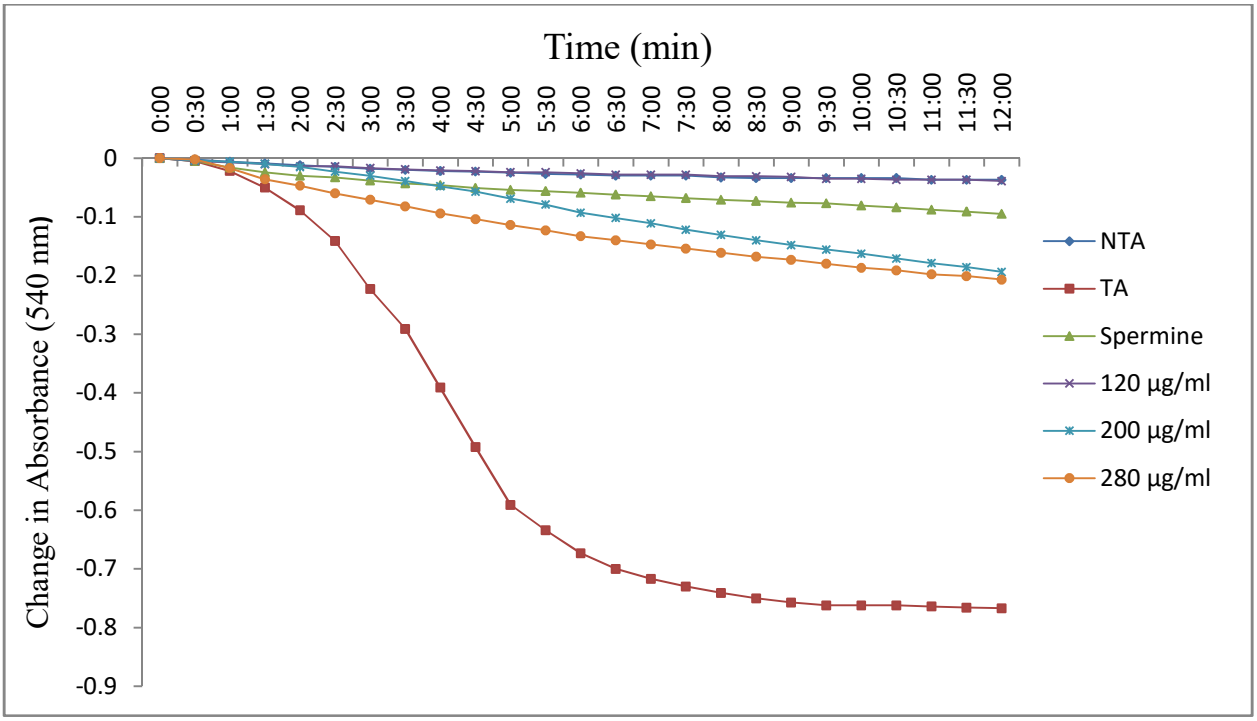


Fig.23: Effect of ethanol: methanol (1:1) subfraction of EFDO on mPTP*in vitro*

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

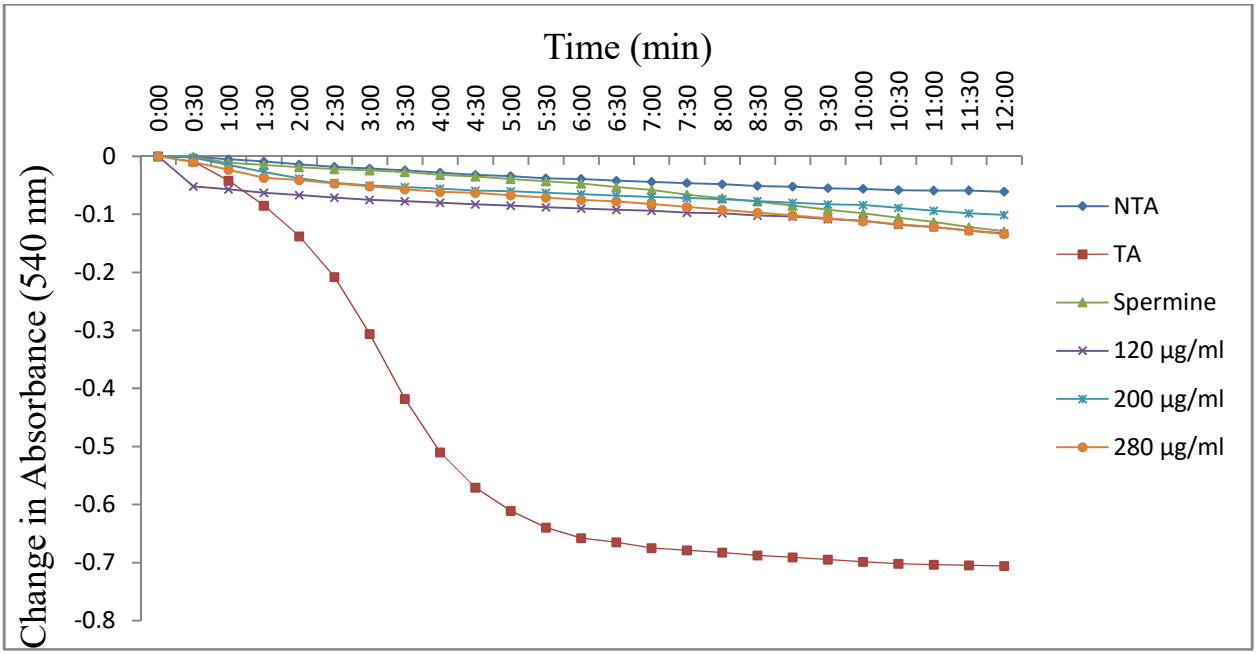


Fig.24: Effect of methanol (100%) subfraction of EFDO on mPTP*in vitro*

TA: Triggering Agent (Ca^{2+})

NTA: Non Triggering Agent

EXPERIMENT 4a

EFFECTS OF ORAL ADMINISTRATION OF CRUDE ETHANOL EXTRACT OF *D.oliveri*STEM BARK FOR 30 DAYS ON MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION, LIPID PEROXIDATION AND ATPASE ACTIVITIES.

EXPERIMENTAL DESIGN

White male rats weighing 70 – 90g were purchased from Veterinary Medicine animal house, University of Ibadan. 15 male rats were kept in cages made of stainless steel at animal house of Biochemistry Department, University of Ibadan. The animals were kept inroom withventilation with 12 h light-dark cycle, and they were freelyallowed access to food and water. The animals were kept for a fortnightfor acclimatizationand standardization of their weight.The animals were then randomlydividedinto three (3) groupsof five (5) animals each as follows:

Group 1: Control (distilled water)

Group 2: 300 mg/kgbdwt of crude ethanol extract

Group 3: 600 mg/kgbdwt of crude ethanol extract

PROCEDURE

Crude ethanol extract of *D. oliveri* stem bark was administered daily by oral administration of 300 and 600 mg/kgbdwt doses for 30days. After 30 days of administration,the animals were deprived of food and water overnight and then sacrificed by dislocation at the cervical. Isolation of mitochondria and assessment of mitochondrial membrane permeability transition pore opening followed the procedure described under materials and methods (section 3.4.1 and 3.4.2 respectively). A modified TBARS assay was used to quantify lipid peroxide formed using mitochondria as media rich in lipid as explained by Ruberto *et al*., (2000). ATPase activity was assessed in accordance with the method of Lardy and Wellman (1953) as modified by Olorunsogo and Bababunmi (1979).

RESULTS

Figure 25 illustrates the mPT pore result of crude ethanol extract after 30 days of oral administration. The control group shows no remarkable ($p > 0.05$) decrease in absorbance (2%). Upon treatment of the control group with triggering agent (Ca^{2+}), it significantly($p < 0.05$) caused a decrease in absorbance (large amplitude swelling) – 70% and this was significantly

reversed by spermine (88%). The two doses of the crude extract administered – 300 and 600 mg/kgbwt remarkably ($p < 0.05$) caused a decrease in absorbance of 41% and 48%, respectively. Figure 26 depicts the ATPase activity results andthe two doses to a significant extent ($p < 0.05$) enhanced ATPase activity by 33.3 and 35.6% respectively, while the standard uncoupler, Dinitrophenol (DNP) enhanced by 42%. Figure 27shows the lipid peroxidation results. The two doses of crude extract to a significantextent ($p < 0.05$) decreased malondialdehyde (MDA) formation by 24 and 33%, respectively when compared to the control.

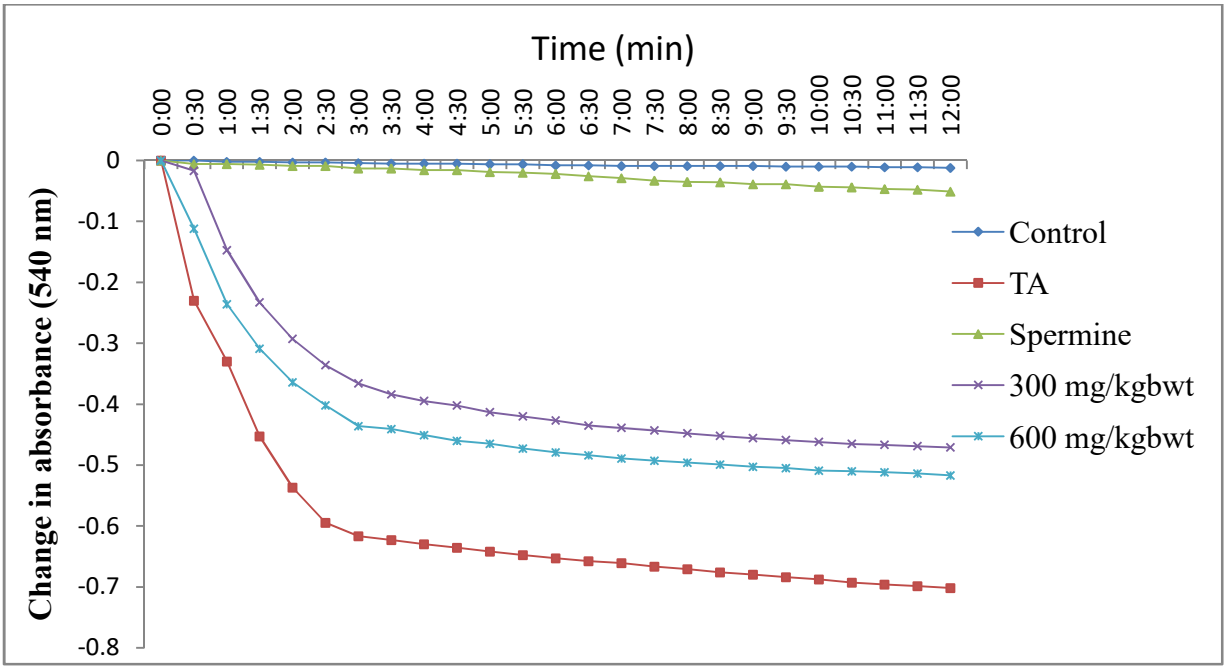


Fig. 25: Effects of crude ethanol extract of *D. oliveri* on rat liver mPT pore *in vivo* after 30 days of oral administration.

TA: Triggering Agent (Ca^{2+})

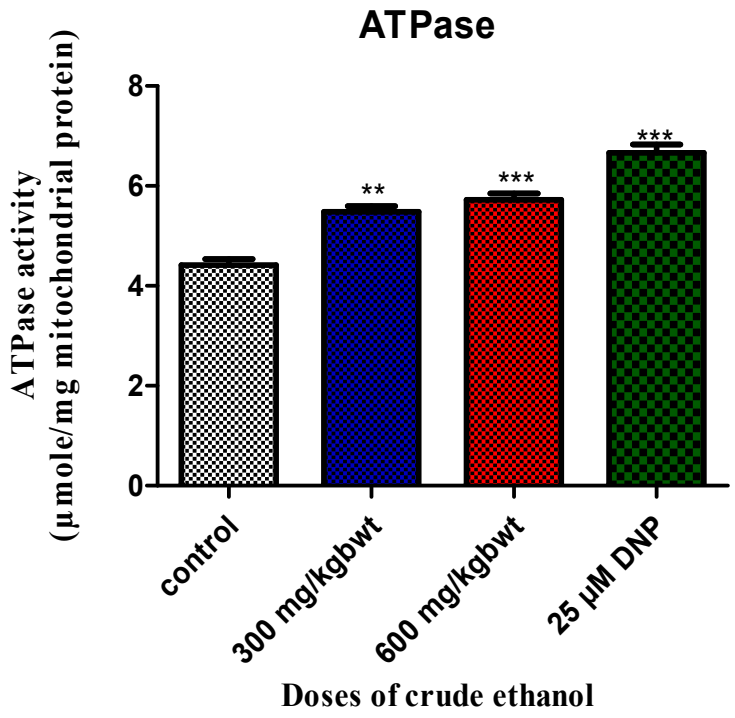


Fig. 26: Effects of crude ethanol extract of *D.oliveri* on ATPase activity *in vivo.*

(*: statistical significance, p< 0.05)**

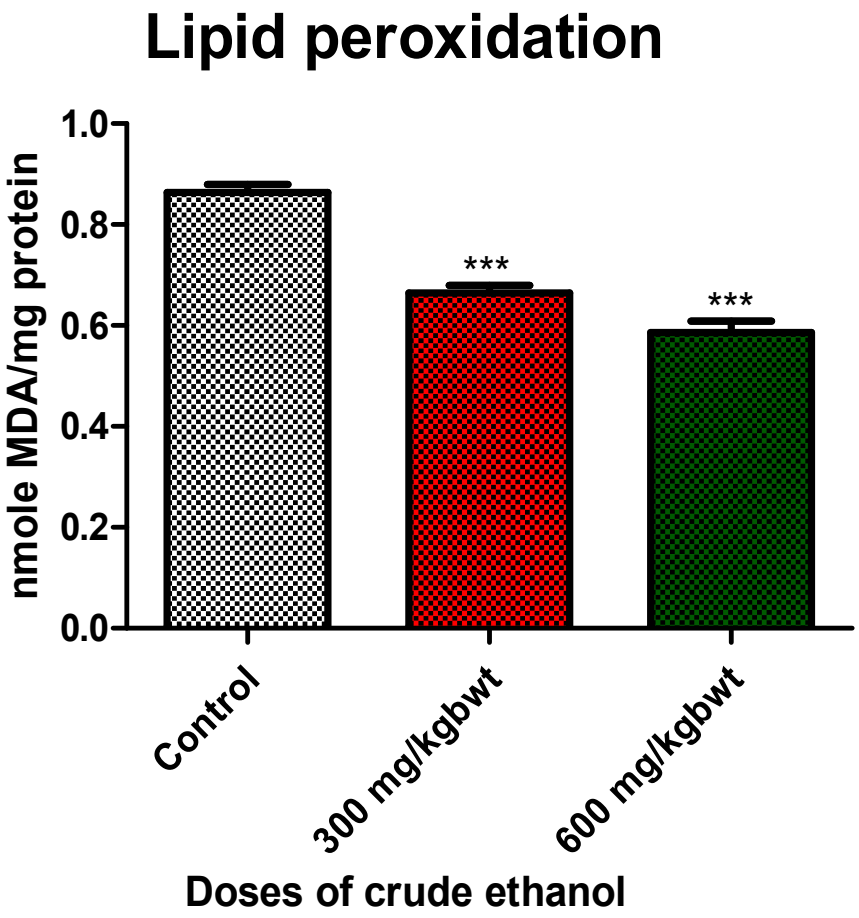


Fig. 27: Effects of crude ethanol extract of *D. oliveri*on lipid peroxidation *in vivo.*

(*:** statistical significance, p< 0.05**)**

EXPERIMENT 4b

ASSESSMENT OF VARYING CONCENTRATIONS OF ETHANOL FRACTION OF *D.oliveri*STEM BARK ON INTACT RAT LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE *IN VIVO*.

INTRODUCTION

The major aim of mitochondria associated chemical treatment of diseases is to induceintrinsicand extrinsic routes of apoptosisto trigger death of cells in abnormal growth and thereby, impede differentiation of cell and tumour development (Fulda *et al.,* 2010). The pharmacological efficacy of anti-tumour drugs is usually elevated by mPTP-targeted agents. Furthermore, the compounds triggering calcium accumulation and ROS production, as well as exhaustion of ATP can in an indirect approach incite mPT pore formation (Fulda and Debatin, 2006; Kroemer *et al*., 2007).

DESIGN OF EXPERIMENT

A total of 30 male albino rats weighing 70 – 90 g were purchased as described under materials and methods (page 43).The animals were then randomly distributed intofive (5) groups of six (6) animals each as follows:

Group 1: Distilled water (Control)

Group 2: 100mg *D. oliveri*/kgbdwt

Group 3: 200mg *D. oliveri*/kgbdwt

Group 4: 300mg *D. oliveri*/kgbdwt

Group 5: 400mg *D oliveri*/kgbdwt

PROCEDURE

Ethanol fraction of *D. oliver* stem bark was administered daily by oral doses for 30days andafter 30 days of administration,the animals were deprived of food and water overnight and then sacrificed by dislocation at the cervical, and mitochondria with low ionic strength were isolated as described by Johnson and Lardy (1969). Isolation of rat liver mitochondria and assessment of mitochondrial membrane permeability transition pore opening followed the procedure described under materials and methods (section 3.4.1 and 3.4.2, respectively).

RESULTS

Figure 28depicts the results of the *in vivo* treatment of ethanol fraction of *D. oliveri* stem bark, respectively on rat liver mPT pore. The result showsthat all the concentrations used in this experiment exhibited no significant($p > 0.05$)inductionin the intact rat liver mPT pore.

SUMMARY

The inability ofethanolfraction to induce mPT pore*in vivo*, despite its positive effects*in vitro*, raises the question of bioavailability, accessability and biotransformation, factors which have up till now impeded the transition of potential drug candidates to preclinical trials.

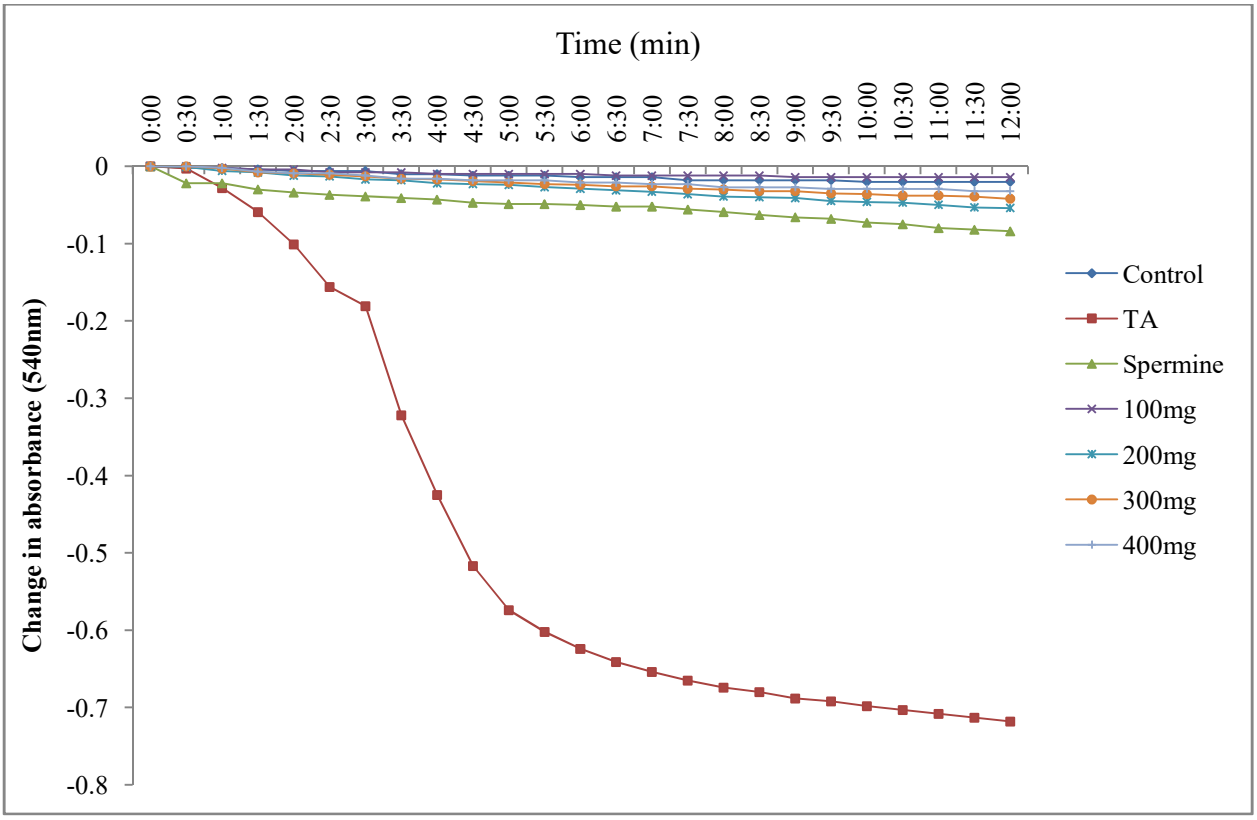


Fig. 28: Effects of ethanol fraction of *D. oliveri* stem bark on rat liver mitochondrial membrane permeability transition pore *in vivo*

TA: Triggering Agent

EXPERIMENT 4c

ASSESSMENT OFINTRAPERITONEAL (IP) ADMINISTRATIONOF VARYING DOSES OF ETHANOL FRACTION OF *Daniellia oliveri* STEM BARK ON INTACT RAT LIVER MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE *IN VIVO.*

EXPERIMENTAL DESIGN

A total of 24 male albino rats weighing 70 – 90 g were purchased as described under materials and methods (section 3.3).The animals were randomly divided into four (4) groups of six (6) animals each as follows:

Group 1: Control (distilled water)

Group 2: 25 mg/kgbdwt of EFDO

Group 3: 50 mg/kgbdwt of EFDO

Group 4: 100 mg/kgbdwt of EFDO

PROCEDURE

Ethanol fraction of *D. oliveri* stem bark wasadministered daily by intraperitoneal administration for 14 days.The animals were then deprived of food and water overnight,sacrificed by dislocation at the cervical, and isolation of rat liver mitochondria and assessment of mitochondrial membrane permeability transition pore opening followed the procedure described under materials and methods (section 3.4.1 and 3.4.2 respectively).

RESULTS

Figure 29 illustrates the mPT pore result of ethanol fraction after 15 days of intraperitoneal administration. The control group shows no noticeable ($p > 0.05$) decrease in absorbance (1.3%). Upon treatment of the control group with triggering agent (Ca^{2+}), there was to a significant ($p < 0.05$) extent decrease in absorbance (large amplitude swelling) – 78% and this was significantly reversed by spermine (83%). The three doses of the EFDO administered – 25, 50 and 100 mg/kgbwt caused a remarkable ($p < 0.05$) decrease in absorbance of 0.28, 0.58 and 0.69 (28, 58 and 69%), respectively. Figure 30 depicts the ATPase activity results and the three doses significantly ($p < 0.05$) enhanced ATPase activity by 62, 66 and 70%, respectively, while the standard uncoupler, dinitrophenyl (DNP) enhanced by 81%. Figure 31illustrates the lipid

peroxidation results. The three doses of EFDO significantly ($p < 0.05$) decreased malondialdehyde (MDA) formation by 9.4, 15 and 30%, respectively relative to control.

