CYTOREMEDIATION OF SODIUM ARSENITE-INDUCED TOXICITIES BY EXTRACTS OF *Adansonia digitata* LINN. IN CELL LINES AND WISTAR RATS

BY

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CERTIFICATION

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DEDICATION

I dedicate this project work to the Almighty God, for the strength He gave me and for keeping me alive all through the entire period of carrying out this research work.

And to my dearly beloved Wife, Mercy Opeyemi Adegoke, for her love, support and care.

Also, to my dear parents, Mr and Mrs J. I. Adegoke, for their endless support, sacrifices and prayers

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LIST OF ABBREVIATIONS

2-AAF - 2-Acetylaminofluorene

A-498 - Human Renal Cancer Cell Line

A-549 - Human Lung Carcinoma Cell line

ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

ACDSF - Anticancer Drug Screening Facility

ACTREC - Advanced Centre for Treatment Research and Education in Cancer

ADH – Alcohol dehydrogenase

ADR - Adriamicin

AEFAD – Aqueous Extract of the Fruit pulp of *Adansonia digitata*

AKR - Aldo-keto reductase

ALDH - Aldehyde Dehydrogenase

ALP - Alkaline Phosphatase Activity

ALT - Alanine Aminotransferase

AML - Acute Myeloid Leukaemia

ANOVA - Analysis of Variance

Apaf-1 - Apoptotic protease activating factor 1

APC - Adenomatous Polyposis Coli

AQF - Aqueous Fraction

AST - Aspartate Aminotransferase

ATP – Adenosine Triphosphate

BrdUrd - Bromodeoxyuridine

CBC - Complete Blood Count

CCK-8 - Cell Counting Kit-8

CDK - Cyclin-Dependent Kinase

CEPM - *O*-carboxyethylcyclophosphoramide Mustard

C-onc - Cellular Oncogenes

CP - Cyclophosphamide

CRF - Chloroform Fraction

CV – Coefficient of Variation

CYP – Cytochrome P_{450}

DI - DNA Index

DMEM- Dulbellco Modified Eagle Medium

DMSO – Dimethyl Sulphoxide

DNA - Deoxyribonucleic acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DPX - Depex

DSBs - Double Strand Breaks

DW - Distilled Water

EAF - Ethyl acetate Fraction

EDTA – Ethylene Diamine Tetraacetic Acid

EI - Electronic Impact

EMEM - Eagle's Minimal Essential Medium

FACS - Fluorescence-Activated Cell Sorting

FBS - Fetal Bovine Serum

FBS - Fetal Bovine Serum

FHI - Forest History Identification

FL - Fluorescent Light of the DNA dye

G1- Gap 1

GC-MS - Gas Chromatography / Mass Spectrometry

GGT - Gamma Glutamyl Transferase

GI50 - Growth inhibition of 50 %

GSH- Reduced glutathione

Hb - Hemoglobin

HPRT - Hypoxanthine-Guanine PhosphoribosylTransferase

IARC- International Agency of Research in Cancer

IC50 – Inhibitory Concentration 50

KB - Human Oral Cancer Cell Line

LC50 - Concentration of Drug resulting in a 50% Reduction in the Measured Protein

LD_{50} – Lethal Dose 50

LDH - Lactate Dehydrogenase

LPO - Lipid Peroxidation

MCF-7 - Human Breast Cancer Cell line

MDA - Malondialdehyde

MDS - Myelodysplastic Syndromes

MEBAD – Methanol Extract of the Stem bark of *Adansonia digitata*

M-phase – Mitotic Phase

MTT - 3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide

NAC - *N*-acetyl Cysteine

NAD - Nicotinamide Adenine Dinucleotide

NADP - Nicotinamide Adenine Dinucleotide Phosphate

NBF - n-butanol Fraction

NCCS - National Centre for Cell Science

NCI - National Cancer Institute

NF-1 - Necrosis factor-1

NF-κB - Nuclear Factor kappa B

NHF - n-hexane Fraction

nMPCEs - Number of Micronucleated Polychromatic Erythrocytes

NTP- National Toxicological Programme

OD - Optical Density

PAGE – PolyAcrylamide Gell Electrophoresis

PARP – Poly (ADP-Ribose) Polymerase

PBS – Phosphate Buffer Saline

PCE - Polychromatic Erythrocyte

PCV – Packed Cell Volume

PE - Plating Efficiency

pH - potential of hydrogen

PI – Propidium Iodide

PUFA - Polyunsaturated Fatty Acids

Rb - Retinoblastoma gene

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RPMI- Roswell Park Memorial Institute

RT - Room Temperature

S - DNA Synthesis phase

S.D – Standard deviation

SA - Sodium Arsenite

SDS - Sodium Dodecyl Sulphate

SF - Surviving Fraction

SOD – Superoxide Dismutase

SPSS - Statistical Package for Social Sciences

SRB – Suphurhodamin B

STD = Standard

T-24 - Human Bladder Cancer Cell Line

TBA - 2-thiobarbituric Acid

TBARS - Thiobarbituric Acid Reactive Substances

TCA - Tricarboxylic Acid

TCA - Trichloroacetic Acid

TGI - Drug concentration resulting in Total Growth Inhibition

TP53 - Tumor Protein p53

V-onc - Viral Oncogenes

WBC – White blood cell

WHO – World Health Organization

ABSTRACT

Arsenicals are proven human and animal environmental clastogens and carcinogens. Research has focused on ameliorating their toxicities using medicinal plants such as *Adansonia digitata* (AD). Although, there are available literatures on the medicinal uses of AD, there is a dearth of information on its use in ameliorating arsenicals-induced toxicities. This study was therefore designed to investigate the cytoremediatory effects of the extracts of AD in cell lines and Wistar rats.

Fruit pulp and stem bark of AD were collected from Ajibode, University of Ibadan (UI), identified at Botany Department and authenticated at Forestry Research Institute, Ibadan (FHI NO.109859). Aqueous and methanol extracts of the plant were obtained by cold maceration. The aqueous extract of the fruit pulp of AD (AEFAD) was partitioned with n-hexane, chloroform, ethyl acetate and n-butanol. Phytochemical screening of the methanol extract of the stem bark of AD (MESBAD) was determined and AEFAD was subjected to GC-MS analysis. The nitrite, 1-1-diphenyl-1-picryl-hydroxyl (DPPH), 2-2-azinibis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) scavenging and reducing power of fractions from AEFAD were assessed spectrophotometrically. Cytotoxicities and antiproliferative potential of fractions of AEFAD and MESBAD were determined in human cancer cell lines: Lung carcinoma (A-549), Breast (MCF-7), Oral (KB), Bladder (T-24), Renal (A-498) using spectrophotometry and crystal violet staining, respectively. Protein expressions of apoptosis (p21 and p53) were determined by western blotting. For the first experiment in the *in vivo* study, rats (100-150g) (n=45, 9 groups of equal rats) were treated thus: distilled water, Sodium arsenite (SA), Cyclophosphamide, AEFAD (200mg/kg), AEFAD 400mg/kg, SA+AEFAD (200mg/kg), SA+AEFAD (400mg/kg), Cyclophosphamide+AEFAD (200mg/kg), Cyclophosphamide+AEFAD (400mg/kg). For second experiment, rats (n=30) were treated thus: distilled water, MESBAD (400mg/kg), SA+MESBAD (400mg/kg), SA+MESBAD (300mg/kg), MESBAD (300mg/kg) and SA only). In both experiments, rats were treated orally for 14-days and sacrificed; samples of blood, bone marrow and liver collected. Alanine and aspartate aminotransferases, γ-glutamyltransferase and lipid peroxidation (LPO) were determined spectrophotometrically. Frequency of micronucleated polychromatic erythrocytes (mPCEs) and liver histology were determined microscopically. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Alkaloid was abundant in MESBAD (330.0±0.0mg/100g), saponins (153.3±2.9mg/100g), flavonoids (121.7±2.9mg/100g) and total polyphenols (121.7±2.9GAE/100g) were significantly high. Seven compounds were identified in AEFAD, namely: Pentadecanoic-acid, Hexadecanoic-Acid, 11-Octadecenoic-acid, Octadecanoic-acid, Oleic-acid, Nonadecanoic-acid and 3,11-Tetradecadiene-1-ol.

N-butanol fraction had the highest reducing power (1.6±0.3) and ABTS scavenging capacities (78.0±2.4%). There was no significant difference between the cytotoxic effects of the fractions

of AEFAD and the negative controls on the cell lines. Aqueous, ethyl acetate and n-butanol fractions conferred apoptotic morphological effects on KB. The MESBAD showed a concentration-dependent cytotoxic and antiproliferative effects on MCF-7 and, also up-regulated apoptotic markers. The AEFAD and MESBAD had hepatoprotective activities by gradual restoration of liver lesions to normal hepatocytes. Cyclophosphamide and SA increased LPO (47.8 and 36.3%, respectively), while co-treatment [(Cychlophosphamide+AEFAD) and (SA+AEFAD)] resulted in 3.1 and 1.7 folds reduction, respectively. Cyclophosphamide and SA increased mPCEs, co-treatment [(Cychlophosphamide+AEFAD), (SA+AEFAD) and (SA+MESBAD)] resulted in 2.5, 5.1 and 2.6-folds decrease, respectively.

Aqueous and methanol extracts of *Adansonia digitata* elicit cytoremediation in sodium arsenite-induced toxicities, while methanol extract possesses anticancer activities.

Keywords: Sodium arsenite, Antitumuor effect, Hepatoprotective activity, Antiproliferative effect

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CHAPTER ONE

1.1: General Introduction:

Cancer is a group of diseases that exhibit unchecked growth and spread of abnormal cells. Cancer could be caused by external factors, such as tobacco, infectious organisms and an unhealthy diet. Also, internal factors such as inherited genetic mutations, hormones, and immune conditions. These factors may act in synergy or in sequence to cause cancer (American Cancer Society, 2016). Current treatments of cancer include surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy (American Cancer Society, 2016). Cancer epidemy has posed a great challenge globally, and mortality is increasing every year. An estimate of 1,685,210 cases, more than 4,600 new cancer diagnoses each day, was projected in the US for the year 2016, with an estimated death of 595,690 about 1,600 deaths per day (Siegel *et al.*, 2016). It accounted for 13% of total deaths worldwide in 2013 and it is estimated that by 2030, deaths from cancer will exceed 13.0 million worldwide (American Cancer Society, 2013). In 2008, 56% of the cancer cases and 64% of the deaths occurred in the developing countries (Ferlay *et al.*, 2008). Scientists around the world have taken up the challenge by working towards the discovery of potent synthetic compounds with little or no side effects to combat cancer and the factors that bring about the initiation of carcinogenesis.

On the other hand, cyclophosphamide (CP) which is an alkylating agent that is prescribed as a chemotherapy in the treatment of some forms of cancer (Baumann and Preiss, 2001); reports indicate that it is cytotoxic (Hales, 1982). The limitation of this drug in the treatment of cancer is majorly the injury to normal tissues and other numerous side effects associated with its use (Fraiser *et al.*, 1991; Bukowski, 1999).

Arsenicals are proven human and animal environmental clastogens and carcinogens; exposure to arsenicals, through the intake of polluted well water has been reported to be associated with lesions of the skin and some forms of cancers (Jayanthika *et al*., 2001).

However, diverse medicinal plants exist in nature, most of which have been used safely for the treatment of various ailments without known deleterious effects. Higher plants are regarded as living chemical factories, providing a vast number of chemical substances that show various potent biological activities (Fasola *et al*., 2011; Udobi and Onaolapo, 2009). *Adansonia digitata L* (baobab) is such an example; it forms an important part of herbal preparations in Nigeria. Locally it is called Ose or Igi-ose by the Yorubas, kukaa by the Hausas in Nigeria. It belongs to the family Bombacaceae (Wickens, 1982) and the genus *Adansonia* (Moiselet, 1998). It is also found in some parts of India in the Asia continent, some local names of the tree in India are: Hindi: Gorakh imli; Marathi: Gorakh chinch; Gujarati: Bukha; Bengali: Gadhagachh and Tamil: Papparappuli.

Adansonia digitata L (Baobab) is known in many African countries as the "tree of life" due to its many traditional, medicinal and nutritional uses (Wickens and Lowe, 2008). The leaves bark and fruits of *Adansonia digitata* are employed traditionally as food stuffs and for medicinal purposes (Etkin and Ross, 1982). Therapeutically, the fruit pulp of *Adansonia digitata* is employed as analgesic, anti-diarrhea and in the treatment of smallpox and measles (Kerharo and Adam, 1974), and to stimulate or counteract immune responses (Ramadan *et al.*, 1994; Ajose, 2007). Fruit pulp extract have been found to have some anti-inflammatory (Vimalanathan and Hudson, 2009), hepatoprotective and anticlastogenic effects (Al-Qarawi *et al.*, 2003; Adegoke *et al.*, 2015). The extract of the plant parts have also been suggested to have anti-tumor action (Elsaid, 2013). In Senegal and Guinea, both a decoction and a

poultice- made from baobab fruit extract were shown to have antitumor activities (Wickens, 2008; Jackson, 2015).

Baobab fruit pulp was reported to have particularly high antioxidant capacity mainly because of its high natural vitamin C content, which is equivalent to 6 oranges per 100g, while baobab leaves contain provitamin A (LFR, 2009; Virtuani, 2002; Jackson, 2015).

Generally, antioxidants prevent cellular damage by reacting with oxidizing free radicals and thereby bringing about their elimination. However, in cancer treatment, a mode of action of certain chemotherapeutic agents involves the generation of free radicals to cause cellular damage and necrosis of malignant cells (VandeCreek *et al.*, 1999). A concern has logically developed as to whether exogenous antioxidant compounds taken concurrently during chemotherapy could reduce the beneficial effect of chemotherapy on malignant cells (VandeCreek *et al.*, 1999). The effects of using antioxidants concurrently with chemotherapy and radiation are synergistic. There is no evidence to date showing that natural antioxidants interfere with conventional cancer therapeutics *in vivo*. Studies have shown that patients treated with antioxidants, with or without chemotherapy and radiation, have many benefits, like tolerating standard treatment better, experience less weight loss, have a better quality of life, and most importantly, live longer than patients receiving no supplements at all (Lamson, 1999).

1.2. Statement of Research Problem:

Cancer is a group of various diseases characterised by uncontrolled growth and spread of abnormal cells, cancer affects most of the populace in both developed and low income countries. It is a leading cause of death worldwide, deaths from cancer worldwide has continued to rise. Continuous exposure of humans to chemicals such as arsenic, through long-term ingestion of contaminated water and its attendant health problems has been widely

reported, also the cytotoxic effects on normal cells following administration of cyclophosphamide in the treatment of certain form of cancer has also been reported. Patients undergoing treatment with cyclophosphamide may also be exposed to *Adansonia digitata*. As a result, patients can be simultaneously exposed to both the drug and the medicinal plant. Arsenic can inhibit the activities of thiol containing enzymes (e.g DNA ligase) and also act in synergy with other mutagenic agents (Mazumder, 2005), resulting in defective DNA replication, repair, recombination and joining of single- and double-stranded DNA breaks (Sunderman, 1984).

1.3. Justification of the Study

The interest in drugs of plant origin is due to the fact that conventional/synthetic drugs can manifest numerous adverse effects, be inefficient and also expensive, making them unavailable to the low-income countries. Although, some medicinal uses of *Adansonia digitata* have been documented, there is little information in literature on the antitoxic, antioxidant and anticlastogenic properties of the extracts of *Adansonia digitata* on sodium arsenite and cyclophosphamide induced-toxicities. Also, there is very limited information on its anticancer potential. Therefore, the idea to investigate the cytoremediatory activities of the extracts of *Adansonia digitata* in wistar rats and cancer cell lines was conceived.

However, there is a dearth of knowledge on the cytotoxic potential of the extracts of *A. digitata* on Wister rats and cell lines used in this study, also the relationship between the cytotoxicity and antioxidant potentials was assessed. The comparative assessment of the simultaneous administration of cyclophosphamide and the fruit pulp of *Adansonia digitata* extracts and the effect of the extracts on sodium arsenite induced toxicities were also investigated.

1.4. Aims and objectives

The aim of the present work was to carry out *in vitro* and *in vivo* assessment of the cytoremediatory activities of the extracts and fractions of *Adansonia digitata*.

1.4.1 Specific objectives

- ❖ To carry out liquid-liquid fractionation of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD) using n-hexane, chloroform, ethylacetate and n-butanol and to determine the antioxidant activities of the crude extract and its fractions
- ❖ To determine the phytochemical contents of the methanol extract of the stem bark of *Adansonia digitata* (MESBAD).
- ❖ To study the *in vitro* inhibitory effects of the fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* on the growth of some cancer cell lines: A-549 (Human Lung carcinoma cell line), KB (Human Oral Cancer Cell Line), T-24 (Human Bladder Cancer Cell Line), A-498 (Human Renal Cancer Cell Line) using SRB.
- ❖ To determine the effects of the fractions on cell cycle distributions of A-549 and MCF-7 cell lines.
- ❖ To assess the effect of AEFAD on liver function enzymes and carry out histopathological analysis on the liver.
- ❖ To assess the effects of AEFAD on lipid peroxidation and heamatological parameters; and to assess the effect of AEFAD on hepatocellular proliferation.
- ❖ To assess the effects of AEFAD and MESBAD on genotoxicity/clastogenicity
- ❖ To study the effect of MESBAD on breast cancer cell lines and some apoptotic protein expression profile.

1.5. Research Questions

The aim and objectives specified above can be achieved by giving attention to the following questions:

What is the fractionation yield of the various fractions of the fruit pulp of *Adansonia digitata*?

What is the antioxidant status of the aqueous extract of the fruit pulp of *Adansonia digitata* and its fractions?

- Does fractionation affect its antioxidant potentials?
- Does the fruit pulp of *Adansonia digitata* and its fractions have cytotoxic effects on the cell lines used at the tested concentrations? Is the extract able to reverse the hepatocellular proliferation induced by sodium arsenite and cyclophosphamide?
- Are the extracts genotoxic or antigenotoxic?
- What effects can its use as supplement have on cancer patients undergoing treatment with cyclophosphamide?
- Does the stem bark have both short term and long term antiproliferative effect on MCF-7 breast cancer cell line?
- What is the effect of MESBAD on the protein expression of certain apoptotic markers?

A review is presented below that will provide a general overview of the key areas of research considered in this thesis.

CHAPTER TWO

2.0: LITERATURE REVIEW

2.1. Cancer

Cancer is a group of various diseases characterised by uncontrolled growth and spread of abnormal cells to other parts of the body, cancer affects most of the populace in both developed and under developed countries (Chakraborti *et al.*, 2002). Cancer could be caused by both internal and external factors, externals factors such as tobacco, infectious organisms, and an unhealthy diet, also internal factors, such as inherited genetic mutations, hormones, and immune conditions. These factors may act synergistically or sequentially to bring about carcinogenesis (American Cancer Society, 2016). Current treatments of cancer include surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy (American Cancer Society, 2016).

The epidemy of cancer has posed a great challenge globally, and mortality is increasing every year. An estimate of 1,685,210 cases, which is an equivalent of more than 4,600 new cancer diagnoses each day, was projected in the US for the year 2016, with an estimated death of 595,690 which is around 1,600 deaths per day (Siegel *et al.*, 2016). Mortality due to cancer accounted for 13% of total deaths worldwide in the year 2013 and it is expected to increase to over 13.0 million by 2030 (American Cancer Society, 2013). In 2008, 56% of the cancer cases and 64% of the deaths occurred in the developing countries (Ferlay *et al.*, 2008). Scientists all over the world have taken up the challenge by working towards the discovery of potent compounds with little or no side effects to combat cancer and the factors that bring about the initiation of carcinogenesis.

2.2. Molecular Basis of Carcinogenesis

The molecular basis of carcinogenesis is found in the defect or even absence of highly important genes that are involved in regulation of cell growth and apparently those involved in cell death that is growth promoting (proto-oncogenes), growth inhibiting (tumor suppressor genes), anti-oncogenes and apoptotic genes (Bishop, 1995).

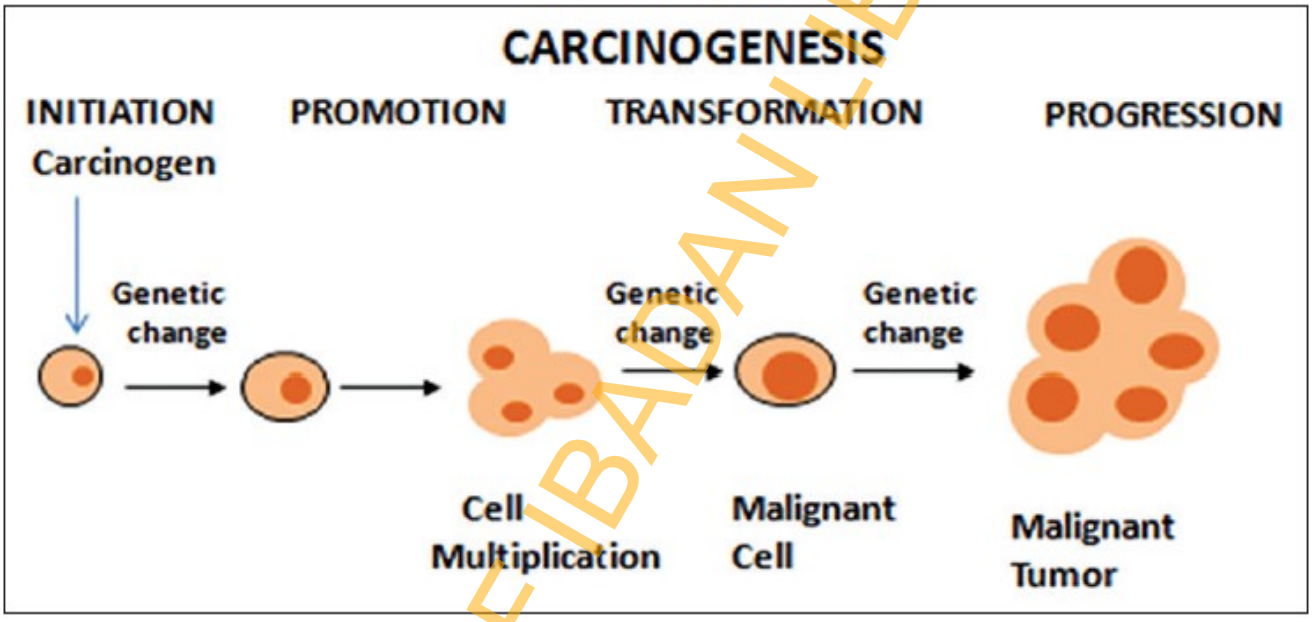


Figure 2.1 Overview of multistage carcinogenesis.

Source: (Kaur *et al.*, 2014).

Oncogenes are derived from proto-oncogenes. They can either be viral oncogenes (v-onc) or cellular oncogenes (c-onc). Oncogenes encode protein products called oncoproteins which resemble the normal products of the proto-oncogenes, with the exception that oncoproteins are devoid of important regulatory elements and their production in the transformed cells does not depend on growth factors or other external signals (Weinberg, 1989). Oncogenes products include; growth factors (c-sis), growth factor receptors (v-erb B), signal-transducing protein (ras protein) and nuclear regulatory protein (c-myc) (Weinberg, 1989).

Tumour suppressor genes are genes whose products control cell proliferation. Mutation or deletion of these genes is like brake failure, permitting unrestrained cellular growth. Examples of these genes include; retinoblastoma (Rb) gene, p53 gene, adenomatous polyposis coli (APC) gene and necrosis factor-1 (NF-1) gene (Russo and Russo, 1998).

Genes regulating apoptosis that prevent or reduce programmed cell death are also important in carcinogenesis. The prototypic gene in this category is bcl-2 and it is found to be over-expressed in certain cancers (Korsmeyer, 1992).

2.3. Clastogens and Clastogenesis

Clastogens can cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged. Therefore, clastogenicity is described as the microscopically visible damages or changes to chromosomes (Testoni *et al*., 1997). This can cause mutation, and lead to cancer formation, as cells that are not killed by the clastogen may become cancerous. Example of clastogens include: Acridine yellow, benzene, ethylene oxide, arsenic, phosphine and mimosine (Testoni *et al*., 1997).

Clastogens generally have low molecular weight (< 10,000 Daltons) (Testoni *et al*., 1997). Apart from causing chromosomal breakage, they can cause gene mutation, sister chromatid exchanges and other chromosomal aberrations. They were first described in the plasma of irradiated persons, but they are also found in hereditary breakage syndromes and chronic reactions (Testoni *et al*., 1997).

2-acetylaminofluorene (2-AAF), besides acting as a mutagen has the capacity of causing chromosomal damage (Ames *et al*., 1972). Another potent and common clastogen is arsenic which may be present in water as contaminants. During cell division, the genetic material replicates and then divides equally between the two daughter cells that are produced. This process is disrupted by clastogens. The effects of clastogens do not allow genetic material to

be incorporated into a new nucleus, and therfore may form its own micronucleus that can be viewed with the microscope. A positive correlation between the degree of clastogenicity and carcinogenicity has already been established (Natarajan, 1984).

2.4. Arsenic

Arsenic is a naturally occurring element found in any of four valence states: -3 (arsine), 0 (elementary arsenic), +3 (arsenites), and +5 (arsenates). Elementary arsenic is a gray metallic-looking crystalline powder; arsine is a colorless gas; arsenites and arsenates are white crystalline powders. The term "arsenic" is used when the valence state is not specified and generally refers to arsenite and/or arsenate (Campbell, 2008). The alkali salts are highly soluble in water but the calcium and lead salts are not. The pentavalent form, As^{5+} (arsenate or organic form) is less toxic than trivalent form, As^{3+} (arsenite or inorganic form) based on lower solubility. Both forms are found in arsenic-contaminated water, and they are inter-convertible once absorbed. Arsenic belongs to the group V elements of the periodic table and has atomic number of 33 and mass number of 76 (Campbell, 2008).

Arsenite is a chemical compound containing an arsenic oxoanion where arsenic has oxidation state +3. Examples of arsenites include sodium arsenite which contains a polymeric linear anion, [AsO_2^-], silver arsenite, Ag_3AsO_3, which contains the trigonal, AsO_3^{3-} anion, sometimes called ortho-arsenite. In fields that commonly deal with groundwater chemistry, arsenite are commonly referred to as As_2O_3. Compounds in this class are acutely toxic, carcinogenic, teratogenic, and mutagenic. They are readily absorbed by various body tissues through the skin, respiratory and intestinal tracts, and transplacentally. They may cause severe irritation of tissues (skin, eyes, mucous membranes, and lungs) (Campbell, 2008). Major uses of arsenic in various forms are as pesticides (insecticides, herbicides, and sheep and cattle

dips) and in drugs. Sodium arsenite is used in the water gas shift reaction to remove carbon dioxide (Campbell, 2008).

2.4.1. Forms and Occurrence of Arsenic

Arsenic compounds are common in nature and most metallic ores contain little quantities. Arsenic is released to the atmosphere from both natural and anthropogenic sources. The principal natural source is volcanic activity, with minor contributions by exudates from vegetation and wind - blown dusts, smelting of metals, the combustion of fuels, principally of low-grade brown coal, and the use of pesticides (Merian, 1984). Arsenic is a component of antifungal wood preservatives, in the United States; products used for wood preservation contain arsenic (ATSDR, 2007). Arsenic trioxide is normally produced in the roasting of metallic ores. This may be carried as a dust along with the smoke, and hence serve as a contaminant of soil, herbage and waste.

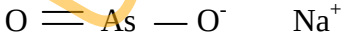
2.4.2 Sodium Arsenite ($NaAsO_2$)

Sodium arsenite is a white-gray powder which is both odorless and tasteless. It is formed from the reaction of arsenic trioxide with caustic soda. It is a trivalent inorganic compound belonging to general class of compound called arsenicals. It is toxic and the most effective agent of the arsenicals. Sodium arsenite is produced from arsenious acid (As_2O_3) by reacting with alkali such as arsenite and water.

$$As_2O_3 + H_2O \longrightarrow 2HAsO_2$$

$$As_2O_3 + 2NaOH \longrightarrow 2NaAsO_2 + H_2O$$

Sodium arsenite is structurally represented as;



It is readily absorbed after ingestion and is distributed to all body tissues especially keratin rich tissues like the hair, skin and fingernails. Animal studies have reported that arsenic can also be transferred to the developing embryo and fetus and as a consequence arsenic has been shown to be both embryotoxic and teratogenic (Martin *et al.,* 2003), when arsenic was administered in multiple doses. The unique chemical and physical properties of sodium arsenite make it an ideal poison and exposure to sodium arsenite represents a much more acute toxic hazard than exposure to less soluble trivalent arsenic compounds (Done and Peart, 1971).

2.4.3. Route of Exposure to Arsenicals

Arsenic is a common environmental toxicant found in soil, water and air. Significant exposure to arsenic occurs via both anthropogenic and natural sources. Arsenic is released into the air by volcanoes and is a natural contaminant of some deep well water (Done and Peart, 1971). Occupational exposure to arsenic is common in the smelting industry (in which arsenic is a byproduct of ores containing lead, gold, zinc, cobalt, and nickel) and is increasing in the microelectronics industry (in which gallium arsenite is responsible). Low level arsenic exposure continues to take place in the general population (as do some cases of high dose poisoning) through the commercial use of inorganic arsenic compounds in common products, such as wood preservatives, ant-killers, herbicide, fungicides, through the consumption of foods and smoking of tobacco treated with arsenic-containing pesticides and through burning of fossil fuels in which arsenic is a contaminant (Done and Peart, 1971).

2.4.4. Uses of Sodium Arsenite

Sodium arsenite is widely used as weed killer (herbicides), dressing of grain for preservation, insect poisons (insecticides), cattle and sheep-dips and wood preservatives and debarking of trees. Reynolds *et al.,* (1999) reported arsenic based compounds that are used to treat diseases like asthma, tuberculosis, diabetes, skin diseases etc.

They are used for the control of Johnson and nusedge grass and weeds in cotton fields as herbicide application. The use of inorganic arsenicals (sodium arsenite and arsenic trioxide) as herbicides has been reduced greatly because of livestock losses, environmental persistence, and their association with carcinogenesis. Arsenic derivatives continue to be available in other parts of the world in wood preservatives and insecticide formulations (Reynolds *et al.*, 1999). These compounds can be hazardous to animals. The highly soluble organic arsenicals (methane arsonate, methyl arsonic acid) can concentrate in pools in toxic quantities after a rain has washed them from treated plants. Arsenicals are used as desiccants or defoliants on cotton, and residues of cotton harvest fed to cattle may contain toxic amounts of arsenic (Reynolds *et al.,* 1999).

2.4.5. Arsenic absorption and fate

The rate of absorption of inorganic arsenicals from digestive tract depends upon their solubility. Sodium arsenite is readily soluble, rapidly absorbed and highly toxic (Calesnick *et al.*, 1966). Accumulation of arsenic is seen in the liver with slow release and distribution to other tissues. Continued administration can cause its disappearance from soft tissues and its long term storage in bones, skin and keratinized tissues (for example, hair and hoof) (Calesnick *et al.*, 1966). Arsenic deposited in hair is irremovable and moves slowly along the hair as it grows. Arsenic is excreted in the urine, faeces, sweat and milk. Pentavalent arsenic is well absorbed through the gut, but the trivalent form is more lipid soluble. Toxicity results

from the arsenite form (As^{3+}), especially by dermal absorption. Arsenic compounds are well absorbed parenterally within 24 hours (Calesnick *et al.*, 1966).

2.4.6. Distribution

Sodium arsenite is distributed to other tissues from the liver where it accumulates and excess can be stored in bone, skin, and keratinized tissues such as hair and hooves. Arsenic initially localizes in the blood bound to globulin. Redistribution occurs within 24 hours to the liver, lungs, intestinal wall, and spleen, where arsenic binds to sulfhydryl groups of tissue proteins only small amounts of arsenic penetrates the blood-brain barrier (Winsk *et al.*, 1995). There is also a significant accumulation of arsenate in the skeleton, presumably by exchange with phosphate (Lindgren *et al.*, 1983). Application of pentavalent arsenic to skin results first in an accumulation of arsenic in the skin, followed by distribution to other organs, and urinary excretion (Lindgren *et al.*, 1983).

2.4.7. Toxicity of Arsenicals

In general, arsenites are much more toxic than arsenates; the oral LD_{50} of arsenates in rats and mice is about 100 mg/kg and that of arsenites about 10 mg/kg; the acute oral LD_{50} of arsenic trioxide is 15 mg/kg in rats and 39 mg/kg in mice. Although rare, acute poisoning is reported in children (Gullen *et al.*, 1995). Acute and chronic effects of arsenic intoxication in man have been summarized (IARC, 1980). They include a burning sensation of mouth and throat; metallic, garlicky odor of breath and feces; difficulty in swallowing; vomiting; diarrhea; and cyanosis. Chronic effects include hyperpigmentation and keratosis (characteristics of prolonged treatment with Fowler's solution), vascular effects ("blackfoot disease"), cirrhosis of the liver, and effects on the hematopoietic system (leukopenia, anemia). The chief toxic effect of inhaled arsine is due to its binding to hemoglobin, resulting in extensive hemolysis

and hematuria followed by jaundice; the usual cause of death is renal failure (Gullen *et al.*, 1995).

