FRACTIONS OF *Drymaria cordata* (Linn)

MODULATE MITOCHONDRIAL-MEDIATED APOPTOSIS IN NORMAL RAT LIVER AND MONOSODIUM GLUTAMATE-INDUCED UTERINE HYPERPLASIA

BY

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ABSTRACT

The mitochondria have become an important component of apoptosis execution machinery. Mitochondrial Permeability Transition (mPT) pore opening constitutes the point of no return for apoptosis to take place when cytochrome C is released into the cytosol. Some dietary components of plant origin induce apoptosis in tumour cells *via* the induction of mPT pore opening. In folkloric medicine, *Drymaria cordata* (DC) is used to treat benign tumours in the liver and uterus. Therefore, this study was designed to investigate the effects of Methanol Extract of DC (MEDC) on mitochondrial-mediated apoptosis in rat liver and its ameliorative potential on Monosodium Glutamate (MSG)-induced uterine hyperplasia.

The *Drymaria cordata* leaf was harvested, authenticated at the Department of Botany, University of Ibadan (UIH: 22555), air-dried, milled and extracted with methanol for 72 hours. The MEDC obtained was partitioned successively using chloroform, ethyl acetate and water to obtain CFDC, EFDC and AFDC fractions, respectively. Thirty female Wistar rats (100.0±8.0 g) were used for *in vitro* study using the fractions (10-90 µg/mL). Another set of 20 female Wistar rats (150.0±10.0 g) were divided into four groups (n=4) and orally treated for twenty-eight days as follows; control (1 mL/kg distilled water), 100 mg/kg CFDC, 200 mg/kg MSG and MSG+CFDC. Rats were sacrificed and the liver mitochondria isolated using differential centrifugation. The mPT pore, mitochondrial ATPase (mATPase), Cytochrome C release (CCR) and nuclear DNA (nDNA) fragmentation were determined by standard methods using a spectrophotometer. Caspases (Casp9 and Casp3) activities, estrogen, progesterone and total cholesterol levels were determined using ELISA, while the fibroblast cell count density was measured histomorphometrically using TS View CX Image® Software. The CFDC was subjected to GC-MS analysis to identify the compounds in the fraction. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The mPT pore opening was induced by MEDC (1.3, 2.7, 4.5, 10.2 and 13.3 folds), CFDC (3.6, 13.4, 15.3, 17.8 and 17.4 folds) at 10, 30, 50, 70 and 90 µg/mL, respectively, when compared with the control. The EFDC and AFDC did not induce pore opening at all concentrations tested. The CFDC enhanced mATPase activity (10.9±0.3 µmol Pi/mg protein/min) relative to control (3.2±0.2 µmol Pi/mg protein/min) and CCR (6.3±0.2 nmol/mg protein) relative to control (2.1±0.1 nmol/mg protein) maximally at 90 µg/mL. The CFDC (50, 100 and 200 mg/kg) increased hepatic nDNA fragmentation (30.1, 37.3 and 48.2%) relative to control (22.4%); increased activation of Casp9 (37.2±2.1, 51.0±3.2, 65.4±3.4 ng/mL) relative to control (29.2±2.3 ng/mL) and Casp3 (9.5±0.2, 12.3±0.2, 15.4±0.4 ng/mL) relative to control (8.5±0.2 ng/mL). The CFDC significantly reduced MSG-induced uterine hyperplasia from 0.009.±0.002 to 0.003±0.001 fibroblast count/µm^2. In addition, the estrogen (142.2±8.3 pg/mL), progesterone (33.1±1.3 ng/mL) and total cholesterol (77.3±2.2 mg/dL) levels in the MSG-treated rats were significantly reduced to 86.4±6.3 pg/mL, 25.4±2.2 ng/mL and 40.6±2.3 mg/dL, respectively. The GC-MS analysis of CFDC revealed the presence of 3-(p-fluorobenzoyl)-propinoic acid (70.2%), bis(2-ethylhexyl) phthalate (12.0%) and *n*- hexadecanoic acid (7.5%).

The chloroform fraction of methanol extract of *Drymaria cordata* induced mitochondrial-mediated apoptosis and protects against monosodium glutamate-induced uterine hyperplasia.

Keywords: *Drymaria cordata*, Apoptosis, Mitochondrial membrane permeability transition, hyperplasia

Word Count: 495

DEDICATION

This work is dedicated to:

The Almighty God, for His grace and presence that saw me through

My beloved parents, for their legacy

My mentors, for training and developing the seed in me

My wife, for her strong care and support through thick and thin

My in-laws, for their endless prayers and support

My children, for their prayers and understanding during different stages of this programme

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All glory, honour and majesty to the most high God who in His infinite mercy has made it possible for me to complete this programme successfully. I am grateful and full of thanks to the almighty God who has been my rock and fortress.

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Ultimately, I thank God, who has been my Alpha and Omega throughout my study. I appreciate Him for His mercy endureth forever.

Adeola Oluwakemi OLOWOFOLAHAN

June, 2019.

CERTIFICATION

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

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Supervisor

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Professor

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ABBREVIATION

MPTP	Mitochondrial Permeability Transition Pore

MMP	Mitochondrial Membrane Potential
Apaf-1	Apoptotic Protease Activating Factors
Smac	Second Mitochondrial-Derived Caspases
IAPS	Inhibitors of Apoptosis Proteins
TRAIL	TNF-Related Apoptosis Inducing Ligand
DISC	Death-Inducing Signaling Complex
DED	Death Effector Domain
IMM	Inner Mitochondrial Membrane
OMM	Outer Mitochondrial Membrane
VDAC	Voltage-Dependent Anion Channel
ANT	Adenine Nucleotide Translocase
HKII	Hexokinase II
CyP-D	Cyclophilin D
PiC	Mitochondrial Phosphate Carrier
MAPK	Mitogen Activating Protein Kinase
ROS	Reactive Oxygen Species
BAX	Bcl-2-Associated X Protein
BAK	Bcl-2antagonist killer 1

DIABLO Direct IAP Binding Protein with Low Pi

DMSO Dimethylsulphoxide

MOMP Mitochondrial Outer Membrane Permeability

CHAPTER ONE

1.0 INTRODUCTION

Apoptosis is one of the potent defence mechanisms by which potentially deleterious and mutated cells are removed from an organism, ensuring proper growth and development, maintenance of tissue homeostasis, differentiation in multicellular organisms, metarmorphosis and embryogenesis (Zamzami and Kroemer, 2001; Lockshin and Zakeri, 2001 and McIlwain *et al*., 2013). Dysregulation of apoptotic signalling has been implicated in various pathologic conditions including cancer, autoimmunity, persistent infections (failure to eradicate infected cells, neurodegenerative diseases like Alzheimers' disease, Parkinson's disease, AIDS (depletion of T lymphocytes) and ischaemia (stroke, myocardial infarction) (Reed, 2004; Rasul *et al*., 2013). Attention was placed on the complex role of mitochondria in apoptosis when biochemical studies identified several mitochondrial proteins that can activate cellular apoptotic programs and the interactions between mitochondrial membrane permeability transition pore components (Verhagen *et al.,* 2000). Mitochondria play a central role in initiation of the intrinsic pathway of apoptosis by releasing mitochondrial proteins, which normally reside in the intermembrane

space, into the cytosol. This causes assembly of the apoptosome and activation of procaspase-9, leading to a cascade of events which ultimately leads to cell death (Kallenberger *et al*., 2014).

The mitochondrion is composed of two distinct membrane systems including an outer membrane in close communication with the cytosol and an inner membrane involved in energy transduction. Permeabilization of the outer membrane is regulated by Bcl_2 family proteins, which control the release of proteins from the mitochondrial intermembrane space; thereby activating apoptosis. Permeabilization of the inner membrane is regulated by the mitochondrial membrane permeability transition pore (mPT), which is activated by calcium and oxidative stress leading to bioenergetic failure and necrosis. Mitochondrial membrane permeability transition pore is an increase in the permeability of the mitochondrial membranes to molecules of greater than 1500 daltons in molecular weight. Mitochondrial membrane permeability transition results from opening of mitochondrial permeability transition pore, which is also known as the mPT pore. This mPT pore is a protein pore formed in the membranes of mitochondria under certain pathological conditions including traumatic brain injury and stroke. Induction of the permeability transition pore can lead to mitochondrial swelling, release of cytochrome c and formation of apoptosome which will ultimately lead to cell death. Various studies have shown that the permeabilization of the inner mitochondrial membrane is a major event in the induction of mitochondrial-mediated apoptosis (MMA) (Crompton, 1999; Wang, 2001). The mPT pore therefore serves as a target for the design of novel drugs which may be needful in diseases where apoptosis needs to be upregulated or downregulated.

Monosodium Glutamate (MSG) is a salt of glutamate which is synthesized from L-glutamic acids and is used as a flavour enhancer in foods for nutritional supplements, (Ikonomidou and Rodriguez *et al*., 1998; Eskes, 1998). Though MSG improves taste stimulation and enhances

appetite, reports indicate that it is toxic to human and experimental animals (Oforofuo *et al*., 1997; Farombi and Onyema 2006; Sharma and Deshmukh, 2015). It has been reported that MSG has neurotoxic effects which result in brain cell damage, retinal degeneration, endocrine disorder and some other pathological conditions such as addiction, stroke, epilepsy, brain trauma, neuropathic pain, schizophrenia, anxiety, depression, Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Eweka *et al*., 2007).

Recent studies have also demonstrated that MSG induces uterine fibroid/tumor in rats by increasing the levels of total protein, cholesterol and estradiol (Eweka *et al*., 2010).

Uterine leiomyomas (or uterine fibroids) are benign smooth muscle tumors that develop in the myometrium. These tumors have an estimated incidence of up to 75% in reproductive age women and are usually characterized by an excess of extracellular matrix (ECM) (Borgfeldt and Andolf, 2000; Baird *et al*., 2003). The most common symptoms for these sex steroid hormone-responsive tumors include: heavy and prolonged menstrual bleeding, anemia (secondary to excessive menstrual blood loss), pelvic pain and pressure, dysmenorrhea (painful menses), urinary incontinence, constipation, lower back pain, sexual dysfunction, infertility, recurrent pregnancy loss and compression of adjacent pelvic organs by large uterine fibroids. Contemporary medical treatment options are gonadotrophin-releasing hormone (GnRH) agonists, progestins, oral contraceptives, levonorgestrel intrauterine devices, selective progesterone receptor modulators and procedural interventions such as myomectomy (uterine preservation with removal of uterine fibroids), uterine artery embolization and MR-guided focused ultrasound treatments. All these treatment options have variable side effect profiles and may not consistently relieve symptoms or prevent disease recurrence (Wu *et al*., 2007). Due to some degree of morbidities that are associated with fibroid and to the ineffective therapies

mentioned ealier above, hysterectomy may be the only option for many women. Fibroids are the most common cause for gynecologic surgery in the USA and accounts for ~600 000 hysterectomies and 60 000 myomectomies annually (Farquhar and Steiner, 2002). The total cost implication related to both surgical and non-surgical leiomyoma treatment is estimated to be between 6 and 35 billion US dollars annually (Cardozo *et al*., 2012).

The cause of fibroid is still unknown, but medically, fibroids are considered to be a women's fibrosis condition that is usually associated with estrogen dominance or excess estrogen. Fibroid tumors can thereby be aggravated by estrogen and their growth depends on estrogen stimulation and may increase with estrogen therapy (such as oral contraceptives) or during pregnancy. The use of plants as medicine dates back to prehistoric period. It is well known that medicinal plants are extensively utilized throughout the world in the management and treatment of several diseases (Schippmann *et al*., 2002; Islam *et al*., 2014). Plants are presently the sources of medicines for many people of different age in many countries of the world, where diseases are treated primarily with traditional medicines obtained from plants. The application of various medicinal plants with potent bbioactive agent have been used in targeting mitochondrial apoptotic machineries through the activation of mPT pore towards efficient and selective treatment of diseases associated with dysregulated apoptosis, such as cancer. They have been shown to be of good sources of natural products that have phytochemicals with many beneficial effects on human health (Martins, 2006).

1.2 RATIONALE

Mitochondria help to maintain cell life and the permeabillization of mPT pore is implicated in apoptosis (Crompton, 1999; Wang; 2001; Xiong *et al*., 2014). The permeabilization of the mPT pore which results in the release of cytochrome c from the intermembrane space, serves as a

point of no return for apoptosis to occur. It is in this light that different investigations have been ongoing in order to pharmacologically design drugs that will target diseases such as neurodegenerative diseases and tumours by modulating the pore opening in dysregulated apoptosis (Martin, 2006). Experimental evidence indicates that certain bioactive agents present in medicinal plants e.g. epigallocatechin gallate, capsaicin in chili pepper, quercetin in onions, resveratrol in grape, organo-sulfur compounds in garlic, and lycopene in tomatoes among many others, have been found to modulate apoptosis, especially at the stage of the permeabilisation of the mitochondrial membrane (Martins, 2006). Therefore, mPT pore serves as a target for the design of novel strategies for blocking pathological cell loss or for killing unwanted cells.

Surgery (myomectomy or hysterectomy) is currently the primary treatment for uterine fibroids (Bachmann, 2006) with uterine artery embolization and focused ultrasound ablation as other emerging options (Stein *et al.,* 2009). Data have shown that the estimated annual direct costs for medical and surgical management of uterine fibroids are much, ranging from approximately 4 to 9 billion US dollars (Cardozo *et al*., 2012). This is very costly for a common man and traumatic. In addition to the associated adverse effect of these procedures, most of these options could result to diminished future fertility (Al-Hendy and Salama, 2006).

At present, available options for leiomyoma treatment are far from satisfactory; therefore, the development of a safe, effective oral treatment method would appeal to many women with symptomatic uterine leiomyomas. It is against this background that the possible presence of bioactive agents in *Drymaria cordata* that could modulate mitochondrial-mediated apoptosis (MMA) in healthy rat liver and monosodium glutamate-induced uterine hyperplasia was investigated.

1.3 GENERAL OBJECTIVE

The ultimate aim of this study was to investigate modulation of mPT pore as a biological mechanism for prevention and management of Uterine Fibroid

SPECIFIC OBJECTIVES

The specific objectives are to:

- determine the phytochemical constituents of crude methanol extract of *Drymaria cordata*
- partition the crude methanol extract into various fractions and determine their potencies
- evaluate the potency of the different solvent fractions of methanol extract of *Drymaria cordata* on normal rat liver mPT pore in the absence and presence of calcium.
- evaluate the effects of these fractions on mitochondrial ATPase activity, lipid peroxidation and cytochrome c release
- investigate the effect of the most potent fraction of the extract on normal rat liver and uterine mPT pore *in vivo*
- assess the effect of the most potent fraction on caspases 9 and 3 activation and other apoptotic biomarkers
- evaluate the effects of the most potent fraction on MSG-induced uterine hyperplasia using histomorphometry
- assess histological changes due to the effect of the most potent fraction on the liver and uterus of MSG-treated rats
- identify, as far as possible, the chemical nature of the most potent bioactive compound present in the most potent fraction
- assess the effect of the compounds on normal rat liver mPT pore and hormonal levels in MSG-treated mice

CHAPTER TWO

LITERATURE REVIEW

2.0 APOPTOSIS

Apoptosis is a form of programmed cell death as a result of signals received by the cell (Tomei and Cope, 1991; Danial and Korsmeyer, 2004). Apoptosis is characterized by several morphologic changes, such as chromatin condensation, nuclear fragmentation and reduction of cell volume (known as pyknosis) (Kroemer *et al*., 2005), as well as biochemical changes that include caspase activation, breakdown of DNA and protein and membrane surface modifications that allow the apoptotic cell to be recognized and engulfed by phagocytic cells (Robbins *et al*.,2009; Fulda *et al*., 2010). When apoptosis is dysregulated, an imbalance between life and death of cells can lead to cancer. (Kerr and Wylliey, 1972; Yang, 1997). Therapeutic interventions targeting molecules involved in apoptotic resistance therefore represent a valid approach to be pursued in order to redirect cells sensitivity to apoptosis and overcome uncontrolled proliferation (Vakifahmetoglu-Norberg and Zhivotovsky, 2010).

2.1 MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN APOPTOSIS

From the morphological point of view apoptotic cells show a characteristic cytoplasmic cell shrinkage, budding of plasma membrane, membrane exposure of phosphatidylserine (PS) on extracellular side, chromatin condensation and DNA fragmentation (Hacker, 2000; Saraste and Pulkki , 2000). The plasma membrane is intact throughout the total process. The expression of PS in the outer layers of the cell membrane allows early recognition of dead cells by macrophages, resulting in phagocytosis without the release of proinflammatory cellular components (Hengartner, 2000). At the later stage of apoptosis, some of the morphological features include membrane blebbing, ultrastructural modification of cytoplasmic organelles and loss of membrane integrity (Kroemer *et al*., 2005). Apoptosis is primarily executed by a family of proteases known as the caspases (cysteinyl, aspartate-specific proteases (Li and Yuan, 2008). Caspases play a central in the mechanism of apoptosis as they are both the initiators (caspase-2, -8, -9 and -10, responsible for the beginning of the apoptotic pathway) and the executors (caspase-3, -6 and -7, responsible for the definite cleavage of cellular components) of cell death (Thomberry and Laxebnik, 1998). After being produced as inactive proteins (zymogens or pro-caspases), the initiator caspases auto-activate through auto-proteolysis, a process that is facilitated by their interaction with specific adapter molecules (Nicholson, 1999). Once they are activated, the initiator caspases cleave off the executors caspases that perform critical cleavage of specific cellular substrates resulting in the final apoptotic cell death (Stennicke and Salvesen, 2000). This caspases activity is responsible for the apoptotic hallmarks, such as chromatin condensation, plasma membrane asymmetry and cellular blebbing. The extensive and irreversible proteolytic activity mediated by executor caspases represents the ultimate outcome of both the extrinsic and the intrinsic apoptotic pathways. Thus, both pathways converge on caspases-3, -6, or -7 that allow disruption of DNA and cellular components inducing the typical

morphological changes in apoptosis (Degterev *et al*., 2003). Caspases activity has been also extended to non-apoptotic functions such as cell differentiation or maturation suggesting the activation of caspase cascade reaction without subsequent induction of an apoptoyic cascade (Pistritto *et al*., 2002; Pistritto *et al*., 2012; Shalini *et al*., 2015).

2.2 EXTRINSIC APOPTOTIC PATHWAY

The extrinsic apoptotic pathway (also known as death receptor-dependent pathway) is initiated by the interaction of cell surface exposed death receptors, belonging to the super family of tumor necrosis factor receptor (TNFR), with their respective protein TNF family ligands (Guicciard *et al*., 2009). Death receptors are structurally defined by an intracellular protein-protein interaction domain, called the death domain (DD), which is critically involved in apoptosis-nducing signalling (Fulda and Debatin, 2003). The more broadly characterized signaling systems of death receptor ligands include TNFR1-TNFα, FAS (CD95, APO-1)- FasL, TRAILR1 (DR4)-TRAIL and TRAILR2 (DR5)- TRAIL.

When the ligand binds to the death receptor, the receptor undergoes oligomerization and a conformational change to reveal its cytoplasmic DD to support interactions with other DD-containing proteins (Guicciard *et al*., 2009). The role of adapter proteins (FADD/TRADD) is to sequester, the initiator pro-caspase-8 and/or -10 resulting in the formation of death-inducing signaling complex (DISC), increasing the local concentration of pro-caspase and promoting the mutual auto-activation (Boatright and Salvesen, 2003). The activation of initiator caspases leads to the processing of the downstream effector caspases-3, -6 and -7 and the activation of these effector caspases leads to the cleavage of essential substrates for cell viability, causing cell death (Degterev *et al*., 2003). Some cells do not die in response to the extrinsic pathway alone. They still require an amplification step that is induced by caspase-8. In this case, capase-8 targets the

BH3-only protein Bid (BH3-interacting-domain death agonist) for cleavage and generate the activated truncated Bid fragment (t-Bid); which then directly activates proapoptotic multi-domain proteins to induce permeabilization of outer mitochondrial membrane, and thus, co-engaging the intrinsic pathway (Robbins *et al*., 2009).

2.3 INTRINSIC APOPTOTIC PATHWAY

The intrinsic apoptotic pathway (also known as mitochondria-dependent pathway) is mediated by intracellular signals converging at the mitochondrial level in response to different stress signals like irradiation, treatment with chemotherapeutic agents, etc.(Green and Kroemer, 2004). Also, internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{+2} and severe oxidative stress can trigger the initiation of the intrinsic mitochondrial pathway (Kroemer and Galluzzi, 2007). Subsequent activation of pro-apoptotic BH3-only members of the Bcl-2 family (Bax, Bak) neutralizes the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, and this leads to disruption of mitochondrial outer membrane causing the release of proteins that are normally confined in the mitochondrial intermembrane space into the cytosol. These proteins include cytochrome-*c*, which plays a crucial role in the activation of mitochondrial-dependent cell death in the cytosol (Danial and Korsmeyer, 2007). Cytochrome-*c* which binds to the cytosolic Apaf-1 (apoptosis protease activating factor-1) and triggers the formation of a complex called apoptosome, then recruits initiator pro-caspase-9 to its caspase recruitment domain (CARD). This allows autoactivation and then proteolysis which in turn activates downstream executor caspases-3, -6 and -7 for cleavage of cellular substrates leading to apoptotic cell death (Slee *et al*.,1999; Kuribayashi *et al*., 2006).

2.4 THE B-CELL LYMPHOMA 2 (BCL-2) FAMILY PROTEINS

The intrinsic pathway is regulated by the B-cell lymphoma 2 (Bcl-2) family of intracellular proteins. This proteins family is involved in the regulation of both pro-apoptotic and anti-apoptotic intrinsic pathways, thereby controlling the alteration of MOMP (Giam *et al*., 2007). Therefore, the Bcl-2 proteins serve as an "apoptotic switch" by mediating permeabilization of the mitochondrial membrane (Danial, 2007). The Bcl-2 proteins are classified into three subgroups.

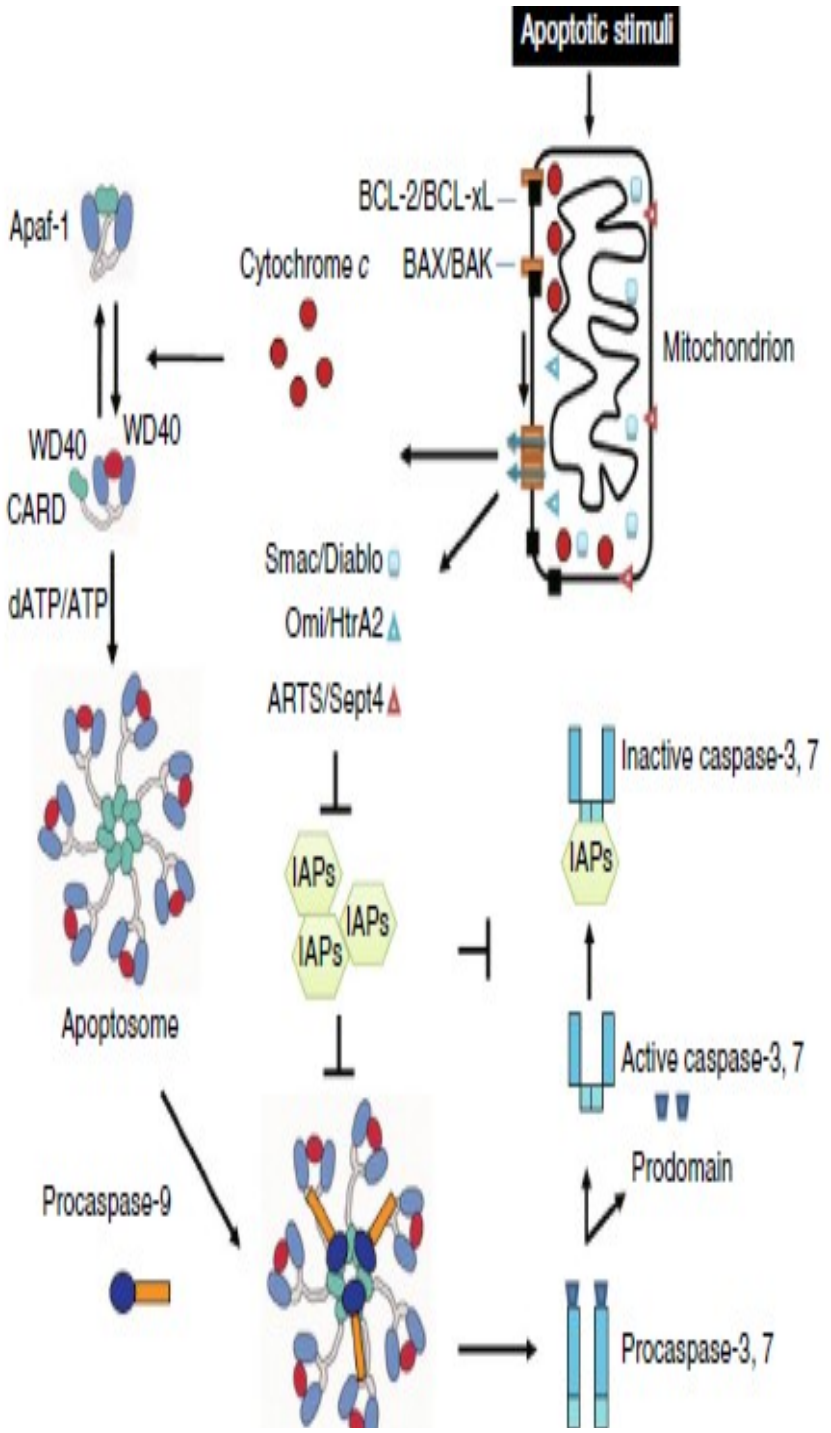

Figure 1: Mitochondrial -mediated pathway of apoptosis (Xiong *et al.,* 2014)

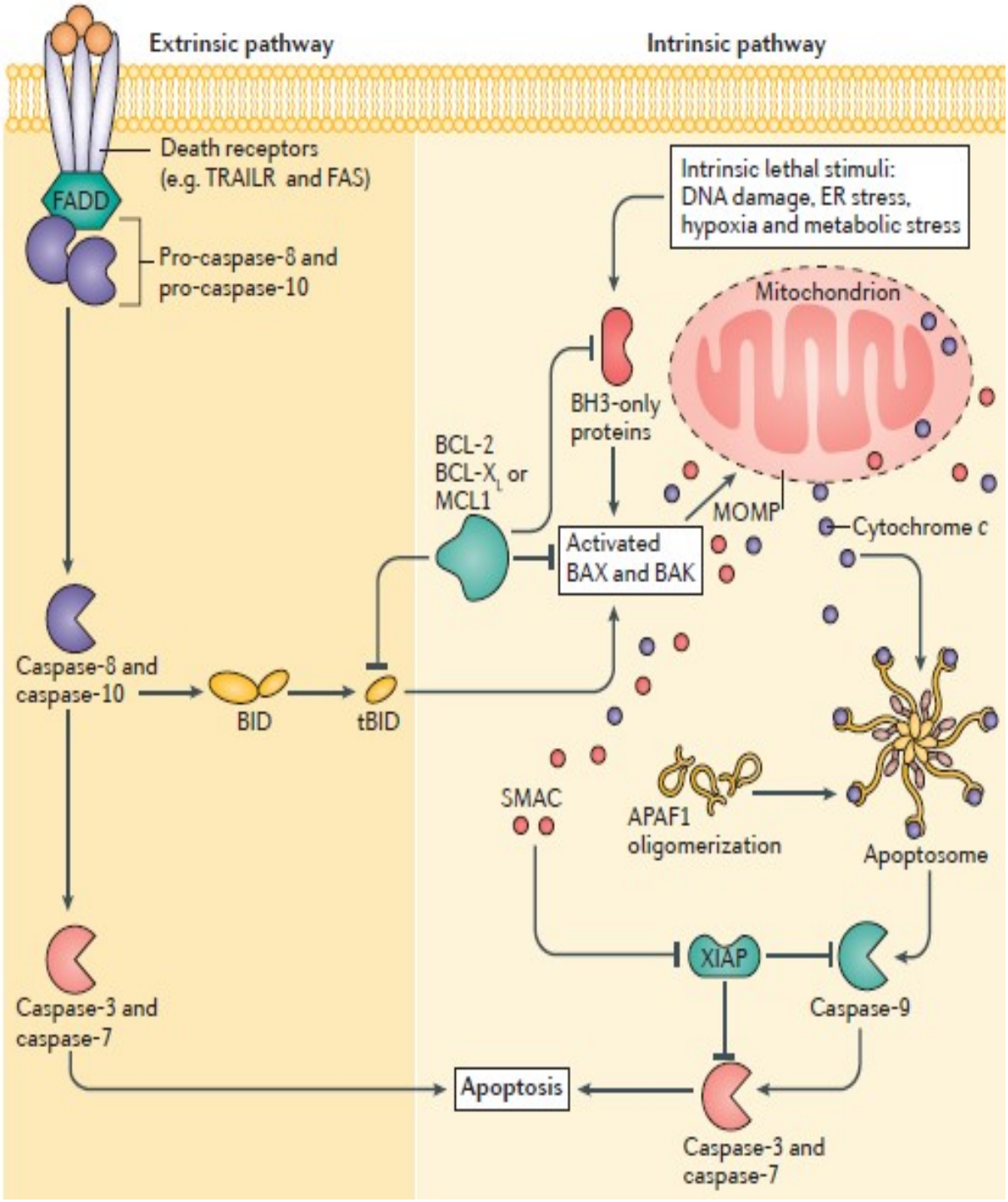

Figure 2: Cross talk between extrinsic and intrinsic apoptotic pathways Czabotar, *et al*., 2014

There is one group with anti-apoptotic and two with pro-apoptotic function, depending on the composition of typical BH (Bcl-2 Homology) domains available from BH1 to BH4 (Danial, 2007; Lomonosova and Chinnadurai, 2009).

The BH1 and BH2 domains of bcl-2 are required for dimerization with pro-apoptotic proteins while the BH3 domain is crucially important to the interaction between pro-apoptotic and anti-apoptotic proteins and is contained by all family members. The amino-terminal BH4 domain is mainly found in the bcl-2 family members with death-repressing activity, but is also present in some pro-apoptotic molecules. The antiapoptotic multi-domain group includes Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1, and Bcl-B, which contain from three to four BH domains. The pro-apoptotic multi-domain group includes Bax, Bak and Bok proteins, which also contain three BH-domains (BH1, BH2 and BH3); and the proapoptotic BH3-only proteins group which includes Bid (BH3 interacting-domain death agonist), Bim (Bcl-2-like protein 11), Bad (Bcl-2-associated death promoter), Puma (p53 upregulated modulator of apoptosis), Noxa, BMF, HRK and BIK (Youle and Strasser, 2008). The anti-apoptotic proteins regulate apoptosis by blocking the release of cytochrome-*c* into the cytosol, while the proapoptotic proteins act by promoting such release. The balance and protein-protein interactions between Bcl-2 protein family members is required to determine the state of the cell for cell survival or apoptosis. The activation of Bax (cytosolic protein that translocates into mitochondria during induction of apoptosis), and Bak (an integral membrane protein located in the mitochondria and endoplasmic reticulum) involves conformational changes which trigger the formation of homo-oligomeric protein complexes, and thereby altering the mitochondrial membrane permeability (Dewson *et al*., 2008; Bleicken *et al*., 2010). The pro-apoptotic BH3-only proteins act by binding with high affinity to anti-apoptotic Bcl-2 family members thereby causing Bax/Bak to elicit MOMP and ultimately leading to

activation of the caspase cascade (Gavathiotis *et al*., 2008; Brunelle and Letai, 2009). Anti-apoptotic multidomain members of the Bcl-2 protein family not only antagonize the pore-forming activity of Bax and Bak by direct inhibitory interactions, but also prevent the generation of proapoptotic cytosolic Ca^{2+} waves either by reducing the endoplasmic reticulum (ER) Ca^{2+} storage, or by interacting with inositol 1,4,5- trisphosphate (IP3) receptor (Oakes *et al*., 2005; Distelhorst and Bootman, 2011). Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Hegde, 2002).

2.5 THE INHIBITORS OF APOPTOSIS PROTEINS (IAPS)

Proteolysis is an irreversible process and therefore, regulation and control of caspases-mediated proteolytic cleavage is necessary to prevent inappropriate cell destruction (Pop, 2009). Negative regulation of caspases function is achieved by IAP proteins family whose principal members in humans are NAIP (BIRC1), cIAP1 (BIRC2), cIAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7), and IAP-like protein 2 (ILP2 – BIRC8) (Salvesen and Duckett, 2002). Their characteristic BIR (baculovirus IAP repeat) domain mediates the interaction with various proteins and enables them to bind and inactivate caspases (Berthelet and Dubrez, 2013). The activities of IAPs, however, may be suppressed by the action of some mitochondrial proteins, such as Omi/HtrA2 and Smac/DIABLO, which are released into the cytosol during apoptosis. These endogenous IAPs antagonists can bind to the BIR domain of IAPs thereby reducing their ability to interact with caspase-3 or -9 and thus, restoring their activity (LaCasse *et al*., 2008).

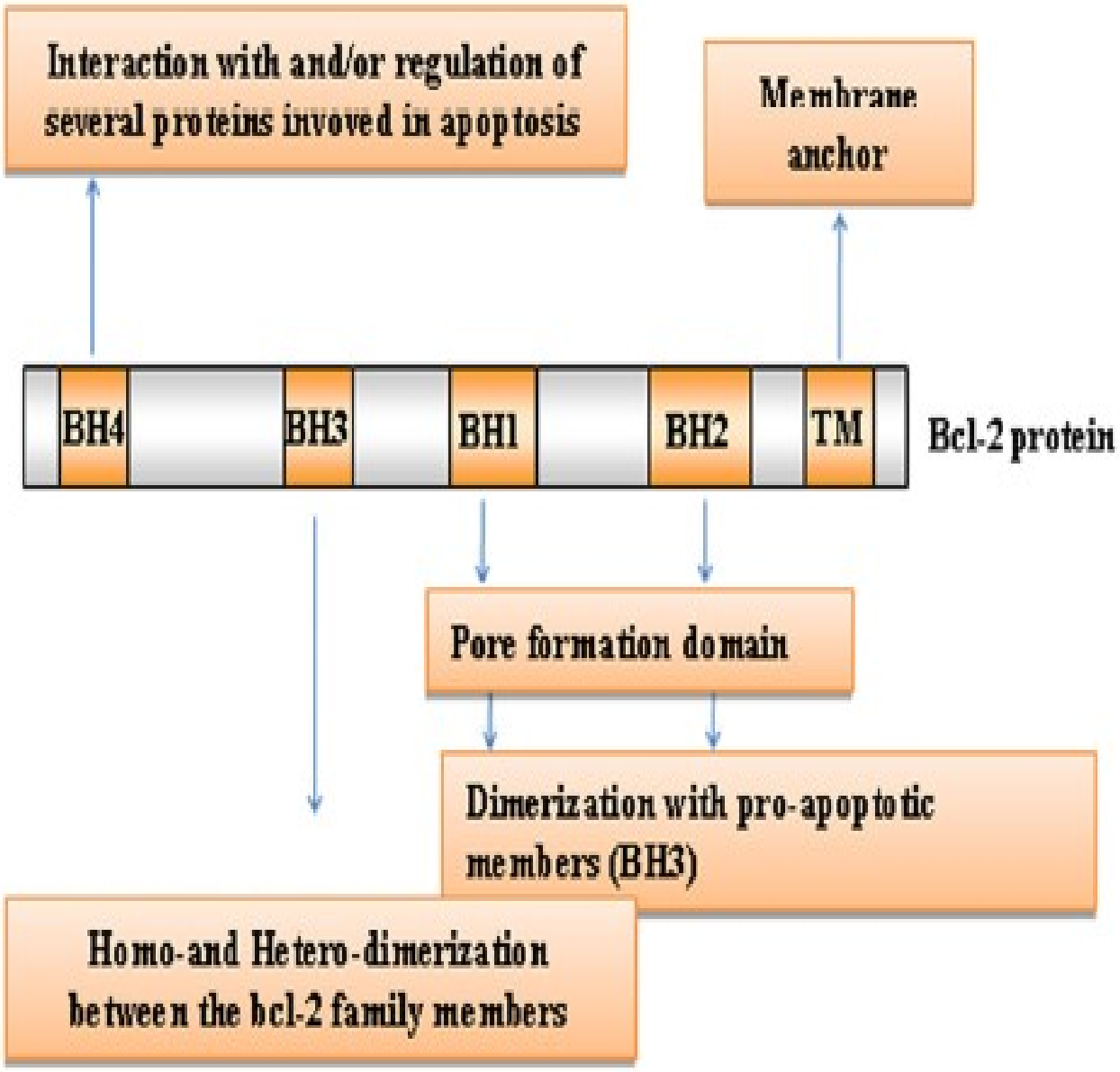

Figure 3: Bcl-2 family members domain composition and function (Giuseppa *et al*., 2016)

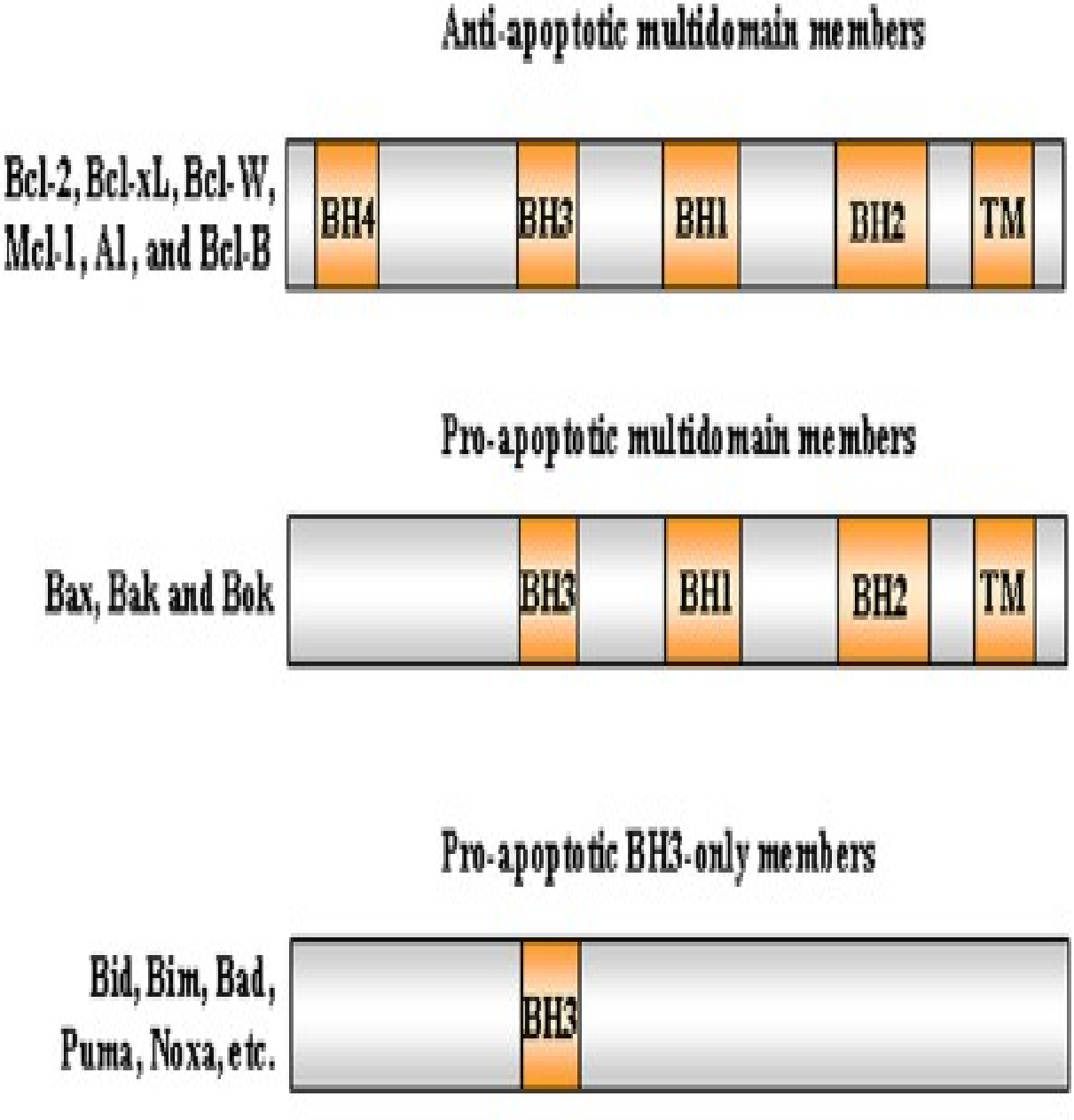

Figure 4: Bcl-2 protein subgroups (Giuseppa *et al*., 2016)

XIAP is the best characterized IAP and is generally recognized to be the most potent endogenous caspase inhibitor. Its action involves inhibition of active executor caspases as well as prevention of initiator caspase-9 activation (Mace *et al*., 2010).

2.6 ALTERATIONS OF THE APOPTOTIC PATHWAYS

There are many ways through which both the extrinsic and the intrinsic apoptotic pathways may be altered, resulting in dysregulated apoptosis. These include impaired death receptor signaling, disrupted balance between proapoptotic and anti-apoptotic proteins, reduced caspase function and impaired p53 function. Alteration of extrinsic apoptotic signaling has been associated with different types of human tumors, as a result of loss of activity of Fas-FasL system **(**Müschen and Beckmann, 2000) or the aberrant expression of cytosolic components of the death receptor apoptotic pathway can predispose to the tumor transformation (Tourneur *et al*., 2007). Several genetic defects have been shown to contribute to the resistance of tumor cells to Fas-mediated apoptosis. Fas transcriptional silencing is a common oncogenic event in the epithelial transformation, while its mutation has been often associated with B-cell germinal center-derived lymphomas (Müschen *et al*., 2002). In acute myelogenous leukemia (AML) reduced or absent expression of FADD has been frequently observed. This results in resistance to chemotherapy and poor patient prognosis (Tourneur *et al*., 2007; Tourneur *et al*., 2005). Moreover, in several cancers including neuroblastoma, medulloblastoma, and small cell lung cancer (SCLC), absent or reduced expression of caspase-8 was reported (Teitz *et al*., 2000; Shivapurkar *et al*., 2002; Zuzak *et al*., 2002). Another mechanism by which a variety of human tumors resist cell death is the overexpression of antiapoptotic protein c-Flip, which is recruited at the DISC level, that prevents the pro-caspase-8 auto-activation, thereby rendering cell resistant to death receptor-mediated apoptosis (Irmler *et al*., 1997; Bagnoli *et al*., 2010; Shirley and Micheau, 2013). Also,

alteration of some cellular components of the intrinsic apoptotic pathway can be a main cause of resistance to chemotherapy in different types of tumors. Distruption in the balance of anti-apoptotic and pro-apoptotic members of the Bcl-2 family protein results in dysregulated apoptosis in the affected cells. This can be attributed to overexpression of one or more of the anti-apoptotic proteins or downregulation of one or more of the pro-apoptotic proteins or both. In several human cancers, including prostate cancer, diffuse large B-cell lymphoma (DLBCL), melanoma, anti-apoptotic Bcl-2 over-expression has been reported. (Gandour *et al.,* 2004; Abramson and Shipp, 2005; Watanabe *et al*., 2013) This results in the evation of cancer cells from apoptosis or inhibition of TRAIL-induced apoptosis (Raffo *et al.,*1995; Fulda *et al*., 2009). In colorectal cancer and Kaposi's sarcoma, overexpression of Bcl-xL has also been reported (Foreman, *et al*., 1996; Krajewska *et al*., 1996). This confers a multi-drug resistance phenotype in the tumor cells and prevents them from being eliminated by apoptosis (Minn *et al*., 1995). Thus, overexpression or high expression levels of antiapoptotic proteins Bcl-2 and Bcl-xL have been reported to correlate with cisplatin resistance and tumor recurrence in different cancers including non-small cell lung cancer (NSCLC), head and neck, ovarian, and breast (Han *et al*., 2003; Erovic *et al*., 2005; Williams *et al*., 2005; Michaud *et al*., 2009; Yang *et al*., 2013).

On the other hand, mutations in the pro-apoptotic Bax gene have been reported in colorectal cancers and this has contributed to resistance to anticancer treatments (Miquel *et al*., 2005). In chronic lymphocytic leukaemia (CLL), increased Bcl-2/Bax ratio has been reported patients (Pepper *et al.,* 1997). Other examples of dysregulation of the intrinsic pathway include reduced expression of the basic component of the apoptosome, Apaf-1, in melanomas (Soengas *et al*., 2001; Baldi *et al*., 2004) as result of promoter aberrant methylation. In addition to this, resistance of cells to apoptosis also occurs as a result of defective mediators that regulate the intrinsic

apoptotic pathway downstream from the apoptosome formation. In this regard, high level of IAPs expression has been found to inhibit the activity of caspases (Schimmer, 2009).

2.7 MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION

The permeability transition (PT) is an increased permeability of the mitochondrial inner membrane to solutes of molecular mass up to 1.5kDa which is caused by opening of the PT pore, leading to depolarization, loss of ionic homeostasis with Ca^{2+}release, and cessation of mitochondrial ATP synthesis. An osmotic imbalance between the matrix and the surrounding medium can lead to mitochondrial swelling, rupture of the outer membrane and release of proapoptotic proteins (Bernardi *et al*., 2015a). Permeability increase in isolated mitochondria was recognized quite early (Raaflaub, *et al*., 1953) and its key functional features were well identified in the 1970s (Bernardi *et al*., 2015b). The permeability change was defined "permeability transition" by Hunter and Haworth, who carried out a systematic characterization of inducers and inhibitors. Further more, they showed that uptake of Ca^{2+} through the mitochondrial calcium uniporter (MCU) is required (De Stefani *et al.,* 2011; Mallilankaraman *et al.,* 2012) and that permeability transition lead to the uncoupling of oxidative phosphorylation.

The awareness in the mPT pore was raised when it was discovered that it could be inhibited by a fungal peptide, cyclosporin A(CsA), when it intereacts with a mitochondrial chaperone, the peptidyl-prolyl cis-trans isomerase cyclophilin D (CyP-D) (Crompton *et al.,* 1988; Halestrap and Davidson, 1990). Hunter and Haworth were the first to clearly propose that mitochondrial permeability transition resulted from the opening of a regulated channel (Hunter and Haworth, 1979) whose molecular nature and component is yet to be clearly defined.

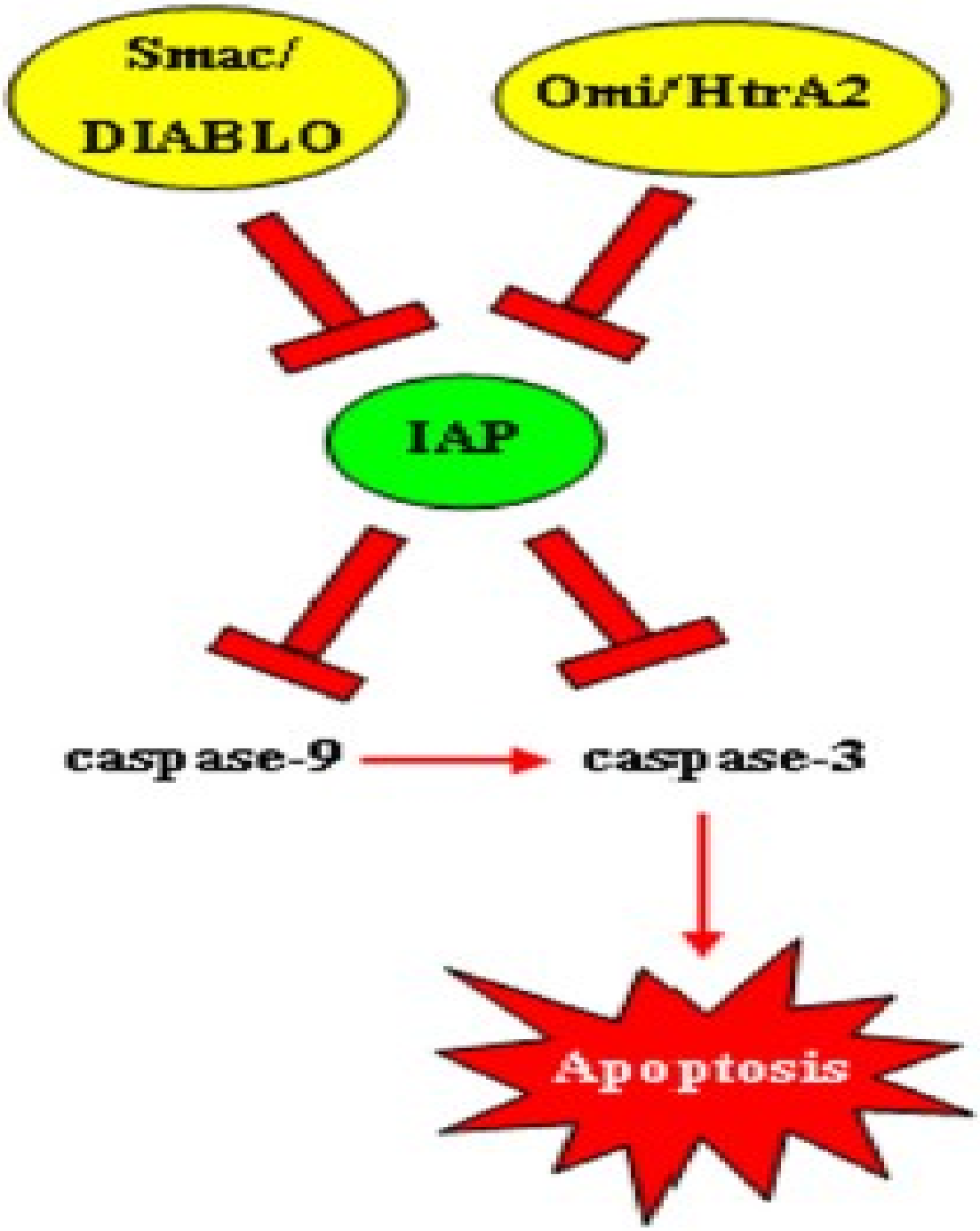

Figure 5: Function of inhibitors of apoptosis (Hegde *et al*., 2002; Tourneur *et al*., 2005)

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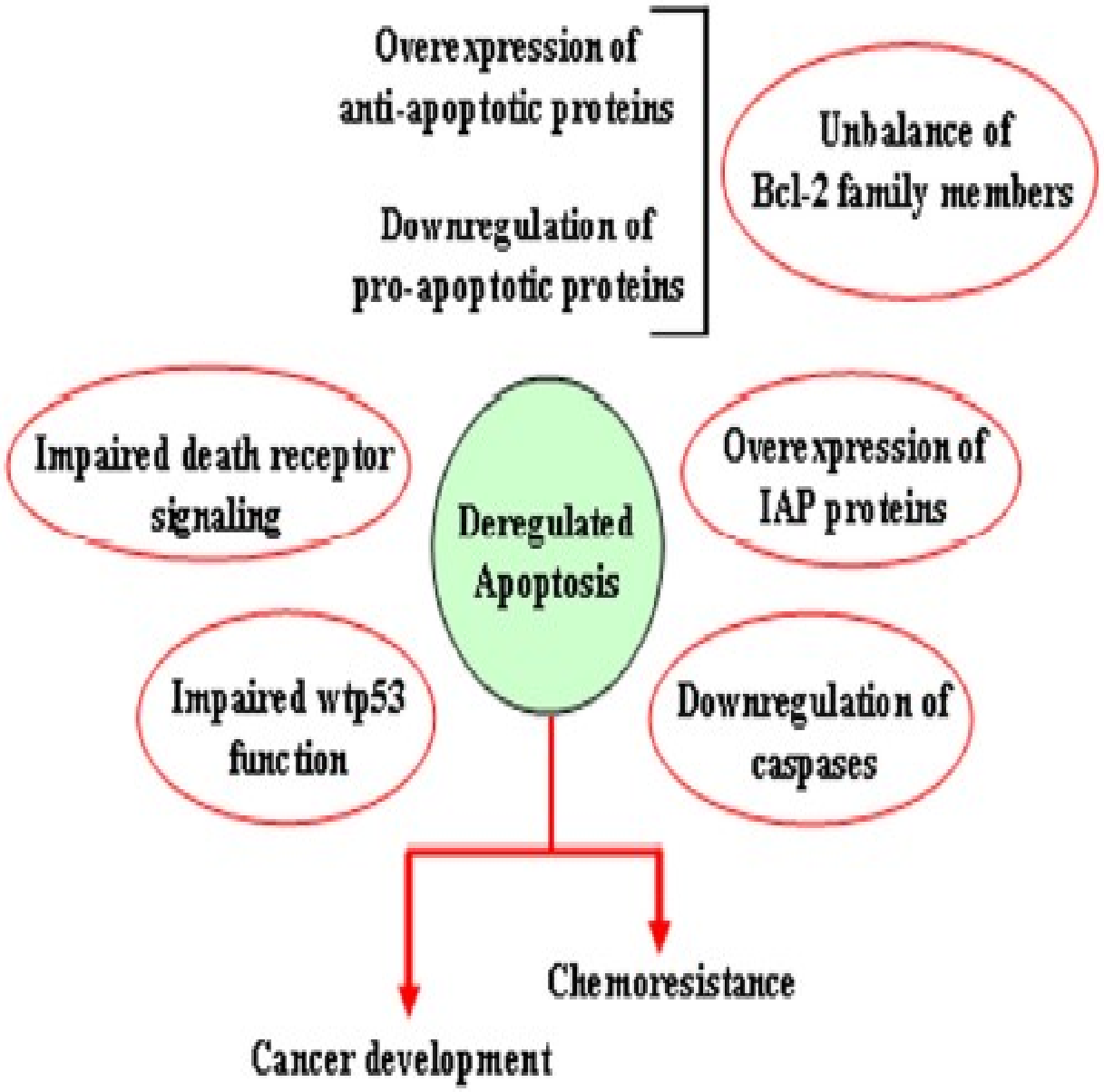

Figure 6: Mechanisms leading to deregulation of apoptosis (Mace *et al*., 2010)

2.8 BACKGROUND ON mPT PORE STRUCTURE

The mPT Pore is the putative pore responsible for the mPT, in which the mitochondrial inner membrane, that is highly impermeable, becomes extremely permeable. The initial model of the mPT pore component proposed that the voltage-dependent anion channel (VDAC), located on the outer mitochondrial membrane and the adenosine nucleotide transporter (ANT), located on the inner mitochondrial membrane were core components of the pore. These proteins are surrounded by a series of regulators including kinases such as hexokinase II (HKII), creatine kinase(CK), and glycogen synthase kinase3 b (GSK3b), the translocator protein(TSPO), Cyp D, and members of the Bcl-2 family (Baines *et al*., 2005; Zhang *et al*., 2010a).

2.9 MODELS FOR PORE FORMATION

There has been a long-standing hypothesis which proposed that the PTP is formed at contact sites between the outer and inner mitochondrial membranes, composed of the adenine nucleotide translocator and/or the phosphate (Pi) carrier in the inner membrane and VDAC in the outer membrane, in association with a variety of regulatory proteins including the peripheral benzodi-azepine receptor and cyclophilin (CyP) D (Zamzami and Kroemer, 2001). This hypothesis has been conclusively disproved by genetic elimination of each candidate protein (Kokoszka *et al.,* 2004; Krauskopf, *et al*., 2006; Baines *et al*., 2007; Gutierrez-Aguilar *et al*., 2014) with the exception of CyPD (Baines *et al*., 2005; Basso, *et al*., 2005; Nakagawa *et al*., 2005). Cyclophilin D (CyPD), which is the target of cyclosporin A (CsA), an inhibitor, is a matrix peptidyl pro-lyl cis-trans isomerase that favors PTP opening but is not a structural pore component. Baines and coworkers later demonstrated that the phosphate carrier (PiC) was also not a core component, though its absence caused the mPT to occur less readily (Gutierrez-Aguilar *et al*., 2014; Kwong

et al., 2014). Conditional elimination of the TSPO did not have any effect on mPT, and thus, disproving earlier pharmacological evidence of its involvement in mPT pore component (Sileikyte *et al*., 2014).

2.10 Cyclophilin D

The only component that survived genetic scrutiny was CypD (Baines *et al*., 2005; Basso, *et al*., 2005; De Marchi *et al*., 2006; Crompton *et al*., 1998; and this has been confirmed in its role as a modulator of the pore's propensity to open in mammals. All attempts to identify the molecular nature of the core, (channel-forming component of the mPTP) have relied on the identification of CypD-interacting proteins. Binding of CypD to the ANT was reported by Crompton's and Halestrap's groups in 1998 (Crompton *et al.,* 1998; Woodfield *et al.,* 1998). Also, its interaction with PiC was demonstrated, confirmed and reported (Leung *et al*., 2008; Gutierrez-Aguilar *et al*., 2014). The hypothesis that the ATPase may form the mPTP arose from the observation that CypD interacted with the lateral stalk (the Oligomycin Sensitivity Conferring Protein, OSCP) of the complex V (Giorgio *et al*., 2009). Spastic Paraplegia Protein 7 (SPG7) also emerged as the top candidate from a proteomics study because it could be co-immunoprecipitated with CypD (Shanmughapriya *et al*., 2015). The cyclophilin D, (Elrod, and Molkentin, 2013) however is a peptidyl prolyl cis–trans isomerase (PPIase), a chaperone, which may be expected to have interaction with several proteins, which may possibly be related or presumably unrelated to mPTP formation or regulation (Alam *et al*., 2015; Fayaz *et al*., 2015).