SUMMARY

Results of the intraperitoneal administration of EFDO stem bark to rats for 14 days show significant induction of mPT pore opening in a concentration-dependent approach. There was also enhancement of ATPase activity and decrease in malondialdehyde (MDA) generation from lipid peroxidation in a concentration-dependent manner.

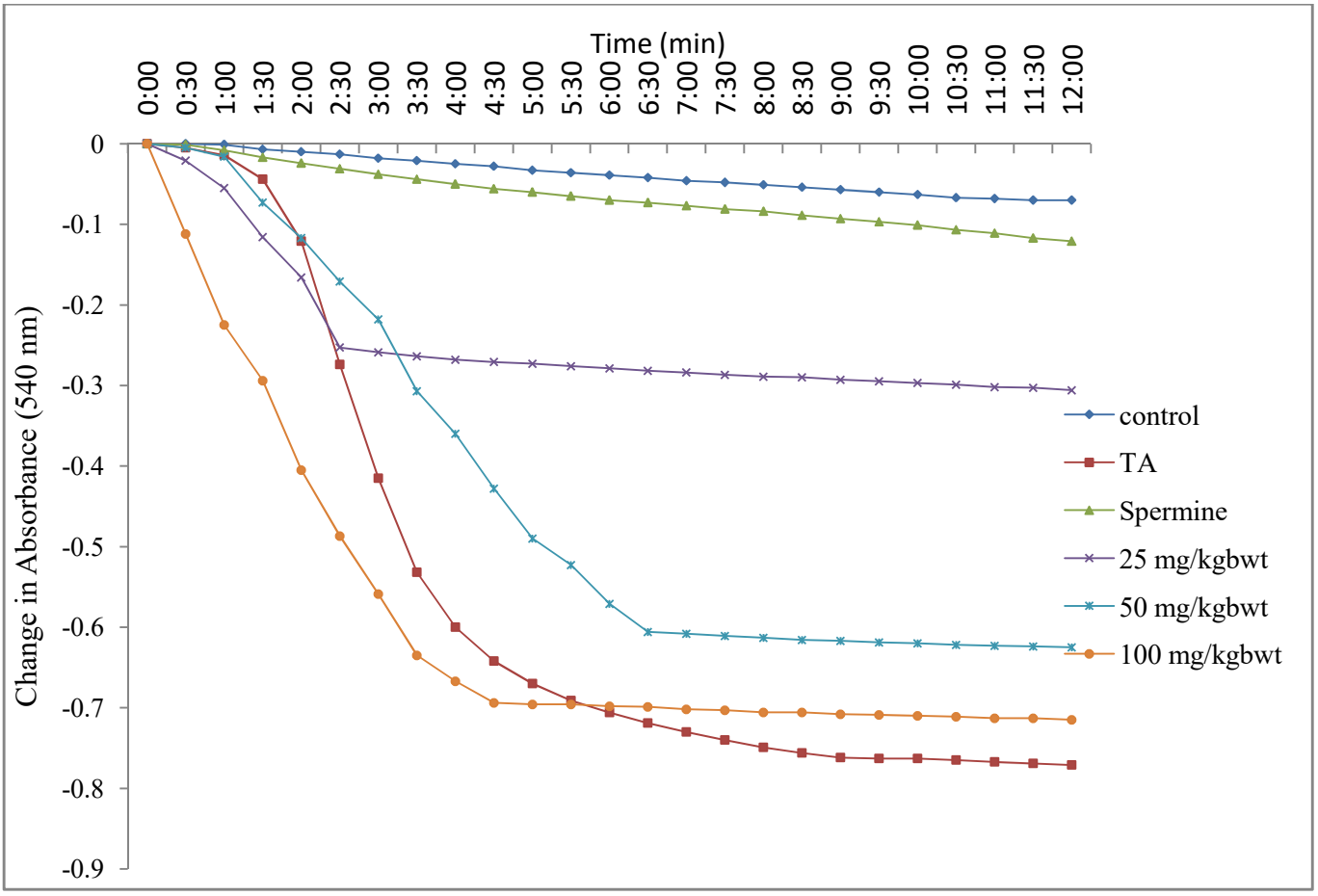


Fig. 29*: In* vivo effect of EFDO on rat liver mPT pore by ip administration

TA: Triggering Agent (Ca^{2+})

EFDO: Ethanol Fraction of *Daniellia oliveri*

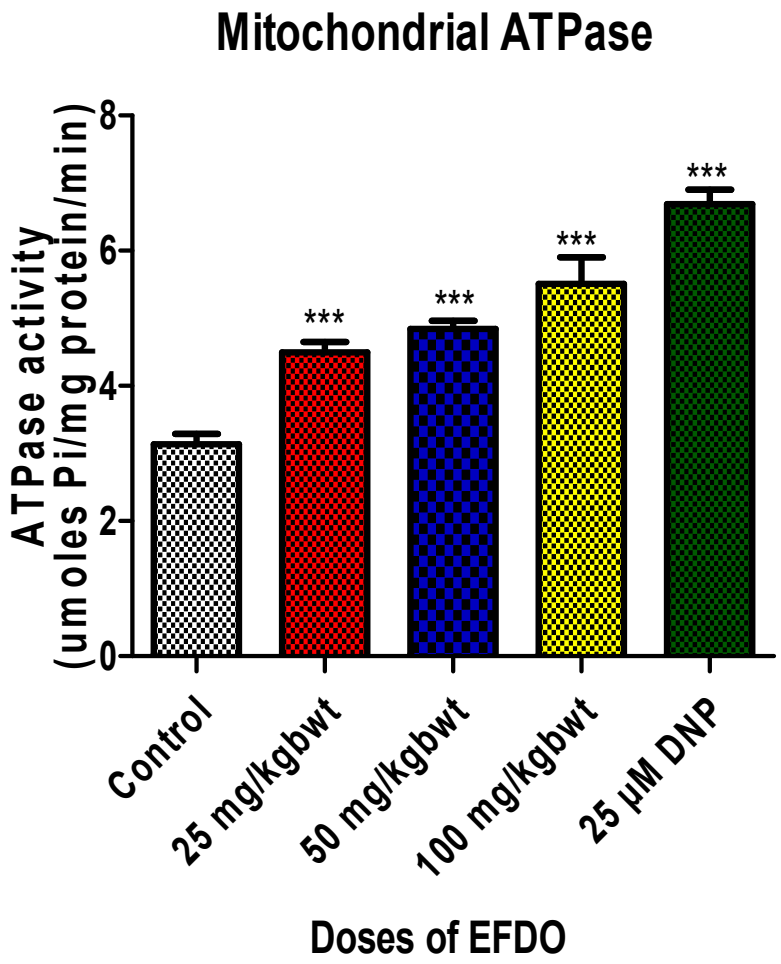


Fig.30: Enhancement of ATPase activity by EFDO on rat liver mitochondria by ip administration.

(***: statistical significance, $p < 0.05$)

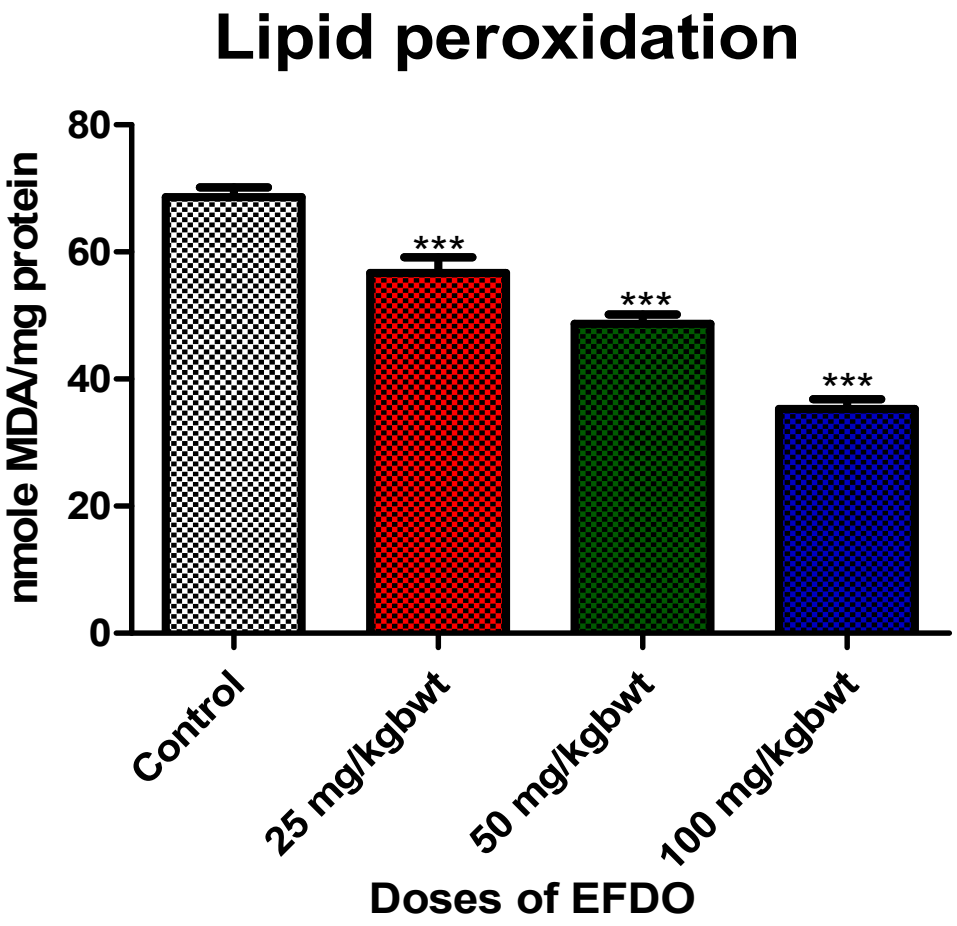


Fig.31: Inhibition of lipid peroxidation by EFDO on rat liver mitochondria by ip administration.

(***: statistical significance, $p < 0.05$)

EXPERIMENT 5

HISTOLOGICAL ASSESSMENT OF VISCERAL ORGANS OFMALE ALBINO RATS AFTER 14 DAYS OF INTRAPERITONEAL ADMINISTRATION OF ETHANOL FRACTION OF *Daniellia oliveri* STEM BARK

INTRODUCTION

Histopathology refers to investigation of tissue usingmicroscope in order to ascertaindiseasemanifestations. This always commences with surgery, tissue is excised and then placed in a fixative which stabilizes the tissues to avoiddecaying. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides. The tissue is then prepared for viewing under a microscope using either chemical fixation or frozen section.

PRINCIPLE

Alum acts as mordant and hematoxylin containing alum stains the nucleus light blue. This turns red in the presence of acid as differentiation is achieved by treating the tissue with acid solution. Bluing step converts the initial soluble red colour within the nucleus to an insoluble blue colour. The counterstaining is carried out by using eosin which imparts pink colour to the cytoplasm.

PROCEDURE

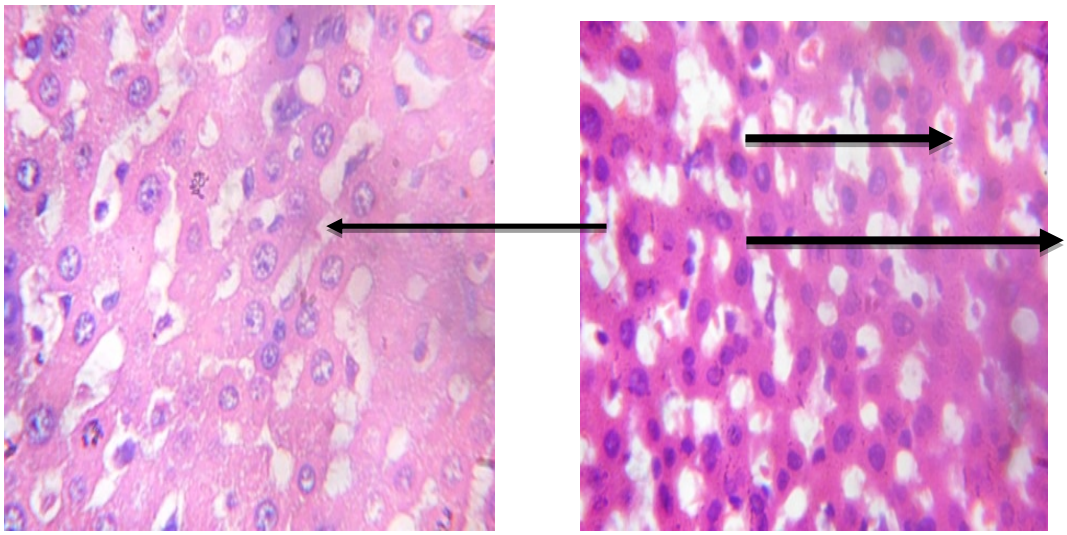
Histopathological investigation of tissues always commenced with surgery and tissue was cut out.This was followed by fixing which makes the tissues stableto inhibitdecaying. Formalin(10% formaldehyde in water) is the frequently used fixative.The tissue is first frozen and a microtome mounted in a below-freezing refrigeration device called the cryostat is then used to slice it into thinly forms. The thin frozen sections prepared are then placed on a glass slide, fixed immediately and briefly in liquid fixative and stained using the similar staining techniques as traditional wax embedded sections.After all other steps were carried out, the tissue section was then mountedunder microscope in mounting media and viewed.

RESULTS

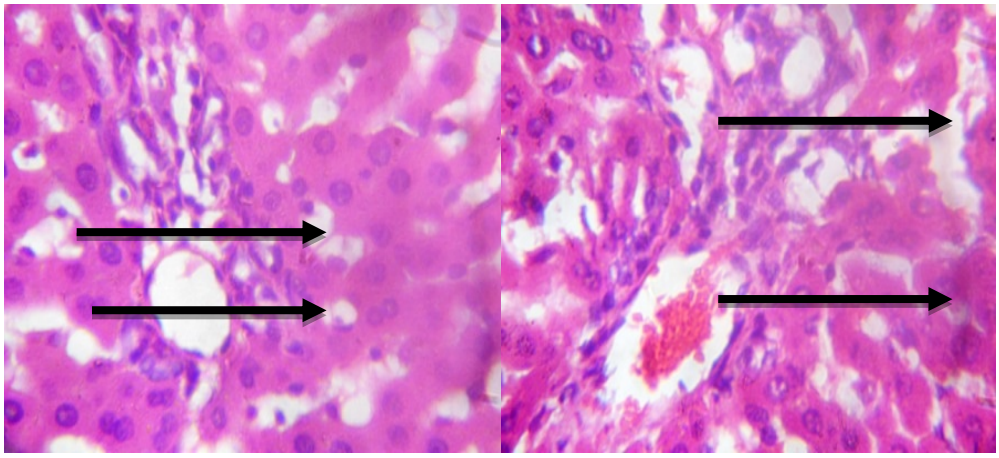
Figure 32 illustrates the photomicrographs of the liver tissue.The control group showedmild congestion and disseminated infiltration by inflammatory cells and lymphoid aggregate. The treated groups (25, 50 and 100 mg/kg bwt) exhibitedmoderate disseminated periportal infiltration by inflammatory cells; disseminated congestion and mild infiltration by inflammatory cells and disseminated congestion, mild infiltration by inflammatory cells and mild steatosis, respectively. Figure 33 depicts the photomicrographs of the kiney tissue. The control group showedmild congestion, mild disseminated infiltration by inflammatory cells and lymphoid aggregate.The treated groups (25, 50 and 100 mg/kg bwt) exhibited mild congestion, focal area of thrombosis and moderate periportal infiltration by inflammatory cells; widespread periportal infiltration by inflammatory cells and mild disseminated congestion, and widespread periportal infiltration by inflammatory cells, mild disseminated congestion and moderate infiltration by inflammatory cells, respectively. Figure 34 illustrates the photomicrographs of the heart tissue. The control group showedmild disseminated steatosis. The treated groups (25, 50 and 100 mg/kg bwt) exhibited moderate disseminated periportal infiltration by inflammatory cells; disseminated congestion and mild widespread periportal infiltration by inflammatory cells, and disseminated congestion, mild disseminated periportal infiltration by inflammatory cells, respectively**.**

SUMMARY

The ip administration of EFDO for 14 days shows mild/moderate congestion and disseminated periportal infiltration by inflammatory cells (cytotoxicity) on the visceral organs investigated.



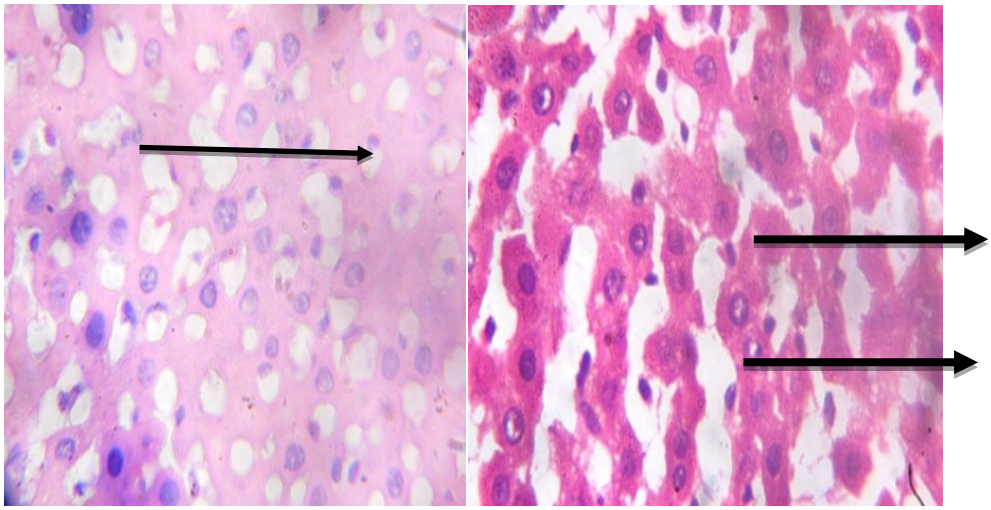
Control 25 mg/kgbwt



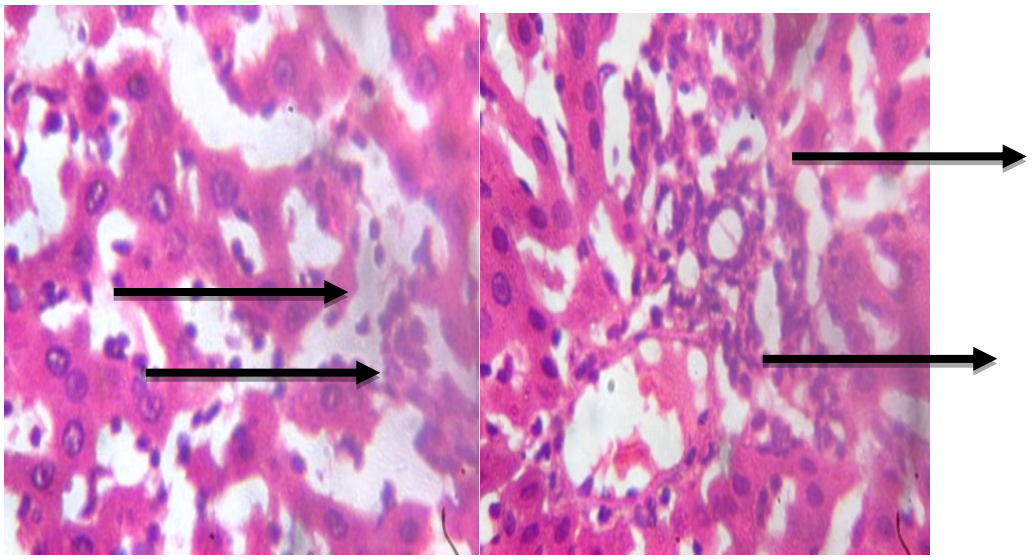
50 mg/kgbwt 100 mg/kgbwt

Fig. 32: Photomicrographs of EFDO on liver tissues of albino rats exposed for 14 days of intraperitoneal administration (x400)

→: Showing area of leision and cytotocity



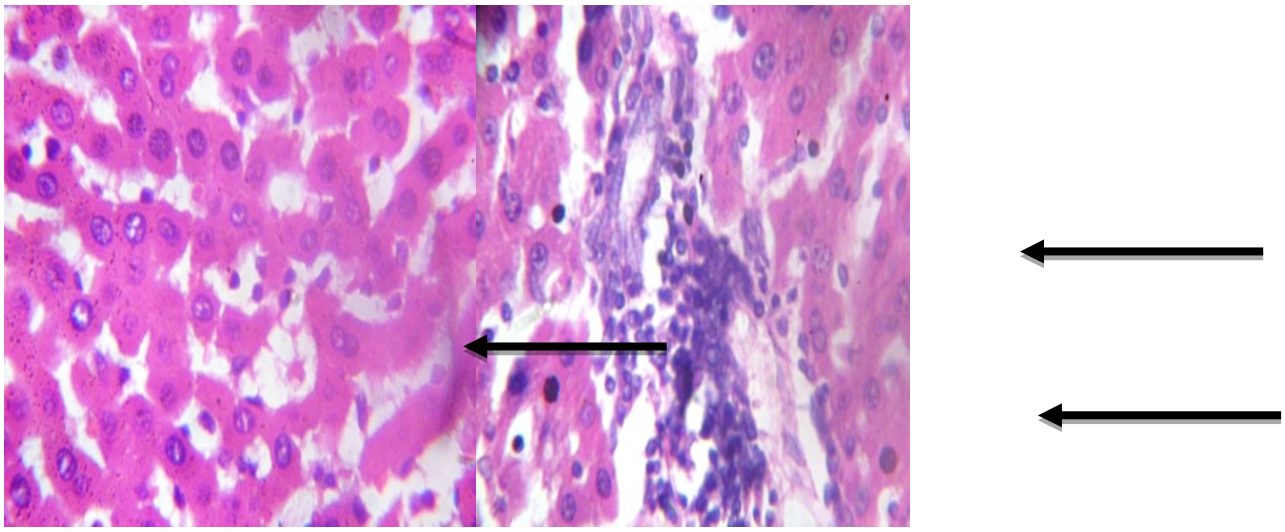
Control 25 mg/kgbwt



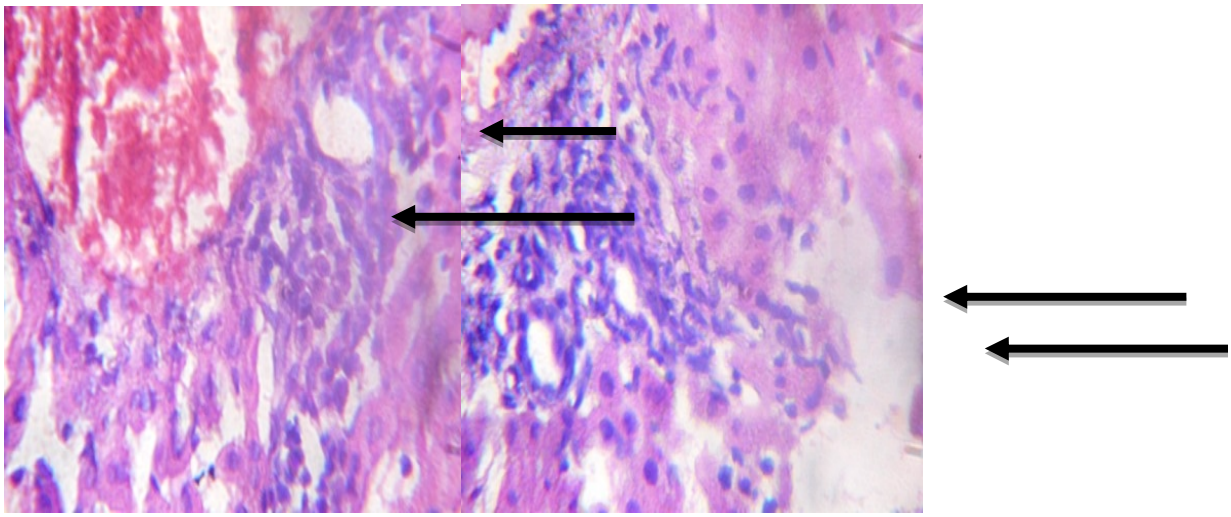
50 mg/kgbwt 100 mg/kgbwt

Fig. 33: Photomicrographs of EFDO on kidney tissues of albino rats exposed for 14 days of intraperitoneal administration (x400).