2.4.8. Basics of Arsenite-Induced Toxicity

Arsenite exerts its cellular toxicity by binding to sulflhydryl groups which results in enzyme inhibition. During arsenic metabolism, oxygen radical may be produced, possibly leading to damage of DNA, proteins, lipids and other molecules. There is a positive correlation between lipid peroxidation and arsenic tissue concentrations in the livers, kidneys and heart of arsenite treated rats. Arsenite induces the body's antioxidant activities in human firoblasts (Lee *et al.*, 1995). It induces heme oxygenase, leading to the heme degradation iron release and decrease in the cytochrome P_{450} biotransformation enzymes important in both endogenous and xenobiotic metabolism (Albores *et al.*, 1989). Because of arsenite affinity for protein sulfhydryls, many side effects can occur from enzyme inhibition. Chronic arsenite toxicity results in mitochondrial changes that block lipoic acid-dependent dehydrogenase, which in turn inhibits glycolysis and results in demand for glucose and subsequently hypoglycemia. Arsenicals also inhibit pyruvate dehydrogenase in gluconeogenesis; carbohydrate depletion caused by gluconeogenesis depletion may therefore aggravate arsenic toxicity (Szinicz *et al.*, 1988).

Increasing evidence indicates that arsenic acts on signaling pathways that regulate cell proliferation rather than causing direct DNA damage because arsenic exhibits its mutagenic activities only at concentrations high enough to also produce cell damage (Jacobson-Kram and Montalbano, 1985). Accordingly, it has been demonstrated that arsenic can induce a moderate increase in keratinocyte cell proliferation, as evidenced by increases in thymidine incorporation (Germolec *et al.*, 1998), cell cycling, labeling of the proliferating cell marker

Ki-67 and expression of oncogenes and growth factors such as c-*fos*, c-*jun*,c-*myc*, and transforming growth factor (Germolec *et al*., 1998).

2.4.9. Carcinogenic Effects of Arsenic

The IARC classify arsenic as a carcinogen for which there is sufficient evidence from epidemiological studies to support a causal association between exposure and skin cancer. Chronic arsenic exposure has also been associated with a greatly elevated risk of skin cancer and possibly of cancer of the lung, liver, angiosarcoma, bladder, kidney and colon cancers (Ellenhern, 1997). They also detected elevation of serum globulins and development of esophaged varices at follow up studies. There is also some evidence suggesting changes in choloestatic function of the liver as shown by conjugated hyperbilirubinemia and elevated alkaline phosphatase activity, which directly relates to the concentration of total arsenic in urine (Ellenhern, 1997). Pulmonary findings of chronic arsenic toxicity include both obstructive and restrictive patterns of pulmonary function tests (Ellenhern, 1997). Sodium arsenite was found to inhibit methyl thymidine uptake in human cells *in vitro*, consistent suppression of DNA synthesis. Chromosomal aberrations were observed in human leucocyte exposed to sodium arsenite. Arsenic has also been suggested to substitute for phosphorus in DNA, causing a weak bond in DNA chain (Petres *et al*., 1977). The toxicity of trivalent arsenic to animals and human beings has also been thought to be caused by its binding to thiol ions, thus inhibiting enzymatic reactions. As late as 1980, it was believed that arsenic compounds were not carcinogenic in experimental animals, and this conclusion was drawn from a summary of largely negative results (IARC, 1980). Since that time evidence has appeared which indicates carcinogenicity in rats (Ivankovic *et al*., 1979). The evidence for carcinogenicity of arsenic compounds in man is more positive, and this has been reviewed (IARC, 1980). A correlation was established between the appearance of skin cancer and

arsenic concentration in the well water in certain regions of Taiwan (Tseng, 1977). Skin cancers were also noted repeatedly in patients after prolonged treatment with Fowler's solution (potassium arsenite) and in vineyard workers employing arsenical pesticides. Lung cancers have been noted in men involved in the production of arsenicals (Mabuchi *et al.*, 1979).

2.4.10. Arsenic poisoning in Bangladesh

Arsenic poisoning in Bangladesh has been described as the largest mass poisoning of a population in history because of the groundwater used for drinking contaminated with naturally occurring inorganic arsenic. It was estimated that of the 125 million inhabitants of Bangladesh between 35 million and 77 million were at risk of drinking arsenic contaminated water (Khan *et al.,* 1997*;* Dhar *et al.,* 1997). The scale of this environmental disaster is greater than any seen before; it is beyond the accidents at Bhopal, India, in 1984, and Chernobyl, Ukraine, in 1986 (Smith, 1997; 1998).

The health effects of ingesting arsenic-contaminated drinking-water appear slowly. For this reason, a more important issue than the number of patients who currently have arsenic-caused diseases is the number who will develop these diseases in the future as a result of past and continuing exposure to arsenic. If the population continues to drink arsenic-contaminated water, then a major increase in the number of cases of diseases caused by arsenic may be predicted (Smith, 1998).

Several disease conditions occur as a result of long term arsenic poisoning, these include: Skin cancer, internal cancers e.g Bladder, Kidney and Lung cancers. Neurological effects e.g Hypertension and cardiovascular disease, diabetes mellitus among others. The latency for arsenic-caused skin lesions in particular keratoses, is typically about 10 years (Guha *et al.*, 1998). In the 1997 consultancy, it was found that the youngest individuals with skin lesions

caused by arsenic were about 10 years old. Other studies have shown that skin lesions also occur in children younger than 10 years (Milton and Rahman, 1999). It was also found that in adults, exposures commenced approximately 10 years before they started to manifest the skin lesions. In some instances, the apparent latency for the appearance of skin lesions from the time of first exposure to contaminated water from the tube-well was much shorter, but as no measurements were available for water from previously used tube-wells, a short latency from first exposure could not be inferred. However, latency that is shorter or longer than 10 years may occur, and the rapidity of the appearance of skin lesions appears to be dose dependent (Guha *et al.*, 1998).

2.5. Cyclophosphamide

Cyclophosphamide (CP) is a drug employed to suppress the immune system. It is used in the treatment of lymphoma, multiple myeloma, leukemia, ovarian cancer, breast cancer, small cell lung cancer, neuroblastoma and sarcoma (ASHP, 2016). CP act by suppressing the immune system, it is administered orally or through the vein (intravenous) (ASHP, 2016). Some usual side effects associated with the use of CP include low white blood cell counts, loss of appetite, vomiting, hair loss, and bleeding from the bladder Other include an increased future development of cancer, infertility, allergic reactions, and pulmonary fibrosis (ASHP, 2016).

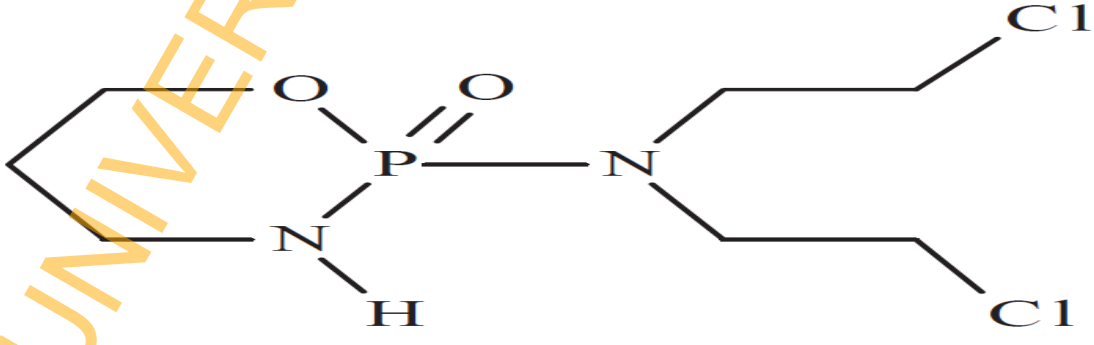


Figure 2.2: Structural formulae of cyclophosphamide (O'Neil, 2006).

The cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, producing cross-links (Hales, 1982). The injury of normal tissues is the major limitation of using CP, which gives rise to numerous side effects (Bukowski, 1999, Fraiser, 1991). It has been reported that oxidative stress mediated disruption of redox balance after CP exposure generates biochemical and physiological disruptances (Das *et al*., 2002; Haque *et al*., 2001). The use of cyclophosphamide in treating cancer patients is limited due to its severe toxicities induced mainly by oxidative stress.

2.5.1 Cyclophosphamide Uses/Exposure

Cyclophosphamide is used as a drug to treat cancer and other medical conditions. In chemotherapy, it may be used alone, but more frequently used concurrently or sequentially with other anticancer drugs (Chabner *et al*. 2001). Cyclophosphamide is available in the United States as 25- or 50-mg tablets, as an oral solution, or in a crystalline hydrate form for injection in strengths of 100 to 2,000 mg. It is used to treat malignant lymphoma, multiple myeloma (bone-marrow cancer), leukemia, breast and ovarian cancer, neuroblastoma (childhood nerve-cell cancer), retinoblastoma (childhood cancer of the retina), and mycosis fungoides (lymphoma of the skin). Cyclophosphamide is also used as an immunosuppressive agent following organ transplants or to treat autoimmune disorders such as rheumatoid arthritis, Wegener's granulomatosis (an inflammation of the blood vessels), and nephrotic syndrome (a kidney disorder) in children (Chabner *et al*. 2001). Researchers have tested cyclophosphamide for use as an insect chemosterilant and in the chemical shearing of sheep (IARC, 1975).

2.5.2 Metabolism of Cyclophosphamide

As a prodrug, Cyclophosphamide is converted to active alkylating mustard by the liver (Fenselau, 1977). The initial activation of cyclophosphamide is 4-hydroxylation at C-4 of oxazaphosphorine ring to form 4-OH-cyclophosphamide (Chang *et al*., 1993). Multiple CYP enzymes including CYP2B6, CYP2C9 and CYP3A4 in the liver are responsible for CP 4-hydroxylation (Chen *et al*., 2004, Chang *et al*., 1993, Ren *et al*., 1997, Huang *et al*., 2000). CYP2B6 is the major contributor (a mean of ~45% of total metabolism) for the activation of CP with the highest intrinsic clearance *in vitro* and *in vivo*, compared with 25% and 12% for CYP3A4 and CYP2C9, respectively (Chen *et al*., 2004, Huang *et al*., 2000, Roy *et al*., 1999). 4-OH-CP is a major circulating metabolite of CP that enters tumor cells and decomposes through its tautomeraldophosphamide (an aldehyde intermediate) by spontaneous β-elimination to form ultimate cytotoxic phosphoramidemustard (*N,N*-bis-2-(2-chloroethyl) phosphorodiamidic acid) and an equimolar amount of the by product acrolein (a highly electrophilic α,β-unsaturated aldehyde) (Fenselau, *et al*., 1977; Hohorst *et al*., 1976, Colvin, 1999). Alternatively, aldophosphamide can be oxidized by alcohol dehydrogenase (ADH) and aldo-keto reductase (AKR1) to generate alcophosphamide. Also, 4-OH-cyclophosphamide is detoxified to *O*-carboxyethylcyclophosphoramide mustard (CEPM) by cytosolic ALDH1A1, and to a much lesser extent, by ALDH3A1 and ALDH5A1 (von Eitzen *et al*., 1994, Sladek *et al*., 1991, Moreb *et al*., 2005). ALDH catalyzes the conversion of a broad range of aldehydes to the corresponding acid *via* NAD+-dependent irreversible reaction. Furthermore, 4-OH-cyclophosphamide is oxidized by ADH to non-toxic 4-keto-cyclophosphamide (Yule *et al*., 1995, Yule *et al*., 2004; Lelieveld and van Putten, 1976). Moreover, 4-OH-cyclophosphamide undergoes reversible dehydration to form iminocyclophosphamide that is further conjugated with intracellular GSH by GSTA1, A2, M1, and P1, giving rise to non-toxic GSCY (Driven *et al*., 1994).

The resultant phosphoramide mustard is a bifunctional alkylator of DNA and the ultimate cytotoxic metabolite of cyclophosphamide. The alkylation involves generation of the intermediate phosphoramide aziridinium ion through an intramolecular nucleophilic attack (cyclization reaction) of the nitrogen on the α-carbon of a chloroethyl chain (Ludeman, 1999). Cellular thiols (e.g GSH) and other nucleophiles react rapidly with phosphoramide aziridinium ions, resulting in thio ether products (Gamcsik *et al*., 1999). CEPM is one of the major chemically stable metabolites of CP, which are easily detected in patient's plasma and urine (Joqueviel *et al*., 1998). However, acroleinis a highly reactive aldehyde that covalently binds to cellular macromolecules and subsequently disrupts the function and causes organ toxicity (Kehrer and Biswal, 2000). It is detoxified by conjugation with GSH *via* GSTs in hepatocytes (Deleve, 1996) and this may cause intracellular GSH depletion and injuries of the hepatocytes (Deleve, 1996). Reaction of GSH with acrolein is via nucleophilic addition at the α-carbon atom, generating stable thioether compounds (Ramu *et al*., 1995).

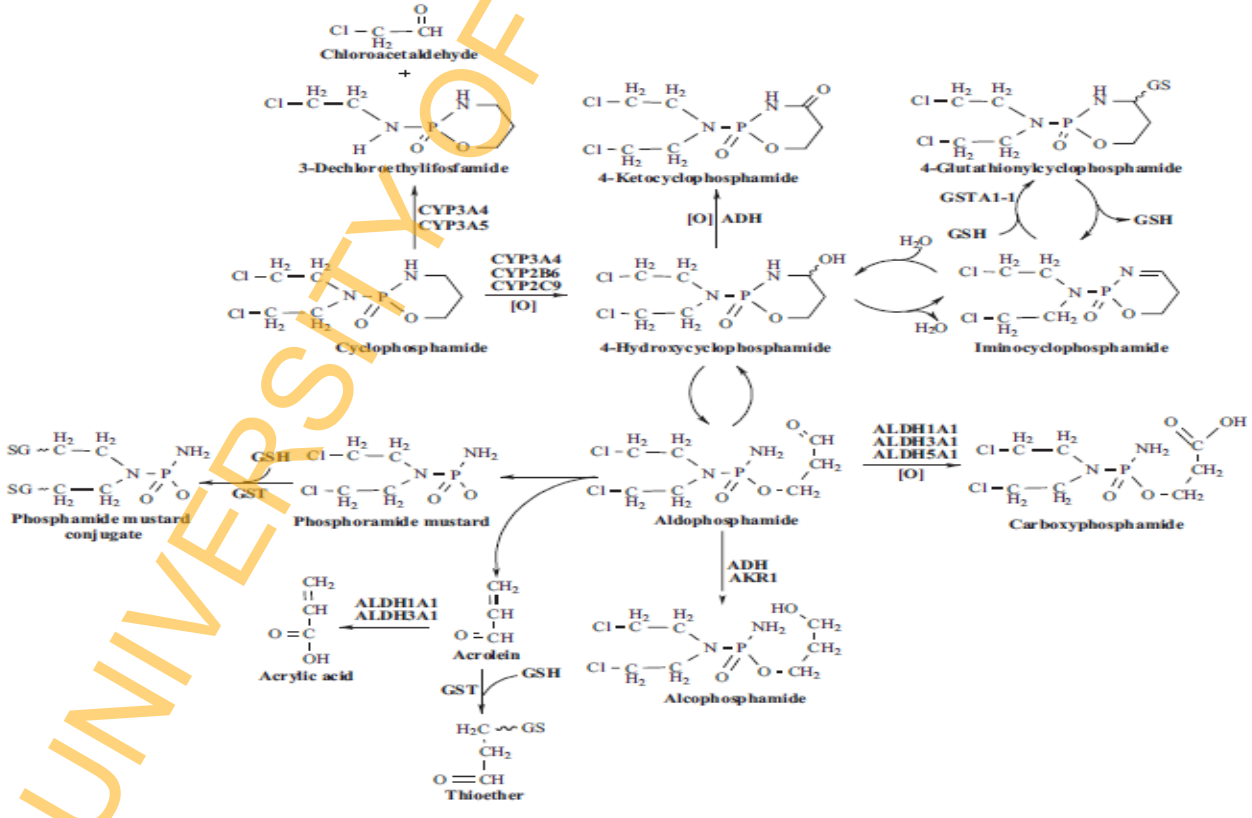


Figure 2.3: Metabolism of cyclophosphamide (Zhang *et al*., 2006).

2.5.3. Carcinogenicity of Cyclophosphamide

The carcinogenicity of cyclophosphamide in humans was established initially on the basis of a large number of case reports, as well as several epidemiological studies (IARC 1981, 1987). The interpretation of the epidemiological studies was limited by the small numbers of cases, the difficulty in separating the role of cyclophosphamide from other agents, or both factors. The most substantial evidence available to previous working groups was a Danish study of 602 patients treated "mainly with cyclophosphamide" for non-Hodgkin lymphoma, in which Nine cases of acute myeloid leukaemia were observed compared to 0.12 expected and a case–control study of leukaemia following ovarian cancer in the former German Democratic Republic where a strong dose–response relationship was observed (Haas *et al.*, 1987). All other studies reported at most three cases of leukaemia or bladder cancer in people who had received cyclophosphamide as the only potentially carcinogenic agent (IARC, 1981; Kinlen, 1985; Greene *et al.*, 1986). There have been several reported cohort studies in which patients treated with cyclophosphamide were followed up, and the occurrence of second cancers investigated. Valagussa *et al.* (1994) followed 2465 women who had received treatment with cyclophosphamide, methotrexate and fluorouracil, a combination in which only cyclophosphamide is considered to have carcinogenic potential in humans. There were three cases of acute myeloid leukaemia observed compared to 1.3 expected, and five cases of bladder cancer compared to 2.1 expected. Statistical significance was not reported but was calculated by the Working Group to be greater than 0.05 for both types of cancer. Smith *et al.* (2003) followed 8563 women who had received cyclophosphamide and doxorubicin as adjuvant therapy for breast cancer and observed 43 cases of acute myeloid leukaemia or myelodysplastic syndromes (AML/MDS). The incidence of AML/MDS overall was seven times higher than expected rates in the general population, and was increased 3-fold in

regimens that contained double the cumulative dose of cyclophosphamide (Smith *et al.*, 2003).

2.5.4. Mechanisms of Carcinogenesis

All of the available evidence indicates that cyclophosphamide exerts its carcinogenic activity via a genotoxic mechanism (McCarroll *et al.*, 2008). The metabolite widely thought to be responsible for the antitumour activity of cyclophosphamide is the phosphoramide mustard (Povirk and Shuker, 1994). This metabolite is also generally considered to be the most genotoxic, but the contribution of acrolein, which is highly toxic, to the genotoxic activity of cyclophosphamide is less clear.

It is well established that the treatment of cancer patients with cyclophosphamide results in inflammation of the urinary bladder (haemorrhagic cystitis), which is not seen with other alkylating agents (Forni *et al.*, 1964; Liedberg *et al.*, 1970). In rats, cyclophosphamide treatment resulted in cystitis as well (Crocitto *et al.*, 1996), and in mice, mutagenic activity has been detected in urine following cyclophosphamide treatment (Te *et al.*, 1997). The ultimate alkylating metabolite of cyclophosphamide, phosphoramide mustard, is metabolized but was not shown to cause cytotoxicity and had minimal morphological effects on the mouse bladder, but an intermediate in the formation of the acrolein metabolite, diethylcyclophosphamide administered by intraperitoneal injection, caused severe cystitis in male rats, and less extensive inflammation in female rats (Cox, 1979). Acrolein administered to rats by intraperitoneal injections increased urothelial cell proliferation (Sakata *et al.*, 1989). Acrolein is the only metabolite of cyclophosphamide that is known to be both reactive and cytotoxic (IARC, 1995).

Collectively, these data indicate that acrolein is the likely causative agent in cyclophosphamide induced cystitis. Cystitis is an established condition associated with the

development of both squamous cell and urothelial bladder cancers (Michaud, 2007). However, intraperitoneal injections of acrolein by itself only induced bladder hyperplasia, not cancer (Cohen *et al.*, 1992), and oral administration studies in mice and rats did not result in carcinogenic effects (IARC, 1995). Thus it is plausible that acrolein-induced cystitis plays a promoting role in cyclophosphamide bladder tumorigenesis that is initiated by other cyclophosphamide metabolites.

The protective effect of *O*6-alkylguanine-DNA alkyltransferase (AGT) against cyclophosphamide mutagenicity (*Hprt* mutations) (Cai *et al.*, 1999), and cytotoxicity (Friedman *et al.*, 1999) in CHO cells implies some involvement of acrolein derived DNA damage. However, mice deficient in this protein (called *O6*-methylguanine-DNA methyl transferase [MGMT] were less susceptible to cyclophosphamide tumorigenesis (Nagasubramanian *et al.*, 2008).

Studies of sister chromatid exchange induced in human lymphocytes by acrolein and phosphoramide mustard suggest that phosphoramide mustard is the more potent genotoxic agent (Wilmer *et al.*, 1990). Furthermore, analysis of *TP53* mutations in cyclophosphamide-associated human bladder cancers suggests that the mutations detected are characteristic of DNA damage caused by phosphoramide mustard, rather than by acrolein (Khan *et al.*, 1998).

2.6. Apoptosis

In many physiological processes of multicellular organisms, cells belong to a community of organised system, where their number is tightly regulated either by cell division or by cell death (Reed, 2000). The cells that are no longer needed are programmed to commit suicide and die; this is referred to as apoptosis. In adult tissues and during fetal development, apoptosis play critical roles (Reed, 2000). Apoptosis also participate in other processes including normal cell turnover, proper development and functioning of the immune system,

hormone-dependent atrophy and chemical-induced cell death. The process of apoptosis is tightly regulated, it is turned on or off when the need arises in a nomal physiological condition, however, inappropriate apoptosis (either too little or too much, that means out of control) has been reported to be a factor in many human diseased conditions such as neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer (Elmore, 2007). In apoptosis, biochemical events lead to characteristic morphological changes, these changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Green, 2011) which are consequences of a coordinated and often energy-dependent pathway(s) that involves the activation of a group of cysteine proteases called — caspases and a complex cascade of events that link the initiating stimuli to the final death of the cell (Elmore, 2007).

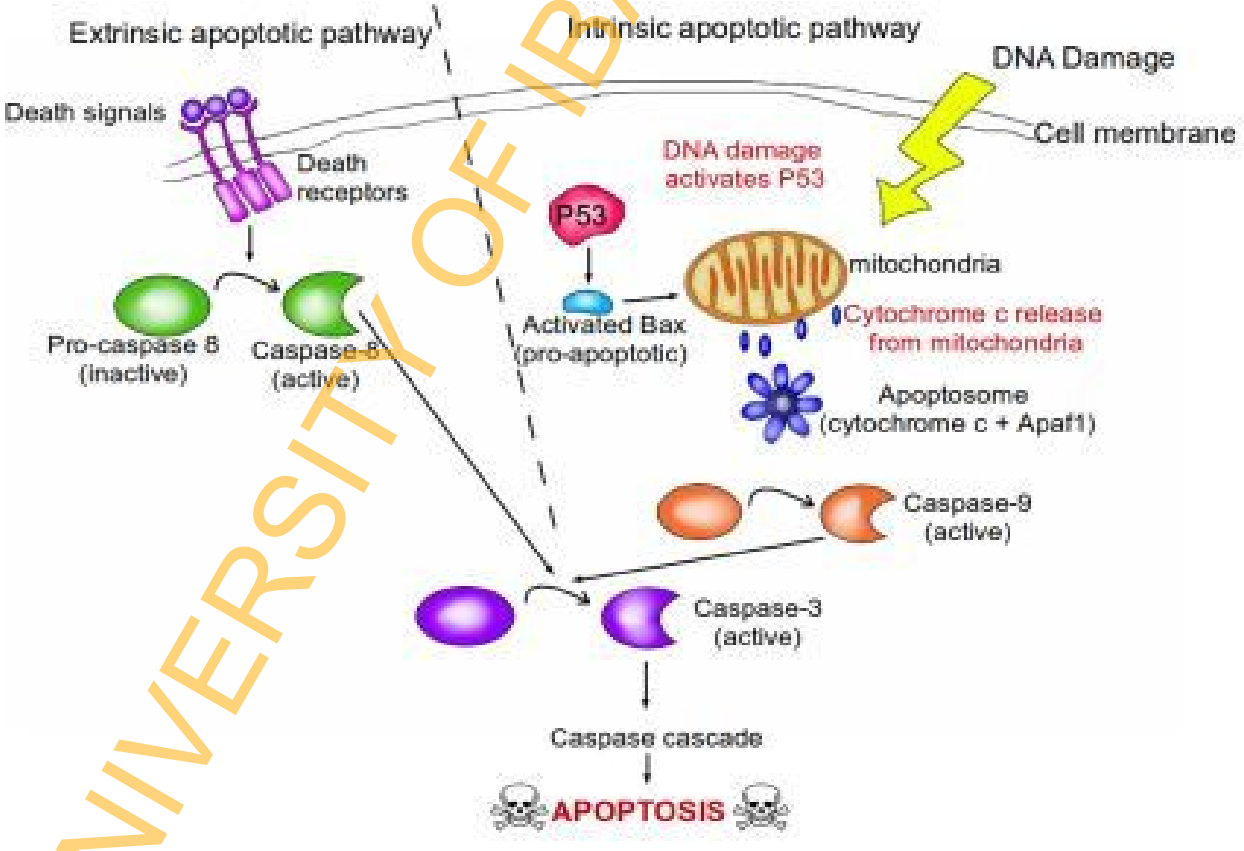


Figure 2.4: A brief overview of the extrinsic and intrinsic apoptotic pathways.

Source: (Samarasinghe, 2013).

In the extrinsic pathway, death signals from the surrounding environment of the cell bind to death receptors on the surface of the cell membrane. This causes the conversion of inactive pro-caspase-8 into active caspase-8. Caspase-8 then goes on to activate caspase-3, which begins the caspase cascade that leads to apoptosis. In the intrinsic pathway, typically initiated by DNA damage, p53 is activated. p53 then activates the pro-apoptotic protein Bax, which initiates the release of cytochrome c from the mitochondria. Apaf-1 and the released cytochrome c combine to form a complex known as the apoptosome. The apoptosome causes the conversion of inactive pro-caspase-9 into active caspase-9. Caspase-9 then goes on to activate caspase-3 in a similar manner to the extrinsic pathway. Activated caspase-3 then leads to the caspase cascade, resulting in apoptosis (Samarasinghe, 2013).

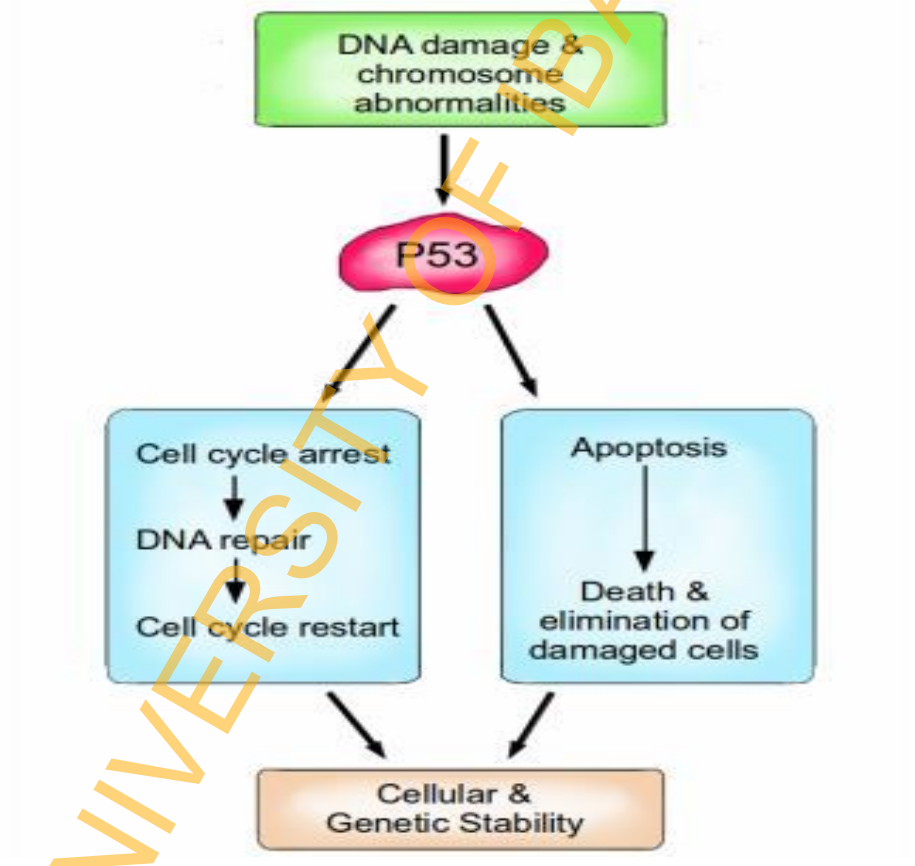


Figure 2.5. Schematic representation of the role of p53 in Apoptosis

Source: (Samarasinghe, 2013).

The tumor suppressor p53 is a vital protein; more than half of all cancers have inactive p53. When activated by either DNA damage or chromosome abnormalities, p53 can halt the cell cycle and initiate DNA repair. If repair is successful, the cell cycle is restarted and cellular and genomic stability is re-established. If repair is not an option because the damage is too great, then p53 can facilitate apoptosis, leading to the death and elimination of the abnormal cell (Samarasinghe, 2013).

2.7. Cell cycle analysis/flow cytometry

The two of the most popular flow cytometric applications has to do with cellular DNA content and the analysis of the cell cycle (Nunez, 2001). Many protocols for DNA measurement have been developed over the years, which include bivariate cytokeratin/DNA analysis, bivariate BrdU/DNA analysis, and multiparameter flow cytometry measurement of cellular DNA content. These analyses are taken into consideration with the development of commercial software for the analysis of cell cycle (Nunez, 2001).

2.7.1. Measurement of Cellular DNA Content by Flow Cytometry

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei (Ross, 1996; Nunez, 2001). The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell (Nunez, 2001). Thereafter, such fluorescence data are considered a measurement of the cellular DNA content. Samples should be analyzed at rates below 1000 cells per second in order to yield a good signal of discrimination between singlets

or doublets. Since the data obtained is not a direct measure of cellular DNA content, reference cells with various amounts of DNA should be included in order to identify the position of the cells with the normal diploid amount of DNA (Ross, 1996; Nunez, 2001). One of the common reference cells often used for DNA measurements is human leukocytes or red blood cells from chicken and trout. Commonly DNA measurements are expressed as a DNA index of the ratio of sample DNA peak channel to reference DNA peak channel. A DNA index of 1.0 represents a normal diploid DNA content, while deviations in cellular DNA content values other than 1.0 indicate DNA aneuploidy (Ross, 1996; Nunez, 2001).

2.7.2. Cell Cycle Analysis

In addition to determining the relative cellular DNA content, flow cytometry also enables the identification of the cell distribution during the various phases of the cell cycle. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content could not be discriminated based on their differences in DNA content. Diverse software containing mathematical models that fit the DNA histogram of a singlet have been developed in order to calculate the percentages of cells occupying the different phases of the cell cycle (Bagwell, 1993; Nunez, 2001).

2.7.3. Pespective to Discriminate Doublets or Cellular Aggregates

A common feature of DNA analysis is the finding of doublets or cellular aggregates. A doublet is formed when two cells with a G1-phase DNA content are recorded by the flow cytometer as one event with a cellular DNA content similar to a G2/M-phase cell. If a sample contains many doublets, which could mistakenly increase the relative number of cells in the G2/M-phase of the cell cycle, yielding to an overestimation of G2/M population (Rabinovitch

1993). In order to correct this error, modern flow cytometers are equipped with a Doublet Discrimination Module that selects single cells on the basis of pulse processed data. The emitted fluorescent light of the DNA dye (FL2) generates an electronic signal that can be recorded as high (FL2H) for the intensity of the staining as well as measured as pulse-area (FL2A) and pulse-width (FL2W) of the samples (Rabinovitch 1993). By plotting the FL2W versus FL2A in a dot plot graph a discrimination of a G1 doublet from a G2/M single can be made. Since the FL2W increases with the diameter of the doublet particle while both the G1 doublet and the G2/M single produce a same FL2A signal is likely to discriminate the doublet from the single. Therefore, in the dot plot graph a gate (G0/G1/S/G2/M) is set around the single population (Nunez, 2001).

2.7.4. Software for Cell Cycle Analysis

Diverse manufacturers such as Becton Dickinson have developed software (CellFit™) for cell cycle analysis. The diverse software provides several mathematical models for fitting the DNA histogram. However, a subtraction of the background is required in order to remove events due to debris and to get a better fit with the models (Rabinovitch, 1993). Before the actual calculation of the phase distributions, two regions that are marked at the left and at the right part of the histogram are examined. Then, the data are fitted into an exponential curve of the form y= e (ax+b), and then the portion of the histogram that includes the two regions is subtracted from this curve. CellFit uses this approach to subtract the background (Nunez, 2001).

However when using modeling for cell cycle statistics, there is a lot of variability and a factor of error because minute variation in the sampling and preparative techniques of the cells can contribute to inaccurate estimates. Therefore, it is essential to be aware of that variability in the analysis and interpretation of DNA content histograms. Also, it is important to evaluate

the advantages of each software package and their respective limitations before using one for the analysis and fitting in the cell cycle models (Nunez, 2001).

2.7.5. Bivariate BrdU/DNA

Flow cytometric analysis of cell DNA content is widely used for the estimation of cell cycle phase distributions. However, this analysis does not provide cytodynamic information such as cycle traverse rates and phase transit times. These parameters can be obtained using autoradiographic techniques, in particular, by determining the fraction of labeled mitosis. By autoradiographic identification of labeled cells during various intervals after labeling, their transition through the subsequent mitotic divisions can be followed, and phase and cycle transit times can be calculated (Nunez, 2001).