2.11 ATP SYNTHASE

Mitochondrial complex V, (ATP synthase) is currently at the fore front in the marathon race towards the identification of the mPTP (Junge *et al*., 2015). There were reports that a

phosphorylated peptide related to the c subunit of the ATPase F_O sector was dephosphorylated under conditions leading to the mPT, and the dephosphorylation was inhibited by CsA (Azarashvily *et al*., 2000; Azarashvili *et al*., 2002; Krestinina *et al*. 2009). The notion that ATPase could be one of the mPT pore components was as a result of the observation that CypD interacts with the OSCP subunit of the peripheral stalk (Symersky *et al*., 2012; Lee *et al*., 2015). Furthermore, Bernardi's group observed that the calcium retention capacity (CRC) of mammalian and yeast mitochondria depended on whether the ATP synthase complexes were synthesizing or hydrolysing ATP, with a higher CRC, i.e. a lower propensity to mPTP induction, in the hydrolytic mode (Hong *et al*., 2008; Ahmad *et al*., 2013).

2.11.1. PTP FORMATION FROM F-ATP SYNTHASE – THE C RING HYPOTHESIS

There is a link between subunits c and PTP formation/regulation and this was suggested by PT induction by a phosphorylated peptide derived from subunit c as well as by subunit c itself (Azarashvili *et al.*, 2002: Azarashvili *et al*., 2014). Recently, the involvement of c ring in the context of PTP formation was provided by two independent studies (Bonora *et al.,* 2013; Alavian, *et al*., 2014). In the protocols of Bonora *et al*.(2013), HeLa cells were treated with siRNA against the c subunit or subjected to over expression of a Myc-tagged c subunit, and the propensity of the PTP to open tested in several paradigms. It was discovered that there was decreased PTP opening and cell death after suppression of the c subunit and increased PTP opening with corresponding increase in cell death after over expression of the c subunit (Bonora *et al.,* 2013). In consonant with the findings of Halestrap and Richardson (2015), their experiments are consistent with a role of the F-ATP synthase in PTP regulation but cannot reveal whether the c ring is directly involved or whether the results are rather a consequence of altered

ATP production or of a misfolded protein response to the over expressed construct. Furthermore, downregulation of the c subunit could have affected the biogenesis of the whole F-ATPsynthase.

2.11.2 PTP FORMATION FROM F-ATP SYNTHASE – THE PERIPHERAL STALK/DIMER HYPOTHESIS

There are inhibitors of the PTP which do not compromise the catalytic activity of F-ATP synthase and those that interfere with its activity (Bernardi *et al*., 2015a). This simply suggests that the catalytic activity and PTP channel formation occur at different regions of the enzyme complex. Studies have shown that gel-excised dimers (but not monomers) of F-ATP synthase rapidly promote PTP-like channels formation in lipid bilayers (Giorgio *et al*., 2013; Carraro *et al*., 2014; Von Stockum *et al*., 2015). This lead to the proposal that channel formation does not involve the c ring (which participates in catalysis) but rather occurs at the interface of two adjacent monomers in the dimmers (Giorgio *et al*., 2013). This is also in consonant with a study in yeast, where ablation of subunits e and g (which are located at the monomer–monomer interface and stabilize the dimers) results in desensitization of the pore to Ca^{2+} (Carraro *et al*., 2014).

The hypothesis which is presently embraced and supported by novel evidence (Giorgio *et al*., 2017), is that opening of the mPT pore is the indirect consequence of Ca^{2+} binding to the metal binding site of the β subunit within F_1 (Giorgio *et al*., 2017), which ultimately leads to a conformational change that transcend to the inner membrane through the peripheral stalk via OSCP. This has recently lead to a proposed mPT pore component to be composed of the either c-subunit rings of the ATP synthase complex (Alavian *et al*., 2014 ; Bonora *et al*., 2013) or the interface between ATP synthase dimers (Giorgio *et al*., 2013 ; Carraro *et al*., 2014).

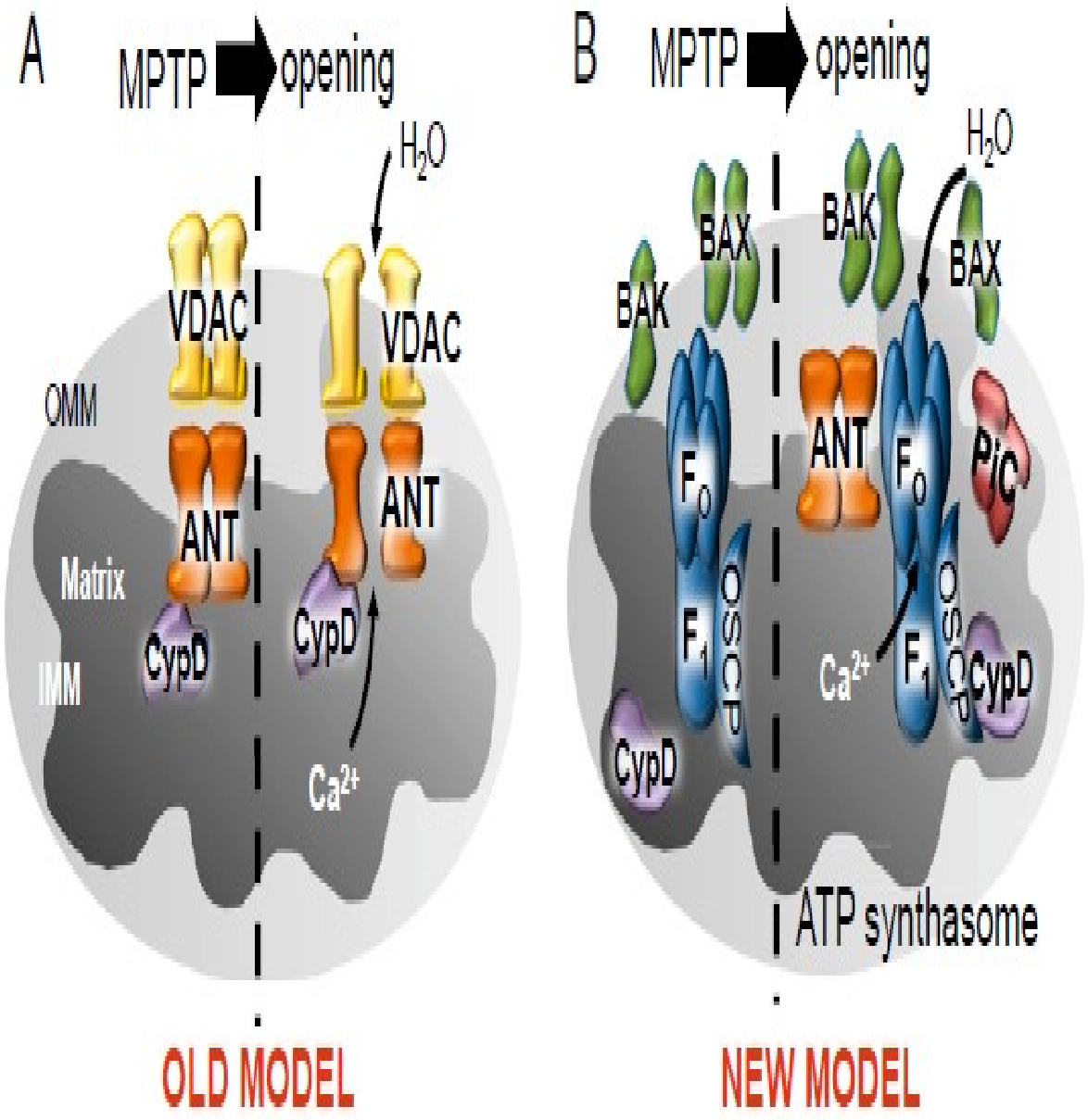

Figure 7: Evolving models of Mitochondrial Permeability Transition pore

(Karch and Molkentin, 2014)

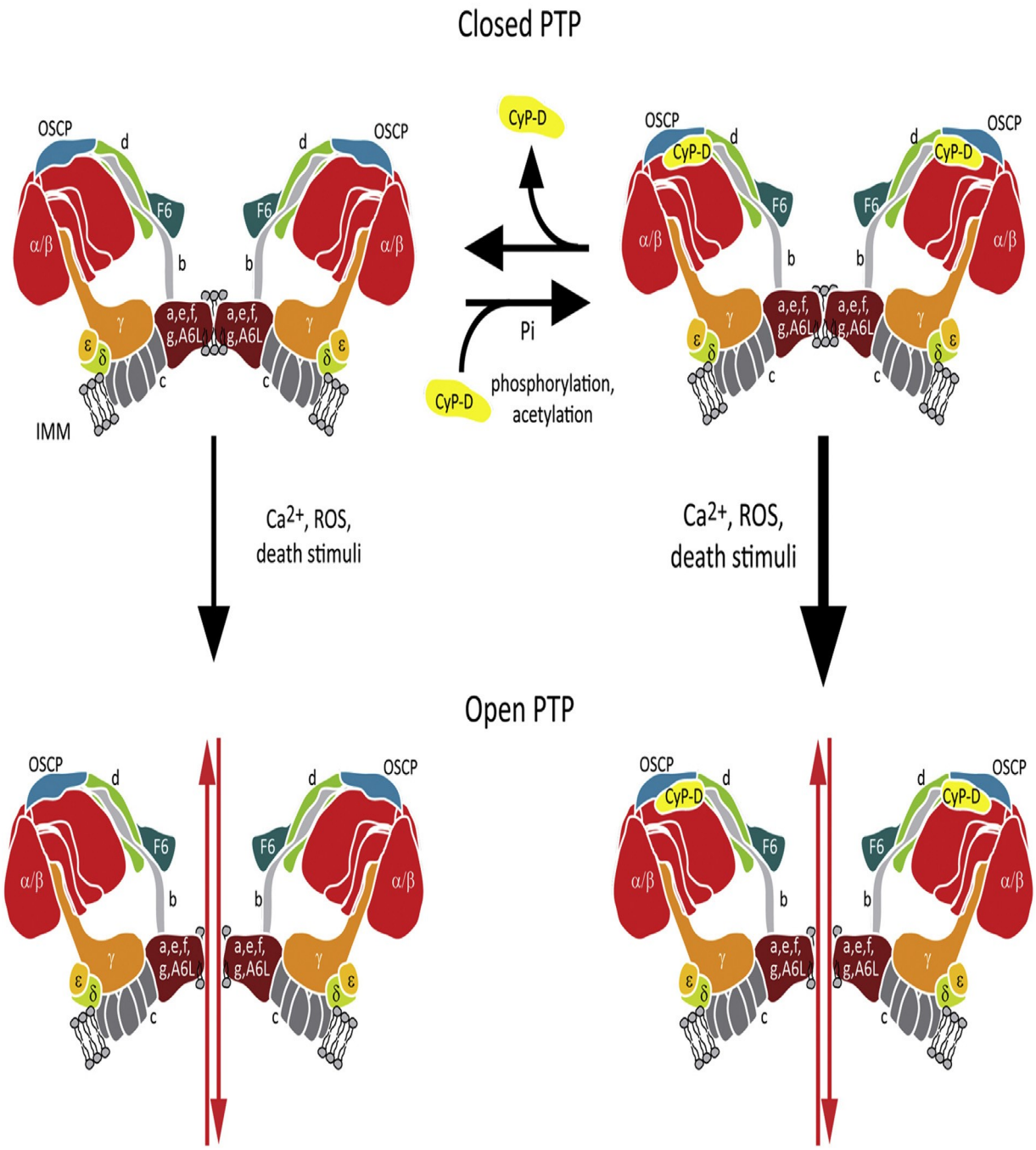

Figure: 8 Evolving model of Permeability Transition Pore structure formed at the interface between two ATP synthase dimers (Andrea and Paolo, 2014)

2.11.3 ATOMISTIC SIMULATIONS ARGUE AGAINST THE C-SUBUNIT RING OF THE ATP SYNTHASE FORMING THE MPT PORE

Studies from the group of Jonas (Alavian *et al*., 2014) showed that the mPT pore forms within the c-subunit ring of the ATP synthase complex. Bonora *et al*., (2013) had earlier proposed that the c-subunits ring form a structural component of the pore. Both studies implied that the lumen of the c-subunits ring form the mPT pore. This claim has been scrutinized by the group of Faraldo-Gomez (Zhou *et al*., 2017), by calculating ion conductance and selectivity in two c-subunit rings of different lumen widths, based on all-atom molecular dynamics simulations. The number of the c-subunits per ring (determining the size of the lumen) varies depending on the species (Watt *et al*., 2010), however, mPT is a phenomenon encountered in almost all species (Azzolin *et al*., 2010), except in Artemia franciscana (Menze *et al*., 2005; Konrad *et al*., 2011; Konrad *et al*., 2012) and perhaps all crustaceans (Chen *et al*., 1974; Tsokos *et al*., 1983). Thus, the choice of the c-subunit ring for atomistic simulations regarding mPT pore should bear no relevance because the lumen of the c-subunit rings is normally not an aqueous pore (Oberfeld *et al*., 2006).

Zhou and colleagues also quantified the probability that it may become hydrated, and thus potentially conducting. Zhou and colleagues calculated the free-energy gain (or cost) associated with hydrating an otherwise, highly hydrophobic, lipidplugged c-ring lumen, and reported that a dehydrated, nonconducting lumen is more probable to exist than a conducting lumen, by multiple orders of magnitude (Zhou *et al*., 2017). They further estimated that if the c-ring lumen were to be filled with water, it would exhibit a resting conductance of 2.5 pS for Kþ and 116 pS for Cl. Such values are not only inconsistent with the purported mPT conductance of 1e1.3 nS but also

the anticipated lack of ion selectivity (Kinnally *et al*., 1989; Petronilli *et al*.,1989). Finally, because Alavian and colleagues reported that the c-subunit rings become the mPT pore upon dissociation of the F1 sector of the ATP synthase complex (Alavian *et al*., 2014), Zhou and colleagues performed atomistic simulation on a particularly large c-subunit ring (largest known to date), that from the bacterium Bacillus pseudofirmus, exhibiting a c13-ring. Again, calculations yielded anion selectivity, albeit a slightly greater conductance, but still no preference for hydration. From their results, it was found that if the the c-subunit rings are correctly folded and assembled, cannot form the mPT pore, even if it detached from the F1 sector of the ATP synthase complex (Zhou *et al*., 2017). However, no matter the results of the simulations, they are still only simulations and the authors admitted that their study does not rule out the hypothesis that the mPT pore molecular structure consists of c-subunits. The definite answer regarding the possibility of the c-subunit rings forming the mPT pore came from Walker laboratory.

2.11.4 MITOCHONDRIA HARBORING VESTIGIAL ATP SYNTHASE COMPLEXES DEVOID OF C-SUBUNIT RINGS EXHIBIT THE MPT

The group of Walker deleted the expression of c subunits in HAP1-A12 cells (He *et al*., 2017), by disrupting ATP5G1, ATP5G2, and ATP5G3 genes, all coding for identical copies of the c subunit protein (Dyer and Walker, 1993; Yan *et al*., 1994). The mitochondria of these cells were found to exhibit a vestigial ATP synthase entailing intact F_1-catalytic and peripheral stalk domains and supernumerary subunits e, f, and g, but devoid of a c-subunit ring as well. It was found that there was induction of mPT in both control cells and in those lacking c-subunit proteins. This was tested by three different, but classical mPT pore-opening protocols. The pore opening was also Cyclosporin A sensitive. There was no statistically-significant difference in the

concentration of Ca^{2+} required for mPT opening in either cell type. These results suggest that the c-subunits ring is not an essential, let alone structural component of the mPT pore. However, it must be noted that 'pore' per se, in the sense of electrophysiological detection of a conductance exhibiting voltage-sensing properties, was not addressed. Thus, it remains possible that the increase in cytosolic calcium detected in the Walker study during mPT protocols could still be due to a low-conductance pore, known to mediate Ca2þ-induced Ca2þ release events (Ichas *et al*., 1997). Nonetheless, due to the reasons highlighted in (He *et al.,* 2017), it is also possible that subunits DAPIT and 6.8PL of the ATP synthase complex might not be part of the mPT event. Finally, since these vestigial ATP synthase complexes were mostly in monomeric form, the fact that there was no statistically measurable difference in the mPT pore opening between cell types creates doubt on the mPT pore model that is formed by ATP synthase dimers (Giorgio *et al*., 2013). This model was also recently disproved by the recent results from the Pinton group (Bonora *et al*., 2017)

2.11.5 DISSOCIATION OF ATP SYNTHASE DIMERS PROMOTES MPT OPENING

Criticism on the concept of ATP synthase dimers as an mPT pore model also appears in the literature (Szabadkai and Chinopoulos, 2013). The proposal of mPT pore appearing in-between ATP synthase dimers is scrutinized from the point of view that this locus is normally occupied by membrane lipids; formation of a pore dictates that at the dimerization interface of a membrane protein dimer, two hydrophobic surfaces should be able to provide a hydrophilic lining while the pore is assembled in order to allow ion flow. Upon pore closure (note that mPT is reversible) these surfaces should regain their hydrophobicity in order to allow membrane lipids interaction (Malkevitch *et al*., 1997; Petronilli *et al*., 1994). To date, there is no such evidence

that this can occur within the ATP synthase dimer. On the contrary, a very recent report by the group of Pinton, showed that stabilization of ATP synthase dimers disfavors mPT opening; by the same token, destabilization of the dimmers triggers mPT, but in a most unexpected twist, this effect required correctly folded c-subunit rings (Bonora *et al*., 2017). The authors argued that because different genetic interventions favoring ATP synthase dimers stabilization limited mPT opening, this implies that "the dissociation of F1FO ATP synthase dimers is a cause, not a consequence, of mPT"; however, the semantics of this claim are not clear to this author, and dimers dissociation may be a consequence, not a cause of mitochondrial reversible swelling (Petronilli *et al*., 1994). Clarification whether dimers dissociation is a cause or a consequence of mPT, is eagerly awaited.

2.12 MITOCHONDRIAL PERMEABILITY TRANSITION PORE STRUCTURE: LOOK ELSEWHERE

Based on all these findings, it can be deduced that the mPT pore is not structured by the c-subunit rings, and perhaps not formed in-between ATP synthase dimers. There is a possibility of mPT being formed by any of the intra membrane ATP synthase subunits b, e, f, and/or g, assisted by the peripheral stalk subunits in a manner integrated by OSCP (Giorgio *et al*., 2017b).

Based on the reports of Zhou *et al*. (2017), He *et al*. (2017) and Bonora *et al*. (2017), the case of c-subunits rings and most likely the membrane space in-between ATP synthase dimers as structural elements of the mPT pore, is closed. The mystery of the pore is still yet to be unraveled. Now the question, where to focus from here? As pointed out in (He *et al*. 2017), other subunits like e, f, and g of the ATP synthase complex could still be examined and scrutinized as structural elements of the pore, as each is predicted to exhibit a single transmembrane a-helix,

thus being capable of participating in a pore structure. The technique used in the experimental protocol for the c-subunit gene distruption strategy could also be employed (He *et al*., 2017).

It is evident that both cyclophilin D and the ANT are modulatory, but not structural components of the pore as gathered from thousands of publications on mPT (Bernardi *et al*., 2015b ; Doczi *et al*., 2016). Thus, common binding partners for these two proteins should be sought.

However, novel technological advances regarding protein-protein interactions, proteomic detection and identification methods, as well as bioinformatics tools, could help to reduce or eliminate false-positive binding proteins and assist in identifying the real partners forming the mPT pore (Kotlyar *et al*., 2015; You *et al*., 2015) . To this end, the fact that the total mitochondrial proteome is known and continuously refined (Calvo *et al*., 2016) should also assist in the search of protein(s) as mPT pore structural elements. Although, the molecular composition of the pore remains uncertain till now, the emergence of Spastic Paraplegia 7 (SPG7), a mitochondrial AAA-type membrane protease which is involved in processing other mitochondrial proteins is fast gaining ground. This is because a gene knock-down (KD) or knock-out (KO) of SPG7 renders mitochondria more resistant to Ca^{2+}-induced mPT, as assessed mainly by the CRC assay using permeabilized cells (Keskin *et al*., 2016).

2.13 PERMEABILITY TRANSITION PORE AND TUMORS

Prolonged mPT pore opening causes the release of proapoptotic proteins especially, cytochrome c into the cytosol and this is a point of no return toward the demise of the cell, committing it either to apoptosis, if there is enough ATP present to activate caspases, or to necrosis, elicited by massive Ca^{2+} release in the cytosol.

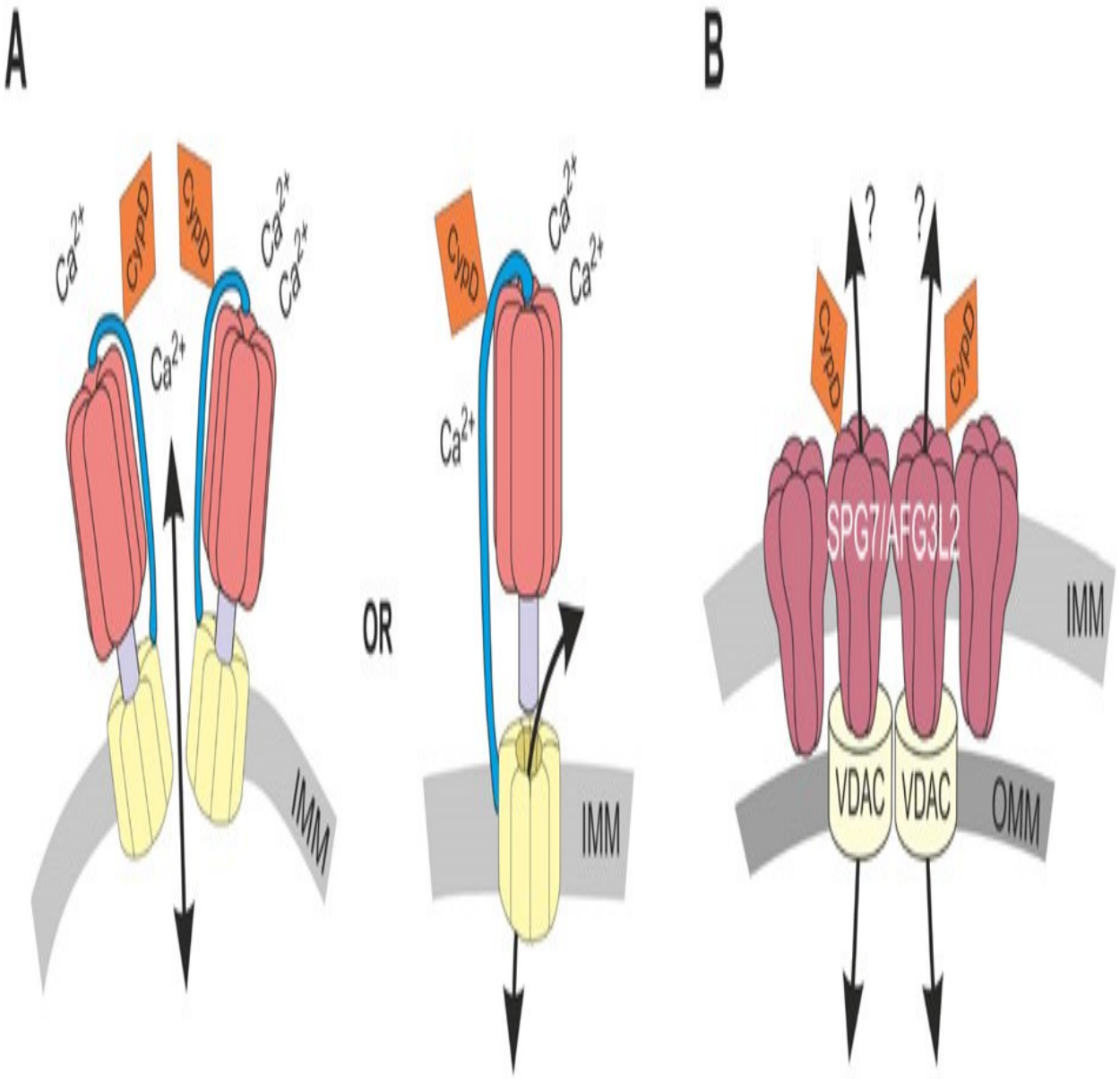

Figure 9: Other Proposed models for Mitochondrial Permeability Transition Pore composition (Lucia *et al*., 2016)

The capability to evade cell death induction after exposure to stress conditions is a platform for cell progression to malignancy (Malik *et al*., 2009; Catherino *et al.,* 2011) and most chemotherapeutic approach focus on the possibility to selectively reinstate apoptotic programs in neoplastic cells. This therefore, calls for a detailed understanding of PTP structure, funtion and of its regulation in cancer as a pharmacological strategy towards developing anti-tumor drug aimed at induction of mPT pore.

2.14 CARCINOGENESIS VIA INTRINSIC SIGNALING DEFECTS

Mitochondria-mediated apoptosis (MMA) is one of the major pathways for execution of apoptosis. Impairment of the apoptotic pathway could lead to inhibition of apoptosis. The central regulators of the intrinsic pathway are the Bcl-2 family proteins. They can cause modulation or changes in mitochondrial membrane permeability required for the release of apoptogenic proteins like cytochrome c, etc (Green and Kroemer, 2004; Motyka *et al*., 2000). Cell survival is promoted by antiapoptotic proteins (Bcl-2, Bcl-xL, Bcl-W, Mcl-1, and Bfl-1/A1) which display sequence homology in all BH1-BH4 domains whereas proapoptotic proteins mediates apoptosis via, extrinsic or intrinsic pathway.

The proapoptotic protein is subdivided into multidomain or BH3-only proteins. The multidomain consists of Bax and Bak, which are required for apoptosis (Reed, 2006). The BH3-only proteins are further divided into two subclasses: The first subclass is the “activators” which include Bim and tBid. This subclass directly activates Bax/Bak to cause permeabilization of mitochondrial outer membrane. The second subclass is refered to as “sensitizers/derepressors” (e.g., Bad, Bik, Bmf, Hrk, Noxa, and Puma). These do not activate Bax/Bak directly but rather neutralize the antiapoptotic proteins (Letai, 2005; Kim *et al*., 2006). The BH3-only proteins do not induce

apoptosis in Bax/Bak-deficient cells. This implies that Bax/Bak play a crucial role in apoptosis (Wei *et al*., 2001; Zong *et al.,* 2001). The antiapoptotic proteins prevent cell death by antagonizing the actions of Bax/Bak. It has been shown that antiapoptotic proteins prevent Bax/Bak activation by inhibiting "activator" BH3-only proteins and/or directly inhibiting Bax/Bak activation (Green, 2006). Also, the "Sensitizer" BH3-only proteins act by displacing the "activator" BH3-only proteins from antiapoptotic proteins, thereby stimulating Bax/Bak activation. Over 50% of all cancers are propably caused by overexpression of antiapoptotic Bcl-2 or Bcl-xL (Kim *et al*., 2008) rendering the tumor cells resistant to apoptotic signal and enabling them to evade cell death (Malik *et al*., 2009).

2.15 TARGETING CANCER CELLS BY MITOCHONDRIAL-MEDIATED APOPTOSIS

Mitochondria play an important role in the intrinsic mechanism of apoptosis in the regulation of cell proliferation and apoptosis (Jiang *et al*., 2013; Rasul *et al*., 2013). The intrinsic or mitochondrial-dependent pathway is mediated by down-regulating the activities of antiapoptotic Bcl-2 protein, translocating Bax/Bak into mitochondrial membrane and permeabilisation of membrane to release proapoptogenic proteins. This leads to cascade of reactions which eventually cause the cell death. The modulation of Bcl-2 family proteins results in dissipation of mitochondrial membrane potential (MMP) and subsequent release of many pro-apoptotic proteins such as cytochrome c, apoptosis inducing factor (AIF), and second mitochondrial activator of caspases (Smac/DIABLO) from the mitochondrial inter-membrane space into the cytosol. Once the cytochrome c is released into the cytosol, it interacts with apoptotic protease activating factor-1 (Apaf-1), leading to the formation of apoptosome which eventually causes the activation of procaspase 9 into active caspase-9. Active caspase-9 then activates caspase-3,

which in turn leads to the degradation of vital cellular proteins and thus apoptosis. Smac/DIABLO promotes activation of caspases by neutralizing the inhibitory effects of inhibitor of apoptosis proteins (xiap and surviving) while AIF and Endo G induce caspase-independent apoptosis by directly causing induction of DNA damage and condensation (Ghavami *et al*., 2009 ; LaCasse *et al*., 2008).

2.16 PHYTOCHEMICALS IN CANCER PREVENTION AND THERAPY

Phytochemicals are secondary metabolites that are naturally found in plants with several roles to play. These types of products could be phenolic compounds, carotenoids, products with nitrogen, alkaloids and organosulfur compounds,etc (Manach *et al*., 2004; De la Rosa *et al*., 2009; Tsao *et al*., 2010). Every class of phytochemicals has its biological active effect (Russo *et al*., 2010). Natural compounds have been associated with antioxidant properties and prevention of free radicals generation. Recently, studies have shown that natural compounds have a more complex protective action at cellular and molecular levels, with important application in disease prevention or treatment (Braicu *et al*., 2011; Petric *et al*., 2015).

The benefits of natural products are also underlined by the so-called “Asian paradox”.The reason for the reduced rates of lung cancer within the Asian population has been attributed to high amount of green tea intake. This is because the catechins within the composition of green tea seems to reduce the risk for pulmonary diseases among others. Also in the case of “French paradox”, high intake of products containing resveratrol has been shown to be the reason underlining the reduced risk of cardiovascular diseases in the population (Braicu *et al*., 2011). These population-based studies are showing the efficacy of polyphenols as a potent pharmacological target for new drug discovery, based on their capability to modulate numerous

pathological processes including malignant transformation and development (Petric *et al*., 2015; Braicu *et al*., 2011). The re-emergence of natural products in the management and treatment of diseases is as a result of increased number of publications which focused on a better comprehension of their biological and beneficial properties in human health. Phytochemicals are now proved to play active role in chemoprevention or chemotherapy (Smith *et al*., 2011; Baker *et al*., 2017). Natural phytochemicals have been used in the prevention and treatment of cancer in folkloric medicine because of they are safe and readily available, from a wide range of natural sources. Recent research techniques have shown that these natural products like polyphenols have impact on human health, having the capacity to modulate gene expression, non-coding RNAs (ncRNAs) or epigenetic processes (Petric *et al*., 2015). This is also supported by the latest progress related to the "omics" approaches (Irimie *et al*., 2015).

2.17 PHYTOCHEMICAL COMPOUNDS AND CANCER

The use of natural compounds in chemotherapy is now gaining ground as a result higher number of clinical trials that have shown that these compounds are potent as they increase the treatment efficiency while decreasing the side effects, cause induction of apoptosis in cancer cells and reduce multidrug drug resistance and the severity of conditions. These compounds can act as pro or anti-oxidant, based on the dose and exposure time because they interfere with key cellular processes (cell cycle regulation, apoptosis, or even angiogenesis, invasion and metastatic processes) (Pandey *et al*., 2009; Wang *et al*.,2012).

2.18 TARGETING APOPTOSIS WITH DIETARY BIOACTIVE AGENTS

Several studies (*in vitro* and *in vivo*) with diet-derived agents have shown modulation of tumor growth via alteration of gene expression and induction of apoptosis (Manson *et al*., 2005).

Dietary phenolic compounds, including ellagic acid and resveratrol, have been shown to modulate several 550 genes after a 48-hr exposure of hormone-dependent human prostate cells, indicating that activation of multiple signaling pathways by these dietary compounds is involved in cellular proliferation and apoptosis (Narayanan *et al*., 2002). Studies have shown that resveratrol reduces cellular proliferation and induces apoptosis in numerous human cancer cells (Lee *et al*., 2004). Also, capsaicin, plants of the ginger family, curcumin, elicit their ability to supress proliferation and clonal expansion of cancer cells by upregulation of apoptosis (Surh, 1999). Some plant extracts and isolated compounds of dietary origin have also been shown to elicit their chemoprotective effect by inducing apoptosis. These include Allium sativum (garlic), silibinin, aloe, and caffeic acid phenyl ester, found in porpolis (Borek, 2001; Dhanalakshmi *et al*., 2002; Furukawa *et al*., 2002; Usia *et al*., 2002).

Polyphenols have also been shown to suppress tumorigenesis, partly through induction of apoptosis (Dorai and Aggarwal, 2004; Lambert *et al*., 2005). Several animal studies have demonstrated that dietary components are capable of inducing apoptosis as a mechanistic means of chemoprevention. For example, curcumin, quercetin, and rutin have been shown to induce apoptosis in azoxymethane-induced rat colon carcinogenesis (Sanaha *et al*., 1997; Yang *et al*., 2000).

Dietary polyphenols from tea has also been found to reduce prostate cancer in mouse model, induce apoptosis in skin tumors of mice exposed to ultraviolet radiation and protect against chemically induced hepatic tumors in mice (Klaunig and Kamendulis, 1999; Lu *et al*., 2000; Gupta *et al*., 2005). Studies have revealed that polyphenols such as resveratrol, EGCG and vanilloids (including capsaicin and curcumin) can execute apoptosis in many different cell types.

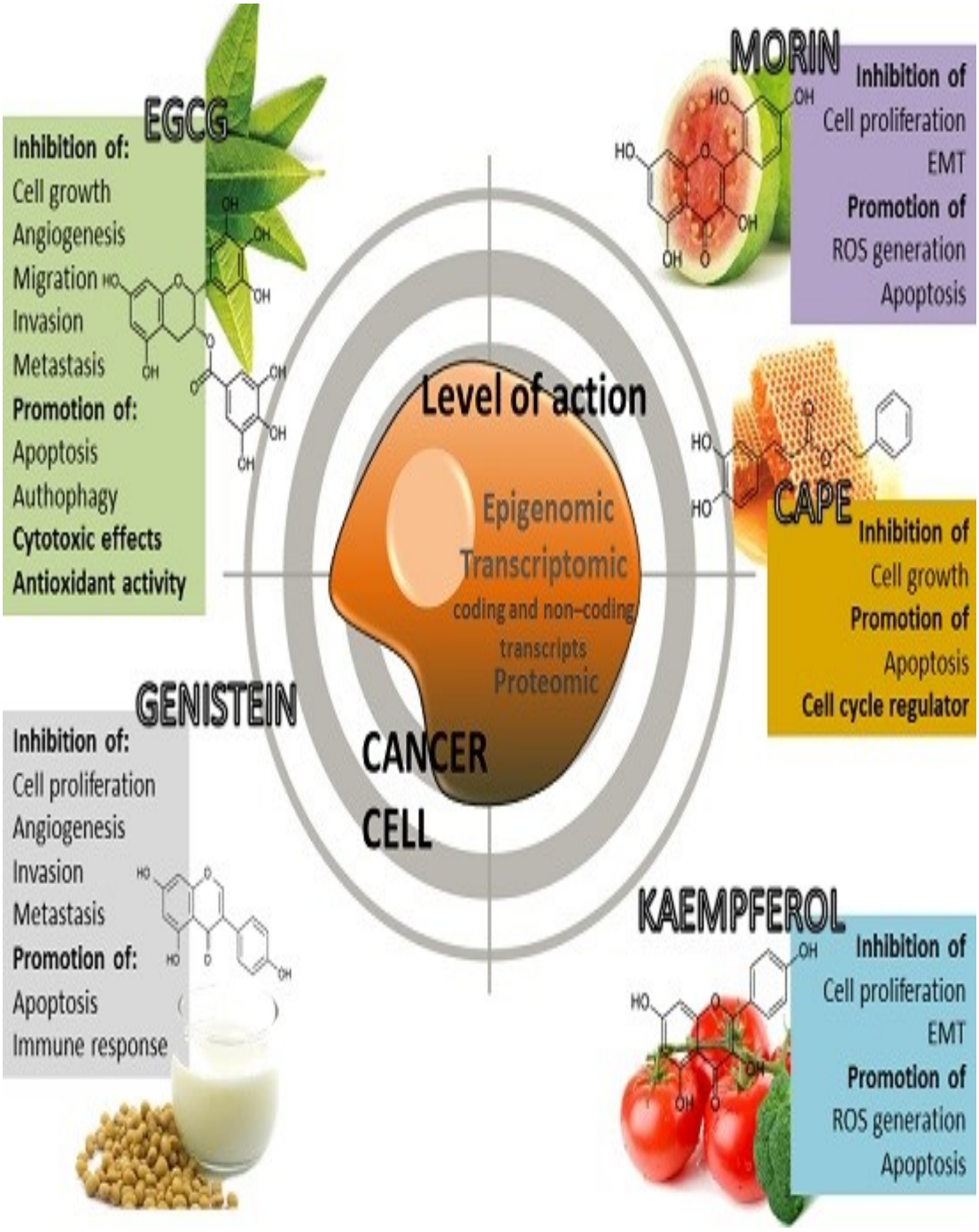

Figure 10: Dietary phytochemicals and their chemical structure and sources

(Md Soriful *et al.*, 2017)

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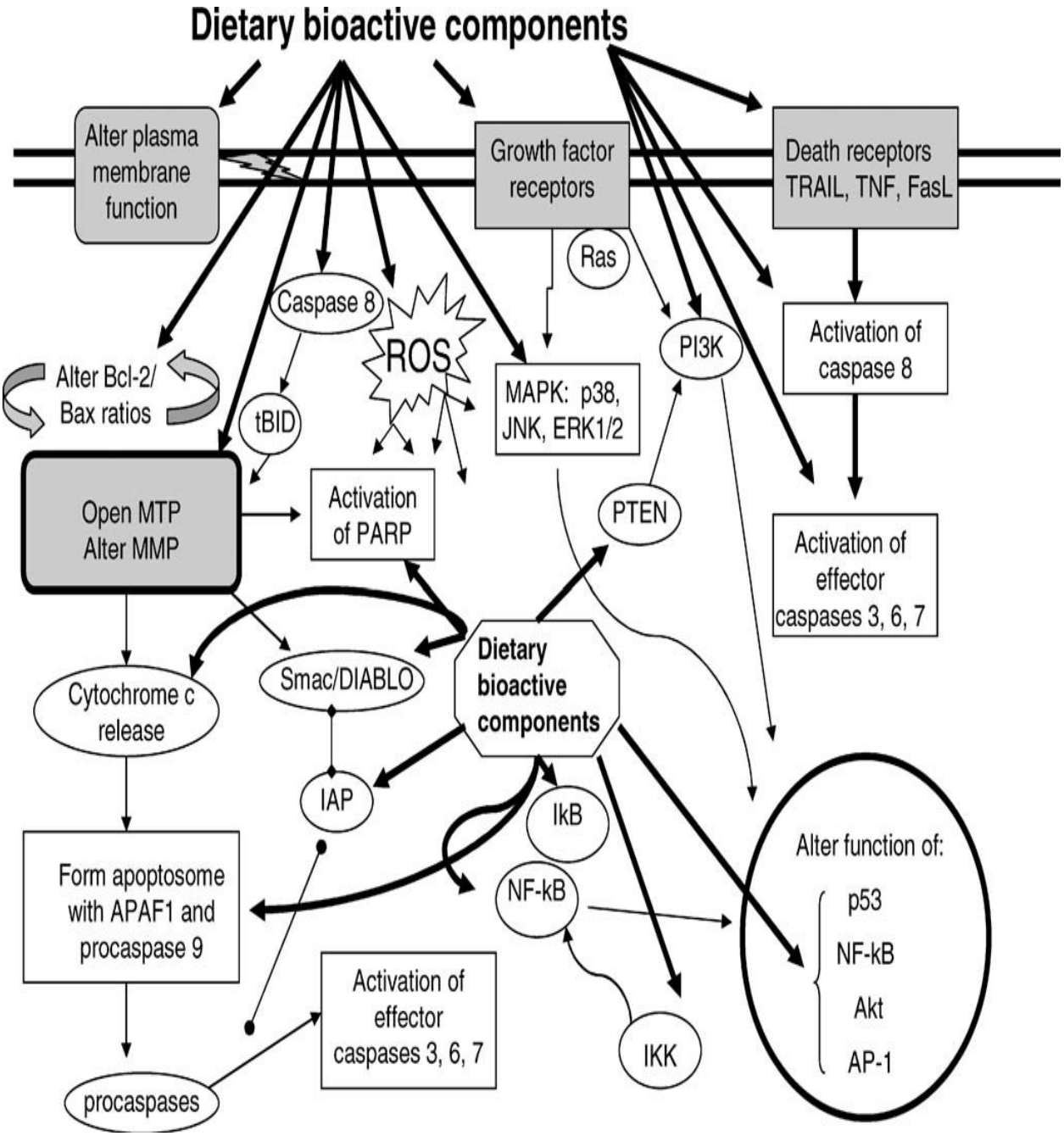


Figure 11: Dietary agents and specific mechanisms of action in apoptosis (Martin, 2006)

These include leukemia cells, colon cancer cells, epidermoid cells, prostate cells, transformed bronchial epithelial cells, and glioma cells (Sundaram *et al*., 2000; Gupta *et al*., 2000; Pan *et al*., 2000; Lee *et al*., 2004). Recently, extracts of tomatoes and associated phytochemical lycopene have been shown to induce apoptosis in prostate cells both in vitro and in vivo (Kim *et al*., 2003; Hwang and Hantz *et al*., 2005). Further more, induction of apoptosis by several dietary agents have been revealed in human trials coupled with improved clinical outcome (Vidya *et al.,* 2012).

2.19 UTERINE FIBROIDS

Uterine fibroids/leiomyomas are benign tumors that emanate from the myometrium of the uterus in premenopausal women (Islam *et al*., 2013a; Islam, 2013). About 20–50% of the affected women show significant symptoms including heavy and prolonged menstrual bleeding, pelvic pressure (urinary frequency and difficulty with urination), pelvic pain, infertility and reproductive dysfunction (Buttram and mReiter, 1981; Sabry and Al-Hendy, 2012). Surgery has been the main approach to treatment of uterine fibroids. This is very expensive and coupled with the fact that it negatively affects the quality of life of women in the society. In the United States, about $5.9–34.4 billion is the annual cost of uterine fibroids management (Cardozo *et al*., 2012). In addition to the fact that the surgery is expensive, current medical treatments of uterine fibroids are also associated with serious adverse side effects and so, we are still in search of effective prevention strategies (Mesquita *et al*., 2010 ; Cardozo *et al*., 2012). The limitations encountered in the medical treatment may be due to poor understanding of the molecular mechanism of uterine fibroids development. However, recently, significant moves to unwrap the precise molecular mechanisms of uterine fibroid development and growth have been undertaken achieved (Islam *et al*., 2013; Varghese *et al.,* 2013; Ono *et al*., 2013; Islam *et al*., 2014).

Uterine fibroids are monoclonal tumors that arise from the uterine smooth muscle tissue (myometrium) and are almost always benign (Parker *et al*., 1994; Varghese *et al*., 2013). Uterine fibroids are almost always benign. A striking feature of uterine fibroids is that their development depend on the ovarian steroids estrogen and progesterone (Ishikawa *et al*., 2010). The activity of ovary is needed for fibroid growth, and this is the reason most fibroids shrink after menopause. The increase and decrease in the production of estrogen and progesterone that are associated with very early pregnancy and the postpartum period have a significant effect on the growth of fibroid (Rosati *et al*., 1992; Laughlin *et al*., 2010; De Vivo *et al*., 2011). Gonadotropin-releasing-hormone (GnRH) analogues, which suppress ovarian activity and thus reduce circulating levels of estrogen and progesterone, have been found to shrink fibroids and reduce associated uterine bleeding (Filicori *et al*., 1983).

2.11.1 CELLULAR ORIGINS OF UTERINE FIBROIDS

The cellular origin of uterine fibroids is still unknown. However, several studies support the notion that each fibroid originates from a transformed single somatic stem cell of the myometrium under the paracrine interaction of ovarian hormones (Linder and Gartler, 1965). This transformed cell undergoes self-renewal and produces daughter cells under the influence of reproductive hormones (Arango *et al*., 2005; Szotek *et al*., 2007; Ono *et al*., 2007; Chang *et al*., 2010). Studies have revealed that the stem cells derived from fibroid tissue (not the myometrium) carry mutated *MED12* gene which regulates transcription initiation and elongation by bridging regulatory elements in gene promoters to the RNA polymerase II initiation complex.

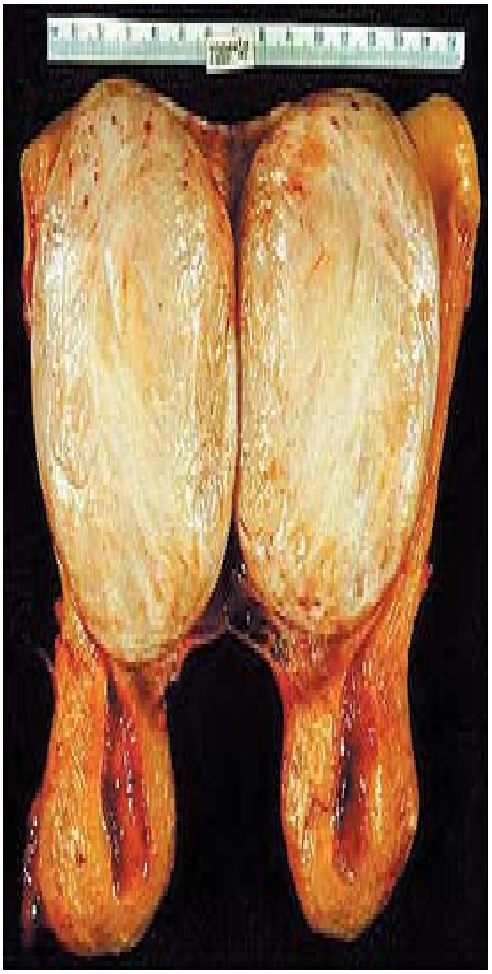
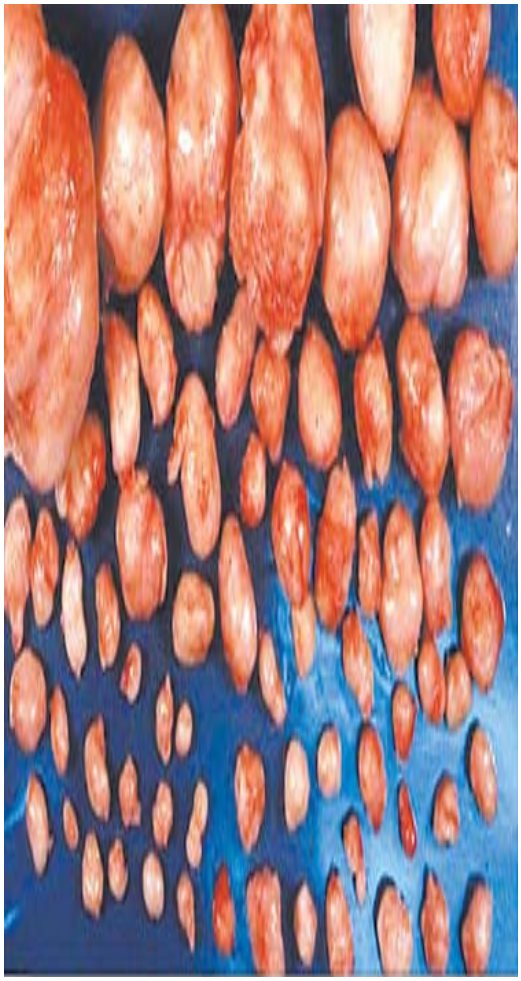


Figure 12: Single fibroid



Fibroids removed from the same uterus

(Serdar and Bulun, 2013)

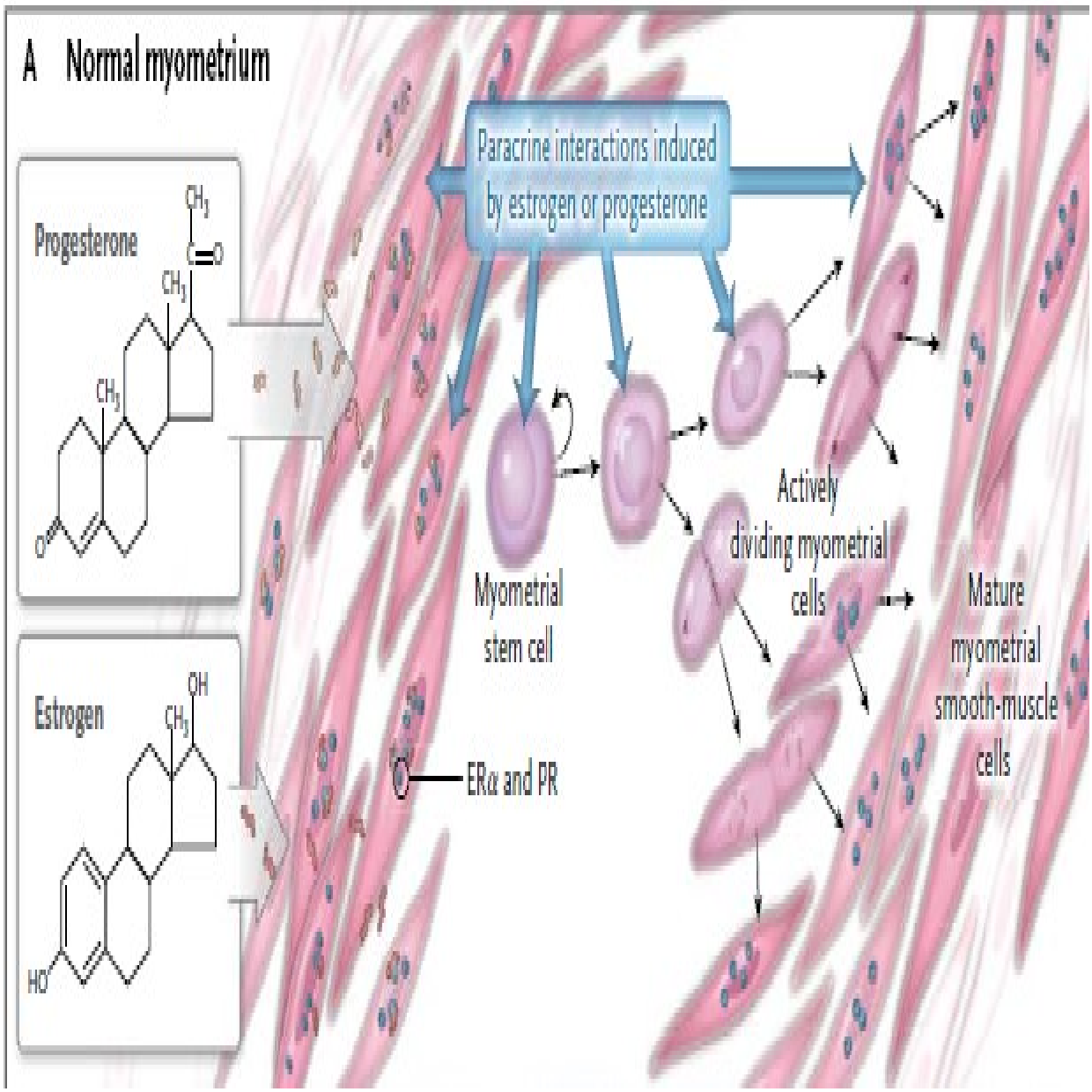


Figure 13: Normal growth in myometrium (Serdar and Bulun, 2013)

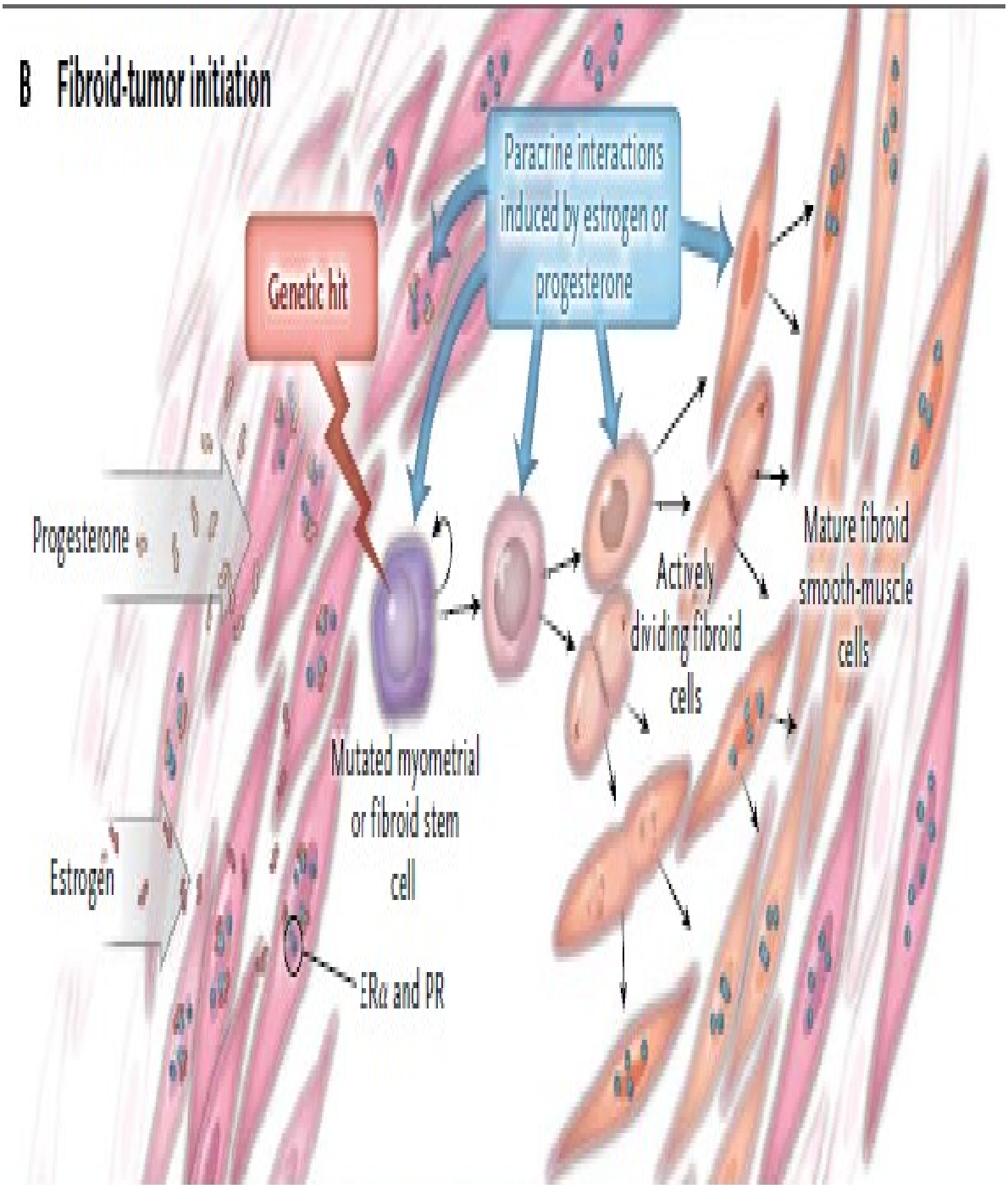


Figure 14: Fibroid tumor initiation (Serdar and Bulun, 2013)

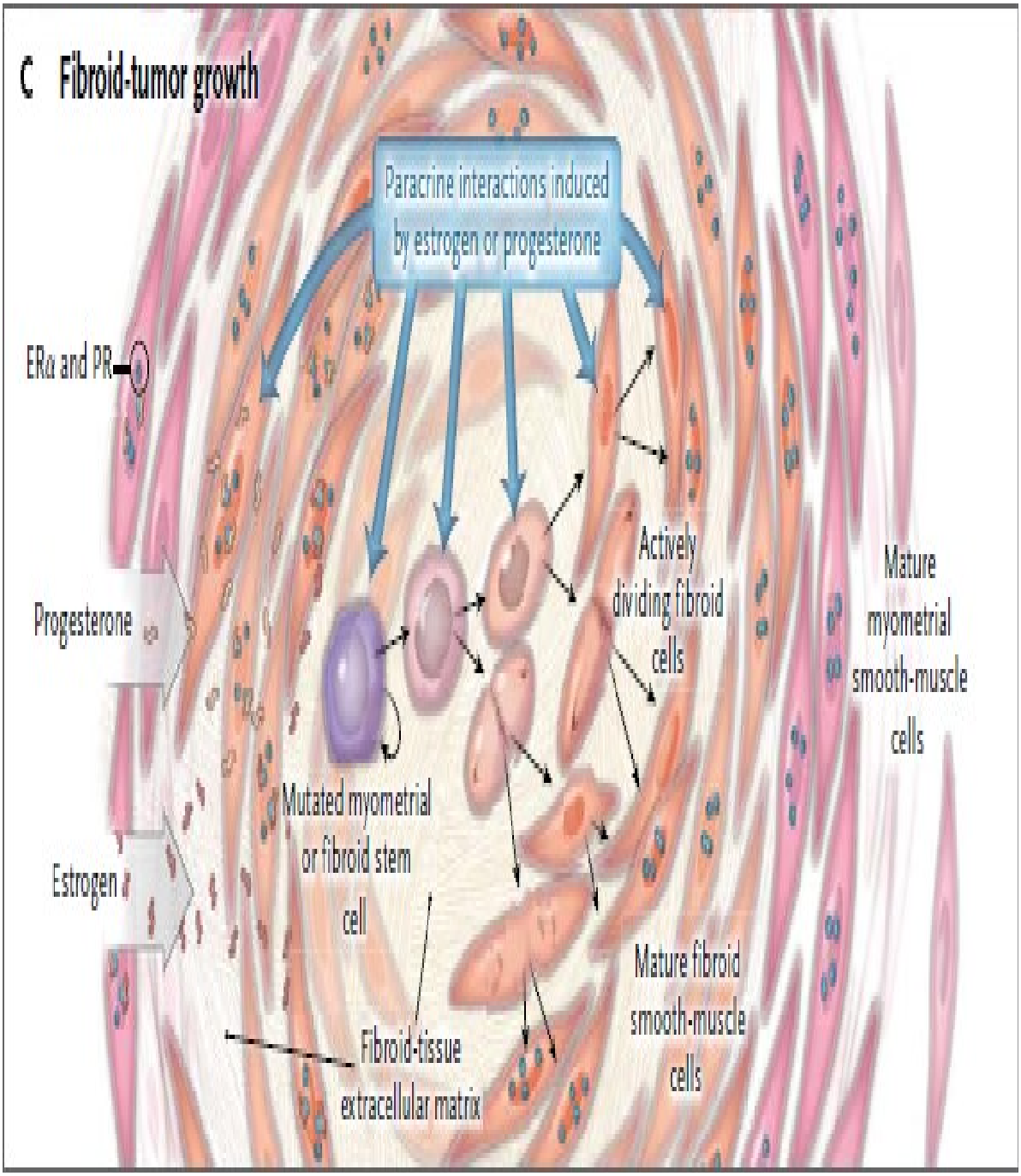


Figure 15: Fibroid tumor growth (Serdar and Bulun, 2013)

This suggests that there is at least one genetic hit which causes transformation of a normal myometrial stem cell, and subsequent interaction with the surrounding myometrial tissue to give rise to a fibroid tumor (Ono *et al*., 2012).