→Showing area of leision and cytotocity



Control 25 mg/kgbwt



50 mg/kgbwt 100 mg/kgbwt

Fig. 34: Photomicrographs EFDO on heart tissues of Wistar albino rats for 14 days of intraperitoneal administration (x400).

→ Showing area of leision and cytotocity

EXPERIMENT 6

ASSESSMENT OF EXPRESSIONS OF APOPTOTIC BIOMARKERS IN RAT LIVER OF ANIMALS EXPOSED TO GRADED DOSES OF ETHANOL FRACTION OF *Daniellia oliveri.*

INTRODUCTION

The Bcl-2 proteins exhibit a crucial function in manipulating the intrinsic channel of cell death and are important for neuronal apoptosis (Blomgren*et al*., 2007). These proteins control a critical step in commitment to apoptosis by regulating MOMP. In the "rheostat" ideal cell deathor survival is a function of ratio of anti- to pro-apoptotic proteins present at the mitochondria (Korsmeyer *et al*., 1993). When there is overexpression of antiapoptotic proteins,the cells are protectedagainst death of cells, whereas, when the proapoptotic proteins are in excess or over expressed, they facilitate the discharge of proapoptotic proteins from mitochondria into the cytosol to stimulate the process of death of cell(Cheng *et al*., 2001). The Bax and Bak proteins are proapoptotic executioners that trigger cell death by forming mPTpores and enhancing liberation of cytochromec.This experiment was thus carried out to investigate the modulatory effects of EFDO on apoptotic biomarkers.

EXPERIMENTAL DESIGN

Male albino rats (24) weighing 80 – 100 g were purchased as described under materials and methods (page 43).Males were chosendue to the fact that females may have likely cardiac protective impacts due to estrogen (Camper-Kirby*et al*., 2001) along with elevated concentrations of telomerase activity, which could raise the tissue rebuilding ability (Leri*et al*., 2000). In fact, male rats displayed higher elevation in pressure-induced myocardial ultrastructure (Soldani*et al*., 1997). The animals were then randomly distributed intofour (4) groups of six (6) animals each as follows:

Group 1: Control (distilled water)
Group 2: 25 mg/kgbdwt of EFDO
Group 3: 50 mg/kgbdwt of EFDO
Group 4: 100 mg/kgbdwt of EFDO

PROCEDURE

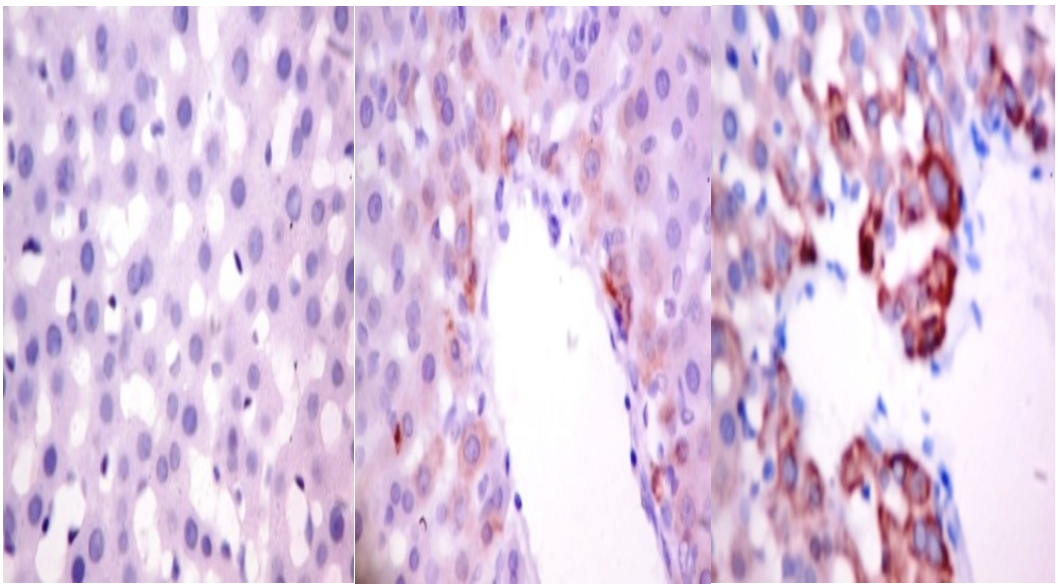
Ethanol fraction of *Daniellia oliveri* stem bark wasadministered daily by intraperitoneal administration for 14 days after which the animals were sacrificed by cervical dislocation and the liver excised, rinsed with and preserved in 10% formalin phosphate Buffer Saline (PBS). Immunohistochemical staining was carried out in accordance with the instructions of the manufacturer.

PRINCIPLE

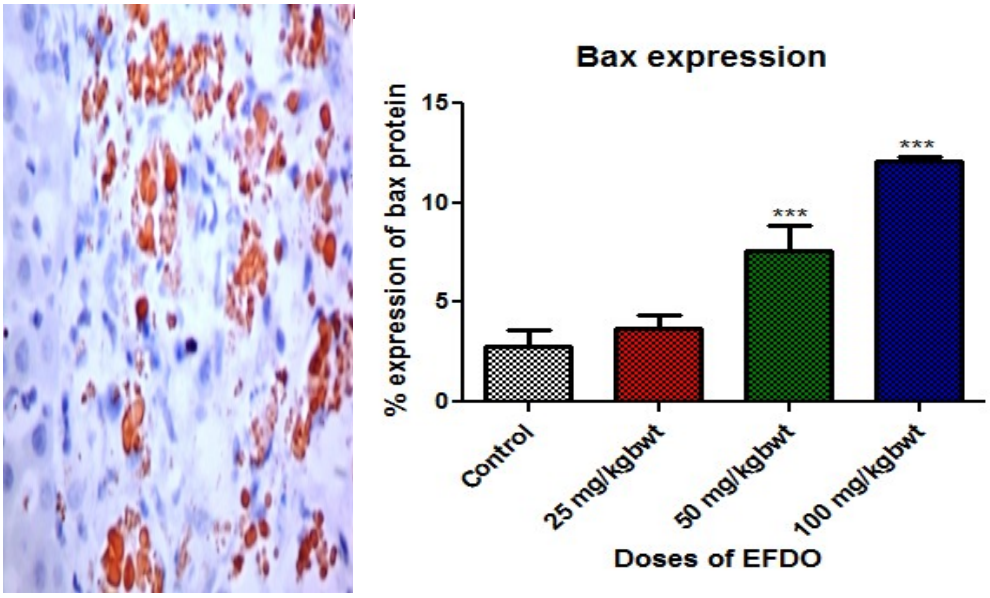
The selection of antibodies for testing immunohistochemistry is made on the basis of their tumour explicitness and the probability that they will react with the tumour under assessment. After incubation oftissue sections with the prospective antibodies, positive reactions (tumour antigen- antibody binding) are identified by means of applying one of manifoldsystems detection. Those that have the greatest sensitivity use a secondary antibody, reactive against the primary antibody, which is conjugated or linked to a marker enzyme. This system tends to bevery sensitive because it permits for the binding of a relatively huge number of molecules of enzyme, such as peroxidase, at thesite of antigen. The reaction colouris assessed byselecting a precipitating chromogen, usually diaminobenzidine (brown) or aminoethylcarbazole (red) which the enzyme reactswith.

Results

Figure 35depicts immunohistochemical expression of Bax protein. The three doses (25, 50 and 100 mg/kg bwt) remarkably ($p < 0.05$) increased Bax expression by35, 200 and 330%, respectively compared to control. Figure 36illustrates immunohistochemical Bcl-2 expression and the doses significantly decreased this protein expression by 5.5, 34.5 and 56.4%, respectively compared to control.Figure 37 shows immunohistochemical expression of cytochrome c and the doses significantly increased its expression by 91.3, 100 and 204%, respectively compared to control. Figure 38 depicts immunohistochemical expression of p53 protein and the doses significantly increased its expression by50, 100 and 300%, respectively compared to control. Figure 39 illustrates immunohistochemical expression of DNA fragmentation (Tunel Assay Method). The three doses significantly increased nuclear DNA fragmentation by 20, 55 and 115%, respectively compared to control.



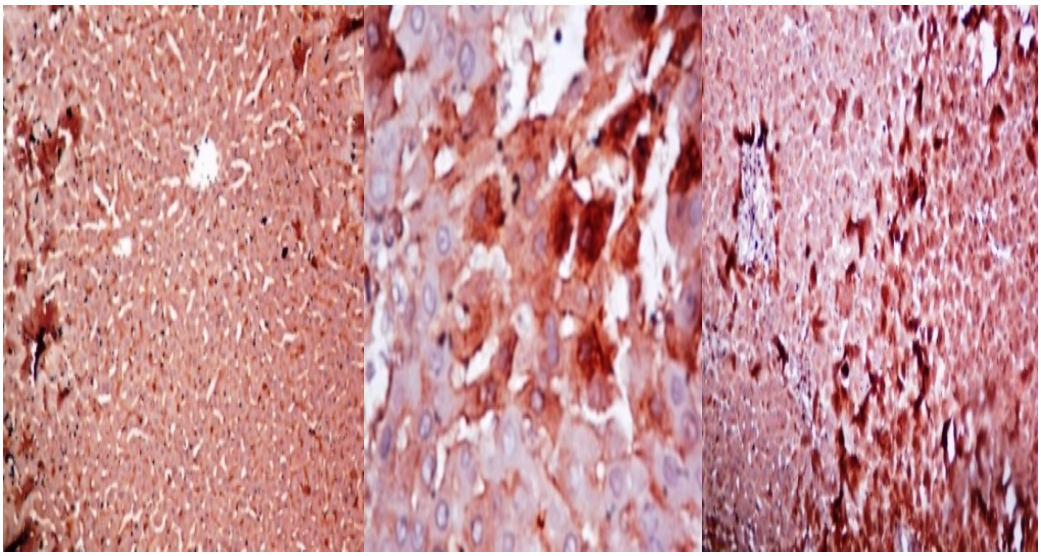
Control25 mg/kgbwt50 mg/kgbwt



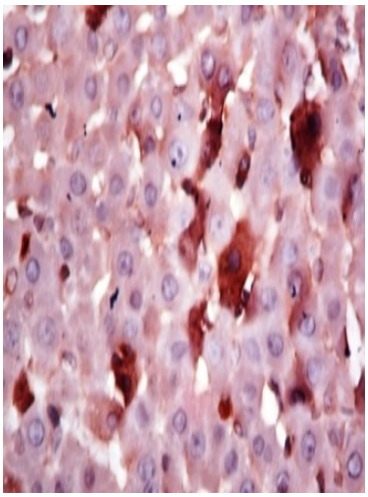
100 mg/kgbwt

Fig. 35: Immunohistochemical expression of Bax protein (x400)

(***: Statistical significance, $p < 0.05$)



Control **25 mg/kgbwt** **50 mg/kgbwt**



100 mg/kgbwt

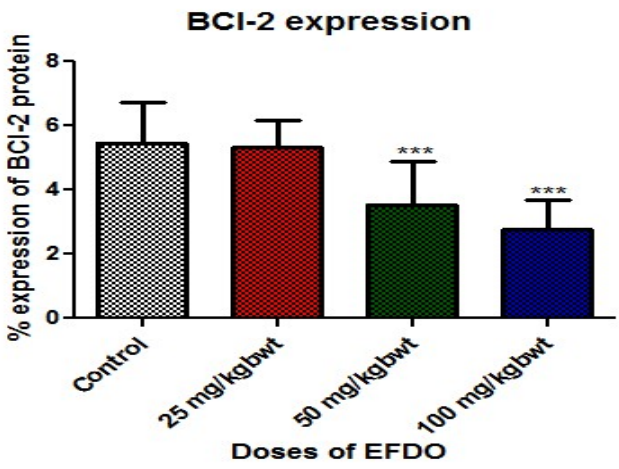
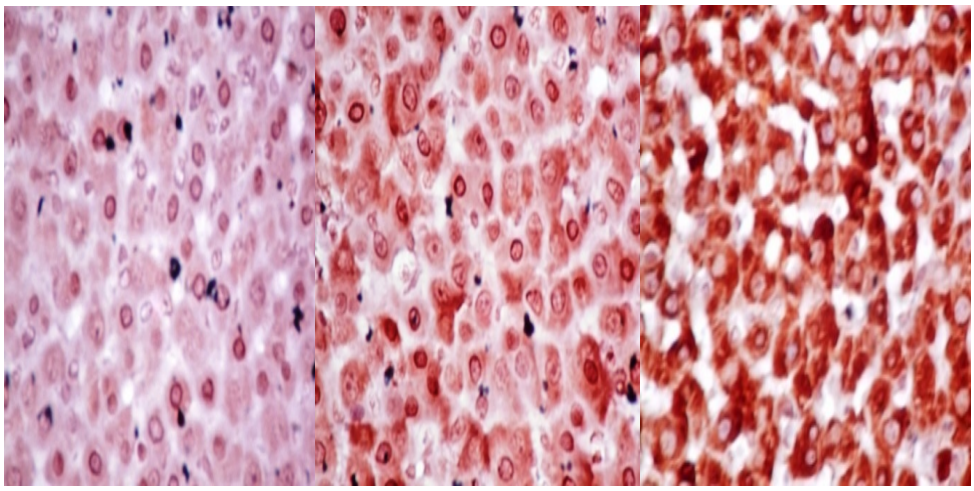
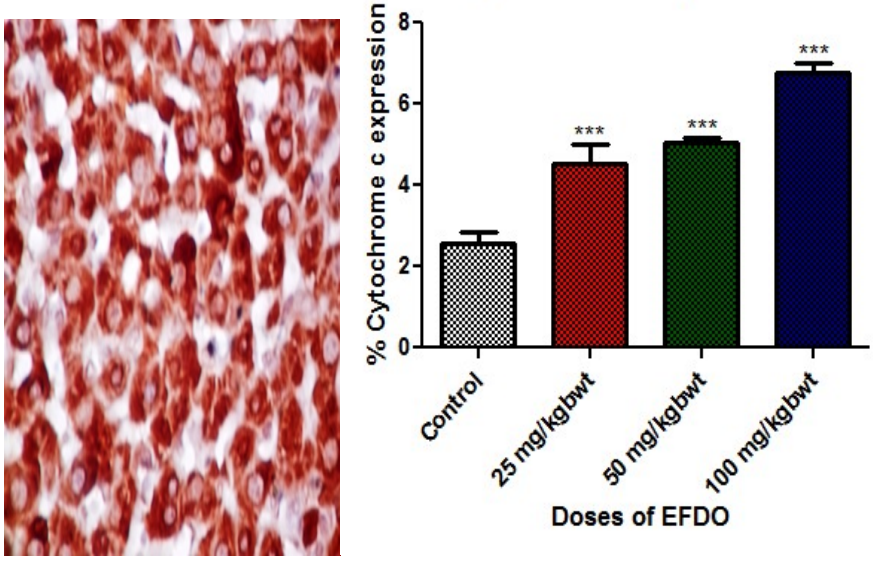


Fig. 36: Immunohistochemical expression of BCl-2 protein (x400)

(***: Statistical significance, $p < 0.05$)



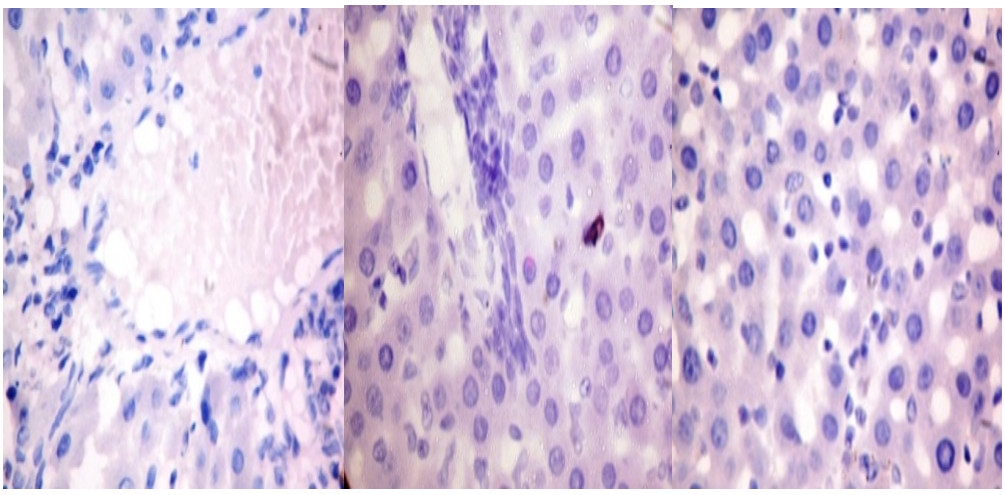
Control **25 mg/kgbwt** **50 mg/kgbwt**



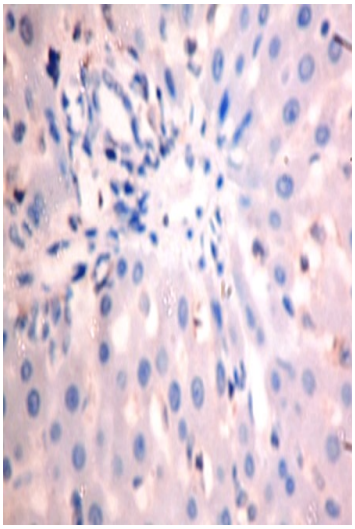
100 mg/kgbwt

Fig. 37: Immunohistochemical expression of cytochrome c protein (x400)

(***: Statistical significance, $p < 0.05$)



Control **25 mg/kgbwt** **50 mg/kgbwt**



100 mg/kgbwt

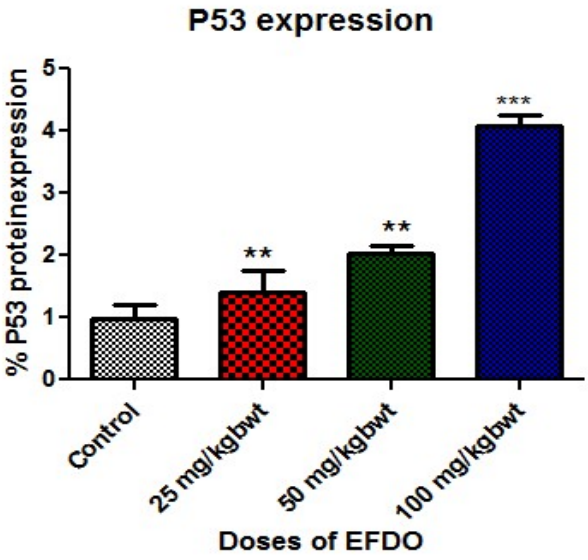
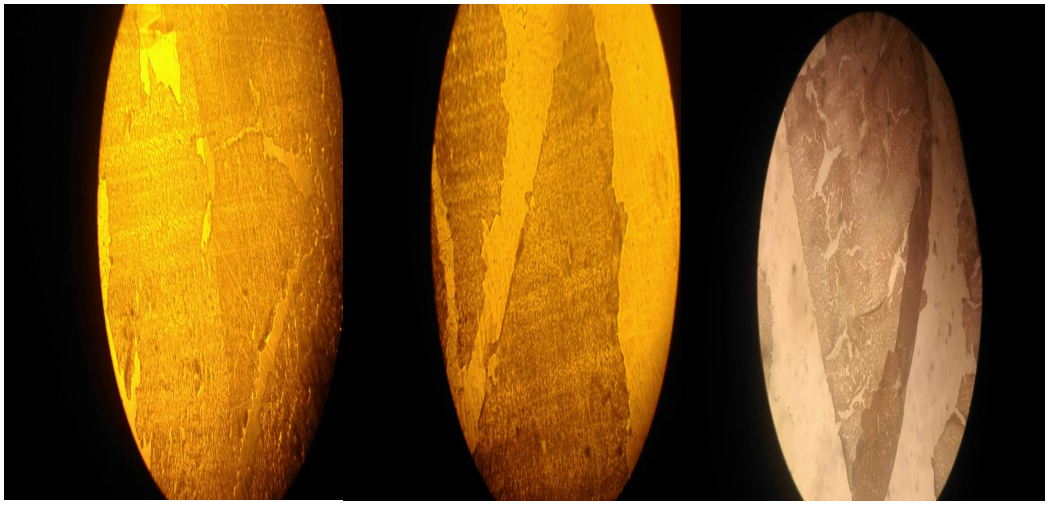
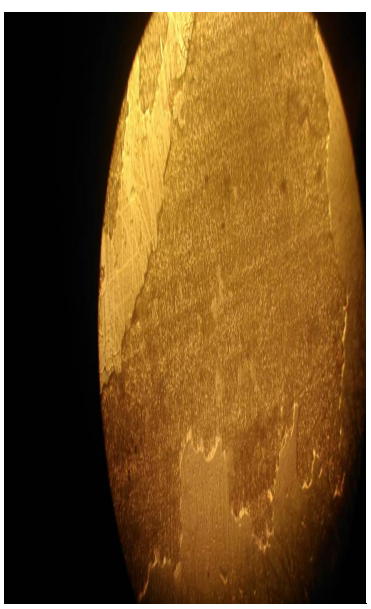


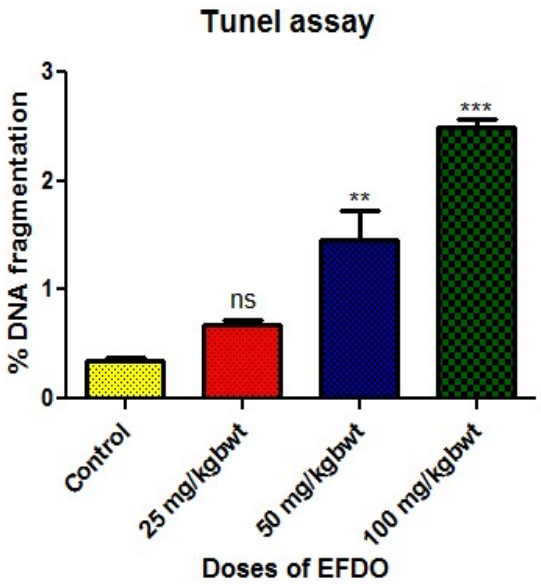
Fig. 38: Immunohistochemical expression of p53 protein (x400)

(***: Statistical significance, $p < 0.05$)



Control **25 mg/kgbwt** **50 mg/kgbwt**





100 mg/kgbwt

Fig. 39: Immunohistochemical expression of DNA fragmentation (Tunel Assay)

(***: Statistical significance, $p < 0.05$)

EXPERIMENT 7

DETERMINATION OF EFFECTS OF ETHANOL FRACTION OF *Daniellia oliveri*ON CASPASE 3 AND 9 ACTIVITIES, AND DNA FRAGMENTATION.