In the last few years, non autoradiographic methods for distinguishing DNA synthesizing cells have been developed. These use monoclonal antibodies to measure the incorporation of bromodeoxyuridine (BrdUrd) into cellular DNA. In an indirect immunocytochemical technique, cells, which have incorporated BrdUrd, can be labeled with a fluorochrome and simultaneously the DNA can be counterstained. Flow cytometry then allows the simultaneous measurement of incorporated BrdUrd as well as the DNA content on a single cell level. In this way the cohort of labeled cells can be followed through the cell cycle (Nunez, 2001).

2.7.6. DNA Content Analysis

In addition to analysis of cellular DNA content in lymphoreticular and hematopoietic neoplasm, flow cytometry has been used to characterize solid tumors. The most frequent tissues analyzed are a) biopsies from breast tumors, and b) tissue derived from products of conception (Shankey *et al.,* 1999).

However, the value of DNA content analysis as a prognostic indicator in the case of tumors has been rather inconsistent and its use is being restricted to specific entities like breast cancer. Staining is with a nucleic acid dye like propidium iodide (PI) and is similar to that described so far for other DNA assays (Bauer and Jacobberger, 1994). However, there are several circumstances in the staining procedures and analysis that require careful attention in order to avoid false interpretations. For example, since RNA would interfere in the staining, the solution should also contain RNAase. Also, the number of cells acquired is critical in order to ensure that adequate cell numbers are collected (Nunez, 2001). In addition, non-specific lowlevel staining must be excluded and only strongly stained should be collected in order to perform an accurate modeling of the data. Also as a part of the analysis, it is important to exclude debris and aggregates, as they will interfere with the measurements. Moreover, in some other circumstances, the G0/G1 peak of one ploidy distribution may be localized in the same area as the S-phase component of another distribution. In those circumstances when there is more than one ploidy distribution it is almost impossible by drawing a few analysis regions to delineate the cell cycle compartments. Therefore, the analysis and quantification of cell cycle compartments is a pretty complex task that requires the use of software for modelling (Bauer and Jacobberger, 1994; Nunez, 2001).

2.7.7. Example of a Typical Analysis

Stained sample is collected on the cytometer and displayed for qualitative assessment. The gating strategy varies depending on the type of software but always includes a step to exclude aggregates. Thereafter, FL2W and FL2A are plotted as either dot plot or contour plot. A gate is set up in the area of cells with 2N DNA content. Moreover, there still exists the possibility that one of the cells containing 4N DNA content could be located inside the single gate, or that doublets with 2N DNA content will be located outside the gate. A one-parameter

histogram of FL2-A (PI fluorescence) is drawn from the cells with 2N DNA content (Sladek and Jacobberger, 1993). Usually two major peaks are observed; one peak is labeled as diploid and included in the region R1 that is colored in red and the other peak is labeled as aneuploid and included in the region R2 that is colored in green. In the further analysis, R1 is always depicted in red and R2 is depicted in green and any cells not associated with these two regions appear white. A SSC/FL2 dot plot is drawn which shows that the debris was stained neither in red nor in green but in white, facilitating its subtraction out of the analysis.

The report shows the separation and quantification of the two ploidy distributions: diploid and aneuploid, as well as the calculation of the cell percentages in each cycle compartment. Also it shows the CV's for the G0/G1 peak of each distribution, and a measurement of the DNA Index (DI), which is the aneuploid/euploid DNA content ratio (Sladek and Jacobberger, 1993; Sramkoski, *et al.*, 1999).

2.8. Anticancer drug screening/MTT, SRB

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity (Skehan *et al.*, 1990). Many have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labeled Thymidine Uptake method, methyl thiazolyl tetrazolium (MTT) and water-soluble tetrazolium (WST) methods, which are used for counting the number of live cells. Trypan Blue is a widely used assay for staining dead cells.

In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments (Skehan *et al.*, 1990). However, Trypan Blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. In the Colony Formation method, the number of cell colonies is counted using a

microscope as a cell viability indicator. In the Tritium-Labeled Thymidine Uptake method, [3H]-thymidine is involved in the cell nucleus due to the cell growth, and the amount of the tritium in the nucleus is then measured using a scintillation counter (Vanicha and Kanyawim, 2006). Though the Tritium labeled thymidine uptake assay is sensitive to determine the influence on the DNA polymerization activity, it requires radioisotope which causes various concerns. The 51Cr method is highly sensitive, and is commonly used to determine low levels of cytotoxicity. However, the use of 51Cr also causes problems in handling, storage, and disposal of the material. Cellular enzymes such as lactate dehydrogenase, adenylate kinase, and glucose-6-phosphate dehydrogenase are also used as cell death markers, and there are several products available on the market. However, adenylate kinase and glucose-6-phosphate are not stable and only lactate dehydrogenase does not lose its activity during cell death assays. Therefore, cell death assays based on lactate dehydrogenase (LDH) activity are more reliable than other enzyme-based cell death assays (Vanicha and Kanyawim, 2006).

Enzyme-based methods using MTT and WST rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is far superior to the previously mentioned methods because it is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests (Keepers *et al.*, 1991). Therefore, this method is suitable for those who are just beginning such experiments. Among the enzyme-based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in the living cells. In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needleshaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals (Keepers *et al.*, 1991). Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture

media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error (Keepers *et al.*, 1991).

Dojindo developed highly water-soluble tetrazolium salts called WSTs. WSTs produce water-soluble formazans and are suitable for cell proliferation and cytotoxicity assays. WSTs receive two electrons from viable cells to generate a yellow, orange, or purple formazan dye. WST-8*, a highly stable WST, is utilized in Cell Counting Kit-8 (CCK-8) (Keepers *et al.*, 1991). The electron mediator used in this kit, 1-Methoxy PMS, is also highly stable. Therefore, CCK-8 is stable for at least 6 months at the room temperature and for one year at 0-5 ^{o}C. Since WST-8, WST-8 formazan, and 1-Methoxy PMS have no cytotoxicity in the cell culture media, additional experiments may be carried out using the same cells from the previous assay (Keepers *et al.*, 1991).

Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenase, NAD (H), NADP (H), and mitochondrial activity. The major difference between CCK-8 and the MTT assay, other than MTT's toxicity, is the enzymes involved. The CCK-8 assay involves most of the dehydrogenase in a cell (Slater *et al.*, 1963). On the other hand, MTT only involves mitochondrial dehydrogenase. Therefore, the MTT assay depends on mitochondrial activity, not the cell itself. Additionally, CCK-8 is far more sensitive than the MTT assay. Since WST-8 formazan is water soluble, it does not form crystals like MTT (Slater *et al.*, 1963). Therefore, after 1-4 hours of incubation with the CCK-8 solution, measurement of O.D. at 450 nm gives the number of viable cells, no extra steps are required. According to the modern science, each drug needs to be scientifically, pre-clinically and clinically evaluated for the global acceptance. To study the various techniques of screening an anticancer activity of the *ayurvedic* preparations by the current researchers, the use of *in vitro* screening techniques is a need of the hour (Slater *et al.*, 1963).

Currently available, *in vitro* screening techniques SRB and MTT assay are the most reliable techniques used to evaluate anticancer activity on the cancer cell lines. Out of which SRB assay is used for qualitative analysis and MTT assay is used for quantitative analysis (Slater *et al.*, 1963). The SRB assay provides a better linearity with cell number and a higher sensitivity and its staining is not cell dependent. It is known that, in contrast to the MTT assay the SRB assay stains recently lysed cells. Cell debris does not get stained by SRB and therefore the drug activity data does not get affected (Keepers *et al.*, 1991).

SRB assay measures whole protein content which is proportional to the cell number. According to cell properties, there are various assays that can be used for this purpose such as Dye exclusion test, MTT assay, florescent assay, SRB assay, Clonogenic assay etc. SRB (Sulphorhodamine B) is a bright pink anionic protein staining dye that binds to the basic amino acids of the cellular proteins. Solvents are needed to extract the herbomineral components for *in vitro* screening (Skehan *et al.*, 1990). Three types of solvents exist - polar, non-polar and mid polar. Water is a universal solvent. Alcohol is the most commonly used solvent for extraction; it has good polarity & easy penetrating power in cell membranes of plant. Methanol is more polar than Ethanol, due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results (Handa *et al.*, 2008). Acetone, DMSO, Ethers etc. are the other solvents used for extraction depending upon the solubility of herbo-mineral components. *In vitro* studies reduce the usage of animals. It helps to test the ability of the compound to kill the cells by taking the advantage of various properties of cell. It is possible to process a larger number of compounds quickly with minimum quantity through *in vitro* studies. It is highly cost effective. Also, the range of concentrations used is comparable to that expected in the *in vivo* studies. However, it is difficult to maintain the cell cultures. They show negative results for the compounds which get activated after body metabolism and *vice versa* (Takhar and Mahant, 2007). It is difficult

to ascertain the pharmacokinetics of the study drugs and to examine the activity, if compound is insoluble in the solvents.

Anticancer activity is evaluated by plotting the graphs and by the following formulae: **i.** GI50: Growth inhibition of 50 % calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50. Drug concentration resulting in a 50% reduction in the net protein increase. **ii.** TGI: Drug concentration resulting in total growth inhibition will be calculated from Ti =Tz. **iii.** LC50: Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50% cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = - 50. If the compound is pure then GI50 value of ≤ 10-6 (i.e. 1 µmole) or ≤ 10µg/ml is Considered indicative of demonstrable activity and in case of extracts, GI50 value ≤ 20µg/ml is considered to demonstrate activity. Drug is considered lethal if it causes death of more than 50% of cells i.e. LC50 values more than 50% at any concentration level. There are number of formulations explained in classics and also in practice that are derived from tribal masses, folklore, cowherds, sages, hunters and other forest dwellers which act on *arbuda, granthi, gandmala, apachee* etc. They show promising effect in clinical practice. It is therefore essential that detailed literature reviews and controlled scientific experimentation with correct methods and currently available techniques is carried out to study these in more detail. It is possible to evaluate or to discover new formulations or herbs which show anticancer activity, with the help of advanced techniques of screening as described here.

2.9. MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the

number of viable cells. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma) assay as described by Mosmann (1983) with little modifications. The human breast cancer cell line (MCF-7) was obtained from University of Cape Town Medical School, Cape Town and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week (Mosmann, 1983). The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95 % air and 100 % relative humidity (Mosmann, 1983). After 24 hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, which resulted in the required final sample concentrations (Mosmann, 1983). Following drug addition, the plates were incubated for an additional 48hrs at 37°C, 5% CO_2, 95 % air and 100% relative humidity. The medium without samples served as control and triplicates were maintained for all concentrations. After 48 hrs of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline PBS) was added to each well and incubated at 37°C for 4hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

$$1\text{- Abs sample \% of cell inhibition} = \frac{\text{Abs sample}}{\text{Abs control}} \times 100$$

Nonlinear regression graph was plotted between % cell inhibition and Log10 concentration and IC50 was determined using GraphPad Prism software.

2.10. Lipid Peroxidation

Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. LPO is broadly defined as "oxidative deterioration of polyunsaturated fatty acids (PUFA)", which are fatty acids that contain more than two carbon-carbon double bonds (Halliwel, 1990). In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor mediated signal transduction, etc.).

Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA resulting in strand breaks and cross-linking. ROS can also induce oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken *et al*, 1994). The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells which are rich in mitochondria and this may include spermatozoa.

The reactions of lipid peroxidation proceed through three main steps- initiation, propagation, and termination (Aitken, 1995; Griveau, 1995). During initiation, the free radicals react with fatty acid chains and release lipid free radicals. This lipid free radical may further react with molecular oxygen to form the lipid peroxyl radical. Peroxyl radicals can react with fatty acids to produce lipid free radicals, thus propagating the reaction (Aitken, 1995; Griveau *et a.l*, 1995). One of the by-products of lipid peroxidation is malondialdehyde (MDA). This byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa (Aitken and Fisher, 1994). There are a lot of other products

of lipid peroxidation such as: conjugated dienes, and secondary peroxidation products, which include saturated and unsaturated aldehydes, ketones, oxo- and hydroxy acids, and saturated and unsaturated hydrocarbons (e.g. ethane, pentane). Lipid peroxidation in biological membranes causes impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions. Moreover, lipid hydroperoxides decompose upon exposure to copper while iron chelates the other factors including metals as haem, haemoglobin or myoglobin. Cytotoxic aldehydes are formed as a consequence of lipid hydroperoxide degradation.

2.11. Antioxidants and Protection against ROS-Induced Damage

To prevent an overload in free radicals and peroxides, aerobic organisms use a sophisticated defense system which operates both in intra- and extracellular aqueous phases and in membranes. Antioxidant defense strategies are committed to counteract the oxidative attack in its early moments i.e., formation of priming radicals, as well as during the initiation and chain propagation processes (Halliwel, 1990).

An antioxidant may be defined as a substance which, when present at low concentrations compared with those of an oxidizable substrate, such as fats, proteins carbohydrates or DNA, significantly delays or prevents the oxidation of the substrate (Halliwel, 1990). Antioxidants, in general, are compounds and reactions which dispose, scavenge, and suppress the formation of ROS, or oppose their actions.

2.11.1. Mechanisms of Action of Antioxidants

Free-radical scavengers, which react with peroxyl radicals before the polyunsaturated fatty acids react with peroxyl radicals, can prevent lipid oxidation. Chain breaking antioxidants donate hydrogen atoms to peroxyl radicals and convert them to more stable and non-radical

products (Decker, 1998). Antioxidant radicals formed from hydrogen-donating antioxidants can react with alkyl, alkoxyl, and peroxyl radicals of PUFA and generate non-radical stable compounds (Decker, 1998).

Metal chelators form complex ions or coordination compounds with metals by occupying all metal coordination sites and preventing metal redox cycling. Metal chelators can convert metal ions into insoluble metal complexes or generate steric hindrance, which can prevent the interactions between metals and lipid intermediates. Metal chelators include phosphoric acid, citric acid, ascorbic acid, polyphenols such as quercetin, carnosine, some amino acids, peptides, and proteins such as transferrin and ovotransferrin (Decker 1995; Halliwell *et al*, 1995).

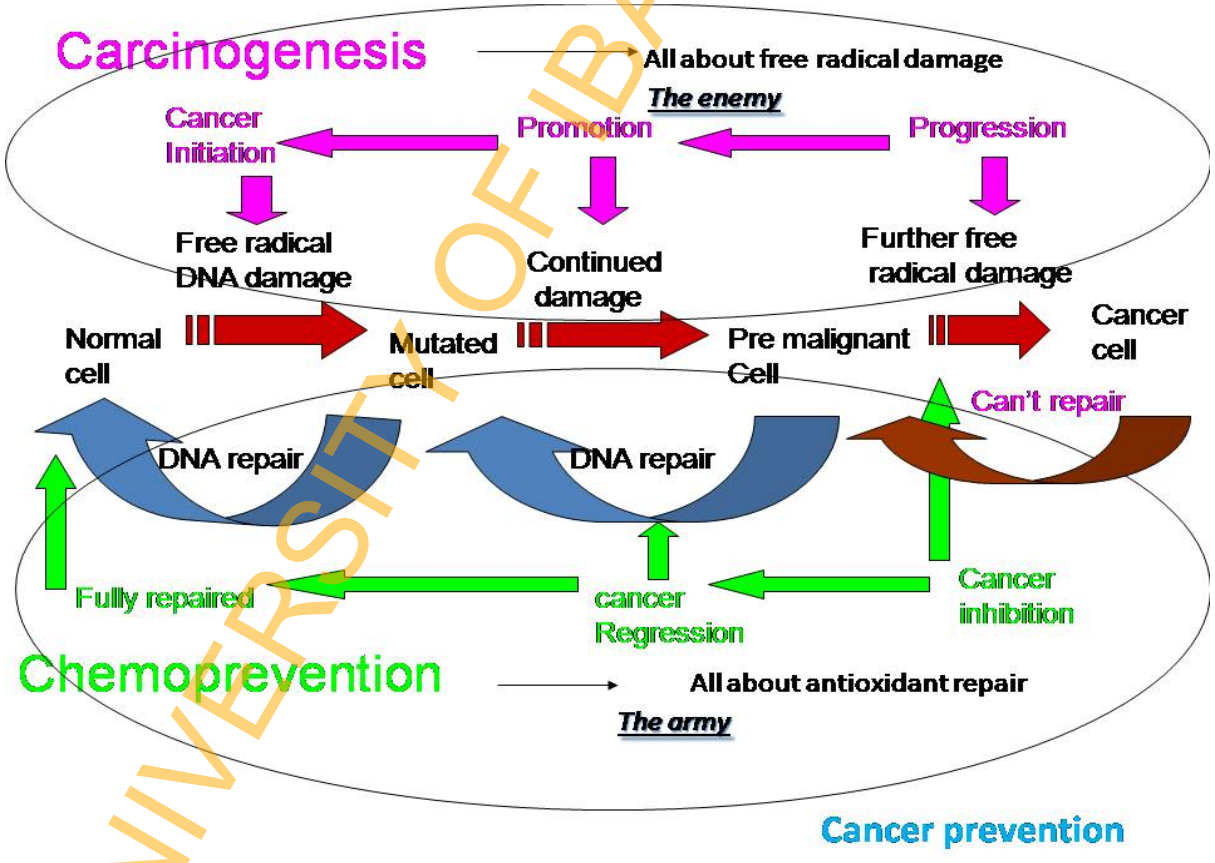


Figure 2.6. A brief overview of Carcinogenesis and Chemoprevention

Source: (http://www.aestheticandyou.com/Anti_Aging_Medicine.html) 04/07/16.

Flavonoids can make complexes with metals and inhibit metal initiating lipid oxidation. Singlet oxygen quenchers work by 2 types of singlet oxygen-quenching mechanisms: physical and chemical quenching. Physical quenching converts singlet oxygen into triplet oxygen by either energy transfer or charge transfer without generating any other intermediates. Chemical quenching is involved with the generation of intermediates, such as oxidized products.

2.11.2. ROS, Antioxidant Status and Cancer

Many of the biological effects of antioxidants appear to be related to their ability not only to scavenge deleterious free radicals but also modulate cell-signalling pathways (Mates *et al.*, 1999). Thus the modulation of cell signalling pathways by antioxidants could help prevent cancer by (i) preserving normal cell cycle regulation; (ii) inhibiting proliferation and inducing apoptosis; (iii) inhibiting tumour invasion and angiogenesis; (iv) suppressing inflammation; (v) stimulating phase II detoxification enzyme activity and other effects. It has been demonstrated that activation of NF-κB by nearly all stimuli can be blocked by antioxidants, including l-cysteine, *N*-acetyl cysteine (NAC), thiols, green tea, polyphenols, and Vitamin E. Because Mn-SOD level seems to be lowered in certain cancer cells, stimulated expression of Mn-SOD appears to suppress malignant phenotypes in certain experimental models, this enzyme has been considered to be a tumour suppressor protein (Pastore *et al.*, 2003). However, the general statement of Mn-SOD as a tumour-suppressor protein is far from clear. Enhanced Mn-SOD expression detected in various primary human cancer tissues and in blood samples from patients with leukemia has been shown to be inconsistent with its proposed tumour suppression function (Pastore *et al.*, 2003). Thus more rational is to assume that over-expression of Mn-SOD may be related to a cellular response to intrinsic oxidative stress in cancer cells. The increased SOD activity decreases superoxide content in the cells and thus reduces the ROS-mediated stimulation of cell growth. It may be hypothesized that Mn-SOD

would decrease cancer cell proliferation indirectly through reduction of ROS, unlike conventional tumour suppressors, which regulate cell growth and decrease expression of cancer cells.

A large number of studies have established an association between cancer incidence and various disorders of GSH-related enzyme functions, alterations of glutathione *S*-transferases (GSTs) being most frequently reported (Pastore *et al.*, 2003). GSTs are a family of enzymes that utilize glutathione in reactions contributing to the transformation of a wide range of compounds, including carcinogens, therapeutic drugs, and products of oxidative stress. GSTs are separated into five classes (α,μ,π,θ and σ) of which μ class is comprised of five different isoenzymes termed GST-M1–GST-M5. Most frequently reported links between cancer and mutations in GSTs concern predominantly GST-M1. The GSH/GSSG ratio measured in the blood of patients with colon and breast cancer has been found to be significantly decreased compared to the control (Pastore *et al*., 2003).

This has been explained by an increased level of oxidized glutathione GSSG, especially in advanced stages of cancer progression. These findings may be explained by increased generation of peroxide, which causes an increased release of GSSG from various tissues within the red blood cells. There exists significant experimental and clinical evidence connecting thioredoxin to cancer (Baker *et al.*, 1997). (i) elevated levels of TRX have been reported in a wide range of human cancers including cervical carcinoma, hepatoma, gastric tumours, lung and colorectal carcinomas; (ii) many cancer cells have been shown to secrete TRX; (iii) TRX is able to stimulate the growth of a wide variety of human leukemia and solid tumour cell lines; (iv) over expression of TRX protected cells from oxidative-stress induced apoptosis and provided a survival as well as a growth advantage to tumours; (v) the elevated levels of thioredoxin in human tumours may cause resistance to chemotherapy (e.g. doxorubicin, *cis*-platin and others).

As it is well known, low-molecular weight antioxidants are involved directly in the conversion of ROS to less reactive species. However, antioxidant protection therapy in cancer patients should be used only with caution since its effects depend on the stage at which it is introduced (Valko *et al*., 2004). Since apoptosis is caused by elevated levels of free radicals, decreased concentrations of free radicals due to the excessive administration of antioxidants might actually stimulate survival of damaged cells and proliferation into neoplastic state and thus rather promote process of carcinogenesis than interrupt it. In addition, antioxidant therapy during the progression stage of cancer might actually stimulate growth of tumours through the enhanced survival of tumour cells. Another important issue which should be taken into consideration is a prooxidant character of some antioxidants which may occur depending on the concentration and environment (oxygenpressure) in which they act (Mortensen *et al*., 2001; Valko *et al*., 2004).

2.12. *Adansonia digitata* LINN.

Adansonia digitata (Linn) belongs to the family *Bombacaceae* and is commonly known as Baobab, Cream of Tartar Tree, Monkey Bread Tree and Lemonade Tree. It is usually found in the savanna region, but it tolerates a wide range of vegetation types (Keay, 1989).

Baobab is a tree found widely throughout Africa and known locally in African countries as the “tree of life” due to its ability to sustain life owing to its water holding capacity, as well as its many traditional medicinal and nutritional uses (Wickens, 2008). Baobab is locally called Ose, Igiose, kukaa, kulambali in Nigeria, belongs to the family Bombacaceae (Wickens, 1982) and the genus *Adansonia*. Generally in Africa the species is indigenous in drier areas found in West Africa, Sudan, Angola, East Africa, Southern Africa up to Transvaal covering about 26 countries (Sidibe and Williams 2002; Wickens 1982). It is a deciduous tree which has four growth phases and produces a fruit consisting of a yellowish-white pulp which has a

floury texture and numerous hard, round seeds, enclosed in a tough shell (Wickens, 2008). The fruit pulp of the *Adansonia digitata* (Linn.), commonly known as baobab is an important human nutrition source in East, Central, and West Africa, including Nigeria. Baobab is used in folk medicine as an antipyretic or febrifuge to overcome fevers. Both leaves and fruit pulp are used for this purpose (Emmy *et al*., 2010).

The fruit pulp of the tree has a high iron; calcium and potassium content (Yazzie *et al*., 1994) and is often consumed as a sweet snack and added to other foods to provide additional nutrients. Furthermore, baobab fruit pulp can contain up to ten times the vitamin C content of oranges (Vertuani *et al*., 2002). The fruit pulp has been evaluated as a substitute for improved western drugs. Fruit pulp has been traditionally a popular material for consumption in various ways, raw or boiled in water, including an anti-diarrhea remedy and various uses to stimulate or counteract immune responses (Ramadan *et al*., 1994; Ajose *et al*., 2007). Fruit pulp extract are also found to be anti-inflamatory (Vimalanathan and Hudson, 2009). The aqueous extract of baobab fruit pulp exhibited significant hepatoprotective activity and, as a consequence, consumption of the pulp may play an important part in human resistance to liver damage in areas where baobab is consumed (Al-Qarawi *et al*., 2003).

Figure 2.7: The *Adansonia digitata* tree (left), leaf and fruits (right)

2.12.1. Uses of *Adansonia digitata*

The baobab tree is an important food, water and shelter source in many African countries, it has a long history of traditional uses as a treatment against fevers, dysentery, and bleeding wounds, and it has a long history of nutritional and medical use in Africa (Gruenwald, 2009). The fruit of the tree, known as "monkey bread", is a popular food source and may be consumed in a number of different ways. It is commonly sucked, chewed or boiled in water to make a drink. It can also be added as a supplement to mix with staple, less nutritious foods such as porridge, as an added source of calcium (Prentice *et al*., 1993). As well as being a source of nourishment, all parts of the baobab tree including the fruit, leaf and stem bark are traditionally used for their medicinal properties. Indeed, the pulp is often consumed as a thickener in gruel (Wickens & Lowe, 2008), in milk, as a refreshing drink with cold water (Bosch *et al*., 2004), as a baking alternative for cream of tartar, and as a rich source of calcium for pregnant women and children (Prentice *et al*., 1993). The fruit can be used for the treatment of fever, diarrhoea, dysentery, haemoptysis and small pox (Greunwald & Galazia, 2005).

Figure 2.9: Baobab fruit (left). The white pulp (right)

2.12.2. Bioactive Compounds associated with the Baobab Fruit Pulp

Due to the high vitamin C content, baobab fruit pulp has a well-documented antioxidant capability (Vertuani *et al*., 2002; Blomhoff *et al*., 2010). It is for this reason that baobab fruit pulp is used as a milk substitute for babies in some African countries (Wickens & Lowe, 2008), in addition to the fact that vitamin C aids the absorption of iron and calcium into the body (Gruenwald & Galizia, 2005). It is well noted that a diet rich in fruit and vegetables, which contain an abundance of antioxidants such as vitamins C and E as well as phenolic compounds and carotenoids, can help prevent oxidative related diseases (Kaur & Kapoor, 2001). The baobab fruit was found to have the highest content of vitamin C at 150-499 mg/100g, out of all fruits investigated. This compared to a vitamin C content of 53 mg/100g in oranges, a well-documented source of vitamin C. Baobab fruit pulp was found to have the highest water-soluble (6.96 mmol/g), lipid-soluble (4.148 mmol/g) and, therefore, total (11.11 mmol/g) antioxidant capacities of all the fruits investigated. The authors of this study, upon examination of the results, suggested that because of the level of antioxidant capacity, baobab fruit is a "new valuable ingredient for food preparation and/or nutraceutical application in the promotion of health" (Vertuani *et al*., 2002).

2.12.3. Anti-pyretic effect (anti-fever effect)

Sufferers of malaria in Africa, India, Sri Lanka and the West Indies are said to consume a mash containing dried baobab bark as a febrifuge in order to treat the fever associated with this illness (Wickens & Lowe, 2008). Fruit pulp and seeds are also widely used for their anti-pyretic properties (Wickens & Lowe, 2008; Ramadan *et al*., 1994). An infusion of the fruit pulp and water is consumed for its anti-pyretic qualities (Wickens & Lowe, 2008) while baobab fruit pulp has also been shown to lower elevated body temperature without affecting normal body temperature (Ramadan *et al*., 1994).

2.12.4. Anti-Inflammatory properties

In Mali, swollen joints are treated by rubbing a paste made with baobab fruit into the affected area causing a cure of the symptoms of this condition known as joint effusion (Wickens & Lowe, 2008). Ramadan *et al*. (1994) found that the fruit pulp of Baobab has similar anti-inflammatory properties to phenylbutazone in rats.

It is proposed that the anti-inflammatory effect of *Adansonia digitata* may be due to the presence of sterols, saponins and triterpenes in the extract. Furthermore, a study carried out by Vimalanathan and Hudson (2009), demonstrated that the pulp extracts had anti-inflammatory properties which the authors say could explain some of the medical benefits attributed to traditional pulp preparations.

2.12.5. Hepatoprotective properties

The hepatoprotective efficacies of *Adansonia digitata* fruit pulp were investigated by Al-Qarawi *et al*. (2003). When extracts of baobab pulp were assessed for their influence on the liver of Wistar male albino rats, it was found that significant hepatoprotection was achieved. Al-Qarawi *et al*. (2003) stated that this may have been as a result of the triterpenoids, β-sitosterol, β-amyrin palmitate, terpenoids, and ursolic acid present in the fruit. The authors also summarised that other bioactivities of Baobab fruit including analgesic, anti-inflammatory and antimicrobial activities could be factors influencing the hepatoprotective activity observed (Al-Qarawi *et al*., 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Models

3.1.1 Cell Culture: The cell lines were obtained from the Anticancer Drug Screening Facility (ACDSF) Laboratory, Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Tata, Memorial Centre, Kharghar, Mumbai, India, and maintained according to the protocols as described by Celis (1998) and Freshney (2000). Human Lung cancer cell line A-549, Human Breast cancer cell line MCF-7, Human Oral Cancer Cell Line KB, Human Bladder Cancer Cell Line T-24 and Human Renal Cancer Cell Line A-498. The cell lines were grown in RPMI 1640 medium, DMEM or EMEM medium modified with 10% (v/v) heat-inactivated FBS and 1% penicillin-streptomycin and incubated at 37°C in 5% CO_2.

3.1.2 Animals A total of 75 Wistar rats weighing 100-150g purchased from the Department of Physiology animal house, University of Ibadan, were used for the *in vivo* study. They were housed at the animal house, in the Department of Biochemistry, University of Ibadan. The animals were fed with commercial pellets obtained from Vita Feeds, Mokola, Ibadan, Nigeria and water *ad libitum* and were allowed to acclimatize for one week. The room temperature was 29 ± 2^{o}C with twelve hours light/dark cycle and relative humidity of 60±5%.

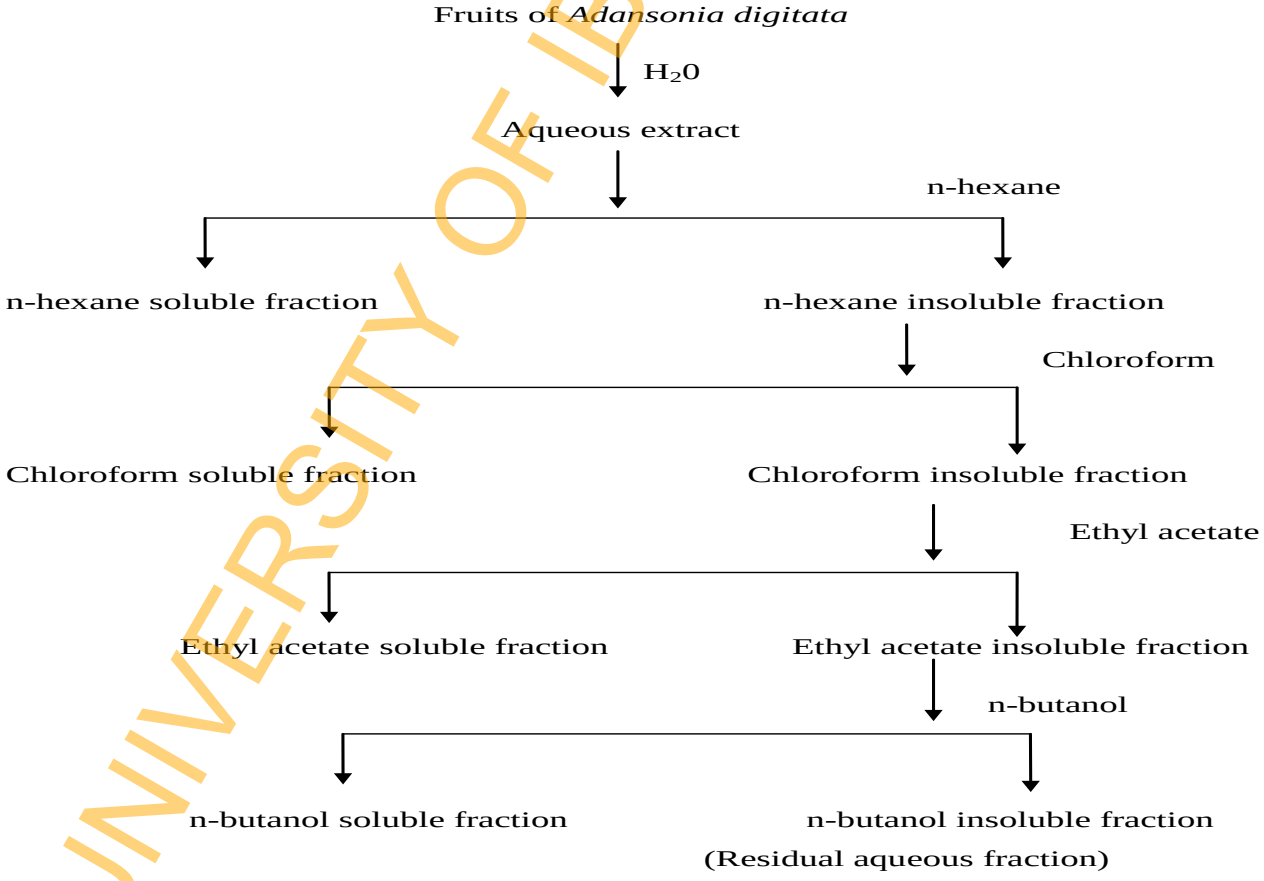
3.2. Plant Identification, Extraction and Solvent Fractionation of AEFAD

3.2.1. Fresh leaves and the matured fruits of the tree, *Adansonia digitata* were used. The identification/confirmation was carried out at the Department of Botany, University of Ibadan and authenticated at Forestry Research Institute, Jericho Ibadan with the FHI NO.:109859. Adequate amount of the fruits and stem bark were harvested. The fruits were broken open and

pulp separated from the seeds with the use of a sieve. The finer particles (i.e the pulp), could pass through the sieve, leaving the seed. Cold extraction was then carried out on the pulp by soaking in distilled water for 72 hours at room temperature. The powdered stem bark was soaked in methanol for 72 hours, the extract was filtered and the filtrate concentrated using a rotary evaporator at temperature of 40^{o}C. The concentrated sample was freeze-dried and the sample kept at room temperature and administered to the experimental animals.