2.12 OESTROGEN

Experimental evidences suggest that estrogen is required for the growth of uterine fibroids through estrogen receptor α (Marsh and Bulun, 2006). The interaction of estrogen and estrogen receptor α in fibroid growth stimulates the induction of progesterone receptor thereby enabling tissue to respond to progesterone (Ishikawa *et al*., 2010). Fibroid tissue is exposed to estrogen produced by the ovaries as well as the one produced locally via the aromatase activity in fibroid cells (Bulun *et al*., 1994). Aromatase P_{450} is the enzyme involved in the convertion of androstenedione to estrogen (Bulun *et al*., 1994). It is likely that aromatase activity is important in the etiology of fibroid growth because fibroid tissue from black women where there is increased risk of incidence or prevalence of uterine fibroid compared with the white women contain high levels of aromatase, resulting in elevated levels of estrogen in tissue (Sumitani *et al*., 2000; Ishikawa *et al*., 2008; Ishikawa *et al*., 2009). High level of aromatase seems to correlates with high risk of uterine fibroid. The use of aromatase inhibitors in shrinking fibroid growth is as effective as GnRH analogues in shrinking fibroid volume, despite stable levels of circulating estrogen. These observations suggest that the inhibition of aromatase is a key mechanism in inhibiting hormone-dependent fibroid growth (Parsanezhad *et al*., 2010).

2.13 PROGESTERONE

Studies have shown that progesterone and its receptor are essential for tumor growth, as they stimulate cell proliferation, accumulation of extracellular matrix and cellular hypertrophy

(Ishikawa *et al*., 2010). The use of progestins in hormone-replacement regimens also stimulates the growth of fibroids in postmenopausal women in a dose-dependent manner, Studies also revealed that the addition of progestins to GnRH agonists caused reduction in the inhibitory potential of these agonists on leiomyoma size (Carr *et al.*, 1993; Friedman *et al*., 1993). Another evidence which is in consonant with the in vivo growth-stimulating effects of progesterone on fibroids emanated from clinical trials of three different antiprogestins. The results show that treatment with each reduced tumor size (Williams *et al*., 2007; Donnez *et al*., 2012).

2.14 DIETARY PHYTOCHEMICALS FOR POSSIBLE PREVENTIVE AND THERAPEUTIC OPTION OF UTERINE FIBROIDS

Dietary phytochemicals are compounds derived from plant origin. They are found in cereals, fruits, vegetables, legumes, herbs, spices, nuts, among others. They have disease-preventive and therapeutic properties on pathological conditions by modulating diverse signaling pathways (Surh, 2003; Kim *et al.,* 2011; Vidya *et al.,* 2012). Evidences have shown that high consumption of green vegetables and fruits has a protective role in reducing the risk of uterine fibroids of US and Italian populations (Chiaffarino *et al*., 1999; Wise *et al*., 2011). This result shows the potential of dietary phytochemicals in the prevention and treatment of uterine fibroids. Dietary phytochemicals like EGCG (epigallocatechin gallate), curcumin, resveratrol, isoliquiritigenin and genistein have been studied in uterine leiomyoma (Catherino *et al.,* 2011; Islam *et al.,* 2014; Wu *et al.,* 2016). Their antiproliferative effects in uterine leiomyoma have been studied. However, their effects on signaling pathways are yet to be investigated in leiomyoma cells except for curcumin (Catherino *et al*., 2011) and genistein (Andrews *et al*., 2012).

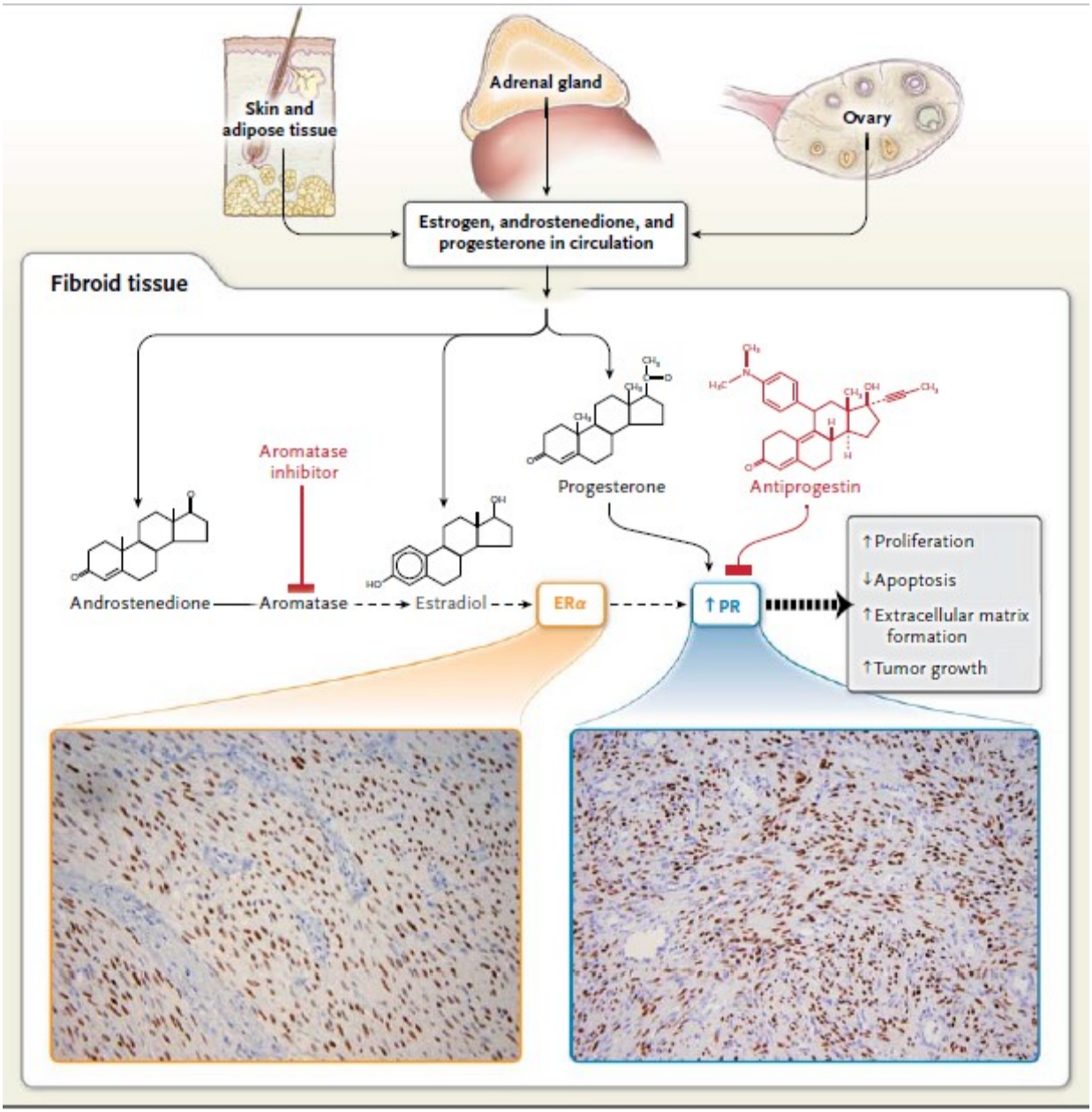


Figure 16: Biological effects of oestrogen and progesterone on fibroid tissue (Serdar and Bulun, 2013)

Figure 17: Dietary phytochemicals and their chemical structure and dietary sources

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Hence, there is room for future research in the area of phytochemical based studies investigating the signaling pathways as therapeutic target.

2.15 *DRYMARIA CORDATA*

Drymaria cordata (Linn.) Willd. (Caryophyllaceae) is a creeping herb which grows in dense patches of moist shady places and also in dry sun-exposed areas. It has green slender stems, cordate leaves, with short petiole and small green flowers. The size of the plant varies in different habitats. Leaves of the plant growing in humid and shady area large and succulent while those growing on exposed rocks and sunny areas have thin and smaller leaves. (Ramashankar and Rawat, 2008). The plant could be found in damp places all over the tropics of Africa, Asia and the Americas where it is being used for various purposes like agriculture and traditional medicine (Burkill, 1985; Asolkar, 1992; Noumi and Dibakto, 2000; Telefo *et al*., 2011). Its anti-inflammatory (Mukherjee, 1998a; Adeyemi *et al*., 2008), antitussive (Mukherjee *et al*., 1997), antibacterial (Mukherjee, 1998b), cytotoxic (Sowemimo *et al*., 2009), anxiolytic (Barua *et al*., 2009a), analgesic, anti-nociceptive and antipyretic properties (Akindele *et al*., 2012; Barua *et al*., 2011; Barua *et al*., 2009b) have been reported.

2.15.1 ETHNOBOTANICAL AND TRADITIONAL USES OF *DRYMARIA CORDATA*

Drymaria cordata is locally used in various parts of the world including Africa and Asia as folk medicine. In tropical Africa, its preparations are used for the treatment of diverse ailments such as cold, headache, coryza, bronchitis, as poultice on sore, leprosy, hepatic benign tumor, uterine tumor,breast tumor and skin tumor. (Burkill, 1985). In west Cameroon, the plant is called “Ton tchikou or Ndougo” (Bangangté) and “Mtokia” (Baham) where it is used to cure peptic ulcer, headaches or nephritis (Noumi and Dibakto, 2000) and female infertility (Telefo *et al*., 2011).

Drymaria cordata is used in Nigerian folkloric medicine in the treatment of sleeping disorders, convulsions, and febrile conditions in children (Adeyemi *et al*., 2008). In some local tribes in India, the plant is used as an antitussive. The herb is put on some big leaves, folded, tied and put over fire and heated. The vapour generated from the smoked leaves is inhaled for the relief of cough and sinusitis or in acute cold attack. The herb is also used for snake bite, burns and skin diseases (Rao *et al*., 1981; Asolkar, *et al*., 1992).

2.15.2 ACTIVE CONSTITUENTS

Plant with active chemical constituents playing a definite therapeutic response in the treatment of various ailments both in humans and animals, is regarded as medicinal plant. *Drymaria. cordata* is found to be rich in saponins especially the pentacyclic triterpenoid types (Trease and Evans, 1989). The presence of alkaloid-like (pyrrolizidine) compounds was also detected in the plant (Murdiati and Stoltz, 1987). The presence of alkaloids and saponins was likewise reported by Volponi, (1985). The principal chemical compound found in this plant is methoxycanthin. However, studies have shown that it also contains starch and other compounds. Other compounds found in the plants include succinic acid , alpha-spinasterol and a mixture containing caproic, caprylic, capric, lauric, myristic, palmitic,stearic, oleic, linoleic and linolenic fatty acids (Adeyemi *et al*., 2008). Cordatamine and the antileukemic substance, cordacin I have also been isolated and characterized from the plant (Tejavathi and Indira, 2011). Several biologically active compounds have been isolated from the leaves of *Drymaria cordata* including norditerpenes and norditerpene glycosides (Tejavathi and Indira, 2011), flavonoid glucosides (Ding *et al*.,1999) and cyclopeptides (Ding *et al.,* 2000).

Figure 18 : **Picture of *Drymaria cordata*** (Akindele *et al*., 2012)`

CHAPTER THREE

MATERIALS AND METHODS

3.0 PLANT MATERIALS

Drymaria cordata (whole plant) was freshly harvested and obtained in the university of Ibadan environment during the raining season. It was authenticated and identified at the Herbarium, Department of Botany, University of Ibadan, Ibadan, Oyo State with a specimen Voucher No.UIH-22555. The freshly harvested *D. cordata* plants were washed with water and air-dried for three weeks in the laboratory after which the dried plants were pulverized into powder with industrial machine (SAISHO, model: S 1841) and weighed.

3.1. PREPARATION OF CRUDE METHANOL EXTRACT OF *Drymaria cordata*

Five (5) kg air-dried, whole plant of *Drymaria cordata* was extracted using 20 Litres of distilled methanol (Sigma Aldrich Chemical) in all- glass jars for seventy-two hours. The filtrate was decanted, filtered and concentrated using a rotary evaporator (Stuart, model : RE 300). The crude methanol extract was heated over a water bath at 40^{o}C to obtain a solvent free extract

3.2 PARTITIONING OF THE CRUDE METHANOL EXTRACT

Five hundred milliliters of water was added to 60 g of the methanol extract (ME). This was poured into a 1 litre separating funnel. n-Hexane (300 mL) was added to remove the fraction that is soluble in n-hexane until exhaustion. The marc was further washed with chloroform (Sigma Aldrich Chemical) until exhaustion. After the chloroform-soluble fraction had been removed, the remaining marc was further subjected to separation by the addition of ethylacetate. After this, the remaining aqueous fraction in the separating funnel was filtered. These various solvent fractions

were concentrated to dryness. All solvent-free fractions were kept in the refrigerator until used. The formula below was used to calculate the percentage yield of each fraction after concentration.

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

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

3.3 EXPERIMENTAL ANIMALS

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

3.4 PHYTOCHEMICAL SCREENING

Phytochemical analysis was carried out qualitatively to detect various natural products present in the crude methanol extract. Phytochemical screening was performed using standard procedures as described by Trease and Evans (1989) and Sofowora (1993).

3.4.1 Test for anthraquinones

A weight of 0.5 g of the methanol extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of 20% ammonia was added. The resulting solution was

observed for colour changes. Observation of a rose pink colour in the aqueous layer indicated the presence of anthraquinone.

3.4.2 Test for terpenoids (Salkowski test)

To 0.5 g of the methanol extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

3.4.3 Test for flavonoids

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

3.4.4 Test for saponins

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

3.4.5 Test for tannins

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

3.4.6 Test for alkaloids

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

3.4.7 Test for steroids

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

3.4.8 Test for cardiac glycosides

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

3.5 VACUUM LIQUID CHROMATOGRAPHY OF CHLOROFORM FRACTION OF *Drymaria cordata*

3.5.1 Packing of the chromatographic column

A prewashed and clean sintered glass Buchner was used in this study. About three-quarters of the column was packed with silica gel 60 (0.040–0.063mm, MERCK), placed on a conical Buchner flask and connected to the vacuum pump. The pump was switched on and 500mL of n-hexane solvent was applied to the column. This was done to further pack the column (Sweety, 2012).

3.5.2 Preparation of the sample slurry

The gel (10 g) and the chloroform fraction (15 g) were mixed together until a homogenous powder was obtained.

3.5.3 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. The flow rate was 25ml/minutes. This was done with 1000ml of the n-hexane solvent. The column was eluted again with n-hexane : chloroform (1:4) , (1:1) and (4:1). This was done until there was a complete exhaustion of the fraction in the column. The column was further eluted with chloroform only (100%) and lastly with chloroform: methanol (4:1). The fractions obtained were concentrated at 40 ^{o} C using rotary evaporator and transferred into pre-weighed all-glass sample bottles with rubber stoppers and labeled. The most potent with respect to induction of pore opening was subjected to column chromatography and thin layer chromatography. Thin Layer Chromatography (TLC) of the sub-fractions were carried out in order to assess the purity and also to identify the phytochemicals present in each of the sub-fractions gotten from the solvent systems. Weight of each fraction was determined as follows:

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

3.6 ISOLATION OF RAT LIVER MITOCHONDRIA

Principle

The principle is based on differential centrifugation. The isolation was carried out as described by Johnson and Lardy (1967).

Reagents

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

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

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

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

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

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

0.25M Sucrose

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

Procedure

The animals were sacrificed by cervical dislocation. Liver was rapidly excised and trimmed. The liver was blotted with blotting paper and weighed. After weighing, it was rinsed with isolation buffer, minced with a pair of scissors and homogenized in a 10% w/v of ice-cold isolation buffer using Potter Elvehjem glass homogenizer.

The homogenate was centrifuged at 2,300 rpm in an MSE centrifuge at 4 ^{o}C for 5 minutes to sediment nuclear fraction and cell debris. This was repeated twice. The supernatant obtained was centrifuged at 13,000rpm for 10 minutes to obtain the mitochondrial pellet. The mitochondrial pellet obtained was washed twice, with washing buffer D at 12,000rpm for 10 minutes. The mitochondrial fraction was resuspended in MSH buffer and dispensed into Eppendorf tubes as aliquots and used within Sx hours. All experiments were carried out on ice to preserve the mitochondrial integrity.

Mitochondria used for assay of mitochondrial ATPase activity was prepared using the procedure described above except that ice-cold 0.25M sucrose was used as buffer for the preparation.

3.7 ISOLATION OF RAT UTERINE MITOCHONDRIA

Principle

The principle is based on differential centrifugation. This was done as described by Costa *et al*. (2006).

Procedure

The animals were sacrificed by cervical dislocation. For the extraction of mitochondria, the tissue of uterus was cleaned of blood and fat, minced and homogenized on ice in 8 ml of

isolation buffer consisting of 70 mM of sucrose, 1 mM of EDTA, and 5 mM of HEPES (pH 7.2). The homogenate was centrifuged for 7 minutes at 1000 g and temperature of 4^{o}C. The supernatant was separated and centrifuged for 7 minutes at 12,000g and temperature of 4^{o}C. The pellet was resuspended in isolation buffer (containing no EDTA) and kept on ice (4^{o}C). Protein content was determined by the method of Lowry *et al*. (1951).

3.8 MITOCHONDRIAL PROTEIN DETERMINATION

Principle

Phenol groups containing amino acids (tyrosine and tryptophan) in a protein will reduce phospho-18-molybdictungstic complex (a mixture of several molecular forms such as $3H_2O.P_2O_5.9MoO_3$. and $3H_2O.P_2O_5.10WO_38MoO_3$) giving a blue colour at alkaline pH. Tyrosine and (or) tryptophan present in the protein are responsible for the reduction of this phosphor-18-molybdictungstic complex giving rise to the blue colouration. The absorbance was read at 750nm. This was done as described by Lowry *et al*. (1951) using bovine serum albumin as standard.

Reagents

Reagent A: 2% Na_2CO_3 in 0.1M NaOH

Sodium trioxocarbonate (IV) (2g) and 4 g sodium hydroxide pellets were dissolved in a small amount of distilled water in a 100 ml standard volumetric flask and made up to the 100 ml mark with distilled water.

Reagent B: 2% Na-K-Tartrate

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

Reagent C: 1% $CuSO_4.5H_2O$

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

Reagent D: Alkaline Copper Solution

Reagents A, B, and C were mixed together in the ratio 100:1:1 respectively.

Reagent E: Folin-Ciocalteau Reagent

The Folin-Ciocalteau Reagent: stock solution of ratio 1:5 was used in this study.

STANDARD PROTEIN CURVE

Bovine Serum Albumin (BSA) (Sigma Aldrich Inc.) (10 ml) of 4mg/ml was used in this study.

Procedure

Reagent D (3ml) was added to protein samples mixed and allowed to stand at room temperature for 10 minutes. Reagent E (0.3ml) was then added to the mixture and then shaken to ensure a thorough mixing. The mixture was left for another 30 minutes for colour development after which the absorbance was read at 750nm using the CamSpec M106 spectrophotometer. The absorbance values obtained were plotted against the respective concentration of the BSA.

TABLE 1: PROTOCOL FOR PROTEIN DETERMINATION

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

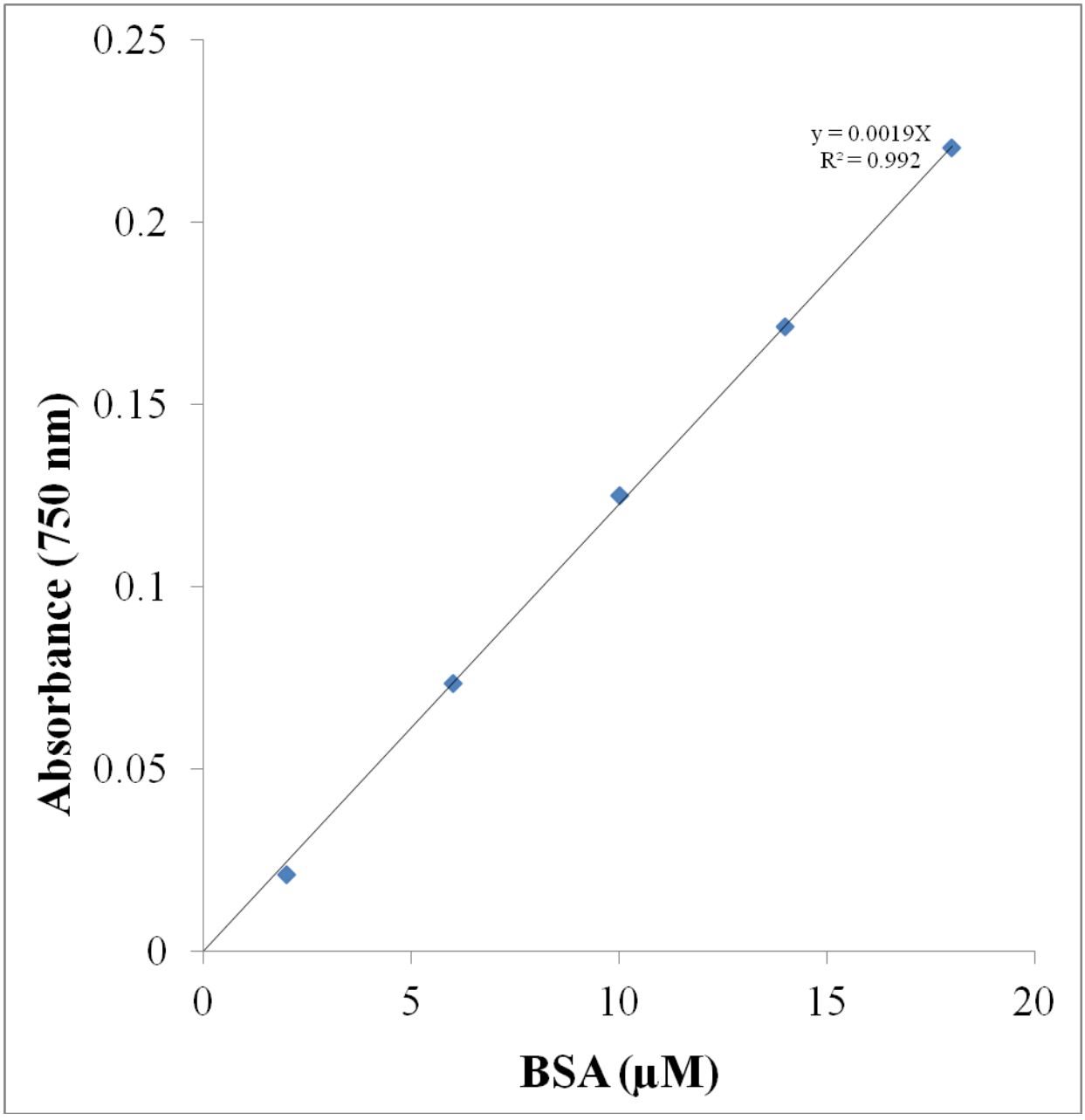


Figure 19 Standard Protein Curve

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

Principle

When mitochondrial membrane is permeabilized and the mitochondrion swells, the refractive index changes in such a way that they scatter less light which can be detected as a decrease in absorbance measured at 520nm in a spectrophotometer (Lapidus and Sokolove, 1993).

Reagents for inducing mitochondrial swelling

80µM Rotenone

Rotenone (Sigma Aldrich Inc., USA) (0.32mg) was dissolved in little quantity of distilled water and made up to the 100ml mark. It is stored in a dark (or amber) bottle because it is sensitive to light.

4mM Spermine

Spermine (Sigma Aldrich Inc., USA) (8.094mg) was dissolved in distilled water and made up to 10ml mark . It was stored in dark bottle because it is photosensitive.

12mM $CaCl_2.2H_2O$

Calcium chloride dihydrate (Sigma Aldrich Inc., USA) (176.4mg) was dissolved in 8ml of distilled water and made up to 10ml with distilled water.

250mM Sodium succinate

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

Procedure for mitochondrial swelling assay

Mitochondria were incubated in the presence of 0.8µM rotenone (10 µl) and swelling buffer (2,200 µl) for 3 minutes after which 250mM sodium succinate (50 µl) was added and incubated for 30 seconds in order to determine the intactness of mitochondria. When exogenous calcium was used as triggering agent, mitochondria were incubated in the MSH buffer and rotenone for 3 minutes, 12mM $CaCl_2$ (25µl) was then added and after 30 seconds, 250mM sodium succinate was also added to energize the mitochondria. The change in absorbance was read as a light scattering effect using a CamSpec M106 spectrophotometer at 540nm. To assay for the inhibitory effect of spermine, mitochondria were incubated with MSH buffer (2,200 µl), 8µM rotenone (10µl) and 4mM spermine (62.5µl) for 3 minutes. Calcium (25µl) was added after 3 minutes and sodium succinate (50µl) was added 30 seconds later. The change in absorbance was spectrophotometrically measured.

3.10 ASSAY FOR MITOCHONDRIAL ATPase ACTIVITY

Principle

When there is opening of mitochondrial membrane permeability transition (MPT) pore, there is lose of membrane potential which causes the mitochondrial ATP Synthase (involved in the ATP synthesis) to be hydrolysing ATP (ATPase). The ATP which is the substrate combines with the enzyme (ATP synthase) to form the enzyme-substrate complex which gives rise to ADP and inorganic phosphate (Lardy and Wellman, 1953).

Procedure

Mitochondrial ATPase activity was determined by a modification of the method of Olorunsogo and Malomo (1985). Instead of 2 mg/ml mitochondrial protein, 1 mg/ml protein was used in this

study. Each reaction mixture contained 65 mM Tris-HCl buffer pH 7.4, 0.5 mM KCl, 1 mM ATP and 25 mM Sucrose. The reaction mixture was made up to a total volume of 2 ml with distilled water. The reaction was started by the addition of mitochondrial suspension and allowed to proceed in a shaker water bath for 30 minutes at 27°C. The reaction was stopped by the addition of 1 ml of 10 percent sodium dodecyl sulphate (SDS) solution. The zero time tube was prepared by addition of mitochondria to the reaction vessel with immediate addition of SDS to stop the reaction while other reaction vessels were stopped at 30 seconds intervals.

Reagents

0.1M Tris-HCl (pH 7.4)

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

0.25M Sucrose

Sucrose (Sigma Aldrich Inc., USA) (6.56g) was dissolved in about 60ml of distilled water and made up to the 100 ml mark with distilled water.

1M KCl

Potassium chloride KCl (7.45g) (BDH Chemicals, England) was dissolved in 60 ml of distilled water and made up to 100ml mark with distilled water.

0.01M ATP

Disodium salt of ATP (0.062g) (Sigma Aldrich Inc., USA) (0.062g) was dissolved in a small quantity of distilled water and the whole volume made up to 10 ml with distilled water. This was stored in a refrigerator.

2% Ascorbic acid

Ascorbic acid (BDH Chemicals, England) (2g) was dissolved in 80ml of distilled water and made up to 100 ml.

5% Ammonium Molybdate

Ammonium molybdate (5.0g) was dissolved in 80 ml of 6.5% H_2SO_4 and made up with the same solvent to 100ml mark and stored in a plastic container at room temperature.

10% Trichloroacetic acid.

Trichloroacetic acid (BDH Chemicals, England) (10g) was dissolved in 80ml of distilled water and made up to 100ml mark with distilled water. This was stored in the refrigerator.

3.11 Determination of Mitochondrial Inorganic Phosphate Concentration

Principle

Inorganic phosphate, in the presence of molybdic acid gives a yellow colour, which can be reduced by ascorbic acid to give a blue colour and the intensity of the colour formed correlates to the concentration of inorganic phosphate.

Reagents

10mM Na_2HPO4

Disodium hydrogen phosphate (142mg) (Sigma Aldrich Inc., USA) was dissolved in 60mL of distilled water and the volume made up to 100mL mark with distilled water.

1.25% Ammonium molybdate in 6.5% H_2SO_4

Ammonium molybdate (6.52g) (Hopkins and Williams Ltd., England) was dissolved in about 450 ml of 6.5% H_2SO_4 and the whole volume made up to 500ml mark with 6.5% H_2SO_4, (that was prepared by adding 32.5ml of concentrated H_2SO_4 to a small volume of distilled water and made up the volume to 500 ml). The molybdate solution was stored at 25°C in a plastic bottle.

9% Ascorbic acid

Ascorbic acid (BDH Chemicals, Poole, England) (22.5g) was dissolved in about 160 ml of distilled water and the volume made up to 250 ml mark with distilled water.

Procedure

Disodium hydrogen phosphate (1mM) was used for preparing a standard curve of inorganic phosphate released. Different concentrations of 1mM Na_2HPO_4 were used for the standard curve (see table 2). Absorbance was read at 660nm after 30 minutes incubation at room temperature. Distilled water was used as the blank.

TABLE 2: PROTOCOL FOR INORGANIC PHOSPHATE CONTENT DETERMINATION

Test tubes in duplicates	Blank	1	2	3	4	5	6	7	8
1mM Na_2HPO_4 (µl)	-	20	40	60	80	100	120	200	300
Distilled water (µl)	1000	980	960	940	920	900	880	800	700
1.25% Ammonium molybdate (ml)	1	1	1	1	1	1	1	1	1
9% Ascorbate (ml)	1	1	1	1	1	1	1	1	1

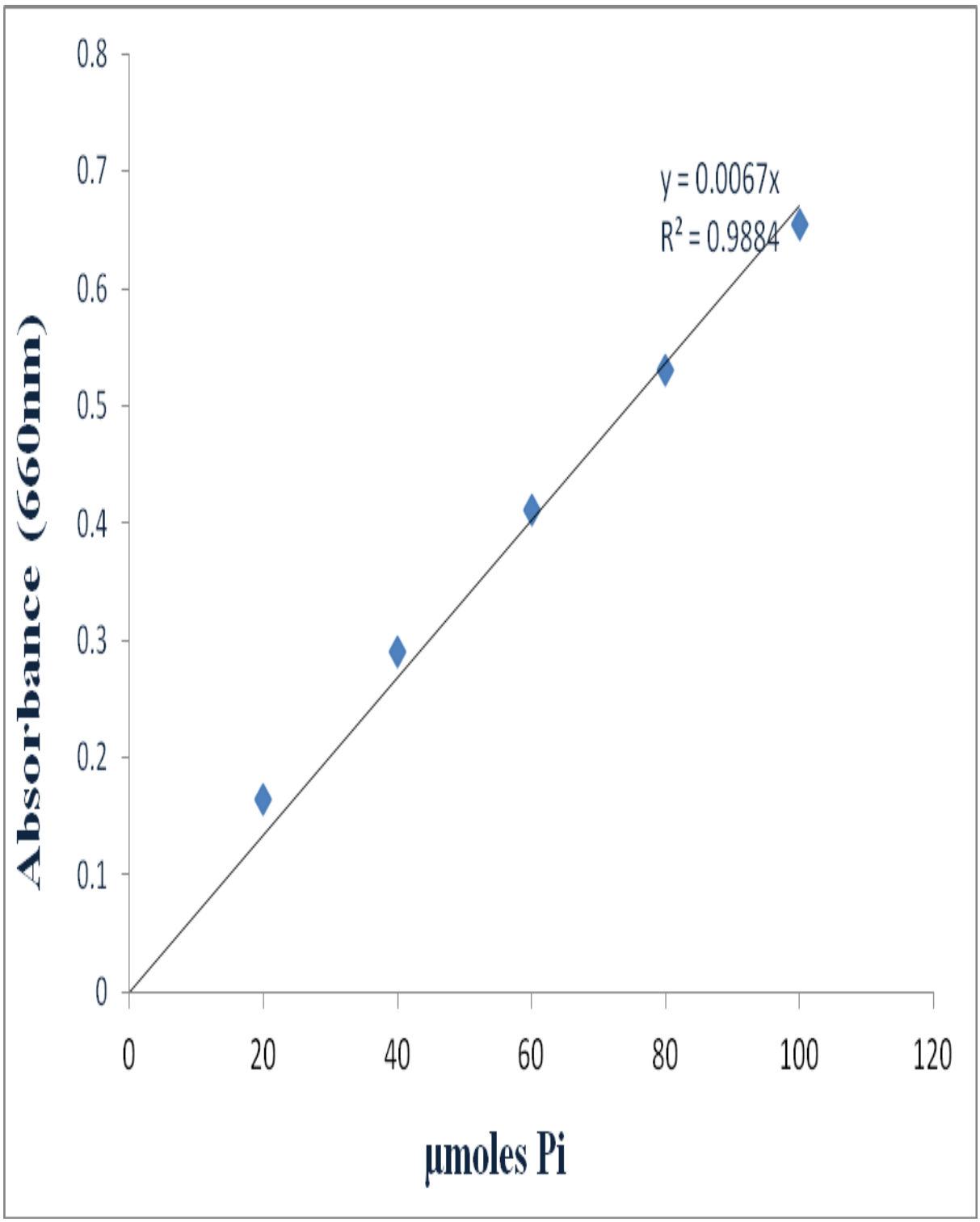


Figure 20: Standard phosphate curve for the determination of inorganic phosphate released

3.12. DETERMINATION OF CYTOCHROME C CONCENTRATION

Principle

When there is opening of mitochondrial permeability transition pore, cytochrome c is released from the mitochondrial intermembrane space into the cytosol. The quantitative determination of cytochrome c released from isolated mitochondria was performed by measuring the Soret (γ) peak for cytochrome c at 414 nm (ε= 100 mM^{-1} cm^{-1}), according to method of Appaix *et al*. (2000).

Reagents

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

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

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

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

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

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

0.25M Sucrose

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

12mM $CaCl_2$. $2H_2O$

Calcium chloride dehydrate (Sigma Aldrich Inc., USA) (0.1764g) was dissolved in 8ml of distilled water and made up to 10ml with distilled water.

Procedure

Mitochondria (1mg protein/ml) were preincubated in the presence of 0.8μM rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 30mins at 27 ^{o}C in the presence of different concentrations of the fractions, using 24mM calcium as the standard. After the incubation, the mixture was centrifuged at 15,000 rpm for 10 minutes. The optical density of the supernatant was measured at 414nm which is the soret (γ) peak for cytochrome c.

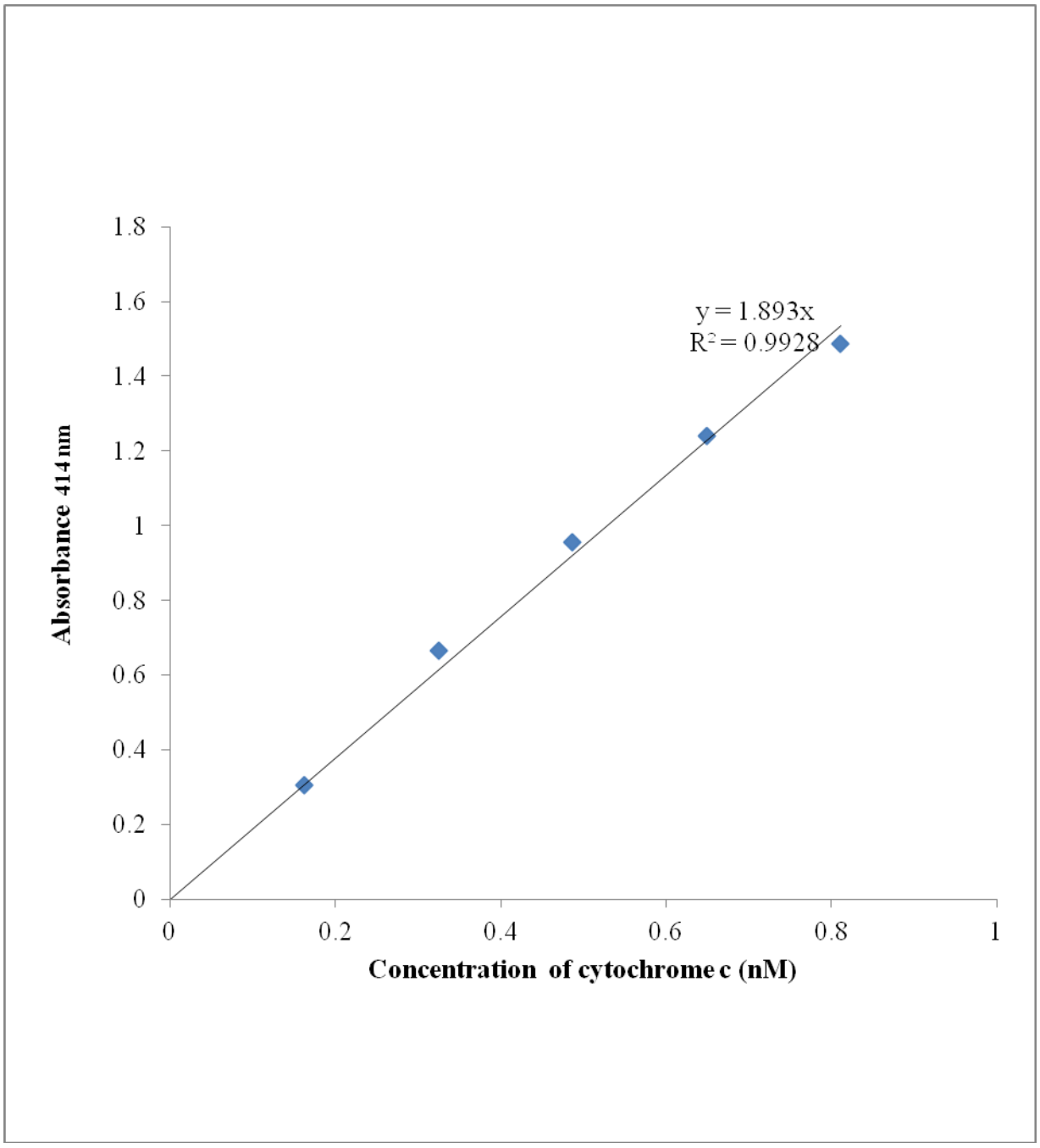


Figure 21 : Standard Curve of Cytochrome c released

3.13 IN VITRO MEASUREMENT OF LIPID PEROXIDATION

Principle

Lipid peroxidation was determined by the method described by Ruberto *et al*. (2000). Malondialdehyde produced during lipid peroxidation reacts with thiobarbituric acid to generate a pink coloured product. The butanol extracts the coloured product and is measured at 532nm in a spectrophotometer.

Reagents

0.8% Thiobarbituric acid

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

1.1% Sodium dodecyl sulphate

8.1 g of sodium dodecyl sulphate (8.1g) (BDH Chemicals, Ltd, Poole, England) was dissolved in 80 ml of distilled water and the solution was made up to 100 ml mark in a volumetric flask using distilled water. Equal volumes of TBA and SDS were mixed and used in the assay.

60µM Ferrous Sulphate

Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Sigma Aldrich Co. St Louis, USA) (0.8341g) was dissolved in a little quantity of distilled water and the solution was made up to 50 ml.

20% Acetic acid

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

Procedure

Mitochondria (2mg/ml) obtained from rat liver and various concentrations (50-800 µg/mL) of fractions were added to each test tube and made up to 1 ml with distilled water. 0.05ml of 60µM $FeSO_4$ was introduced into the reaction medium and incubated for half an hour at 37°C. 1.5 mL of 20% acetic acid and 1.5 ml of TBA in SDS were added and mixed with the aid of vortex machine and heated at 95°C for 1 hour. It was allowed to cool after which it was thoroughly mixed with 3 ml of butan-1-ol and centrifuged at 3000 rpm for 10 minutes. The optical density of the top organic layer was taken at 532 nm.

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

Where:

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

3.14 IN VIVO MEASUREMENT OF LIPID PEROXIDATION

Principle

Lipid peroxidation was determined by the method described by Varshney and Kale (1990). Under acidic conditions, malondialdehyde (MDA) produced from the peroxidation of fatty acids reacts with the chromogenic reagent 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm.

Reagents

1. 30% Trichloroacetic acid (TCA)

Trichloroacetic acid (TCA) (CCl_3COOH) (4.5g) was dissolved in distilled water and made up to 15 ml with the same.

2. 0.1M Hydrochloric acid (HCl)

Concentrated HCl (36.5-38%) (13µl) was added to distilled water and the volume made up to 15 ml with the same.

3. 0.75% Thiobarbituric acid (TBA)

Thiobarbituric acid (TBA) (0.1125g) was dissolved in 0.1 M HCl and made up to 15 ml with 0.1 M HCL . Dissolution was aided by stirring in a hot water bath (50˚C).

4. 0.15M Tris-KCl buffer (pH 7.4)

Potassium Chloride (KCl) (0.559g) and 0.909 g of Tris base were dissolved in 45 ml of distilled water, the pH was adjusted to 7.4 with HCl and the volume made up to 50 ml with water.

Procedure

An aliquot of 0.4 ml of the test sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% TCA was added followed by 0.5 ml of 0.75% TBA and incubated in a water bath for 45 minutes at 80^oC. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm.

Calculation

The MDA level was calculated using an extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$ (Adam-Vizi and Seregi, 1982) as follows:

$$\text{Lipid peroxidation (nmole MDA/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample} \times \text{mg protein/ml}}$$

3.15 PREPARATION OF SERUM

The Wistar strain albino rats that were orally administered CFDC for 30 days were sacrificed 24 hours after the last dose by cervical dislocation. They were quickly opened up and blood was collected from the heart into plane sample bottles for serum preparation. The blood was allowed to clot and after 30 minutes, the blood samples were centrifuged at 3,000rpm for 20 minutes. The serum obtained was separated from the clotted blood using pasteur pipette and used for the hormonal assay (Henry, 1979; Thavasu, 1992).

3.16 TISSUE PREPARATION FOR HISTOPATHOLOGY

Liver and uterus were quickly removed and trimmed and were placed in 10% formalin for about five days for proper fixation, dehydrated by ascending grades of isopropyl alcohol for an hour. The dehydrated organs were cleared in xylene and transferred into two changes of liquid paraffin wax. The tissue sections were stained in Ehrlich's hematoxylin for eight minutes, washed in tap water and dipped in acid alcohol to remove excess stain. These were counter stained in 10% aqueous eosin, incubated and mounted for photomicrography (Hopwood, 1996; Carson, 2007)

3.17 DETERMINATION OF CASPASES 9 AND 3 ACTIVITIES

Principle

The principle of sandwich ELISA is the quantification of a specific protein through its containment in a sandwich of specific antibodies conjugated to the colorimetric TMB (3,3',5,5'-tetramethylbenzidine) substrate, whose intensity is proportional to the protein quantity and it is specific to the protein being assayed for. A secondary biotin-linked antibody specific to the

protein (caspases 9 and 3) recognizes and binds to the primary antibody forming a “sandwich” of specific antibodies around the desired protein in the cell lysate i.e. several secondary antibodies will bind to primary antibody, hence enhancing the signal. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible

The streptavidin-HRP (Horse-radish peroxidase) complex was then used to bind the biotin-linked secondary antibody through its streptavidin portion. The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes colour during reaction. The HRP domain reacts with the added TMB (3,3’,5,5’-tetramethylbenzidine) substrate, which is converted by the enzyme-HRP to elicit a chromogenic signal forming a coloured product that was measured at 450 nm by a plate reader (ChroMate-4300, FL, USA) after which the reaction was terminated by the addition of stop solution which is usually acidic (*Engvall, 1972; Schmidt et al., 2012*).

3.18 SAMPLE PREPARATION AND ANALYSIS OF CASPASES 9 AND 3 USING ELISA TECHNIQUE

The rat liver was excised, weighed and rinsed with phosphate buffered saline thoroughly until a clear wash was obtained. The liver was homogenized on ice and the homogenate centrifuged at 8,000 rpm for 5 minutes. The supernatant thus obtained was transfered into sample bottles and frozen. After freezing for two days, the samples were allowed to thaw. This was done to enable

the cells to break. This was done twice after which the samples were assayed for caspases 9 and 3.

PROCEDURE

The manufacturer's instruction (Elabscience kit) was followed in the course of the experiment. Samples/Standards (50µL) were added to the wells followed by the addition of 50µL antibody mix/cocktail (Component of Elabscience kit). The microplate was sealed and incubated for an hour at room temperature (25 °C) on a plate shaker set to 400rpm, the wells were washed thrice to remove unbound material. Complete removal of liquid at each step was ensured for good performance . After the last wash the microplate was inverted and blotted against a clean paper towel to remove excess liquid. Thereafter, 100 µL of TMB substrate was added and incubated for 10minutes in the dark. This reaction was stopped by the addition of 100µL stop solution completing the colour change from blue to yellow.

3.19 ASSAY OF DNA FRAGMENTATION BY DIPHENYLAMINE METHOD

Principle

The DNA fragmentation assay was employed for the quantitative determination of fragmented nuclear DNA according to the method of Wu *et al*. (2005). This method is based on the notion that extensively fragmented double-stranded DNA can be separated from chromosomic DNA upon centrifugal sedimentation. Diphenylamine (DPA) ; a light sensitive molecule reacts and binds to the deoxyribose sugar of DNA molecule to form a blue coloured complex in acidic medium. The absorbance of the complex at 620nm and the percentage of DNA fragmentation is calculated relative to the amount of intact DNA molecule.

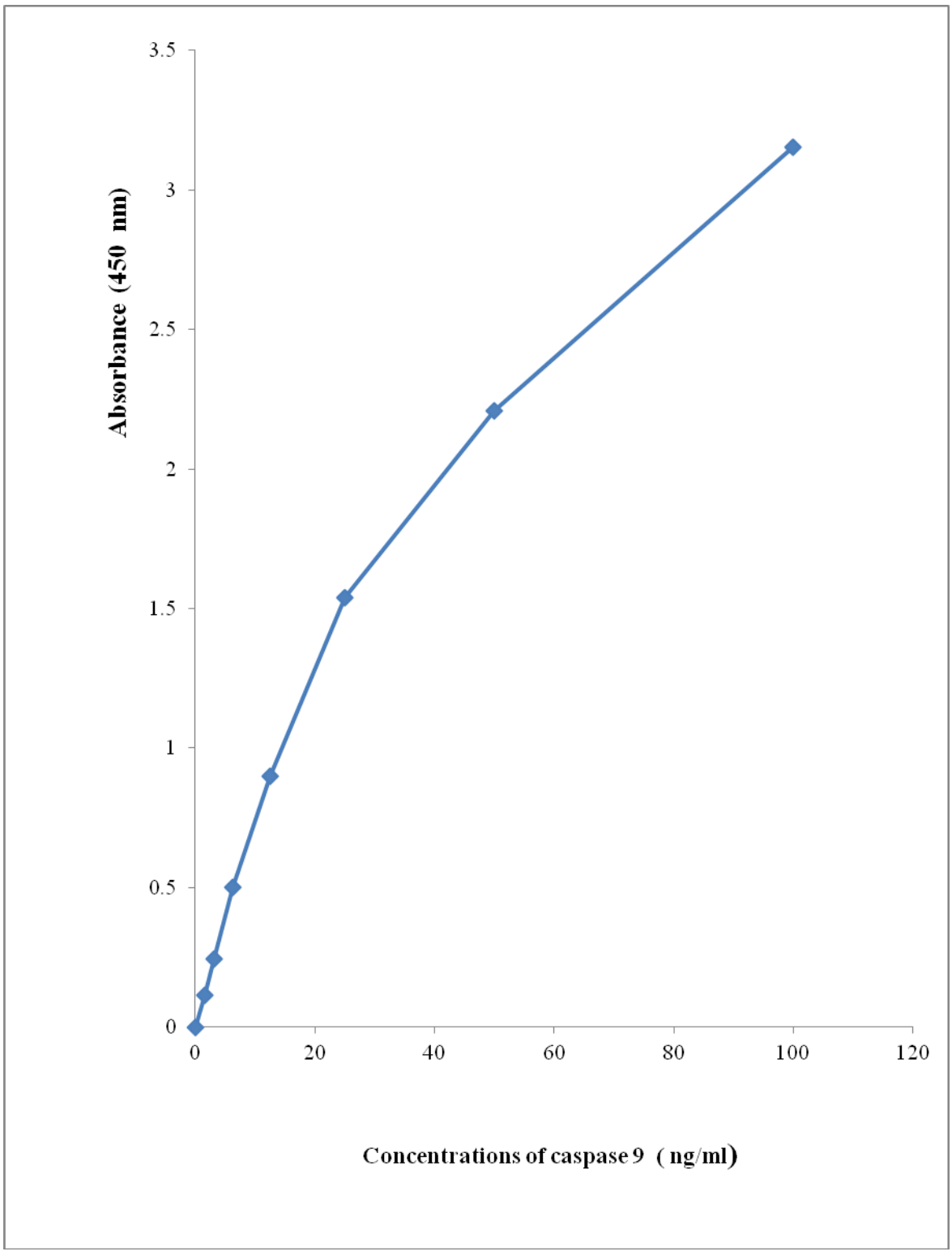


Figure 22: Caspase 9 standard curve

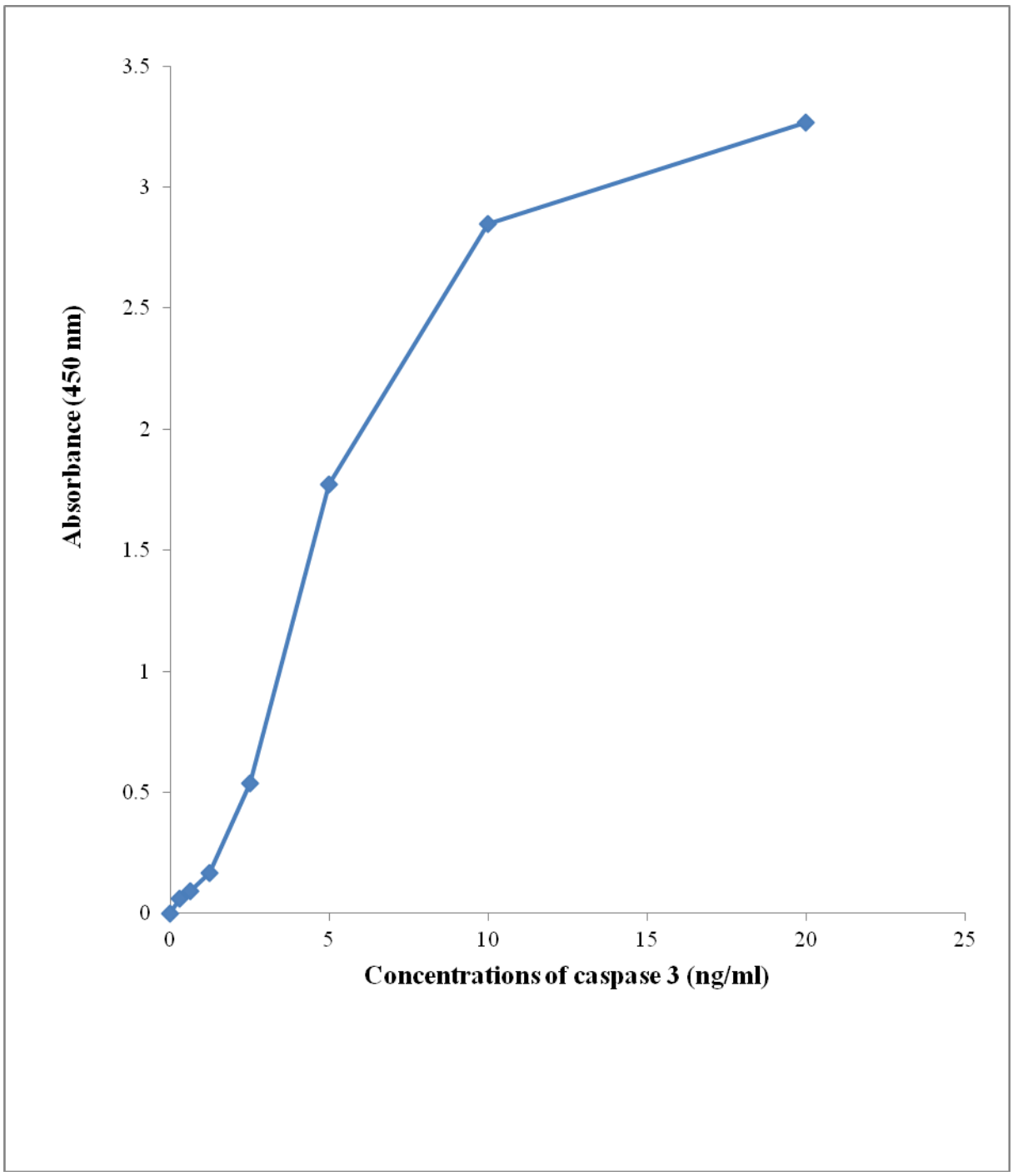


Figure 23: Standard curve for caspase 3

Reagents

5mM Tris, 20mM EDTA, TRITON (TET) buffer PH 8.0

Tris-HCL (0.61g), 7.45g of EDTA and 2ml of Triton-X100 solution were dissolved in a little quantity of distilled water and made up to the mark of 1L with distilled water in a volumetric flask after adjusting the pH to 8.0.

5mM Tris, 20mM EDTA (TE) buffer PH 8.0

Tris-HCL (0.61g) and 7.45g of EDTA were dissolved in a little quantity of distilled water and made up to the mark of 1L with distilled water in a volumetric flask after adjusting the pH to 8.0.

Diphenylamine (DPA) solution

Diphenylamine (1.5g) was dissolved in 0.1 L of acetic acid. 1.5ml of concentrated H_2SO_4 was added to the solution.

Procedure

Rats was cervically dislocated. Liver and uterus were excised and trimmed. The liver and uterus were blotted with blotting paper and weighed. The tissues were sliced and homogenized in 10 volumes of Tri-EDTA Triton buffer(TET) pH 8.0. The homogenate was centrifuged at 27,000g for 20mins to separate intact chromatin pellet from the fragmented supernatant. The intact chromatin pellet was suspended in Tri-EDTA (TE) buffer. Aliquot amount (5ml) of each sample (pellet and supernatant) was placed in separate test tubes and then 3ml of freshly prepared diphenylamine solution was added and incubated at 37^{o}C for 20 hours. Absorbance was taken at 620nm.

Calculation

Quantity of fragmented DNA was estimated by using the formula:
% fragmented DNA = B/(A+B) X 100
Where A = Intact DNA
B= Fragmented DNA

3.20 IMMUNODETECTION OF CASPASE 3, CASPASE 9, CYTOCHROME C AND BCL-2 IN RAT LIVER AND UTERUS

Principle

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The antibody-antigen binding can be visualized in different manners. Enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color-producing reaction (Clifton, 2011).

3.21 Preparation of Immunohistochemistry samples

Liver and uterus sections were immersed in 10% phosphate buffered formalin and processed in the laboratory.

Reagents

Ethanol

Varying grades of ethanol was prepared by making certain volume of absolute ethanol up to 100 ml.