DETERMINATION OF DNA FRAGMENTATION (Assay via Diphenylamine)

INTRODUCTION

The gradual breakdown of DNA in thenucleus into small nucleosomal subunits is one of the distinguishing characteristic of death of cell. This process takes place as result of different apoptotic stimuli inseveraltypes of cell. Explicit DNase, caspase-activated DNase (CAD) that disintegrates chromosomal DNA in a caspase-dependent mode has been identified from molecular characterization of this process. The DNA breakdown acts a major function in the process ofapoptosis. It is a natural breakdown phenomenon which cells carry outand it is a biochemical characteristic of death of cell. The fragmentation of DNA during apoptosis is being used as a marker of cell death. This assay was thus performed to ascertain the effects of EFDO on fragmentation of DNA.

PROCEDURE

Preparation of liver tissue for DNA fragmentation assay followed the procedure as described under materials and methods (section 3.4.12).

$$\% \text{ DNA fragmentation} = \frac{\text{Absorbance of supernatant}}{\text{Absorb.of supernatant + pellet}} \times 100$$

ASSAY FOR CASPASE 3 ACTIVITY

Caspases act essentially in triggering apoptosis and caspase 3 is an important factor. It possesses many functions in several routes through which apoptosis signals transduce. It is often present in the cytosol inform of a pro-enzyme and during the starting stage of cell death, it is actuated. Stimulated caspase-3 is made of two subunits, that is, 17 kD and 12 kD subunits, and it cleaves substrate in cytosol and nucleus. The activity of caspase-3 significantly reduces during the ending stage of apoptosis.

PROCEDURE: Assay was performed in accordance with the instructions of the manufacturer as explained under materials and methods (section 3.4.13).

PRINCIPLE

Activated caspase 3 can explicitly split substrate by breaking peptide bond. Based on this reaction, a short peptide coupled with fluorescent dye was designed. In covalent coupling, Aminomethylcoumarin (AMC) can't be actuated to eject fluorescence. After the short peptide is hydrolyzed in which AMC is liberated, free AMC can be actuated to eject fluorescence. According to the fluorescence intensity discharged by AMC, caspase 3 activity can be measured at 460 nm.

RESULTS

Figure40ilustrates theeffects of EFDO on caspase 9 activity and the two doses (25 and 50 mg/kg bwt) used significantly increased caspase activity by 15.8 and 68.4% respectively, compared to control.Figure 41 depicts effects of EFDO on caspase 3 activity, which was significantly increased by 87.5and 150%, respectively relative to control.Figure 42 shows the effects of EFDO on hepatic DNA fragmentation and this was significantly increased by 60 and 92%, respectively compared to control.

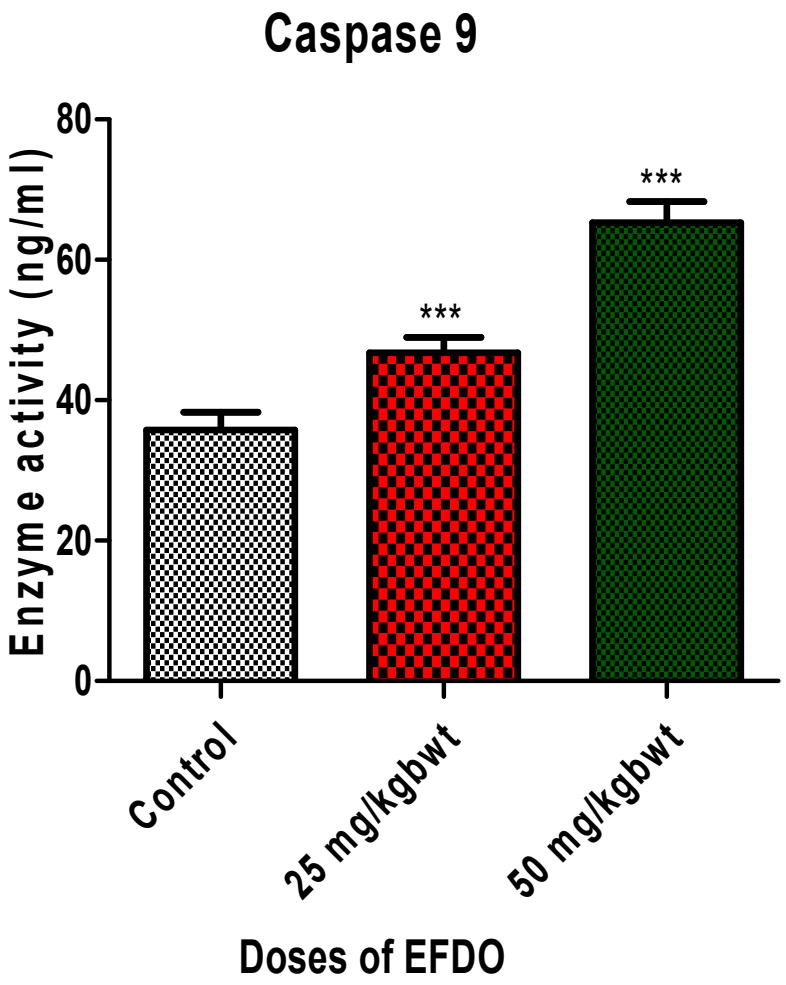


Fig. 40: Effects of EFDO on caspase 9 activity

(*: statistical significance, $p < 0.05$)**

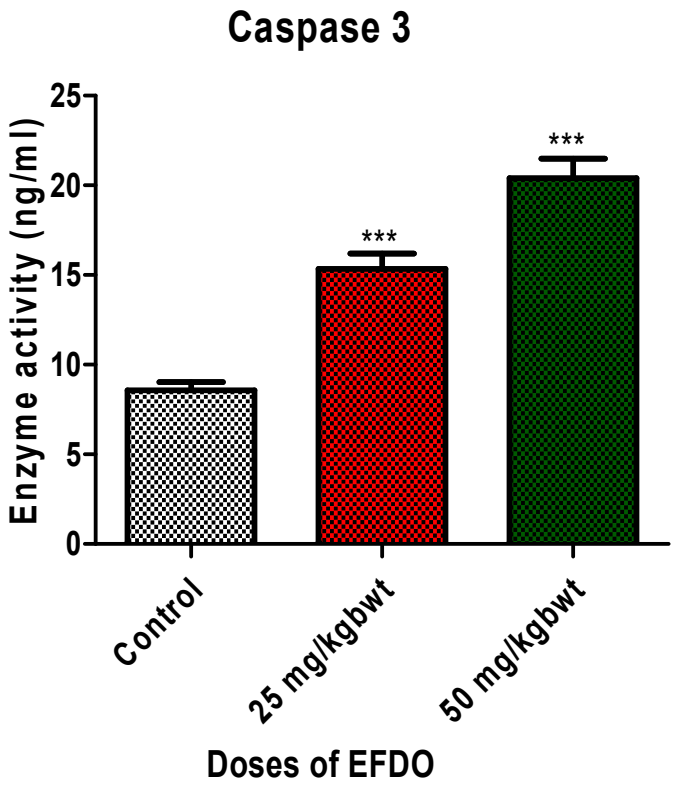


Fig. 41: Effects of EFDO on caspase 3 activity

(***: statistical significance, $p < 0.05$)

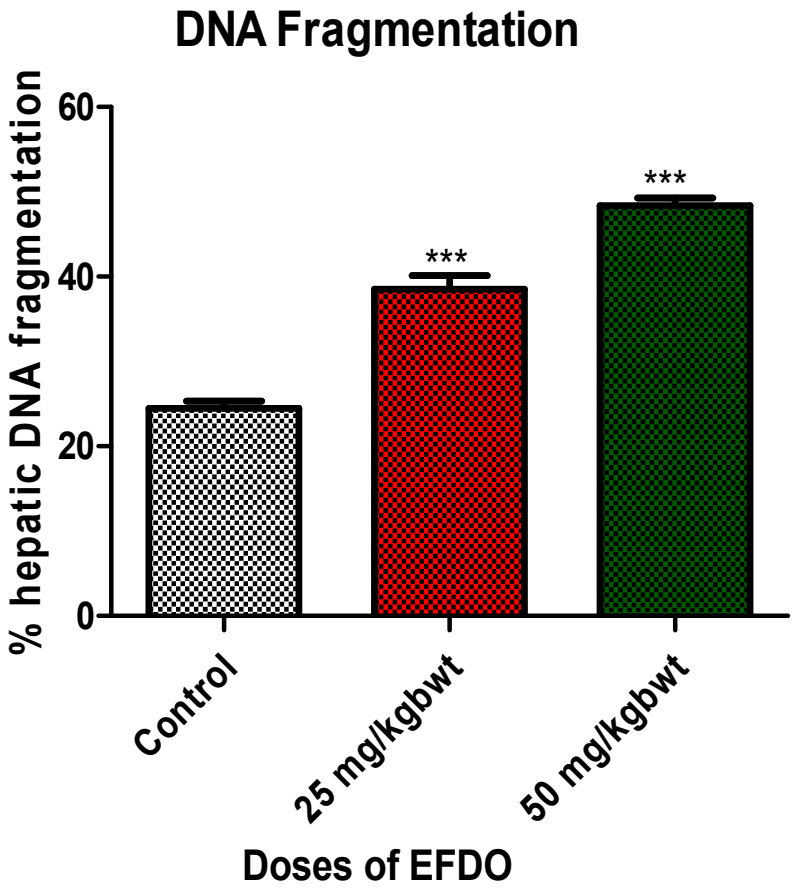


Fig. 42: Effects of EFDO on DNA fragmentation

(***: statistical significance, $p < 0.05$)

EXPERIMENT 8

CHARACTERIZATION OF ETHANOL FRACTION OF *Daniellia oliveri* STEM BARK

INTRODUCTION

Column chromatographyis a technique applied to identify and purify individual componentsfrom mixtures and it is always used for preparative applications.This separation technique is fundamentally a kind of adsorption chromatography methods and the isolation of components is dependent on the degree of adsorption to stationary phase. The stationary phase used is a powdered silica gel of mesh size 60 – 20 often packed in a vertical glass column.This separation techniqueis essential foridentification and purification of liquids and solids. It is a solid - liquid method in which themobile phase is a liquid and stationary phase is a solid. The common adsorbents applied in this technique include silica, calcium carbonate,alumina, magnesia etc., and selection of solvent is determined by the nature of adsorbent and solvent. The rate of separation of a mixture is dependent on the activity of polarity of the solvent andthe adsorbent. For instance, high activity for the adsorbent and very low polarity for the solvent will give very slow but good isolation.

PROCEDURE

Two methods are commonly applied in the preparation of a column namely, the dry and the wet method.Preparation of the column followed the procedure described under materials and methods (section 3.4.14)

PRINCIPLE

Introduction of mixture of mobile phase and sample from top of the column will cause each components of mixture to travel with varying speeds.

Table 8: Pooled fractions from column chromatography

Fraction	Tubes	Solvent system
A	9 – 11	100% EA
B	13 – 16	95% EA : 5% Acetone
c	17 – 23	90% EA : 10% Acetone
D	37 – 41	70% EA : 30% Acetone
E	42 – 49	65% EA : 35% Acetone
F	56 - 82	50% EA : 50% Acetone
G	83 - 87	45% EA : 55% Acetone
H	88 – 90	40% EA : 60% Acetone
I	91 – 93	30% EA : 70% Acetone
J	101 – 109	20% EA : 80% Acetone
K	120 – 124	95% Acetone : 5% Ethanol
L	134 – 139	80% Acetone : 20% Ethanol
M	156 – 162	45% Acetone : 55% Ethanol
N	166 – 175	10% Acetone : 90% Ethanol
O	181 - 185	100% Ethanol

Table 9: Induction fold of pooled fractions

FRACTION	INDUCTION FOLD
A	0.20
B	0.22
C	0.25
D	0.15
E	0.32
F	0.27
G	0.21
H	0.42
I	0.18
J	0.22
K	0.44
L	0.26
M	0.38
N	0.44
O	0.86

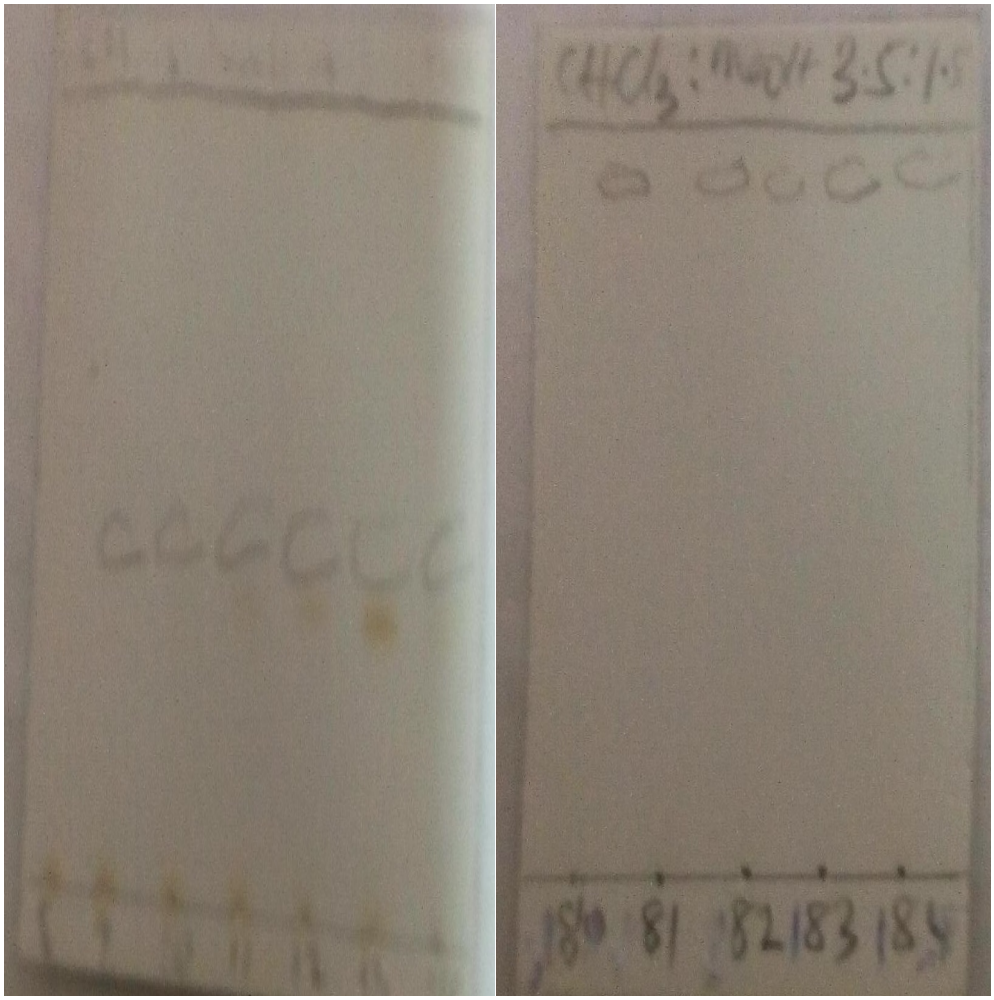


Fig. 43: Spotted fractions on TLC plate

GC-MS principle

The model of the instrument used for the analysis is Agilent technologies 7890 GC system and the model of the detector is Agilent technologies 5975 MSD (Mass Spect. Detector). The principle behind the GC-MS analysis is separation techniques. In separation techniques, there are two phases – the mobile and the stationary phase. The mobile phase is the carrier gas (Helium, 99.99% purity), while the stationary phase is the column. The model of the column is HP5 MS with length 30 m, internal diameter 0.320 mm, while the thickness is 0.25 μm. The oven temperature program is initial temperature of 80^{o}c to hold for 1 minute. It increases by 10^{0} per minute to the final temperature of 240^{0}c to hold for 6 minutes. The injection volume is 1 microlitre and the heater or detector temperature is 250^{0}C.

Operation: The sample extracted is put in a vial bottle and the vial bottle is placed in auto injector sample compartment. The automatic injector injects the sample into the liner. The mobile phase pushes the sample from the liner into the column where separation takes place into different components at different retention time. The MS interpret the spectrum MZ (mass to charge ratio) with molar mass and structures.

RESULTS

The GC-MS result from eluent tubes 181 – 185 revealed the presence of oleic and palmitic acids.

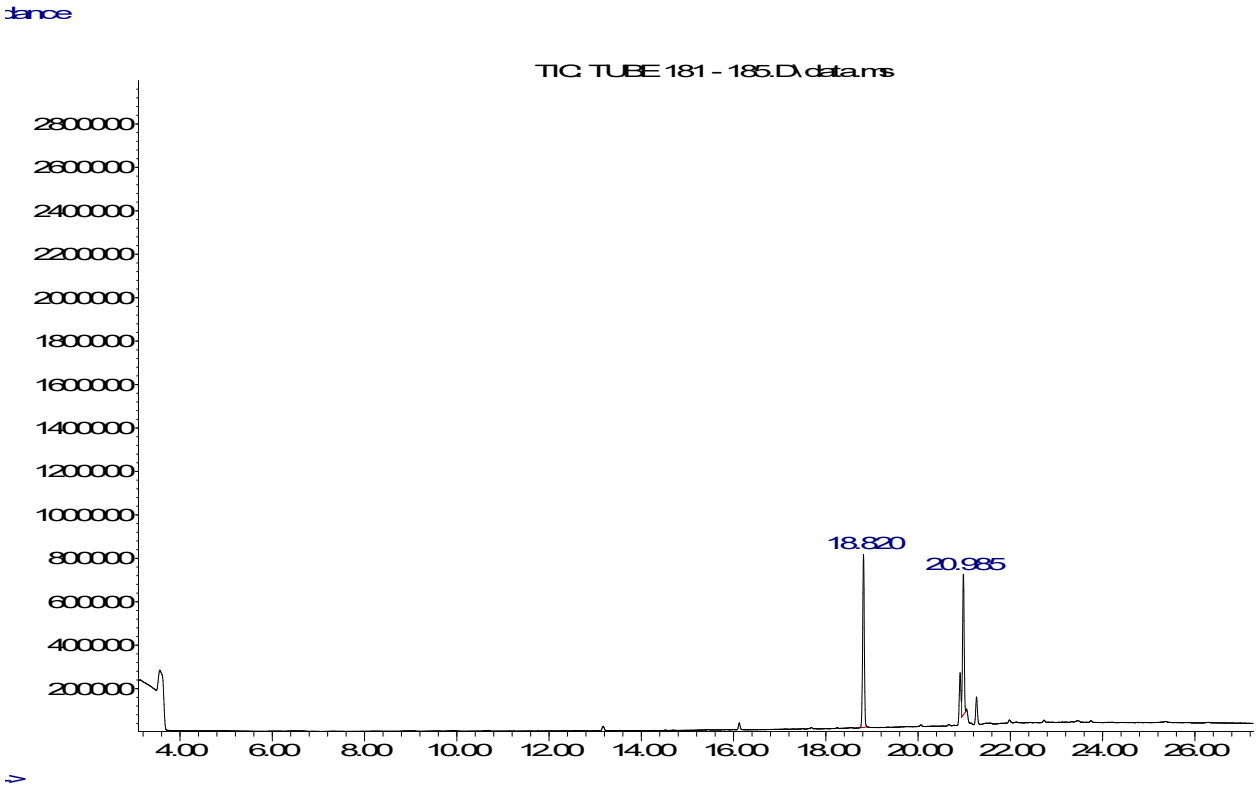


Fig. 44: GC-MS chromatogram of EFDO eluted from tubes 180 - 185

Hexadecanoic acid (palmitic acid)

9- octadecenoic acid (oleic acid)