The crude lyophilized aqueous extract (105.6g) of AEFAD was thereafter resuspended in distilled water (200 ml) and sonicated for 5 min. The mixture was then transferred to a 1-L separating funnel and sequentially partitioned with n-hexane (3 x 200 ml), chloroform (3 x 200 ml), ethyl acetate (3 x 200 ml) and n-butanol (3 x 200 ml).

Scheme of Extraction and Fractionation



The resulting fractions and residual aqueous fraction (AQF) were then concentrated on a rotary evaporator under reduced pressure to get n-hexane fraction (NHF), chloroform fraction (CRF), ethyl acetate fraction (EAF), n-butanol fraction (NBF) and aqueous fraction (AQF).

The fractions were pooled and evaporated under a vacuum and then allowed to dry, to obtain the dry residues. The dry residues were stored at -20°C until required for further use.

3.2.2. Methanol extract preparation by cold maceration: The fresh stem bark were air dried at room temperature in the laboratory and grinded into fine powder using a grinder, 500g of the powder was stirred in 2000ml of 70% methanol and was left for 72 hours, the extract was filtered and the filtrate concentrated using a rotary evaporator at temperature of 40^{o}C. The concentrated sample was then freeze-dried and the sample kept at room temperature and administered to the experimental animals.

3.3 Antioxidant determinations

3.3.1 Determination of DPPH Radical Scavenging Activity

Analysis of DPPH radical-scavenging activity was carried out according to the Blois method (Blois, 1958).

Principle

This is based on the ability of a substance to reduce the DPPH free radicals.

Reagents

DPPH and methanol were purchased from Sigma-Aldrich, USA.

Procedure: DPPH was added to each sample, after incubation for 30 minutes in the dark at room temperature, the absorbance was measured at 518 nm using a microplate reader. Ascorbic acid was used as a positive control. Percentage reduction of the DPPH radical was calculated in the following way: inhibition concentration (%) = 100 - ($A_{sample}/A_{control}$) × 100, where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the test sample. Tests were carried out in triplicate. For the final results, IC_{50} values (the concentrations required for 50% inhibition of DPPH by 30 minutes after starting the reaction) were calculated from the absorbance diminished by 50%. The experiment was performed in triplicate.

3.3.2. Determination of Reducing Power Assay

This was determined according to the method of Oyaizu (1986).

Principle

This is based on the reduction of ferric ion to ferrous ion which invariably indicates the reducing power.

Reagents

1 % potassium ferricyanide, phosphate buffer (0.2 M, pH 7.4), 10% tetrachloroacetic acid solution and 0.1 % ferric chloride.

Procedure: The fractions were added to phosphate buffer (0.2 M, pH 7.4) and incubated with 1% potassium ferricyanide at 50^{o}C for 20 mins. The reaction was terminated by adding 10 % tetrachloroacetic acid solution, centrifuged at 3000 rpm for 10 min and the supernatant was mixed with 0.1 % ferric chloride, the absorbance was measured at 700 nm. The experiment was performed in triplicate.

3.3.3. Determination of ABTS radical cation scavenging activity

ABTS radical cation scavenging activity was carried out according to the method of (Re *et al.*, 1999).

Principle

The ABTS assay was based on the ability of different fractions to scavenge the ABTS radical cation in comparison to a standard (ascorbic acid).

Procedure: The radical cation was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1 v/v) and leaving the mixture for 24 h until the reaction was completed and the absorbance was stable. The ABTS radical solution was diluted with PBS to an absorbance of 0.7 ± 0.02 at 732 nm. The photometric assay was conducted with 180 µL of ABTS radical solution and 20 µL of samples; measurements were taken at 732 nm after 1 min. The antioxidant activity of the tested samples was calculated by determining the decrease in absorbance. The experiment was performed in triplicate; the free radical scavenging capacity was expressed by IC50.

3.3.4. Determination of Nitrite-scavenging capacity

Nitrite-scavenging capacity was evaluated by the method of hydrochloric acid naphthalene ethylenediamine coloration.

Procedure: One milliliter of sample or 1 mL of 50% ethanol (blank) was mixed with 1 mL of 5 mg/L nitrite solution and 1 mL of citric acid buffer (pH 3). After reacting for 30 min at 37°C, 1 mL of 4 g/L amino benzene sulfonic acid sodium (in 20% hydrochloric) was added in the mixture, and then 0.5 mL of 2 g/L hydrochloric acid naphthalene ethylenediamine (in

water) was also added after 3 min. The mixture was reacted for 15 min and measured at 538 nm. Ascorbic acid was used as positive control. Nitrite-scavenging ratio (%) = (1 – Asample/Ablank) × 100. The experiment was performed in triplicate.

3.4. Determination of Phytochemicals

3.4.1 Determination of Total Phenol: The total phenol content was determined by the method of (Singleton *et al.*, 1999).

Procedure: 0.20ml of the extract (1 mg/ml) was mixed with 2.5 ml of 10 % Folin ciocalteau's reagent and 2.0 ml of 7.50 % sodium carbonate solution. The reaction mixture was subsequently incubated at 45^0C for 40 mins and the absorbance of the coloured mixture was read at 700 nm using UV visible spectrophotometer. Garlic acid was used as standard phenol.

3.4.2. Determination of Total Flavonoid: The total flavonoid content was determined using a colorimeter assay developed by (Bao, 2005).

Procedure: 0.20 ml of the extract (1 mg/ml) was added to 0.30 ml of 5% $NaNO_3$ at zero time. After 5 mins, 0.60 ml of 10 % $AlCl_3$ was added and 6 mins later, 1M NaOH solution was added to the mixture, followed by the addition of 2.10 ml of distilled H_2O. Absorbance was read at 510 nm. Quantification was performed based on a standard curve with quarcetin.

3.4.3. Determination of Alkaloid: The alkaloid content was determined by the method of (Harborne, 1973).

Procedure: 5.0 g of the sample extract (1 mg/ml) was made to react with 200 ml of 10 % solution of acetic acid in ethanol in a 250 ml beaker. This was covered with aluminium foil and left to stand for 4 hrs. This was later filtered and the extract concentrated on a water bath.

Concentrated ammonium hydroxide was added drop wise to complete the precipitation process. The resulting solution was allowed to settle and the precipitate was collected and washed with distilled water. The residue was weighed as the total alkaloid content.

3.4.4. Determination of Tanins: The Tanins content was determined using the method described by Van-Burden and Robinson (1981).

Procedure: 500 mg of the sample was weighed into a plastic bottle. 50 ml of distilled water was added and shaken for 1 hour in a mechanical shaker. This was filtered into a 50 ml volumetric flask and the volume made up to the mark. 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.10m $FeCl_3$ in 0.10N HCl and 0.008M potassium ferrocyanide. The absorbance was then measured at 120 nm.

3.4.5. Determination of Saponins

2.0 g of the sample was weighed into a 250 ml flask and 100.0 ml of 20 % aqueous ethanol was added. The mixture was heated over a water bath for 4 hours with constant shaking. The mixture was filtered and the residue re-extracted with a further 200 ml of 20 % ethanol. The concentrate was recovered and the ether layer discarded. 60 ml of n-butanol was added and the solution washed with 5% aqueous sodium chloride solution. The sample was then evaporated to dryness on a water bath and the drying completed in the oven.

3.4.6. Determination of Carotenoids

2 g of each sample was weighed into a flat bottom reflux flask, 10 ml of distilled water was added shaking carefully to form a paste. 25 ml of alcoholic KOH solution was added and a reflux condenser attached. The above mixture was heated in boiling water bath for 1 hour

shaking frequently. The mixture was cooled rapidly and 30 ml of water was added. The hydrolysate obtained was transferred into separating funnel. The solution was reextracted 3 times with 25 ml quantity of chloroform. 2 g anhydrous Na_2SO_4 was added to the extract to remove any traces of water, the mixture was then filtered into 100 ml volumetric flask and made up to the mark with chloroform. Standard solution of B-carotene vitamin A of range 0-50 mg/ml with chloroform by dissolving 0.003 g of standard B-Carotene in 100 ml of chloroform. The above gradient of different standard prepared were determined with reference to the absorbance from which average gradient was taken to calculate Vitamin A (B-carotene in mg/100g). Absorbance of sample and standard were read on the spectrophotometer at 328nm on a spectronic 21D spectrophotometer (Digital)

$$\text{Carotenoid or vitamin A } (\mu g/100g) = \frac{\text{Absorbance of sample x gradient factor x 10}}{\text{Wt. of sample x 1000}}$$

Conversions 6 mg of B-carotene = 1 retinol equivalent

12 mg of other biological active carotenoids =1:1 retinol equivalent

1 retinol equivalent of vitamin A activity = 1mg retinol

1 retinol equivalent = 3.1.U.

3.4.7. Determination of Terpenoids: Terpenoid was determined according to the method earlier described by (Ejikeme *et al.*, 2014).

Procedure: 0.30 g of each sample powder was weighed into a beaker**,** 30 cm^3 of distilled water was added and the mixture was allowed to stand for 2 hours. 5 cm^3 of each extract was then measured and mixed in 2 cm^3 of chloroform and 3 cm^3 of concentrated tetraoxosulphate

(VI) acid was thereafter added to form a layer. A reddish brown colouration at the interface is indicative of positive results for the presence of terpenoids.

3.4.8. Determination of Oxalate: This was carried out using the method reported by Ejikeme *et al*., 2014; Munro and Bassir, 1969.

Procedure: 20 cm^3 of 0.3 M HCl in each sample (2.50 g) was extracted three (3) times by warming at a temperature of 50°C for 1 hour with constant stirring using a magnetic stirrer. For oxalate estimation, 1.0 cm^3 of 5M ammonium hydroxide was added to 5.0 cm^3 of extract to ensure alkalinity. Addition of 2 drops of phenolphthalein indicator, 3 drops of glacial acetic acid, and 1.0 cm^3 of 5% calcium chloride to make the mixture acidic before standing for 3 hours was followed by centrifugation at 3000 rpm for 15 minutes. After discarding the supernatant, the precipitate was washed three times using hot water by mixing thoroughly each time of centrifugation. Then, to each tube, 2.0 cm^3 of 3M tetraoxosulphate (VI) acid was added and the precipitate dissolved by warming in a water bath at 70°C. Freshly prepared 0.01 M potassium permanganate ($KMnO_4$) was titrated against the content of each tube at room temperature until the first pink colour appears throughout the solution. The solution was allowed to stand until it returned colourless, after which it was warmed on an electric hot plate at 70°C for 3 minutes, and retitrated again until a pink colour appears and persists for at least 30 seconds. Titration reaction of oxalate in sample was thereafter calculated.

3.4.9. Determination of Anthocyanin content: Anthocyanin content was determined using a method earlier described by (Connor *et al.,* 2002).

Procedure: Each sample was diluted (5:95, v/v) in 1% HCl in methanol to obtain an absorbance between 0.500 and 1.000 at 530 nm. The values were expressed as mg cyanidin-3-

glucoside (3 g) equivalents per 100 g fresh weight using a molar extinction coefficient of 27.900. All determinations were performed in triplicates.

3.4.10. Determination of Steroid

Analytical method used is according to (Ejikeme *et al.*, 2014). Each sample (0.30 g) weighed into a beaker was mixed with 20 cm^3 of ethanol; the component was extracted for 2 hours. To the ethanolic extract of each sample (5 cm^3) was added 2 cm^3 acetic anhydride followed with 2 cm^3 of concentrated tetraoxosulphate (VI) acid. A violet to blue or green colour change in sample(s) indicates the presence of steroids.

3.4.11. Determination of Protease inhibitor: Activity of protease inhibitor against protease was assayed according to the procedure described by Kunitz with slight modifications (Kunitz, 1947).

Procedure: TCA soluble fractions formed by action of trypsin on the protein substrate Hammerstein casein was measured by the change in absorbance at 280 nm. The residual caseinolytic activity of the trypsin in the presence of inhibitor, at 37°C, was used as a measure of inhibitory activity; appropriate blanks for enzyme, inhibitor, and substrate were also included in the assay along with the test.

3.4.12. Determination of Cyanogenic Glycoside: Cyanogenic glycoside was determined according to the method earlier described by (Amadi *et al.*, 2004).

Procedure: One gram of the sample powder was weighed into a 250 cm^3 round botton flask and 200 cm^3 of distilled water was added and allowed to stand for 2 hours for autolysis to occur. An antifoaming agent (tannic acid) was added and full distillation carried out in a 250

cm^3 conical flask containing 20 cm^3 of 2.5% NaOH (sodium hydroxide). To 100 cm^3 of each distillate containing cyanogenic glycoside, 8 cm^3 of 6M NH_4OH (ammonium hydroxide) and 2 cm^3 of 5 % KI (Potassium Iodide) was added, mixed and titrated with 0.02M $AgNO_3$ (silver nitrate) using a micro-burette against a black background. Permanent turbidity indicates the end point. Cyanogenic glycoside content of the sample was calculated appropriately.

3.5. Blood Collection and Serum Preparation

On the completion of the period of administration the rats were sacrificed by cervical dislocation. Blood was collected into non heparinised bottles. The blood samples were kept for up to two hours to allow for clotting. The blood samples were then centrifuged for 30 minutes at 3000 g to obtain serum as the supernatant, the sediments were discarded and the serum decanted into another set of labeled bottles for liver enzyme assay. The serum samples were stored in ice until required for use.

3.6 Haematology Assays

Haematology is the study of blood and blood forming tissues. It is the study of blood disorders such as leukemia, anemia and hemophilia as well as diseases of the organs that produce blood including the lymph nodes, bone marrow and spleen. Laboratory based tests are used to diagnose a variety of disorders. Of particular importance are blood tests that provide information about the cellular components of a patient's blood. The most common test, called a complete blood count (CBC), indicates the number of red blood cells, white blood cells and platelet in a given unit of blood. Haematological parameters analysed include: haemoglobin concentration, red blod cell count, white blood cell count, platelet and packed

cell volume (haematocrit). For this study blood was collected via retro-orbital bleeding and was taken to the haematology unit, Department of Veterinary medicine, University of Ibadan for analysis.

3.7. Extraction of Tissue Samples

The abdominal and thoracic regions of the sacrificed animals were dissected opened exposing the liver. All livers of experimental animals were harvested one after the other. Part of the harvested liver was cut off and stored in 10 % buffered formalin for histological examination after being appropriately designated. While the other part of the liver was placed each in a beaker of about 5 ml chilled KCL in preparation for enzyme assays.

3.8. Histological Analysis

A portion of the liver from each sacrificed animal was excised, blotted and then perfuse with potassium chloride (1.15 %) in order to remove all traces of haemoglobin which might contaminate the tissues. The liver samples were then preserved and fixed in 10 % buffered formalin and were processed for paraffin sectioning. Sections of about 5 µm thickness were stained using Haematoxylin and Eosin staining according to the method of (Chayen *et al.*, 1973), the liver sections were left for 24 hours for fixation of the organs after which cross-sections of the organs were cut and placed in a processor overnight. In the processor, the tissues were first placed in 70 % alcohol for 2 hours, followed by 90 % alcohol for another 2 hours, xylol for 4 hours, and finally, in wax for 5 hours. The tissues were removed, embedded in molten fibro wax and allowed to solidify under a running tap. The tissues were brought out and mounted on wooden blocks and then chilled in ice. Sections of the tissue were cut at a thickness between 3 and 5 mm using the rotary microtome and then allowed to

float in 20 % alcohol, followed by water at 58^{o}C (an incubator), placed on albumized glass slides and dried on a hot plate at 60^{o}C. The slides so prepared were initially placed in xylol and washed with decreasing concentration of absolute alcohol, 90 % alcohol, 80 % alcohol and finally, 70 % alcohol. They were washed in water stained with Cole's haematoxylin, washed again with water, followed by 1 % hydrochloric acid, running tap water and rinsed in saturated lithium carbonate. These glass slides were transferred to 1 % aqueous solution of eosin for 2 minutes, and washed in a running tap. They were cleaned, mounted on Depex after treatment in absolute alcohol. The slides were finally allowed to dry on the bench at room temperature and then evaluated by the pathology department of University of Ibadan.

3.9. Cells per Millimeter squared Assay

The slides prepared for histological analysis from liver tissue samples were used to count the number of cells in a millimeter squared area under the microscope at magnification X 40 with the aid of microscope grid (Gulubova *et al.*, 2005; Aller *et al.*, 2008). The average numbers of cells per squared millimeter in test groups were then compared with those from the control group.

3.10 Determination of Lipid Peroxidation

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. Livers were washed in ice cold 1.15 % potassium chloride (KCl) and weighed. They were then homogenized in 4 volumes of homogenizing buffer containing 50 mM Tris-HCl mixed with 1.15 % KCl using a Teflon homogenizer. The homogenate was centrifuged at 10,000 g for 20 minutes. The supernatant which is the post- mitochondria fraction was stored at -20^{o}C until when needed for analysis.

An aliquot of 0.4 ml of the sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30 % TCA was added. Then 0.5 ml of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000 rpm for 15 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^{5} $M^{-1}Cm^{-1}$.MDA (units/mg protein) = Absorbance x volume of mixture E532 nm x volume of sample x mg protein.

3.11. Determination of Hematological Parameters

Whole blood examination includes total leukocytes (WBC), hemoglobin (Hb) content, hematocrits (PCV), lymphocytes and Neutrophils. Haematocrits (PCV) were determined immediately after sampling, using a microhaematocrit centrifugation (10, 500 ×g for 5 min). Capillary tube was centrifuged for 5 minutes at 10, 500 rpm in a micro-haematocrit centrifuge and volume PCV was measured using microhaetocrit reader. Hemoglobin concentrations (mg/dl) were determined by the cyanhaemoglobin method using a wavelength of 540 nm. The blood plasma was obtained by centrifuging the heparinized blood at 4100 ×g for 10 min at 4°C and the blood cells (erythrocytes and leucocytes) were separated into eppendorf tubes. All blood parameters were performed within 12 h. The parameters were determined as described by Jane (1986).

3.12. Liver Function Enzymes

3.12.1 Aspartate Aminotransferase (AST) Aspartate Aminotransferase (AST) like ALT is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from

aspartate to α-oxoglutarate to form oxaloacetate and glutamate. The diagnostic implications of this enzyme in the serum include liver cirrhosis, myocardial infarction (becoming evident 4-8 hours after the onset of pain and peaking after 24-36 hours), muscular dystrophy and paroxysmal myoglobinuria.

Principle Aspartate Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine and NaOH at absorbance 546nm. 2-oxoglutarate + L-aspartate → L-glutamate + pyruvate (Reitman and Frankel, 1957)

Reagents 1.) R Substrate

2.66 g of L-aspartate (BDH Chemicals Ltd., England) and 30mg alpha-oxoglutaric acid was dissolved in 20.5 ml of normal sodium hydroxide buffer with gentle warming. The resultant solution was made to 100ml with phsphate buffer, 7.4. The substrate was kept at 4^{o}C and a drop of chloroform was added.

(2.) 0.1M Phosphate buffer, pH 7.4

a. First 0.1M Na_2HPO_4 $12H_2O$ (MW=358.22) was prepared by dissolving 7.1628 g in 200 ml of distilled water.

b. 0.1M NaH_2PO4. $2H_2O$ (MW=156.03) was prepared by dissolving 1.5603 g in 100 ml of distilled water. Finally 0.1M phosphate buffer was prepared by adding 200 ml of (a) to 100 ml of (b) and the pH adjusted to 7.4 using pH meter with drops of concentrated HCl or NaOH as the case may be. This is stable indefinitely unless mold forms. If crystals develop during storage at 4^{o}C, heating may dissolve these.

(3.) 2, 4- dinitrophenyl hydrazine (1mM)

200 mg of 2, 4- dinitrophenyl hydrazine (Hopkins and Williams Ltd. England) was dissolved in 100 ml of 1N HCl with warming.

(4.) Sodium hydroxide (0.4N)

This is prepared by dissolving 16 g of sodium hydroxide pellet (Hopkins and William Ltd., England) in distilled water. The volume was then made up to 1 litre.

Procedure: 0.1 ml of diluted sample was mixed with phosphate buffer, L-aspartate and α-oxoglutarate, and the mixture incubated for exactly 30 min at 37^0C. 0.5ml of 2, 4-dinitrphenylhydrazine was added to the reaction mixture and allowed to stand for exactly 20 min at 25^0C. Then 5.0 ml of NaOH was added and the absorbance read against the reagent blank after 5 min at 546 nm.

Calculation The activity of AST in the serum was obtained from the below table using the standard curve.

Table 3.1. Standard AST values

Absorbance	U/l	Absorbance	U/l
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

Source: (Reitman and Frankel, 1957)

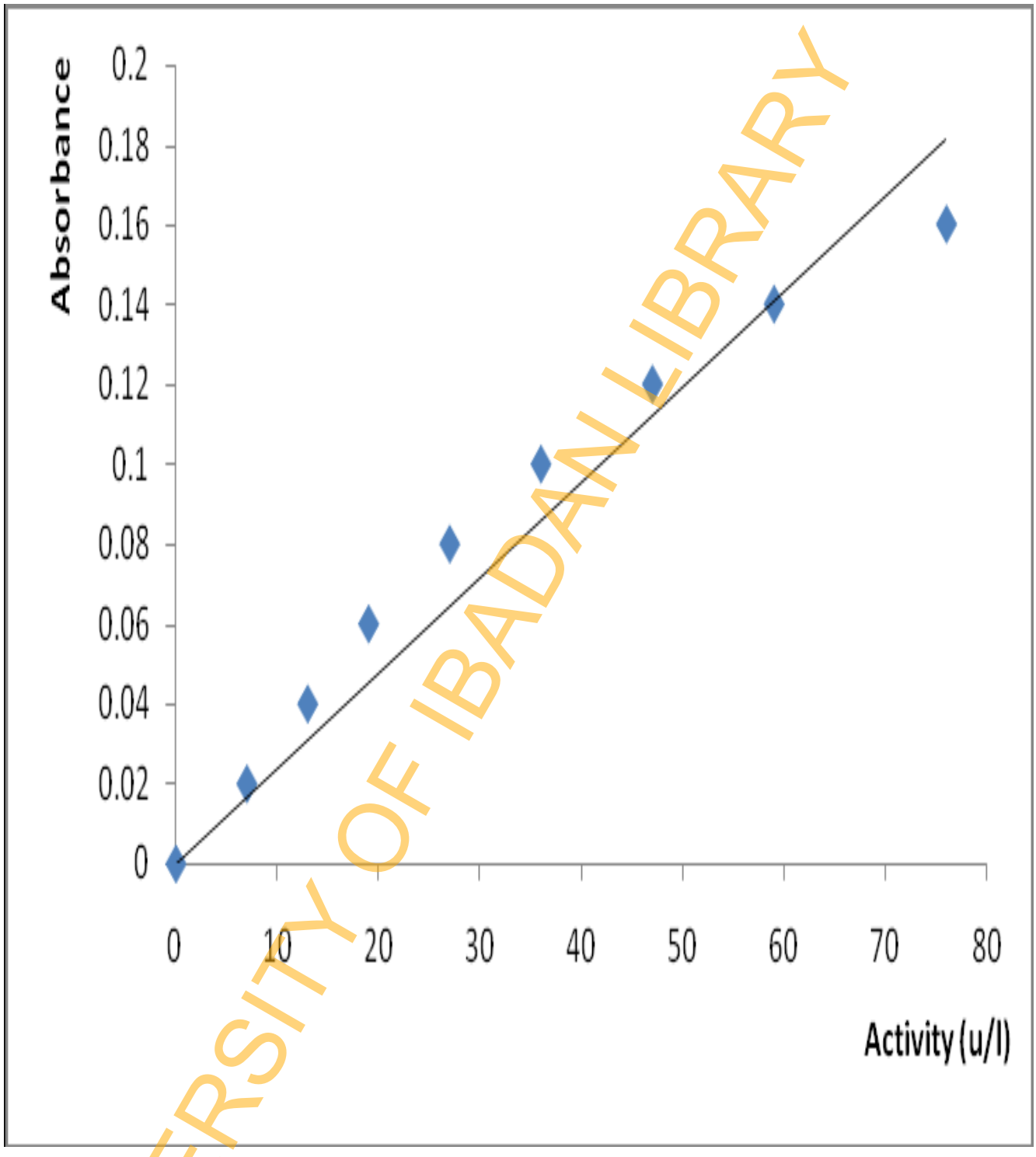


Figure 3.1: Standard Aspartate Aminotransferases (AST) curve.

(Reitman and Frankel, 1957)

3.12.2 Alanine Aminotransferase (ALT) Alanine Aminotransferase (ALT) is an intracellular enzyme involved in amino acid and carbohydrate metabolism. It is present in high concentrations in the liver and muscle. It is involved in the transfer of amino group from alanine to α-oxoglutarate to form pyruvate and glutamate. The diagnostic implications of this enzyme in the serum include hepatitis and other liver diseases in which the level is often higher than that of AST. Elevated level is also found in metastatic or primary liver neoplasm.

Principle: Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine and NaOH at absorbance 546nm. 2-oxoglutarate + L-alanine → L-glutamate + pyruvate (Reitman and Frankel, 1957)

Reagents

(1.) substrate

1.78 g of L-alanine (BDH Chemicals Ltd., England) and 30 mg alpha-oxoglutaric acid was dissolved in 20 ml of buffer to which 0.5 ml 1N sodium hydroxide had been added. The resultant solution was made to 100 ml with the buffer. The substrate was kept at $4^{o}C$ and a drop of chloroform was added

(2.) 0.1M Phosphate buffer, pH 7.4 a. First 0.1M Na_2HPO_4 $12H_2O$ (MW=358.22) was prepared by dissolving 7.1628 g in 200 ml of distilled water.

b. 0.1M NaH2PO_4. $2H_2O$ (MW=156.03) was prepared by dissolving 1.5603 g in 100 ml of distilled water. Finally 0.1M phosphate buffer was prepared by adding 200 ml of (a) to 100 ml of (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case may be. This is stable indefinitely unless mold forms. If crystals develop during storage at $4^{o}C$, heating may dissolve these.

(3.) 2, 4- dinitrophenyl hydrazine (1mM)

200 mg of 2, 4- dinitrophenyl hydrazine (Hopkins and Williams Ltd. England) was dissolved in 100 ml of 1N HCl with warming.

(4.) Sodium hydroxide (0.4N)

This is prepared by dissolving 16 g of sodium hydroxide pellet (Hopkins and Williams Ltd., England) in distilled water. The volume was then made up to 1 litre.

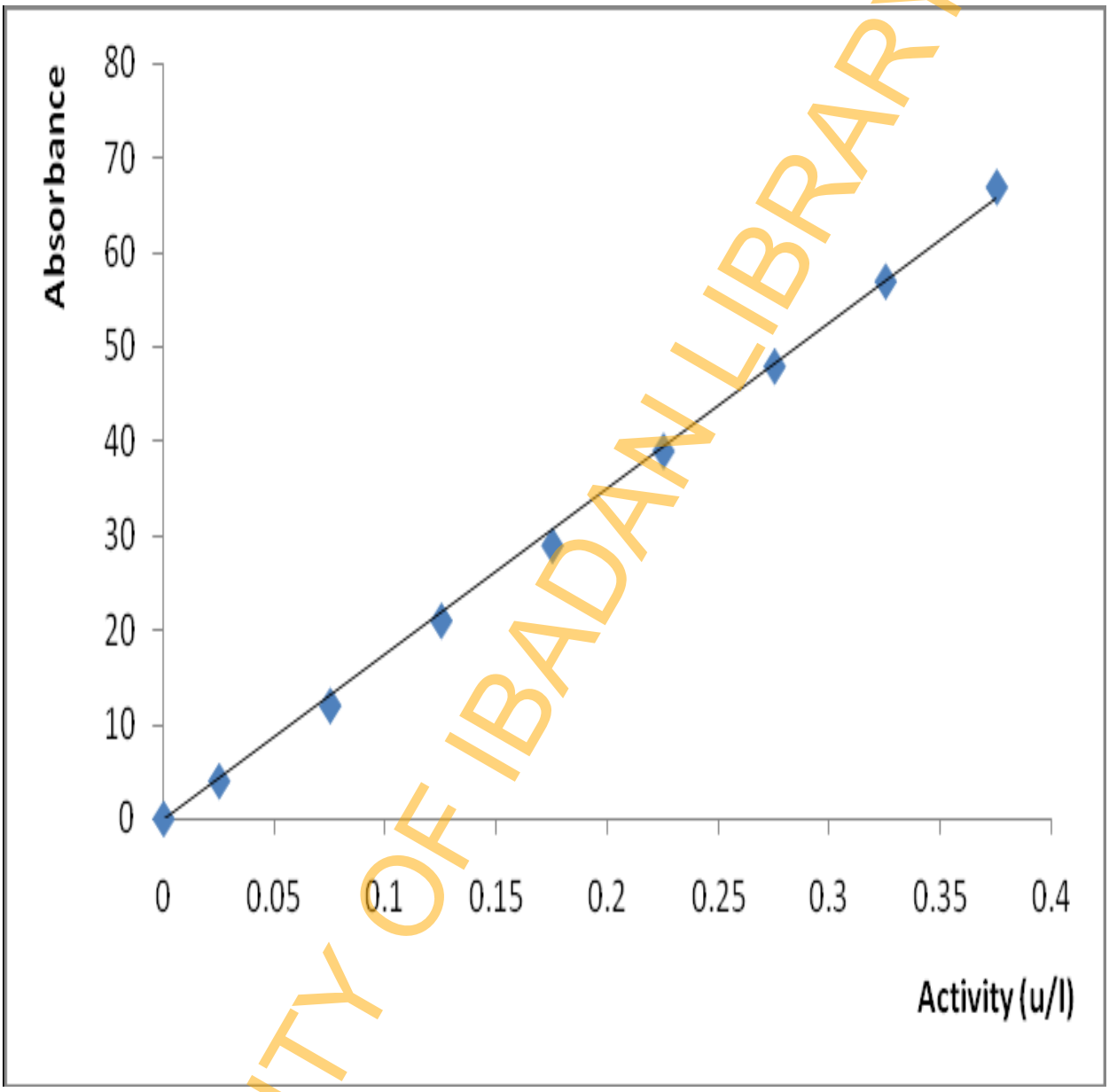
Procedure: 0.1 ml of diluted sample was mixed with phosphate buffer, L-alanine and α-oxoglutarate, and the mixture incubated for exactly 30 min at 37^{o}C. 0.5 ml of 2, 4-dinitrphenylhydrazine was added to the reaction mixture and allowed to stand for exactly 20 min at 25^{o}C. Then 5.0 ml of NaOH was added and the absorbance read against the reagent blank after 5 min at 546 nm.

Calculation The activity of ALT in the Serum was obtained from the below table using the standard curve.

Table 3.2. Standard ALT values

Absorbance	U/l	Absorbance	U/l
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

Source: (Reitman and Frankel, 1957)



Source: (Reitman and Frankel, 1957)

Figure 3.2: Standard Alanine Aminotransferases (ALT) curve.

3.12.3. Gamma Glutamyl Transferase GGT activity was determined following the principle described by Szasz (1969).

Principle Gamma glutamyl transferase (GGT) catalyses the transfer of the glutamyl group from glutamyl peptide to an amino acid of another peptide, glylglycine to yield a cleavage product, p-nitro-anilide, which absorbs UV light at 405 nm, thus making a direct kinetic determination possible. Gamma Glutamyl transferase (E.C. 2.3.2.2.) has been known to be more sensitive and hence more reliable than the serum amino transferases.

L-gamma-glutamyl-p-nitoranilide + glycylglycine gamma-GT L-gamma glutamylglycylglycine + p-nitroaniline

Reagent and substrate preparation. 0.7 g of Glycylglycine (mol. Wt. 132g); 0.15 g of Magnesium chloride (mol. Wt. 95 g) and 0.12 g of -glutamyl-p-nitroanilide were dissolved in 0.005M Tris base (mol. Wt. 121.14) and the solution made up to 100ml with 0.005M Tris base. The solution was prepared and stirred at 50^{o}C and the pH was adjusted to 8.2. This serves as the substrate.

Procedure: 0.1 ml of the serum samples were added to 2.9 ml of the substrate solution in a test tube. The solution was mixed and incubated at 37^{o}C for 1 minute and the absorbance was taken at 405 nm for 5-8 minutes interval. The blank solution that contains the substrate alone was incubated at 30^{o}C for 10 minutes and the curve of change in absorbance per minute 405 nm/min was plotted. 405 nm/min was calculated from the linear portion of the curve.

Calculation The GGT activity (U/L) = 1158 x ΔA 405 nm/min

3.12.4. Alkaline Phosphatase Activity ALP was determined according to the optimized recommended method of Deutsche Gesellschaft fur Klinische Chemie (Rec. GSCC DGKC), 1972.