Phosphate buffer saline (PBS) pH 7.4

Phosphate buffer saline pH 7.4 was prepared by dissolving 2g of potassium chloride (KCL), 80g of sodium chloride (NaCL), 2g of potassium dihydrogen phosphate and 11.6g disodium hydrogen phosphate in one litre of distilled water.

10% Buffered formalin

10% Buffered formalin was made by making 125 ml of 40% formalin up to 500mls with Phosphate Buffer Saline

0.3% Hydrogen peroxide

Hydrogen peroxide (0.3%) was prepared by dissolving 10mls of 30% hydrogen peroxide in 1000mls Phosphate Buffer Saline.

Procedure

The method used in this study is Avidin Biotin Complex (ABC) procedure also referred to the Avidin biotin Imunoperoxidase method. The antibody dilution factor used were 1:100 dilution for all the antibody markers. The processed tissue were sectioned at 2micons on the rotary microtome and placed on the hot plate at 70 degree for at least 1hour. Sections were brought down to water by passing through 2 changes of xylene, 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the sections by heating them on a citric acid solution of PH 6.0 using the Microwave at power 100 for 15minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5minutes for the section to cool. Peroxidase blocking were done on the sections by simply covering section with 3% hydrogen peroxide (H_2O_2) for 15minutes. Sections were washed with Phosphate Buffer Saline and protein blocking were performed using avidin for 15minutes. Sections were washed with Phosphate Buffer Saline and endogenous biotin in tissue were blocked using biotin for 15minutes. After washing with Phosphate Buffer Saline, sections were incubated with the respective diluted primary antibody for 60 minutes.

Excess antibody were washed off with Phosphate Buffer Saline and a secondary antibody were applied on section for 15minutes. Sections were washed and the horsradish peroxidase (HRP) were applied on the sections for 15minutes. A working DAB solution is made up by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate . This working solution is applied on sections after washing off the HRP with PBS for at least 5minutes. The brown reactions begins to appear at this moment especially for a positive target. Excess DAB solution and precipitate are washed off with water. Sections are counterstained with Haematoxylin solution for at least 2minutes and blued briefly. Sections are dehydrated in alcohol, cleared in xylene and mounted in DPX. Cells with specific brown colours in the cytoplasm, cell membrane or nuclei depending on the antigenic sites are considered to be positive. The haematoxylin stained cells without any form of brown colours are scored negative. Non specific binding/brown artifacts on cells and connective tissue are disregarded.

3.22 DETERMINATION OF OESTROGEN

Principle

In this competitive ELISA test, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to the microtitre well which is coated with antigen. The more the antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. After the well is washed, enzyme conjugated secondary antibody specific for isotype of the primary antibody is added to determine the amount of primary antibody bound to the well. The higher the concentration of antigen in the sample, the lower the absorbance. The intensity of signal is inversely proportional to the amount of estradiol in the sample and the intensity is measured at 450 nm.

Procedure

The samples and the Estradiol-HRP conjugate are added to the wells, where estradiol in the sample competes with the added Estradiol-Horse Radish Peroxidase for antibody binding. After incubation, the wells are washed to remove unbound material and TMB substrate is then added which is catalyzed by Horse Radish Peroxidase to produce blue coloration. The reaction is terminated by addition of stop solution which stops the color development and produces a color change from blue to yellow.

3.23 DETERMINATION OF PROGESTERONE

Principle

Rat PGR (Progesterone Receptor) ELISA Kit from Elabscience Biotechnology Inc. was used in this study for the quantitative measurement of progesterone in rat serum. It is a solid phase competitive ELISA. The samples and Progesterone enzyme conjugate are added to the wells coated with anti-Progesterone monoclonal antibody. Progesterone in the sample competes with a Progesterone Enzyme Conjugate for binding sites. Since the number of sites is limited, as more sites are occupied by progesterone from the sample, fewer sites are left to bind PROG-HRP conjugate. The more the antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. The intensity of signal is inversely proportional to the amount of estradiol in the sample and the intensity is measured at 450 nm.

Procedure

The procedure for the determination of progesterone level is similar to that of oestrogen as described on page 87.

3.24 DETERMINATION OF TOTAL CHOLESTEROL

Principle

The coated well immunoenzymatic assay for the quantitative measurement of Total Cholesterol utilizes a polyclonal anti-TC antibody and anTC-HRP conjugate. The intensity of color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the TC concentration since TC from samples and TC-HRP conjugate compete for the antiTC antibody binding site. Since the number of sites is limited, as more sites are occupied by TC from the sample, fewer sites are left to bind TC-HRP conjugate.

Procedure

The assay sample and buffer are incubated together with TC-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow (Hanaa and Eetmad, 2015).

3.24. THIN LAYER CHROMATOGRAPHY (TLC)

Principle

The principle is based on differential mobilities of compounds due to their differences in attraction to the stationary phase and solubility in the mobile phase. By modifying the solvent system, the separation can be adjusted by measuring the R_f value (Sweety, 2012).

Procedure

Fractions obtained using Vacuum Liquid Chromatography (VLC) were subjected to column chromatography (CC) and thin layer chromatography (TLC) by using the coated plates

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

3.25 Gas Chromatography Mass Spectrum

Principle

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at a molecular level (the MS component). The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of the analyte molecule.

Procedure

The compounds are propelled by an inert carrier gas such as argon, helium or nitrogen. As the components become separated, they elute from the column at different times, which is generally referred to as their retention times. Once the components leave the GC column, they are ionized by the mass spectrometer using electron or chemical ionization sources. Ionized molecules are

then accelerated through the instrument's mass analyzer, which quite often is a quadrupole or ion trap. It is here that ions are separated based on their different mass-to-charge (m/z) ratios. The final steps of the process involve ion detection and analysis, with compound peaks appearing as a function of their m/z ratios. Peak heights, meanwhile, are proportional to the quantity of the corresponding compound. A complex sample will produce several different peaks, and the final readout will be a mass spectrum. Using computer libraries of mass spectra for different compounds, researchers can identify and quantitate unknown compounds and analytes.

STATISTICAL ANALYSIS

Mean, standard deviation and 95% confidence interval were computed. Comparison between groups was performed using the Turkey's test. Multiple comparisons were performed using the Graphpad Prism software. Comparison of the variables were made using the ANOVA and *P-value* of < 0.05 was considered as statistically significant.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT 1: DETERMINATION OF PHYTOCHEMICALS IN *Drymaria cordata*

INTRODUCTION

Many phytochemicals have been shown to possess anti-tumour property. The experiment was aimed at determining the phytochemicals present in the whole plant of *Drymaria cordata* in order to have insight into the constituents of the plant that may be responsible for its traditional use in the treatment of uterine fibroid (Burkil, 1985).

PROCEDURE

The plant *Drymaria cordata* was collected and extracted as earlier described on page 54. The plant extract was screened for the the presence of some secondary metabolites as described on pages 55-57.

RESULTS

Table 3 shows the secondary metabolites obtained from the crude methanol extract of *Drymaria cordata*. The crude extract contains alkaloids, saponins, tannins, antraquinones, steroids, flavonoids and cardiac glycosides. The cold extraction method employed in this work showed that the crude methanol extract yield was 1.5%. When partitioned, the methanol extract yielded 15.6%, 3.2%, 5.1% and 6.1% for hexane fraction, chloroform fraction, ethylacetate fraction and aqueous fraction respectively.

CONCLUSION

The crude extract contained phytochemicals which are beneficial to humans. The reported role of *Drymaria cordat* in traditional treatment of fibroid may be as a result of the presence of some of these phytochemicals.

Table 3: Phytochemicals of the crude methanol extract of *Drymaria cordata*

Chemical Constituents	MEDC
Alkaloids	+
Antraquinones	+
Cardiac glycosides	+
Flavonoids	+
Saponins	+
Steroids	+
Tanins	+

MEDC: methanol extract of *Drymaria cordata*

EXPERIMENT 2: EVALUATION OF THE EFFECT OF CALCIUM AND SPERMINE ON RAT LIVER MPT PORE

Inner mitochondrial membrane can undergo mitochondrial permeability Transition (mPT) due to increased matrix calcium levels, inorganic phosphate concentration, increased temperature, oxidative stress or chemicals. This permeability transition is Ca^{2+}-dependent and cyclosporin A (CsA)-sensitive. Given the fact that mitochondria are delicate organelles, their isolation should be carried out with care. Thus, the aim of this experiment was to ascertain the integrity of the isolated mitochondria.

PROCEDURE

Male albino rats (Wistar strain) having a mean weight of 100g were used for this experiment. The isolation was carried out using the protocol of Johnson and Lardy (1967) with slight modifications. The mitochondrial protein used in this study was increased from 1mg protein/ml to 2mg protein/ml.

RESULTS

The data presented in Figure 24 show that the mitochondria were intact and suitable for use. As seen from the figure, addition of calcium caused highly significant increase in mPT pore opening which was reversed by spermine.

CONCLUSION

The result shows that exogenous calcium caused induction of mPT pore opening while spermine, a potent inhibitor significantly reversed the Ca^{2+}-induced opening of the pore. This indicated that the membrane integrity of the liver mitochondria were not compromised, hence, the mitochondria were intact and suitable for use.

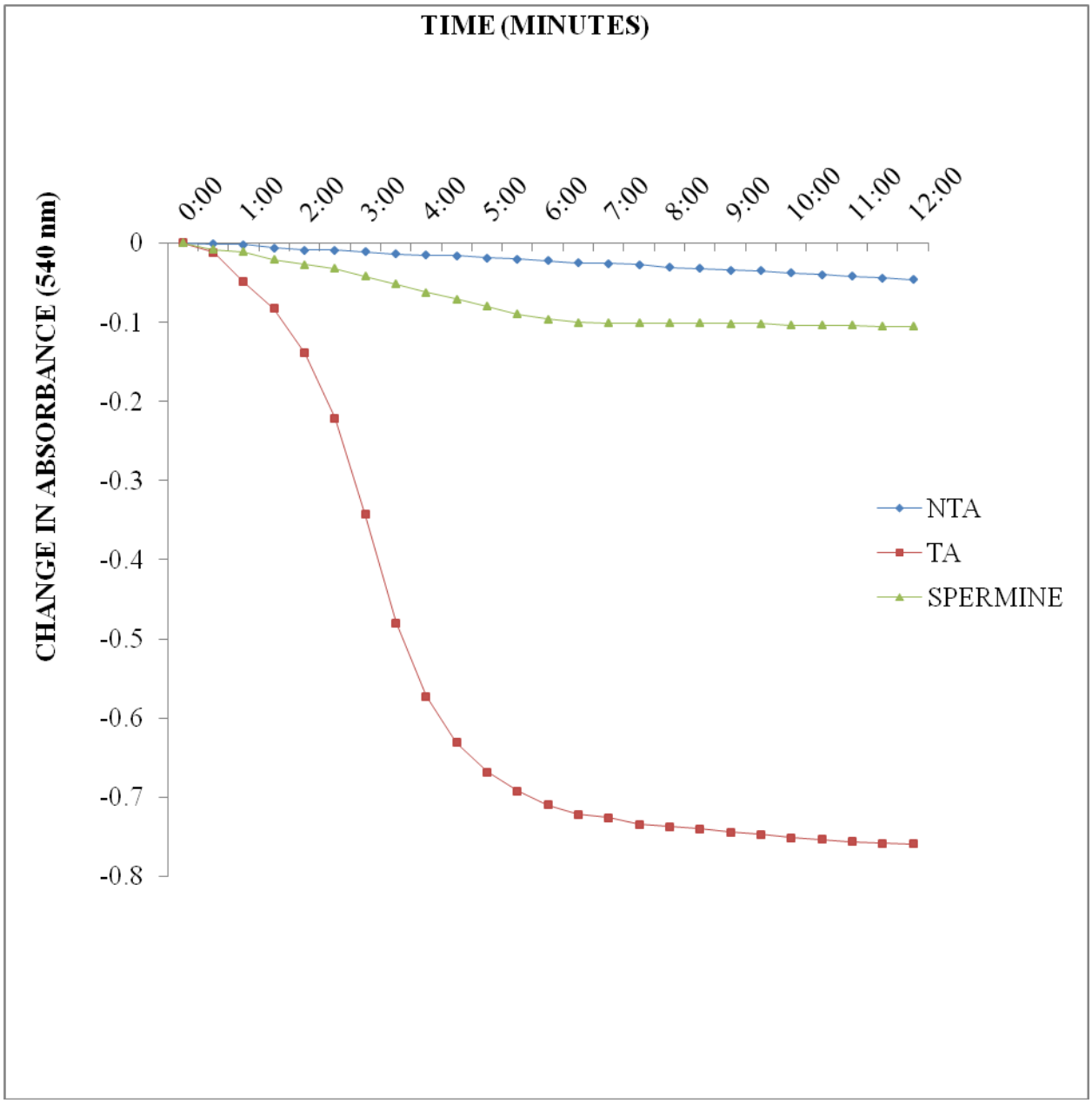


Figure 24: The intactness of mitochondria using calcium and spermine

NTA: No triggering agent (without calcium)

TA: Triggering agent (with calcium)

Spermine: Standard inhibitor

EXPERIMENT 3: EFFECTS OF DIFFERENT SOLVENT FRACTIONS OF *Drymaria cordata* ON RAT LIVER MPT PORE IN THE ABSENCE OF CALCIUM

INTRODUCTION

Studies have shown that some dietary components of plant origin induce apoptosis by inducing mPT pore opening. Several natural compounds have been shown to modulate the mPT pore in order to elicit their pharmacological effect (Millimouno *et al.,* 2014). This experiment was aimed at investigating the influence of varying concentrations of the different solvent fractions of *Drymaria cordata* on mPT pore.

PROCEDURE

The mitochondria were isolated as described on page 60 while the swelling assay was carried out as described on page 66. Different concentrations of the solvent fractions used in this experiment were 10, 30, 50 and 70 and 90 µg/ml.

RESULTS

The effects of various concentrations of MEDC on mPT pore in the absence of calcium was depicted in Figure 25. From the results, all the concentrations induced pore opening by 2.5, 4.6, 10 and 13 folds, respectively, relative to control. Maximum induction (13 folds) was seen at (90µg/ml) and the least induction fold (2.5) was obtained at (30µg/ml) when compared to the control. In Figure 26, the influenc of varying concentrations of CFDC on the pore in the absence of calcium were clearly depicted. Here, different concentrations of the fraction exhibited a significant induction of pore opening by 3.6, 13, 15, 18 and 17 folds, respectively, at all the

concentrations used. The pattern of induction by the various concentrations of the different solvent fractions was concentration-dependent.

Like calcium-induced pore opening, the MEDC and CFDC induction of pore opening was reversed by spermine. Figures 27 and and 28 show the effects of EADC and AFDC on mPT pore in the absence of calcium. At lower concentrations (10μg/ml, 30μg/ml, 50 μg/ml, 70 μg/ml), both fractions had no significant effect on pore opening. However, at 90 μg/ml, both fractions had significant inductive effect of 3.1 and 1.8 folds, respectively.

CONCLUSION

All the solvent fractions induced mPT pore with CFDC being the most potent with respect to induction of pore opening. The potency of induction is in the order CFDC > MEDC > AFDC>EFDC.

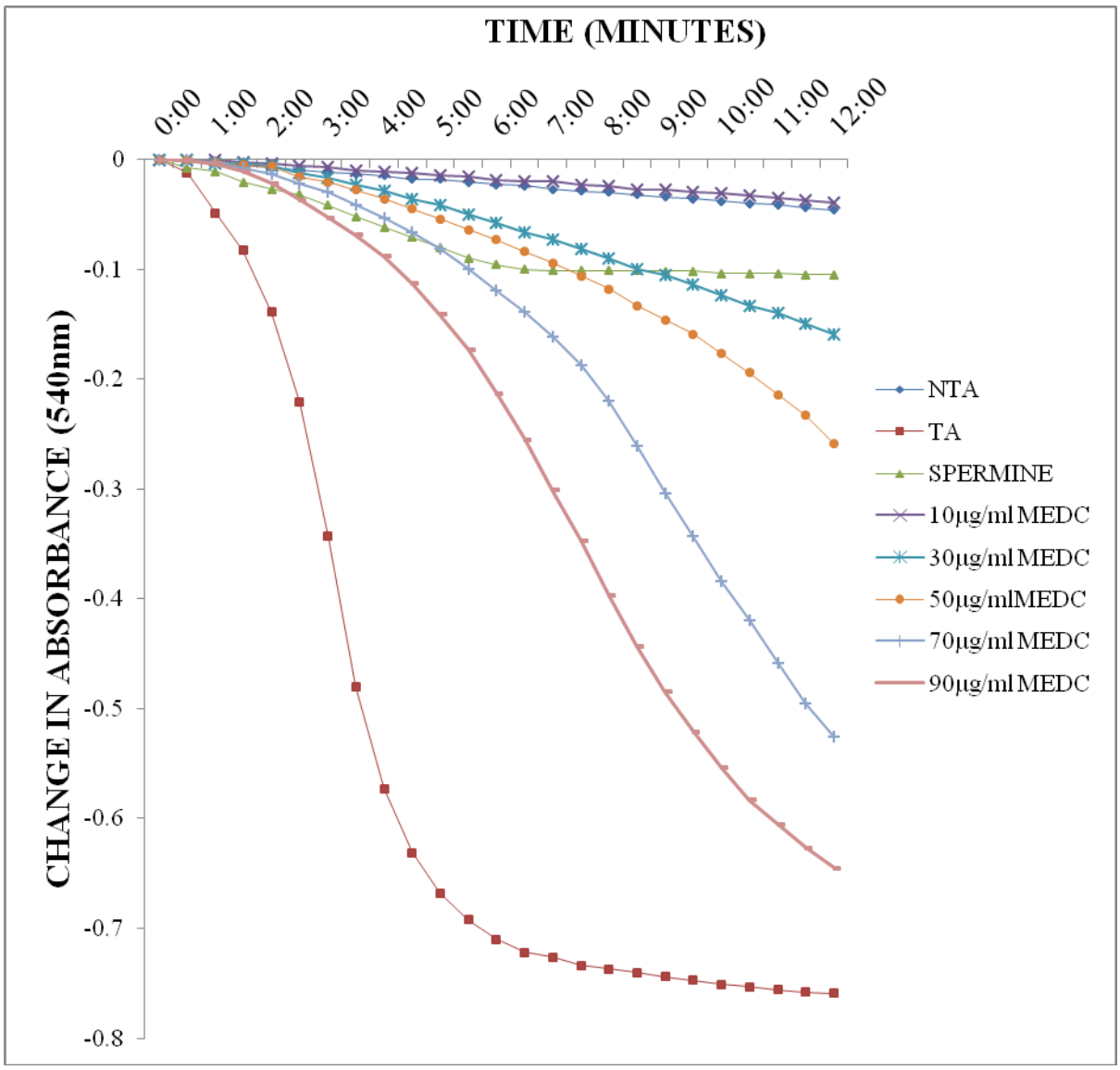


Figure 25: Methanol extract of *Drymaria cordata* (MEDC) induced mPT pore in the absence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

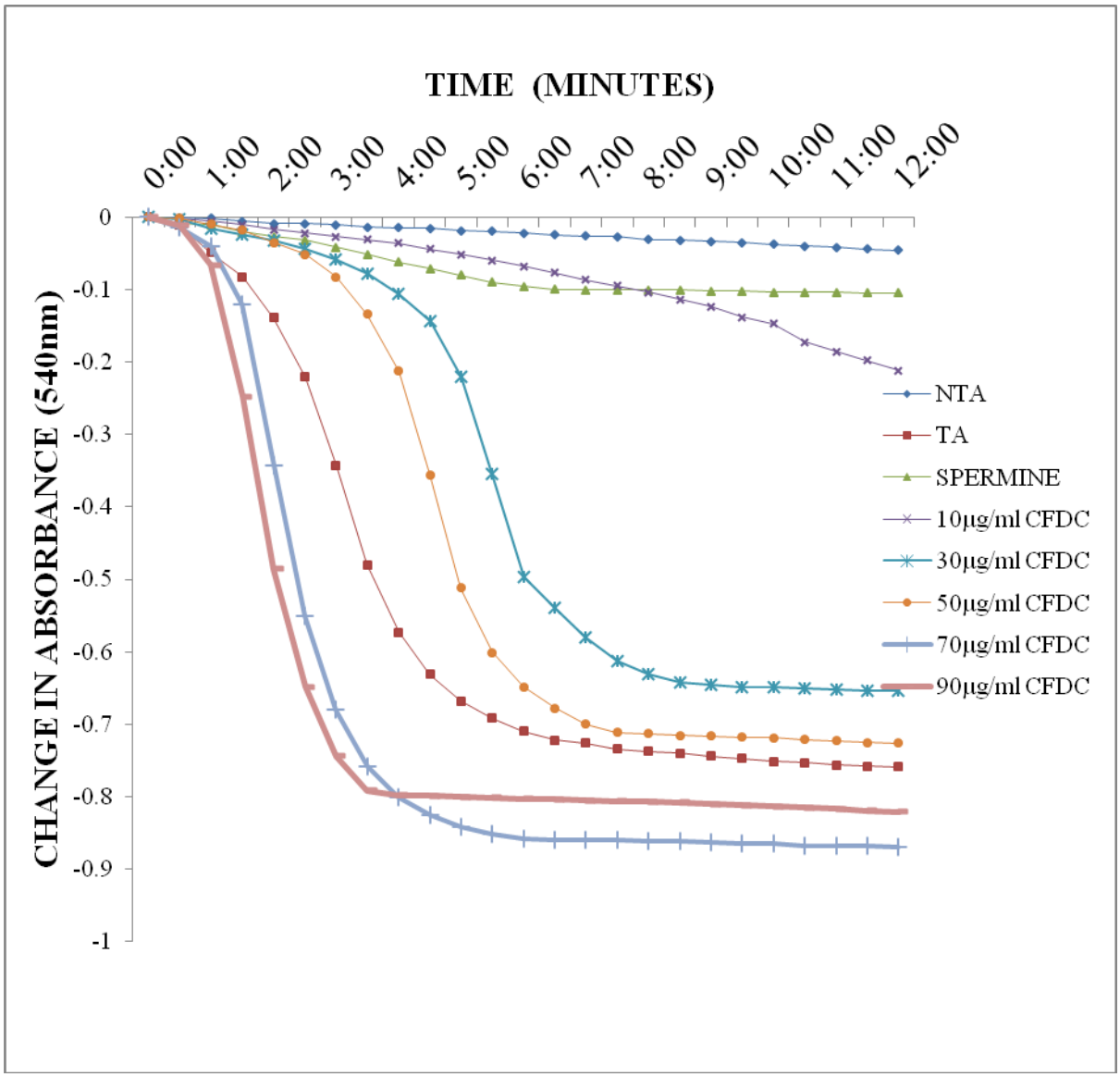


Figure 26: Chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) induced mPT pore opening in the absence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

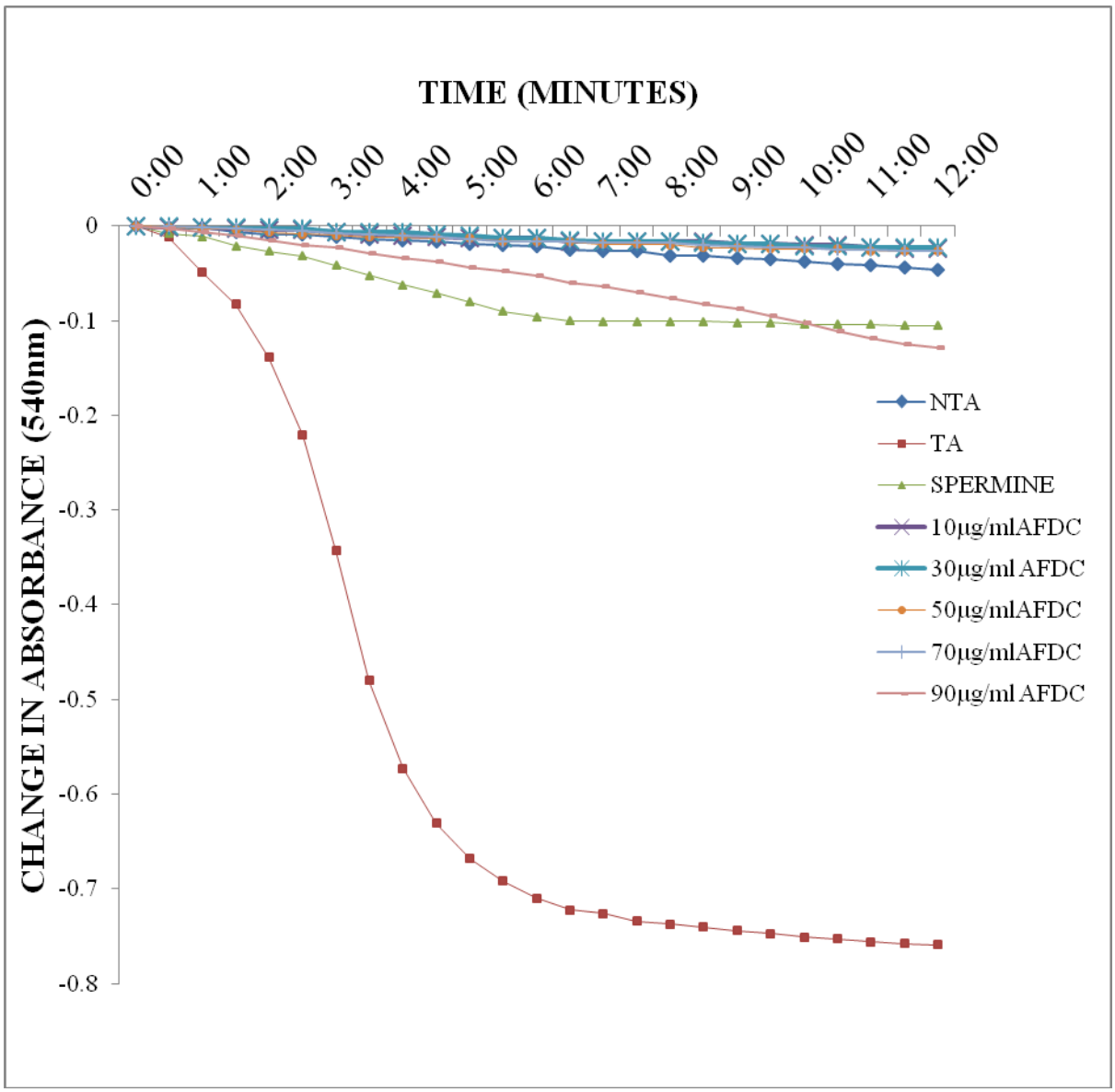


Figure 27: Effect of varying concentrations of aqueous fraction of methanol extract of *Drymaria cordata* (AFDC) on mPT pore in the absence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

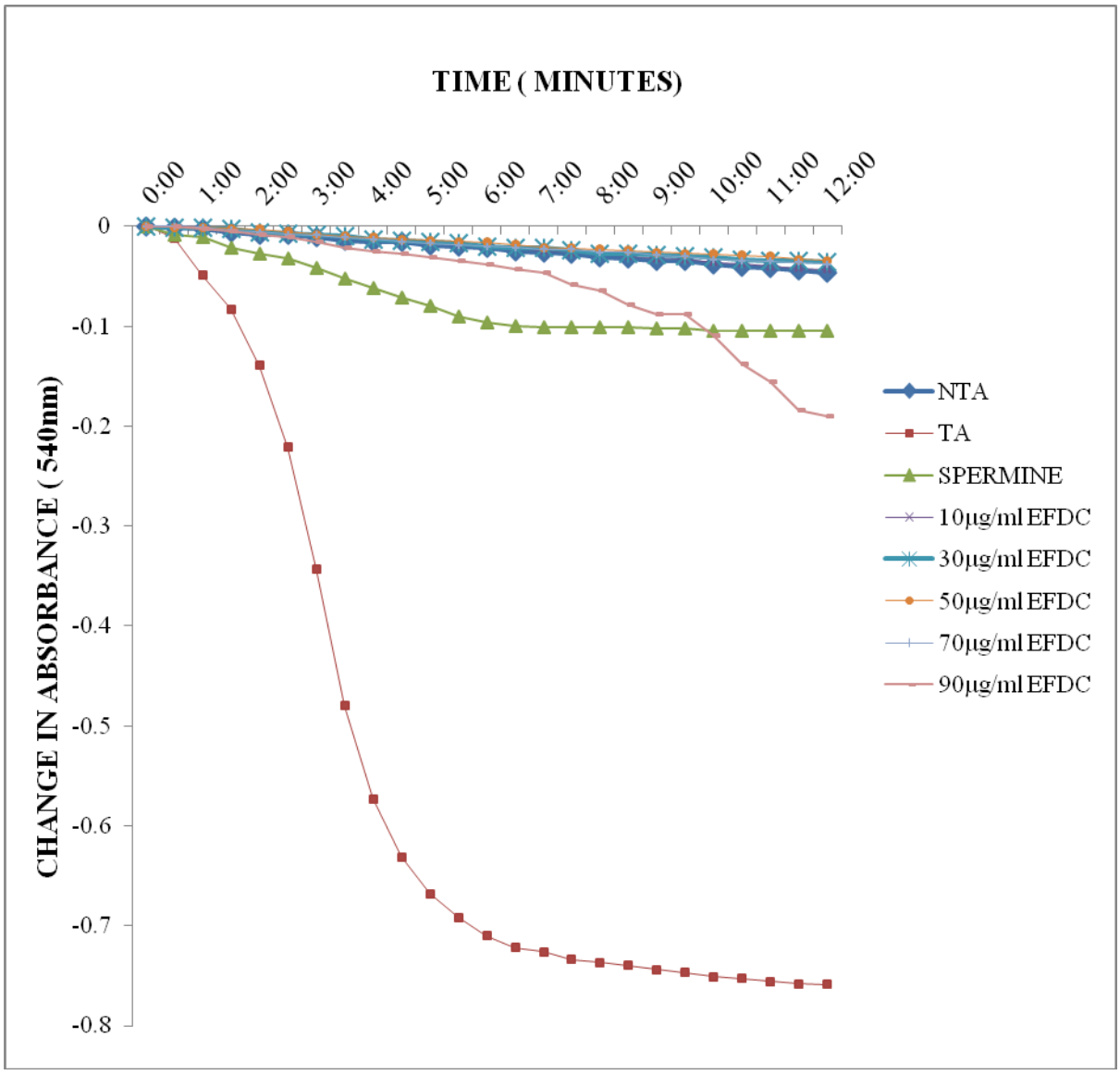


Figure 28: Effects of varying concentrations of ethylacetate fraction of methanol extract of of *Drymaria cordata* (EFDC) on the mPT pore in the absence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

EXPERIMENT 4: EFFECTS OF DIFFERENT SOLVENT FRACTIONS OF *DRYMARIA CORDATA* ON RAT LIVER MPT PORE IN THE PRESENCE OF CALCIUM

INTRODUCTION

Mitochondrion is an organelle that supports life of a cell and could also be an agent of cell suicide. The level of mitochondrial calcium determines the mPT induction. This experiment was aimed at assessing the effect of varying concentrations of solvent fractions of the *Drymaria cordata* on mitochondrial permeability transition using calcium as triggering agent.

PROCEDURE

Mitochondria (2mg/ml protein) was pre incubated in the MSH buffer (2,200µl), 0.8µM rotenone (10µl) for 3 minutes after which 12mM Ca^{2+} (25µl), the triggering agent was added, and 30 seconds later, 5mM succinate (50 µl) was added to energize the reaction. The light scattering effect was measured at 540nm over a period of 12 minutes at 30 seconds interval in a CamSpec M106 spectrophotometer.

RESULTS

The results as shown in Figures 29 and 30 show that calcium-induced opening was inhibited. In this regard, MEDC at all concentrations inhibited calcium-induced opening respectively by 38, 48, 50, 57 and 65% while CFDC at the same concentrations reversed pore opening by 7, 14, 15, 28 and 34%, respectively. Spermine, a standard inhibitor, showed 81% inhibition. However, similar concentrations of EFDC and AFDC did not inhibit calcium-induced opening as shown in Figures 31 and 32.

CONCLUSION

CFDC and MEDC inhibited calcium-induced pore opening in a concentration-dependent manner while EFDC and AFDC did not inhibit calcium-induced opening. .

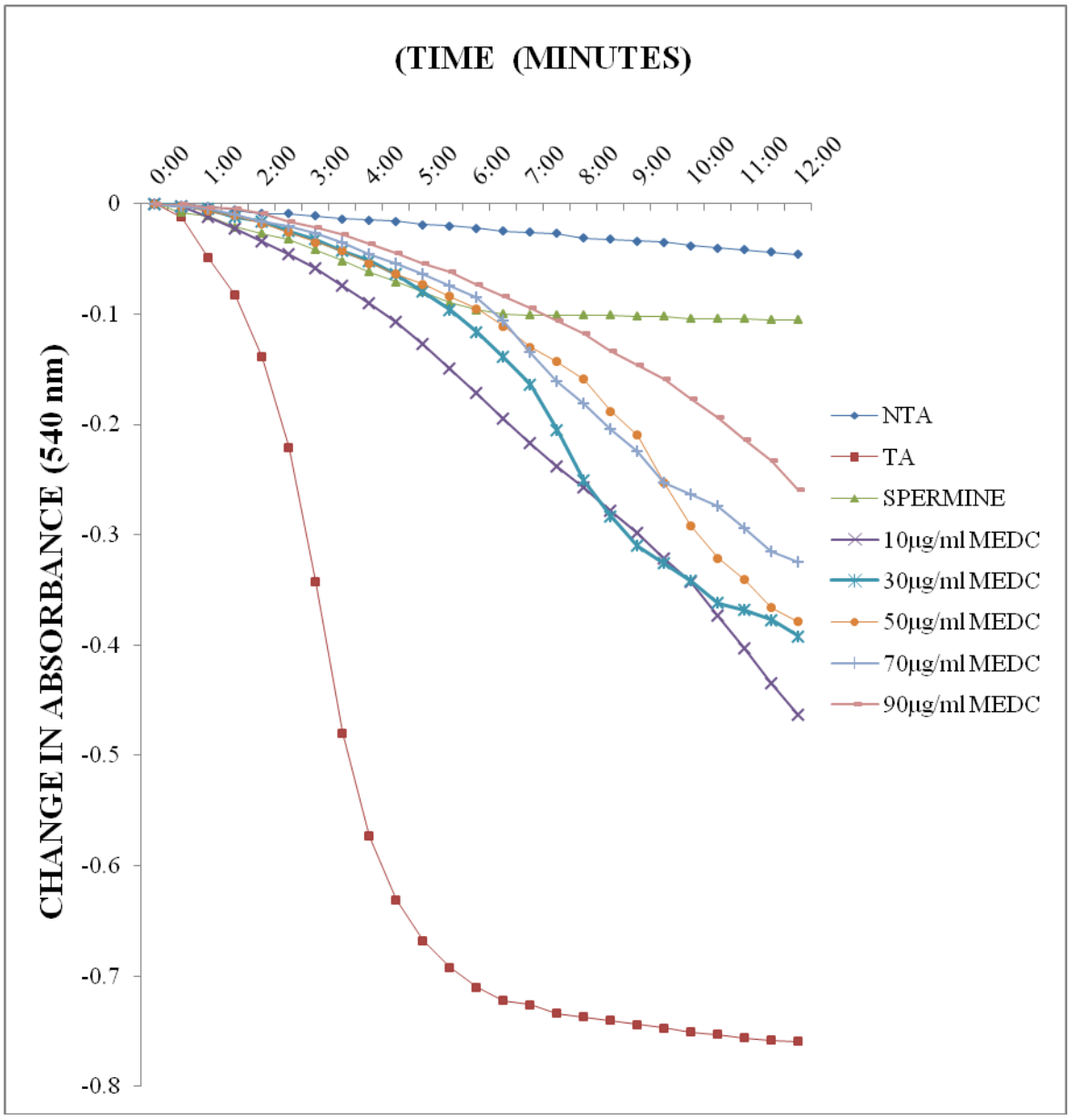


Figure 29: Inhibition of calcium-induced mPT pore opening by methanol extract of *Drymaria cordata* (MEDC) in the presence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

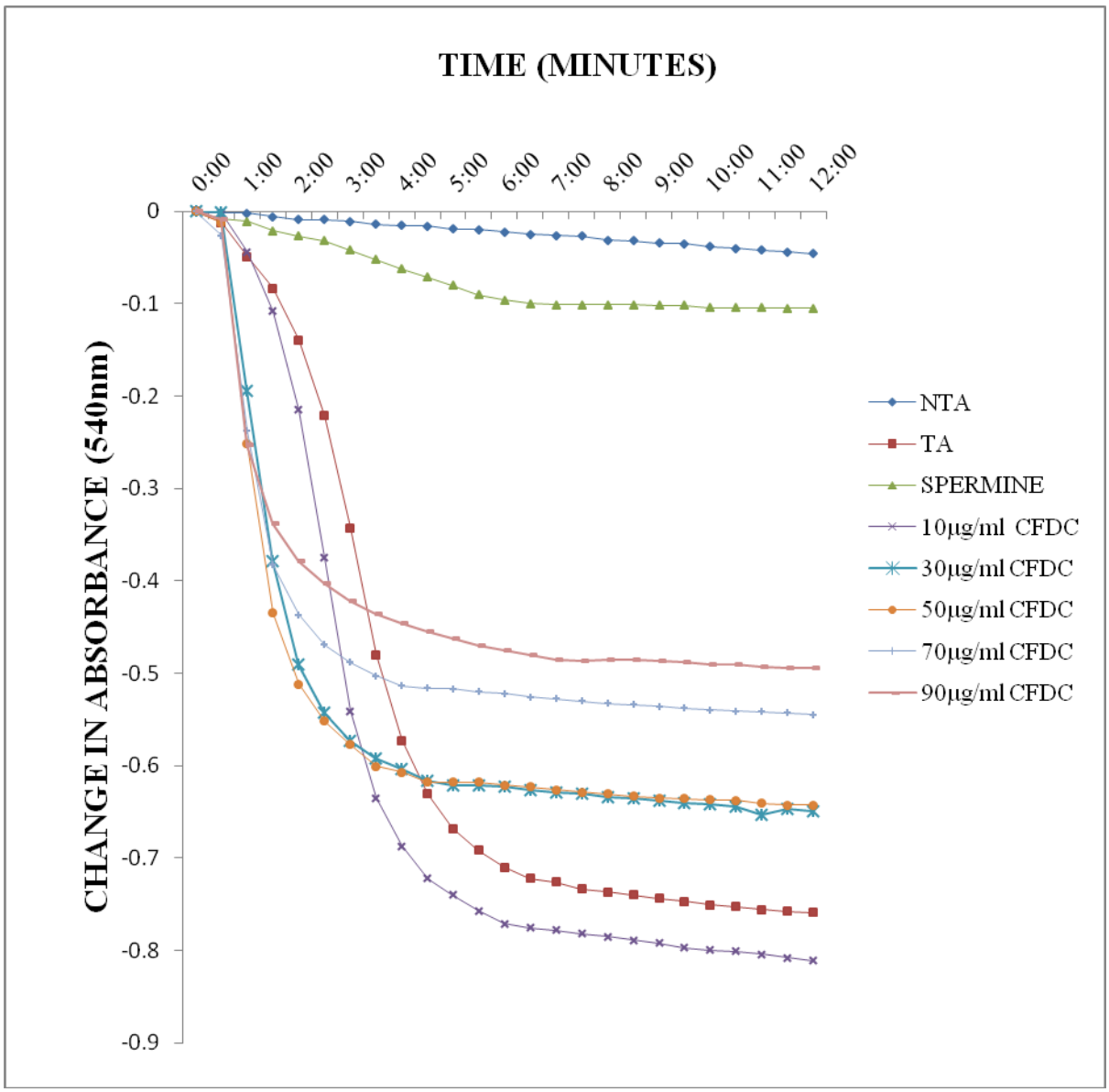


Figure 30: Inhibition of calcium-induced mPT pore opening by chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) in the presence of calcium.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

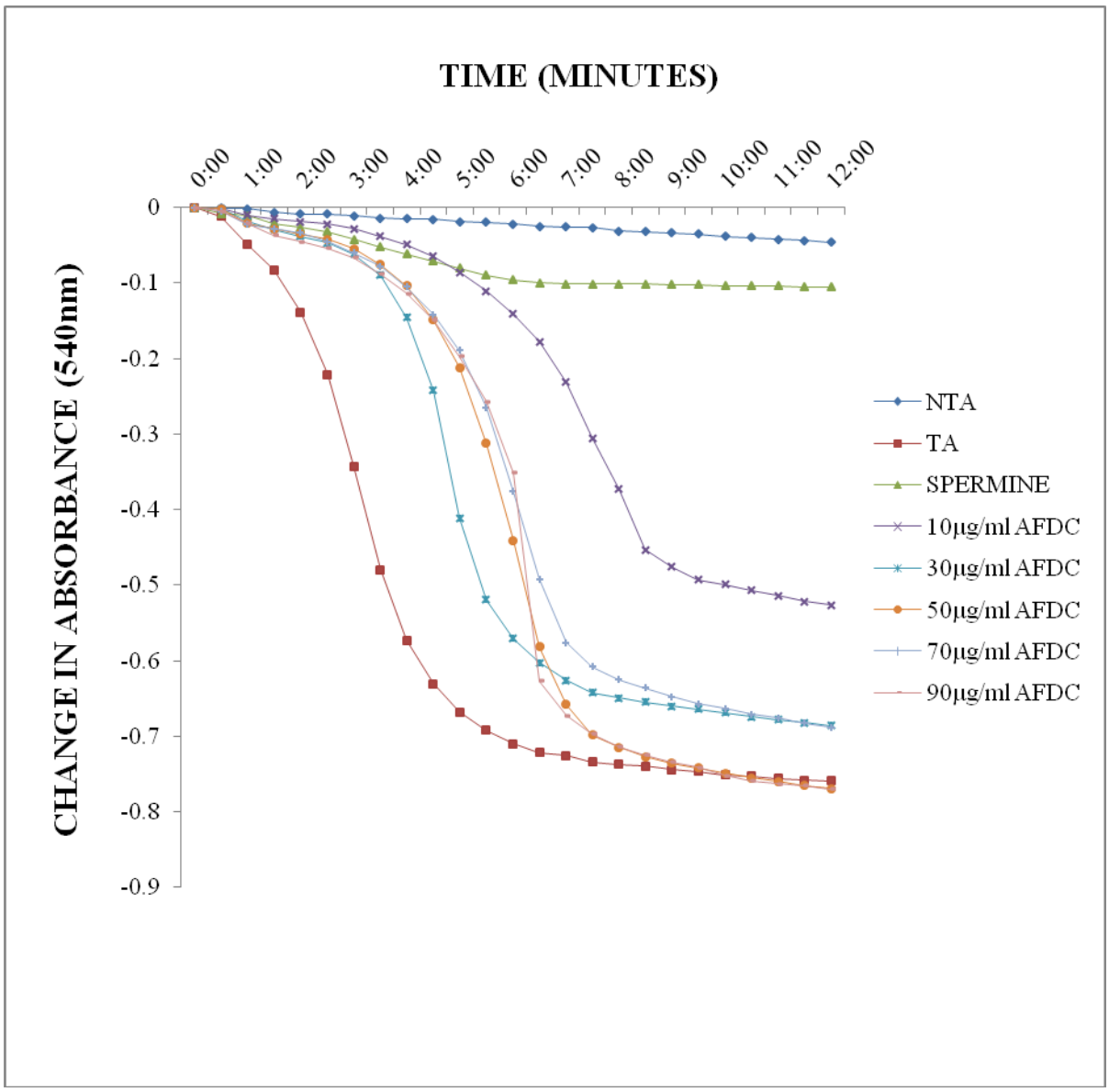


Figure 31: Effect of varying concentrations of aqueous fraction of methanol extract of *Drymaria cordata* (AFDC) on the mPT pore in the presence of calcium

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

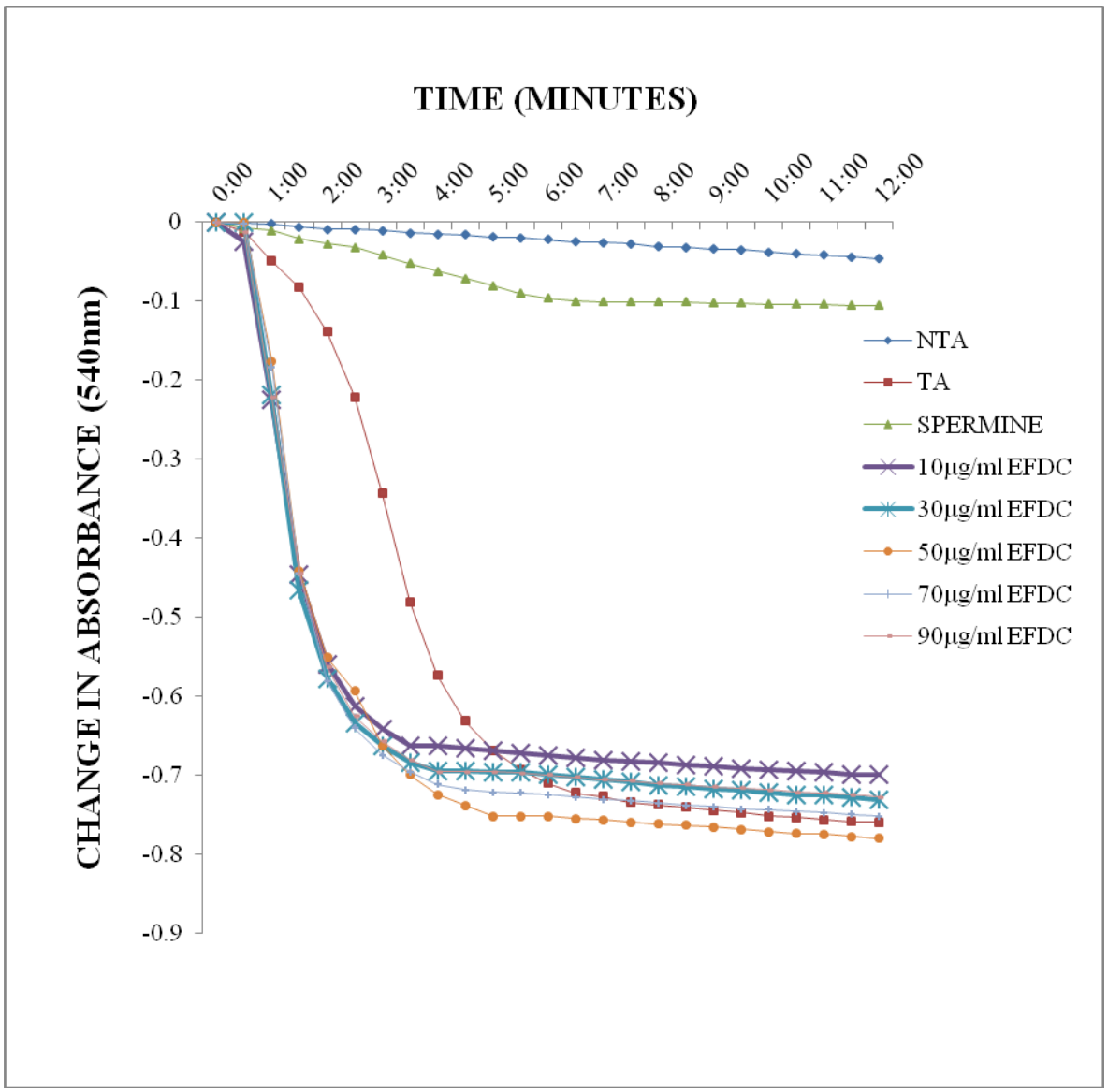


Figure 32: Effect of varying concentrations of ethylacetate fraction of methanol extract of *Drymaria cordata* (EFDC) on the mPT pore in the presence of calcium

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

EXPERIMENT 5: EFFECTS OF SPERMINE ON CHLOROFORM FRACTION OF *Drymaria cordata*–INDUCED OPENING OF MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN THE PRESENCE OF CALCIUM

INTRODUCTION

One of the primary factors that cause the opening of pore is Ca^{2+} (Elustondo *et al*., 2015). This experiment was aimed at investigating the influence of spermine, a standard inhibitor of pore opening on the potent chloroform fraction–induced mPT pore opening in the presence of calcium.

PROCEDURE

Mitochondrial swelling involved preincubation of the mitochondria in the presence of MSH buffer (2,200µl), 0.8µM rotenone (10µl) for 3 minutes before addition of varying concentrations (10, 30, 50, 70 and 90µg/ml) of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) and 12 mM calcium (25µl) after which 5mM succinate (50µl) was added to energize the reaction.

Addition of 4mM spermine (63µl) was done immediately following addition of 0.8µMrotenone and just before the addition of mitochondria (2mg protein/ml).

RESULTS

The results as shown in Figure 33 depicts the effect of spermine on chloroform fraction-induced mPT pore opening in the presence of spermine and calcium. The induction of pore opening by CFDC was reversed by spermine. This shows that the integrity of the mitochondria is not

damaged/compromised as a result of pore opening by CFDC due to its reversal by spermine, a standard inhibitor. This is also in accordance with the results obtained in calcium-induced opening, which was also reversed by spermine.

CONCLUSION

The induction of pore opening by CFDC was reversed by spermine showing that the integrity of the mitochondria was still maintained.

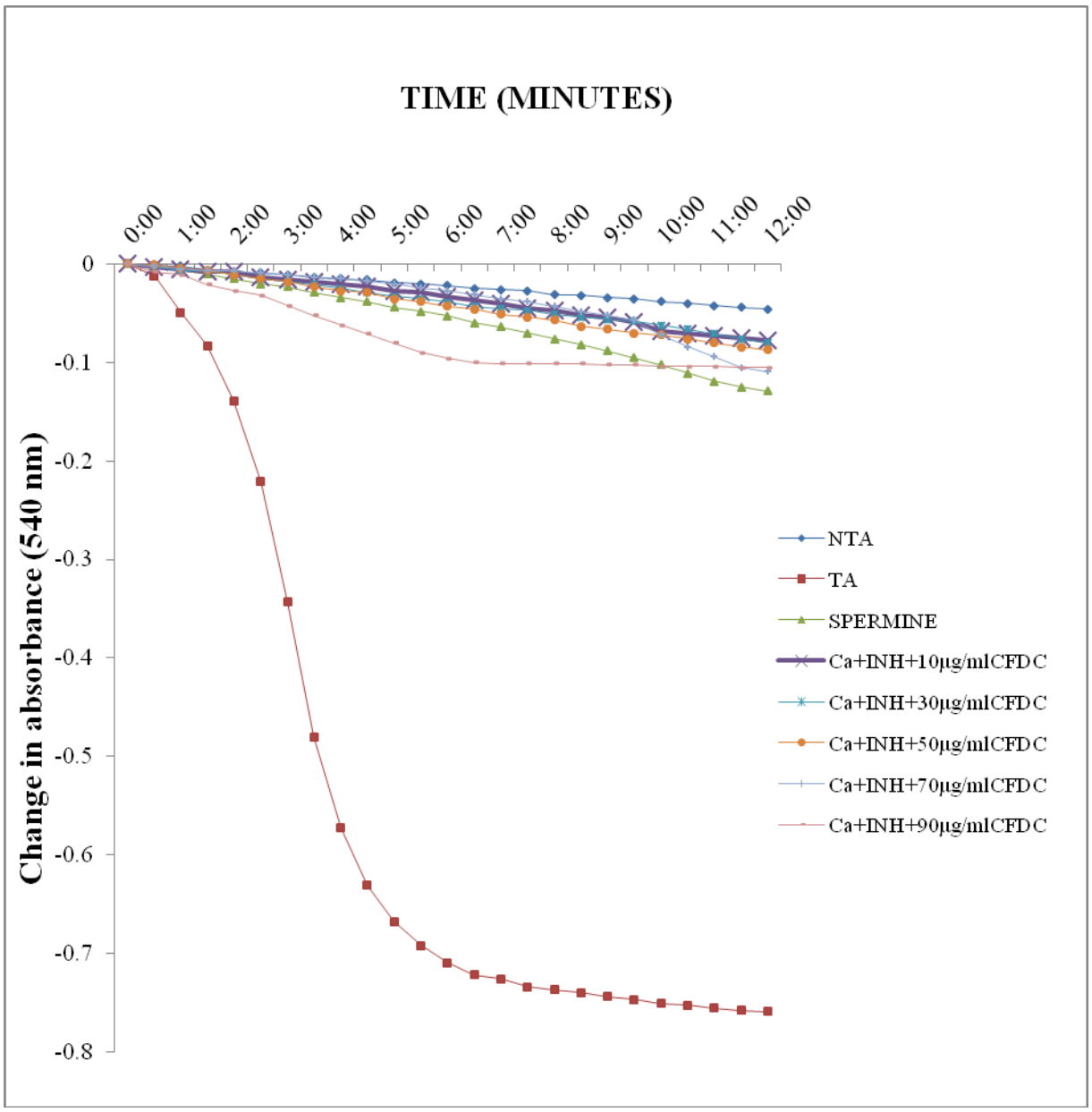


Figure 33: Effects of spermine on chloroform fraction of methanol extract of *Drymaria cordata* (CFDC)-induced opening of mPT pore in the presence of calcium

NTA: No triggering agent (without calcium)

TA: Triggering agent (Calcium)

Spermine: Standard inhibitor

EXPERIMENT 6: EFFECTS OF DIFFERENT SOLVENT FRACTIONS OF *Drymaria cordata* ON MITOCHONDRIAL ATPase ACTIVITY

INTRODUCTION

Complex V catalyses the synthesis of ATP in the mitochondria. It can also function in hydrolyzing ATP (Bason *et al*., 2014). This experiment was therefore designed to test the different solvent fractions of *Drymaria cordata* on mitochondrial ATPase activity in order to ascertain the status of the mPT pore of mitochondria using concentration of inorganic phosphate released as an index of enhancement of mitochondrial ATPase activity, 2, 4 dinitrophenol was used as a classical inducer of ATPase activity.

PROCEDURE

The reaction medium contained 65mM Tris-HCL buffer pH 7.4, 0.5mM KCl, 1mM ATP and 0.25M sucrose in a total volume of 2 ml. Distilled water or the test compound was added accordingly. The mitochondrial fraction was added to start the reaction and allowed to proceed for 30 minutes with constant shaking at 37^{o}C. Aliquot amount (1ml) of sodium dodecyl sulfate (SDS) was immediately put in the zero time test tube to stop the reaction after the addition of the mitochondria while the reaction in the other tubes was stopped at 30 seconds interval by the addition of 1ml of sodium dodecyl sulfate (SDS). Aliquot amount (1ml) of the reaction media was taken across board into a clean test tube. Ammonium molybdate (I ml) was put into all the test tubes. L-ascorbate (1 ml) was then added to the test and after 20 minutes, the absorbance was taken at 660nm.

RESULTS

Figure 34 shows the effects of varying concentrations of the solvent fractions on mitochondrial F_0F_1 ATPase activity. The data obtained show that MEDC, CFDC, EFDC and AFDC significantly ($P<0.05$) stimulated the ATPase activity to varying degrees. Furthermore, the results show that CFDC had the highestt stimulatory effect followed by MEDC. However, the stimulatory effects shown by EFDC and AFDC were very low and also statistically not significantly different from each other.

CONCLUSION

Both MEDC and CFDC significantly enhanced mitochondrial ATPase activity with CFDC being more potent.