Fig. 45: Identified compounds from EFDO

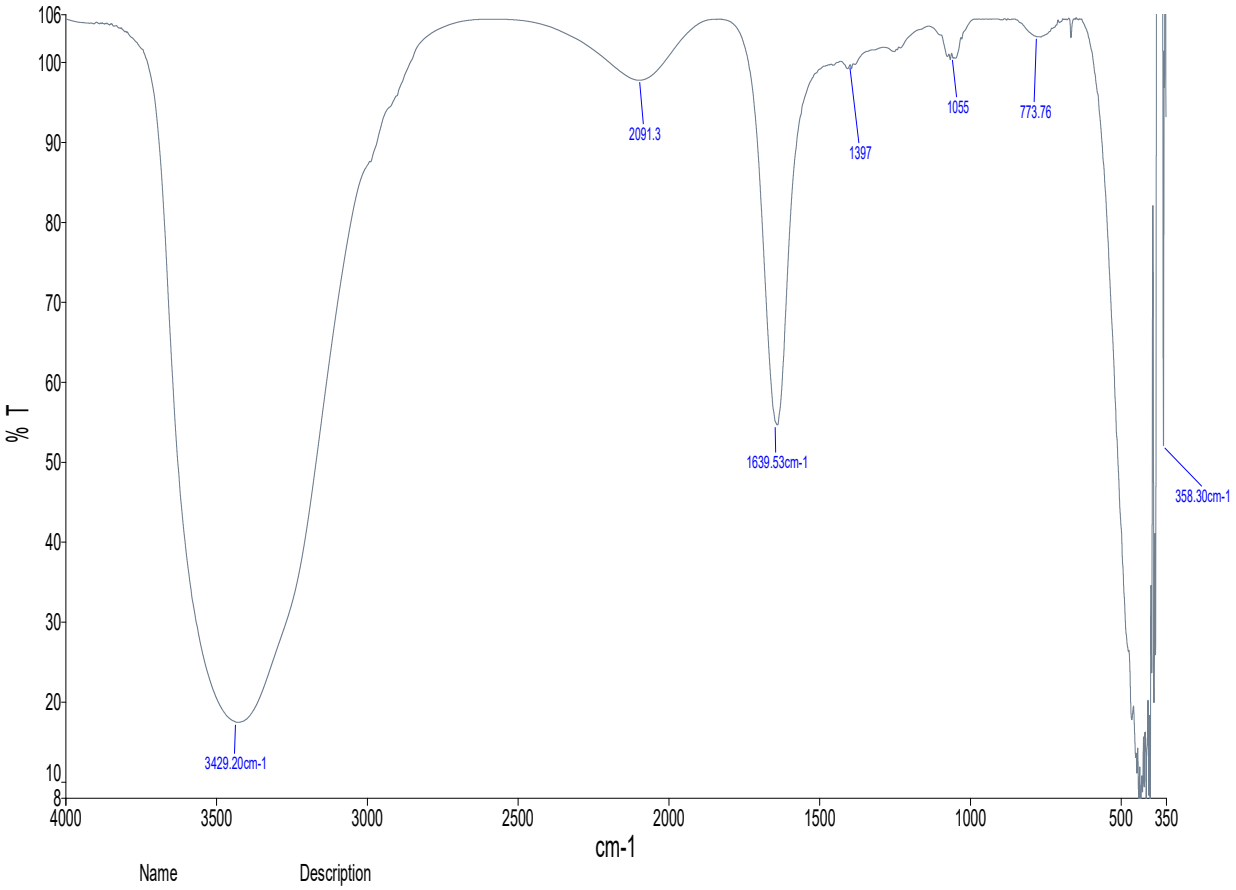


Fig.46:Infra red spectrometry of identified compounds

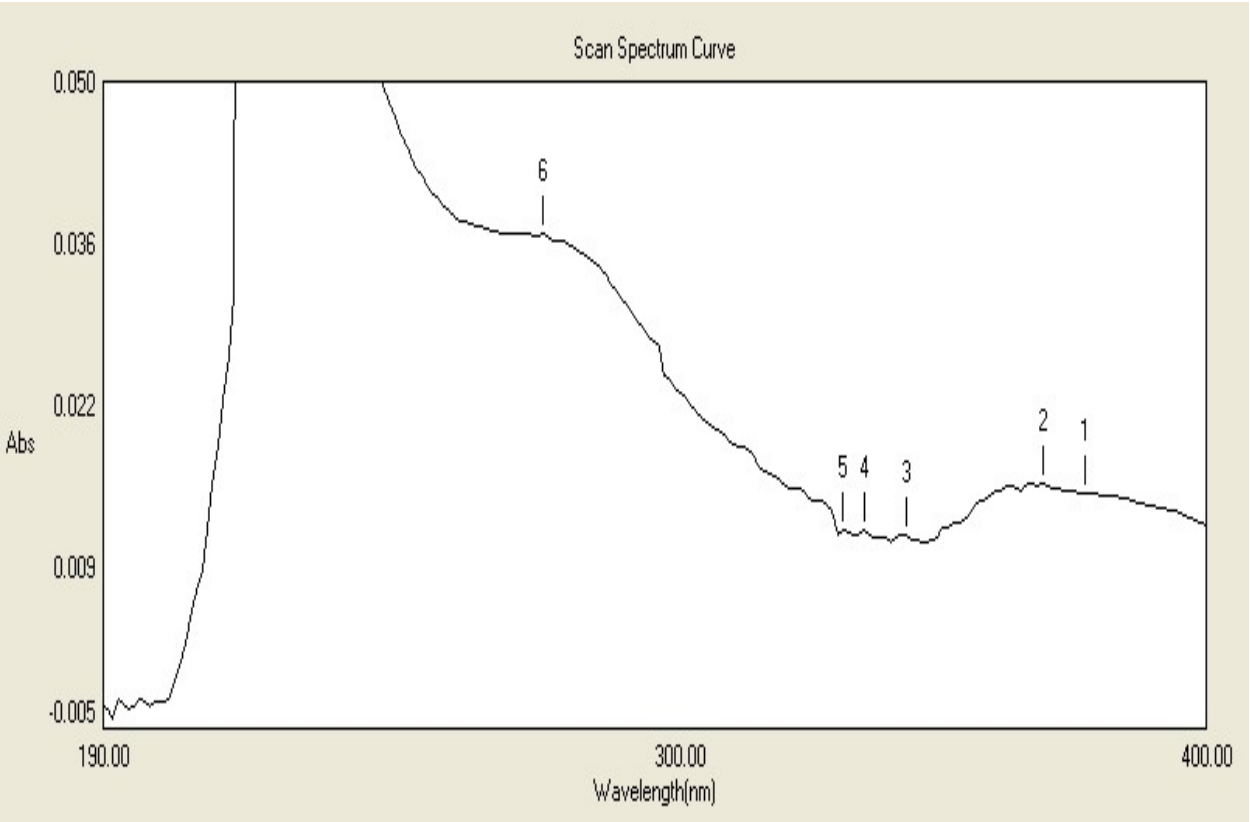


Fig.47: Uv Spectrometry of identified compounds

EXPERIMENT 9

ASSESSMENT OF IDENTIFIED COMPOUNDS ON MMPT, ATPASE ACTIVITY, LIPID PEROXIDATION AND RELEASE OF CYTOCHROME C OF RAT LIVER MITOCHONDRIA *IN VITRO.*

INTRODUCTION

The eluents from which oleic and palmitic acids were identified were treated with mitochondria to ascertain their effects on mPT pore, ATPase activity, lipid peroxidation and cytochrome c release *in vitro.*

PROCEDURE

Isolation of rat liver mitochondria, assessment of mitochondrial membrane permeability transition pore opening, lipid peroxidation assessment, ATPase activity determination and cytochrome c release quantification followed the procedures described under materials and methods (section 3.4.1; 3.4.2; 3.4.5; 3.4.6 and 3.4.8), respectively.

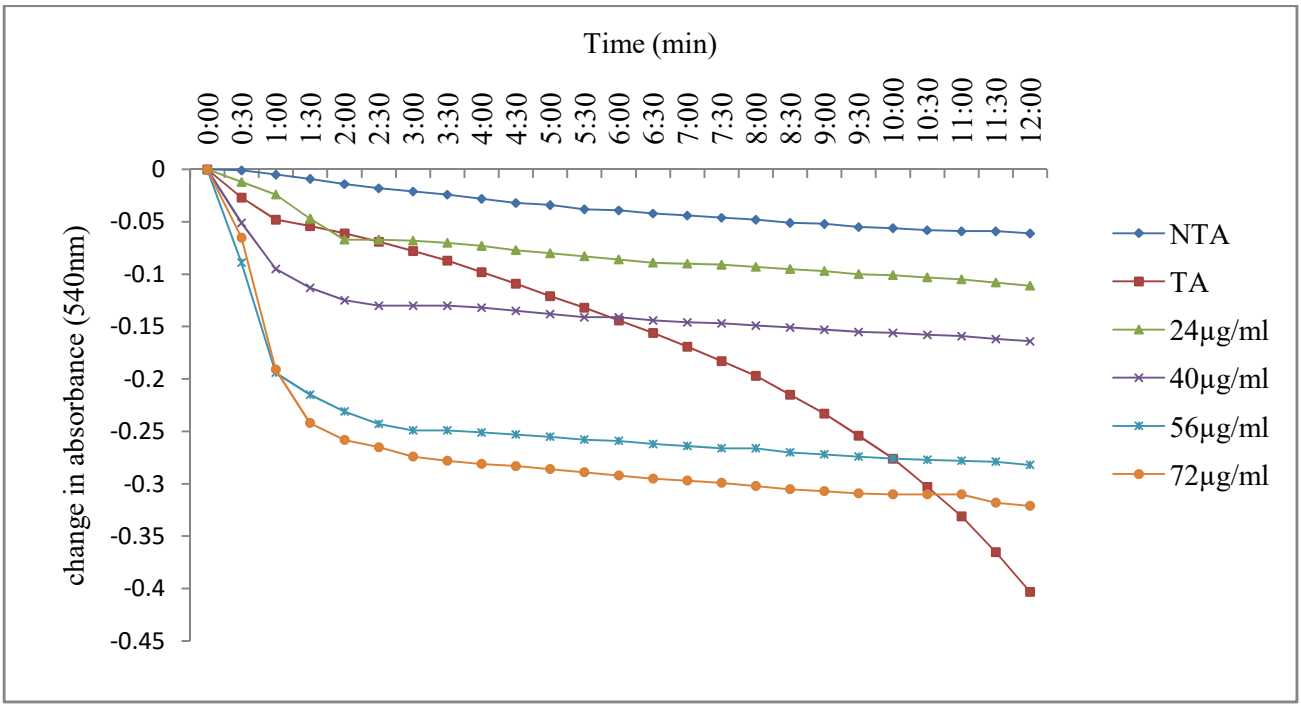


Fig. 48:Effect of identified compouds from EFDO on rat liver mPTpore *in vitro*

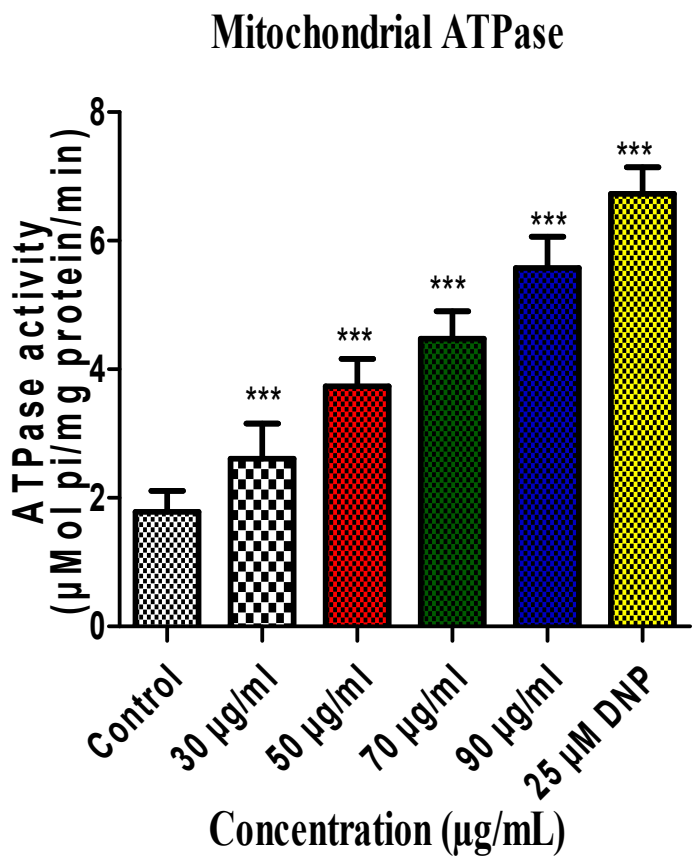


Fig. 49:Effect of identified compouds from EFDO on rat liver ATPase *in vitro*

(***: Statistical significance, $p < 0.05$)

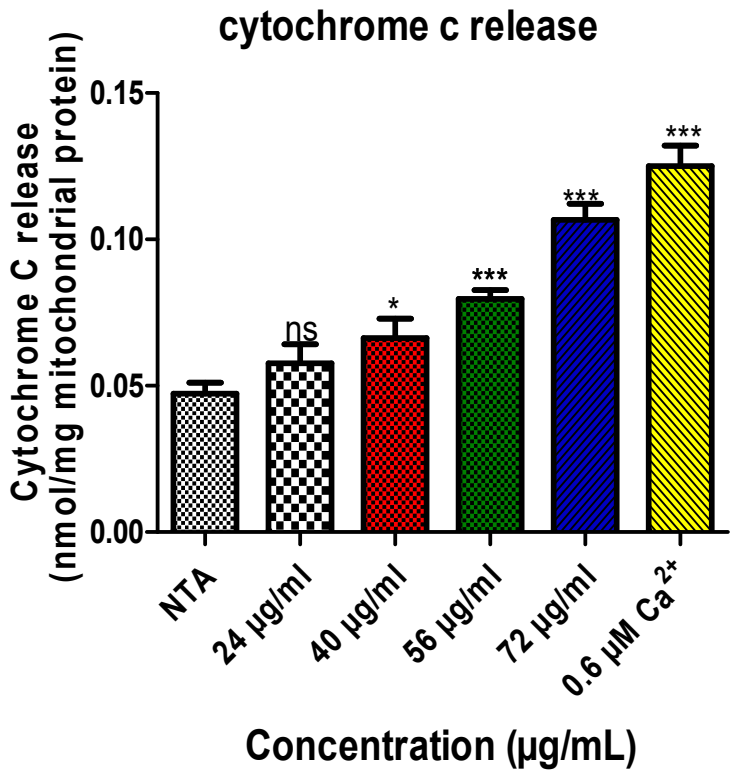


Fig. 50: Effects of identified compounds from EFDO on release of cytochrome c*in vitro*

(***: statistical significance, $p < 0.05$)

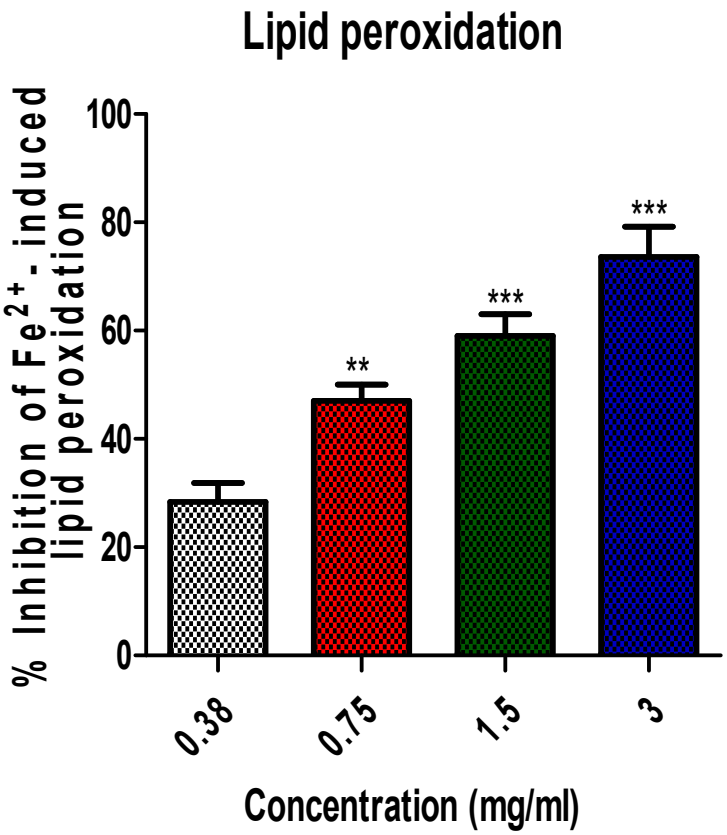


Fig. 51: Effects of identified compounds from EFDO on lipid peroxidation*in vitro*

(***: statistical significance, $p < 0.05$)

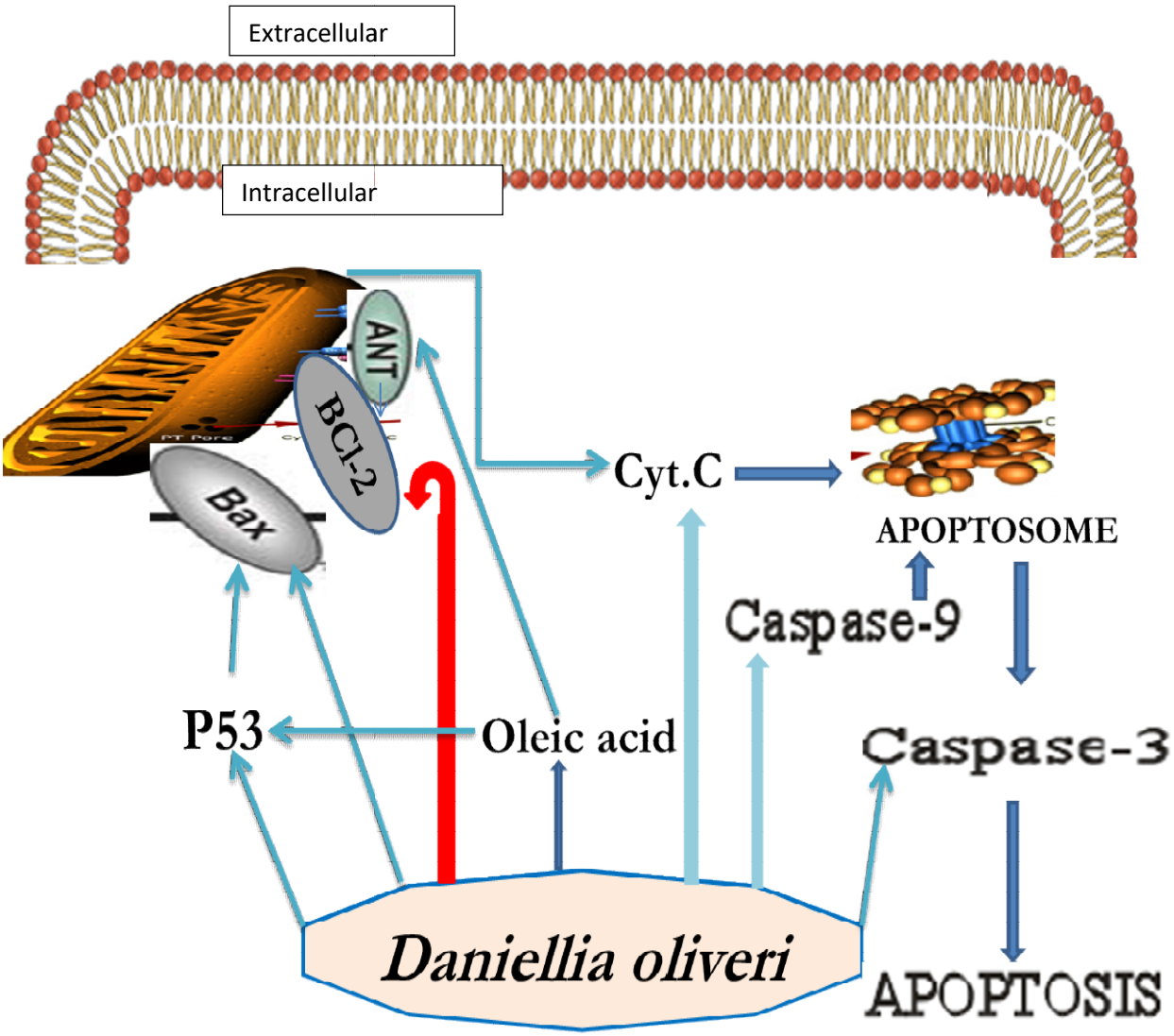


Fig 52: Proposed Mechanism of action of *D. oliveri* on Mitochondrial-Dependent Cell Death

CHAPTER FIVE

DISCUSSION

Natural products from time immemorial have been used in the prevention and treatment of sicknesses like stroke, cancer, diabetes, cardiovascular and neurodegenerative disorders, e.t.c. (Lund *et al*., 2005; Yang *et al*., 2009). Plants which are rich in antioxidants have been recommended by various epidemiological investigations to improve health status generally(Milner, 1999), and eating them ameliorated danger of several diseases including cancer, heart sicknesses,stroke and hypertension (Diaz *et al*., 1997; Wolfe *et al*., 2003). Vitaminsand polyphenols are the mainphytochemicalgroups that often enhance the antioxidant capacity of plants. Although phenolic compounds of plants origin may not contribute to nutrient quality of plant,they are useful in the maintenance of human well being. They are derivatives fromcinnamicand benzoic acids through hydroxylation reaction and have been demonstrated to exhibitanticarcinogenic andantioxidative effects. Flavonoids aresubstances derived from phenol and they are crucial in plant defense processes against environmental stress andbacterialinvasion(Ndhlala*et al*., 2007; Wallace and Fry, 1994). Flavonoids are equally known to exhibit anti-inflammatory and anti-proliferative activities (Wallace and Fry, 1994; Kuda*et al*., 2005; Sharma*et al*., 1994).

Several epidemiological studies indicate that a diet rich in fruits and vegetables is associated to the reduction of cancer risk in humans, suggesting that certain dietary constituents may thus be effective in preventing cancer. Phenolic compounds, abundant in vegetables and fruits ubiquitous in diet, were described to play an important role as chemopreventive agents. Phenolic compounds constitute one of the most numerous and ubiquitous groups of plant metabolites, and are an integral part of the human diet. For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Plants have been used in medicine for their natural antiseptic properties. Thus, research has developed into investigating the potential properties and uses of terrestrial plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer. Many plant species are already being used to treat or prevent development of cancer (Sivaraj *et al*., 2014).

Reports from various investigations have demonstrated thatmedicinal plants antioxidant capacitycould be connected to the levels of their phenolic components including flavonoids, alkaloids and anthocyanins (Djeridane*et al*., 2006). These compounds exhibit high value in impeding the initiation and progression of severalhumanailments(Kim*et al*., 2003). Antioxidants from plants possesshealth-promoting capacitywhich is thought to emanate from their protection by scavaging ROS (Wong*et al*., 2006). Antioxidants are compounds that play roles in delaying and inhibitingoxidation of lipid and when introduced to foods reduce rancidity and slow down generation of harmfulproducts of oxidation (Fukumoto and Mazza, 2000). These investigationshave resulted to an increased attention on the prevention and treatment of cancer diseases in which these phytochemicals are greatly utilized (Keith, 2000).

Preliminary phytochemical screening of the fractions of *D. oliveri* stem bark carried out in this study showed thatdifferent classes of phytochemicals are presentas shown in Table 7. The screening could play essential role in identifyingbioactive agents which could subsequently lead to drug discovery and development. Furthermore, their quantitative value, qualitative isolation and locating the source of active componentspharmacologically could be madeeasy by these tests (Varadarajan *et al.,* 2008). The screening of EFDO, CFDO and EAFDO showed the presence of manifold phytochemicals as shown in table 7.The presence of glycoside moieties like flavonoids,saponins and cardiac glycosides are of pharmacognostic importance. For instance, flavonoids, cardiac glycosides and alkaloids have been shown to impede tumour growth and as well serve to guard against gastrointestinal infections. The presence of these bioactive agents could be connected to the use of *D. oliveri*plant in ethnomedicine (El-Mahmood, 2009).

Steroids were found to be present in all the fractions, while alkaloids which were absent in all the fractions of *D. oliveri* screened were present in the crude extract. The absence of some phytochemicals in one fraction and its presence in the other can be connected with fractionation and the solubility of the various phytoconstituents in some solvents and its insolubilityin others.These results are in accordance with Babayi *et al.,* (2004) who revealed thatphenols, saponins, steroids and cardiac glycosidesare absentin fractions of *Terminalia catappa* although thecrude extract was initiallyfound to possess them(El-Mahmood *et al*., 2008).