Principle: Alkaline phosphatase catalyzes the hydrolysis of p-nitrophenylphosphate into phosphate and p-nitrophenol which absorbs UV light at 405 nm, thus making a direct kinetic determination possible.

p-nitrophenylphosphate + H_20 $\xrightarrow{ALP}$ phosphate + p-nitrophenol (Tietz *et al.*, 1983).

Reagents:

1.) R1a. Buffer 1M Diethanolamine buffer pH 9.8 0.5 mM $MgCl_2$

2.) R1b. Substrate 0.01M p-nitrophenylphosphate Procedure The 0.02 ml of sample was added into cuvette followed by the 1.0 ml of reagent. The initial absorbance of the mixture was read and the timer was set simultaneously. The mixture was read again after 1, 2 and 3 minutes.

Calculation: The ALP activity (U/L) = 2760 x ΔA 405 nm/min

3.13. Assessment of Lipid Peroxidation: Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation by following the procedure decribed by Rice- Evans *et al.*, 1986.

Principle This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532 nm and which is

extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of free MDA produced.

Reagents 1. 30% Trichloroacetic acid (TCA)

9 g of TCA (CCl3COOH) was dissolved in distilled water and made up to 30 ml with same.

2. 0.75 % Thiobarbituric acid (TBA) This was prepared by dissolving 0.23 g of TBA in 0.1M HCl and made up to 30 ml with same.

3. 0.15M Tris-KCl buffer (pH 7.4)

1.12 g of KCl and 2.36 g of Tris base were dissolved separately in distilled water and made up to 100 ml with same. The pH was then adjusted to 7.4 with pH meter.

Procedure: An aliquot of 0.4 ml of the sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30 % TCA was added. Then 0.5 ml of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000 g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 105 M-1Cm-1. MDA (units/mg protein) = Absorbance x volume of mixture E532nm x volume of sample x mg protein

3.14. The Micronucleus Induction Assay Procedure

3.14.1 The Micronucleus Induction Assay

Micronucleus refers to a chromosomal fragment which is lagging in anaphase for various reasons. It is therefore not incorporated into the daughter nuclei at the time of cellular division. In telophase stage of cell division, this material becomes inserted into one of the daughter cells, and either fuses with the main nucleus or forms one or several secondary nuclei (if any). These are significantly smaller than the main nucleus and hence are called micronuclei. They can be observed in any dividing cell population, which has lost some chromosomal fragments (Heddle and Salamone, 1981).

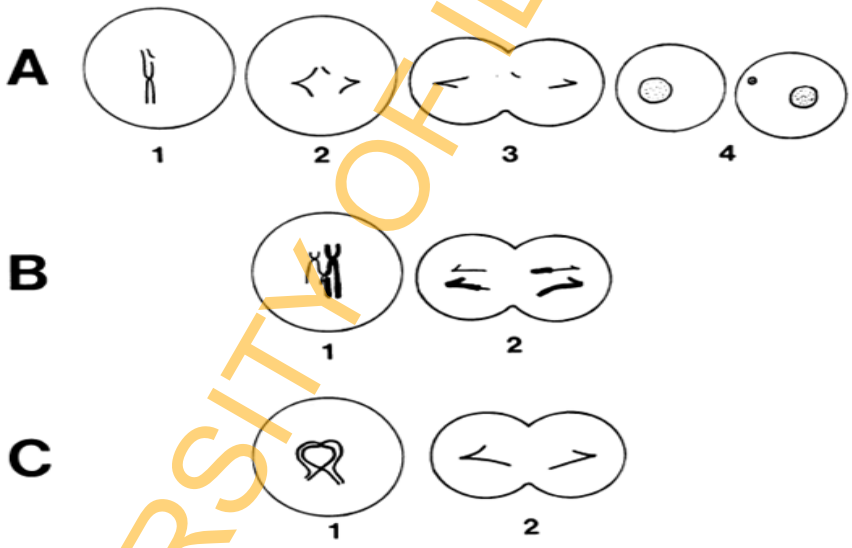


Figure: 3.3. Chromatid deletions and its fate (Heddle and Salamone, 1981)

(A) Schematic diagram of a chromatid deletion and its fate (1) at metaphase, (2) at anaphase, (3) at telophase and (4) the resultant micronucleus at interphase. **(B)** A chromatid translocation at metaphase which may segregate either (1) in a balanced form or (2) in an unbalanced form giving a duplication for one region and a deficiency for the other in each daughter cell. **(C)** A Chromatid inversion (assymetrical chromatid interchange) (1) at metaphase (2) at anaphase.

3.14.2. Detection of Micronucleus

The micronucleus assay is developed for detection of *in vivo* chromosomal breakage more conveniently than the traditional cytogenetic methods (Heddle, 1973). It has been used to detect *in vivo* genetic activity in bone marrow cells (Sai *et al*., 1992). The micronucleus test has also been employed to detect *in vitro* chromosomal aberration. The majority of the micronuclei are found in the polychromatic erythrocyte (PCE) cells and this offers an advantage for the use of the micronucleus assay for screening mutagens (Von Le De Bur and Schmidt, 1973).

Positive result with the micronucleus test is just an indication of chromosomal damage; it does not conclude the tested agent as a mutagen or carcinogen. However, most studies have proved the agent being tested as mutagenic or carcinogenic agents (Salamon *et al.*, 1980).

Principle

The principle of the micronucleus assay is based on the fact that polychromatic erythrocyte (PCE) cells have a staining property that is different from the normal mature erythrocyte (normocyte). The polychromatic staining property results from the presence of ribosomal RNA 24 hours prior to the formation of the cell. As PCEs develop into mature erythrocytes, they lose the ribosomal RNA and the staining property. In mammals, mature erythrocytes expel their nuclei 8-12 hours after the last mitosis preceding the formation of an erythrocyte. The micronuclei for some reasons are not expelled completely. Micronuclei are not normally found in the circulating erythrocytes in blood because they are filtered out by the spleen (Schalm, 1965).

Reagents

-Bovine serum albumin (BSA)

-Absolute methanol

-Xylene

-Depex (DPX) mountant.

-0.4 % May Grunwald Stain

-5 % Giemsa Stain.

-0.01M Phosphate buffer pH 6.8

3.14.3. Preparation of Bone marrow Smears

The method of Schmid (1975) was adopted in the preparation of bone marrow smears. After the rats were sacrificed by cervical dislocation and the femur of each rat was removed and stripped clean of muscle. A pair of scissors was used to make an opening in the iliac region of the femur. A small pin was then introduced into the marrow canal at the epiphyseal end. As the pin was pushed inside the canal, the marrow exuded through the hole at the iliac end.

The marrow was placed into a slide and a drop of bovine serum was added to the smear using a Pasteur pipette. The whole content was mixed to become homogeneous by using a clean edge of another slide. The homogeneous mixture was then spread on the slide as a smear and allowed to dry.

3.14.4. Fixing and Staining of Slides.

The procedure for fixing and staining of the slides was followed in a stepwise manner: The prepared slides were fixed in methanol for 5 minutes, air dried to remove methanol and thereafter stained with 0.4 % May Grunwald stain 1, then in stain 2 of May Grunwald. The May Grunwald stained slides were air dried and stained in 5 % Giemsa for at least 30 minutes, they were thereafter rinsed in phosphate buffer for about 30 seconds and also rinsed in distilled water. The slides were air dried and fixed in xylene for 20 minutes, air dried again and mounted with cover slips in DPX (a natural mountant).

3.14.5. Scoring of the Slides

The fixed cells on the slides were viewed under a Nikon Compound microscope to detect the presence of micronucleated polychromatic erythrocytes. Tally counter was used for counting. The slides were first screened at medium magnification to get suitable regions for scoring, and scored at X 40 magnification. PCEs and micronuclei stain blue while normal mature erythrocytes stain red.

3.15. Preparation of Tissues for Biochemical and Histological analyses

Reagents: 1. Homogenizing buffer (50mM Tris-HCl, 1.15% KCl, pH 7.4)

7.80 g of Tris (hydroxymethyl) amino ethane (Sigma chemical Co., St Louis U.S.A) and 11.5 g of potassium chloride were dissolved in 900 ml of distilled water. The pH was adjusted to 7.4 and then made up to 1 liter with distilled water.

2. 1.15 % Potassium Chloride 1.15 g of potassium chloride (BDH Chemical Limited, England) was dissolved in distilled water and made up to 1000ml and stored at 4°c.

Procedure: The animals were sacrificed by cervical dislocation, the liver and kidney tissues of the animals were excised and immediately rinsed in ice cold 1.15 % KCl solution and blotted dry. They were then homogenized in 4 volumes of the homogenizing buffer (pH, 7.4) using a Tefflon homogenizer. The liver and kidney homogenates were centrifuged at 10,000 g, 4°C for 10 minutes. These post-mitochondrial supernatant fractions were collected and processed for biochemical estimations. A portion each of kidneys and livers samples were cut and fixed in formalin fixative, sectioned and stained routinely for histopathological examination.

3.16. Cell lines experiments

3.16.1. Maintenance of Cell Lines

The cell lines were maintained according to the protocols as described by Celis (1998) and Freshney (2000). They were thawed and mixed with culture medium followed by centrifugation and determination of cell concentration and viability as described in subsequent sections.

3.16.2. Thawing of the Cell lines

Cryovials containing cell lines were placed in water bath (37^{o}C) for 2-3 minutes and contents were immediately transferred into centrifuge tubes (15 ml) containing washing

medium (RPMI-1640/ DMEM: 98 %, FBS: 1%, GPSS: 1%). After gentle mixing, the tubes were balanced and centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded; the cell pellet was dislodged by gentle tapping and re-suspended in washing medium (1 ml) for counting and determination of viability. All the cell lines were placed individually in flasks ($25cm^2$) in culture medium {(RPMI-1640/ DMEM supplemented with heat-inactivated FBS (10%), L-glutamine (2 mM), streptomycin (100 µg/ml), penicillin (100 U/ml} and placed in a humidified atmosphere of CO_2 (5%) at 37^oC, after loosening the caps for the exchange of gases and to maintain the desired pH (7.2-7.4). The normal color of the culture medium was pink because of phenol red. When the medium became acidic, the color changed to orange and dark yellow due to the production of pyruvic and lactic acids and CO_2 as the byproducts of normal metabolism of the cells. The cells were examined daily for any change in their morphology, color of the medium and contamination and the culture medium was changed after every 2-3 days.

3.16.3. Trypsinization of Monolayer

The culture medium was discarded and 1x PBS (phosphate buffer saline, 10 ml) was added to remove serum and dead cells followed by addition of trypsin-EDTA (0.05 %, 5 ml for 25 cm^2 flask) at room temperature. Cell detachment is dependent on the nature of cell line used (2-6 minutes). The trypsin was inactivated by the addition of washing medium (7 ml) for 25 cm^2 flask. The cell suspension was centrifuged at 1000 rpm for 10 minutes at room temperature and the cell pellet was resuspended in washing medium (1 ml).

3.16.4. Cell Culture and Treatments

Cells were grown in either RPMI/DMEM medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 100 U penicillin and 100 µg/ml streptomycin at 37^{o}C in a humidified atmosphere with 5 % CO_2. The cells were passaged twice weekly. AEFAD fractions and MESBAD were initially dissolved in DMSO and then with culture medium at a final concentration of 10 % ($^{v}/_{v}$) and the mixture was filter-sterilized using 0.22 µm syringe filter unit (Millipore, USA). The AEFAD fractions and MESBAD were added to cells in complete medium after 24 hrs of growth and were diluted at a final concentration of 10, 20, 40, 80 µg/ml for AEFAD fractions and 50, 100, 250, 500 and 1000 µg/ml for MESBAD. The experiments were in triplicates/duplicates and repeated at least three times.

3.16.5. Cell viability Assessment by Trypan blue exclusion Assay

The cell viability was assessed by trypan blue dye exclusion method (Rosengard and Cochrane, 1983) using hemocytometer.

Principle: This is based on the principle that, trypan blue is not excluded by dead or dying cells but is exlcuded by living cells; therefore after reaction with trypan blue, dead cells absorb trypan blue dye and turn deep blue while living cells remain white.

Reagents 0.4 % Trypan blue solution

Procedure: Trypan blue solution (10 µL, 0.4 % in 0.9 % saline) was mixed gently with the cell suspension (10 µL) and the sample (10 µL) was examined within 30 seconds under the light microscope at 10x magnification. The blue stained cells were counted as non-viable while the unstained, bright and translucent cells as viable. The concentration of cells was

calculated as follows: Cells per mL = N x 5 x D.F. / volume Where, N is the total number of cells counted in 5 squares, D.F. is the dilution factor and volume is the depth of the counting chamber (0.1 mm^3 = $1x10^{-4}$). Therefore, cells / mL = N x 5 x D.F. x 10^4. Dilution factor was calculated by dividing the total volume used (20 µL) with the cell suspension volume (10 µL) so the dilution factor was 2. % live and dead cells were calculated as follows: % live cells = number of live cells X 100 Total number of cells % dead cells = number of dead cells X 100 Total number of cells

3.16.6. MTT (3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) Assay

Principle

The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dye, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

Procedure: Cytotoxicity of the various fractions of AEFAD were evaluated in 96-well plates by using standard MTT (3-[4, 5-dimethyl thiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay (Mosmann, 1983) on MCF-7 and SRB assay on others. Exponentially growing cells were harvested, counted with hemocytometer and diluted with a medium. Cell culture with the concentration of 1×10^4 cells/mL were prepared and

introduced (100 µL/well) into 96-well plates. After overnight incubation, medium was removed and 200 µL of fresh medium was added with different concentrations of MESBAD and fractions of AEFAD. After 48 hrs, 20 µL MTT (2 mg/mL) was added to each well after aspiration of media and incubated further for 4 hrs. Subsequently, 100 µL of DMSO was added to each well. The extent of MTT reduction to formazan within cells was calculated by measuring the absorbance at 570 nm, using a micro plate reader. The cytotoxicity activity was recorded as concentration causing 50% growth inhibition (IC_{50}). The percent inhibition was calculated by using the following formula:

% inhibition = 100 - (mean of Absorbance of sample/mean of Absorbance of control) × 100

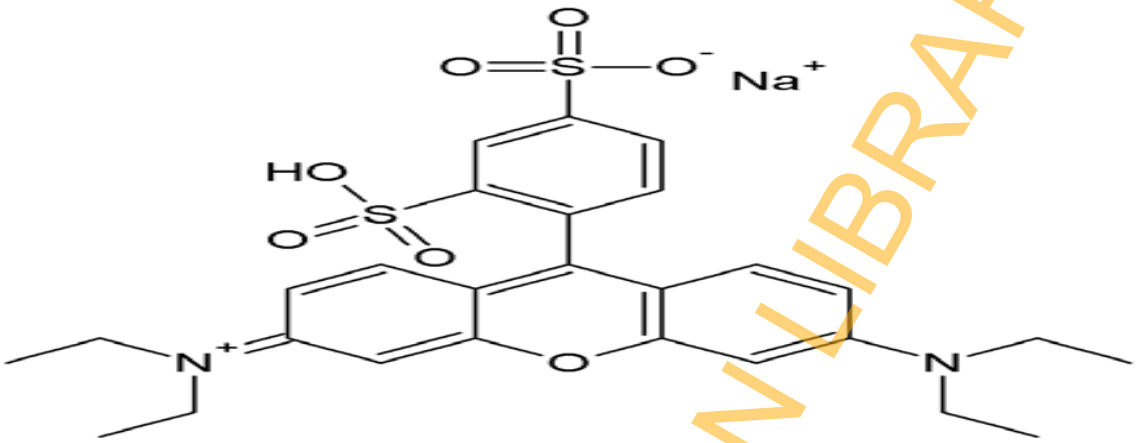
Figure 3.4: MTT assay reaction (Mosmann, 1983).

3.16.7. Cell cytotoxicity assay (Sulforhodamine - B Assay)

Introduction: SRB Cell cytotoxicity Assay is an accurate and reproducible assay based upon the quantitative staining of cellular proteins by sulforhodamine B (SRB). This assay has been used for high-throughput drug screening at the National Cancer Institute (NCI)

Principle: Sulforhodamine B is an anionic aminoxanthene dye that forms an electrostatic complex with the basic amino acid residues of proteins under moderately acid conditions,

which provides a sensitive linear response. The colour development is rapid and stable and is readily measured at absorbances between 560 and 580 nm.



Other required Items

Microplate reader capable of readout between 550 and 580 nm

96 well tissue culture plates

Protocol

The optimal conditions for monitoring cytotoxicity are to have the cells in the log phase of growth and not to exceed 10^6 cells/cm^2. It is recommend that each test is performed in a final volume of 200 µl and includes a 200 µl control sample of cell free medium to be used to blank absorbance readings.

Following treatment with appropriate test agents, the 96-well plates were removed to a sterile tissue culture hood and gently layered with 50 µl 30 % of TCA onto each well. During and after the fixative reagent step, the plates were disturbed as little as possible (Sudden shaking or jolting could dislodge cells and result in inaccuracies in protein

quantity). Incubation of the plate was done for 1 hour at 4°C, and the wells were washed 3-4 times with water and plates vigorously flicked between washes to remove excess water. Thereafter, the plates were washed to remove excess fixative and serum proteins.

Air drying of the plates was done overnight, 50 µl 0.4 % SRB dye Solution was added to cover the culture surface of the wells and then incubated for 20 minutes at room temperature in the dark. The steps above were repeated up to the wash step (Step 3) using 1 % acetic acid X5 times and air-drying of the plate was thereafter done as in Step 4. 100 µl 10 mM Tris buffer was added to each well. This was mixed by pipetting up & down or gentle agitation to dissolve the dye completely for 10 minutes. Absorbance was measured at 540 nm, with reference to 690 nm is the absorption maximum, with a microplate reader. Mean values and CV from 3 replicate wells were calculated automatically. Curves and statistical analysis were performed using Excel 7.0 software for Windows.

3.16.8. Calculation of results

The percentage cytotoxicity for a given experimental treatment was calculated by using the average absorbance values from experimental cell control. Ensuring that absorbances were blanked with the cell free medium as controls.

% Cytotoxicity= (100 x (Cell Control – Experimental) ÷ (Cell Control)

3.17. Apparatus/Instrument.

(A) Materials for the preparation of test substances.

Beakers, measuring cylinders, glass stirrer.

(B) Materials for the administration of Test substances

Oral intubators syringe and needles.

(C) Materials for sacrificing Animals, collection of blood and Serum, liver samples and bones marrow smear.

Dissecting set, Dissecting board, disposable syringe, disposable needles, precleaned slides, heparinized capillary tubes, cotton wool, sample bottles, Pasteur pipette and centrifuge.

(D) Materials for staining.

Cover glasses, rectangular tray, staining jar, precleaned slides and petri dishes.

(E) Apparatus for scoring of slides.

Nikon Compound microscope and Tally counter.

(F) Apparatus for Liver Function Test.

Micropipette, time, test-tubes, test-tube rack, incubator, spectrophotometer.

(G) Disposables.

Aluminium foil, Centrifugation tubes, Hand Gloves.

(H) Computer-softwares.

MS. PowerPoint Microsoft Corporation, USA, MS Word Microsoft Corporation, USA

Internet Explorer Microsoft Communications, USA.

(I) Other equipments.

Refrigerator and freezer, pH-meter, cuvettes, sterile work bench, Stop watch.

3.18. Preparation of Reagents

1.15 % KCl.

1.15 g of potassium chloride (KCl) was dissolved in distilled water and the volume made up to 100 mL. This was used as a rinsing buffer during the harvesting of liver samples.

10% Buffered Formalin.

10 ml of formalin was added to 90 ml of phosphate buffer saline.

0.4M NaOH

16 g of NaOH was dissolved in distilled water and the volume made up to 1000 mL. It was used for ALT and AST assays.

0.01M Phosphate Buffer (PH 6.8)

0.71 g of disodium hydrogen phosphate (Na_2HPO_4) (BDH Chemicals Ltd Poole England--30132) and 0.68 g of potassium dihydrogen phosphate (KH_2PO_4) (BDH Chemicals Ltd Poole England--29608) was dissolved in water and made up 1000 ml, and then the pH was adjusted to 6.8. This was used to prepare 5 % Giemsa and to rinse the slides while washing.

5 % Giemsa Stain

5 g of Giemsa (Kiran Light Lab. Mumbai, India –G4890) was dissolved in phosphate buffer (pH 6.8) and the volume made up to 100 mL. It was used for micronucleus assay.

0.04 % Colchicine

0.04 g Colchicine was dissolved in distilled water and the volume made up to 100 mL. It was used to inject the rats (1 ml/100 g body weight) two hours prior to the sacrifice to arrest the metaphase stage of cell division.

0.4 % May-Grunwald stains 1

0.4 ml of May-Grunwald stain was dissolved and made up to 100 ml with absolute methanol.

0.4 % May-Grunwald stains 2

1:1 dilution was made using stain 1 and distilled water. They were used in staining of slides.

3.19. Reagents and Kits

Sodium arsenite ($NaAsO_2$; BDH chemicals Ltd poole England) was dissolved in distilled water and administered at a dose of 2.5 mg/kg body weight corresponding to $1/10^{th}$ of the oral LD_{50} of the salt (Preston *et al*., 1987). Cyclophosphamide ($C_7H_{15}Cl_2N_2O_2P.H_2O$) (Cyclocel^{TM} 500) CELON Laboratories limited, India, administered at a dose of 10 mg/kg body weightsKits for aspartate amino transferase (AST), alanine amino transferase (ALT), and gamma glutamyl transferase (γGT) were obtained from Randox Laboratories, UK. All other chemicals and reagents were of analytical grade and were products of Sigma Chemical Co. St. Louis, MO., USA.

3.20. Experimental Animals for the Experiments

3.20.1 Experimental Animals for the Stem bark Experiment

The extract was administered to the experimental animals as described below.

Thirty Male Wistar albino rats (100-150 g) were purchased and housed in the experimental animal house, Department of Biochemistry, University of Ibadan, Nigeria at 29 ± 2^{o}C, maintained on water and standard diet (Ladokun Livestock Feeds Limited, Ibadan, Nigeria) *ad libitum*. The albino rats were allowed to acclimatize for one week prior to the commencement of the study. The rats were randomly distributed into eleven groups of six animals each based on the treatment received. **Group 1:** These served as control and were administered distilled water only. **Group 2:** The rats in this group were treated daily with 400 mg/kg body weight extract. **Group 3:** The rats in this group were orally treated daily with 400 mg/kg extract + sodium arsenite. **Group 4:** The rats in this group were orally treated daily with 300 mg/kg extract + sodium arsenite. **Group 5:** The rats in this group were orally treated daily with 300 mg/kg extract alone. **Group 6:** The rats in this group were administered 2.5 mg/kg body weight sodium arsenite corresponding to 10% of the oral LD_{50} of the salt (Preston *et al.*, 1987).

3.20.2. Experimental Animals for the Pulp Experiment

Forty five Male Wistar albino rats (100-150 g) were purchased and housed in the experimental animal house, Department of Biochemistry, University of Ibadan, Nigeria at 29 ± 2^{o}C, maintained on water and standard diet *ad libitum*. The rats were randomly distributed into eleven groups of six animals each based on the treatment received.

Group A: Animals in this group served as control and were administered distilled water only. **Group B:** The rats in this group were orally treated daily with 2.5 mg/kg body weight sodium arsenite corresponding to 10% of the oral LD_{50} of the salt (Preston *et al.*, 1987) for 14 days. **Group C:** The rats in this group were treated daily with 10 mg/kg body weight (6.25% of the LD_{50}) cyclophosphamide for 14 days via ip. **Group D:** The rats in this group were treated with 200 mg/kg body weight *A.digitata* fruit pulp. **Group E:** The rats in this group were treated with 400 mg/kg body weight plant. **Group F:** The rats in this group were orally treated with 2.5 mg/kg body weight sodium arsenite and 200 mg/kg body weight plant extract. **Group G:** The rats in this group were administered 2.5 mg/kg body weight sodium arsenite and 400 mg/kg body weight plant extract. **Group H:** The rats in this group were administered 10 mg/kg body weight cychlophosphamide for 14 days (ip) and orally treated with 200 mg/kg body weight plant extract. **Group I:** The rats in this group were treated with 10 mg/kg body weight cychlophosphamide for (ip) and orally treated with 400 mg/kg body weight plant extract simultaneously. Unless otherwise stated, all administration was done orally and daily for fourteen (14) days. All the animals were kept in the animal house of our department, and were fed with commercial rat feed and water *ad libitum*. They were kept with 12 hours light/ dark cycle and temperature of 29 $\pm$ 2 ^{o}C. These conditions were maintained throughout the duration of the experiment. Experimental animals were treated and sacrificed following 'Guiding Principles in the Use of Animals' as spelt out in the 'Standard Rules on the Treatment of Experimental Animals' laid down by the University of Ibadan Ethics Committee. They were allowed to acclimatize with the environment for one week and thereafter divided into groups

3.21. Cell cycle analysis:

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. Some anticancer agents exert cytotoxicity by inducing DNA double strand breaks (DSBs) leading to cell cycle arrest and cell death. To investigate the effects of the various fractions of AEFAD on breast and lung cancer cell growth, their effects on the cell cycle profile was therefore tested

Principle:

The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content.

3.21.1. Cell Viability

The cell viability was assessed by trypan blue dye exclusion method (Rosengard and Cochrane, 1983) using hemocytometer. Cells per ml were calculated as follows: Cells per ml = N x 5 x D.F. / volume. Where, N is the total number of cells counted in 5 squares, D.F. is the dilution factor and volume is the depth of the counting chamber (0.1 mm^3 = $1x10^{-4}$). Therefore, cells / ml = N x 5 x D.F. x 10^4. Dilution factor was calculated by dividing the total volume used (20 µl) with the cell suspension volume (10 µl). The percent cell viability was determined as follows: Viable cells x 100 / Total number of cells

3.21.2. Cell Cycle Analysis by Fluorescence-activated Cell Sorting (FACS)

The DNA content and cell cycle distribution of A-549 (Human Lung carcinoma cell line) and MCF-7 (Human Breast cancer cell line were assessed using propidium iodide (PI) staining, the cell lines were collected washed in PBS and fixed in ice- cold 70 % ethanol at -20^{o}C. Fixed cells were then washed and resuspended in staining buffer containing 50µg/ml PI, 0.1% sodium citrate, 0.1 % triton X-100, and 100 µg/ml RNase A. The cell suspensions were incubated at 37^{0}C for 30 minutes and thereafter analyzed by flow cytometer.

3.21.3. Cell cycle Analysis by Propidium Iodide Staining

Principle This is based on univariate analysis of cellular DNA content after staining cell with propidium iodide (PI). Three major phases of the cycle (G1, S and G2/M) and apoptotic cells with fractional DNA content (subG1) are represented in cytofluorohistogram.

Reagents

1.) Propidium iodide (PI) working concentration

30 µl of PI (Sigma-Aldrich, USA) was pipetted from stock (500 µg/ml), into 1.5 ml of 1X PBS to make 10 µg/ml

2.) RNAse stock concentration: 1 mg/ml

1mg RNAse was measured into 1 ml of 1X PBS and the solution was boiled for 1minute to inactivate DNAse.

3.) Triton-X-100 working concentration: 0.2 %

0.02 ml Triton-X-100 was measured into 10 ml 1X PBS

4.) 70% ethanol

7 ml absolute ethanol was measured into 10 ml tube and was made up to mark with distilled water

5mM EDTA 15 mg of EDTA was dissolved in 10 ml of distilled water

Working solution 1 ml of PI (10 µl/ml) was measured into a 10 ml tube, 100 µl of RNAse (1 mg/ml) and 500 µl of triton X-100 were added. The volume was made up to 2 ml with PBS. Final concentration of reagent was therefore: 5 µg /ml PI, 50 µg /ml RNAse and 0.05 % Triton X-100

Procedure: Cells were seeded at 20 x 10^4 cells per well in 6 well plate and were treated correspondingly After treatment, the cells were harvested and washed twice with cooled PBS, and resuspended with 100 µl PBS, vortexed to separate them into single cells after which they were fixed in 1ml of 70 % ethanol for 6 h at 4°C. The fixed cells were washed twice with cooled PBS to remove the excess ethanol and were re-suspended in PBS, RNAse was added at a final concentration of 50 µg/ml and incubated at 37°C for 1 hr. After 1hr, EDTA was added at a final concentration of 5 mM to inactivate the RNAse activity, PI was added to the cell suspension at a final concentration of 5ug/ml and incubated at 25°C for 15 minutes in the dark. The stained cells were diluted with 400 µl of PBS and the cells were analysed by flow cytometer**.**

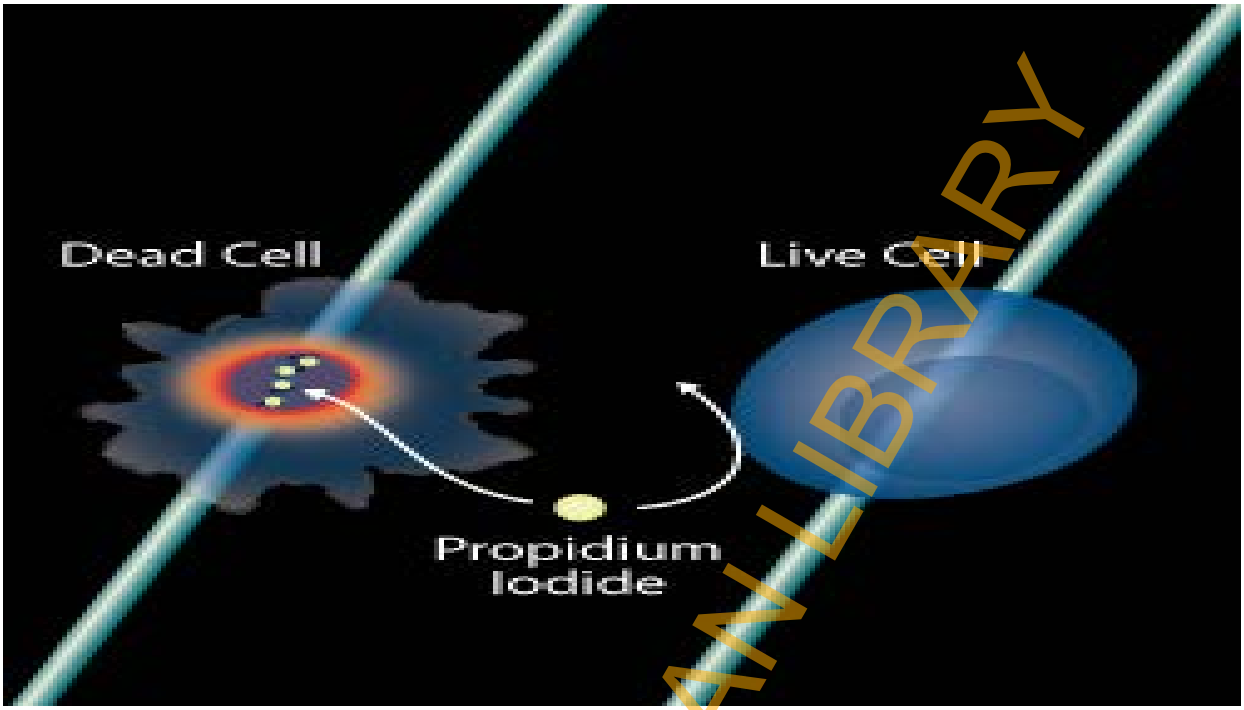


Figure 3.5: Principle of cell viability analysis.

(https://www.rndsystems.com/resources/protocols/flow-cytometry-protocol-analysis-cell-viability-using-propidium-iodide) 05/09/2017

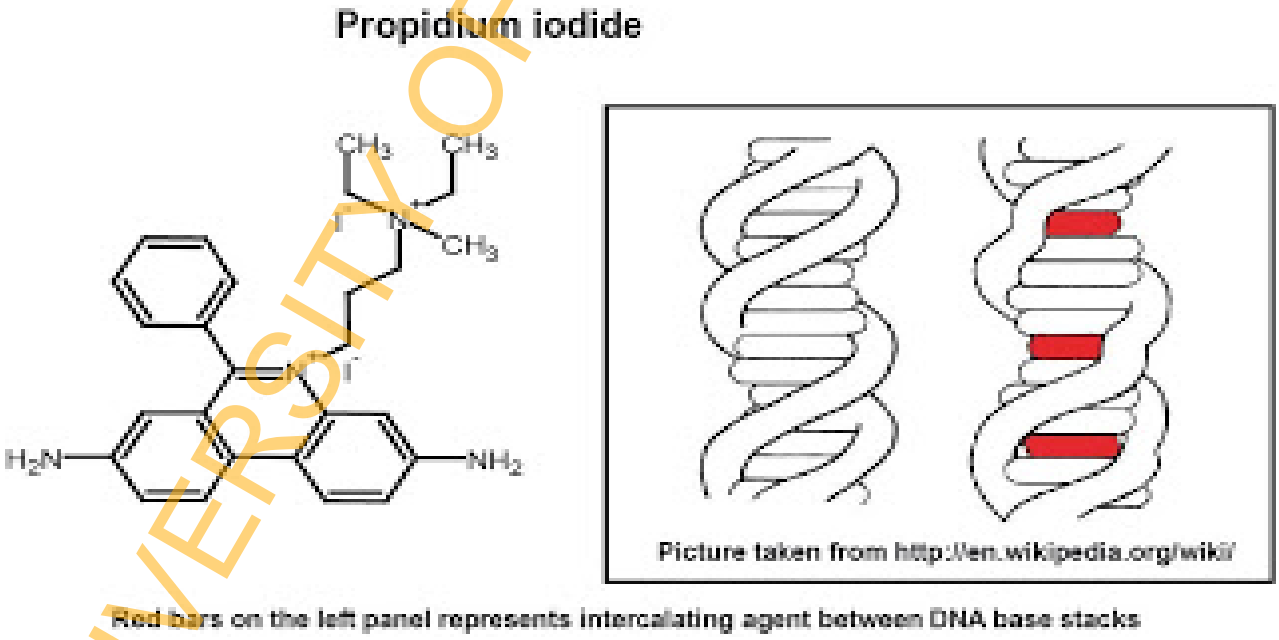


Figure 3.6: Intercalation of DNA by Propidium Iodide.