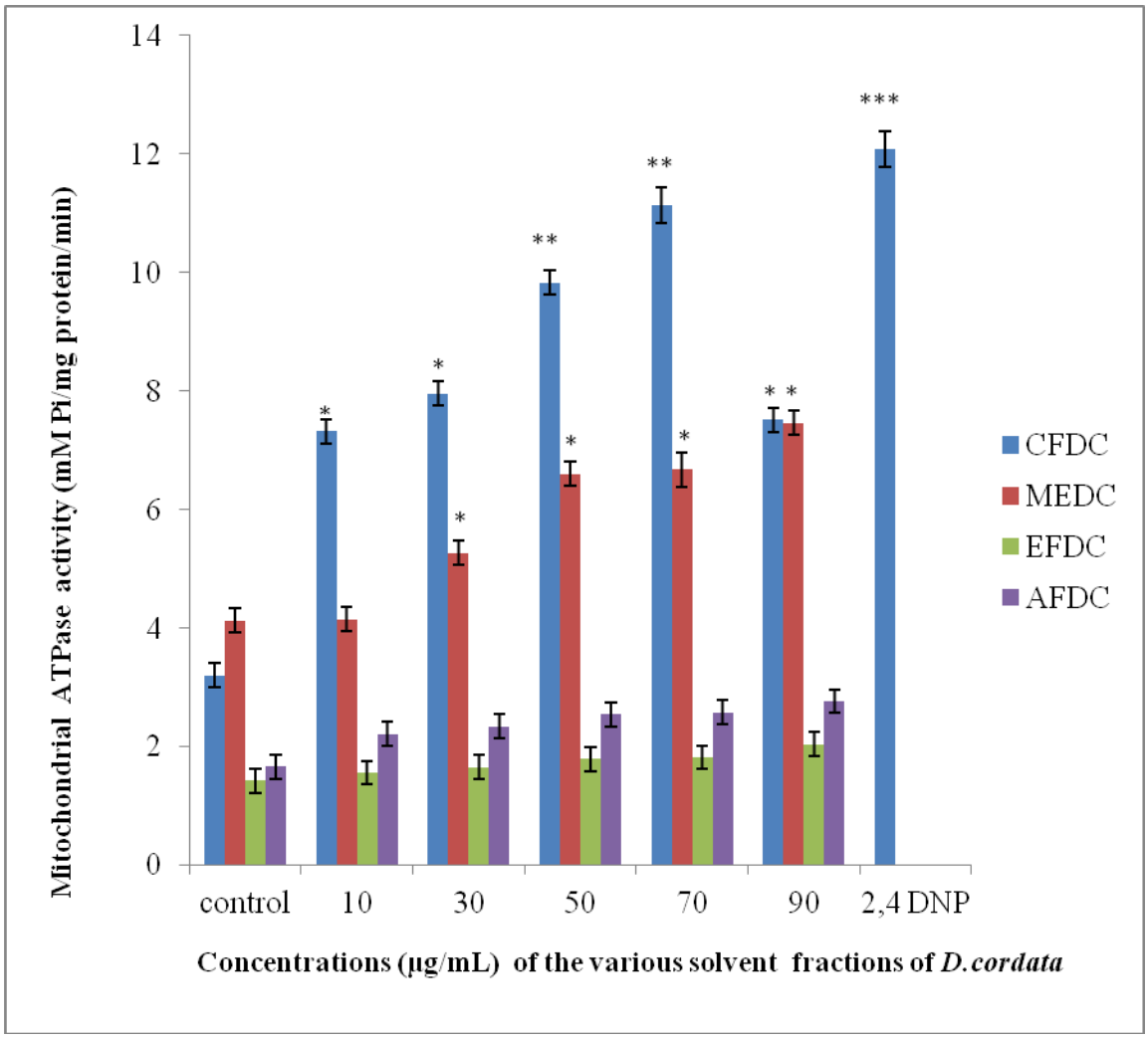


Figure 34: Effects of varying concentrations of solvent fractions of methanol extract of *Drymaria cordata* on mitochondrial ATPase activity

CFDC: Chloroform fraction of methanol extract of *Drymaria cordata*

MEDC: Methanol extract of *Drymaria cordata*

EFDC: Ethylacetate fraction of methanol extract of *Drymaria cordata*

AFDC: Aqueous fraction of methanol extract of *Drymaria cordata*

EXPERIMENT 7: EFFECTS OF DIFFERENT SOLVENT FRACTIONS OF *Drymaria cordata* ON Fe^{2+}-INDUCED MITOCHONDRIAL LIPID PEROXIDATION (LPO)

INTRODUCTION

Some phytochemicals have been identified to mop up free radicals and also inhibit lipid peroxidation. Reactive oxygen species can attack unsaturated fatty acid and thus cause lipid peroxidation and this can cause several human pathological disorders. This experiment was carried out to investigate if the solvent fractions of *Drymaria cordata* are associated with the generation of malondialdehyde.

PROCEDURE

The experiment was done as described by Ruberto *et al*. (2000). Aliquot portion of liver mitochondria (30µl) was added to varying concentrations of the test sample in test tubes. Iron sulphate (0.07M) was added to induce lipid peroxidation and the mixture incubated for 30 minutes at 37 °C. This is followed by the addition of 20% acetic acid (1.5 ml) and 0.8% thiobarbituric acid (0.5 ml) in 1.1% sodium dodecyl sulphate (SDS) (1 ml) and well mixed , after which it was heated at 95 °C for 60 minutes. The medium was cooled, butan-1-ol (3 ml) was added and centrifuged at 3000 rpm for 10 minutes. The absorbance of the upper organic layer was measured at 532nm**.**

RESULTS

Effect of the fractions of *Drymaria cordata* on ferrous-induced LPO is shown in Figure 35.The results show that MEDC, CFDC, EFDC and AFDC ameliorated ferrous-induced lipid peroxidation in a concentration-dependent manner.

CONCLUSION

All the solvent fractions inhibited iron-induced mitochondrial lipid peroxidation.

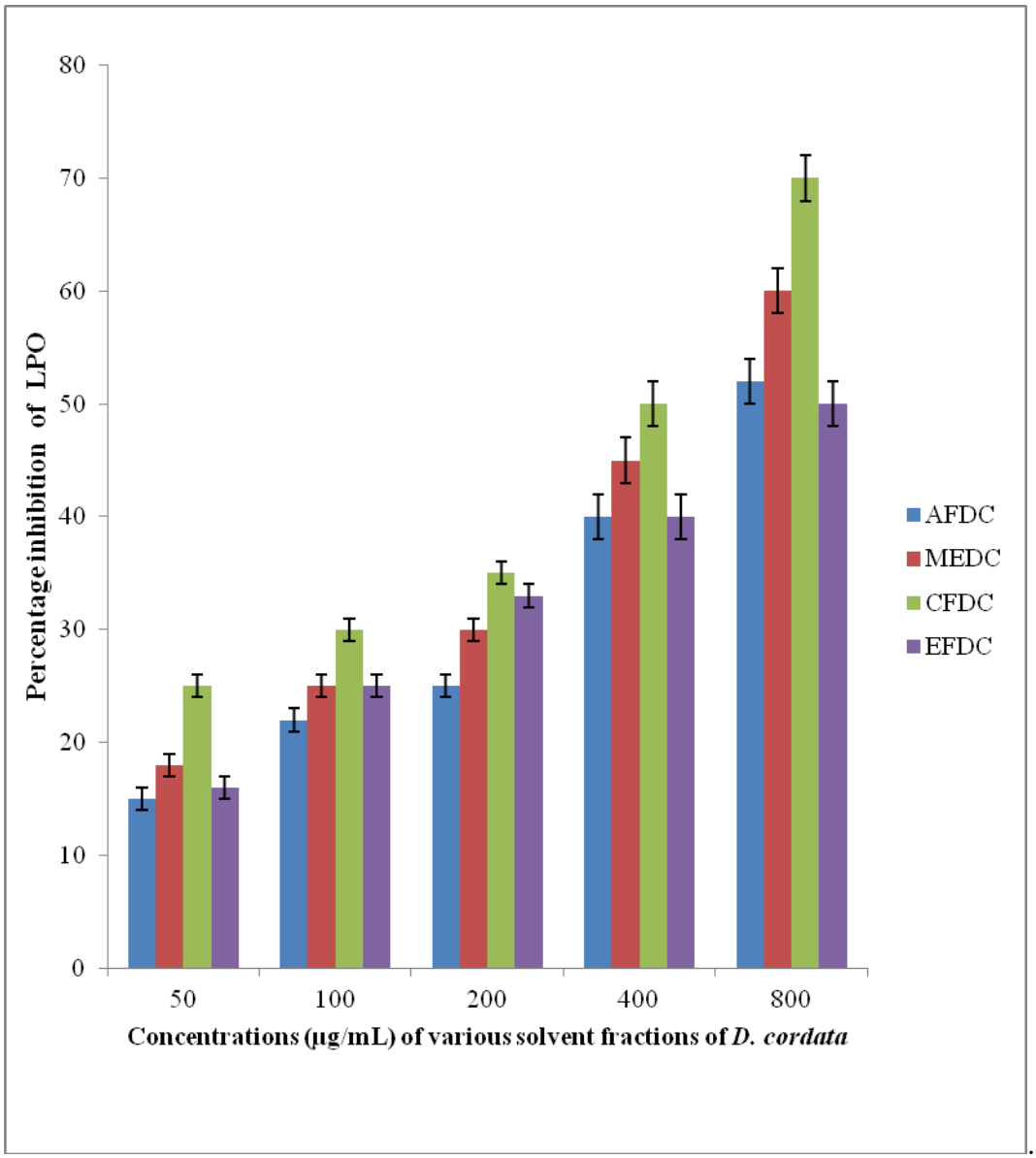


Figure 35: Inhibition of ferrous-induced LPO by fractions of *Drymaria cordata*

CFDC: Chloroform fraction of methanol extract of *Drymaria cordata*

MEDC: Methanol extract of *Drymaria cordata*

EFDC: Ethylacetate fraction of methanol extract of *Drymaria cordata*

AFDC: Aqueous fraction of methanol extract of *Drymaria cordata*

EXPERIMENT 8: EFFECTS OF DIFFERENT SOLVENT FRACTIONS OF *Drymaria cordata* ON CYTOCHROME C RELEASE

INTRODUCTION

When the mitochondria permeability transition pore opens, cytochrome c is released. This activates caspases and ultimately cell (Eleftheriadis *et al.,* 2016). This experiment was aimed at determining the effect of the solvent fractions on cytochrome c release as a consequence of pore opening and ATPase activity enhancement.

PROCEDURE

Mitochondria (1mg protein/ml) were preincubated in the presence of 0.8μM rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 30minutes at 27 ^{o} C in the presence of different concentrations of the fractions, using 24mM calcium as the standard. After the incubation, the mixture was centrifuged at 15,000 rpm for 10 minutes. The optical density of the supernatant was measured at 414nm which is the soret (γ) peak for cytochrome c.

RESULTS

The effects of varying concentrations of the solvent fractions of *Drymaria cordata* on cytochrome c release are shown in Figure 36. As seen from the results, there was significant release of cytochrome c following exposure to these fractions; the extent of release being CFDC > MEDC while EFDC and AFDC are not significantly different from control.

CONCLUSION

There was significant induction of cytochrome c release by MEDC, CFDC with CFDC being more potent than MEDC. These results are also in consonants with the mPT and mATPase results.

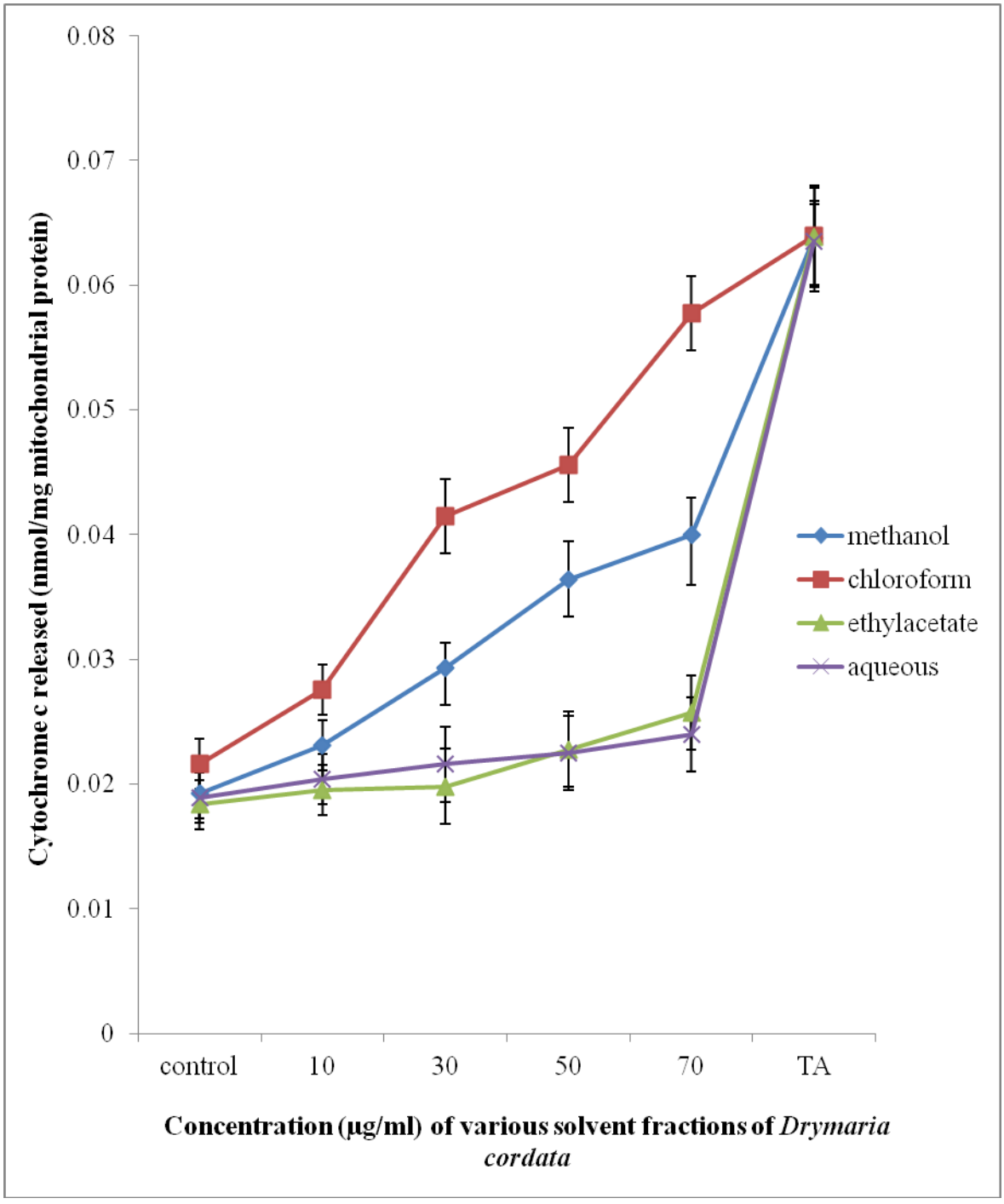


Figure 36: Effects of solvent fractions of methanol extract of *Drymaria cordata* on cytochrome c release.

EXPERIMENT 9: EFFECTS OF 14 AND 28 DAYS ADMINISTRATION OF CHLOROFORM FRACTION OF *Drymaria cordata* (CFDC) ON RAT LIVER AND UTERINE MITOCHONDRIAL PERMEABLILITY TRANSITION PORE IN THE ABSENCE OF CALCIUM

INTRODUCTION

Apoptosis, a form of programmed cell death, is a crucial physiologic process in the development and homeostasis of multicellular organisms (Baehrecke, 2002). Perturbation of this vital process leads to a range of diseases, such as ischemia, cancer, neurodegeneration, and autoimmunity (Saikumar *et al*., 1999).

This study was carried out to investigate the effect of oral treatment of CFDC on the rat liver and uterus mPT pore.

PROCEDURE

Wistar strain of albino rats used in this study were acclimatized for two weeks after which they were treated with Chloroform fraction of methanol extract of *Dymaria cordata* (CFDC) for 14 and 28 days.

Dose Regimen:

Grp 1 : Control (Corn oil containing 5% DMSO)

Grp 2: 50mg/kg bd wt CFDC

Grp 3: 100mg/kg bd wt CFDC

Grp 4: 200mg/kg bd wt CFDC

The animals were sacrificed 24 hours after the last dose for 14 and 28 days daily administration of chloroform fraction of methanol extract of *Drymaria cordata*. The liver and uterus were excised, minced, homogenized and then centrifuged to isolate the liver and uterine mitochondria as described on pages 60. The protein content and mitochondrial swelling were carried out as described on pages 62 and 66.

RESULTS

The data presented in Figure 37 shows the representative profile of the effect of calcium and spermine on liver mitochondria of rats following oral administration of CFDC for 14 and 28 days. There was no significant change in the volume of mitochondria respiring on succinate in the presence of rotenone in the mitochondria of control animals after oral administration for 14 and 28 days.

Calcium caused significant increase in mPT pore opening and was reversed by spermine (Figure 37). Figure 38 shows the effects of various doses of CFDC on liver mPT pore after 14 days of treatment. Administration of 50mg/kg bd wt showed no significant induction of pore opening while 100 and 200mg/kg bd wt induced pore opening by 3.3 and 5.5 folds, respectively, when compared with the control. Exposure to the CFDC for 28 days (Figure 39) at 50, 100 and 200mg/kg bd wt significantly induced mPT pore opening by 2.7, 5.1 and 7.7 folds, respectively. Also, the outcome of CFDC treatment observed in the case of uterine mitochondrial permeability transition showed dose-dependent induction of pore opening after 28 days of exposure. Induction fold of 1.8, 4.2 and 6.2 were recorded at doses of 50, 100 and 200mg/kg bd wt, respectively, as shown in Figure 40.

CONCLUSION

Oral administration of CFDC caused induction of rat liver and uterine mPT pore opening in a dose-dependent manner. Prolonged treatment caused increase in fold of induction of pore opening.

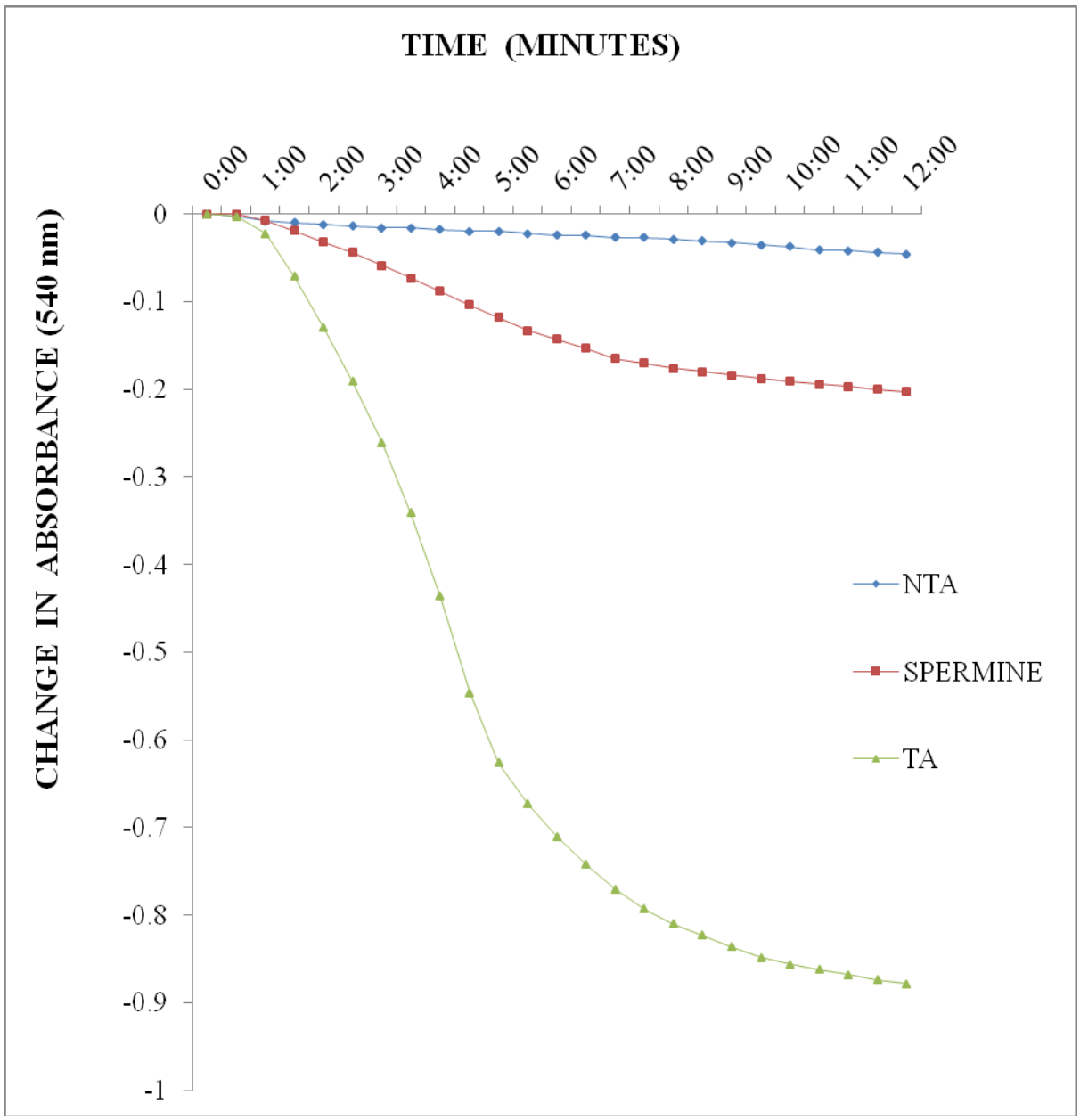


Figure 37: Profile of calcium-induced mPT pore opening in control rat liver mitochondria and its reversal by spermine after 14 and 28 days

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

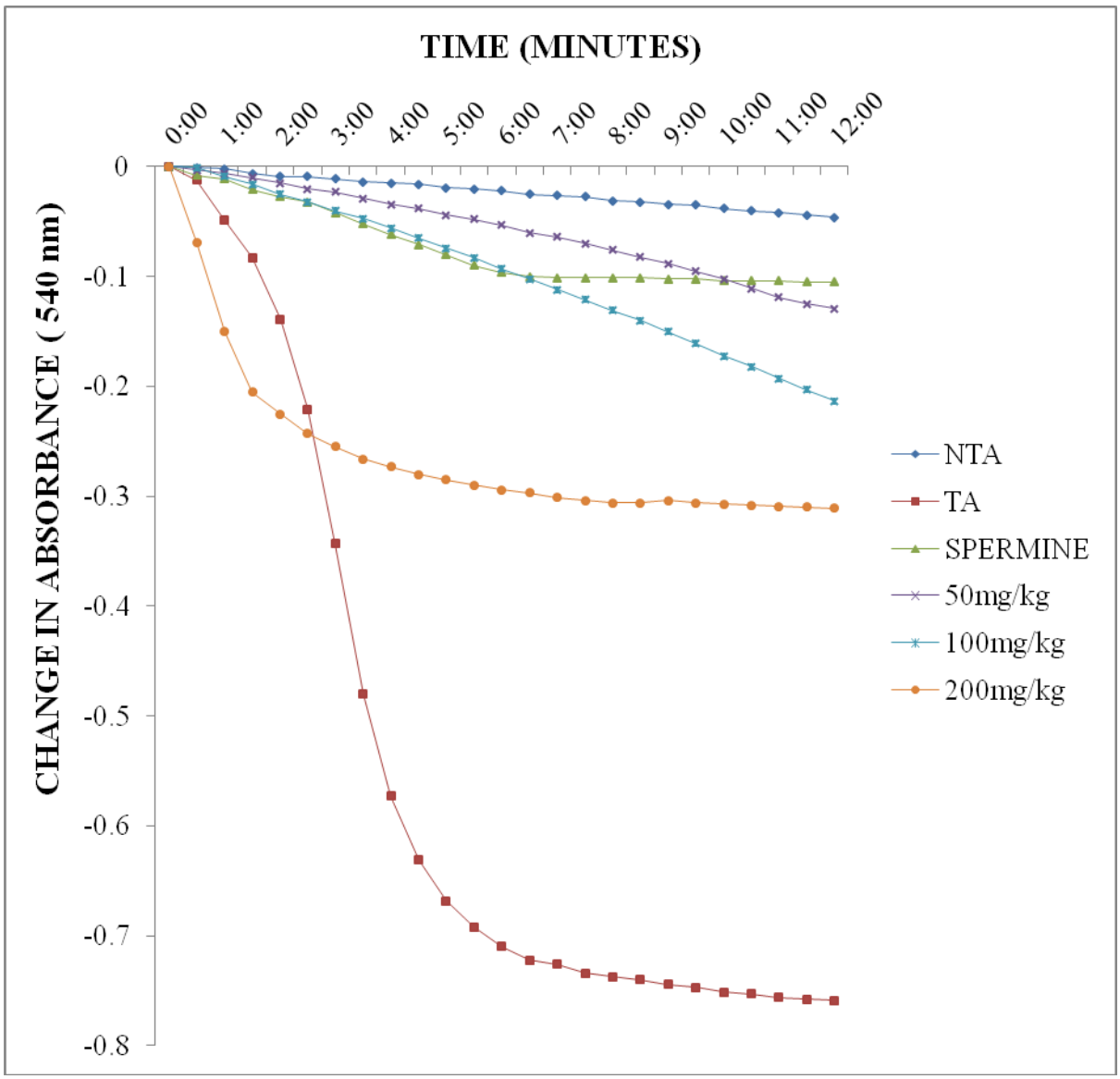


Figure 38: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) for 14 days on rat liver mPT pore.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

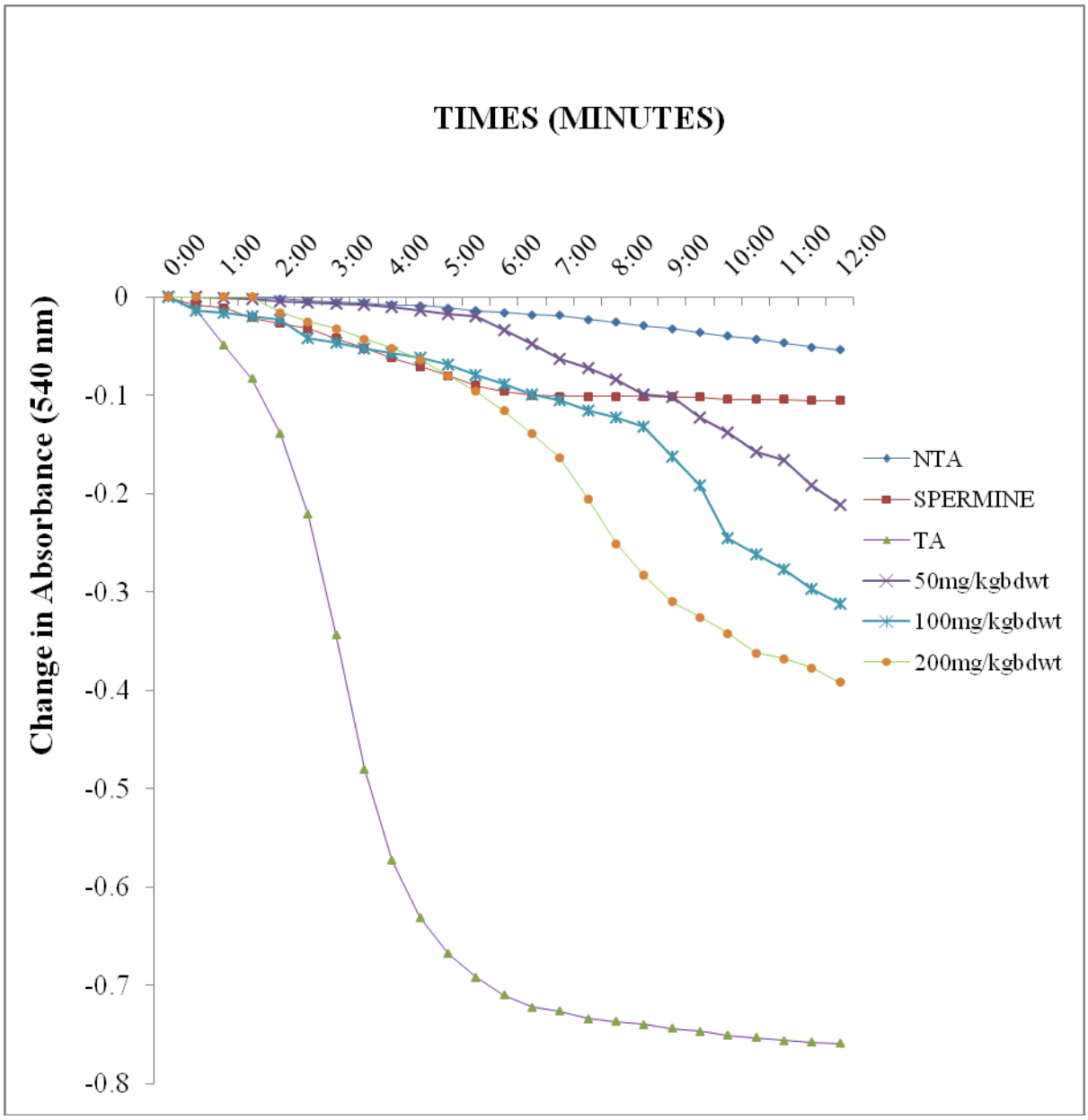


Figure 39: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) for 28 days on rat liver mPT pore

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine:Standard inhibitor

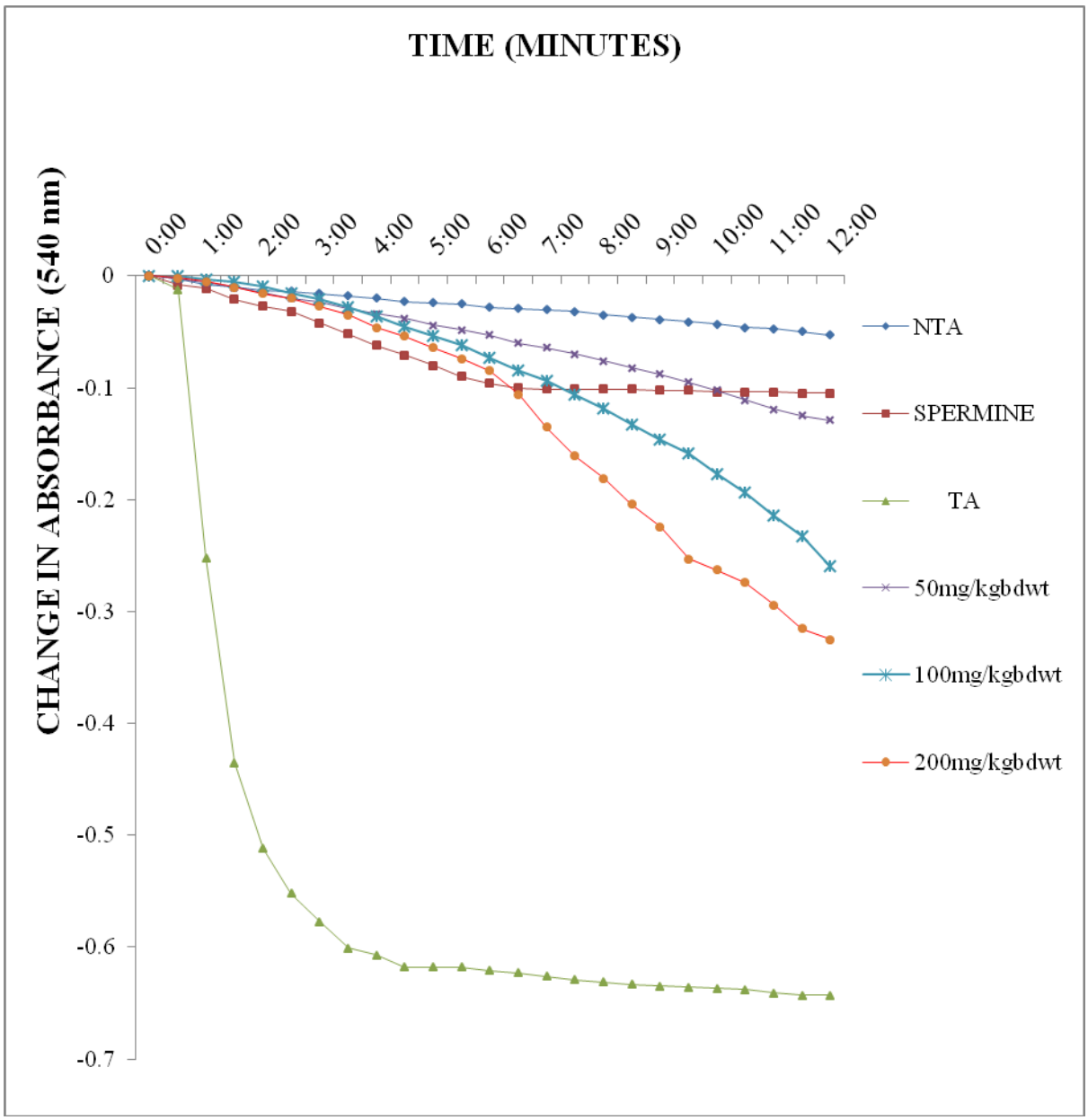


Figure 40: Effects of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* for 28 days on rat uterus mPT pore

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

EXPERIMENT 10: EFFECT OF 28 DAY ADMINISTRATION OF MONOSODIUM GLUTAMATE ON RAT LIVER AND UTERINE MITOCHONDRIAL PERMEABLILITY TRANSITION PORE IN THE ABSENCE OF CALCIUM

INTRODUCTION

Monosodium glutamate (MSG) is used in many countries as additive in different canned and packed food (Beyreuther *et al*., 2007). Its toxic effects have been reported by many studies (Sharma and Deshmukh, 2015). This experiment was designed to investigate if MSG would cause induction of mPT pore opening in the liver and uterus of rat exposed to MSG treatment for 28 days.

PROCEDURE

Wistar strain albino rats used in this study were orally treated for 28 days as shown below:

Dose Regimen:

Grp 1: Control (Corn oil containing 5% DMSO)

Grp 2: 50mg/kg bd wt MSG

Grp 3: 100mg/kg bd wt MSG

Grp 4: 200mg/kg bd wt MSG

After the last day of administration, the animals were sacrificed by cervical dislocation and the liver and uterus excised, minced, homogenized and then centrifuged to isolate the liver and

uterine mitochondria as described on pages 60. The protein content and mitochondrial swelling were carried out as described on pages 62 and 66.

RESULTS

The data presented in Figure 41 shows the pattern of the effect of calcium and spermine on mitochondria of control animals following oral administration of MSG for 14 and 28 days. There was little change in the volume of the mitochondria of control animals after oral administration for 14 and 28 days. Calcium caused induction of mPT pore opening and were reversed by spermine (Figure 41). Figure 42 shows the effects of various doses of MSG on rat liver mPT pore after 14 days of treatment. From the results, there was no induction of pore opening at all the dosages. However, administration of 100 and 200mg/kg bd wt for 28 days showed induction of pore opening by 4.2 and 6.2 folds, respectively, while 50mg/kg bd wt did not induce pore opening as shown in Figure 43. Also, oral administration of MSG did not cause any significant induction of uterine mPT pore opening at 50mg/kg bd wt while there was induction of pore opening at 100 and 200mg/kg bd wt by 4.3 and 7.5 folds, respectively, as depicted in Figure 44.

CONCLUSION

Oral administration of MSG did not induce pore opening at lower dosE but caused significant induction of rat liver and uterine mPT pore opening in a dose dependent manner at higher doses. Also, prolonged treatment with MSG caused increase in fold of induction of pore opening.

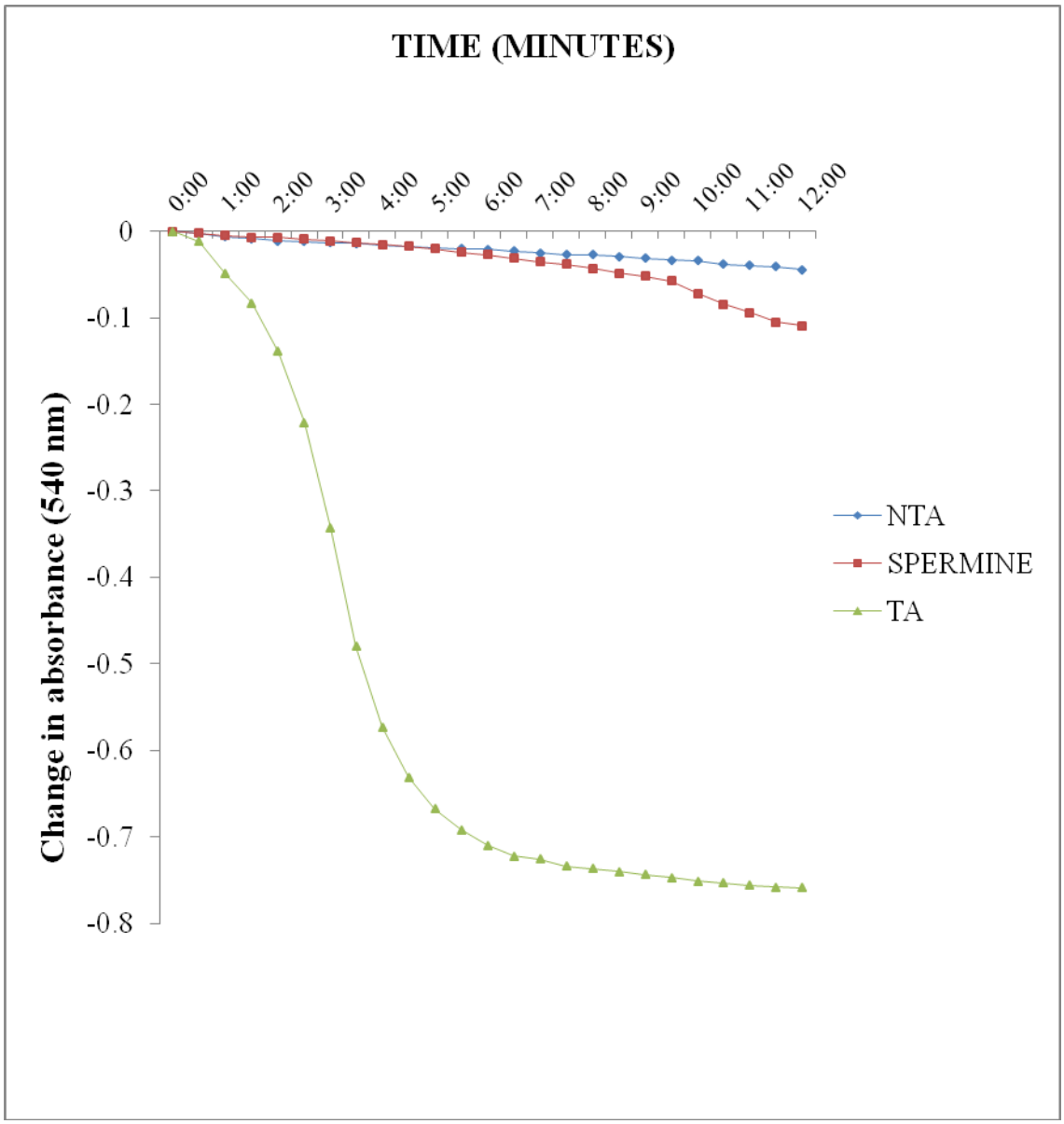


Figure 41: Profile of mPT pore induction by calcium and its reversal by spemine in control rat liver and uterus after 14 and 28 days.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

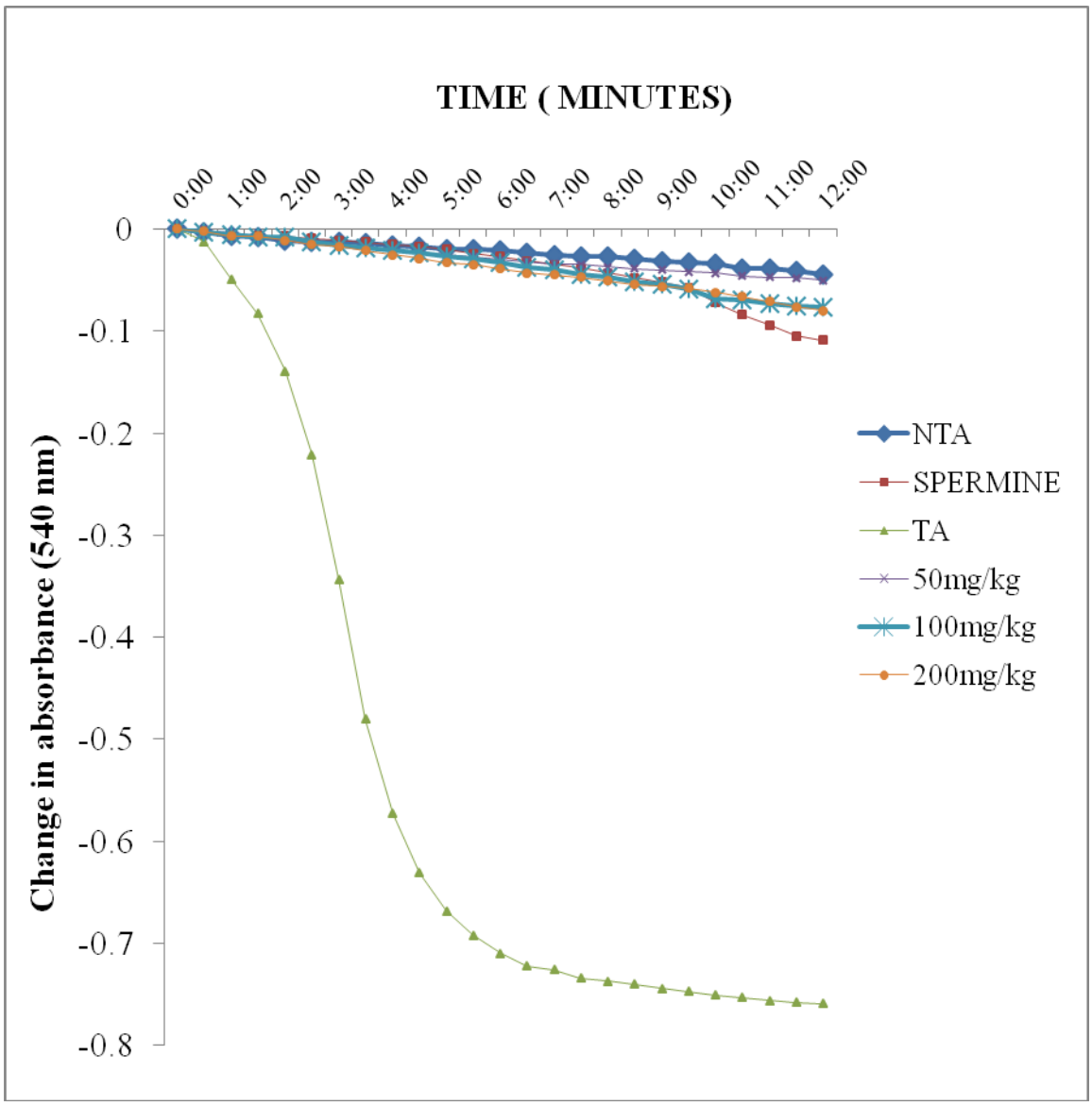


Figure 42: Effect of oral administration of monosodium glutamate (MSG) for 14 days on rat liver mPT

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

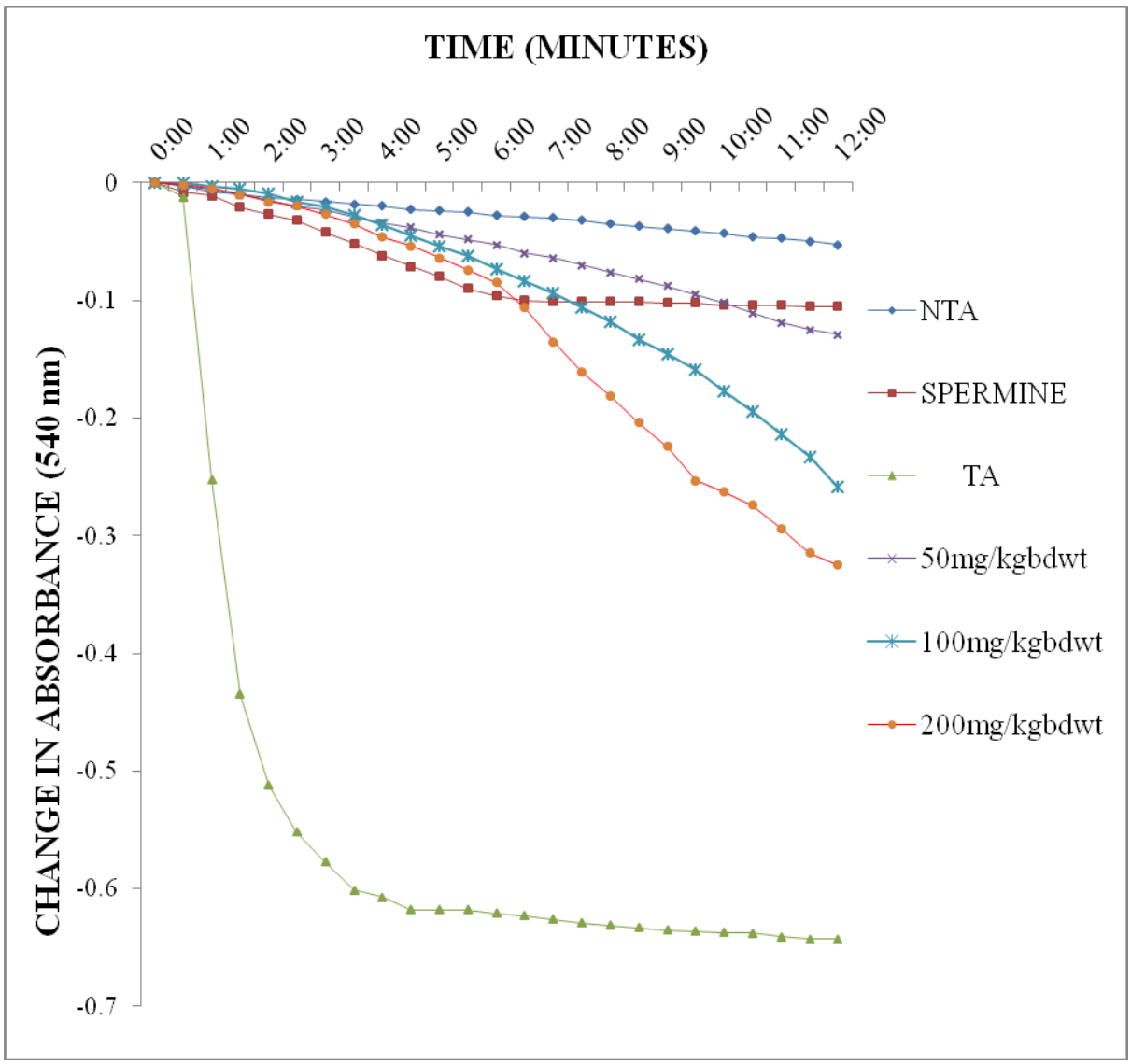


Figure 43: Effects of oral administration of monosodium glutamate (MSG) for 28 days on rat liver mPT

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

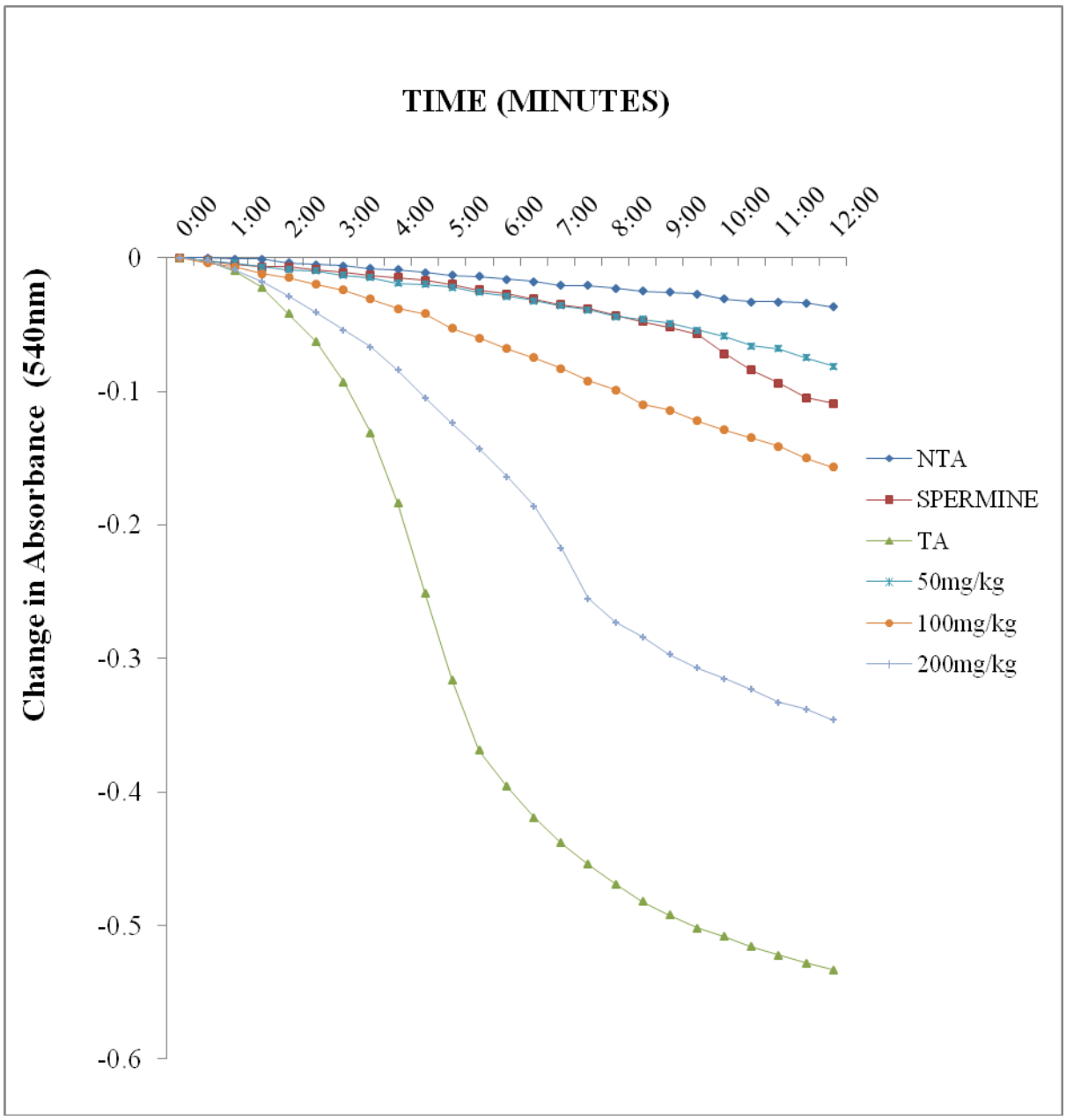


Figure 44: Effects of oral administration of monosodium glutamate (MSG) on rat uterus mPT pore for 28 days.

NTA: No triggering agent (without calcium)

TA: Triggering agent (calcium)

Spermine: Standard inhibitor

EXPERIMENT 11: EFFECTS OF VARYING DOSES OF CHLOROFORM FRACTION (CFDC) ON RAT LIVER MITOCHONDRIAL ATPase ACTIVITY

INTRODUCTION

The opening of the mPT pore can initiate the intrinsic pathway of apoptosis (Dietze *et al*., 2001). This encourages the hydrolysis of ATP to ADP and Pi rather than its synthesis. We therefore investigated the effect of chloroform fraction of *Drymaria cordata* on mitochondrial ATPase activity using inorganic phosphate released as an index of enhancement of ATPase activity and 2,4 dinitrophenol as a classical inducer of ATPase activity.

PROCEDURE

The method of Lardy and Wellman (1953) was used to assay for mitochondrial Adenosine triphosphate activity. Mitochondrial protein (0.5mg/ml) was used for this assay.

To each test tube in duplicate, 0.25 Msucrose, 5mM KCl, 0.1M Tris-HCl (pH 7.4) were added. The solutions were made up to 2000 µl accordingly with distilled water. The 0.01M ATP (pH 7.4) was added to the designated tubes and the whole set up was incubated in a shaking water bath at 27^{o}C. Mitochondria isolated from the control group were added to the zero time tube (and the reaction was immediately stopped by the addition of 10% Sodium Dodecyl Sulphate) while the mitochondria isolated from other test groups were added to the rest of the test tube except the blank at every 30 seconds for 30 minutes. Dinitrophenol (DNP) was added to the uncoupler tube immediately mitochondria were added. Mitochindria isolated from other test groups were added to the rest of the test tubes except the blank at every 30 seconds for 30 minutes. SDS was added to each test tube (except the zero time tube) every 30 seconds to stop the reaction. Aliquot amount (1ml) of the reaction medium was dispensed into separate test tubes after which

ammonium molybdate (1.25%) and freshly prepared 9% ascorbic acid were added. The set up was incubated for 20 minutes. The intensity of the blue colour was read at 660nm using Camspec M105 spectrophotometer.

RESULTS

Figure 45 shows the effects of varying doses of CFDC on mitochondrial F_0F_1 ATPase activity. The data obtained show that CFDC caused enhancement of mitochondrial ATPase activity by 25, 38 and 63%, at 50, 100 and 200mg/kg, respectively, and it is significant ($P<0.05$) at 100 and 200mg/kg bd wt when compared with the control group.

CONCLUSION

Varying doses of CFDC enhanced ATPase activity in a dosage dependent manner statistically significant at 100 and 200mg/kg bd wt.

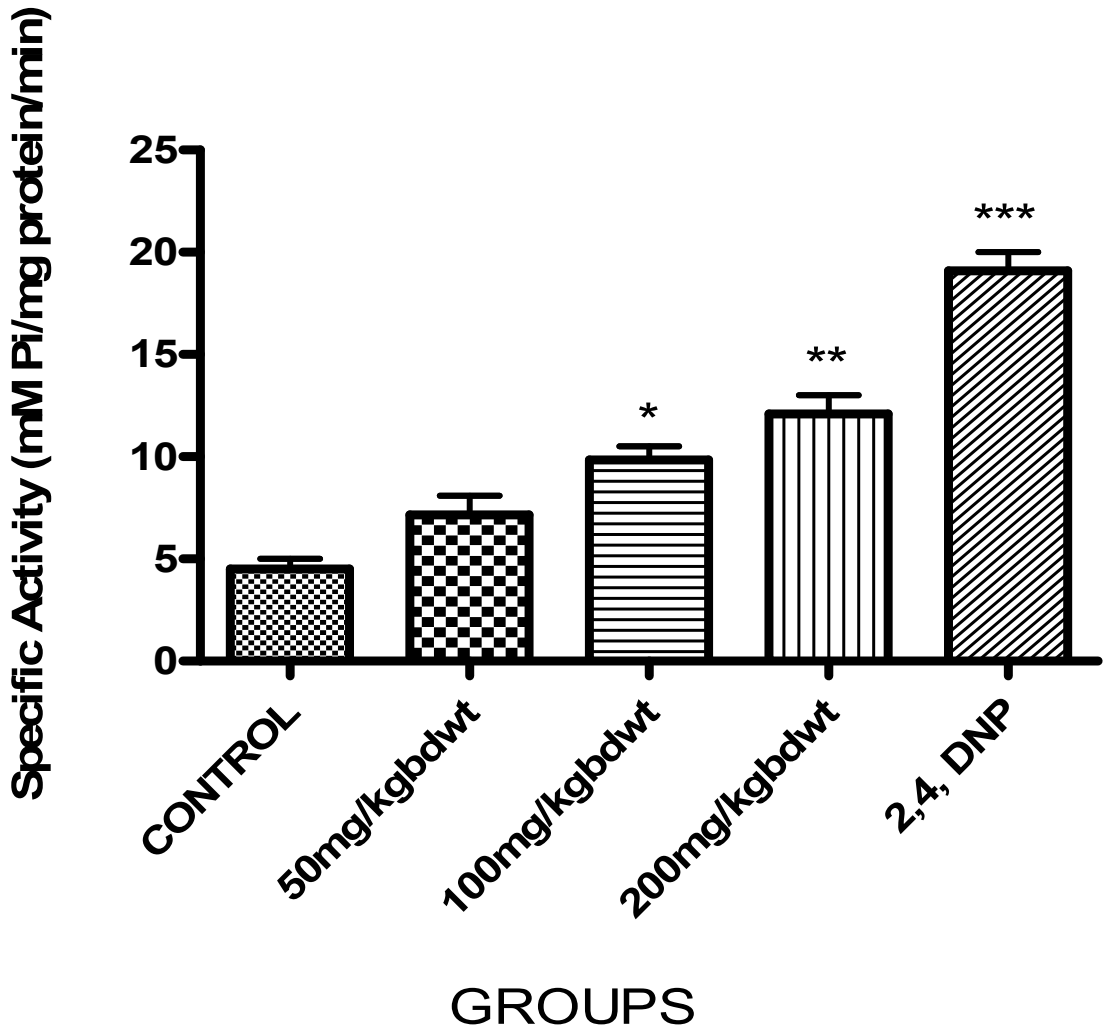


Figure 45: In vivo effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on ATPase activity

*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

***** Each value is statistically significant at p<0.001, compared with control using the one-way ANOVA**

EXPERIMENT 12: INFLUENCE OF CHLOROFORM FRACTION (CFDC) ON CASPASES 9 AND 3 ACTIVATION USING ELISA TECHNIQUE

INTRODUCTION

As a result of the induction of mPT pore opening, cytochrome c release and mitochondrial ATPase activity caused by CFDC, this experiment was therefore carried out to investigate if all these pharmacological properties would activate caspases 9 and 3 which is crucial in the activation of mitochondrial-mediated apoptosis.

PROCEDURE

Analysis of caspases 9 and 3 were carried out using an ELISA kit, a product of Elabscience biotechnology Ltd., Technology Industry Park, WuHan, Peoples Republic of China. This kit uses Sandwich-ELISA as the method. A microplate reader (DNM-9602A from China) was used to read the optical density at 450nm wavelength. The assay was carried out according to manufacturer's instruction as described on page 80.

RESULTS

Effects of oral administration of CFDC on caspases 9 and 3 are shown by Figures 46 and 47. CFDC caused induction of caspase 9 activity by 5% 37%, 63% and caspase 3 activity by 33%, 53%, 67% at doses of 50, 100 and 200mg/kg bd wt, respectively which was significant ($P<0.05$) at 100 and 200mg/kg.