Polyphenolic compounds include flavonoids, tannins, curcumin, resveratrol and gallacatechins and are all considered to be anticancer compounds (Azmi *et al*., 2006). The cytotoxicity of polyphenols on a range of cancer cells has been demonstrated and their antioxidant properties determined (Siriwantanmetanon *et al*., 2010; Heo *et al*., 2014). Polyphenols are thought to have apoptosis inducing properties showing anticancer properties which can be utilized. The mechanism in which polyphenols are thought to carry out apoptosis initiation is through regulating the mobilization of copper ions which are bound to chromatin inducing DNA fragmentation. In the presence of Cu(II), resveratrol was seen to be capable of DNA degradation (Azmi *et al*., 2006). Other properties plant polyphenols show is their ability to interfere with proteins which are present in cancer cells and promoting their growth.

Flavonoids are from the polyphenolic compounds and constitute a large family of plant secondary metabolites with 10,000 known structures (Cao *et al*., 2013). They are physiologically active agents in plants and becoming of high interest scientifically for their health benefits (Agati *et al*., 2012; Huntely, 2009). Various plants have been investigated for their flavonoid content and how these compounds affect cancer cells, such as fern species and plants used in traditional Chinese medicines like the litchi leaf (Cao *et al*., 2013; Wen *et al*., 2014). Flavonoids have been found to demonstrate cytotoxicity on cancer cells and to have high free radical scavenging activity. Purified flavonoids have also shown anticancer activities against other human cancers. The flavonoids extracted from *Erythrina suberosa*stem bark (4'-Methoxy licoflavanone (MLF) and Alpinumi soflavone (AIF)) were shown to have cytotoxic effects in HL-60 cells (human leukaemia) (Kumar *et al*., 2013). MLF and AIF induced apoptosis through intrinsic and extrinsic signalling pathways. The mitochondrial membrane potential is significantly reduced due to the induction of apoptotic proteins. With mitochondria damage to cells the cancer cells cannot survive (Kumar *et al*., 2013).The presence of flavonoids in solvent fractions of *D. oliveri* stem bark substantiates the induction of mPT pore by the plant in this study.

Tannins and flavonoids are phenolic compounds commonly found in plants and they serve primarily as antioxidants or ROSscavengers.The presence of these bioactive agents in *D. oliveri* fractions could be accountable for the antioxidant capacity of *D. oliveri.* Several plant species that contain wealth of flavonoids have been demonstrated to ameliorate risk of diseases and exhibit treatment/curative properties. This observation is specifically essential since flavonoids are major components of vegetables and fruits and eating them can mitigate the danger of cancer

(Kanadaswami *et al*. 2005). Induction of mPTP activity shown by *D. oliveri*fractionsin this study could be due to the presence of certain phytochemicals.These bioactive agents foundin medicinal plants are responsible for their folkloric applications. For instance, saponins have been reported to exhibit cardio-depressant and hypotensive properties (Olaleye, 2007).

Bioactive compounds from various plants have been shown to induce different phases of cell cycle arrest (Kuo and Lin, 2003), and also induce apoptosis in many human cancer cells (Meng *et al*., 2004).Certain phytochemicals of medicinal plants have been shown to induce apoptosis via mPT pore. For instance, resveratrol, a polyphenolic compound from grapes and wine, can inhibit mitochondrial ATP synthesis and trigger MOMP (Fulda *et al*., 2010).Betulinic acid, a natural pentacyclic triterpenoid of the lupane class, is known to trigger mitochondrial apoptosis in cancer cells (Fulda *et al.*, 2010). Danthron, a naturally occurring component isolated from the root and rhizome of *Rheumpalmatum* L has been shown to depolarize the membrane potential and stimulate the opening of mPT porein human gastric cancer cells (Jo-Hua *et al*., 2011). Aloe-emodin (AE), a natural anthraquinone derivative has also been demonstrated to promote the loss of mitochondrial membrane potential in human U87 malignant glioma cells (Ismail *et al*., 2013).Recent results from our laboratory also present evidence that crude extracts of *Brysocarpus coccinues* and *Cnestis ferruginea* can induce mPT pore opening (Adedosu *et al.,* 2012; Adisa *et al*., 2012).

Cardiac glycosides have been reported to exhibit anti-inflammatory activity (Shah *et al.,* 2011). The presence of cardiac glycosides in EFDO, CFDO and EAFDO corroborate the anti – proliferative use of *D. oliveri* in traditional medicine. Cichewicz and Thorpe (1996) demonstrated the inhibitory and terpenoids effect on membrane disruption against bacteria and fungi. Terpenoids are found to be present in EFDO, CFDO and EAFDO and this could be responsible for the significant induction of mPTP by EFDO observed in this study.Reports from various researches illustrated that saponins exhibit anti-mutagenic and antitumour properties and can diminish human cancersrisk by inhibiting cancer cells proliferation (Nafiu *et al.,* 2011). Saponins exhibit foaming and cell membranepermeabilization properties. Their soapy characteristic is attributed to their surfactant properties (Noudeh *et al.,* 2010).Phytochemical screening of *D. oliveri* shows the presence of saponins in crude extract and EFDO and this

substantiate induction of mPT pore by *D. oliveri* in this study, the traditional use of *D. oliveri* in treating breast tumour and its use as an anti-cancer substance.

Tannins have been identified in the extracts of *Praecitrullus fistulosus* (Tinda) and *Cucumis sativa* (Cucumber),and they have been reported to display astringent activities. Tannins are potentialbiological antioxidant and metal ion chelator (Okonkwo, 2009). Results of *D. oliveri*phytochemical screening reveals presence of tannins in crude extract, EFDO and EAFDO and this corroborate literature reports of the antioxidant properties of *D. oliveri*.

Alkaloids are known to be among the important bioactive agents in plants and some have been developedinto chemotherapeutic drugssuccessfully.For instance, topoisomerase I (TopI) inhibitor (Huang*et al*., 2007), camptothecin (CPT) and vinblastine, which interact with tubulin. Many alkaloids possess remarkable biological activities, such as analgesic property of morphineand the anticancer properties of vinblastine (Wang and Liang, 2009; Lee, 2011; Benyhe, 1994). *Berberine,* an isoquinoline alkaloid found in herbs, hasa wide range of bioactivities like anti-bacterial, -inflammatory, -ulcer, -diabetes, expansion of blood vessels, neuroprotective and hepatoprotective effects (Han*et al*., 2011; Kulkarni and Dhir, 2010; Ji, 2011). Different investigations revealed that *Berberine* possesses anti-cancer capacity through interference of tumourigenesis (Sun*et al*., 2009; Diogo*et al*., 2011).

Tetrandrine, a bisbenzylisoquinoline alkaloid obtained from *Stephania tetrandra*root,exhibits a wide range of pharmacological activities such as immunomodulation, anti-portal hypertension, anti-arrhythmic, antihepatofibrogenetic,neuroprotective and anticancer activities (Li, *et al*., 2001). *Tetrandrine* triggersarrestof several cell cyclephases, depending ontype of cancer cell (Kuo and Lin, 2003), and also initiates cell death in different cancer cellsof human including hepatoma,colon, bladder and lung (Meng*et al*., 2004).Presence of alkaloid in crude ethanol extract of *D. oliveri* substantiates the traditional use of this plant in treating breast tumour and literature reports of the anti-cancer and anti-inflammatory properties of *D. oliveri*.

Growing proof suggests that mitochondria participate actively in stimulation of cell death programme (Kroemer *et al*., 1997). Numerous stimuli can trigger a rise in permeability of mitochondrial membrane which is attributed to the creation of a pore, the PTP, which occurs at

the point of contact sites between the innermost and the outermostmembranes of mitochondria(Zoratti and Szabo, 1995). The PTP opening triggers mitochondrial membrane potentialcollapse, the inhibition of ATP productionand respiration impairment. It liberates apoptogenic substances and can lead todeath of cells (Crompton, 1999). Hence, novel anti-tumour modalities now center on mitochondria. Formation of the pore results to mitochondrial failure through uncoupling of oxidative phosphorylation and enhancing ATP hydrolysis. Pore formation signals towards death of cell (through necrosis), mitochondrial autophagy and cell death (through rupture of OMM).

The effects of certain solvent fractions of *D. oliveri* stem bark on mPT pore opening were carried out *in vitro* in this study and the results are presented in Figures 8 - 15. The assay results in this research shows that 60, 180, 300 and 420 µg/ml of EEDO stem bark in the absence of Ca^{2+} exhibited an induction fold of 0.03, 0.09, 0.20 and 0.65, respectively and in the presence of triggering agent exhibited induction folds of 0.58, 0.54, 0.48 and 0.42, respectively. The results also revealed that 60, 180, 300 and 420 µg/ml of EFDO exhibited induction folds of 0.04, 0.10, 0.35 and 0.68, respectively in the absence of the triggering factor while in the presence of Ca^{2+},the same concentrations exhibited induction folds of 0.64, 0.62, 0.56 and 0.52,respectively. The CFDO and EAFDO exhibited no significant induction in the absence of Ca^{2+}.

An increasing number of researches on different cell lines and tumour models illustrated that instigation of mPTpore opening by pharmacological application triggers death of cell and inhibits cell gradual formation in tumourigenesis which is an inveterate method with specific building up of tissue and broken down reprogramming (Fantin and Leder, 2006; Armstrong, 2007). The ROS overproduction andCa^{2+} accumulation in the mitochondrial matrix along sides ATPexhaustion and accumulation of phosphate are among the primary metabolic changes in cardiac Ischemic Reperfusion (IR) that favour creation of mPTpore.Stimulation of cell death in tumour cells is deemed to be important in the control, therapy and inhibition of neoplasia (Ram and Kumari, 2001; Hu*et al*., 2007).

The mPT pore was initially brought to view by Haworth and Hunter (1979) and has been found to contribute to hepatotoxicity, neurodegeneration, cardiac necrosis and wasting of muscularandnervous tissues (Fiskum, 2000; Baines, 2010; Bernardi and Bonaldo, 2008). Under

sundry conditions like presence of calcium together with inorganic phosphate, isolated mitochondria undergo the mPT. This process is marked by an increasein Ca^{2+}-dependent permeabilization of the interior mitochondrial membrane, causing loss of membrane potential,mitochondrialenlargement and bursting of the exterior mitochondrial membrane (Halestrap*et al*., 2002).

The induction of mPT pore opening by EFDO stem bark shown in this work supports data from Literatures that bioactive agents from plantsare biologically active against different strains of bacteria and manifold human cancer cell lines (Havsteen, 2002; Min, 2000). During the last two years, evidence has been accumulating that mitochondrial alterations, which previously only had been associated with necrotic mode of cell death, are as well implicated in apoptosis. It has become obvious that mPT pore opening (also called "megachannels") might form a critical event of apoptosis and constitute part of the "central executioner" (Kroemer and Martinez-A,1995; Nakajima *et al*., 1995). The present investigations confirmed that *D. oliveri* inducedPTP opening and also guarded mitochondria againstROS accumulation andmembranes lipidperoxidation.Thus, in our experimental situations induction of mPTP opening cannot be attributed to the generation of ROS which was insinuated to incite apoptosis (Bhaumik *et al*., 1999).

Results on*in vivo* oral administration of EFDO and EEDO on rat liver mPT pore are as illustrated in Figures 24 and 27. The results of EEDO show that there was significant induction in mPTP opening at all the doses used, whereas the results of EFDO show no noticeableinduction at all doses used. The failure of EFDO fraction to induce mPT pore opening*in vivo* despite the fact that it illicit induction *in vitro* could not be unconnected with fractionation, bioavailability, accessability and biotransformation, factors which have up till now impeded the transition of potential drug candidates to preclinical trials.

Cytochrome c is commonly thought to be found mainly in thespace ofintermembrane of the mitochondriawhen there is no physiological alteration (Neupert, 1997). Whenever this protein is liberated to the cytosol from the mitochondria, it promotes stimulation of the proteasescaspase family and this is thought to be primary trigger bringing about initiation of cell death (Kroemer *et al*., 1998). Quantifying the concentration of cytochrome c enteringinto the cytosolis a sensitive

process to manipulate the level of cell death (Waterhouse and Trapani, 2003). One way by which apoptosis is activated in cell is by liberation of cytochrome c into the cytosol (Loo *et al*., 2013).

Various bioactive phytochemicals present in food were traditionally believed to exert health-promoting effects and currently the number of scientific reports investigating their action in cell culture and animal models has been growing rapidly. Some reports point out that the ROS–related effects may contribute to the antiproliferative and proapoptotic activity of Epigallocatechin-3-Gallate(EGCG) (Valenti *et al*., 2013; Kil *et al*., 2011). Another flavonoid with potential chemopreventive and neuroprotective properties is quercetin, broadly distributed in many plants and vegetables of the human diet. Quercetin was found not only to prevent the reactions to oxidative stress (Carrasco *et al*., 2012), but also actively mount up in mitochondria in biologically active form in cells, during treatment with micromolar concentrations (10–50 μM) (Fioran*iet al*., 2010). New formulations containing genistein were more cytotoxic, largely through destabilization of mitochondrial membrane and induction of cytochrome c release leading to apoptosis (Pham *et al.*, 2013).

Green tea plyphenols (catechins, epigallocatechins, and their derivatives), quercetin, genistein from soybean and allicin from garlic are among the most extensively studied compounds. Green tea polyphenols, particularly EGCG have been shown to act as potent chemopreventive and anticancer agents. Their mode of action concentrates on induction of mitochondrial apoptotic pathway, such as mitochondria depolymerization, cytochrome c release and activation of caspases, in various cancer cell lines (Kazi *et al*., 2002).

We assessed the effects of *D. oliveri* fractions on isolated intact mitochondria in order to ascertain the quantification of cytochrome c release *in vitro* in this research and the results obtained is as depicted in Figure 18.Graded concentrations (0.75, 2.25, 3.75, 4.25 and 6.75 μg/mL) of EFDO, CFDO and EAFDOincreased CCR by 0.7±0.04, 0.8±0.03, 0.9±0.04, 1.1±0.06, 1.5±0.04; 0.6±0.03, 0.7±0.03, 0.8±0.06, 0.9±0.02, 1.0±0.04 and 0.6±0.05, 0.7±0.02, 0.8±0.04, 0.9±0.03, 1.1±0.02 nmol/mg protein respectively relative to control (0.5±0.01).All the solvent fractions of *D. oliveri* used in this assay induced the discharge of cytochrome cwhich is dependent on concentration and EFDO is seen to have induced the highest liberationof this protein. The ability of fractions of *D. oliveri* to instigate liberation of cytochrome cwhich could

be attributed to the presence of secondary metabolites gave credence to its significant induction on rat liver mPT pore opening investigated earlier in this study.

During normal physiological conditions, cytochrome c is attached to cardiolipinin the interior membrane of the mitochondria, thus anchoring its presence and inhibiting its release from the mitochondria. At the onset of cell death, ROS production in the mitochondria is triggered and this is followed by oxidation ofcardiolipin by a peroxidase.This will lead to dissociation of the hemoprotein from interior membrane of the mitochondriaand followed by its extrusion into the soluble cytosol through pores in the exterior membrane (Orrenius and Zhivotovsky, 2005). Our present study thus demonstrates that liberation of cytochrome ctakes place by two-step processes, i.e., dissociation of cytochrome c from the interior membraneand permeabilization of the exterior membrane and the liberation of cytochromecinto the extra-mitochondrial surroundings (Martin *et al.,* 2001).

Cytochrome c is reported to serve an essential function in electron transport chain anddeath of cell. Studies, nonetheless, has revealed that it can as well serve as an anti-oxidative enzyme in the mitochondria; and it perform this by detaching hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) from mitochondria(Bowman and Bren, 2008). Therefore, in the mitochondria cytochrome c is not only needed for cellularrespiration, but it is equally required to limit generation of ROS (Bowman and Bren, 2008).It has been revealed by a recent study that cells guard themselves from cell death by impeding cytochrome c dischargefrom the mitochondria using Bcl-x_L (Kharbanda *et al*., 1997). The ability of fractions of *D. oliveri*stem bark to promote the liberation of cytochrome c, which can subsequently act as an antioxidative enzyme in the mitochondria by removing O^{2-} and H_2O_2 also proved that mPT pore induction in our earlier experiment cannot be attributed to the production of ROS but due to the presence of certain bioactive agents present in *D. oliveri* stem bark.

Cytochrome cis bound to the interiormembrane of the mitochondriaby anionic phospholipids, basically cardiolipin. This attachmentrequirestwo structures namely, a freely attached form offered by electrostatic interaction withnegatively charged phosphate groups of cardiolipin and positively charged lysine residues of cytochrome c(Nichols, 1974), and a tightly bound conformationbringing about incomplete embedment of the protein in the membrane which could be due tohydrophobic interactions follow by loosening of the tertiary structure (Cortese *et al*.,

1998; Gorbenko, 1999). Because of association of cytochrome c with cardiolipin in both conformations, it is deemed thatexterior membrane permeabilization alone, would not be enough to trigger liberation of cytochrome c. Thus,an interruption of theinteraction betweencardiolipin and cytochrome c would have to take placesimultaneouslyor before, withouter membrane permeabilization in order to initiatedischarge of cytochrome c(Martin *et al.,* 2001).Changes in membrane conformation by cardiolipinperoxidation will cause the release of tightly bound protein into the cytosol. Therefore, the form which cytochrome c will adoptis dependent on its ability or inability to build an electrostatic interaction with cardiolipin (Martin *et al.,* 2001).

The main source of ATP for cellsis mitochondria, and for execution of the apoptotic processsuccessfullyenergy is needed (Leist *et al*., 1997). Recent study has shown the capacity of ATP to fasten and induce a structural alteration in cytochrome cwhich impededcardiolipininteractionwith this proteindespite the fact that partof this energy necessity is required for apoptosome formation(Tuominen *et al*., 2001). Hence, any interruption in membrane potential and/or oxidative phosphorylation that brings about a reduction in ATP synthesis could cause impairment in moving from one stage to the other of this type of cell death. Treated mitochondria with oligomeric Bax, during severeconditions of non-mPT, promotes a restorable submaximal change in membrane potential and the liberation of portion of this protein and the larger part of it is retainedwithin the the mitochondria making it possible for them to sustain their ability to produce ATP. On the other hand, MPT, usually connected with final-stage of apoptosis (Chen *et al*., 2000) or necrosis (Crompton,1999), is related with a continuous decrease in membrane potential, exhaustion of ATP anddischargeof cytochrome c. Under this condition, dischargeof this protein is just an outcome of decrease of mitochondrial probity and does not possess an important way on investigation of the type of death cells undergo(Martin *et al.,* 2001).

Lipid peroxidation can be described as a sequence of reaction started by hydrogen withdrawal or oxygen radicaladdition, leading to oxidative destruction of polyunsaturated fatty acids.Itis the changing process by which ROS will lead to oxidative destruction of lipids, which perhaps to a large extent alter cell membrane structure and function.Epidemiologic findings have demonstrated the correlation between the plant antioxidants and amelioration of inveterate diseases (Sasikumar *et al*., 2010; Lieu, 2003). These advantages are considered to come from the

antioxidant constituents of plant origin including vitamins, flavonoids and carotenoids (Rice-Evans, 2001). Researches in few years past have indicated that phenolic compounds in plants mop upROS and essentially inhibit oxidative cell destruction (Divya and Mini, 2011). Application of herbal products could be a better way to satisfy the objective of finding an appropriate treatment for decreasing the free radicals production.

In this research, we investigated the inhibitory effects of certain solvent fractions of *D. oliveri* stem bark on Fe^{2+}-induced lipid peroxidation on rat liver mitochondria *in vitro* (Figure 16). All fractions of *D. oliveri* stem bark used in this assay significantlyinhibited Fe^{2+}-induced lipid peroxidation in amanner that is concentration-dependent. The 0.75, 1.5, 3, 6 and 12mg/ml of ethanol fraction used inhibited Fe^{2+}-induced lipid peroxidation by 13.2, 33.1, 52.5, 79.3 and 133.5%, respectively. Chloroform fraction inhibited by 3.4, 14.4, 39.4, 51.1 and 57.8% and ethyl acetate fraction by 3.9, 25.5, 31.9, 65.8 and 103.4%, respectively.

The initiation and continuous occurrence of peroxidation of lipid within membranes is connected with variations in physical and chemical properties and with changes of biological role of proteins and lipids. The Polyunsaturated Fatty acids (PUFA)and their break down products exhibit physiological functions such asprovision of energy, structure of the membrane fluidity and discriminative permeation of membranes, and cell signalling andgene expressionregulation (Catala, 2006).

Several aldehydes, for instance, Malondialdehyde (MDA), 4-hydroxynonenal (HNE) etc are generated bydecomposition of unsaturated fattyacids through oxidative peroxidation. Studies have shown recently that lipid peroxidationproduct causing destruction to cell is HNE. The HNE exhibits various effects such as acting as intracellularsignal to control gene expression, enlargement of cell, gradual formation of cells or tissuse and apoptosis.

Oxidative pressure is a conspicuousprocess of damage to cell that transpires with elevatedlipid peroxidation of cell phospholipids and that has been associated with numerousabnormalities of cell(Catala, 2006). Aldehydes are known to highly react with large molecules like proteins, DNA and lipids producing intra- and intermolecularproducts.The physiological levels of these adducts are small; nevertheless, larger quantitieswill result topathologic conditions. Hence, injury to DNA caused by peroxidation of lipid end products could proffer inspiring markers for

prognostication of disease risk andpreventive measures focus. The HNE andMDA have beendemonstrated to modify bases of DNA, producingpromutagenic lesions and contributing to generation of cancer cells (Repetto *et al*., 2012; Fridovich and Porter, 1981).

The biological generation of ROS mainly $O_2^{\cdot-}$ and H_2O_2 possess the ability of causing damage tobiochemicalmolecules like aminoand nucleicacids. Proteins exposure to ROS causes denaturation, loss of function, gathering togetherand disintegration of collagen tissues (Chance *et al*., 1979). Cardiolipin oxidation could be one of the important factors inducing cell death by releasing cytochrome c frominner membraneof the mitochondria and enhancing permeabilization of exterior membrane. The liberation of cytochrome c triggers a proteolytic sequence that finally ends in cell death through apoptosis(Navarro and Boveris, 2009). The inhibitory actions of fractions of *D. oliveri* stem bark on Fe^{2+} - induced lipid peroxidation shown in this study revealed that induction of mPT pore opening by *D. oliveri* stem bark couldn't be due to ROS, which are known to induce mPT. Thus induction of mPT pore opening by *D. oliveri* stem bark could be attributed to certain phytochemicals present in it.

Morphological injury to membranes and production of secondary products are the two main consequences of lipid peroxidation(Catala, 2006). These effects are harsh to biological systems, cause destruction to membrane function, inactivation of enzymes and harmful effects on division and function of cell.The inhibition of peroxidationof lipid exhibited by certain solvent fractions of*D. oliveri* stem bark in this work supports the fact that different bioactive agents present in *D.oliveri*have been indicated to exhibitdifferent activities, which could aid in protecting against pathologies like cancer, control inflammatory and immune response and guard against lipid peroxidation (Hollman and Katan 1997).

The mechanism of lipid peroxidation has been insinuated to progress througha free radical sequence reaction (Halliwell, 1989), which has been connected to cell injury in biomembranes(Usuki*et al*., 1981).The injury has been demonstrated to precipitate various ailments like diabetes,cancerand cardiovascular diseases. Incubation of rat liver homogenate withferrous sulphate ($FeSO_4$) causes remarkable elevation in lipid peroxidation.