(https://bio-ggs.blogspot.com.ng/2010/05/) 05/09/2017

3.22. Clonogenic Assay to Test for anticancer agent

Introduction: Clonogenic assay is a useful tool to test whether a given anticancer agent can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells that can often only be determined microscopically.

Principle: The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. A cell that retains its ability to synthesize proteins and DNA and go through one or two mitoses, but is unable to divide and produce a large number of progeny is considered dead. This is very commonly referred to as loss of reproductive integrity or reproductive death and is the end point measured with cells in culture. On the other hand, a cell that is not reproductively dead and has retained the capacity to divide and proliferate indefinitely can produce a large clone or a large colony of cells and is then referred to as "clonogenic." The ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce; this is what is determined by the clonogenic assay

Materials and Reagents

1. Cell culture medium

2. Phosphate buffered saline (PBS)

3. Fetal bovine serum (FBS)

4. Trypsin/ EDTA (Life Technologies, Invitrogen™, catalog number: 25200-056)

5. Crystal violet (Sigma-Aldrich, catalog number: C3886)

6. Methanol (Sigma-Aldrich, catalog number: 34860)

7. Glacial acetic acid (Sigma-Aldrich, catalog number: 320099)

8. Fixation solution

9. Colony fixation solution

10. Crystal violet solution

Equipment

1. Cell culture petri dishes or six-well plates (Thermo Fisher Scientific)

2. Hemocytometer (Hausser Bright-Line) (Thermo Fisher Scientific

3. Stereomicroscope (*e.g.*, Nikon Eclipse, model: TS100)

4. Incubator

Procedure

A. Cell preparation:

1. Cells were cultured according to the requirements

2. The medium was removed, and cells rinsed with 10 ml PBS.

3. 4 ml 0.25 % trypsin was added to the cells and incubated at 37°C for 1-5 min

4. 10 ml medium with 10% FBS was added, and cells were detached by pipetting.

5. Cell count was done using a hemocytometer.

6. Desired seeding concentration was prepared, and then cells were seeded into dishes or 6-well plates.

B. Assay setup:

Cells were plated after the treatment.

a. Cells were harvest after treatment. Up to 50 x 10^4 cells were plated. Serial dilutions with different numbers of cells were prepared.

b. Cells were thereafter incubated in a CO_2 incubator at 37°C for 13 days until cells in control plates have formed colonies with substantially good size (50 cells per colony is the minimum for scoring).

C. Fixation and staining:

1. The medium was removed, and cells rinsed with 10 ml PBS.

2. PBS was removed and 2-3 ml of fixation solution was added and the dishes/plates were left at room temperature (RT) for 5 min.

3. Fixation solution was removed.

4. 0.5 % crystal violet solution was added and incubated at room temperature for 2 h.

5. 10 ml medium with 10 % FBS was added, and the cells detached by pipetting.

6. Crystal violet was carefully removed and the dishes/plates immersed in tap water to rinse off crystal violet.

7. The dishes/plates were thereafter air-dried on a table cloth at room temperature for up to a five days.

D. Data analysis:

1. The number of colonies was counted with an inverted microscope.

2. The stained cells were exposed on the bench at room temperature for about five days before pictures were taken

3. Specialized camera was used to take the picture of the cell culture petri dishes after staining; the petri dishes were prepared in triplicate

Recipes

1. Colony fixation solution

Acetic acid/methanol 1:7 (vol/vol)

2. Crystal violet 0.5% solution

3.23. Western blot analysis

3.23.1. Isolation and purification of proteins from MCF-7 cells for gel electrophoresis and western blotting techniques. The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to

specifically detect its antigen) it is a routine technique for protein analysis. The technique involves:

a) **Electrophoretic Separation of Proteins:** Gel electrophoresis is a technique in which charged molecules, such as protein or DNA, are separated according to physical properties, they are forced through a gel by an electrical current. Proteins are commonly separated using polyacrylamide gel electrophoresis (PAGE) to characterize individual proteins in a complex sample or to examine multiple proteins within a single sample. When combined with Western blotting, PAGE is a powerful analytical tool providing information on the mass, charge, purity or presence of a protein.

b) **Blocking Nonspecific Sites:** The membrane supports used in Western blotting have a high affinity for proteins. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block free sites on a membrane. The blocking buffer should improve the sensitivity of the assay by reducing background interference and improving the signal to noise ratio.

c) **Primary and secondary antibodies:** Most commonly, the transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic precipitate on the membrane for colorimetric detection. The antibodies used were detected

with the aid of super signal detection solution in the dark room. The intensity of the signal correlates with the abundance of the antigen on the membrane.

3.23.2. Protein Harvesting Protocol for Western Blotting

MCF-7 cells were treated with MESBAD in 6 cm dishes; the cells were trypsinised, spinned down and washed with PBS before cell lysis. The heating block was set at 100°C, the medium containing the dead and dying cells was removed from the dish and placed in 10 ml tube using the automatic pipette. 750 µl Trypsin/EDTA was added to 6 cm dish, swirled and tilted side-to-side. The trypsin/EDTA was thereafter removed and added to 10 ml tube, another 750 µl Trypsin/EDTA was added to 6cm dish, swirled, tilt side-to-side and left for cells to detach (checked under microscope), after cells have detached, 1 ml of dead-cell containing medium from 10 ml tube was added to inactivate trypsin in the 6cm dish containing trypsin/EDTA and the cells were washed down. Trituration was done and contents added to 10 ml tube, this was spinned down at 2000 rpm for 2 minutes and medium removed by sucking off, leaving a bit to resuspend pellet. 500 µl of 1% PBS was added to the 10 ml tube and pellet resuspended gently, the resuspended pellet was placed into an eppendorff tube (on ice) and spinned down for ±1minute. Medium was removed as much as possible, being careful not to suck off pellet. Another 500 µl 1% PBS was added, resuspended, spinned down for ±1 minute and medium removed as much as possible.

Volume of 2X boiling blue was added according to the size of pellet, boiling blue was added and placed in heating block at 100°C to boil for 5-10 minutes (depending on volume).

Eppendorff tube was allowed to cool at room temperature (condensation appeared on lid), the eppendorff tube was centrifuged briefly and stored at -20°C until it was used for western blotting.

3.23.3. SDS-PAGE Preparation

The reagents needed were placed on the working bench; the front plates and back plates were washed with detergent and allowed to dry on the rack. They were later sprayed with ethanol and allowed to dry completely. The SDS-PAGE apparatus was set up: Front plate and back plate into clamp and into stand clip. Resolving and stacking gels were prepared according to acrylamide gel percentage and gels were poured accordingly.

Table 3.3: Preparation of Resolving Gel

Gel % acryamide	**10 %**		**15 %**	
Total vol.	10 ml	20 ml	10 ml	20 ml
H_2O	4.8 ml	9.6 ml	3.5 ml	7 ml
40 % acrylamide	2.5 ml	5 ml	3.8 ml	7.6 ml
1.5M Tris (pH 8.8)	2.5 ml	5.0 ml	2.5 ml	5.0 ml
10 % SDS	100 µl	200 µl	100 µl	200 µl
10 % APS	100 µl	200 µl	100 µl	200 µl
TEMED	10 µl	20 µl	10 µl	20 µl

Table 3.4: Preparation of Stacking Gel

Gel % acry.	**5 %**	
Total vol.	8 ml	4 ml
H_2O	5.8 ml	2.75 ml
40 % acrylamide	1 ml	650 µl
1.5M Tris (pH 6.8)	1ml	500 µl
10 % SDS	80 µl	40 µl
10 % APS	80 µl	40 µl
TEMED	8 µl	4 µl

Resolving Gel (first)

A pipette was set up to right amounts to add APS and TEMED, autopipetter was also set up for pouring gel as well as 1ml pipette for adding isopropanol, TEMED was taken and left to the side. APS was added to the glass bottle containing other resolving gel reagents and mixed gently, TEMED was added and mixed by inverting glass jar a few times, using the autopipetter the gel was poured up to the bottom of the green bar, isopropanol was quickly added on top of resolving gel up to the top edge of the front plate, and left for 10-15 minutes to set. Isopropanol was poured off and rinsed gently with water. Filter paper was thereafter used to dab off water.

Stacking Gel (second)

Combs were prepared, sprayed with ethanol and left to dry, same procedure as resolving gel preparation was followed using a P1000 pipette instead of the autopipetter, gel was poured up to rim of front plate and combs were inserted at an angle with front of comb facing the front plate. Gel was allowed to set for a minimum of 10-15 minutes and used immediately or stored over night in running buffer.

3.23.4. Running SDS-PAGE

The running apparatus was set up**:** Gels were placed in cassettes with front plates facing inwards. Cassette containing gels was placed in tank with electrodes in right place black to black and red to red. Dam was filled until overflowing with running buffer. Combs were taken out carefullly and straight up, while buffer was poured in to dam from side-to-side to wash out and tank was filled up to 1/2 with running buffer. Samples were loaded appropriately in preplanned order (one gel at a time) and 5 µl marker was added as a guide while 4-5 µl boiling blue was added in empty lanes. Tank lid was placed firmly and correctly (black to black and red to red) and plugged into power and voltage set appropriately for complete separation.

3.23.5. Transfer to Nitocellulose Membrane

Nitrocellulose membrane was prepared accordingly, by cutting top left corner, bottom and top labeled and covering was remobed with tweezers and placed in container with "old" transfer buffer. Baking dish was filled with transfer buffer; holders were set up with black side in the dish and clear side open up. Sponges were soaked and placed on clear and one on black holder surfaces. A piece of whatman paper was also soaked and placed on top of sponge on black side, running cassette was removed with gel from electrophoresis tank, dam running buffer was poured out into the tank. Using the separator the back plate was pushed down to lift the front plate and each side separated to loosen the gel, Using fingers, the edges of gel were placed on whatman paper with marker on the right hand side and cover with transfer buffer to prevent from drying out. Using tweezers the nitrocellulose membrane was placed over the gel with cut corners on the right hand side, the second piece of soaked whatman paper was placed on top of the nitrocellulose membrane and gently across with finger to smoothen out any bubble. The soaked sponge was placed on top of whatman and holder and holder latch were closed.

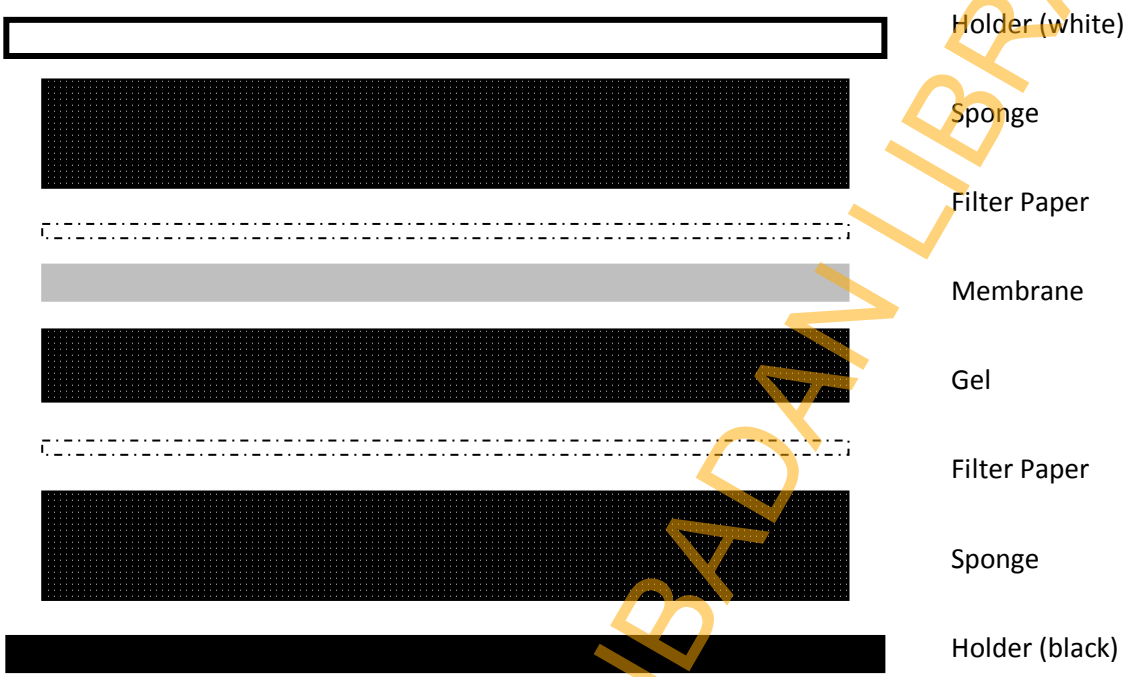


Figure 3.7: Nitrocellulose Membrane Transfer Setup

The holder was placed in cassette (BLACK TO BLACK), and placed in transfer tank (RED TO RED & BLACK TO BLACK ELECTRODES). Tank was filled up to half with chilled freshly made transfer buffer with ice pack in tank, and lid connected (matching correct electrodes) and connected to power pack, and voltage was set to 100 V and allowed to run for 1hr (thin gel) and 1hr 30 minutes (thick gel), it was checked for bubbles to see transfer taking place. Transfer tank was placed in ice box with ice packed at bottom and sides.

3.23.6. Detection

Holders were removed from transfer assembly and nitrocellulose membrane was placed correctly into container with PBS/T - 2 x brief PBS/T washes, Ponso S stain was added to visualise bands and to determine where to cut membrane, and then rinsed with distilled water until single bands were visualized. Membrane was placed on whatmann paper and covered with some distilled water, bands of interest were separated and place in labeled containers containing PBS/T and rinsed twice on shaker until Ponso S stain was removed. Incubation on shaker was for 1hr at room temperature with blocking buffer (primary antibody specific) - PBS/T + 5 % milk or TBS/T + 5% milk. Incubation was done overnight with primary antibody on shaker in 4°C fridge. The following day, membrane was washed with PBS/T or TBS/T**,** 2 X quick washes**,** 2 X 5 minutes washes (shaker)**,** 2 X 10 minutes washes (shaker)**,** thereafter there was Incubation on shaker for 1 hour at room temperature with secondary antibody**.** Washes were repeated as above, keeping membranes in last wash until visualisation in darkroom.

3.23.7. Visualizing Membrane

Dark Room: Tray was filled up with water**,** developer and fixer were put inside separate trays, ensuring solutions were clear colour, autorad was taken out and cut accordingly. Detection reagents were mixed in a 50:50 ratio, into small glass bottle (e.g. 800 µl each), membrane was placed in shallow black container, using pipette, 1ml of the detection reagent was placed over membrane and allowed to wait for 1-2 minutes before checking for signal. Membrane was transferred to transparent pocket with flash on it, the transparent pocket was

closed from one side and smoothen out to remove any bubble. Transparent pocket was thereafter placed into cassette.

Exposure: A piece of autorad was placed over membrane (outside of pocket) and cassette closed and exposed for 1 or 2 minutes, the cassette was opened and autorad was immediately immersed in the developer in the tray. As soon as bands become visible, film was rinsed in water and place into fixer for at least 2 minutes. Autorads was rinsed under running water, rubbing with fingers and left on the stand to dry. After drying, autorads were labeled by matching to flash on transparency pocket and relevant bands were labelled and named. The primary antibody was indicated with dilution (e.g. 1:1000, 1:500), blots were scanned and stored in a systematic manner. Membranes were rinsed in PBS/T and stored in a well labeled whatman envelope

3.24. Gas chromatography-mass spectrometry analysis

Principle

This is based on the separation of sample into relative 'pure' eluents and subsequently followed by the combination of ionization, fragmentation and separation processes which results in characteristic 'mass spectra.

Procedure

The analysis was carried out on a Shimadzu GCMS-QP2010 PLUS Gas Chromatography/ Mass Spectrometry (GC-MS) Japan using helium gas and 5 % diphenyl/95 % dimethyl

polysiloxane as mobile and stationary phases, respectively. The oven and injection temperature were 100^0C and 250^0C. The injection mode was split and helium at 1.75 mL/min was used as carrier gas. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning the 50–700 *m/z* range. The source and interphase temperature were 200°C and 250°C, respectively.

Statistical Analysis

The results were expressed as mean ± Standard deviation. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 10.0.1. P-values less than 0.05 were considered statistically significant for differences in mean and Sidak's *post hoc* test using Prism version 6 (GraphPad Software Inc.) USA. P-values less than 0.05 were considered statistically significant for differences in mean.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

Experiment 1: Phytochemical screening of Methanol Extract of the stem Bark of *Adansonia digitata* (MESBAD) and characterization of the aqueous Extract of the Fruit pulp of *Adansonia digitata* (AEFAD).

Introduction: A wide array of plants used in ethno medicine are found to contain various phytochemicals, plants have been used for the treatment of various diseases since the existence of man (Suriyavathana *et al.,* 2010). In Africa and some other developing countries, medicinal plants are employed as the major sources of medicine. The potency of medicinal plants have been linked to the inherent phytochemicals, these include: Alkaloids, flavonoids, phenolic compounds, tannins etc (Hill, 1952). Plants have also displayed various abilities to scavenge free radicals; this can be one of the major reasons for their use as medicinal agents particularly against diseases resulting from oxidative stress. *Adansonia digitata* is a plant that has also displayed some notable medicinal properties. The aim of this experiment therefore, was to determine the qualitative and quantitative phytochemicals present in MESBAD and to determine the free radical scavenging abilities of AEFAD and its fractions *in vitro* and also to carry out partial characterization of AEFAD to determine the inherent compounds

Procedure: MESBAD was subjected to phytochemical screening, while AEFAD was subjected to *in vitro* antioxidant determinations as well as gas chromatography/mass

spectrometry (GC/MS) analyses to determine the compounds present, following the standard procedure.

Results: The residual yield of the solvent fractions of aqueous extract of the fruit pulp of *Adansonia digitata* is shown in table 4.2. Other results of are shown in tables 4.1, 4.3, 4.4 and figures 4.1. Tanins, saponins, alkaloids, terpenoids, flavonoids, oxalates, total carotenoids, anthocyanins, cyanogenic glycosides, steroids, protease inhibitors and total polyphenols are present in MESBAD, alkaloid is relatively abundant among the identified phytochemicals 330.00±0.00 mg/100g (table 4.1) while saponins, flavonoids and total polyphenols are significantly high 153.33±2.89, 121.67±2.89 and 121.67±2.89 respectively, (table 4.1). AEFAD and its fractions revealed potent free radical scavenging activities using the DPPH, ABTS, reducing power and nitrite scavenging assays. N-butanol fraction had the highest reducing power (1.6±0.3) and ABTS scavenging capacities (78.0±2.4%) among all the fractions tested (table 4.3 and figure 4.1). The GC/MS analysis of AEFAD clearly revealed that it contains seven bioactive chemical compounds namely: Pentadecanoic-acid, Hexadecanoic-Acid, 11-Octadecenoic-acid, Octadecanoic-acid, Oleic-acid, Nonadecanoic-acid and 3,11-Tetradecadiene-1-ol (table 4.4).

Conclusion: MESBAD contain important phytochemicals, which account for the medicinal properties. The free radical scavenging activities of AEFAD and its fractions is a proof that they are capable of modulating oxidative stress related diseases. Out of the seven compounds present in AEFAD, hexadecanoic acid is known to possess antitumor effect; this may be responsible for anticancer activity.

Table 4.1: The concentration of some phytochemicals in methanol extract of the stem bark of *Adansonia digitata*

S/N	PARAMETER	QUALITATIVE RESULTS	QUANTITATIVE RESULTS MEAN+S.D
1	Tannins (mg/100g)	+	43.33±2.89
2	Saponins (mg/100g)	+	153.33±2.89*
3	Alkaloids (mg/100g)	+	330.00±0.00**
4	Terpenoids (mg/100g)	+	10.00±0.00
5	Flavonoids (mg/100g)	+	121.67±2.89*
6	Oxalates (mg/100g)	+	23.33±2.89
7	Total Carotenoids (mg/100g)	+	83.33±2.89
8	Anthocyanins (mg/100g)	+	7.83±0.00
9	Cyanogenic glycosides (mg/100g)	+	0.02±0.00
10	Steroids (mg/100g)	+	5.00±0.00
11	Protease inhibitors (mg/100g)	+	1.00±0.00
12	Total Polyphenols (GAE/100g)	+	121.67±2.89*

Each value represents mean ± S.D of 3 determinations * indicate a significantly high amount

** indicate abundance

Table 4.2 The residual yield of the solvent fractions of aqueous extract of the fruit pulp of *Adansonia digitata*

Solvent Fractions	**Residue yield (grams)**
n-hexane fraction (NHF)	0.28
Chloroform fraction (CRF)	0.44
Ethyl acetate fraction (EAF)	0.35
n-butanol fraction (NBF)	2.69
Aqueous fraction (AQF)	24.58

Table 4.3 Total antioxidant capacities of the crude and various solvent fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD).

Solvent Fraction	DPPH (%) (100 µg/ml)	ABTS (%) (100 µg/ml)	Nitrite-scavenging capacity (%) (100 µg/ml)
CAQ	72.86 ±2.24	63.55±3.85	53.04±2.30
NHF	35.33 ±2.87	33.28±2.64	29.33±1.76
CRF	44.02 ±4.73	55.46±7.49	40.25±2.97
EAF	69.41±1.47	62.03±2.49	59.12±3.18
NBF	70.95±0.85	78.12±2.36	47.82±2.54
AQF	60.93±1.13	42.58±1.03	56.18±3.39
STD	70.95±1.38	75.08±1.27	76.01±4.67

Each value represents 3 determinations at concentration of 100µg/ml test samples. Values are expressed as % inhibition of the DPPH radical, ABTS radical or Nitrite scavenging capacity. CAQ = Crude aqueous extract, NHF= n-hexane fraction, CRF= Chloroform fraction, EAF= Ethyl acetate fraction, NBF= n-butanol fraction, AQF= Aqueous fraction, STD = Standard (ascorbic acid)

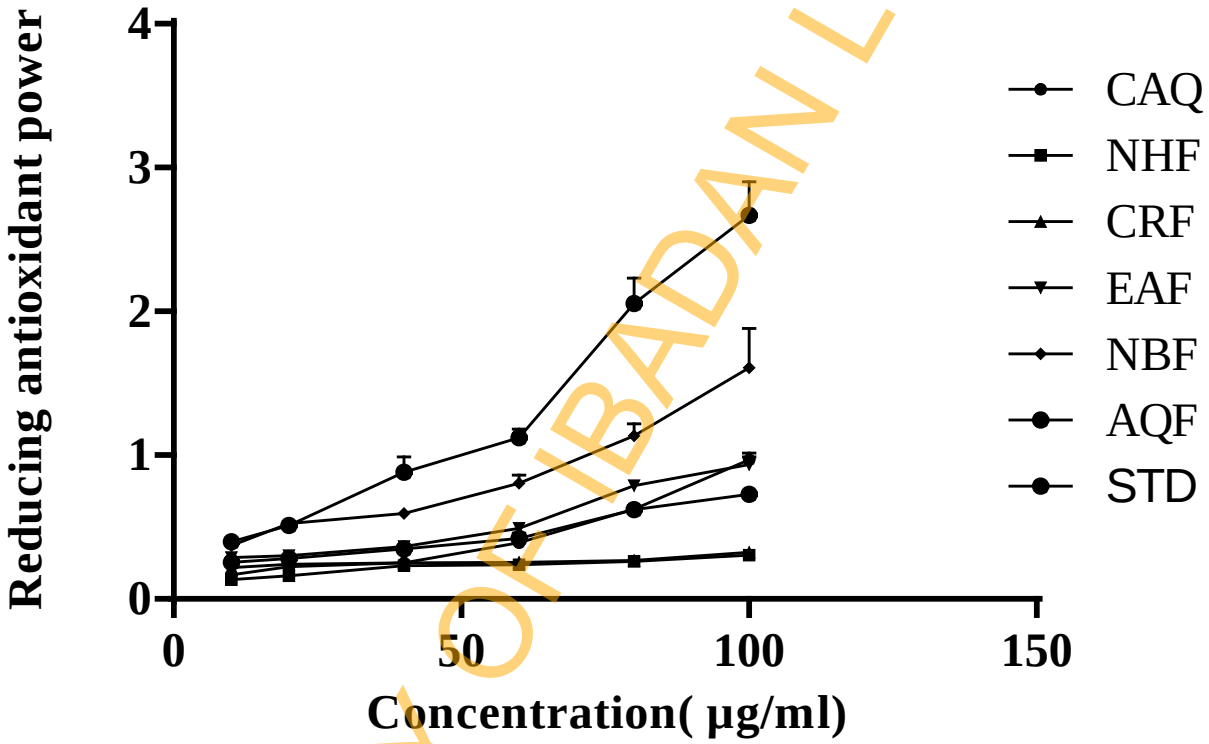


Figure 4.1 Reducing antioxidant power of the crude and various solvent fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD). Each value represents mean ± S.D of 3 determinations. CAQ= Crude aqueous extract, NHF= n-hexane fraction, CRF= Chloroform fraction, EAF= Ethyl acetate fraction, NBF= n-butanol fraction, AQF= Aqueous fraction, STD= Standard (ascorbic acid).

Table 4.4. The GC/MS Analysis of the bioactive compounds in Aqueous Extract of *the* fruit pulp of *Adansonia digitata* (AEFAD).

S/N	Retention time	Compound name	Molecular formular	Molecular weight	Peak area (%)
1	17.30	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	96.17
2	18.71	Hexadecanoic Acid	$C_{16}H_{32}O_2$	256	39.45
3	20.30	11-Octadecenoic acid	$C_{19}H_{36}O_2$	296	51.45
4	20.60	Octadecanoic acid	$C_{19}H_{38}O_2$	298	92.56
5	21.50	Oleic acid	$C_{18}H_{34}O_2$	282	35.98
6	21.7	Nonadecanoic acid	$C_{19}H_{38}O_2$	298	34.40
7	24.70	3,11-Tetradecadiene-1-ol	$C_{14}H_{26}O$	210	39.60

Experiment 2: Hepatoprotective and anticlastogenic effects of AEFAD on Sodium Arsenite and Cyclophosphamide-induced toxicities in Wistar rats

Introduction:

Many compounds which occur naturally in medicinal plants have been proven to have abilities to mitigate several chemically-induced hepatotoxicity and clastogenicity. Sodium arsenite (SA) is a known hepatotoxic and clastogenic agent; it has been listed to be a probable human carcinogen (IARC, 1994). Cyclophosphamide is an anticancer agent, but the cytotoxicity on normal cells is the major limitation of the drug (Hales, 1982). Due to unavoidable exposure of human to SA especially through underground water, and also the toxicity experienced by cancer patients undergoing treatment with cyclophosphamide, researches that address amelioration of their toxicities and the understanding of their mechanism of toxicity become inevitable. Reports indicate that following exposure of rats to SA, the biomarkers of hepatotoxicity and clastogenicity were significantly increased (Mallick *et al*, 2003; Odunola *et al.*, 2011; Gbadegesin *et al.*, 2014; Gbadegesin *et al.*, 2009), suggesting that SA exposure can increase the risk of hepatotoxicity and clastogenicity. Also, previous reports on cyclophosphamide indicated its clastogenicity and hepatotoxicity (Singh *et al.*, 2015; Chakraborty *et al.*, 2009; Zarei and Shivanandappa, 2013). From previous experiments, AEFAD was shown to possess some bioactive components that may be responsible for the amelioration of hepatotoxicty and clastogenicity. However, it is not established whether AEFAD could ameliorate SA and CP induced hepatotoxicity and clastogenicity. The aim of this experiment therefore, was to

investigate the possible ameliorative effects of AEFAD on SA and CP-induced hepatotoxicity and clastogenicity in male rats.

Procedure:

45 male rats (Wistar Stain) were used for this experiment. The animals were divided into nine groups. Group I served as control and animals in group II were administered 2.5mg/kg body weight of sodium arsenite (SA) corresponding to 10% of the oral LD_{50} of the salt (Preston *et al.*, 1987). Rats in group III received 10 mg/kg body weight (6.25% of the LD_{50}) cyclophosphamide, while rats in group IV received 200 mg/kg body weight AEFAD, group V received 400 mg/kg body weight AEFAD, group 6 got 2.5 mg/kg body weight sodium arsenite and 200 mg/kg body weight AEFAD, group 7 received 2.5 mg/kg body weight sodium arsenite and 400 mg/kg body weight AEFAD. Group 8 got 10 mg/kg body weight cyclophosphamide and 200 mg/kg body weight AEFAD, while group 9 received 10 mg/kg body weight cyclophosphamide and 400 mg/kg body weight AEFAD simultaneously. All treatments were for 14 days, SA and AEFAD were administered orally while cyclophosphamide was administered intraperitoneally.

All animals were fed with normal rat diet and water *ad libitum.* At the end of the experiment (24 hours after the last administration), animals were sacrificed by cervical dislocation and dissected. Blood, liver and femur samples were obtained for hematological analysis, serum enzyme determinations, liver histology, hepatocellular proliferation, lipid peroxidation and micronucleus induction assay, as earlier described.

Result: Administration of SA resulted in significant (P <0.05) increase in the mean serum AST and ALT activities (figures 4.2 a and b) as compared with the negative control group.

Also there was over fourfold increase in the mean γGT activities in the group of animals treated with SA only as compared with the observed mean γGT value in the negative control group (figure 4.2 c). Administration of CP at 10 mg/kg body weight also resulted in significant increase in the mean serum AST and ALT activities, and over fivefold increase in the observed mean serum γGT activities as compared with the observation made in the negative control groups (figures 4.2 a,b and c). Simultaneous treatment with AEFAD during exposure to SA or CP resulted in reduction in the activities of AST and ALT and a significant reduction in the mean γGT activities as compared with the respective groups treated with each of the two toxins alone (figures 4.2 a,b and c). The results of histopathology examinations also support the trends of the liver function enzyme results (plate 4.1). The sample slides for scoring micronucleated polychromatic erythrocytes are shown in figure 4.3. Number of micronucleated polychromatic erythrocytes (nMPCEs) per 1000 PCEs scored in the bone marrow (figure 4.4) is significantly ($p<0.05$) higher in the groups of rats administered SA or CP (Groups B and C) compared with the nMPCEs scored in the control (Group A). The increase in the nMPCEs was reduced close to 80% in the group administered both SA and the 200 mg/kg body weight AEFAD and reduced close to 50% in the group administered both CP and 200 mg body weight AEFAD (figure 4.4) In the groups treated with AEFAD alone, there was no significant ($P <0.05$) increase in the nMPCEs when compared with the negative control (figure 4.4). Lipid peroxidation level increased significantly ($P <0.05$) in the group or rats treated with SA alone or CP alone when compared with the negative control (figure 4.5). There was also increase in malondialdehyde production after SA treatment. The malondialdehyde level observed in the groups treated simultaneously with extract and CP reduced significantly ($P <0.05$) and also

extract and SA reduced significantly (P <0.05) when compared with the toxicants groups (figure 4.5). The result of hepatocellular proliferation indicated that SA and CP intoxicated groups significantly (*P* <0.05) induced increase in hepatocellular proliferation (figure 4.6), when compared with the negative control Group (A). The group that received 200 mg/kg body weight AEFAD alone has a value almost similar to that of the negative control. There was a slight increase in hepatocellular proliferation in group E which received 400 mg/kg body weight AEFAD but not significant (*P* <0.05) when compared with the intoxicated groups. Groups which received AEFAD and toxicants all showed reduction in the mean rate number of hepatocellular proliferation when compared with the intoxicated groups (figure 4.6).

The following haematological parameters: Total leukocytes (WBC), hemoglobin (Hb) content, hematocrits (PCV), lymphocytes and Neutrophils, were tested in the treated and control groups of experimental rats. Exposure to CP caused a significant (*P* <0.05) reduction in PCV and (fugure 4.7a). There was also a significant (*P* <0.05) increase in the WBC count of the SA only treated group as compared with control, which was not restored even with the administration of the extract (figure 4.7b).

Conclusion Findings from this study therefore reaffirmed the hepatoxicity and clastogenicity of SA and CP and revealed that AEFAD can ameliorate these toxicities in rats. Moderate consumption of AEFAD may play an important role in human hepatoprotection and resistance to liver damage. AEFAD supplementation may possibly influence the response to chemotherapy and ameliorate/ reduce development of adverse side effects that result from the treatment of cancer with CP in patients.

a.