CONCLUSION

The results show that CFDC is an inducer of mitochondrial-mediated apoptosis via induction of caspases 9 and 3 activities.

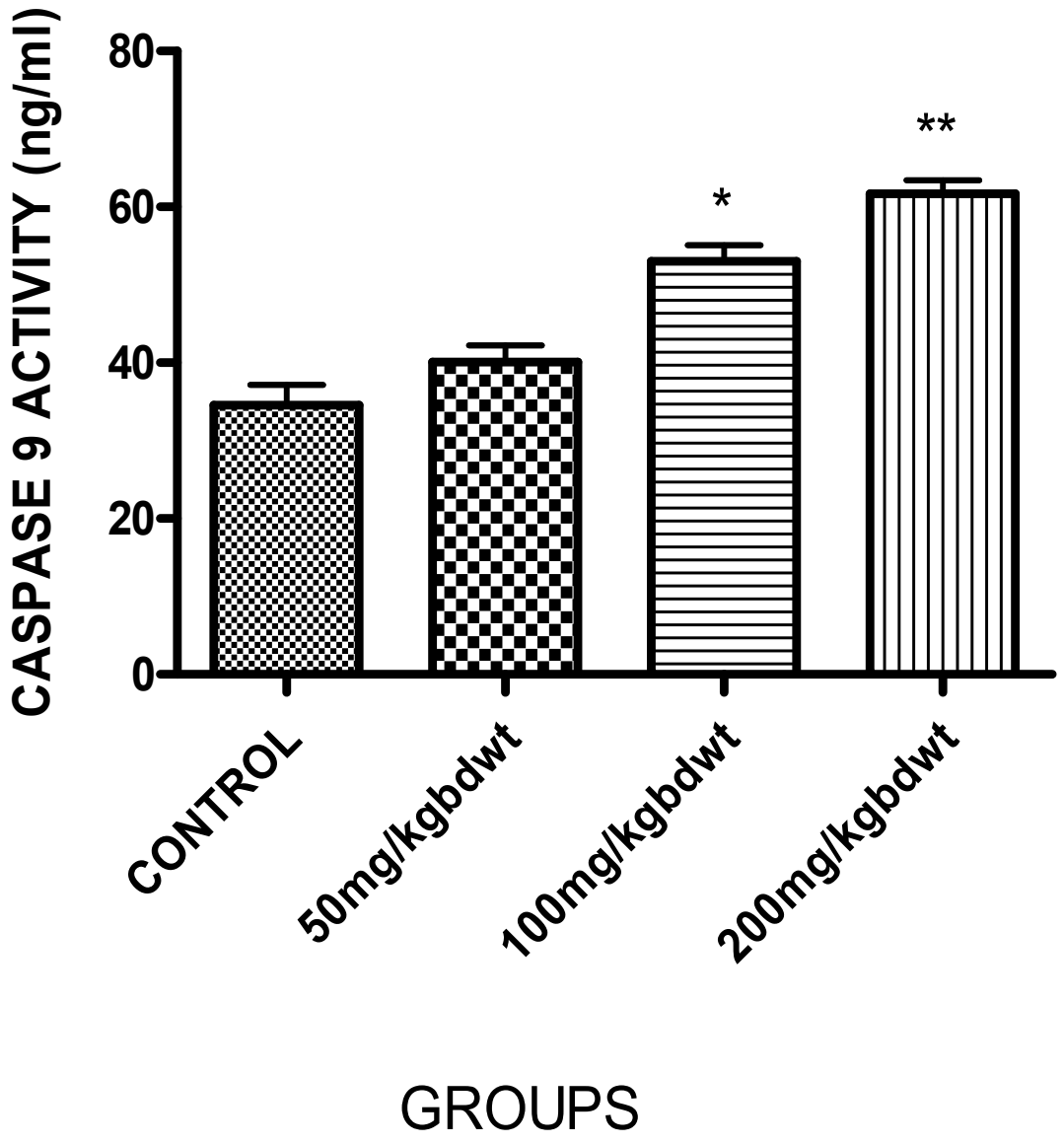


Figure 46: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on caspase 9 activation

*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

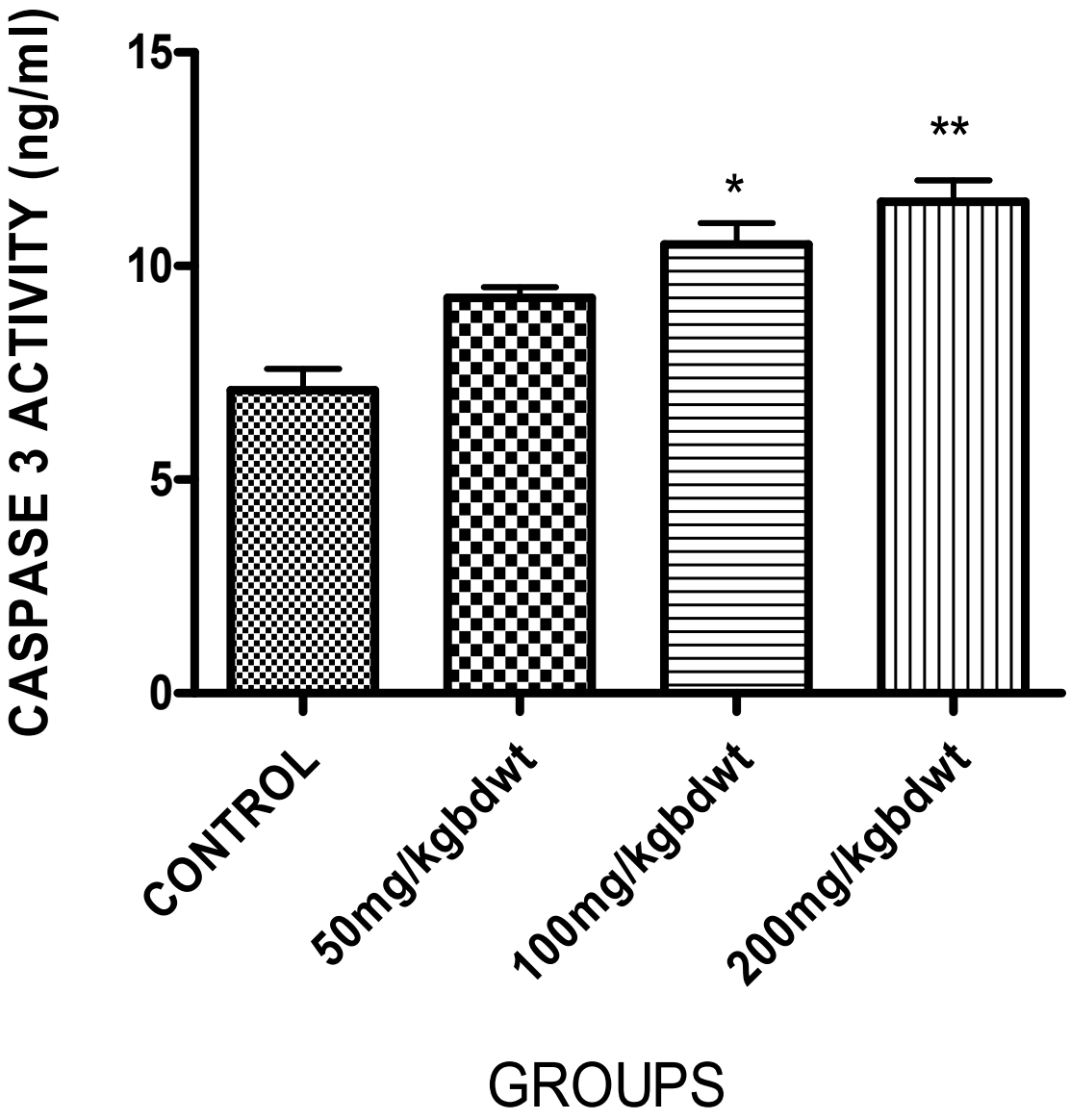


Figure 47: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on caspase 3 activation

*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

EXPERIMENT 13: EFFECTS OF ORAL ADMINISTRATION OF CHLOROFORM FRACTION (CFDC) ON HEPATIC AND UTERINE DNA FRAGMENTATION

INTRODUCTION

DNA fragmentation is one of the hallmark of apoptosis. As a consequence of the activated caspases 9 and 3 by CFDC, this experiment was carried out to see if the induction of caspases caused by oral administration of CFDC caused DNA fragmentation by upregulation of caspase-activated deoxyribonuclease (CAD).

PROCEDURE

The procedure of Wu *et al*. (2005) was followed as described on pages 83.

RESULTS

As shown in Figures 48 and 49, oral administration of CFDC at the tested doses caused induction of hepatic DNA fragmentation by 20%, 37% and 60% as well as uterine DNA fragmentation by 15%, 50% and 75% when compared with the control group. DNA fragmentation at 100 and 200mg/kg were significant.

CONCLUSION

Oral administration of CFDC caused induction of hepatic and uterine DNA fragmentation

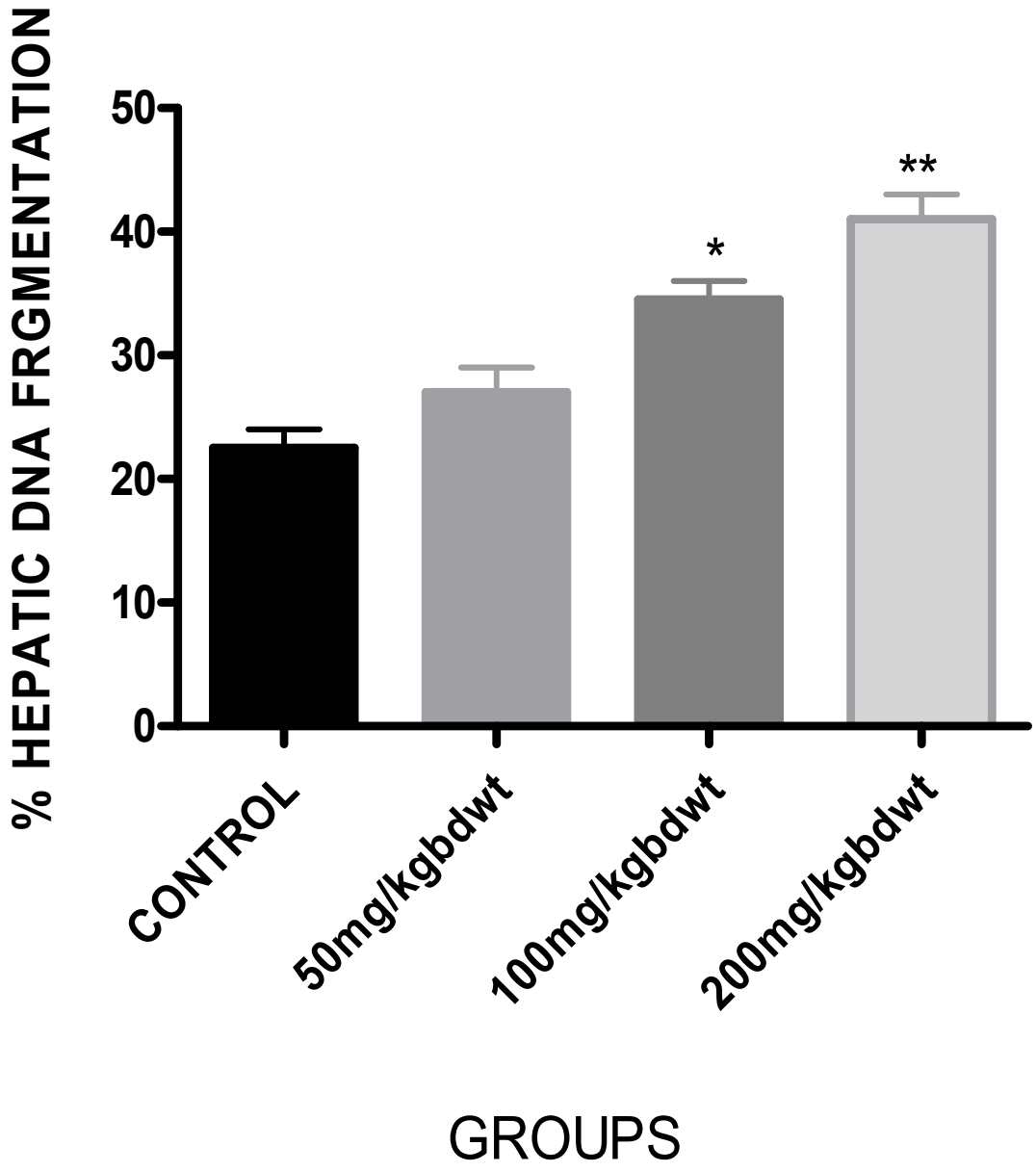


Figure 48: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on hepatic DNA fragmentation

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

**** Each value is statistically significant at $p<0.01$, compared with control using the one-way ANOVA**

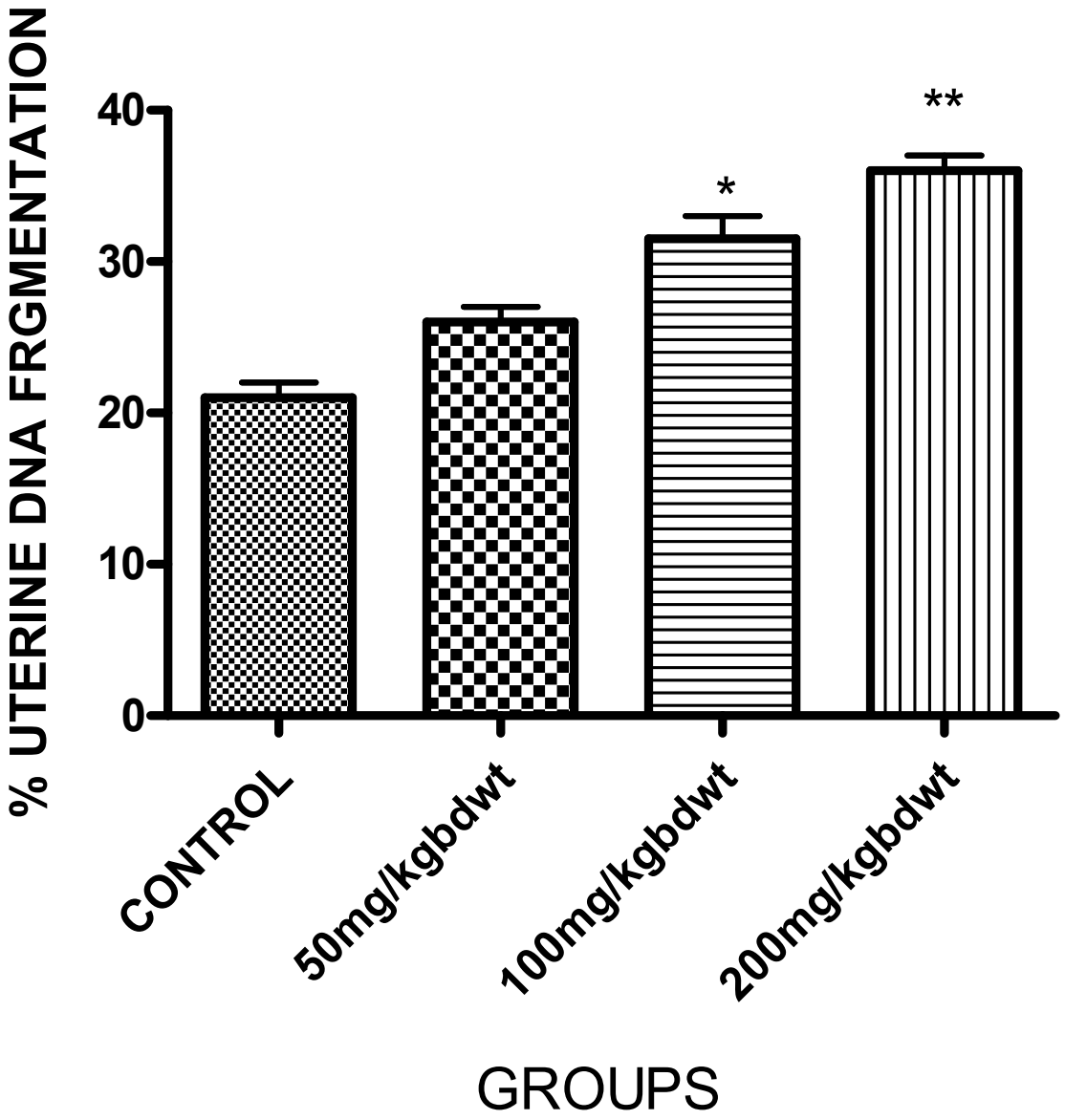


Figure 49: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on uterine DNA fragmentation

*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

EXPERIMENT 14 IMMUNODETECTION OF CYTOCHROME C IN THE LIVER OF RATS ORALLY TREATED WITH CHLOROFORM FRACTION OF *Drymaria cordata* FOR 28 DAYS

INTRODUCTION

When mPT is induced, cytochrome c is released. This is a crucial step in the activation of mitochondrial-mediated apoptosis. This experiment was designed to investigate if oral administration of CFDC will cause expression of cytochrome c in the liver tissue since there was induction of cytochrome c in the *in vitro* study.

PROCEDURE

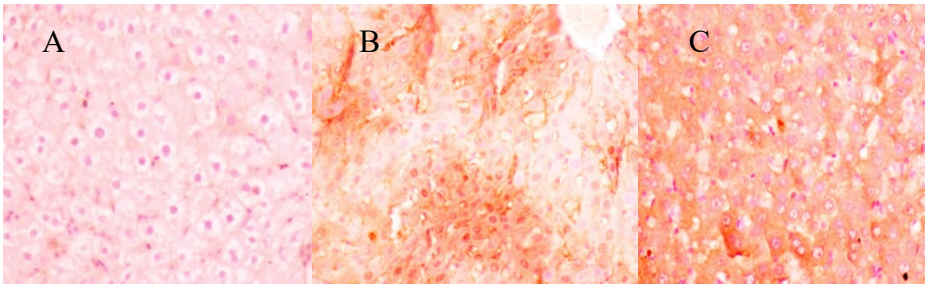
The procedure was carried out according to the manufacturer's instruction as described on page 85-86.

RESULTS

The results in Figure 50 show that there was negative expression of cytochrome c in the control rat liver. However, in the groups treated with 50 and 100mg/kg bd wt CFDC, positive expression of cytochrome was observed as indicated by the brown stain in the rat liver. The immunoreactivity was more expressed in the 100mg/kg bd wt than 50mg/kg bd wt.

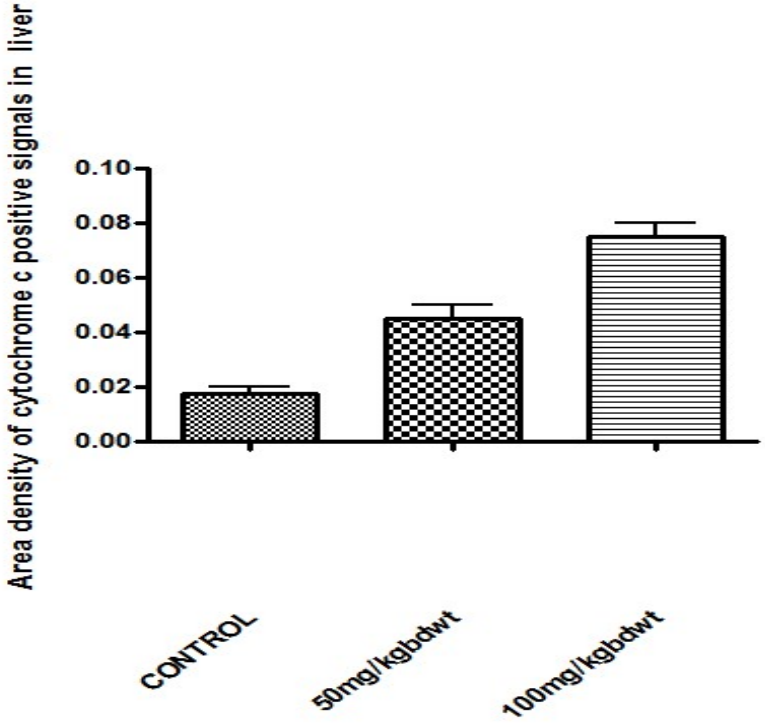
CONCLUSION

Oral administration of CFDC caused an increase in cytochrome c immuno-positive expression in the hepatocytes of the rat liver.



Control **50mg/kg bd wt** **100mg/kg bd wt**

Figure 50: Immunohisto activity of rat liver showing the immunohistochemical effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on cytochrome c release (Mag x400)



A : (control) showed mild/negative expression of cytochrome c

B: (50 mg/kg bd wt) showed positive expression of cytochrome c compared with the control

C: (100 mg/kg bd wt) showed more positive expression of cytochrome c compared with control and 50mg/kg bd wt

EXPERIMENT 15: IMMUNODETECTION OF CASPASES 9 AND 3 PROTEIN IN THE LIVER OF RATS ORALLY TREATED WITH CHLOROFORM FRACTION OF *Drymaria cordata* FOR 28 DAYS

ITRODUCTION

The caspases, which are cysteine proteases homologous, are critical in the apoptotic-signaling-network . This experiment was carried out in order to investigate whether oral administration of CFDC will cause immune-expression of caspases 9 and 3 in the liver tissue as it caused the induction of caspases 9 and 3 using ELISA technique.

Principle

The principle is as described on page 85 by exploiting the principle of antibodies binding specifically to antigens in biological tissues. Enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color-producing reaction. The absorbance is read at 450 nm (Clifton, 2011).

PROCEDURE

The procedure used in this study is Avidin Biotin Complex (ABC) procedure also referred to as Avidin biotin Imunoperoxidase method as described on pages 85-86.

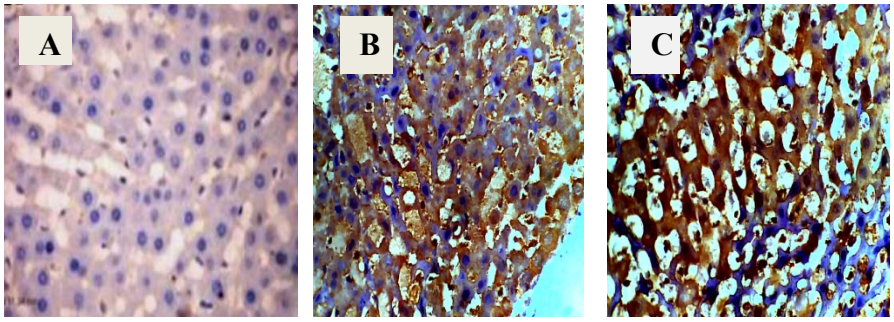
RESULTS

As shown in Figure 51, the control shows a negative expression for caspase 9 while the 50 and 100mg/kg bd wt show a mild and strong expression, respectively, for caspase 9. Caspase 9 is more expressed in the 100mg/kg bd wt. Also, the expression of caspase 3 as indicated Figure 52,

shows that there was negative expression of caspase 3 in the control group while there was mild expression in the 50mg/kg bd wt and strong expression was noticed in the 100mg/kg bd wt group as indicated by the brown stain in the rat liver.

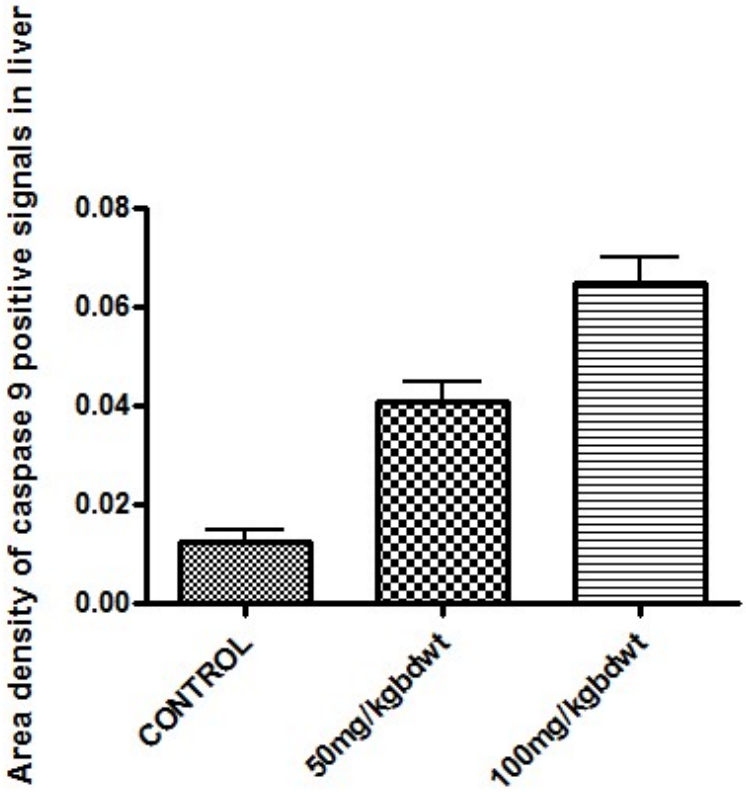
CONCLUSION

Oral administration of CFDC caused a significant elevation in caspases 9 and 3 immuno-positive expression in the hepatocytes of the rat liver.

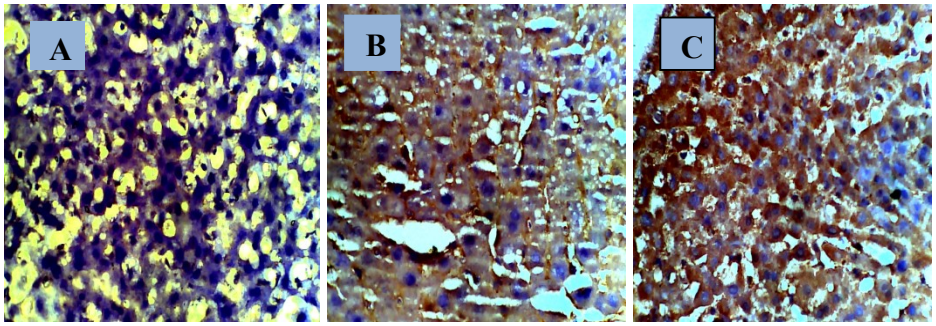


Control **50mg/kg bd wt** **100mg/kg bd wt**

Figure 51: Immunohisto activity of rat liver showing the effect of oral administration of chhloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on caspase 9 activation (Mag x400)

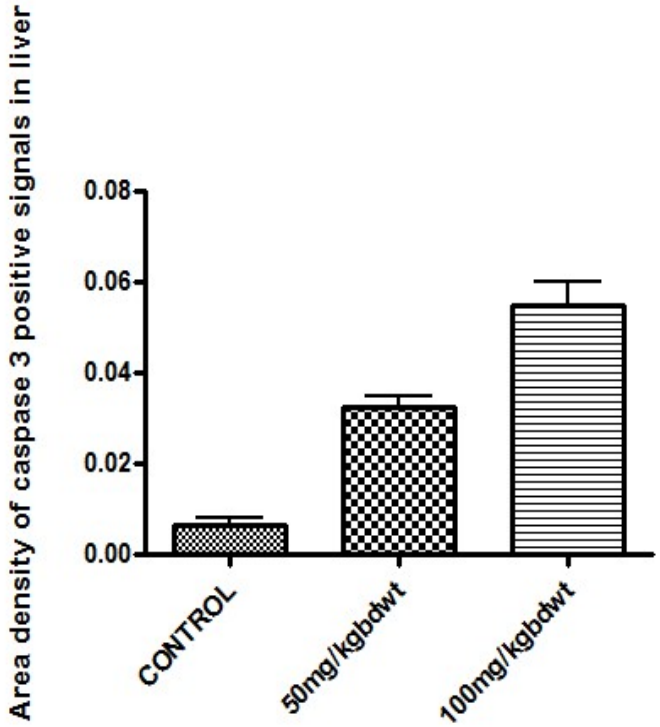


A : (control) showed mild/negative expression of caspase 9 activity
B: (50 mg/kg bd wt) showed weak expression of caspase 9 activity compared with control
C: (100 mg/kg bd wt) showed strong expression of caspase 9 activity compared with control and 50mg/kg bd wt



Control **50mg/kg bd wt** **100mg/kg bd wt**

Figure 52: Immunohisto activity of rat liver showing the effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on caspase 3 activation (Mag x400)



A : (control) showed negative expression of caspase 3 activity
B: (50 mg/kg bd wt) showed weak expression of caspase 3 activity compared with control
C: (100 mg/kg bd wt) showed strong expression of caspase 3 activity compared with control and 50mg/kg bd wt

EXPERIMENT 16 : IMMUNODETECTION OF ANTI-APOPTOTIC BCL-2 PROTEIN IN THE LIVER AND UTERUS OF RATS ORALLY TREATED WITH CHLOROFORM FRACTION OF *Drymaria cordata* FOR 28 DAYS

INTRODUCTION

Based on the results of the previous study which showed that CFDC caused immune-expression of caspases 9 and 3, this experiment was designed to know whether the expression/activation of caspases 9 and 3 is coupled to downregulation/repression of anti-apoptotic BCL-2 protein, following oral treatment with CFDC for 28 days.

PROCEDURE

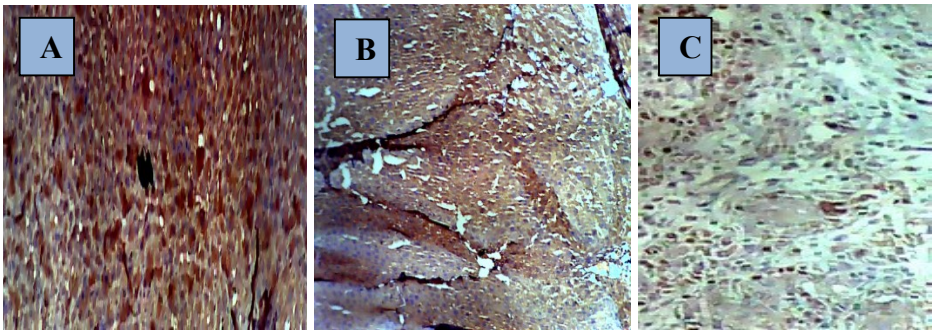
The procedure was carried out according to the manufacturer's instruction as described on pages 85-86.

RESULTS

The results as shown in Figures 53 and 54 revealed a moderate immune positive expression of anti-apoptotic BCL-2 in the control group both in the hepatocytes and the uterus. However, there was reduced expression in the group treated with 50mg/kg CFDC while negative expression was recorded in the 100mg/kg both in the liver and uterus

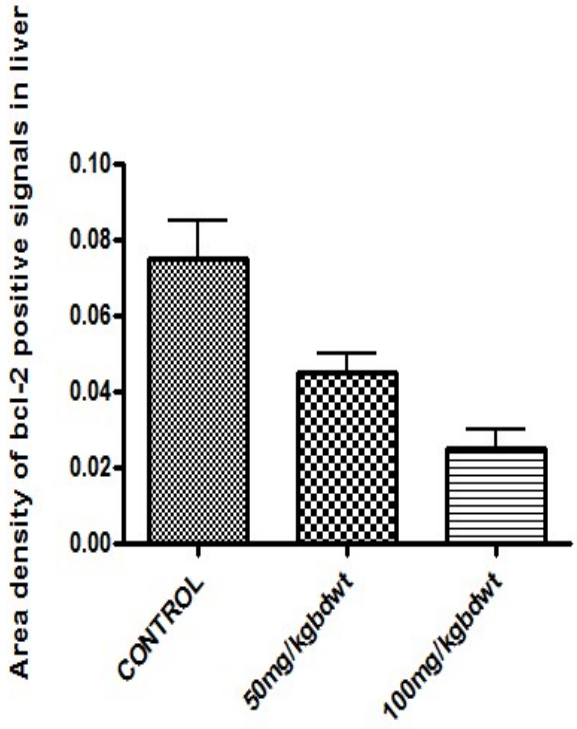
CONCLUSION

Oral administration of chloroform fraction of *D.cordata* caused downregulation of BCL-2 protein both in the liver and the uterus

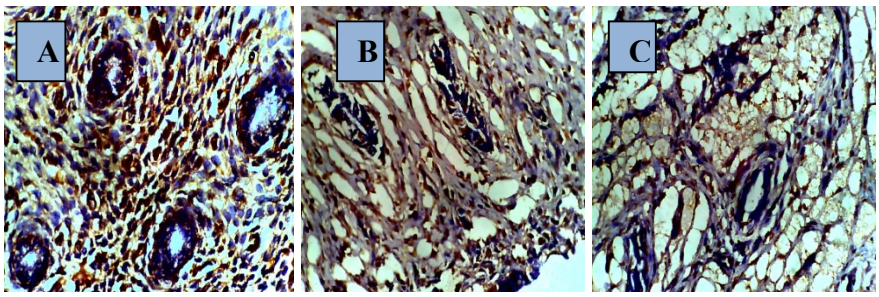


Control **50mg/kg bd wt** **100mg/kg bd wt**

Figure: 53: Immunohisto activity of rat liver showing the effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on Bcl-2 protein expression (Mag x400)

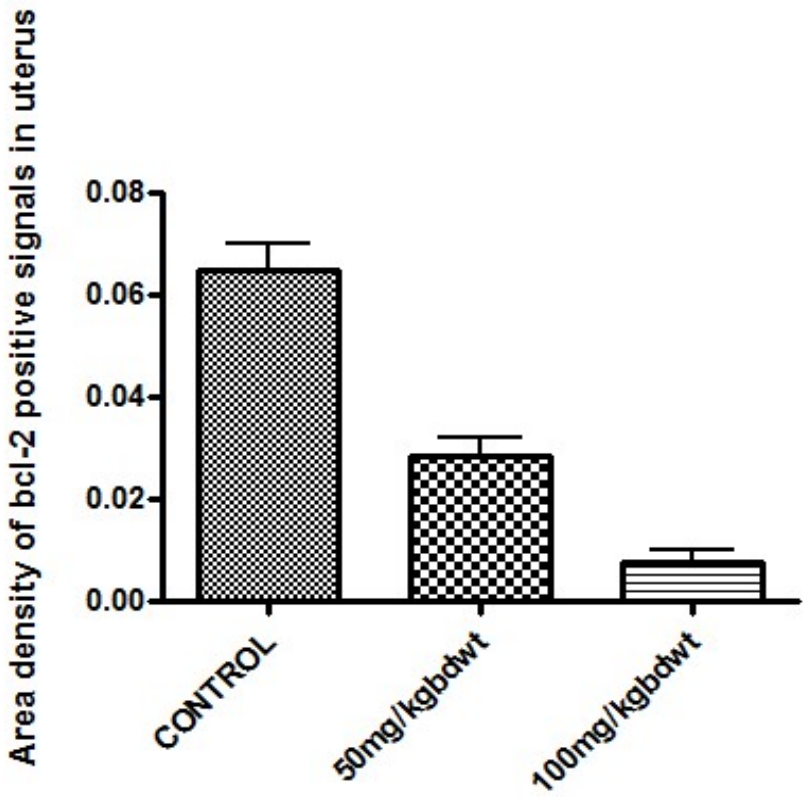


A : (control) showed strong/moderate expression of anti-apoptotic bcl-2 protein
B: (50 mg/kg bd wt) showed weak expression of anti-apoptotic bcl-2 protein compared with control
C: (100 mg/kg bd wt) showed negative/mild expression of anti-apoptotic bcl-2 protein compared with control and 50mg/kg bd wt



Control **50mg/kg bd wt** **100mg/kg bd wt**

Figure 54: Immunohisto activity of rat uterus showing the effect of oral administration of chloroform fraction of *Drymaria cordata* (CFDC) on Bcl-2 protein expression (Mag x400)



A : (control) showed strong/moderate expression of anti-apoptotic bcl-2 protein
B: (50 mg/kg bd wt) showed weak expression of anti-apoptotic bcl-2 protein compared with control
C: (100 mg/kg bd wt) showed negative/ expression of anti-apoptotic bcl-2 protein compared with control and 50mg/kg bd wt

EXPERIMENT 17 EFFECT OF ORAL TREATMENT OF CHLOROFORM FRACTION OF D. CORDATA ON MSG-INDUCED UTERINE HYPERPLASIA IN RATS

INTRODUCTION

Folklorically, *Drymaria cordata* is used in the treatment of hepatic, uterine, breast and skin tumour. Our interest in this study is in the area of uterine tumour. Experimental evidences have shown that monosodium glutamate can induce uterine tumour in experimental rats by increasing the level of oestrogen, progesterone and cholesterol (Eweka *et al.,* 2010). This experiment was therefore designed to investigate the effect of oral administration of chloroform fraction of *D.cordata* on MSG-induced uterine fibroid using the levels of oestrogen, progesterone, total cholesterol, uterine cellular proliferation and fibroblast count/μm^2 as indices of uterine fibroid/hyperplasia.

PROCEDURE

Healthy matured female rats (control) having a mean weight of 170g were used for this experiment. They were grouped as shown below and treated for 28 days

Dose Regimen:

Control 5% DMSO in distilled water

MSG 200mg/kg bd wt

CFDC 100mg/kg bd wt

CFDC & MSG (100 & 200) mg/kg bd wt, respectively.

The rats were orally treated daily with CFDC for 28 days. Twenty four hours after the final exposure, the animals were sacrificed. The rats were cardiac punctured and blood was collected into plain sample bottles. Serum was prepared by centrifugation (3000xg, 20 min) and used for

the analysis of total estradiol (estrogen), progesterone and total cholesterol. The uteruses were harvested, cleaned and dispensed in 10% formalin after which it was processed for masson's trichome staining, a differential stain for connective tissue and the cellular precursors. The Histological pictures were taken with a Digital Microscope, VJ-2005 DN MODEL BIOMICROSCOPE ®. The morphometrical analyses of the density of spindle shaped cells within the endometrial submucosa were done using TS View CX Image® Software, File version 6.2.4.3 Motic Image 2000 (China). The oestrogen, progesterone and the total cholesterol levels were determined as described on pages 89-90 according to the manufacturer's instruction.

RESULTS

Figure 55 shows the results of the effect of chloroform fraction of *Drymaria cordata* (CFDC) on oestrogen level of the female rat treated with monosodium glutamate. The investigation showed that the animals treated with MSG presented a significant ($p<0.05$) increase (87.5%) in the levels of estrogen (estradiol) in the animals treated with 200 mg/kg MSG relative to control. The groups co-treated with CFDC were able to ameliorate the oestrogen level to about 25.5%, relative to the MSG-treated group. Also, the value of progesterone level in the MSG-treated groups was significantly increased by about 59.0% as shown in Figure 56 when compared with the control group while the co-administration with the fraction significantly reduced the progesterone level to 27.2% , relative to MSG-treated group. Figure 57 depicts the level of total cholesterol which also showed a similar pattern. There was increase in the level of total cholesterol in the MSG-treated group and this was reduced in the group co-administered with CFDC relative to MSG-treated group ($P<0.05$). Figure 58 shows the cell count density obtained from the myometrium of the uterus of the control and treated female rats. The histology results

of the uterus using histomorphometric technique shows the cell counts of stained spindle shaped connective precursor cells elevated in the MSG-treated group when compared with the control group. In contrast, the cell counts in the co administered group shows a mitigated level when compared with the MSG-treated group. The cell counts density recorded in the MSG-treated group was 3.6 folds compared with the control group. This was reduced by the co-administration with CFDC having 56% reduction , when compared with the MSG-treated group ($P<0.05$).

Figures 59a-d showed the photomicrograph of the masson trichrome-stained sections of myometrium of the connective tissue and precursor cells within the endometrial submucosa and the endometrial glands. Figure 58a which is the control uterine section shows normal architecture of the connective tissues and the precursor cells. Similar pattern of results were noticed in the CFDC treated groups. However, in Figure 58c which is the group treated with 200 mg/kg bd wt MSG, there was a severe hyperplasia of spindle shaped precursor cells. The hyperplasia was mitigated in the group that received co administration of CFDC as shown in Figure 59d

CONCLUSION

These results show that CFDC contains bioactive agents that can reduce the levels of oestrogen, progesterone and total cholesterol in MSG-treated rats which has been implicated in the development of uterine fibroid . The findings from the histological studies using masson richome stain showed that CFDC protected against MSG-induced uterine hyperplasisa in rats.

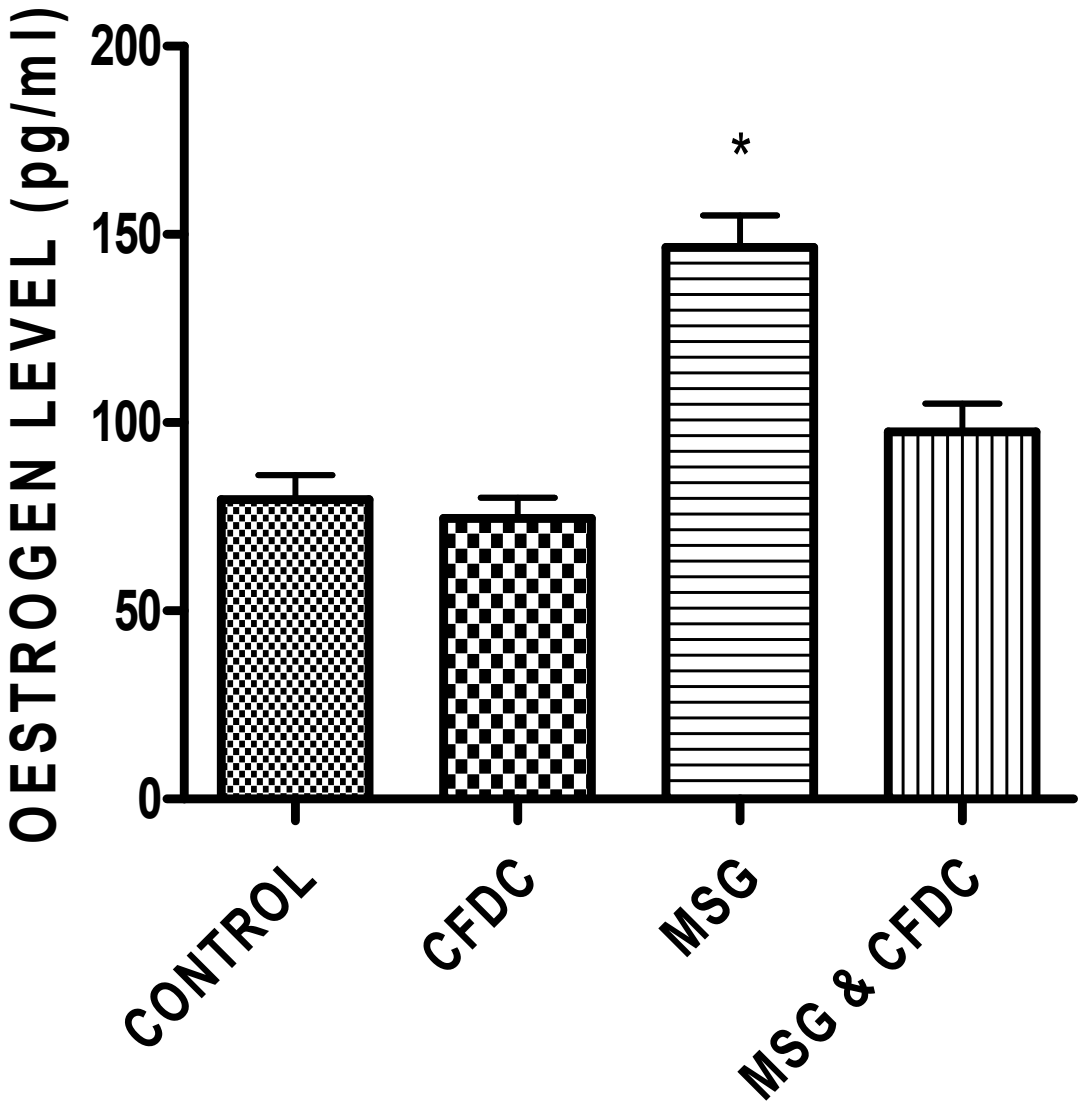


Figure 55: Effects of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on ostrogen level in normal and monosodium glutamate (MSG)-treated female rats

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

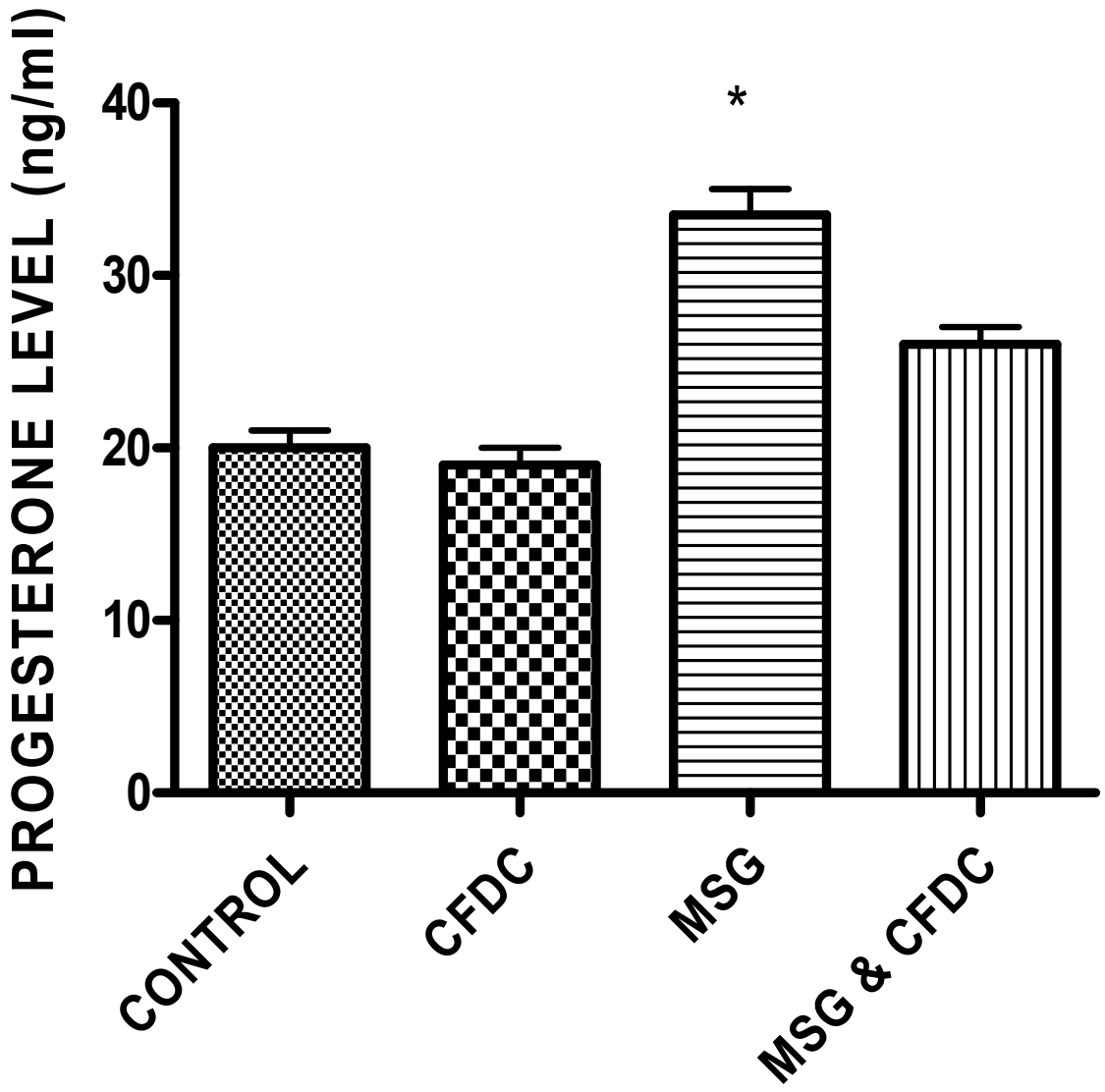


Figure 56: Effects of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on progesterone level in normal and monosodium glutamate (MSG)-treated female rats

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

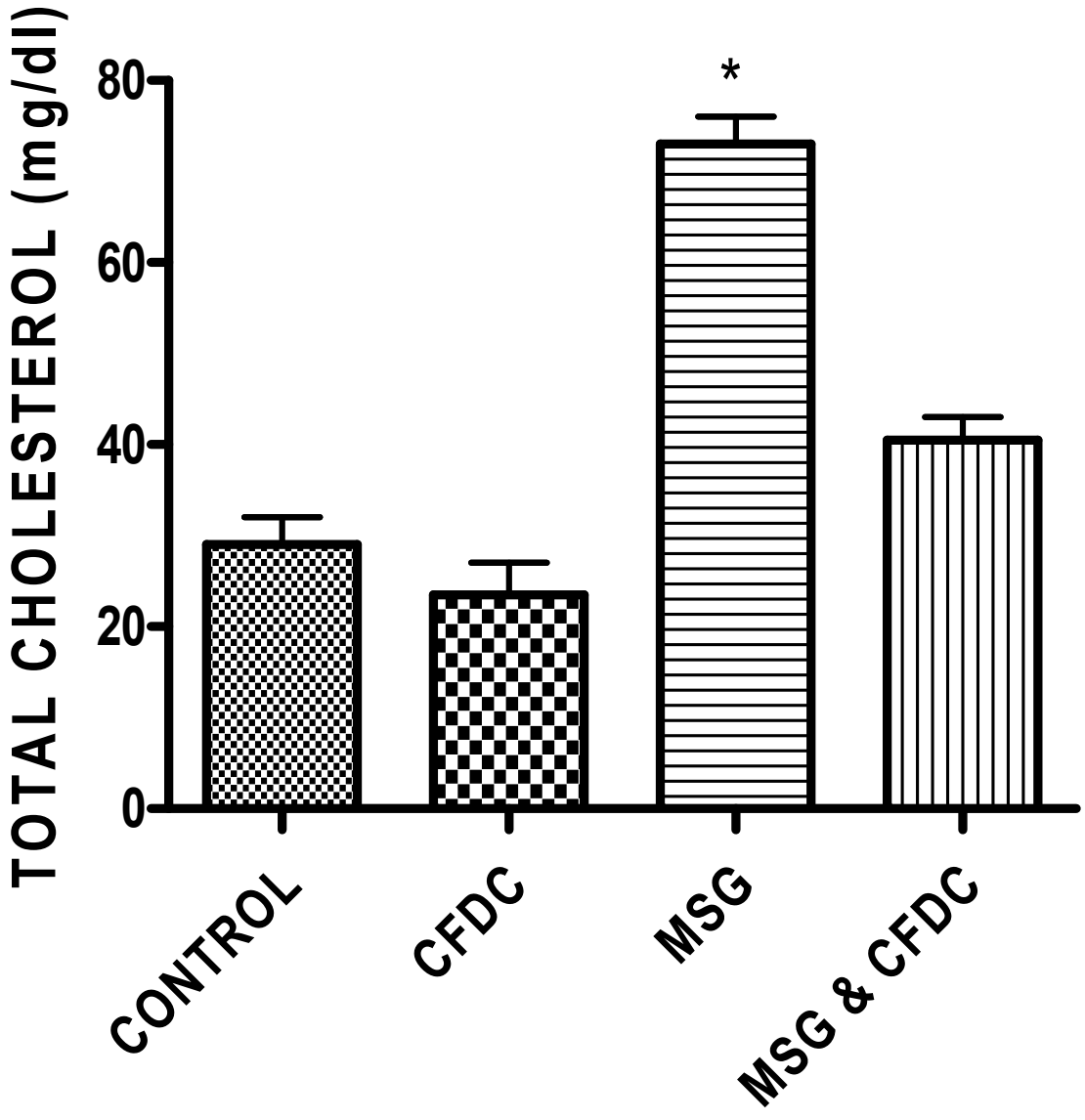


Figure 57: Effects of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on total cholesterol level in normal and monosodium glutamate (MSG)-treated female rats

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

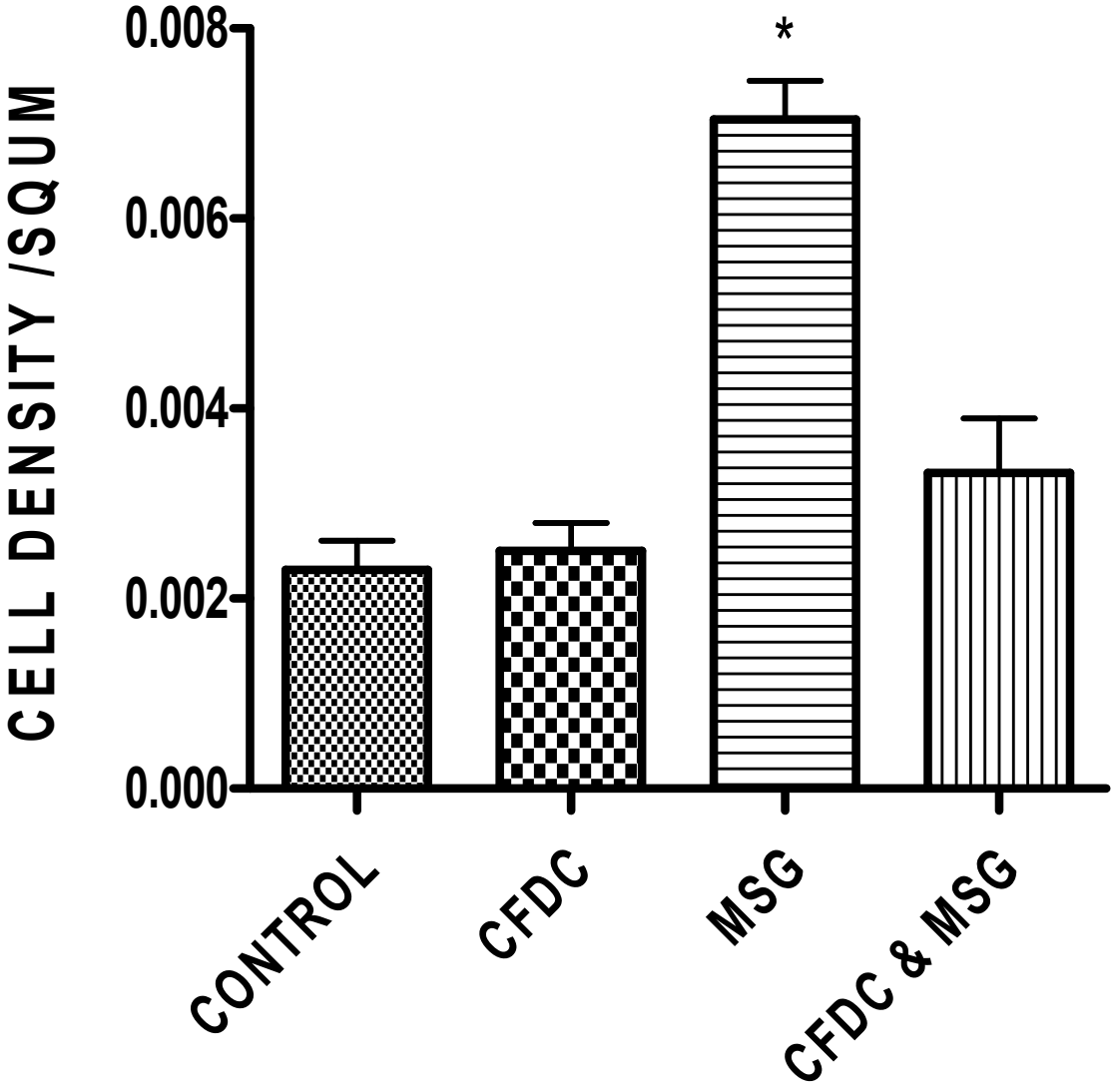


Figure 58: Cell count density obtained from the myometrium of uterus of female wistar rats treated with monosodium glutamate (MSG) and chloroform fraction of methanol extract of *Drymaria cordata* (CFDC)

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

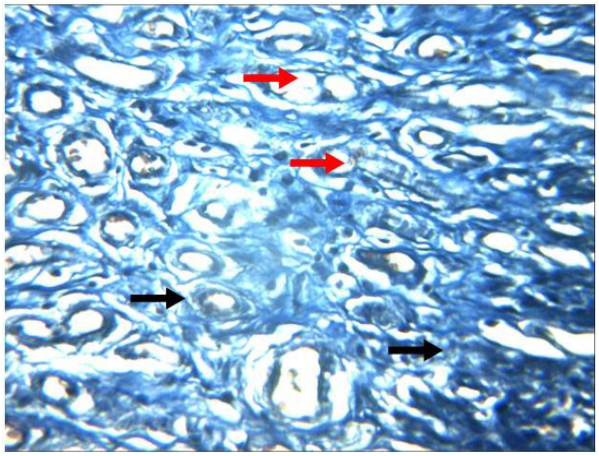


Figure 59a: Photomicrograph of stained section (control) showing the connective tissue and precursor cells within the endometrial submucosa (black arrows) and the endometrial glands (red arrow) MT (Mag. x400). There was normal deposition of collagen fibers.