The abilities of certain solvent fractions of*D. oliveri*stem bark to inhibit lipid peroxidation were assessed applying the procedure of Ruberto *et al*. (2000). This result suggests that*D. oliveri*stem bark solvent fractions could act to guard the physiochemical properties of membrane bilayers from free radical induced severedysfunction of cells. Different supportive reports underscore the positive relationship between phenolic content and the efficacy of antioxidant (Kukic *et al*. 2006; Buricova and Reblova, 2008). A positive correlation between antioxidant activity and polyphenol content were investigated, proposing that the antioxidant capacity of*D. oliveri* stem bark is attributed to its polyphenols (sasikumar *et al*. 2009; Kiselova *et al*. 2006). Strong positive relations between antioxidant activity and flavonoid and phenol contents suggests that the antioxidant ability of the stem bark fractions of*D. oliveri*is due to a great extent of bioactive agents like flavonoids and other phenols.

Adenosine triphosphatase (ATPase) is a group of enzymes responsible for hydrolyzingATP into ADP and phosphate ions. The FoF1 ATPase is found in the interior membrane of the mitochondria and serves as the powerhouse by generating ATP. This enzyme could as well operate in the reverse direction during adverse conditions, hydrolyzing ATP to ADP and phosphate ions. Hydrolysis of ATP will cause generation of ROS which have been associated with many cellular processes like apoptosis and protection ofcell.The ATP is important for functioning of the cell because it supplies the energy needed formany reactions of the cell. Consequently, ATP exhaustion quickly results to destruction of the cell's ion balance and as a consequence, numerous cellularprocesses cannot work effectively, and the cell dies.Interferences in the ion levels are oftennoticed at the beginning stage of cell death (Rosser and Gores 1995).

In this research, we investigated the effects of certain solvent fractions of *D. oliveri* stem bark on activities of mitochondrial F0F1-ATPase/ATP synthase. All solvent fractions of *D. oliveri* stem bark used in this assay remarkably enhanced ATPase activities compare to control in a concentration – dependent manner (Figure 17). 0.75, 2.25, 3.75, 4.25 and 6.75 mg/ml of ethanol fraction used enhanced ATPase activities by 42.0, 53.8, 55.3, 58.9 and 63.1% respectively, while dinitrophenyl (DNP) enhanced by 68.6%. Chloroform fraction enhanced it by 42.1, 45.0, 46.3, 47.9 and 47.6% respectively and DNP enhanced it by62.7%. Ethyl acetate fraction enhanced it by 34.4, 35.7, 37.0, 38.2 and 45.7%, respectively and DNP by 56.7%.

Our research, therefore, could proffer a potential process for the actions of*D. oliveri*stem bark via targeting of the F0F1-ATPase/ATP synthase.Our findings indicated that enhancement of F0F1-ATPase activity of the mitochondria could be an endowed mechanism imparting to the various effects of dietary polyphenols (Moser *et al*., 1999).Effects of*D. oliveri* stem bark on ATPase activity could remarkably affect function of the mitochondriaand influence ATP concentration, mitochondrial transmembrane potential and production of ROS, which are known to be connected with many processes of the cell such as protection of the cell, cell death, O_2 sensing and ageing (Wallace, 1999).

Reports from recent researches propose that the α and β subunits of F1-ATPase could be found on the periphery of human umbilical vein endothelial cells and are binding points for angiostatin, a proteolytic portion of plasminogen that is a potent antagonist of angiogenesis and a suppressor of neoplasia development (Moser *et al*., 1999). Increase in the activity of ATPase by *Daniellia oliveri* stem bark solvent fractionsalso collaborated induction of mPT pore opening by this plant, and could thus enhance its anti-tumour activity and could be potential therapeutic targets for cardiovascular diseases, diabetes and cancers etc.

Duringopening of mPT pore, uncoupling of mitochondria occurs and ATPaseoperates in reversed direction, decomposition of ATP. With decreased ATP concentrations, thestructural and functional integrityof cell are compromised, including ion homeostasis resulting in irreversible injury and cell death, majorly by necrosis. Apoptosis could take place when only a portion of the mitochondria undergoes opening of MPTP and the cells still possess enough concentration of ATP to guide cell death viaroute of apoptosis(Sabzali *et al*., 2009).

The Bcl-2 proteins influence an extremely essential step in commitment to apoptosis by regulatingMitochondrial Outer Membrane Permeabilization (MOMP). This family of protein is divided into three groups relatedby structure and function: pro-apoptotic proteins like Bax and Bak that directly permeabilize the MOM; BH3 proteins, for instance, Puma thatdetectstresswithin the celland activate(directly or indirectly) the proapoptotic members; andtheanti-apoptoticmembers like Bcl-2, Bcl-xL that impede the generalmechanism by impeding the BH3 andthe proapoptotic proteins. Moreover,these constant interactions cause structural alterations in Bcl-2 proteins that regulatetheir function in apoptosis, supplying extra potential means of manipulation. The ratio of anti-apoptotic to pro-apoptotic Bcl-2 proteins therefore

appears to influence the comparative sensitivity or resistance of various typesof cells to stimuli that lead to apoptosis.

In this research, we assessed the effects of EFDO stem bark on the apoptotic biomarkersexpression including Bax, Bcl-2, cytochrome c, p53 using immunohistochemical technique and DNA fragmentation applying Tunel assay technique. Figure 34 illustrates the results of immunohistochemical expression of Bax protein. Relative to the control group the 25, 50 and 100 mg/kg body weight significantly up-regulate Bax proteins by 35, 200 and 330%, respectively. The result of immunohistochemical expression of Bcl-2 is as shown in figure 35. Compared to the control group, 25, 50 and 100 mg/kg body weight sbstantially down-regulate Bcl-2 proteins by 5.5, 34.5 and 56.4%, respectively. Figure 36 depicts the result of cytochrome c expression. The 25, 50 and 100 mg/kg body weight doses used significantly up-regulate cytochrome c expression by 91.3, 100 and 204%, respectively, relative to control group. Figure 37 illustrates the results of p53 immunohistochemical expression. Compare to the control group, the doses used remarkably increase p53 expression by 50, 100 and 300%, respectively. The nuclear DNA fragmentation result is as shown in figure 38. Also, the doses used significantly increased DNA fragmentation by 20, 55 and 115% respectively, relative to control group.

It has been observed that approximately one-third of advanced carcinomas of the breast experience a marked reduction in Bax immunostaining compared with normal breast epithelium (Krajewski*et al*., 1995). Thus, reductions in Bax as opposed to elevations in Bcl-2, Bcl-XL, or Mcl-1 may represent an alternative process for dysregulating cell death mechanisms in some types of cancer.Growing of tissue relies on the comparative speeds of reproduction and death of cell. Reducing thedegree of cell death could enhance tumour formation and progression by promotingsurvival of cell. Elevated Bax expression not only compensates the rise in cell enlargement rate but as well permits the removal of cells with permanentinjuries to genes, which perhaps decline the result of carcinogens on the epithelium (Sousa *et al*., 2009). In this research *D. oliveri* was observed to up-regulate Bax expression, and therefore it could serve to protect against cell proliferation, thereby corroborating its use as antitumour agent by indigenous people.

Both anti- and pro-apoptotic Bcl-2 are associated with regulatory proteins of apoptosis. The Bcl-2 could form homodimers, Bcl-2 – Bcl-2and heterodimers, Bcl-2 – Bax. During

physiologicalconditions, Bcl-2 forms heterodimers with Bax and impedes activation of Bax. Over expressionof Bcl-2 will favour Bcl-2 homodimers formation and will have influenceon cells by protecting against death of cell. In contrast, overexpression of Bax enhanced cell death, and the contrasting functions proposed a rheostat model, whereby the comparative levels of proapoptotic and antiapoptotic Bcl-2 member dtictatefate of cell.Abundance of Bax will result in Bax homodimers formation and will have influence by allowing cells togo through apoptosis. The ratio of Bcl-2⁄Bax manipulates liberation of cytochrome c. The following activation of caspase and proteolytic sequence will result indeath ofcells(Zhang*et al*., 2009). Our results obtained from the immunohistochemical assay in this research indicated that *D. oliveri* stem bark elevated the expression of Bax and suppressed that of Bcl-2. Thus, *D. oliveri* stem bark posseses the potential to enhance cell death through Bax upregulation and subsequent Bax homodimers formation at the MOM.

Bax and Bak regulate pro-death role at the MOM, where they oligomerize and permeabilize the MOM, bringing about the dischargeof Intermembrane Space (IMS) proteins like cytochrome c, SMAC and endonuclease G (Kuwana and Newmeyer 2003). The pro-death function of Bax is stimulated in response to various harmful stimuli within or outside the cell, causing Bax to go through structural alterations, membrane-insertion, and oligomerization to form a route in the outermost membrane of the mitochondria. This is commonly believed to be the channel of exitof cytochrome c to evoke caspase stimulation and death of cell (Kim *et al*. 1997; Rosse *et al*. 1998; Kluck *et al*. 1999).

Bax exhibitsan immense attraction for anti-apoptotic proteins, Bcl-2 and Bcl-XL, while Bak possesses a great attractive force for anti-apoptotic proteins, Mcl-1 and Bcl-XL. Bak is locatedconstantly attached to the MOM regardless of physiological demand, whereas Bax is mainly found in the cytosol but translocates to the MOM in response tostimuli that cause apoptosis to occur (Griffiths *et al*. 1999).It has been proposed that triggered Bax could gather an intricate protein termed the PTP to form an opening running across both mitochondrialmembranes, and eventually resulting to rupture of MOM due toswelling of matrix of the mitochondria (Schwarz *et al*. 2007). Opening of the pore will take place after stimulated Bax bind to VDAC1, bringing about a structural alteration in this preexisting route, such that it is connected to ANT (Shimizu *et al*. 1999). The *D. oliveri* could thus induce mitochondrial

permeability transition pore opening through the promotion of Bax protein as seen in our immunohistochemical assay and the subsequent binding of Bax to VDAC, thus causing a conformational modification by linking VDAC to ANT.

Electrophysiological researches applying patch clamping established a pore that was called the Mitochondrial Apoptosis-induced Channel (MAC). The MAC contains oligomeric Bax or Bak, supplying the first evidence that these proteins can create a proteinaceous pore (Dejean *et al*. 2005). At the mitochondria, Bax can homodimerize or heterodimerize with Bak or truncated Bid, thus interrrupting the integrity of the OMM through formation of pores on the mitochondria and enhancing its permeabilization. These pores can then result in the discharge of apoptogenic proteins (Vyssokikh*et al*., 2002). Some reports as wellhave recommended that Bax reacts cooperatively with proteins from the PTPC to incite MOMP (Marzo*et al*., 1998).The results obtained from our immunohistochemical assay thus, suggest that *D. oliveri* can create pores in the MOM and subsequent liberation of cytochrome c through promotion of Bax protein.

Bcl-2 is a 26 kDa constitutingmembrane protein that is found on the exterior mitochondrial and ER membrane. The Bcl-2 attracted immense interest when it was found to enhance cell survival through impairment of apoptosis (Vaux *et al*., 1998).Elevation of Bcl-2 is a distinguishing feature of chronic lymphocytic leukemia (CLL) (Rong *et al*., 2009).In the "rheostat" apoptosis fashion, cell survival or death is determined by the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family at the mitochondria (Korsmeyer*et al*., 1993). Bcl-2 proteins have been linked to signalling implicated in ROSgeneration (Krishna*et al*., 2011; Low*et al*., 2011). There are some evidences forantiproliferative effects of Bcl-2, Bcl-xL and Mcl-1 in the physiological setting (Janumyan, 2003). In this case, a survival benefit of cells less prone to apoptosis is maintained at least in part on the expense of proliferation.In this research we investigated the effects of EFDO on the immunohistochemical expression of Bcl-2 and our results showed that this solvent fraction down – regulated Bcl-2 thus, corroborating the induction of mPT pore opening earlier in this study and the traditional use of *D. oliveri* as anti – tumour plant.

One of the major activities of Bcl-2 is its interaction with pro-apoptotic family members, like Bax, Bak and the BH3-only members. By reacting with pro-apoptotic proteins, Bcl-2 impedes themfrom oligomerizing and creating pores in the mitochondrial exterior membrane, hence

inhibiting release of cytochrome c and stimulating a sequence of caspase activation, eventually preventing cell death. Thus the ability of *D. oliveri* to down – regulate Bcl-2 in this study shows that *D. oliveri* has the potential to enhance cell death through induction of mPT pore opening.

Studies have demonstrated that Bcl-2 and Bcl-XLguard the cells by engaging withproteins of the mitochondria includingANT or VDAC, therefore hindering them from creatingopenings at the outer membrane of the mitochondria, maintaining membrane integrity, and preventing discharge of factors causing apoptosislike cytochrome c (Brenner *et al*., 2000).Suppression of Bcl-2 protein by *D. oliveri* in this study shows that this plant has the potential to ameliorate tumour growth and cancer cells, thus corroborating the use of this plant in treating tumour by indigenous people.

Cytochrome c is acomponent of electron transfer sequence of the mitochondria, which starts caspase stimulation when liberated during apoptosis. Discharge of cytochrome c is regarded as the commitment stage in the process of apoptosis. Our immunohistochemical results also show remarkable elevation in expression of cytochrome c relative to control. This indicates that *D.oliveri*could exert its apoptotic effects through cytochrome c expression.

The p53 protein is a transcription agent that plays an important functionas a tumour suppressor. Manifold triggers such as damage to DNA, activation of oncogene and erosion of telomere could result in actuation of p53. The P53 protein has been shown to influence death of cell in both transcription-dependent and -independent manner(Moll*et al*., 2005). Dependent on transcription channel, p53 incites various proapoptotic proteinsexpression like PUMA, Bax and BID, which participated in controllingcell death through intrinsic route and also promotion of cluster of differentiation 95 (first apoptosis signal /Apo1) and DR5 receptors, which brings aboutcell deaththrough extrinsic signals. Apart from transcriptionalactuation of proteinsthat are pro-apoptotic, p53 as wellis reported to keep lowproteins that are antiapoptotic like Survivin (Nakano*et al*., 2005). The p53 acts in the transcription-independent pathway by localizing to the mitochondria sequel toimpulsethat induces apoptosisfor physical engagement with Bcl-2 and/or Bcl-XL and opposes their anti-apoptotic role. This interaction discharges Bax and Bid to carry out their downstream effects. Furthermore, p53 is illustrated to engage with Bak hence,disengaging it from Mcl-1 neutralizing hold (Leu*et al*., 2004). Our immunohistochemical

results indicatedsignificant elevation in p53 expression suggesting that*D. oliveri* could have induced mPT pore opening (apoptosis) via promotion of p53 expression.

Caspasesare family of protease enzymes that are actively engaged in cell deathand inflammation.Caspases are the executioners of apoptosis.Stimulation of caspases during apoptosis brings about splitting of critical cellular substrates so precipitating the dramatic structural alterations of apoptosis.

Investigation have shown that plant extracts with a combination of anticancer compounds were able to have killing activity which was specific to cancer cells and showed no effect on normal human lymphocytes and fibroblasts. This makes plant extracts more desirable as therapeutic agents than those that are chemically derived which cause toxic complications in cancer treatment. The plant extracts induced apoptosis which was demonstrated by an increased sub-G1 phase population of cells with lower DNA content and condensation of chromatin. Also an increase in caspase 3 activation was seen after extract treatment which is a key stage in apoptotic cell death (Solowey *et al*., 2014).

During this research we assessed effects of EFDO on activities of caspases 9 and 3 using Enzyme – Linked Immunosorbent Assay (ELISA) method and the results are as shown in Figure 39 and 40, respectively. The various doses (25, 50 and 100 mg/kg body weight) used significantly increased caspases 9 and 3 activities by 15.8, 68.4, 88.2%; 87.5, 150 and 180.4%, respectively. This indicated that *D. oliveri* could incite apoptosis via enhancing the activities of caspases 9 and 3.

The biochemical distinguishing feature of apoptosis is DNAdisintegrationby endogenous DNases. Internucleosomal disintegration has been illustrated with well-distinguished morphology of apoptosis in different conditions and type of cells(Bortner*et al*., 1995). The enzymesof DNase responsible for the degradation during cell death include DNA Fragmentation Factor (DFF40) (Liu*et al*., 1998), Caspase Activated DNase (CAD) (Sakahira*et al*., 1998). Exposing nuclei to activated CAD or DFF40 is enough to incite morphologic alterations of the nuclear typical of apoptosis (Enari*et al*., 1998).In this research the effects of EFDO on DNA fragmentation was investigated using Tunel Assay method and the result is depicted in Figure

41. The manifold doses (25, 50 and 100 mg/kg body weight) administered intraperitoneally significantly ($p < 0.05$) increased nuclear DNA fragmentation by 20, 55 and 115%, respectively. The ability EFDO to promote DNA fragmentation reveals that *D. oliveri* cause occurrence of apoptosis through nuclear DNA fragmentation.

Evidence has been accumulated indicating that fatty acids are involved in permeabilization of the inner membrane, through different mechanisms, e.g., Induction of permeability transition pore (PTP): Free Fatty Acids (FFA) interaction with mitochondria isolated has been reported to induce PTP opening with consequent matrix swelling (Marco and Michele, 2006).Various processes have been recommended for the antiproliferative effect of oleic acid. Moon *et al*., 2014 demonstrated thatoleic acid could cross-regulate the Adenosine Monophosphate Kinase(AMPK/S6) axis and up-regulate genes of tumour suppressor (p53, p21, and p27) in esophagealcancer cells. Fu *et al*. (2016) as well discovered that OA could lead to production of reactive oxygen species andup-regulation of NOX4 protein. In short, the effects of OA on cancer cells include effects on the cell membrane,apoptosis, autophagy, mitochondria, proteasome inhibition, cell adhesion and glycolysis (Fontana *et al*., 2013, Menendez *et al*., 2005).Palmitic acid has been reported to induce apoptosis. Treatment of palmitic acid with human granulosa cells was marked by a dramatic reduction in the expression of Bcl-2 and promotion of Bax (Yi-Ming *et al*., 2001). Our GC-MS assay result indicated that oleic and palmitic acids are present in EFDO. Hence, the induction of mPT pore opening earlier by this fraction of *D. oliveri* could be due to the availability of these fatty acids. This also corroborates the traditional application of *D. oliveri* in the treatment of tumour.

CONCLUSION

Mitochondria possess pivotal opposing roles in energy production for survival of cell and release of cytochrome c for cell death viaapoptosis. The result obtained in this study shows that certain solvent fractions of *D. oliveri*stem barkcontains a number of bioactive compounds that can effectively scavenge ROS and thus could exhibit anti-cancer, anti-inflammatory, anti-ageing and anti-tumor activity. Enhancement in the activity of mitochondrial ATPase by *D. oliveri* stem bark substantiates its anti-tumor activity and could be potential therapeutic targets for mitochondrial – mediated cell death.The inhibition of Fe^{2+} - induced lipid peroxidation results from this research showed that *D. oliveri*has potentials as free radical scavengers and so the induction of mPT pore observed in this study could not be attributed to ROS generation but due to some bioactive compounds present in the stem bark of this plant.This result also suggests that *D. oliveri*stem bark can act functionally to protect the physiochemical properties of membrane bilayers from free radical induced cellular dysfunction through excessive apoptosis. The ability of fractions of *Daniellia oliveri* to increase release of cytochrome c corroborates induction of mPT pore earlier in this research.The promotion of Bax, cytochrome c and p53 proteins and suppression of bcl-2 protein in the immunohistochemical assay substantiate the induction of mPT pore, cell death and anti-tumor properties of *D. oliveri*.The promotion of caspases 3 and 9 activities by ELISA method and increase in hepatic DNA fragmentation also corroborate the anti-tumor property of *Daniellia oliveri*.Oleic acid, which is known to induce apoptosis through up-regulation of p53 protein andgeneration ofROSwas identified in *D.oliveri.*

CONTRIBUTION TO KNOWLEDGE

- The Ethanol Fraction of *Daniellia oliveri*(EFDO) stem bark induced mitochondrial membrane permeability transition pore opening *in vitro* and *in vivo* in rat liver.
- Fractions of *Daniellia oliveri* stem bark inhibited Fe^{2+}- induced lipid peroxidation.
- Fractions of *Daniellia oliveri* stem bark enhanced mitochondrial ATPase activitiesand induced release of cytochrome c.
- The Ethanol Fraction of Daniellia oliveri (EFDO) stimulated the expression of apoptotic biomarker proteins.
- Oleic and palmitic acids were identified in ethanol fraction of *Daniellia oliveri* stem bark.