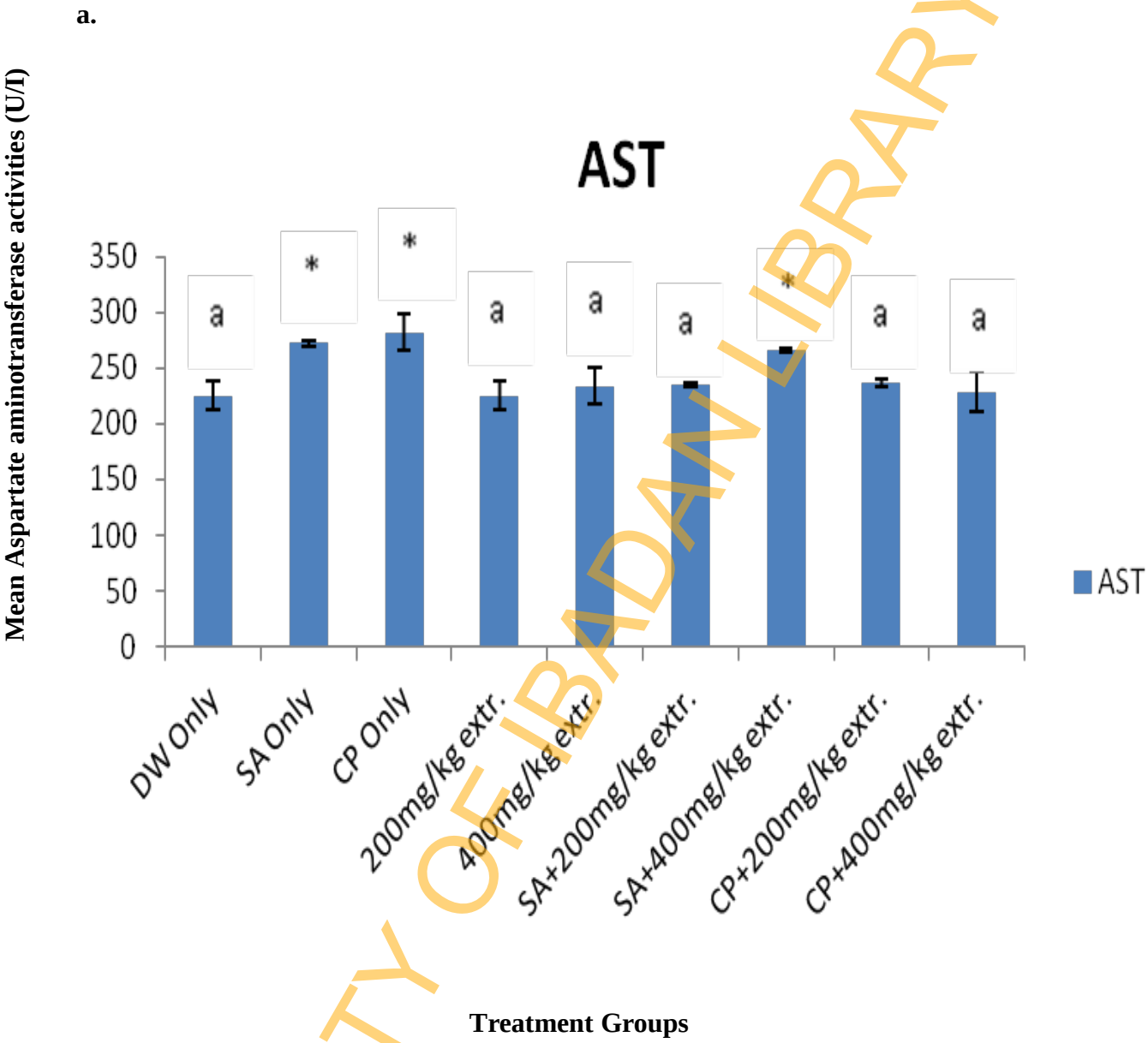


Figure 4.2 a : Levels of aspartate aminotransferase (AST) in sera of rats treated with aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD), sodium arsenite (SA) or cyclophosphamide (CP). Data are expressed as mean ± sd. (n=5): SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups.

b

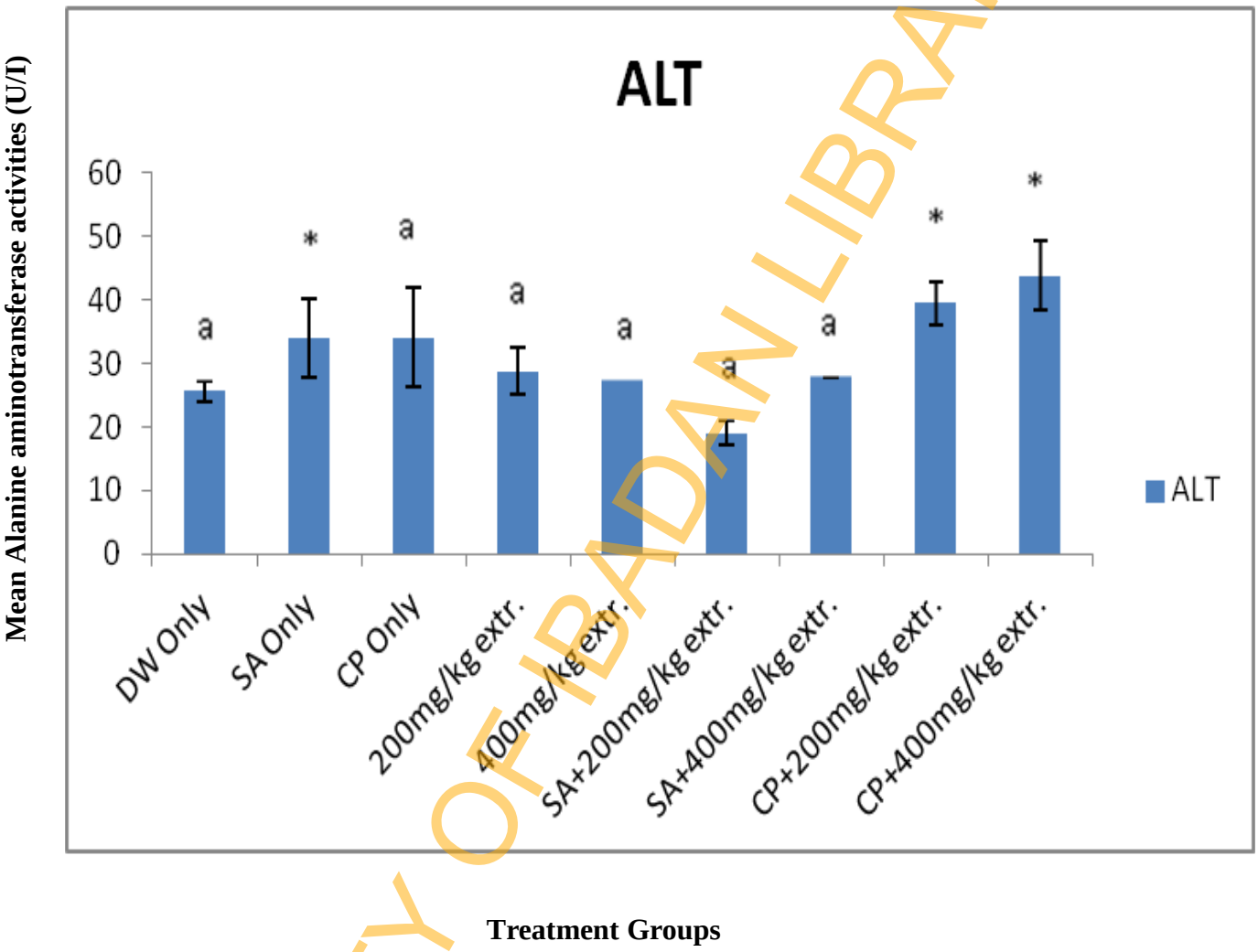


Figure 4.2 b : Levels of alanine aminotransferase (ALT) in sera of rats treated with aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD), sodium arsenite (SA) or cyclophosphamide (CP). Data are expressed as mean ± sd. (n=5): SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups.

C

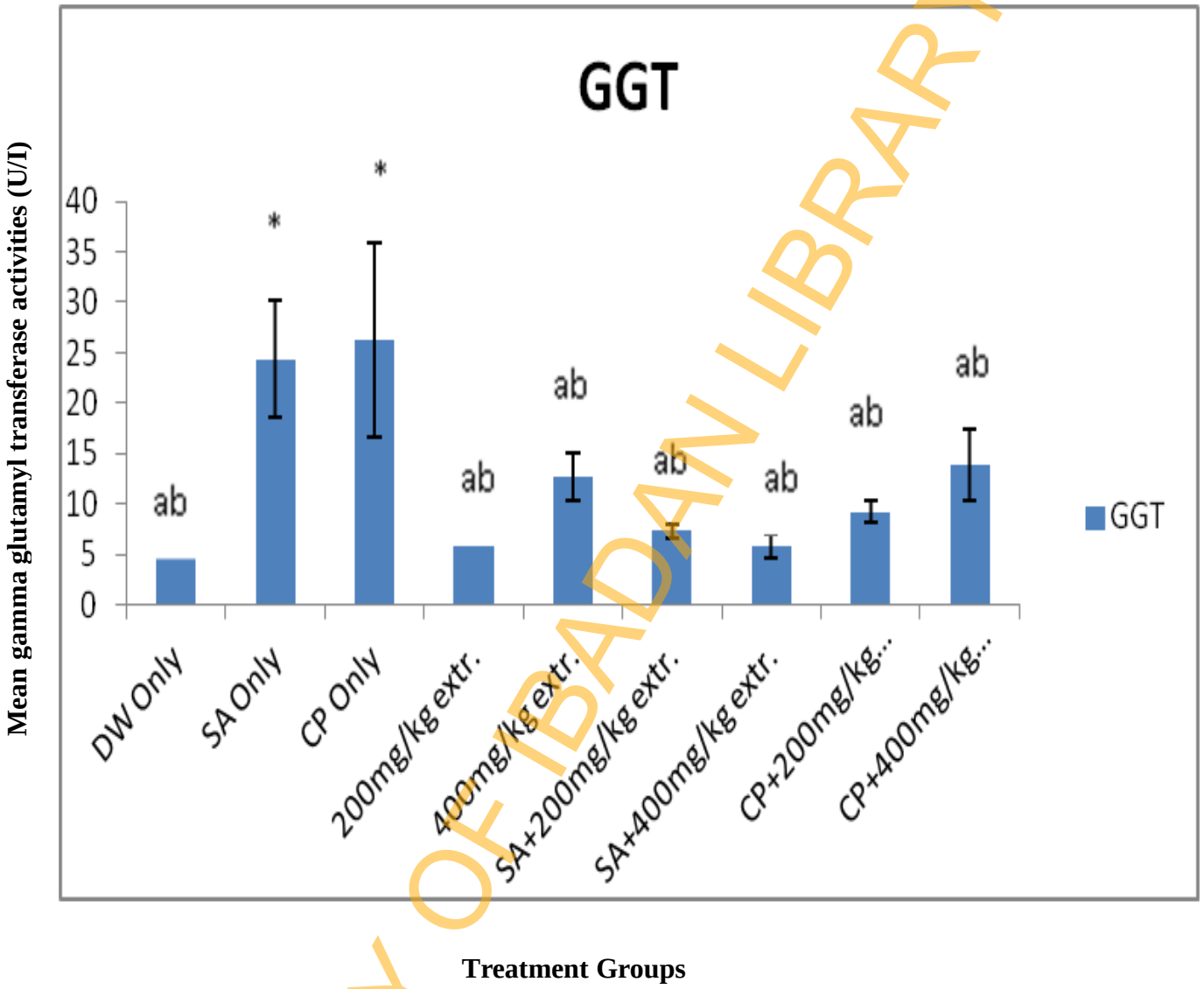


Figure 4.2 c: Levels of gamma glutamyltransferase (GGT) in sera of rats treated with aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD), sodium arsenite (SA) or cyclophosphamide (CP). Data are expressed as mean ± sd. (n=5): SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups. [b] mean difference is significant ($p< 0.05$) when compared with CP only group.

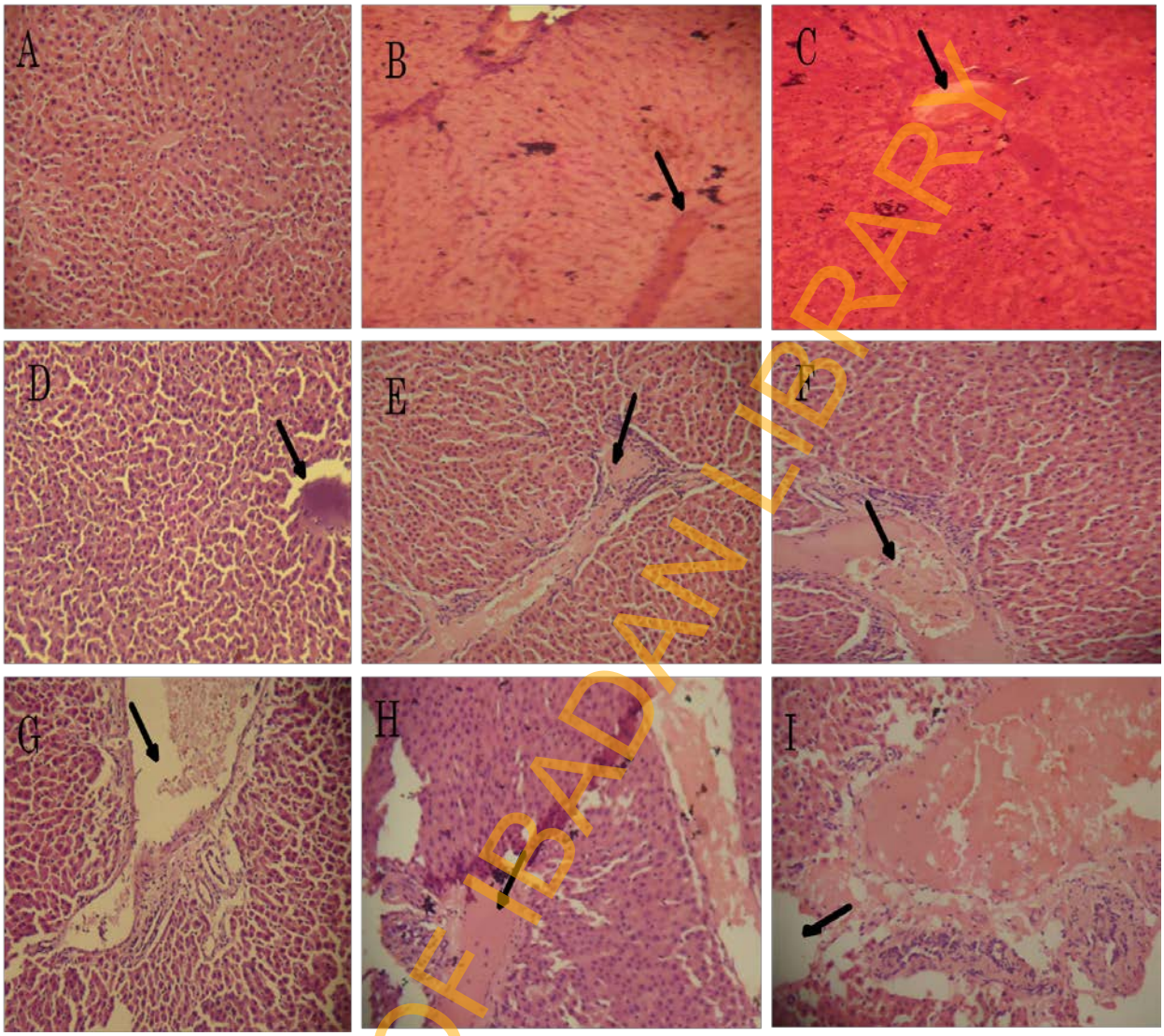
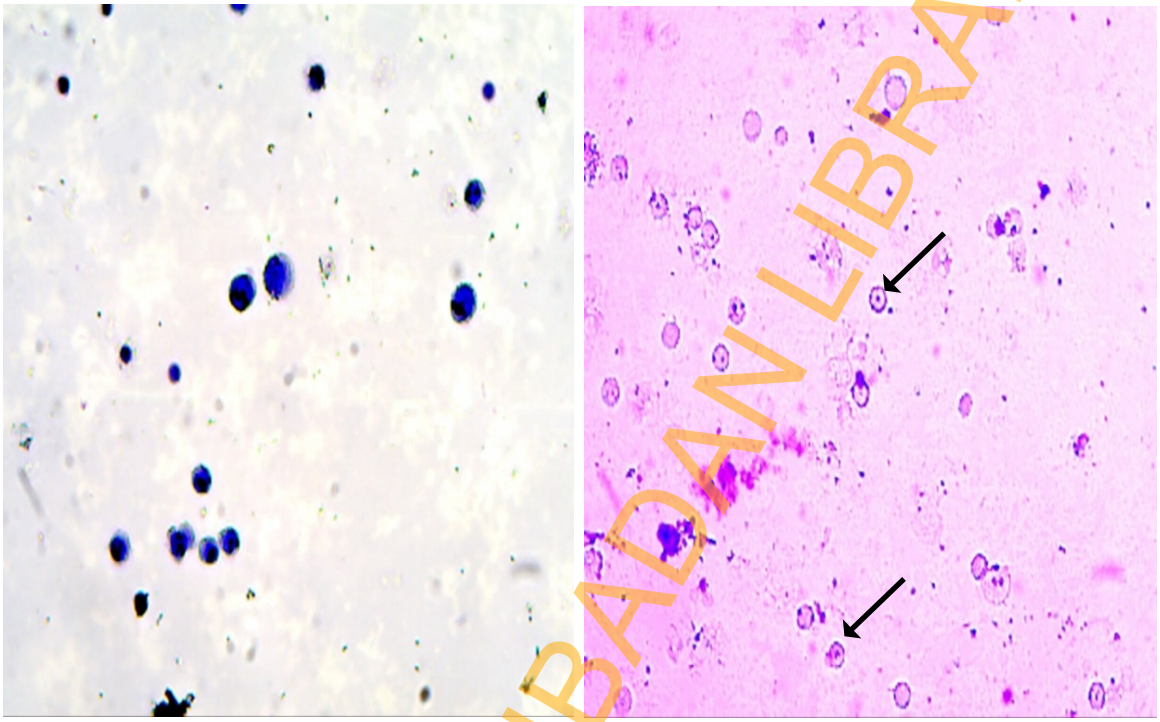


Plate 4.1: Photomicrograph of Limited Histopathology Examination (x400) of liver of rats for the pulp experiment. Negative control (A), sodium arsenite SA only (B), cyclophosphamide only CPA (C), 200mg/kg extract only (D), 400mg/kg extract (E), 2.5mg/kg SA+200mg/kg extract (F), 2.5mg/kg SA+400mg/kg extract (G), 10mg/kg CPA+200mg/kg extract (H), 10mg/kg CPA+400mg/kg extract (I). Arrows indicate the sites of lesions

A No visible lesion, **B** There is diffuse portal and sinusoidal congestion, **C** There is diffuse portal and sinusoidal congestion, **D** There is very mild portal congestion, **E** There is cellular infiltration by mononuclear cells, **F** There is a moderate portal and central venous congestion. There is also a mild periportal cellular infiltration by mononuclear cells **G** There is severe portal congestion, with moderate periportal hepatic necrosis, mild fibroplasia and cellular infiltration by mononuclear cells, **H** There is moderate portal congestion, and mild periportal hepatic necrosis and cellular infiltration by mononuclear cells, **I** There is moderate portal congestion, and mild periportal hepatic necrosis and cellular infiltration by mononuclear cells.



Control slide **Micronucleated slide**

Figure 4.3 Slides for micronucleus induction assay (Mag. * 40). Arrows indicate micronucleated polychromatic erythrocytes

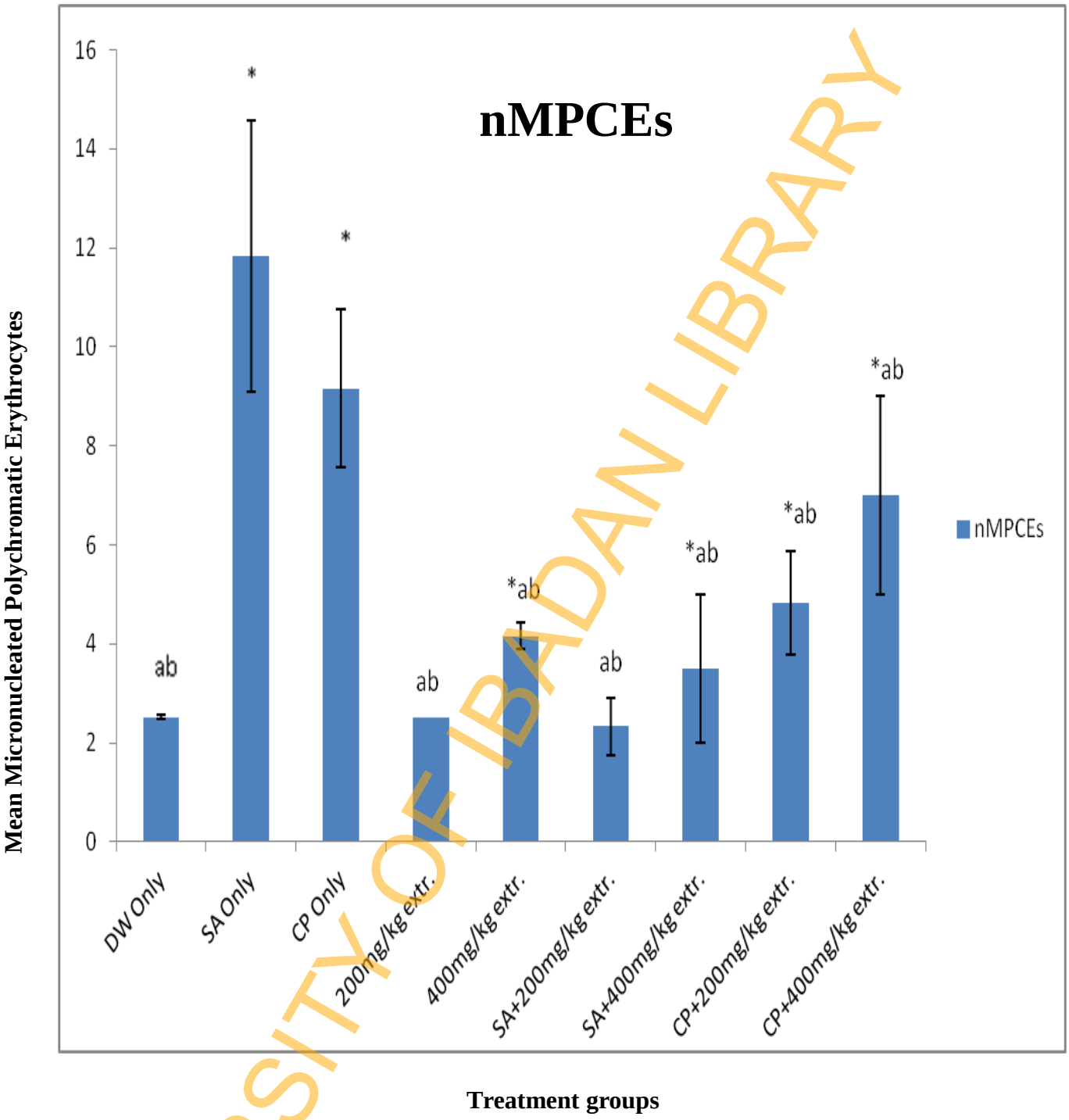


Figure 4.4: Frequency of induction of micronucleated polychromatic erythrocytes (mPCEs) in rat bone marrow cells after treatment with *AEFAD*, sodium arsenite (SA) or cyclophosphamide (CP). Data are expressed as mean ± sd. (n=5): SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups. [b] mean difference is significant ($p< 0.05$) when compared with CP only group.

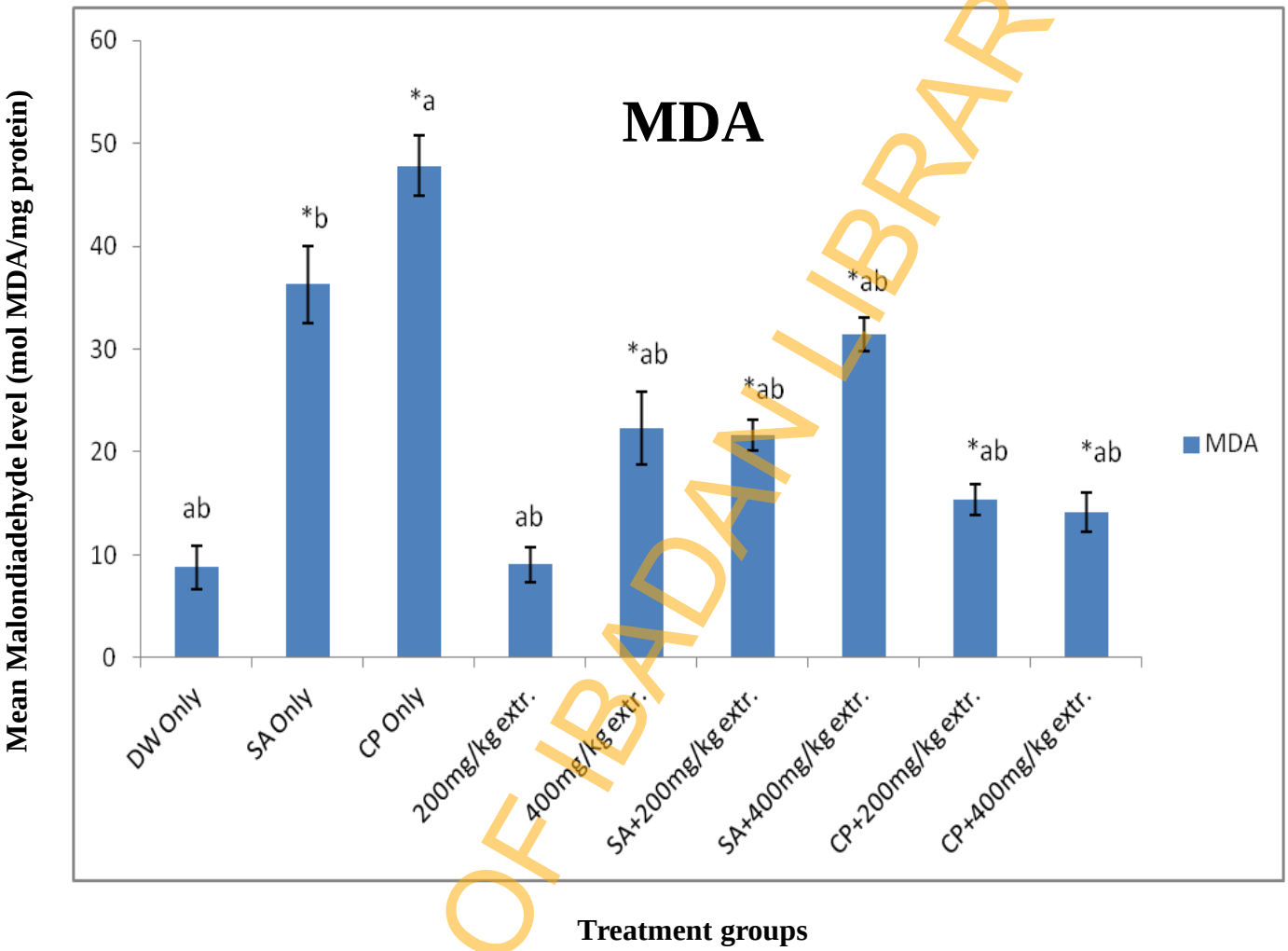


Figure 4.5: Effects of sodium arsenite (SA), cyclophosphamide (CP) and *AEFAD* on Malondialdehyde (MDA) level in the liver of treated rats and control. Data are expressed as mean ± sd (x 10^{-8}) (n=5). SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p < 0.05$) when compared with DW only group, [a] mean difference is significant ($p < 0.05$) when compared with SA only groups. [b] mean difference is significant ($p < 0.05$) when compared with CP only group.

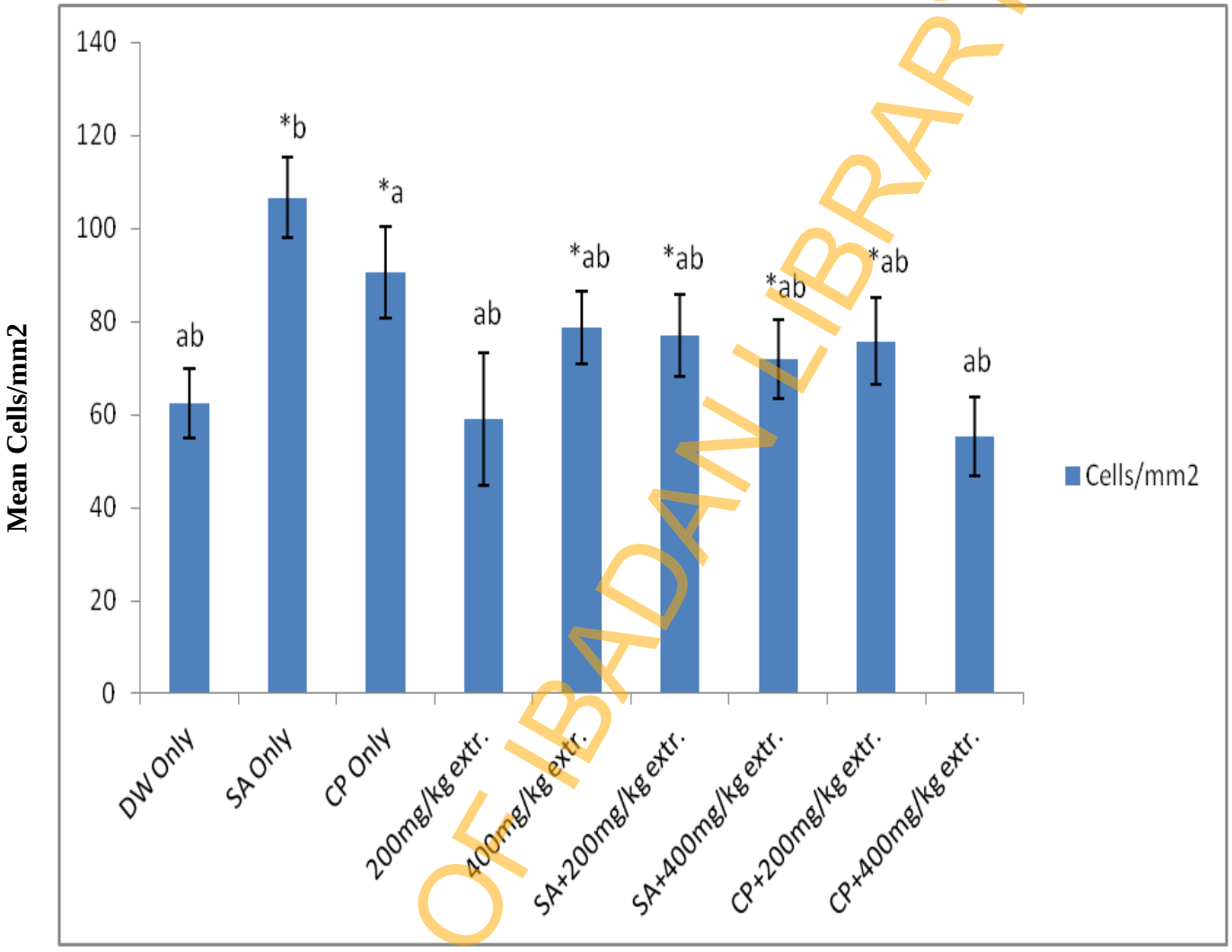


Figure 4.6: Effects of sodium arsenite (SA), cyclophosphamide (CP) and *AEFAD* on cells/mm^2 analysis in the liver of treated rats and control. Data are expressed as mean ± sd. (n=5). SA and CP were administered at 2.5mg/kg and 10mg/kg respectively* mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups. [b] mean difference is significant ($p< 0.05$) when compared with CP only group.