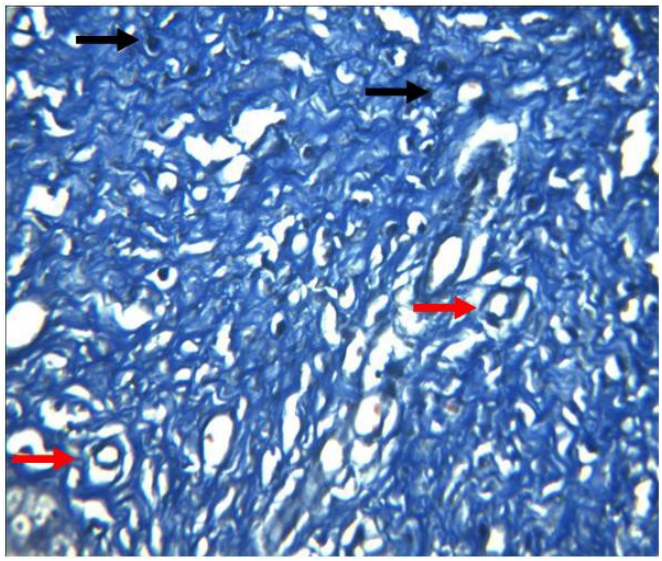


Figure 59b: Photomicrograph of stained section (treatment) of the myometrium (CFDC) showing the connective tissue and precursor cells within the endometrial submucosa (black arrows) and the endometrial glands (red arrows) MT (Mag. x400). There was normal deposition of collagen fibers

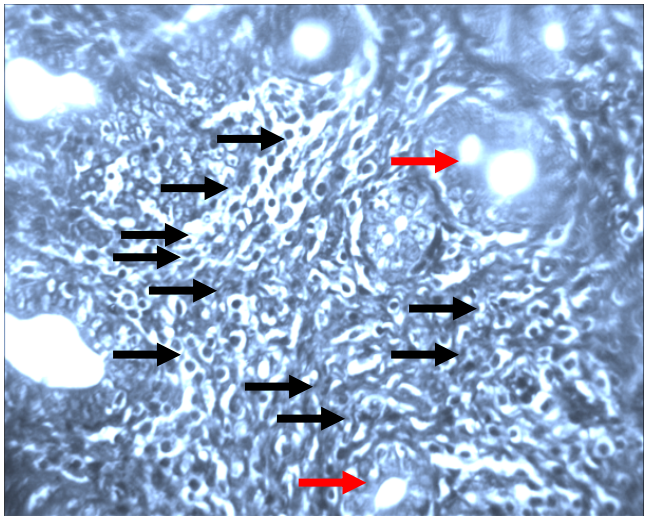


Figure 59c: Photomicrograph of stained section (treatment) of the Uterus (200 mg/kgbdwt MSG) showing the connective tissue and precursor cells within the endometrial submucosa (black arrows) and the endometrial glands (red arrows) MT (Mag. x400). There was a severe hyperplasia of spindle shaped precursor cells

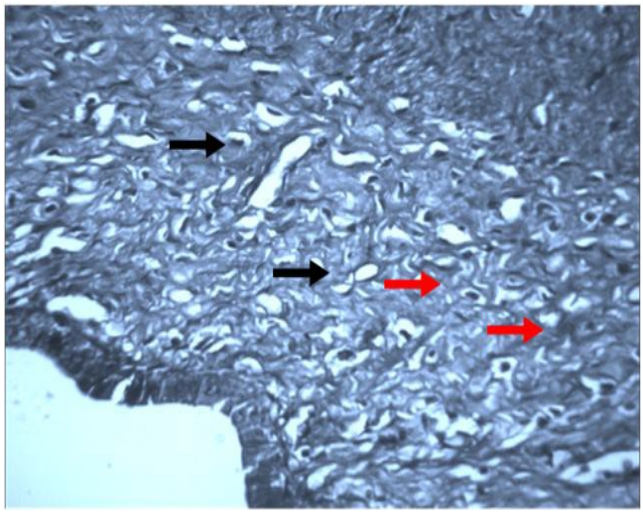


Figure 59d: Photomicrograph of stained section (treatment) of the endometrium (200 mg/kg bd wt MSG & 100 mg/kg bd wt CFDC) showing the connective tissue and precursor cells within the endometrial submucosa (black arrows) and the endometrial glands (red arrows) MT (Mag. x400). There was a reduction of hyperplasia compared with MSG-trated group

EXPERIMENT 18: HISTOLOGICAL ASSESSEMENT OF ORAL ADMINISTRATION OF CFDC ON THE LIVER AND UTERUS IN NORMAL AND MSG-TREATED RATS

INTRODUCTION

Having shown that oral administration of MSG in rats resulted in uterine hyperplasia, the effect of CFDC administration on MSG-treated rats using histological technique was investigated.

PROCEDURE

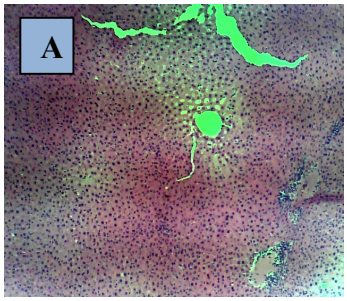
The procedure was carried out as described on page 78.

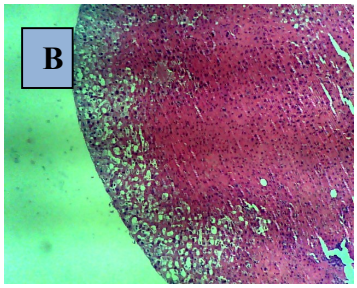
RESULTS

In the control liver, the hepatocytes are normal while the control uterus is also showing a normal myometrium. In the CFDC-treated groups, the the chloroform fraction of *D.cordata* did not show any adverse effect on the architechture of both the hepatocytes and the myometrium. MSG administration caused an onset of necrosis and complete loss of hepatic lobules with cytoplasmic degeneration in the hepatocytes while there was atretic or fibrosed glands loosing its architechture coupled with stromal inflamation in the case of myometrium. Co-administration with CFDC showed moderate hepatic distortion and moderate distortion of endolining in the myometrium.

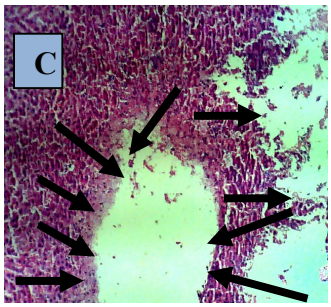
CONCLUSION

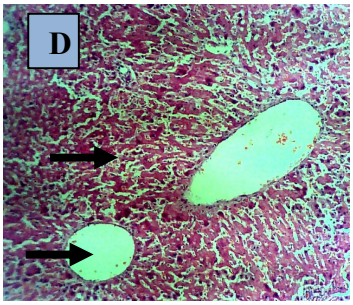
Oral administration of MSG caused hepatic and uterine damage in the female rats while co-administration ameliorated level of damage caused by MSG administration. The administration of chloroform fraction of *D.cordata* protected against MSG-induced damage in the liver and uterus of the rats.





Control **CFDC**

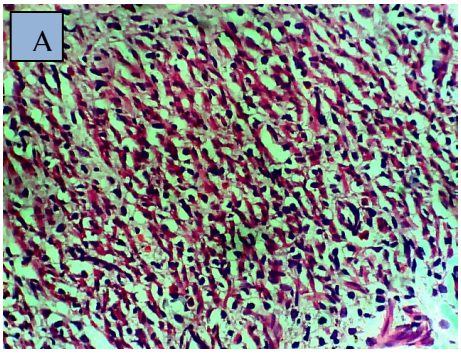


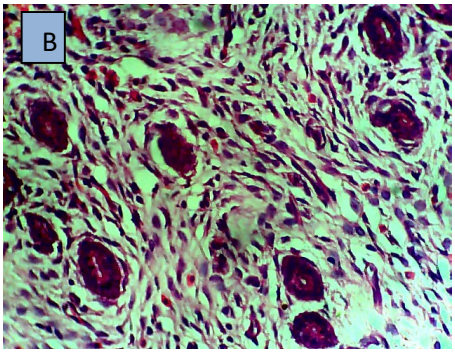


MSG **MSG & CFDC**

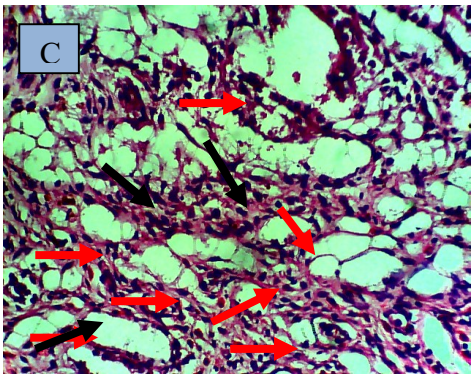
Fig 60: Photomicrograph of cross section of hepatic plate showing the effect of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on normal and monosodium glutamate (MSG)-treated rats (Mag x 100)

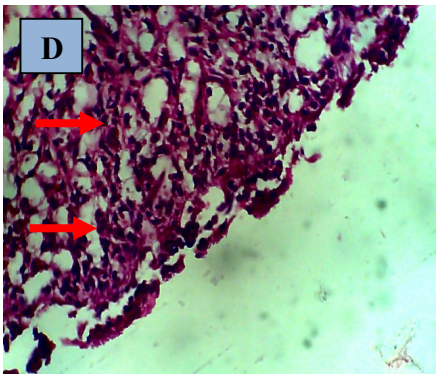
A: (control) showed normal architecture
B: (CFDC) showed normal architecture
C: (MSG) showed necrotic damage
D: (MSG & CFDC) showed moderate hepatic distortion





CONTROL **CFDC**





MSG **MSG & CFDC**

Figure 61: Photomicrograph of cross section of uterine plate showing the effect of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on normal and monosodium glutamate (MSG)-treated rats (Mag x 400)

A: (control) showed normal architecture
B: (CFDC) showed normal architecture
C: (MSG) showed fibrosed glands with stromal inflamation
D: (MSG &CFDC) showed moderate distortion

EXPERIMENT 19: EFFECT OF ORAL ADMINISTRATION OF CFDC ON LIPID PEROXIDATION, DNA FRAGMENTATION, CASPASES 9 AND 3 ACTIVATION AND BCL-2 PROTEIN EXPRESSION IN NORMAL AND MSG-TREATED FEMALE RATS

INTRODUCTION

In other to further investigate the pharmacological properties of CFDC, its effect on MSG-induced cellular injury in rats was investigated.

PROCEDURE

The LPO was assessed according to the method described by Varshney and Kale (1990), while caspases 9 and 3 were determined using ELISA kits and DNA fragmentation determined by the method of Wu *et al*. (2005) as described on pages 77, 80 and 83, respectively.

RESULTS

The results of the lipid peroxidation as depicted in Figure 62 shows an elevated level of malondialdehyde produced in the MSG-treated rats relative to control group. The result in the CFDC-treated rats was similar to the control. Interestingly, in the co-administered group, there was a reduction in the level of malondialdehyde produced relative to the MSG-treated group.

Also, Figure 63 depicted a significant increase in percentage uterine DNA fragmentation in the MSG-treated rats compared with the control while it was lowered in the co-administered group.

As shown in Figures 64 and 65, there was significant upregulation of caspases 9 and 3 in the MSG-administered rats relative to control and this was ameliorated in the group co-administered with CFDC and MSG, but there was no significant difference between the MSG-treated group and the co-administered group. As shown in Figure 66, CFDC-treated rats, MSG-treated rats and

the co-administered group showed negative immunoreactivity for anti-apoptotic bcl-2 protein. However, there was moderate expression in the control rats.

CONCLUSION

The results show that chloroform fraction of *Drymaria cordata* has an antioxidant property which enables it to reduce lipid peroxidation induced in MSG-treated rats. It also shows that CFDC posseses bioactive agents that can protect against MSG-induced apoptotic damage. The results also show that MSG is an inducer of apoptosis.

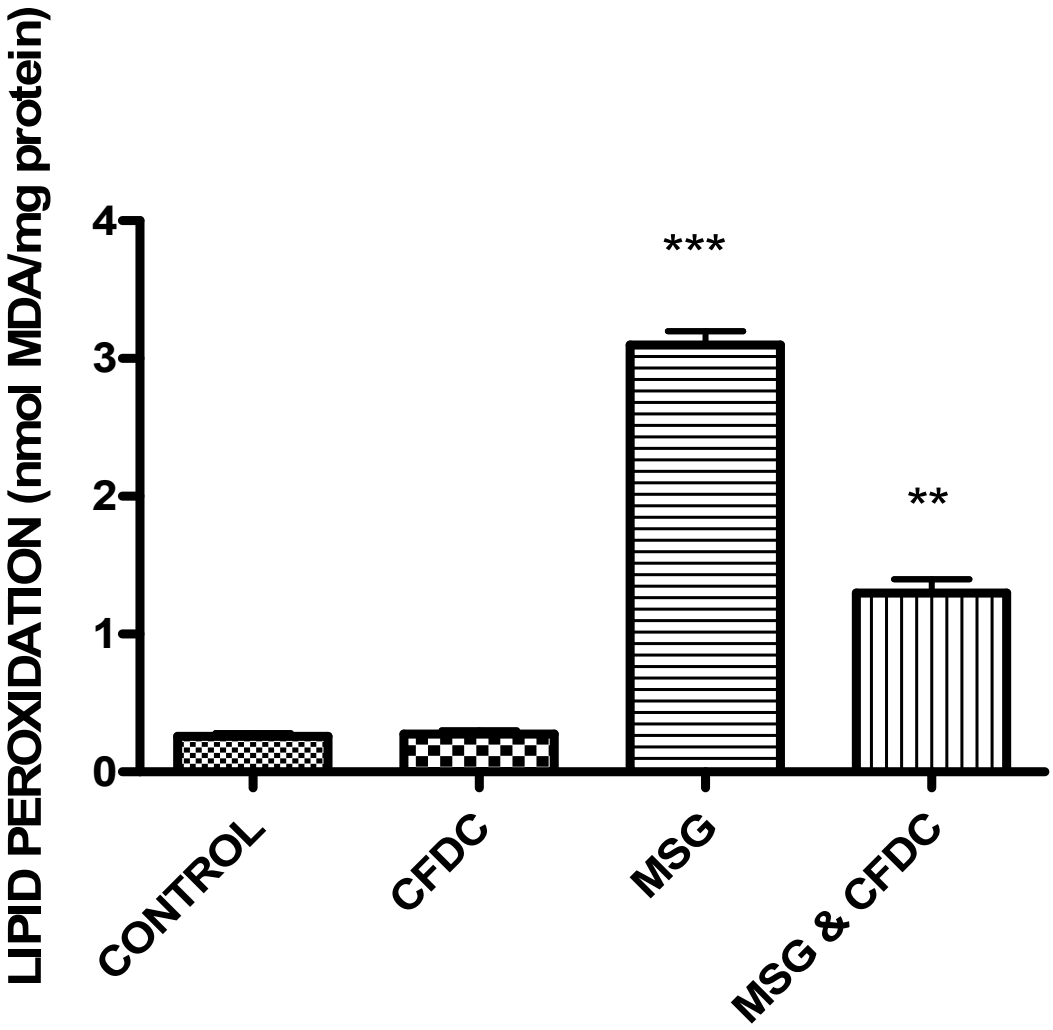


Figure 62: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on lipid peroxidation in normal and monosodium glutamate (MSG)-treated rats

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

***** Each value is statistically significant at p<0.001, compared with control using the one-way ANOVA**

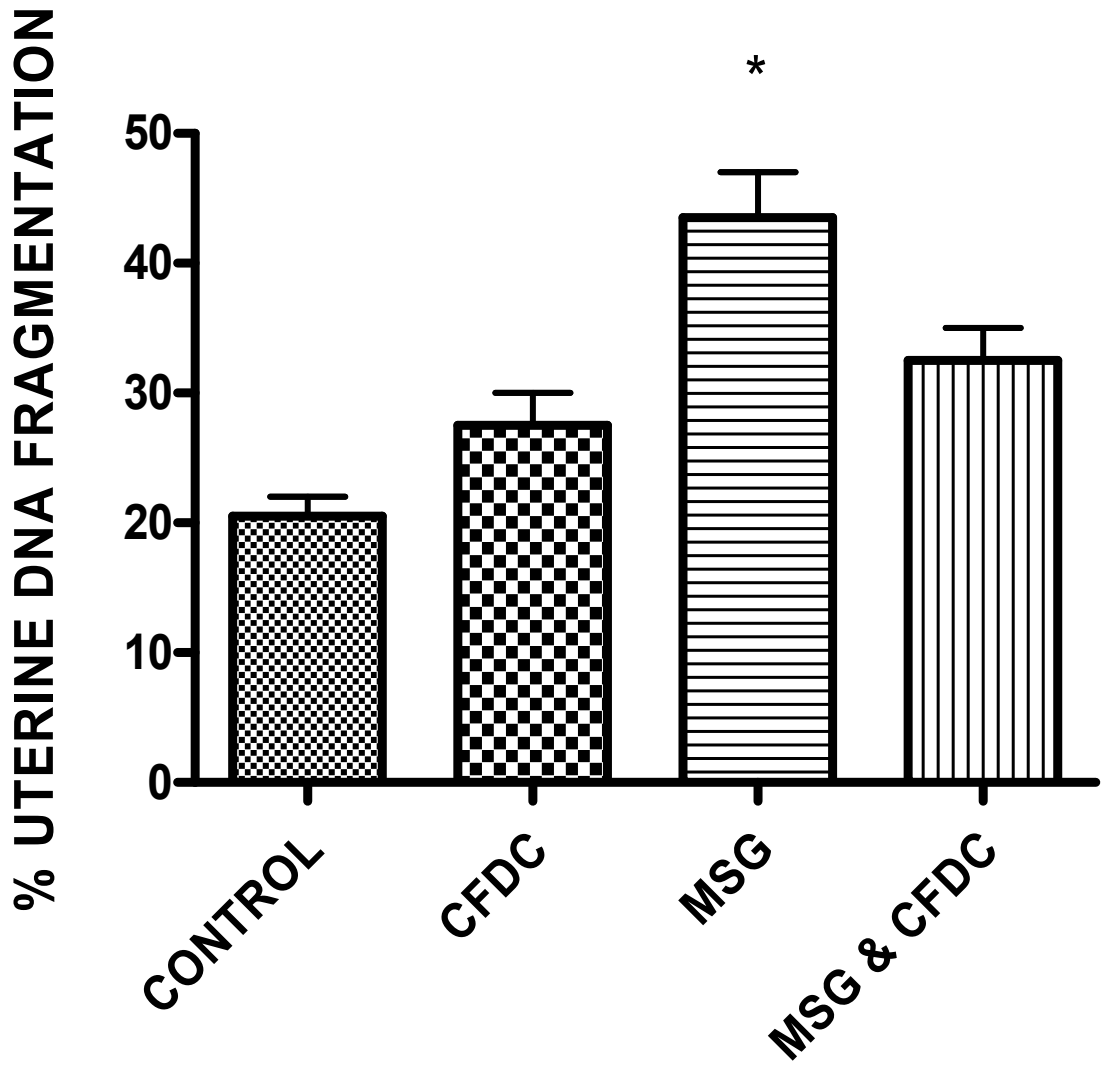


Figure 63: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on uterine DNA fragmentation in normal and monosodium glutamate (MSG)-treated rats

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

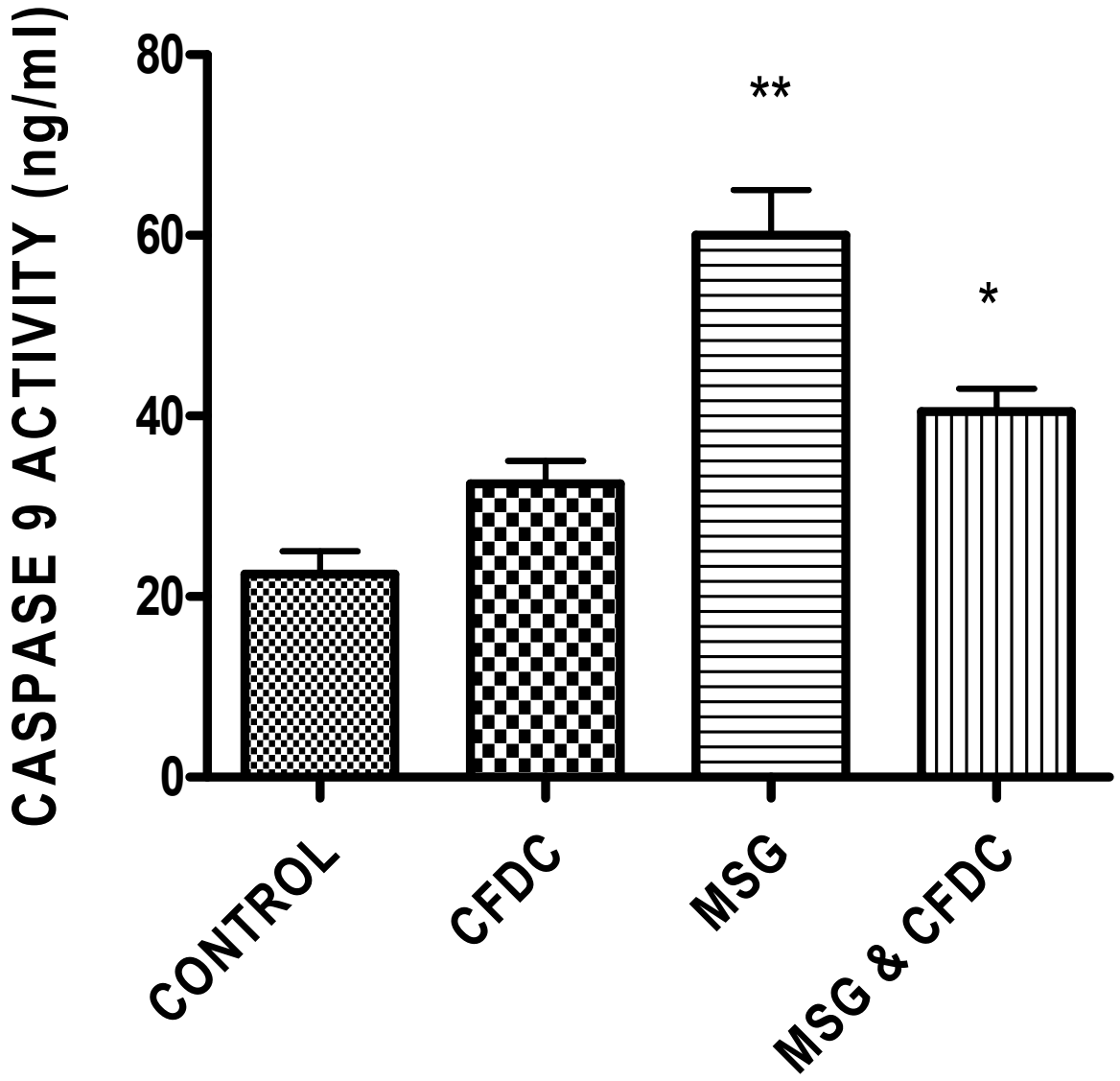


Figure 64: Effect of oral administration of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on caspase 9 activation in normal and monosodium glutamate (MSG)-treated female rats

*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**

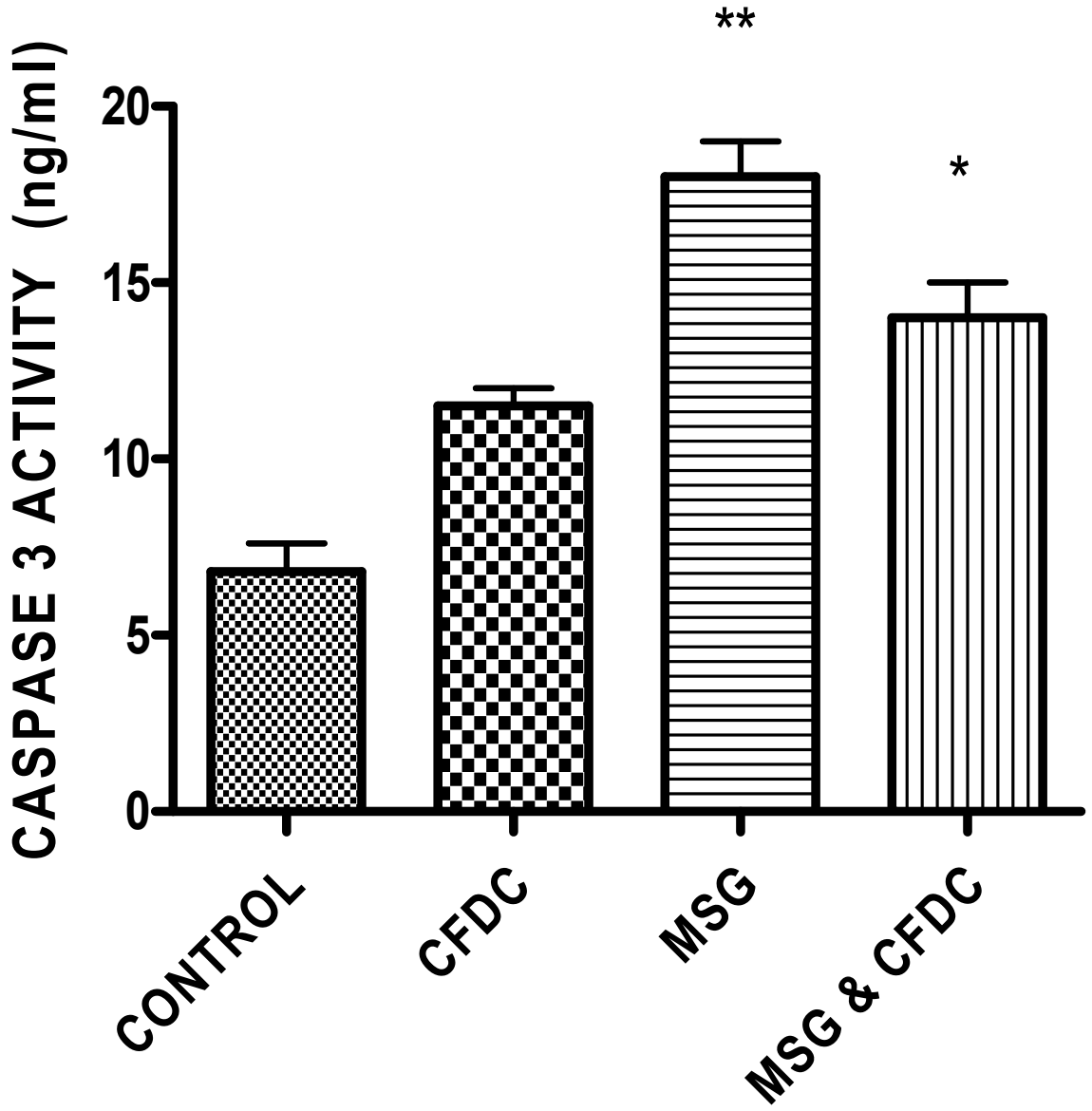
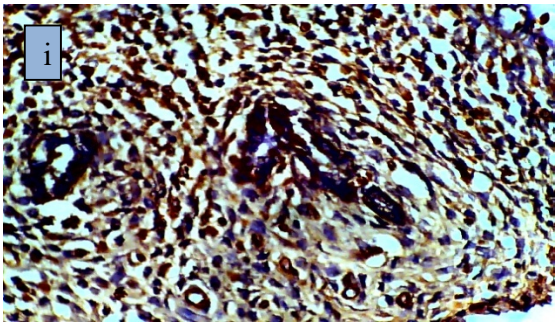
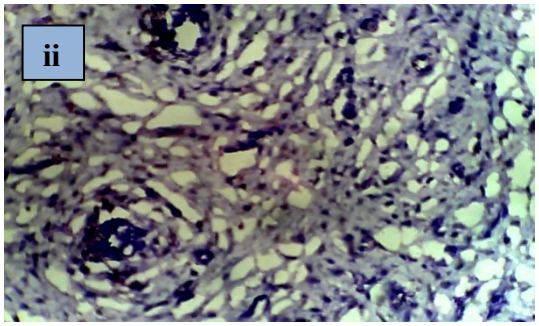


Figure 65: Effect of oral administration of chloroform fraction of methanol extract of D*rymaria cordata* on caspase 9 activation in normal and monosodium glutamate (MSG)-treated rats

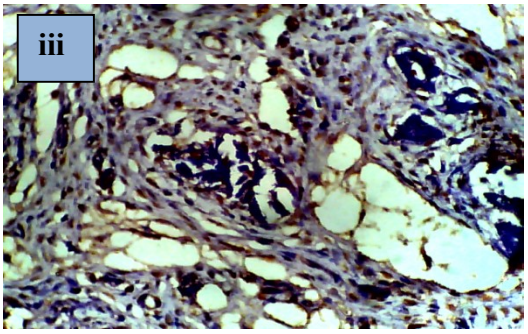
*** Each value is statistically significant at p<0.05, compared with control using the one-way ANOVA**

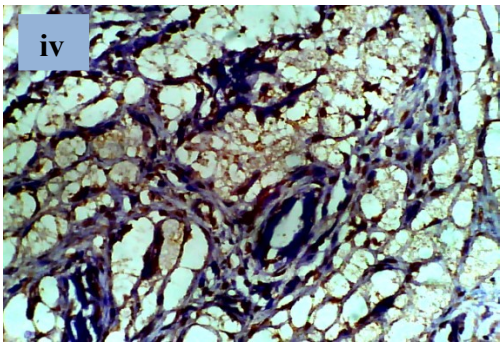
**** Each value is statistically significant at p<0.01, compared with control using the one-way ANOVA**





CONTROL **CFDC**





MSG **MSG & CFDC**

Figure 66a: Effect of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on Bcl-2 protein in normal and monosodium glutamate (MSG)-treated rat uterus (Mag x400)

i : (control) showed moderate expression of anti-apoptotic bcl-2 protein

ii: (CFDC) showed negative expression of anti-apoptotic bcl-2 protein

iii: (MSG) showed negative expression of anti-apoptotic bcl-2 protein

Iv: (MSG & CFDC) showed negative expression of anti-apoptotic bcl-2 protein

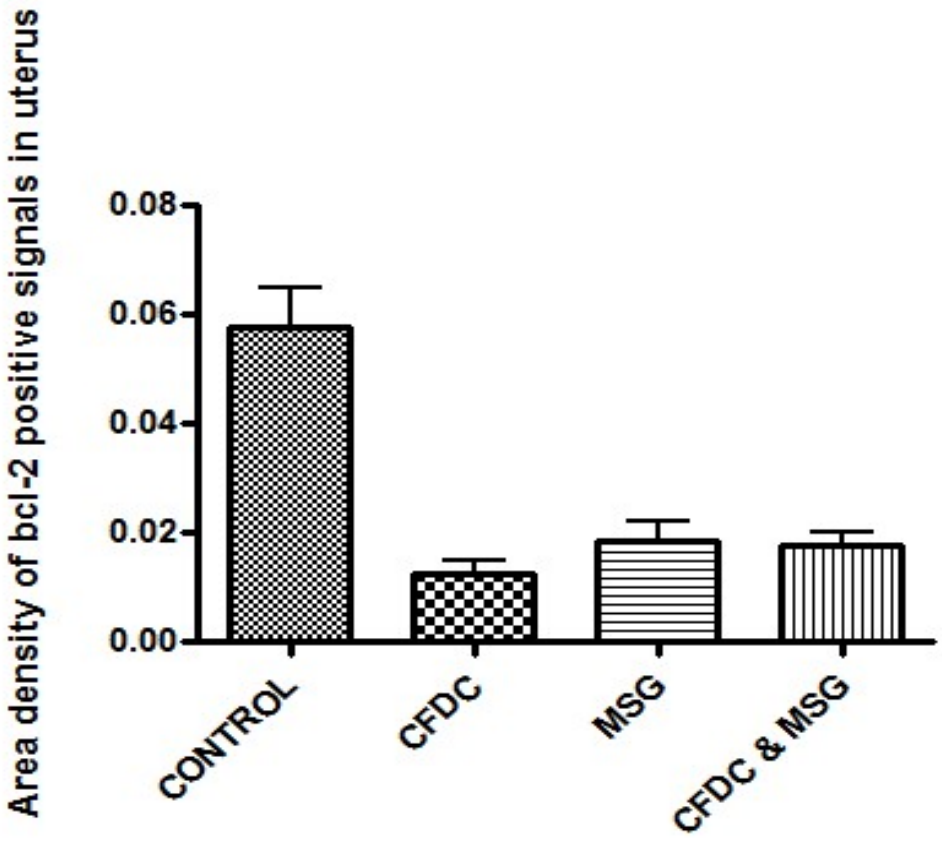


Figure 66b: Graphical representation of the effect of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) on Bcl-2 protein in normal and monosodium glutamate (MSG)-treated rat uterus

EXPERIMENT 20: IDENTIFICATION AND PARTIAL PURIFICATION OF BIOACTIVE COMPOUNDS PRESENT IN THE CHLOROFORM FRACTION OF *Drymaria cordata*

INTRODUCTION

Phytochemicals are a fascinating yet mysterious group of thousands of chemicals found in plants. Some protect against cancer when isolated, some are not associated with cancer at all, and many have yet to be discovered. It is known that many phytochemicals, when kept in their natural food forms, can protect us from cancer as they interact with other phytochemicals and the cells in our bodies (Liang *et al.* 2001). Epidemiological studies showing a protective effect of diets rich in fruits and vegetables against cancer have focused attention on the possibility that biologically-active plant secondary metabolites exert anti-carcinogenic activity. This huge group of compounds, now collectively termed 'phytochemicals', provides much of the flavour and colour of edible plants and the beverages derived from them. Many of these compounds also exert anti-carcinogenic effects in animal models of cancer, and much progress has been made in defining their many biological activities at the molecular level. Such mechanisms include the detoxification and enhanced excretion of carcinogens, the suppression of inflammatory processes such as cyclooxygenase-2 expression, inhibition of mitosis and the induction of apoptosis at various stages in the progression and promotion of cancer (Houston *et al.* 2005; He *et al*. 2006).

PROCEDURE

The dried and milled plant material (5kg) was extracted with methanol at room. After soaking for one week, it was sieved and concentrated. The concentrate consists of an oily upper part and a

brown residue which were separated by decantation. The crude methanol extract was subjected to VLC chromatography as described on page 57 to obtain the chloroform fraction.

The chloroform fraction obtained was further partitioned into six sub-fractions using the following solvent system by VLC technique.

N-hexane	100%	(A)
N-hexane:chloroform	80:20	(B)
N-hexane:chloroform	50:50	(C)
N-hexane:chloroform	20:80	(D)
Chloroform	100%	(E)
Chloroform:methanol	80:20	(F)

The most potent sub fraction (D) with respect to induction of pore opening was adsorbed on silica gel in a column packed with silica gel using n-hexane. The column was eluted using the following solvent systems:

- N-hexane 100%
- N-hexane :chloroform 95:5
- N-hexane :chloroform 90:10 * tubes 21-35 (G)

- N-hexane :chloroform 85:15
- N-hexane :chloroform 80:20
- N-hexane :chloroform 75:25 * tubes 85-105 (H)
- N-hexane :chloroform 70:30
- N-hexane :chloroform 65:35 * tubes 152-183 (I)
- N-hexane :chloroform 60:40

Fractions that showed similar components on TLC were pulled together. The samples G, H and I which showed a single band on the TLC plates were further subjected to micro-column chromatography for a better resolution and more purified sample using the same solvent system.

Sample G - tubes 10 & 11

Sample H - tubes 10, 11, 12 and 13

Sample I - tubes 8, 9 and 10

The samples obtained were analysed using GC-MS.

RESULTS

The results show the presence of hexadecanoic acid methyl ester (11.30%), n-hexadecanoic acid (14.71%) and oxalic acid, 2-ethylhexyl isobutyl ester (63.66%) in sample G and 3-(p-fluoro-benzoyl) propinoic acid (70.24%) and Bis(2-ethylhexyl) phthalate(11.96%) in sample H. Other compounds identified in sample I include hexadecanoic acid methyl ester (16.11%), hexadecanoic acid ethyl ester (37.18%) and 9-octadecenoic acid (44.14%)

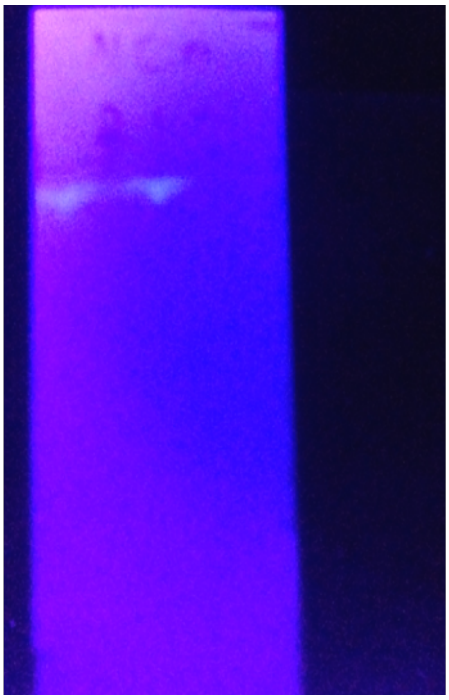


Figure 67 : TLC plate of sample G

Sample G: Partially purified sub fraction of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) containing hexadecanoic acid methyl ester, n-hexadecanoic acid and oxalic acid, 2-ethylhexyl isobutyl ester

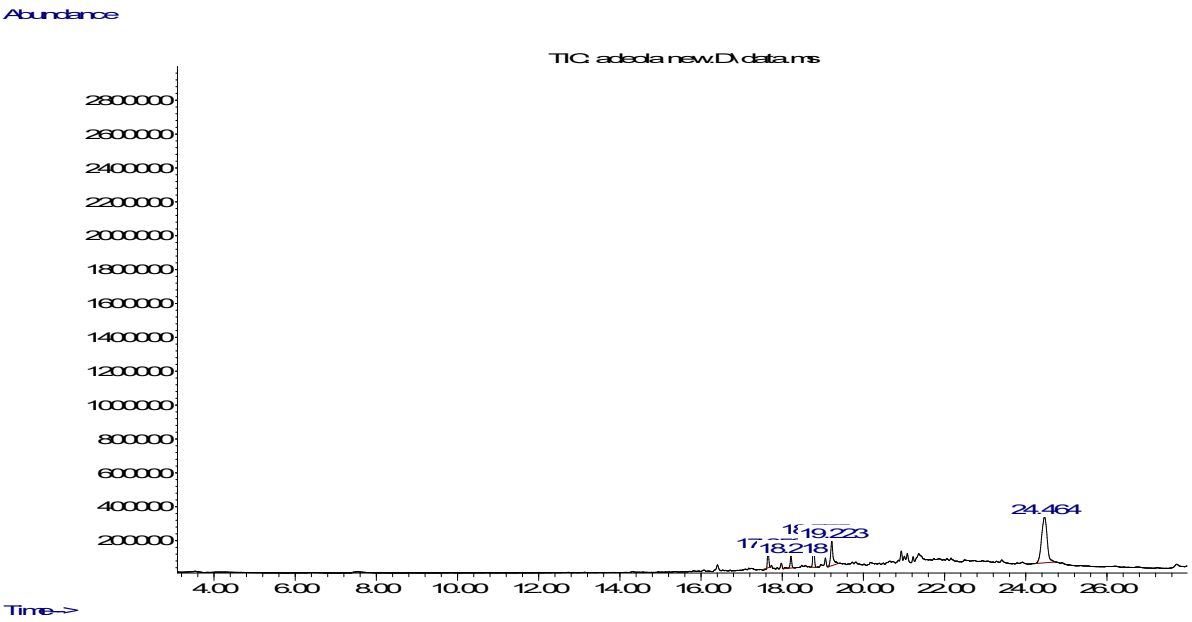


Figure 68a: Chromatogram of sample G partially purified from chloroform fraction of *Drymaria cordata* (CFDC)

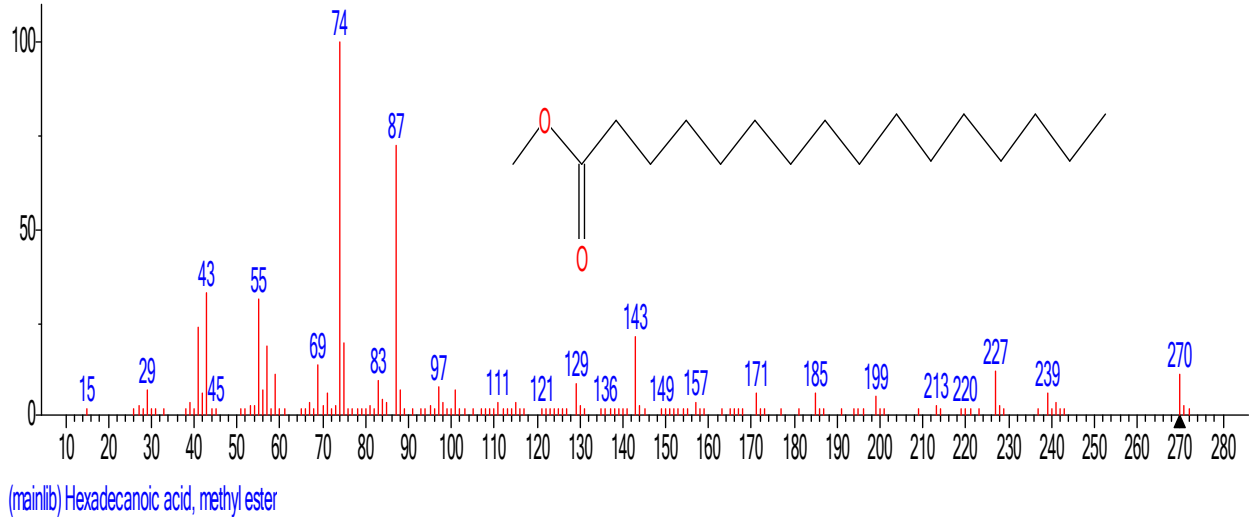


Figure 68b: Retention peak and mass spectrum obtained by GCMS of sample G containing hexadecanoic acid methyl ester (11.30%)

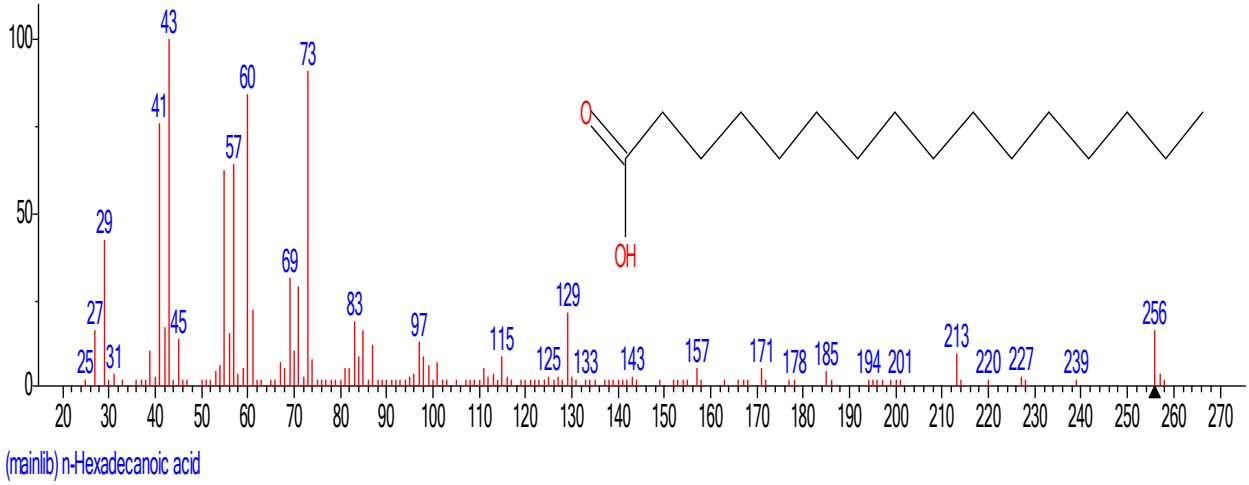


Figure 68c: Retention peak and mass spectrum obtained by GCMS of sample G containing n-hexadecanoic acid (14.77%)

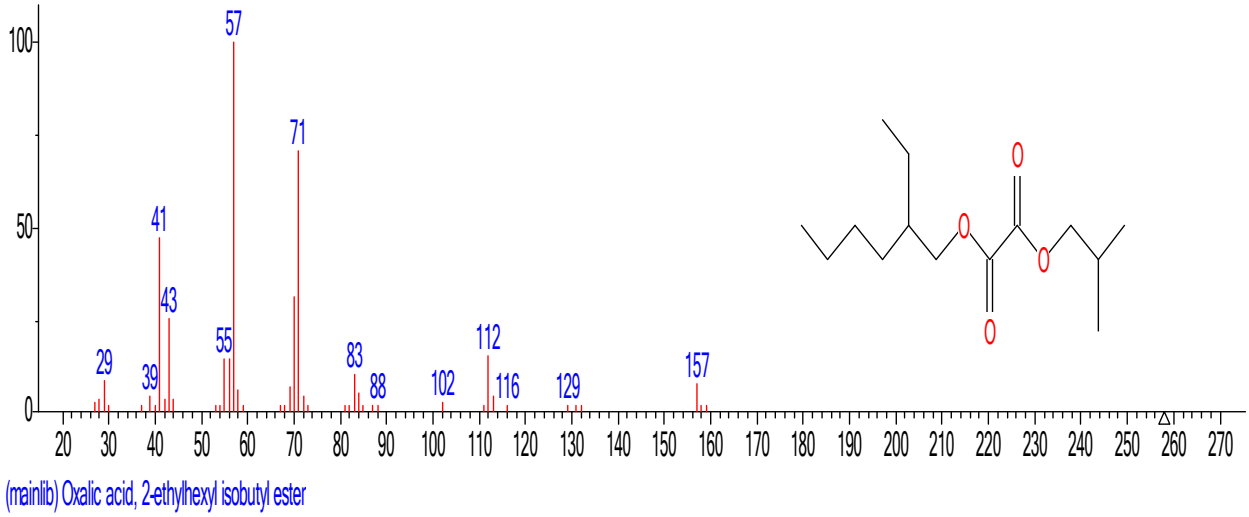


Figure 68d: Retention peak and mass spectrum obtained by GCMS of sample G containing oxalic acid, 2 ethylhexyl isobutyl ester (63.66%)

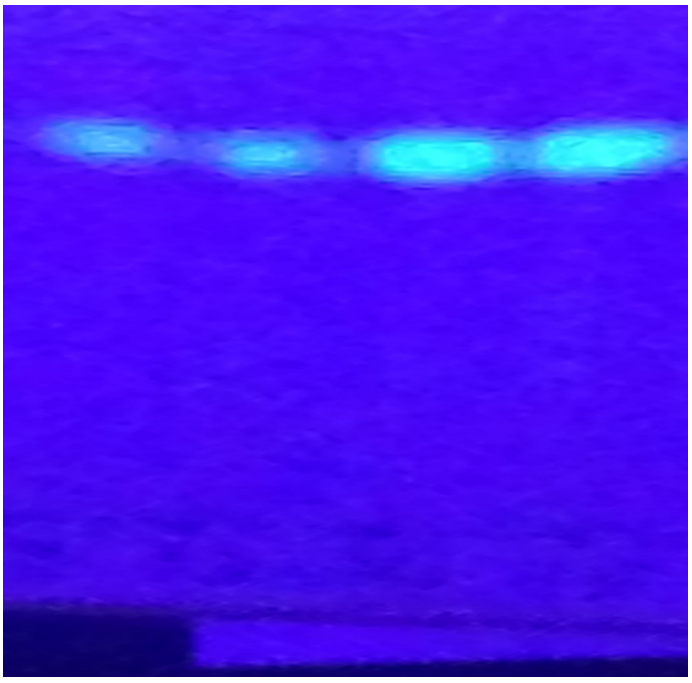


Figure 69: The TLC plate of sample H

Sample H: Partially purified sub fraction of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) containing hexadecanoic acid methyl ester, Bis (2-ethylhexyl) phthalate and 3-(p-Fluorobenzoyl)-propionic acid

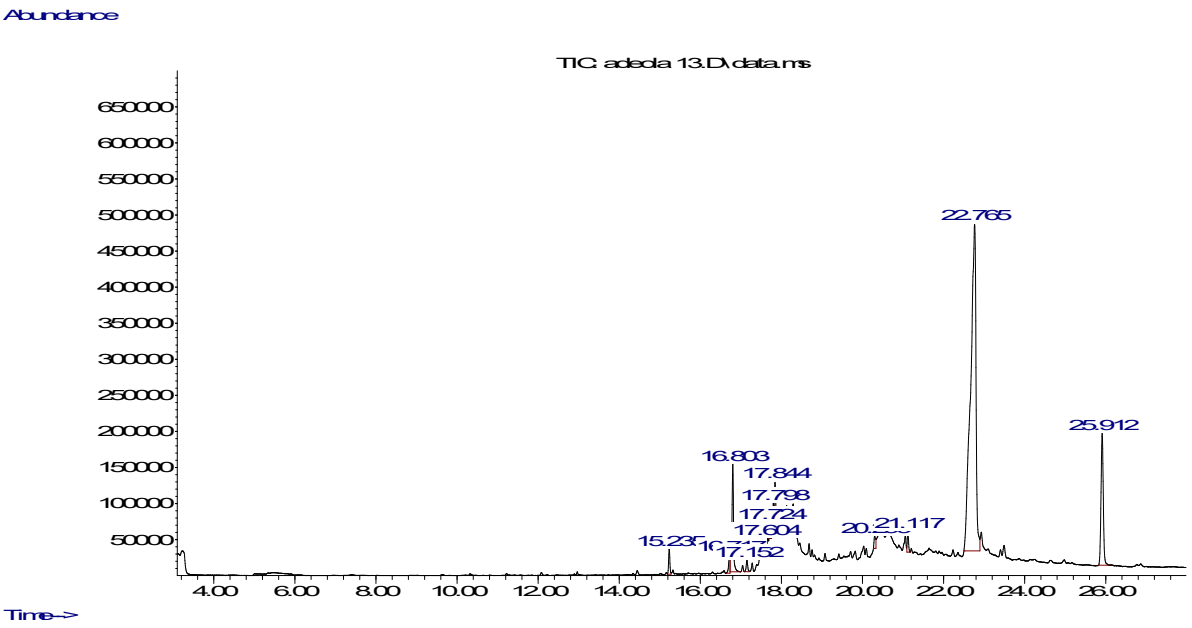


Figure 70a: Chromatogram of sample H partially purified from chloroform fraction of methanol extract of *Drymaria cordata* (CFDC)

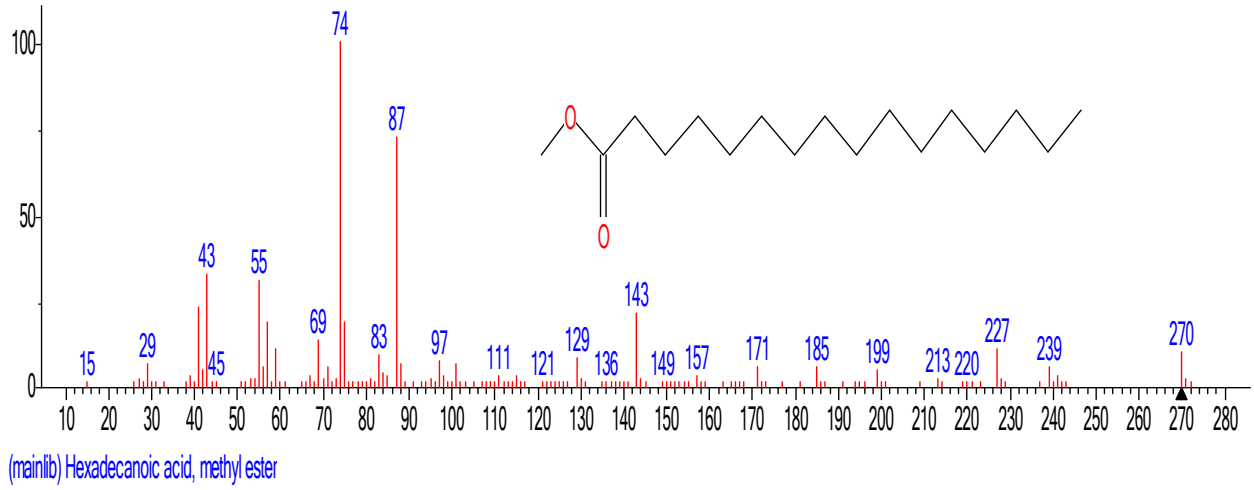


Figure 70b: Retention peak and mass spectrum obtained by GCMS of sample H containing hexadecanoic acid (2.35%)

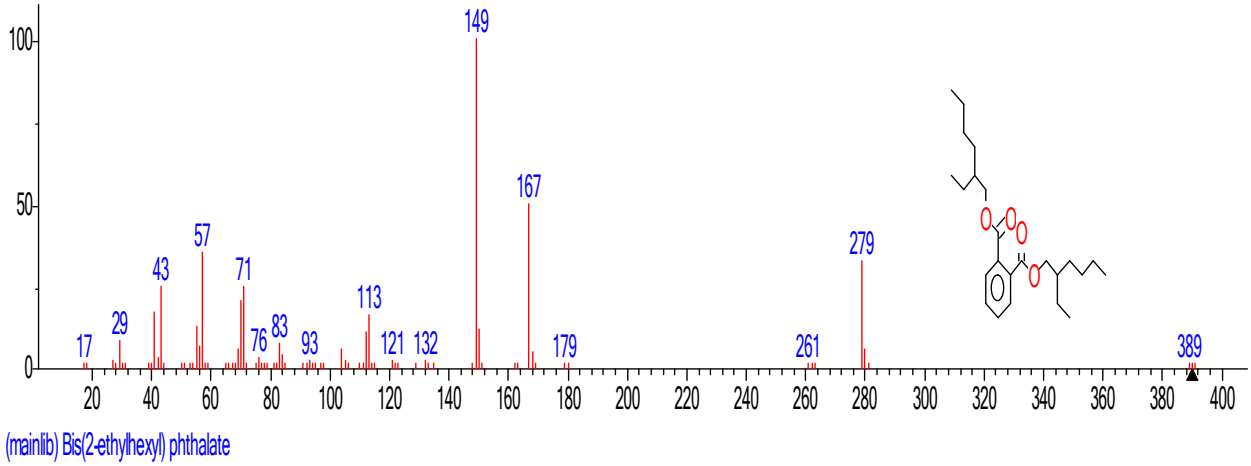


Figure 70 c: Retention peak and mass spectrum obtained by GCMS of sample H containing Bis (2-ethylhexyl) phthalate (11.96%)

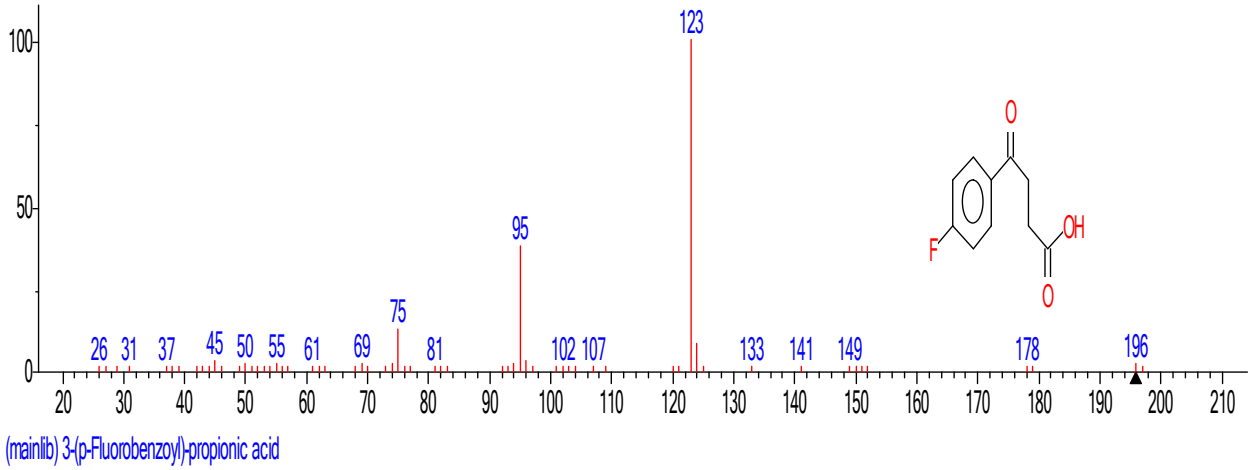


Figure 70d: Retention peak and mass spectrum obtained by GCMS of sample H containing 3-(p-Fluorobenzoyl)-propionic acid (70.24%)

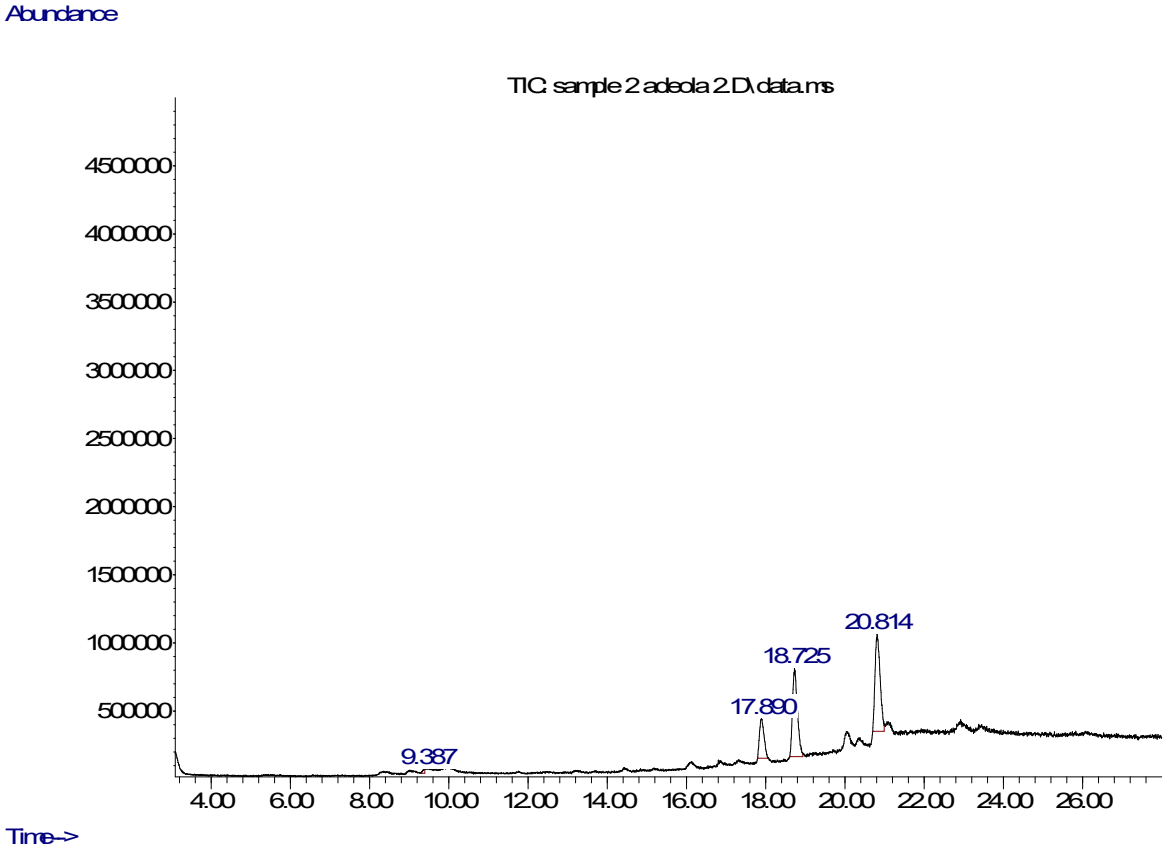


Figure 71a: GCMS chromatogram of sample I partially purified from chloroform fraction of methanol extract of *Drymaria cordata* (CFDC)

Sample I: Partially purified sub fraction of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) containing hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester and 9-octadecenoic acid

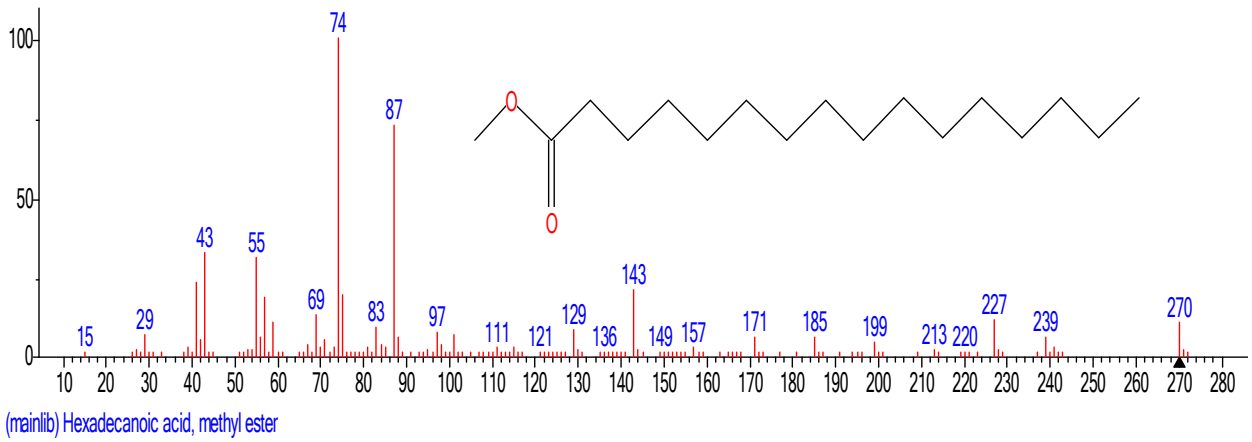


Figure 71b: Retention peak and mass spectrum obtained by GCMS of sample I containing hexadecanoic acid methyl ester (16.11%)

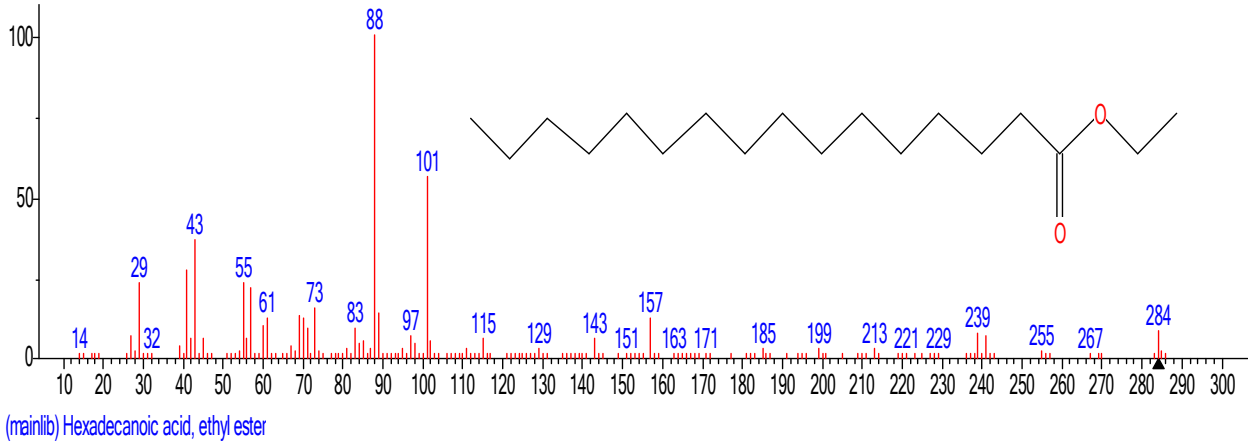


Figure 71c: Retention peak and mass spectrum obtained by GCMS of sample I containing hexadecanoic acid methyl ester (38.98%)

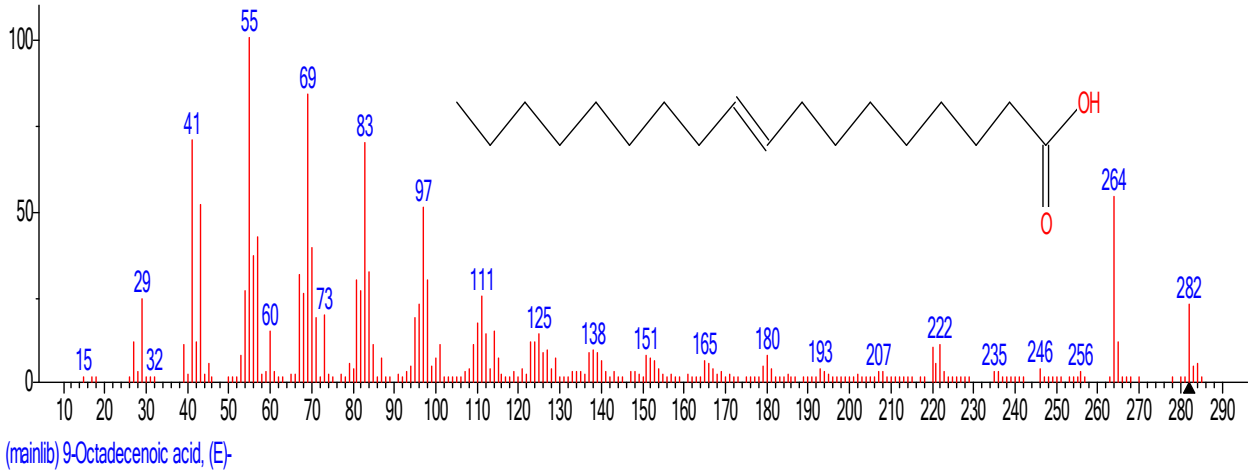


Figure 71d: Retention peak and mass spectrum obtained by GCMS of sample I containing 9-octadecenoic acid (44.14%)

CONCLUSION

The partially purified samples from CFDC contains phytochemicals compounds which might be responsible for its pharmacological activities.