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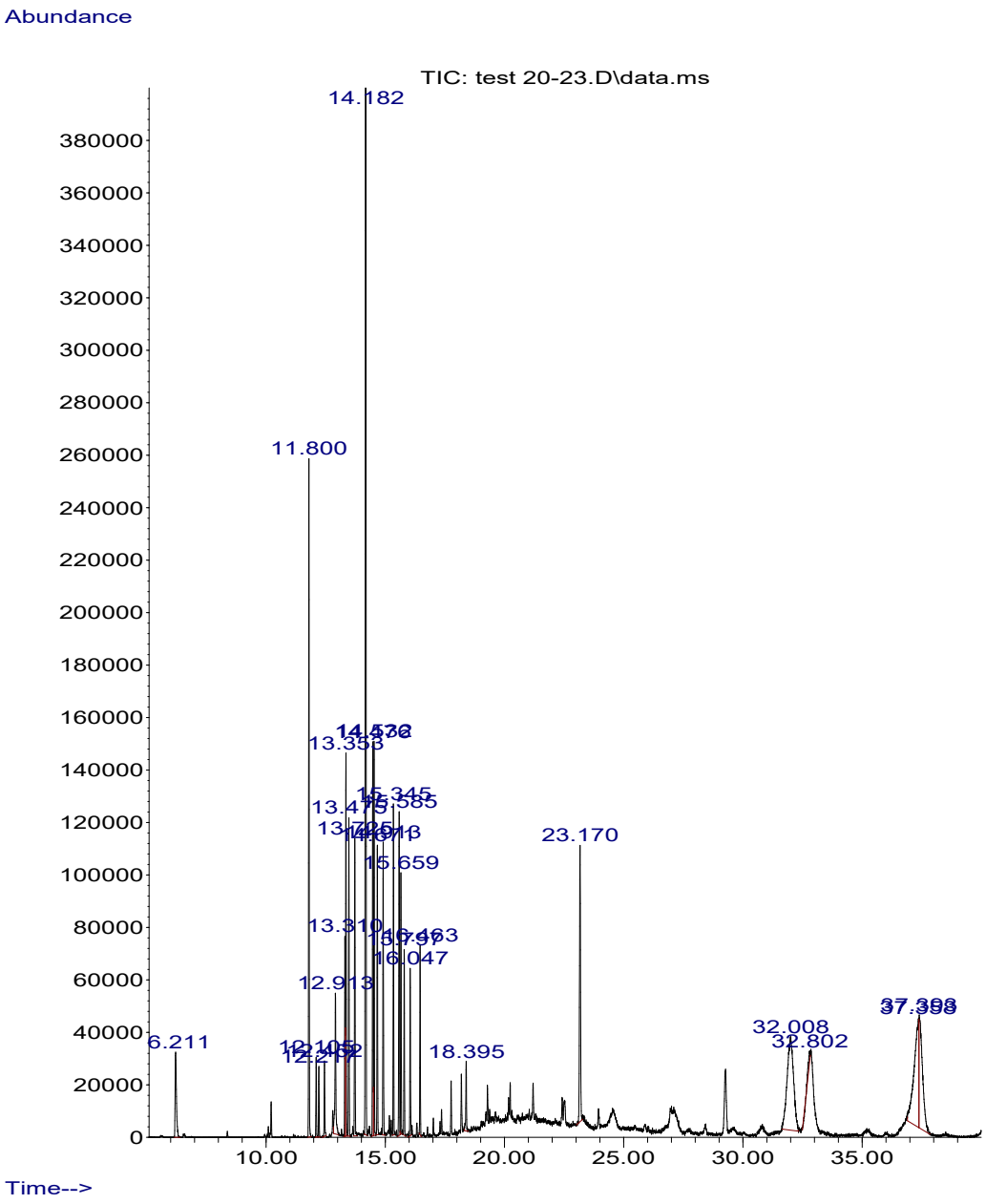
APPENDIX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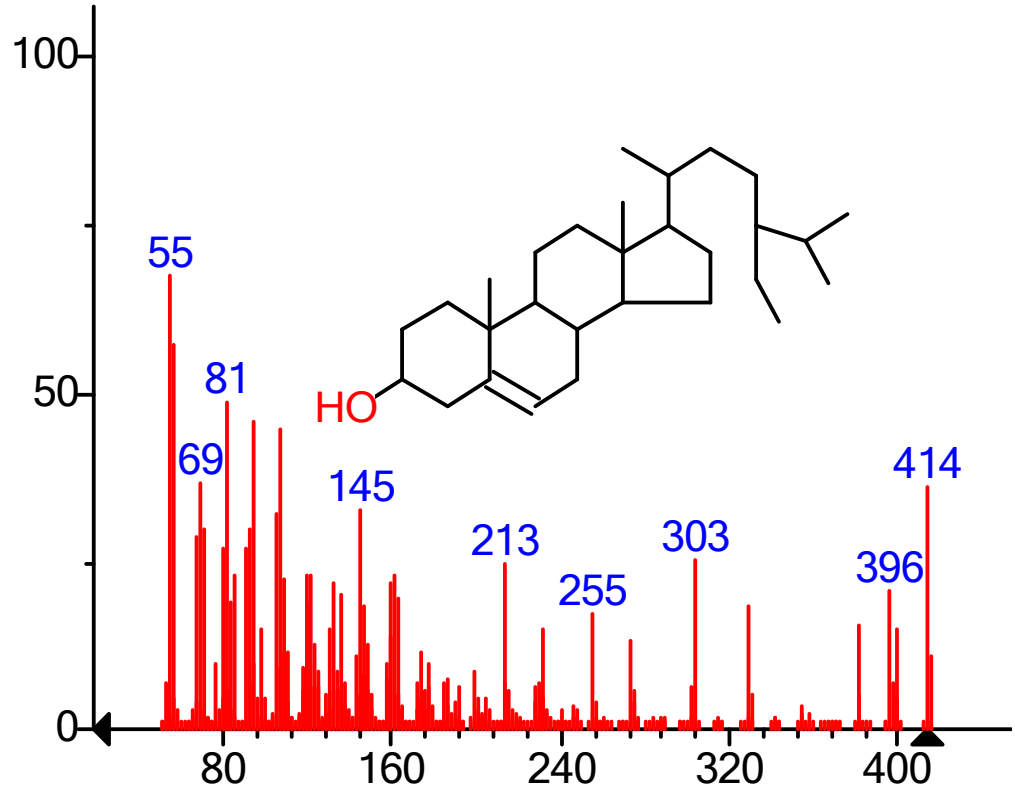

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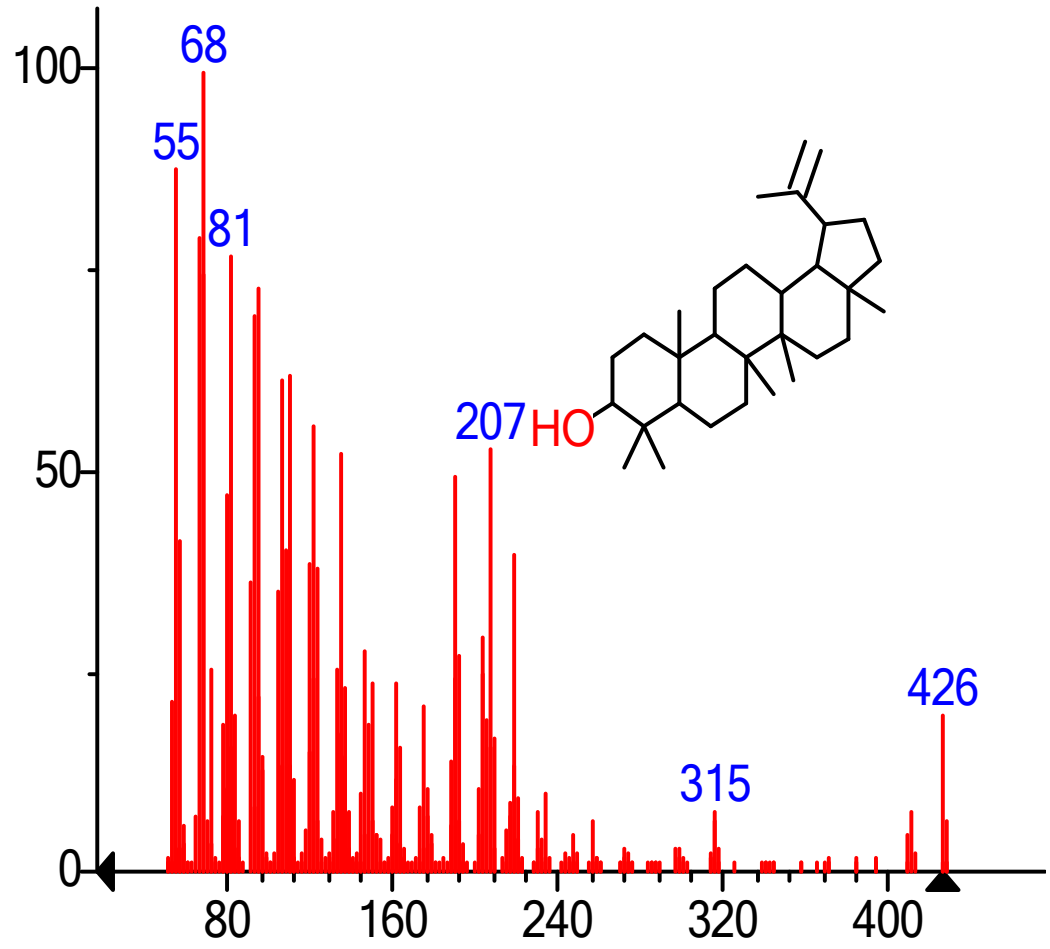

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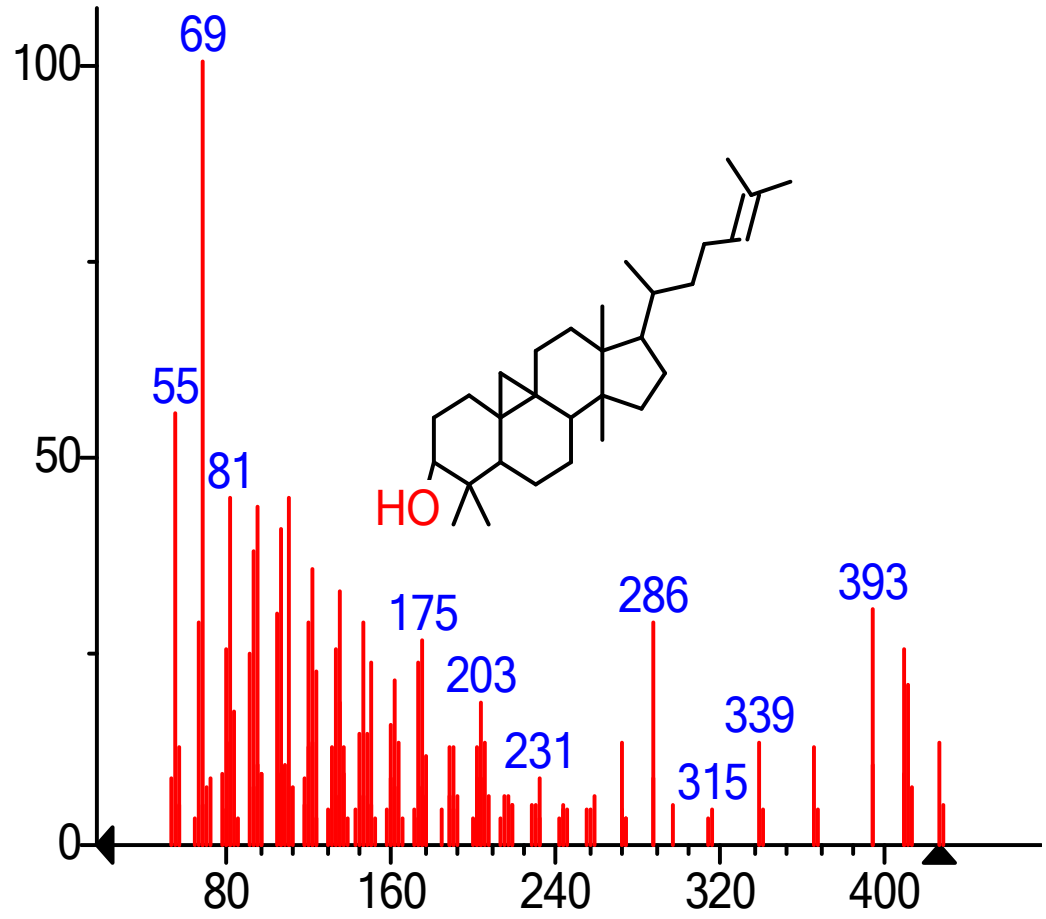

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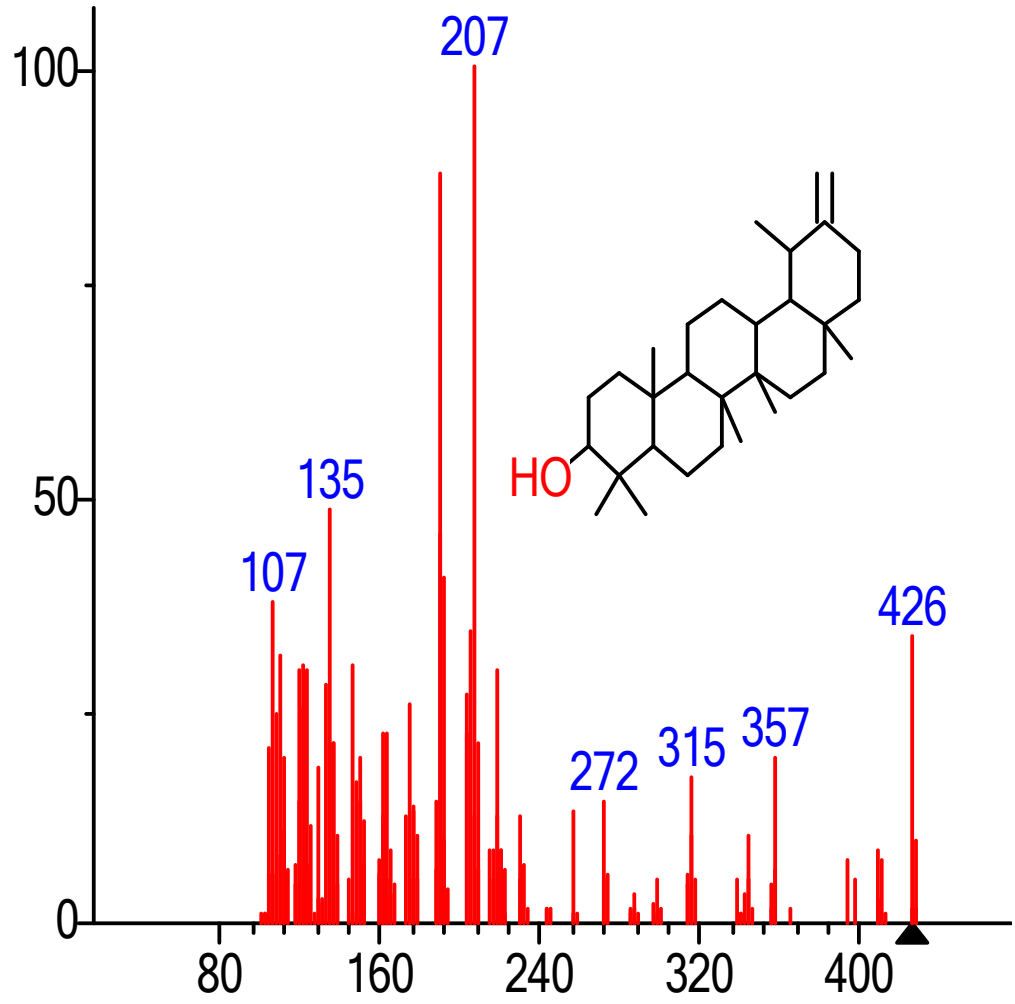

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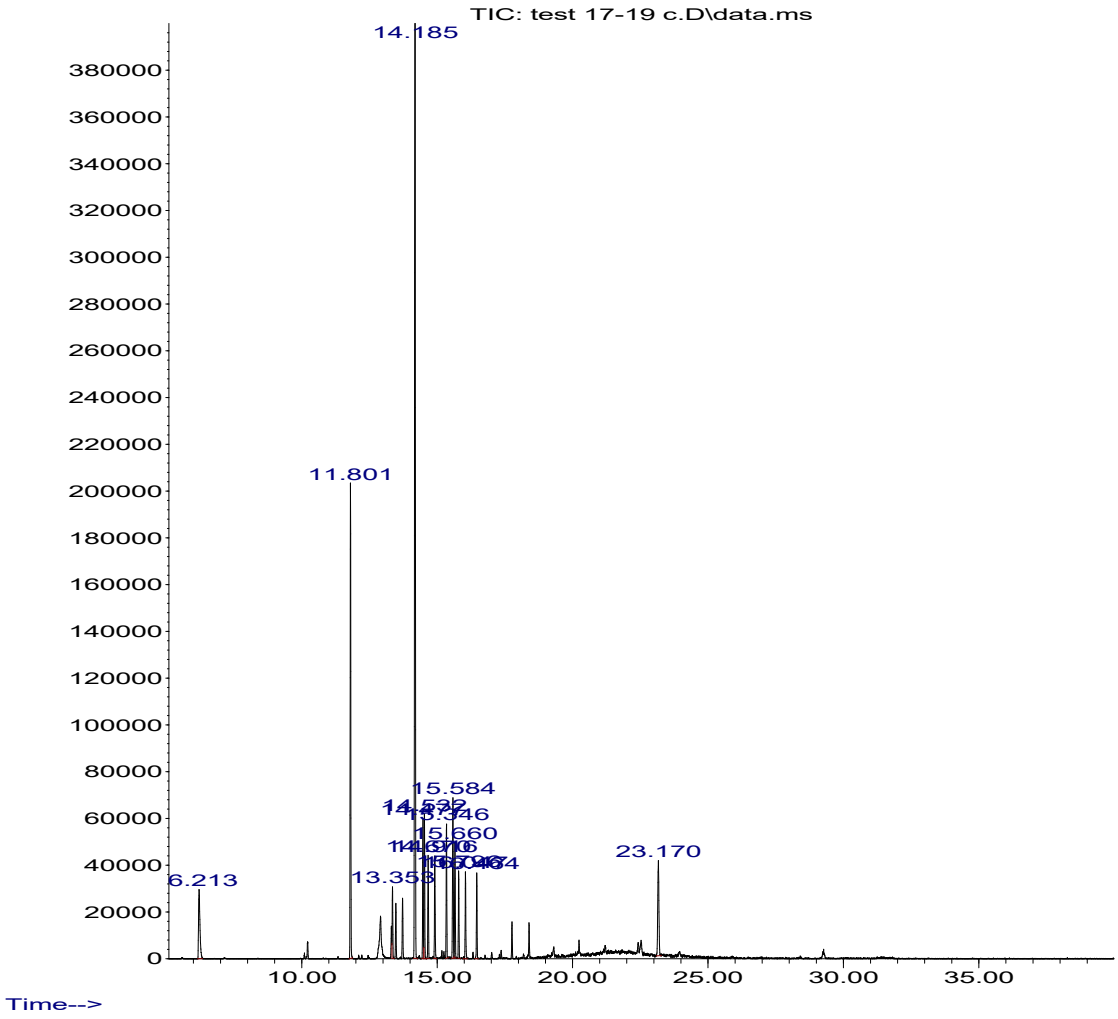
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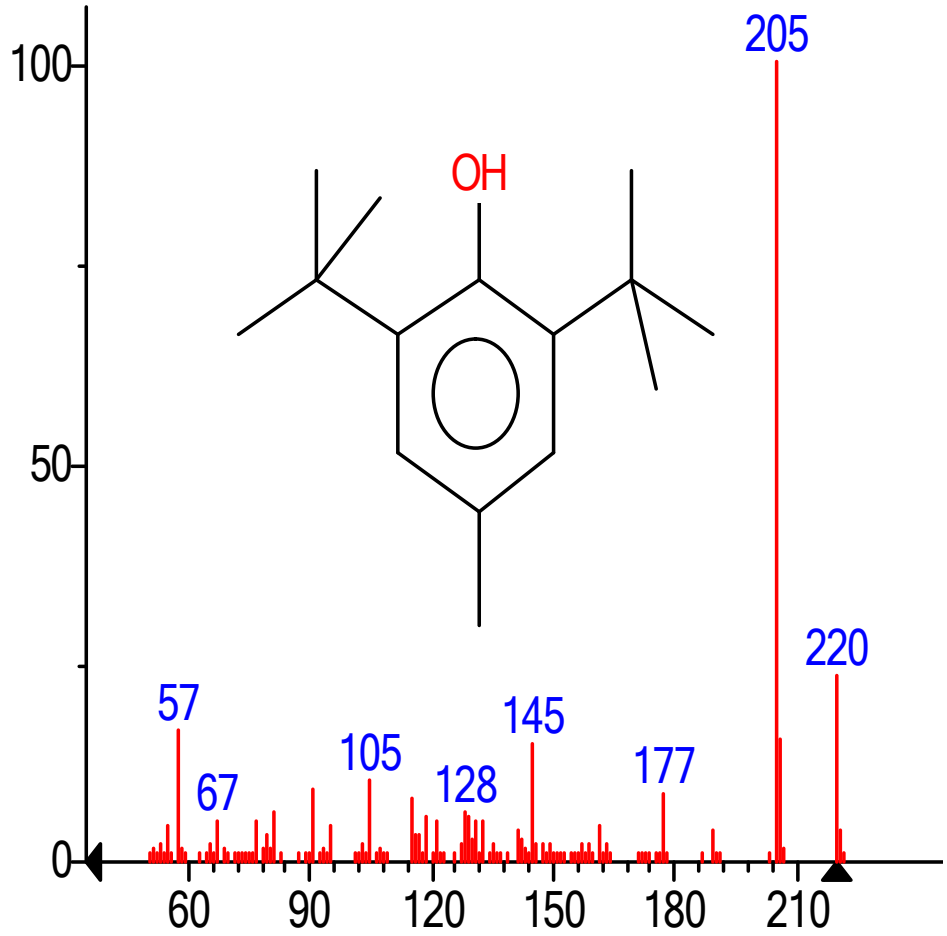

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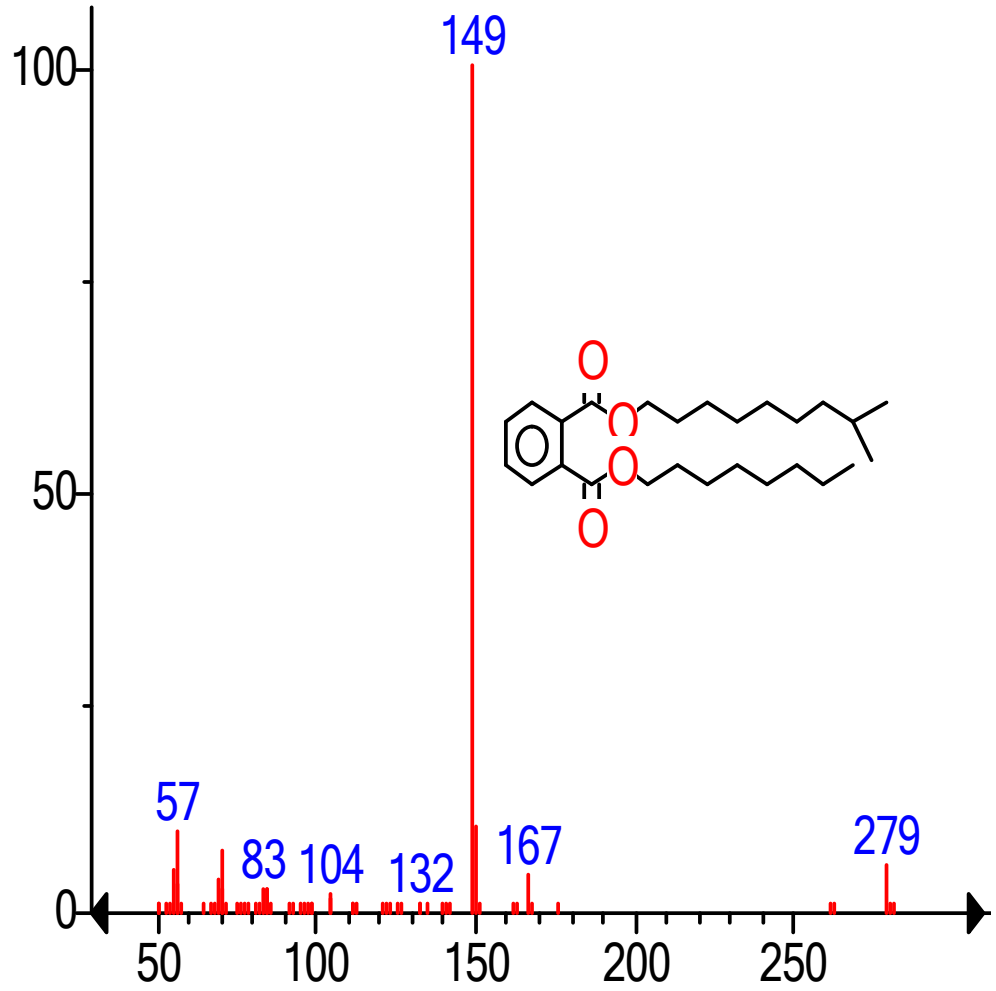

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