EXPERIMENT 21: ASSESSMENT OF THE EFFECTS OF OXALIC ACID, 2-ETHYLHEXYL ISOBUTYL ESTER AND 3-(P-FLUOROBENZOYL) PROPIONIC ACID ON MPT PORE OPENING , OESTROGEN, PROGESTERONE AND TOTAL CHOLESTEROL LEVELS IN NORMAL AND MSG-TREATED MICE

INTRODUCTION

The pathogenesis of several chronic diseased conditions, including cancer, have been attributed to dysregulated apoptosis (Zhang, 2004). The inability of the diseased cell to die as a result of defective apoptotic pathway seems to be a universal occurrence in cancer ((Moore *et al*., 2007). Interestingly, studies have shown that there are some naturally-occurring compounds of plant origin which can modulate signal transduction pathways, apoptosis and cell cycle progression, supporting them as a potential candidate in cancer management and therapy (Catherino *et al.,* 2011). This experiment was therefore designed to test the partially purified compounds on mPT pore opening and the levels of hormones that are implicated in the development of fibroids using mice.

Procedure

The procedure was carried out as previously described on pages 60, 62, 66, 87 and 88.

RESULTS

Figure 72 showed that the mitochondria were intact. Addition of calcium caused highly significant increase in mitochondria swelling which was almost totally reversed by spermine, showing that the mitochondria used in this investigation were intact (Figure 74).

Figures 73 and 74 show the effect of partially purified oxalic acid, 2-ethylhexyl isobutyl ester and 3-(p-fluorobenzoyl)-propinoic acid, respectively, on mPT pore. At concentrations 0.8µg/ml and 1.6µg/ml, Oxalic acid, 2-ethylhexyl isobutyl ester gave an induction fold of 1.5 and 2.8 respectively while 3-(p-fluorobenzoyl)-propinoic acid, gave an induction fold of 4.1 and 7.8 respectively. Also, 3-(p-fluorobenzoyl)-propinoic acid reversed the MSG–induced increase in oestrogen, progesterone and total cholesterol levels in MSG-treated mice

CONCLUSION

The partially purified fractions induced mPT pore opening. Therefore, the purified fractions may find therapeutic use in conditions requiring induction of cell death such as cancer. Also, 3-(p-fluorobenzoyl)-propinoic acid reversed the levels of hormones associated with development of fibroid in MSG-treated mice. The presence of this compound in CFDC may be one of the reasons for its potential to reverse MSG-induced uterine hyperplasia.

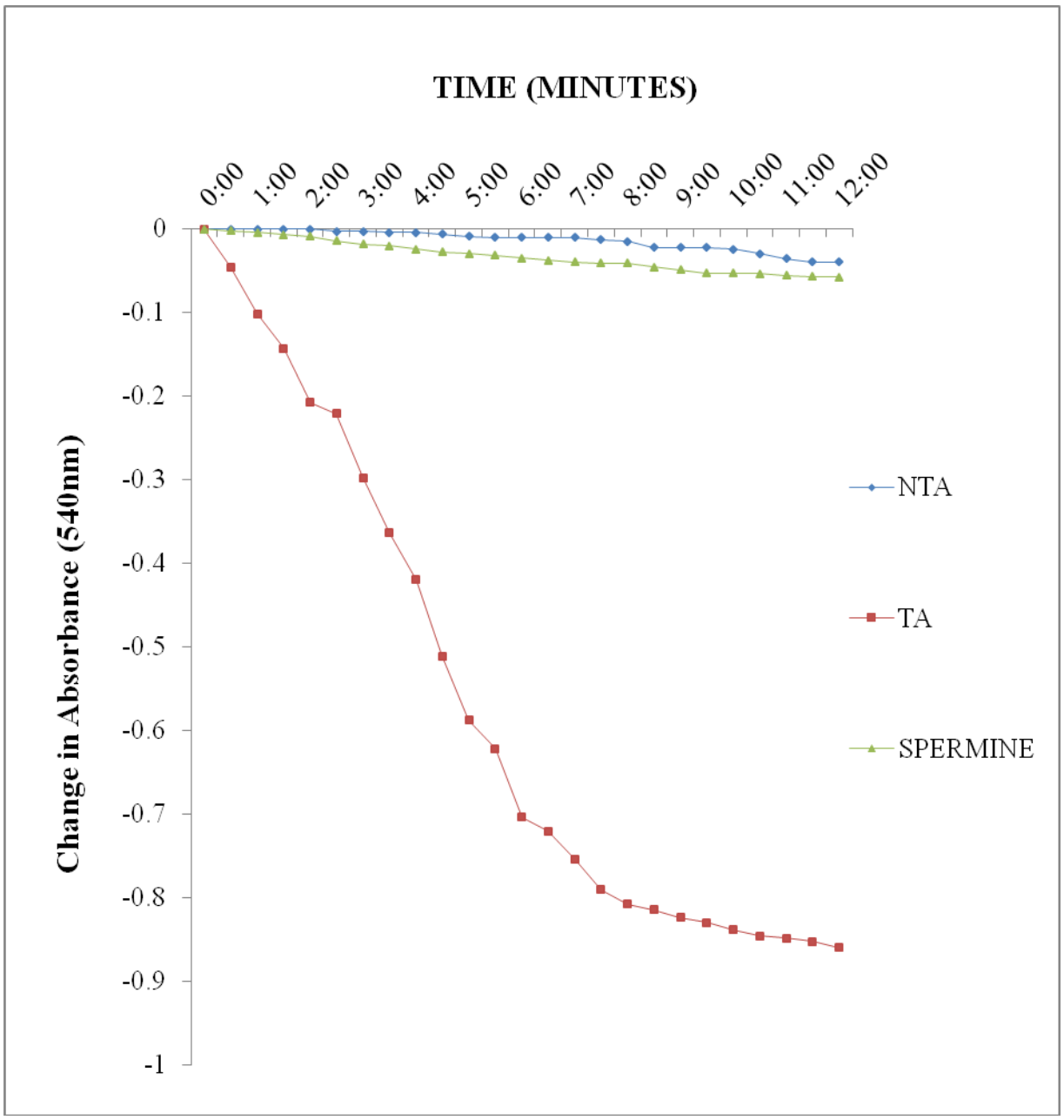


Figure 72: Determination of intactness of the mitochondria

NTA: No triggering agent (without calcium)

TA: Triggering agent (with calcium)

SPERMINE: Standard inhibitor

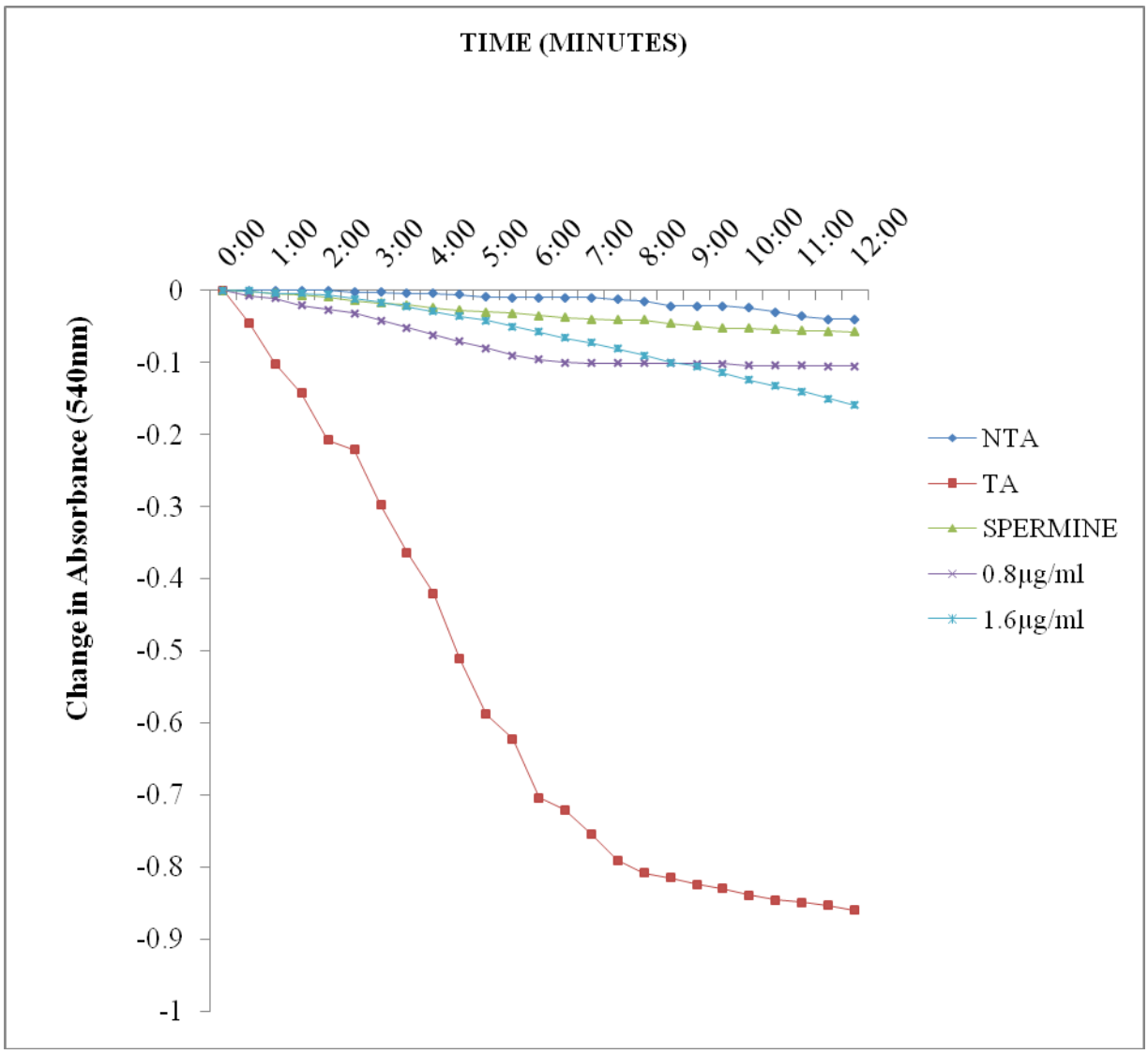


Figure 73: Effect of sample G on rat liver mPT pore

NTA: No triggering agent (without calcium)

TA: Triggering agent (with calcium)

SPERMINE: Standard inhibitor

Sample G: Partially purified sub fraction of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) containing hexadecanoic acid methyl ester, n-hexadecanoic acid and oxalic acid, 2-ethylhexyl isobutyl ester

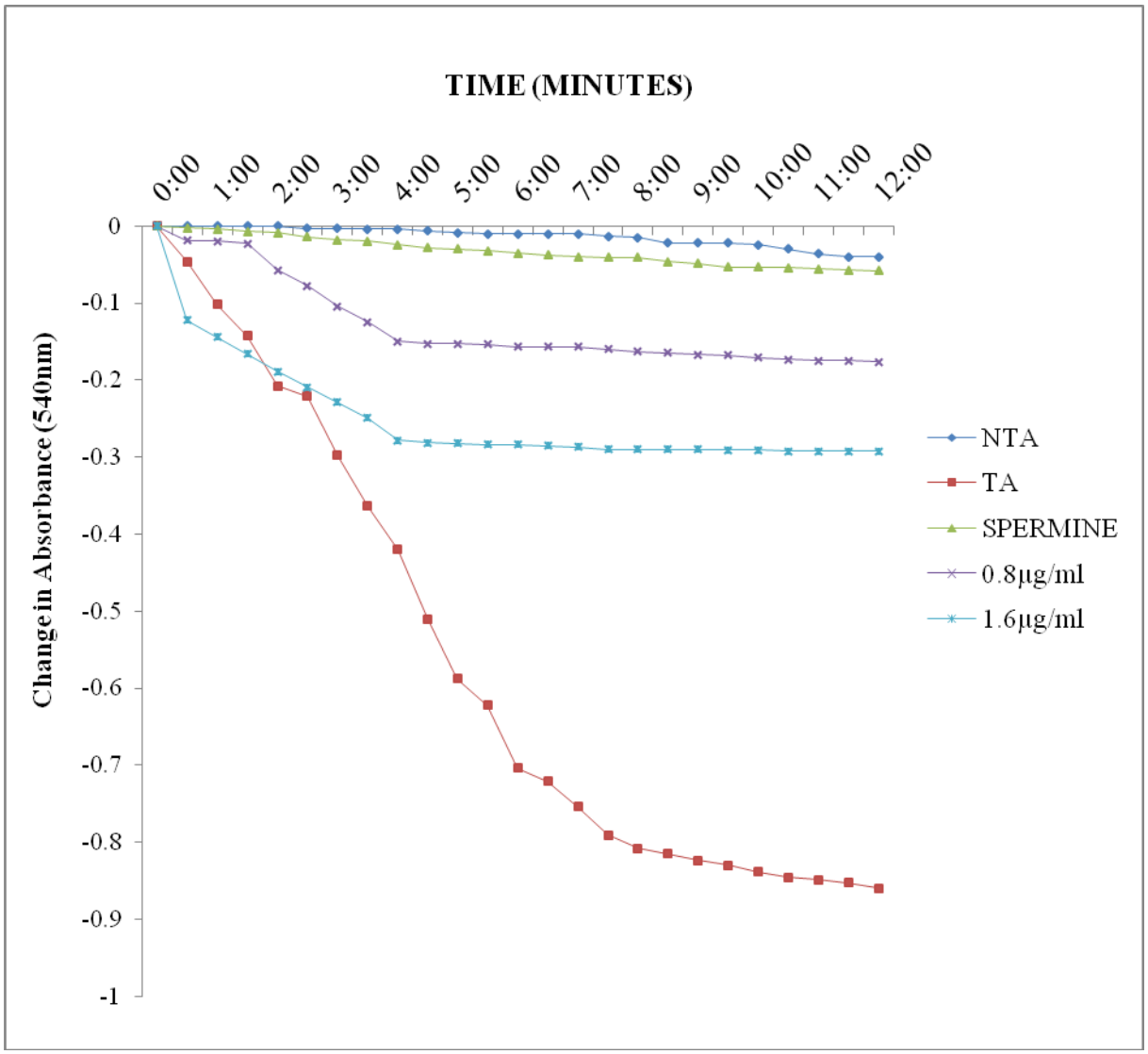


Figure 74: Effect of sample H on MPT pore

NTA: No triggering agent (without calcium)

TA: Triggering agent (with calcium)

SPERMINE: Standard inhibitor

Sample H: Partially purified sub fraction of chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) containing hexadecanoic acid methyl ester, bis (2-ethylhexyl) phthalate and 3-(p-fluorobenzoyl)-propionic acid

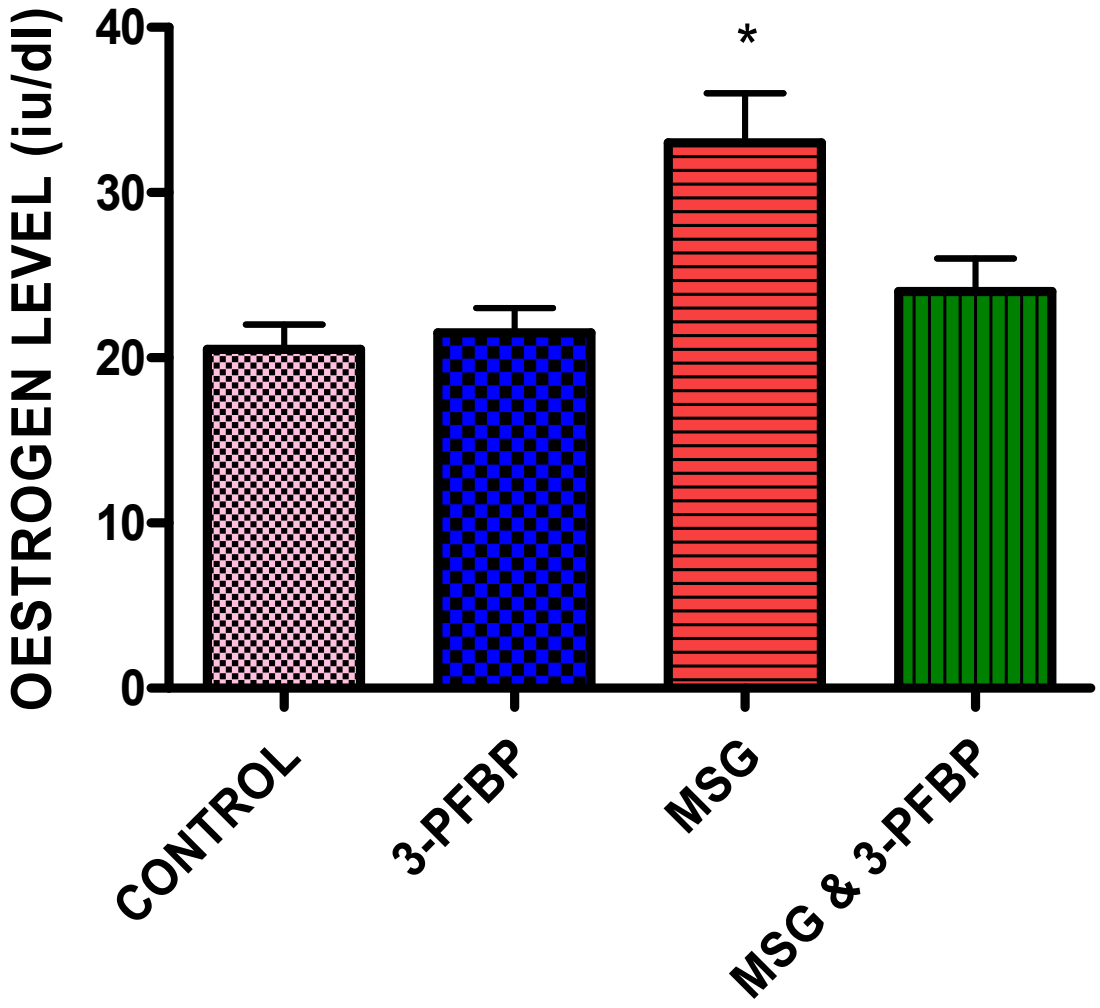


Figure 75: Effect of 3-(p-fluorobenzoyl) propionic acid on oestrogen level in normal and monosodium glutamate (MSG)-treated mice

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

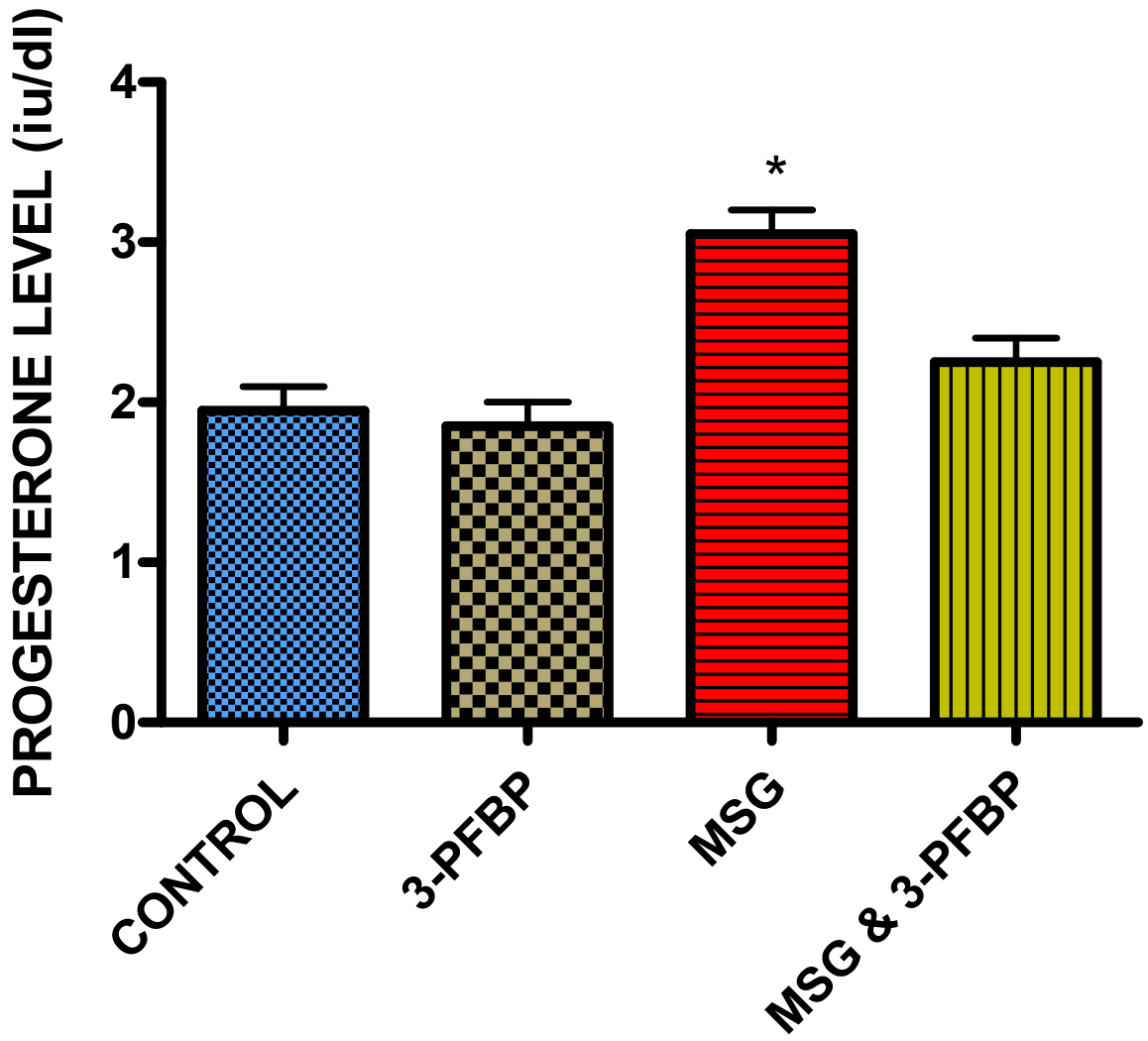


Figure 76: Effect of 3-(p-fluorobenzoyl) propionic acid on progesterone level in normal and monosodium glutamate (MSG)-treated mice

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

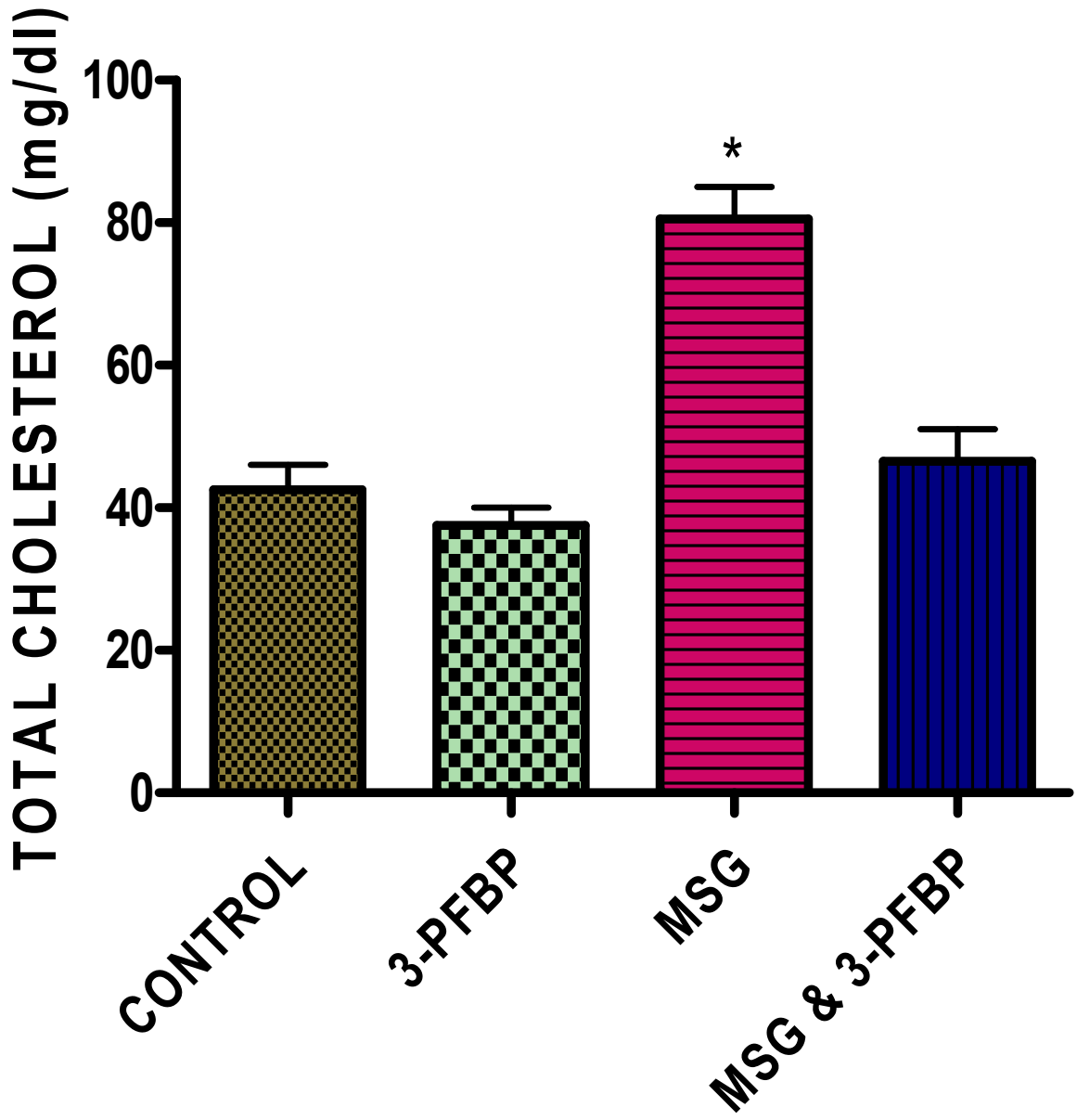


Figure 77: Effect of 3-(p-fluorobenzoyl) propionic acid (3PFBPA) on total cholesterol level in normal and monosodium glutamate (MSG)-treated mice

*** Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA**

CHAPTER FIVE

DISCUSSION

To allow for cellular homeostasis, apoptotic mechanism should not be dysregulated in a normal cell. When there is a compromise and the cell also fails to activate cell death, such cell is prone to cancer development. Though there are several signaling pathways that lead to cancer vulnerability, perharps one of the best approaches to specifically eliminate cancer is to target the cancerous cells via apoptosis. The mitochondrion which is a life supporting organelle is also an agent of cell death (Hopkins-Donaldson *et al*., 2003). Mitochondrion has been made a pharmacological target for drug design and therapeutic manipulation because of its dual role. Defect in the apoptotic pathways has been a factor in cancer development. Drugs or therapeutic agents that have the ability to normalize the defective apoptotic pathways can protect against cancer development. Since cancer cells are using the opportunity of the the defective signals to stay alive, any therapeutic approach/strategy that can reverse the impaired apoptotic signaling pathways could eliminate cancerous cells. Recently, many important discoveries have lead to the development of dietary phytochemicals as anticancer drugs. Dietary phytochemicals are plant based chemical compounds with disease-preventive properties. These phytochemicals can elicit their therapeutic effects on several pathological conditions by modulation of diverse signaling pathways (Le Marchand *et al*., 2000). For example, the incidence of several cancers have been shown to be ameliorated by foods rich in flavonoids and quercetin. Green vegetables and fruit consumption also show protective potential on uterine fibroid (Wise *et al*., 2011). Green tea intake has also been shown to lower the risk of breast, prostate, ovarian and oesophageal cancer (Song *et al*., 2012). A combination of *Drymaria cordata* and *Dissotis*

rotundifolia is used in the traditional treatment of uterine fibroid. *Drymaria cordata* is used traditionally to treat liver and uterine tumour. However, paucity of scientific information to validate the rationale for the use of the herbal medication stimulated further investigation on the potential of *Drymaria cordata* as a possible anti tumour agent with particular emphasis on evasion of the apoptotic signal which is a hallmark in many forms of cancer (Yang *et al*., 2013). Research has shown that certain bioactive agents which are present in medicinal plants elicit their chemoprotective and therapeutic effects by inducing the mPT pore and thereby activate mitochondrial-mediated apoptosis (MMA) which might prove useful in diseased conditions where apoptosis needs to be upregulated (Tascilar *et al*., 2006). It is not known whether any of the phytochemical constituents of the *Drymaria cordata* would have modulatory effect on intrinsic pathway of apoptosis via mitochondrial permeability transition pore opening. In the *in vitro* study, the mitochondrial intactness was established as the exogenous calcium caused induction of the mPT pore opening, while spermine a potent inhibitor significantly reversed the calcium-induced pore opening. This may indicate that the the membrane intergrity of the liver mitochondria were not compromised, hence, the mitochondria were suitable for use in this study (Lapidus and Sokolove,1993; Javadov and Karmazyn, 2007). When intact mitochondria were exposed to MEDC and CFDC in the absence of calcium, the mPT pore openend significantly compared to NTA. Moreover, the opening of the pore was reversed by spermine showing that MEDC and CFDC induction of pore opening had no deleterious effect on membrane integrity. These therefore suggest that MEDC and CFDC may contain certain bioactive agents that could interact with the components of the pore, cause the opening of the pore and ultimately apoptosis. Moreover, since the effect was concentration-dependent, it may be deduced that the active principle increased with increase in concentration of the fraction used. Also, the fact that MEDC

and CFDC inhibited lipid peroxidation shows that the mechanism of induction of pore opening is not via damage of the lipid bilayer by peroxidation. This further suggests that the fractions could protect against lipd peroxidation-induced damage. The observation that EFDC and AFDC had no effect on mPT pore at lower concentrations and little effect at the highest concentration used indicates the presence of low concentration of this active principle in the these fractions compared to MEDC and CFDC. These studies show that the active component responsible for the opening of the pore is highest in the chloroform fraction of the MEDC. Conversely, when calcium is present, MEDC and CFDC reversed calcium-induced pore opening when compared to spermine. This might result from the presence of some agents which have calcium chelating property thereby reducing the concentration of calcium available to induce pore opening. The EFDC and AFDC had no significant effect on calcium-induced pore opening.

Cytochrome c release due to opening of pore has been shown to be critical for for the induction of mitochondrial-mediated apoptosis (Green and Kroemer, 2004). CFDC effected the highest release of cytochrome c compared to other fractions. This result suggests that there was mPT pore opening which resulted in cyt c release. This is *sine qua non* for apoptosis. This result conforms with the mPT result where CFDC had the highest effect. However, the stimulatory effect shown by EFDC and AFDC is very low and statistically not significant.

There are some hypotheses showing the involvement of ATP synthase in the formation of PT pore. Some researchers even reported that it is the c ring that forms the pore. (Morciano *et al.,* 2015), and there is another hypothesis that the mPT pore arises from the c-ring of the F_0 sector of the mitochondrial ATPase (Bernadi and Di Lisa, 2015). All theses hypotheses have been scientifically disproved recently (Zhou *et al*., 2017). Though, when there is prolonged opening of

the pore, the ATP synthase starts hydrolyzing ATP and inorganic phosphate is released. The release of inorganic phosphate (Pi) is an indication of uncoupling of phosphorylation in the mitochondrion and this happens during pathological conditions (Rasul *et al*., 2013). The inorganic phosphate released is used as an index to measure the ATPase activity. The status of mitochondrial F_0F_1-ATPase was therefore investigated in the presence of the various solvent fractions of *Drymaria cordata* (MEDC, CFDC, EFDC and AFDC) using 2,4-Dinitrophenol (DNP), as a standard. This was determined using spectrophotometer by following the rate of release of inorganic phosphate from the ɣ-position of ATP during the ATPase action. The results from this study showed that chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) and the methanol extract of *Drymaria cordata* (MEDC) significantly caused the release of inorganic phosphate in a concentration-dependent manner as measured by their ATPase activity. The EFDC and AFDC did not show any significant effect on the ATPase activity. This may be due to lack of the active component in the EFDC and AFDC. The highest effect on ATPase activity was observed with CFDC, followed by MEDC. This result is in accordance with the mPT result from this study. This result confirms that certain phytochemicals present in these fractions could interact with the pore and as a result, caused mPT pore opening and subsequent cytochrome c release. Also, the result is consistent with reports that mPT pore opening causes uncoupling of oxidative phosphorylation by inhibiting uptake of inorganic phosphate. This converts mitochondria to ATP consumers instead of producers, thus,elevating inorganic phosphate level (Bernardi and Di Lisa, 2015).

Mitochondrion produce ROS as "side products" of respiration (Zorov *et al.,* 2014). The target of the free radicals is the phospholipid. The results from the lipid peroxidation show that all the fractions of *Drymaria cordata* prevented lipid peroxidation-induced damage. Compounds that

inhibit membrane phospholipids peroxidation may be relevant in preventing free radical-induced damage (Ruberto *et al.,* 2000). Mitochondria exposed to Fe^{2+} in the presence of ascorbate exhibit lipid peroxidation by the decomposition of lipid hydroperoxides to yield alkoxy or peroxyl radicals, which eventually caused chain reaction of lipid peroxidation (Recknagel *et al*.,1989). Extracts of *Drymaria cordata* ameliorated mitochondrial lipid peroxidation to varying degrees. This also shows that the induction of opening shown by the solvent fractions was not via lipid peroxidation as the fractions were able to inhibit lipid peroxidation. The *in vitro* study showed chloroform fraction to be the most potent with respect to mPT pore opening. The putative agent causing the induction of pore opening might be highly resident in the non polar fraction. These results necessitated our curiosity to find out if the chloroform fraction would induce pore opening *in vivo* and if the signalling pathways of mitochondrial-mediated apoptosis are involved in the real mechanism of interaction of CFDC. Owing to the relevance of bioavailability of the compound of interest at the target site and to determine the safety dose regimen for the potent chloroform fraction of *D. cordata,* female abino rats were orally admistered different doses of CFDC for 14 and 28 days while control animals received the vehicle (5% DMSO distilled water). The mitochondrial permemability transition was mornitored spectrophotometrically in the animal subjects at 540nm. The results obtained showed that the intergrity of the mitochondria isolated from control animals were not compromised thus, showing that they were suitable for further use. The effects of various dosages of CFDC on mPT pore after 14 days of treatment showed that at 50mg/kg bd wt, there was no significant induction of pore opening while 100 and 200mg/kg bd wt induced pore opening by 3.3 and 5.5 folds, respectively when compared with the control. After exposure to the CFDC treatment for 28 days, 50, 100 and 200mg/kg bd wt significantly induced pore opening by 2.7, 5.1 and 7.7 folds, respectively. The increase in

induction fold after 28 days of treatment might be due to increase in bioavailability of the CFDC at the target site and prolonged exposure to treatment. The induction of rat liver mPT pore opening by CFDC *in vivo* is also in consonance with the *in vitro* data. Also, the results of CFDC treatment observed in the case of uterine mitochondrial permeability transition show dosage-dependent induction of pore opening after 28 days of exposure. Induction fold of 1.8, 4.2 and 6.2 were recorded at doses of 50, 100 and 200mg/kg bd wt, respectively.

These results suggest that CFDC was available at the target site to elicit inductive effect. Also, the induction was dosage-dependent showing that the induction of mPT pore opening increased with increase in doses of administration. The *in vivo* effect of oral administration of CFDC on mitochondrial F_0F_1 ATPase activity was also in consonance with our *in vitro* results as CFDC caused enhancement of mitochondrial ATPase activity by 25, 38 and 63%, at 50, 100 and 200mg/kg bd wt, respectively, and it is significant ($P<0.05$) at 100 and 200mg/kg bd wt relative to control. The enhancement was also dose-dependent showing that increase in the bioavailability of the fraction was commensurate with increase in the ATPase activity.

When the pore is permeabilised and cyt c is released, caspases are activated (Qin *et al*., 2001). The level of caspases 9 and 3 were determined using ELISA technique. Data showed that CFDC caused dose-dependent upregulation of the caspases 9 (initiator) and 3 (executioner) activity. The activation was significant at 100 and 200mg/kg of CFDC. The activation of caspase 9 activity by CFDC suggests that CFDC has potential to induce mitochondrial-mediated apoptosis (MMA). DNA damage is one of the features of mitochondrial-mediated apoptosis.

In this study, the percentage hepatic and uterine DNA fragmentation was determined using diphenylamine (DPA) method (Wu *et al*., 2005), after the exposure of the rats to varying doses of CFDC for 28 days. The data show that there was induction of hepatic DNA fragmentation by

20%, 37% and 60% and uterine DNA fragmentation by 15%, 50% and 75% at doses of 50, 100 and 200mg/kg bd wt, respectively, and statistically significant ($P<0.05$) at 100 and 200mg/kg bd wt relative to control.

Having established the potency of CFDC as an inducer of mitochondrial-mediated apoptosis, we also demonstated the effect of CFDC on some apoptotic parameters using immunohistochemistry technique. The data showed that CFDC caused an elevated immunoreactive expression of cytochrome c, caspase 9 and caspase 3, which are critical to the activation of MMA. However, anti-apoptotic bcl-2 protein was downregulated. Upregulation of Bcl-2 protein has been implicated in many cancers as they stabilise $\Delta\psi$, prevent mPT and block cytochrome c release (Elmore, 2007).

Their down regulation could be a therapeutic strategy in combating cancerous cells. In folkloric medicine, *Drymaria cordata* is used in the treatment of fibroid; a benign tumor. Based on this folkloric use, a rat model of fibroid was mimicked by treating the animals with monosodium glutamate (MSG). Several studies have shown MSG as in inducer of fibroid (Obochi *et al*., 2009; Eweka *et al*., 2010) and hyperplasia (George *et al*., 2013) in rats. In this study, MSG was used to induce fibroid and the effect of CFDC was tested on MSG-treated rats. The markers of fibroid development which are oestrogen, progesterone and total cholesterol were assayed for, using ELISA technique while the histological assessment of the myometrium was carried out using haematoxylin, eosin and masson trichome stains.

masson trichome stain) was carried out. Masson trichome stain is specific for collagen fibre. During fibroid growth, there is increase in the extra cellular matrix (ECM) proteins ; collagen fibre (Norian *et al*., 2009) within the myometrium. Thus justifying the assessement of

development of fibroid growth in the uterus at the cellular level. At the end of the study, MSG administration at 200mg/kg for 28 days caused significant increase in the levels of oestrogen, progesterone and total cholesterol levels implicated in uterine fibroid relative to control while co-treatment with CFDC significantly ameliorated this effect. The histological assessment also revealed uterine hyperplasia in the MSG-treated group which was remarkably lowered in the co-administered group. The uterine hyperplasia noticed in the MSG-treated female rats could be as a result of cellular proliferation due to high doses and long exposure to MSG treatment. These are in consonant with Obochi *et al*. (2009) who investigated the effect of green tea extract on MSG-induced fibroid in Wistar rats and Muhammad *et al*. (2014) who worked on the effect of monosodium glutamate on the serum estrogen and progesterone levels in female rat and prevention of this effect with diltiazem. CFDC caused reduction in the level of oestrogen and progesterone which play a crucial role in the development of uterine fibroid. This could be due to the ability of the phytochemicals present in CFDC to interact and inhibit some hormonal and signaling pathways, thus, bringing down the level of oestrogen implicated in uterine fibroid development. Aromatase catalyses the synthesis of oestrogen from androgens (Simpson *et al*., 1997). Aromatase is found in breast,skin, brain, adipose, muscle and bone (Smith *et al*., 2002). The concentration of estrogens is higher in breast cancer tissues compared with the circulating plasma and this implies that there is high aromatase activity around or within the cancerous tissues (Simpson *et al*., 2000). Blocking the action of aromatase enzyme would reduce the estrogen level in the body. Phytochemicals that can inhibit aromatase could be an agent for cancer chemotherapy or chemoprevention (Brueggemeier, 2006). Several dietary agents of plant origin have the ability to reduce the oestrogen level via inhibition of aromatase and thus shrink fibroid growth.

The clearance of the uterine hyperplasia by CFDC could be due to several factors. It could be due to upregulation of some proapoptotic proteins like cytochrome c, caspase 9, caspase 3 and downregulation of Bcl-2 protein, thereby activating mitochondrial-mediated apoptosis by phytochemicals present in CFDC. The reversal of MSG-induced hyperplasia by CFDC could also be probably due to the presence of some antioxidants which could exert antifibroitic effects through regulation of ROS and antioxidant signaling pathways. Several dietary phytochemicals such as crocetin, butein, betulinic acid, naringin and chlorogenic acid (Li *et al*., 2015) exert antifibrotic property via regulation of ROS and antioxidant signaling pathways. Crocetin, an important carotenoid obtained from the dry stigmas of the plant *Crocus sativus* L protects against cardiac hypertrophy by blocking the ROS-dependent MAPK/ERK signaling pathway (Cai *et al*., 2009).

In fibroid cells, the levels of ECM (collagen) is elevated relative to the normal cell (Chen *et al*., 2013a). Dietary phytochemicals such as apigenin and emodin block integrin and its downstream kinase, ERK (Xu *et al*., 2015). Apigenin, flavonoid, effect its antitumor properties by disrupting actin cytoskeleton organization (Franzen *et al.,* 2009). Based on all these findings, possible mechanism of the clearance of MSG-induced uterine hyperplasia by CFDC could be via disintegration of the ECM, inhibition of MAPK signaling pathway, inhibition of aromatase enzyme activity, upregulation of some proapoptotic proteins like cytochrome c, caspase 9, caspase 3 and downregulation of Bcl-2 protein. The curiosity to further investigate the effect of CFDC on MSG treatment gave a clearer picture of the possible mechanism. The effect of oral administration of CFDC on LPO, DF, caspases 9 and 3 activities and anti-apoptotic Bcl-2 protein expression in normal and MSG-treated female rats was investigated. It was found that MSG administration caused significant elevation of LPO, increase in DNA fragmentation and caspases

9 and 3 activities and decrease in anti-apoptotic Bcl-2 while co administration with CFDC treatment ameliorated the MSG-induced abberation. Many phytochemicals like epigallocatechin gallate, catechin, and curcumin induce DNA damage at higher concentrations while they protect against DNA damage at lower concentrations (Md Soriful *et al*., 2017). This result is similar to Eman (2016), who reported that MSG caused upregulation of caspase 3 expression and downregulation of anti-apoptotic Bcl-2 in rat liver and testes but was normalized by *Annona muricata* Linn. (Annonaceae) leaf extract. Also, Nia *et al*. (2016) reported MSG-induced apoptosis in the brain cells of zebrafish (*Ddanio rerio*).

MSG was chosen for this research due to its wide consumption in our diet and due to the experimental reports on it, causing hypertrophy and uterine fibroid in rats (Obochi *et al*., 2009). It is intriguingly surprising that an inducer of apoptosis (MSG) can also be an agent of fibroid/uterine hyperplasia. This event was even corroborated when we found that MSG (though did not induce pore opening at lower concentrations/dose and short exposure) caused significant induction of pore opening at high dosage after 28 days of exposure. This simply shows that the mechanism by which CFDC cleared MSG-induced uterine hyperplasia in rats is not via upregulation of apoptosis but rather by other mechanism different from mitochondrial-mediated apoptosis. Though, CFDC is an inducer of mitochondrial-mediated apoptosis as seen in this study, MSG also has been shown to cause induction of DNA fragmentation, caspase 9 and 3 activities. This confirms that though CFDC induced apoptosis, its mechanism of action in the amelioration of MSG-induced uterine hyperplasia/fibroid is not via mitochondrial-mediated apoptosis. As mentioned earlier, the possible mechanisms could be inhibition of aromatase enzyme, inhibition of MAPK signaling pathway and perharps distruption of some extracellular matrix (ECM) proteins found to be over expressed in the uterine hyperplasia.

The chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) was further subjected to purification process using column chromatography (CC), thin layer chromatography (TLC) and gas chromatography mass spectroscopy (GCMS). The analysis of one of the partially purified samples reveal the presence of hexadecanoic acid methyl ester (11.3%), hexadecanoic acid (14.71%), and oxalic acid, 2-ethylhexyl isobutyl ester (63.66%). Another sample was found to contain 3-(p-fluorobenzoyl)-propinoic acid (70.24%), hexadecanoic acid methyl ester (2.35%) and bis(2-ethylhexyl)phthalate (11.96%). Other compounds found in another partially purified sample of CFDC include hexadecanoic acid methyl ester (16.66%), hexadecanoic acid ethyl ester (38.98%) and 9- octadecenoic acid (44.14%). According to Hyeon *et al*. (2006), 3-(p-fluorobenzoyl)-propinoic acid (70.24%) has been shown to block MAPK signaling pathway. The presence of this compound in CFDC could be responsible for its potency to reduce uterine hyperplasia via inhibition of MAPK signaling pathway. The partially purified compounds were tested on mPT and were found to induce pore opening . The 3-(p-fluorobenzoyl)-propinoic acid (70.24%) was more potent than oxalic acid, 2-ethylhexyl isobutyl ester (63.66%) with respect to induction of pore opening. These suggest that the partially purified compounds are potent inducers of mitochondrial-mediated apoptosis like the parent chloroform fraction. Furthermore, from the results, the degree of potency of the partially purified compounds with respect to induction of mPT pore opening showed that purification enhanced the activity when compared with the potency of the parent chloroform fraction. The 3-(p-fluorobenzoyl)-propinoic acid (70.24%) was found to reverse MSG-induced oestrogen, progesterone and total cholesterol elevation in mice. The 9-octadecenoic acid and hexadecanoic acid methyl ester have anti-cancer and antioxidant properties (Gomathi *et al*., 2015). The presence of these phytochemical compounds in the sample of partially purified CFDC correlates with its anticancer property of

the plant as it is used folklorically to treat uterine, liver, skin and breast tumour. This study shows that CFDC is rich in phytochemical compounds that can induce mitochondrial-mediated apoptosis making it a potent anticancer agent and which may ameliorate MSG-induced uterine hyperplasia making it a therapeutic agent against uterine fibroid.

Based on these findings, the folkloric use of *Drymaria cordata* in the treatment of uterine fibroid is justifiable due to the presence of some compounds, especially in *Drymaria cordata*, that can inhibit some hormonal and signaling pathway involved in the development of uterine fibroid. The clearance of the MSG-induced uterine fibroid may not be via mitochondrial-mediated apoptosis but some other pathways which may include inhibition of aromatase activity, MAPK signaling and inhibition of some extracellular matrix (ECM) proteins involved in the development of uterine fibroid.

CONTRIBUTIONS TO KNOWLEDGE

• *Drymaria cordata* contains bioactive agents that can induce mPT pore opening with the consequent release of cytochrome c and enhancement of mitochondrial ATPase activity.

• Bioactivity guided assay shows that chloroform fraction of methanol extract of *Drymaria cordata* is the most potent with respect to mPT pore opening.

• Oral administration of the chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) induced mitochondrial-mediated apoptosis via the opening of the mPT pore, downregulation of Bcl-2 expression with the consequent release of cytochrome c, activation of caspases 9 & 3 and induction of DNA fragmentation .

• MSG induced apoptosis by the same mechanism in the uterus and liver.

• Co-administration with CFDC modulated this effect of MSG by reducing the upregulated caspases 9 &3 activities and induction of DNA fragmentation in the MSG-treated female rats.

• Oral administration of MSG elevated the levels of oestrogen, progesterone and total cholesterol

• Co-administration with CFDC reduced the levels of these hormones in animals that received MSG.

• CFDC protected against MSG-induced damage in the liver and uterus

• MSG caused hyperplasia in the uterus and this was lowered by co-administration with CFDC

• GC-MS analysis revealed the presence of hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester, n-hexadecanoic acid, oxalic acid, 2-ethylhexyl isobutyl ester,bis(2-ethylhexyl) phthalate, 9-octadecenoic acid and 3-(p-fluorobenzoyl)-propinoic acid in CFDC.

• The partially purified 3-(p-flourobenzoyl)-propinoic acid and oxalic acid, 2 ethylhexylisobutyl ester induced mPT pore opening in mice liver.

•The partially purified 3-(p-fluorobenzoyl)-propionic acid lowered the oestrogen, progesterone and total cholesterol levels elevated in MSG-treated female mice.

FURTHER STUDIES

(1) To investigate the effect of chloroform fraction of methanol extract of *Drymaria cordata* on mitochondrial-mediated apoptosis in normal and oestradiol valerate- induced uterine fibroid via oestrogen receptor/β catenin signaling pathway.

(2) To investigate the effect of some of the identified compounds (hexadecanoic acid methyl ester, 9-octadecenoic acid and oxalic acid, 2-ethylhexyl isobutyl ester) on oestradiol valerate- induced uterine fibroid as some fatty acid ester have been shown to have anticancer property.

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