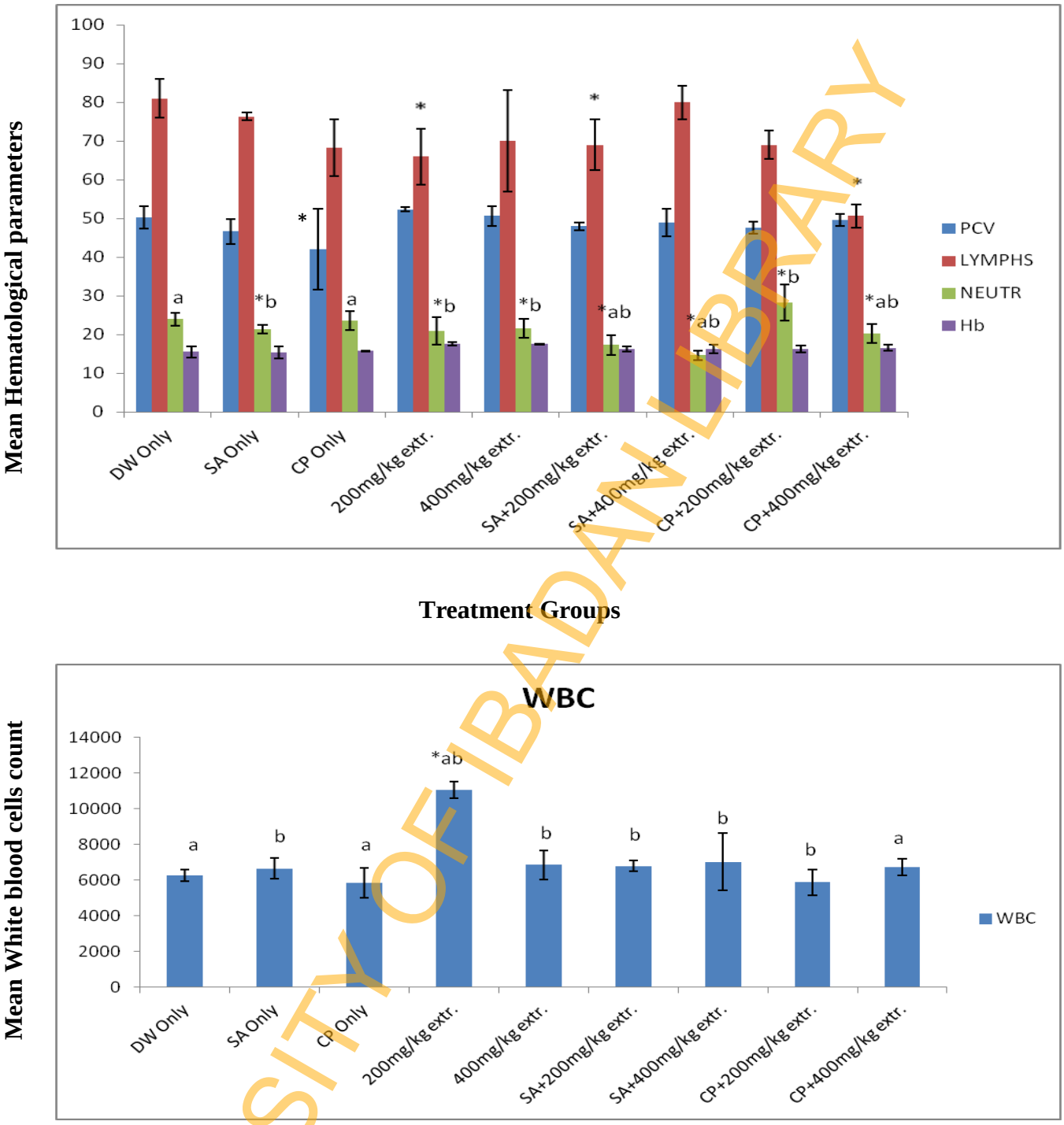


Figure 4.7 a and b: Effects of sodium arsenite (SA), cyclophosphamide (CP) and *AEFAD* on hematological parameters of treated rats and control. Data are expressed as mean ± sd. (n=5): SA and CP were administered at 2.5mg/kg and 10mg/kg respectively. * mean difference is significant ($p < 0.05$) when compared with DW only group, [a] mean difference is significant ($p < 0.05$) when compared with SA only groups. [b] mean difference is significant ($p < 0.05$) when compared with CP only group.

Experiment 3: Hepatoprotective and antigenotoxic effects of Methanol Extract of the stem Bark of *Adansonia digitata* (MESBAD) on Sodium Arsenite-induced toxicities in Wistar rats.

Introduction:

Bioactive compounds in medicinal plants have been proven over the years to possess abilities to mitigate chemically-induced hepatotoxicity and clastogenicity. Sodium arsenite (SA) is an established hepatotoxic and clastogenic agent and also listed to be a probable human carcinogen (IARC, 1994). Human and animals are unavoidably exposed to SA, especially through drinking water in contaminated arears, diverse research that address amelioration of these toxicities and the foundamental understanding of the mechanism of toxicity become necessary. Some reports had earlier indicated that following exposure of rats to SA, SA significantly induced hepatotoxicity and clastogenicity (Odunola *et al*., 2011, Gbadegesin *et al.*, 2014), suggesting that SA exposure can increase the risk of hepatotoxicity and clastogenicity. However, it is not established whether MESBAD could ameliorate SA induced hepatotoxicity and clastogenicity. The aim of this experiment therefore, was to investigate the possible ameliorative effects of MESBAD on SA induced hepatotoxicity and clastogenicity in male rats.

Procedure:

30 male albino rats (Wistar Strain) were used for this experiment. The animals were divided into 6 groups. Group I served as control (distilled water only) and animals in group II were administered 400mg/kg body weight MESBAD, group III received 400 mg/kg body weight MESBAD and 2.5mg/kg SA, while group IV received 300 mg/kg body weight MESBAD

and SA, group V received 300 mg/kg body weight MESBAD only while group 6 got 2.5 mg/kg body weight sodium arsenite only. All treatments were for 14 days, administrations were done orally. All animals were fed with normal rat diet and water *ad libitum*. At the end of the experiment (24 hours after the last administration), animals were sacrificed by cervical dislocation and dissected. Serum and femur samples were obtained for serum enzyme determination and micronucleus induction assay, as earlier described, liver tissues were also processed for histology.

Result

There was no significant difference in the mean liver and relative liver weights of the treatment groups across board when compared with the control group, which was administered distilled water only and the group administered the toxicant, SA only (figure 4.8). Serum AST activity in Group 6 (SA only) was not higher when compared with the negative control (figure 4.9), but GGT activities was higher (figure 4.11) while ALP activities was significantly higher when compared with the negative control (DW only) (figure 4.10). Administration of the extract alone at both 300 and 400 mg/kg body weight did not produce any significant increase in the activities of the enzymes except in the AST activities in the group administered 400mg/kg MESBAD. Co administration of the MESBAD with SA significantly ($p<0.05$) reduced the elevated activities of AST and ALP (figures 4.9 and 4.10). The histological studies also support the liver function enzyme assays by presenting a clearer description of the hepatotoxicity of SA and the hepatoprotective role of the extract (plate 4.2).

Administration of SA to animals in Group 6 significantly ($P < 0.05$) increased the frequency of mPCEs in the bone marrow of rats by about 6 folds when compared with the control while co-administration of the extract and SA at 300 mg/kg body weight gave a sharp and statistically significant ($P < 0.05$) decrease in the frequency of mPCEs by about 3 folds, when compared with SA only group (figure 4.12).

Conclusion: Taken together, the MESBAD has the potential to mitigate or remediate toxicities resulting from SA intoxication in rats, and it may offer protection in circumstances of co-exposure and cases of arsenicosis.

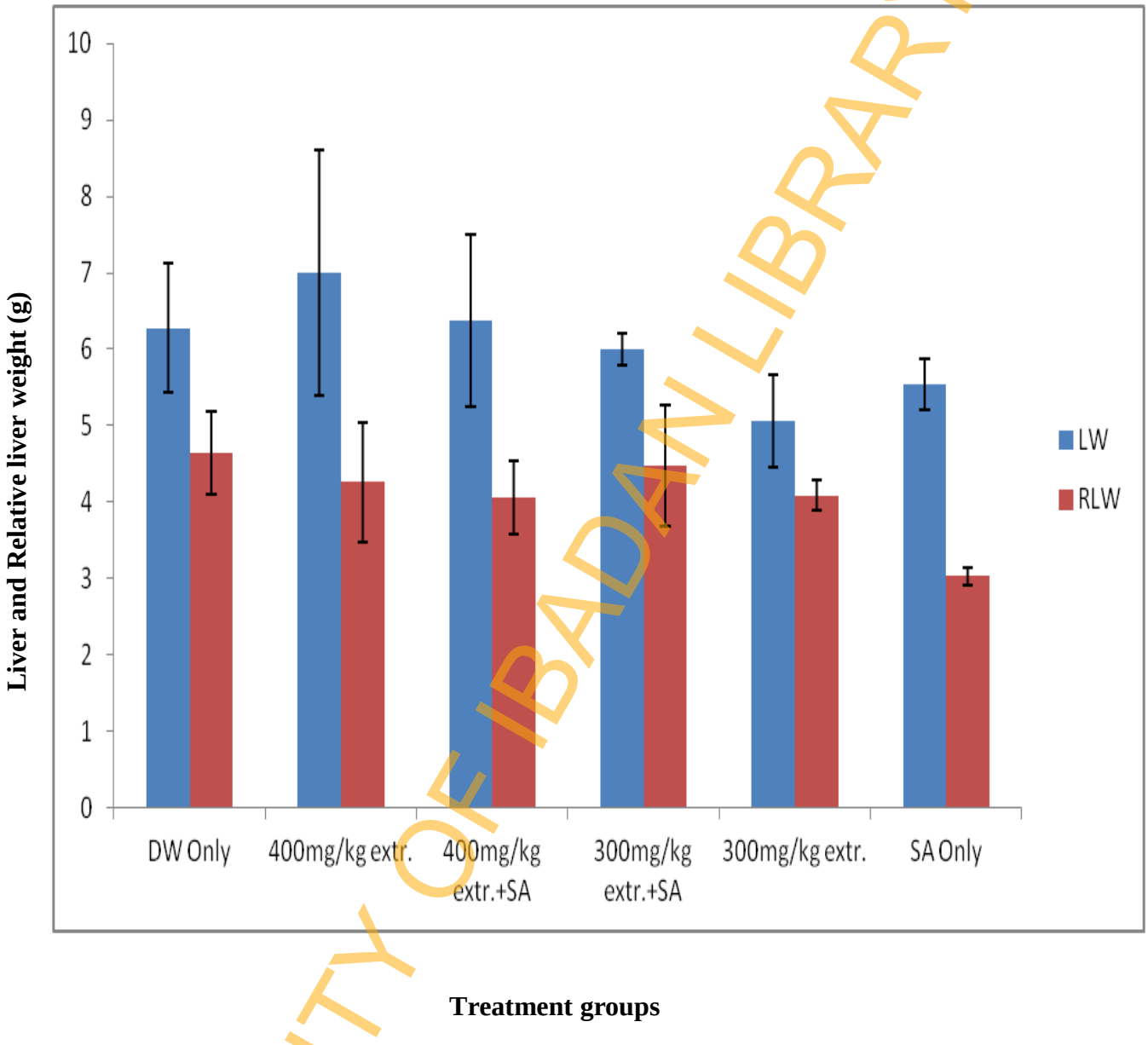


Figure 4.8: Liver weight and relative liver weight of the experimental animals after treatment with *Adansonia digitata* stem bark extract or sodium arsenite (SA). Data are expressed as mean ± sd. (n=5): DW=Distilled water, SA=Sodium arsenite.

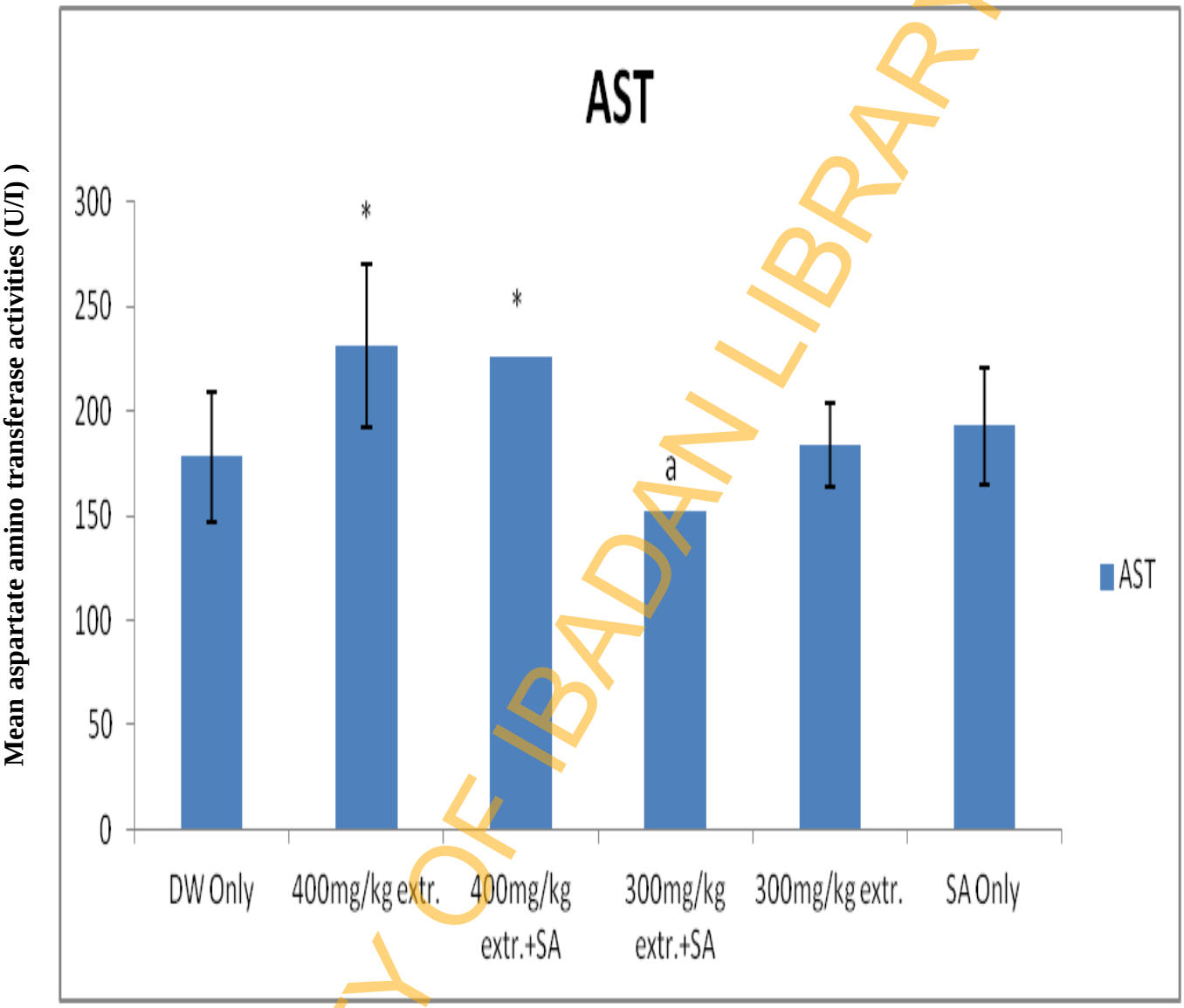


Figure 4.9: Serum aspartate aminotransferase (AST) activities of the experimental animals after treatment with *Adansonia digitata* stem bark extract or sodium arsenite (SA). Data are expressed as mean ± sd. (n=5): DW=Distilled water, SA=Sodium arsenite. * mean difference is significant ($p < 0.05$) when compared with DW only group, [a] mean difference is significant ($p < 0.05$) when compared with SA only groups.

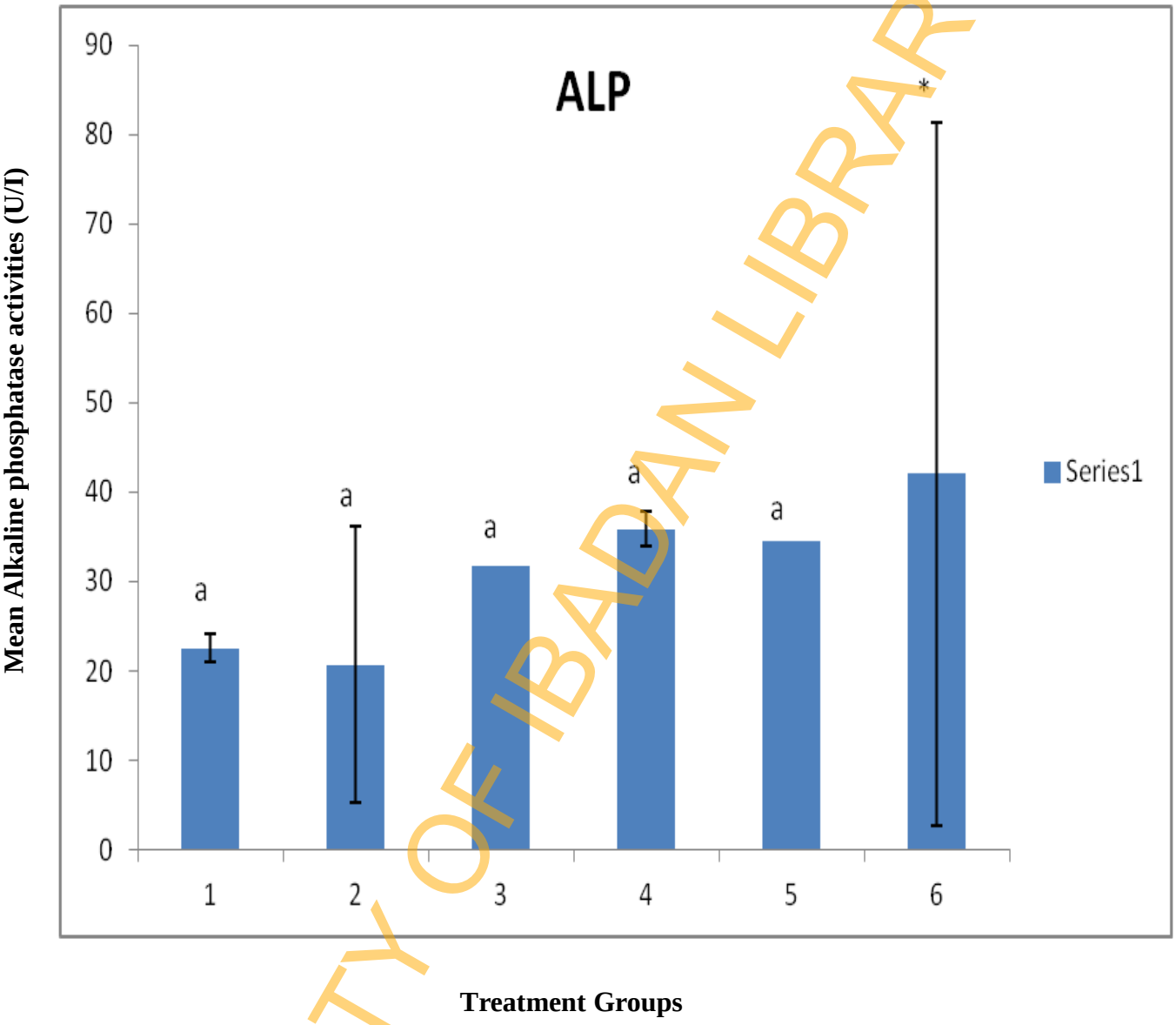


Figure 4.10: Serum alkaline phosphatase (ALP) activities of the experimental animals after treatment with *Adansonia digitata* stem bark extract or sodium arsenite (SA). Data are expressed as mean ± sd. (n=5): DW=Distilled water, SA=Sodium arsenite. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups.

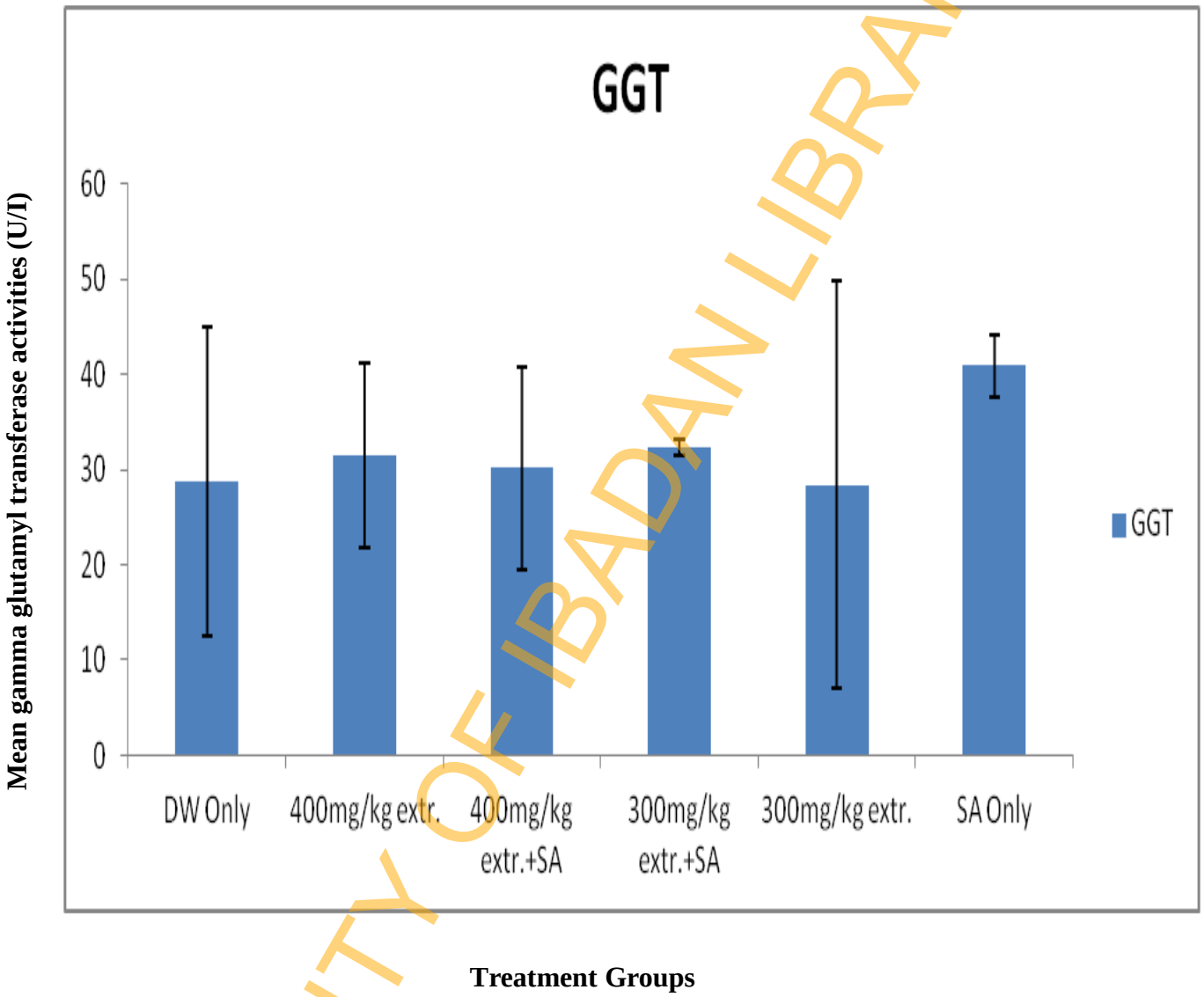


Figure 4.11: Serum gamma glutamyl transferase (GGT) activities of the experimental animals after treatment with *Adansonia digitata* stem bark extract or sodium arsenite (SA). Data are expressed as mean ± sd. (n=5): DW=Distilled water, SA=Sodium arsenite. * mean difference is significant ($p < 0.05$) when compared with DW only group, [a] mean difference is significant ($p < 0.05$) when compared with SA only groups.

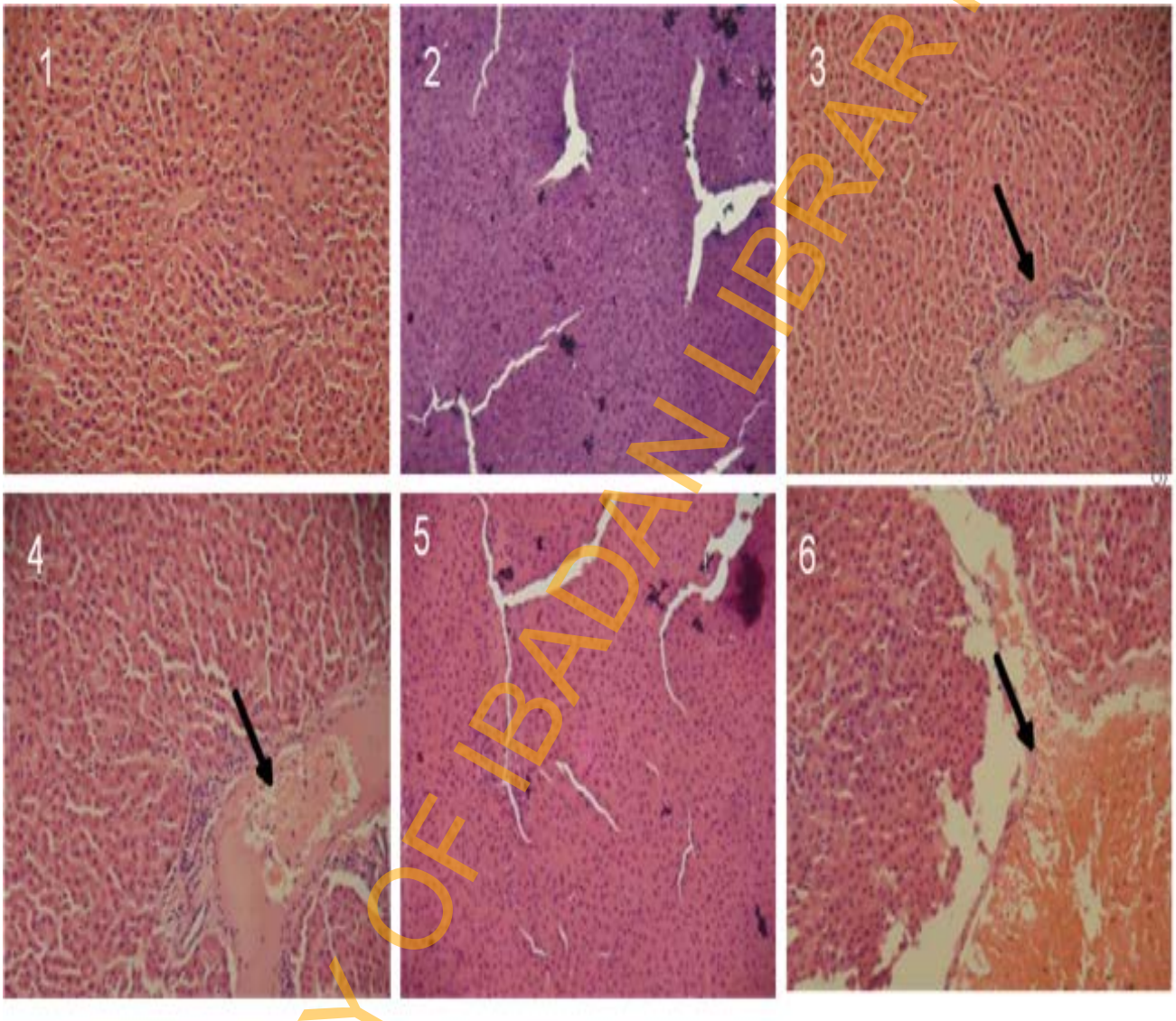


Plate 4.2: Photomicrograph of the histological examination of the liver
1.Distilled water only 2. 400mg/kg MESBAD 3. 400mg/kg MESBAD+SA 4. 300mg/kg MESBAD+SA 5. 300mg/kg MESBAD alone 6. SA alone
1. Normal hepatocytes, no visible lesion, 2. Mild portal congestion 3. Normal hepatocytes 4. Very mild portal congestion, diffuse periportal cellular infiltration by mononuclear cells, 5.No visible lesion 6. Severe portal and central venous congestion. **H&E, Mx400**

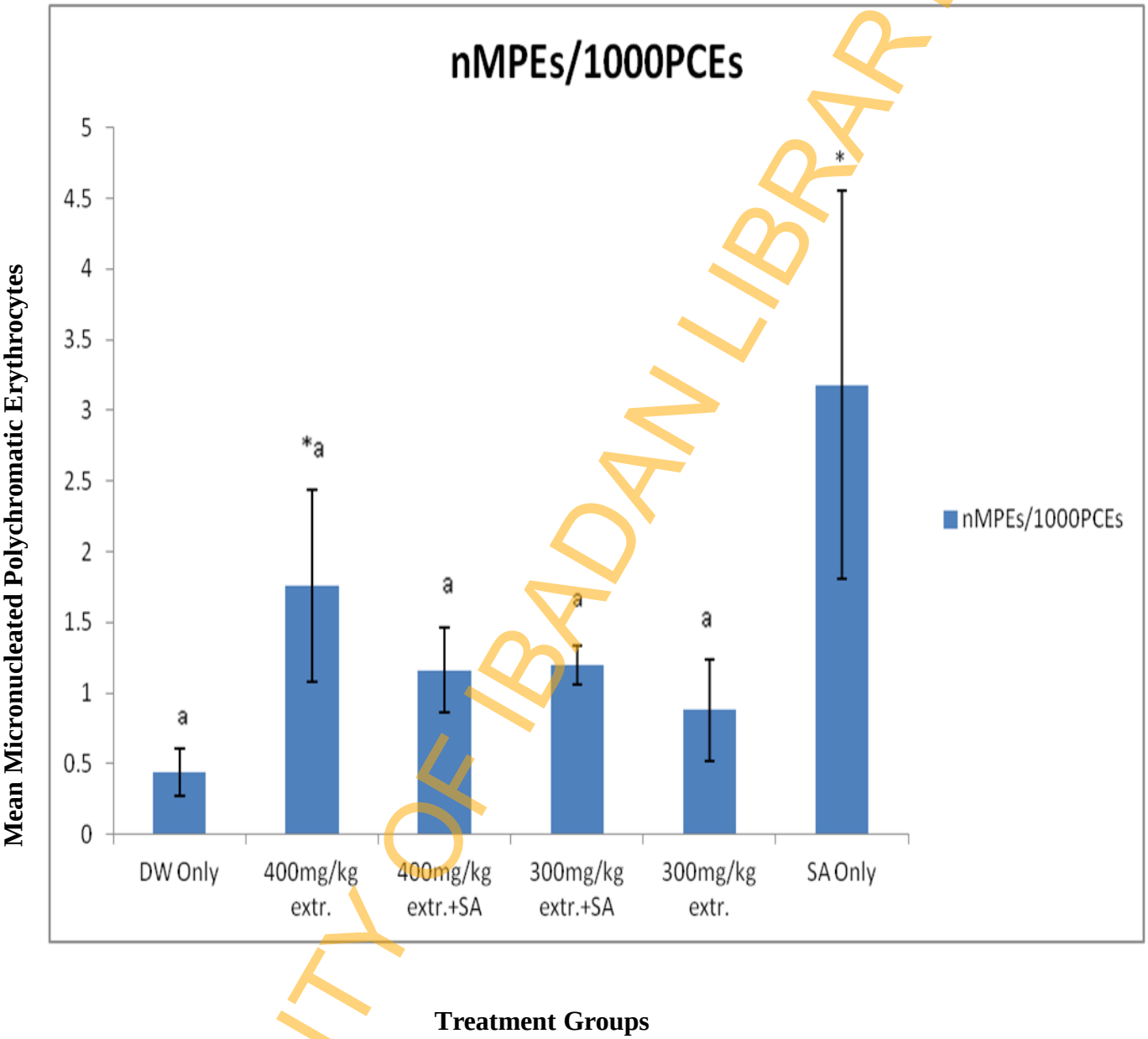


Figure 4.12: Mean number of micronucleated polychromatic erythrocytes (nMPCEs) of the experimental animals after treatment with *Adansonia digitata* stem bark extract or sodium arsenite (SA). Data are expressed as mean ± sd. (n=5). DW=Distilled water, SA=Sodium arsenite. * mean difference is significant ($p< 0.05$) when compared with DW only group, [a] mean difference is significant ($p< 0.05$) when compared with SA only groups.

Experiment 4. The effects of duration of exposure of various fractions of AEFAD on some cancer cell lines using the SRB assay.

Introduction: The search for potent anticancer agents from plant origin becomes necessary, owing to the high cost and toxicity of synthetic drugs which has made it difficult for people from developing countries to have good access to these drugs. There is a great increase in researches geared towards understanding the mechanism of carcinogenesis and ways of carrying out treatment by employing the use of natural bioactive agents from medicinal plants. Although, there are some reports on the folkloric use of *Adansonia digitata*, very little is known about the anticancer potential of this forest tree. The aim of this experiment therefore, was to assess the cytotoxic effect of exposure of the various fractions of the fruit pulp of *Adansonia digitata* on some selected cancer cell lines using the sulphurhodamine B (SRB) assay.

Principle: Sulforhodamine B is an anionic aminoxanthene dye that forms an electrostatic complex with the basic amino acid residues of proteins under moderately acid conditions, which provides a sensitive linear response. The colour development is rapid and stable and is readily measured at absorbances between 560 and 580nm.

Procedure The cell lines were cultured at appropriate densities, employing the right media as earlier indicated in the methods section, for 24 hours, after 24 hours, they were treated with different concentrations (10, 20, 40, 80) µg/ml of the fractions for 48 hours. At the end of the experiment, cell viability was determined. Cytotoxicity of the fractions was determined by SRB assay as earlier described and the morphological changes in the cells were observed by phase contrast microscopy.

The SRB assay was carried out as described by (Skehan *et al.*, 1990; Skehan, 1995). 70 μL 0.4% (w/v) sulforhodamine B in 1% acetic acid solution were added to each well and left at room temperature for 20 min. SRB was removed and the plates washed 5 times with 1% acetic acid before air drying. Bound SRB was solubilized with 200 μL 10 mM unbuffered Tris-base solution and plates were left on a plate shaker for at least 10 min. Absorbance was read in a 96-well plate reader at 492 nm subtracting the background measurement at 620 nm. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value ('blank' is the mean optical density of the background control wells). Mean values and CV from 3 replicate wells were calculated automatically. Curves and statistical analysis were performed using Excel 7.0 software for Windows.

Results: The results of cytotoxicity are presented in figures 4.13, 4.14, 4.15 and 4.16. The various fractions did not show significant ($p<0.05$) decrease in cell viability based on the concentrations tested and the duration of exposure. The morphological observations are presented in plates 4.3, 4.4, 4.5, and 4.6. The morphological observation shows that Aqueous, ethyl acetate and n-butanol fractions conferred some apoptotic morphological effects on KB (plate 4.3).

Conclusion: The results from this experiment revealed that there was no statistically significant difference between the cytotoxic effects of the fractions of AEFAD and the negative controls on the cell lines. Aqueous, ethyl acetate and n-butanol fractions conferred some apoptotic morphological effects on KB.

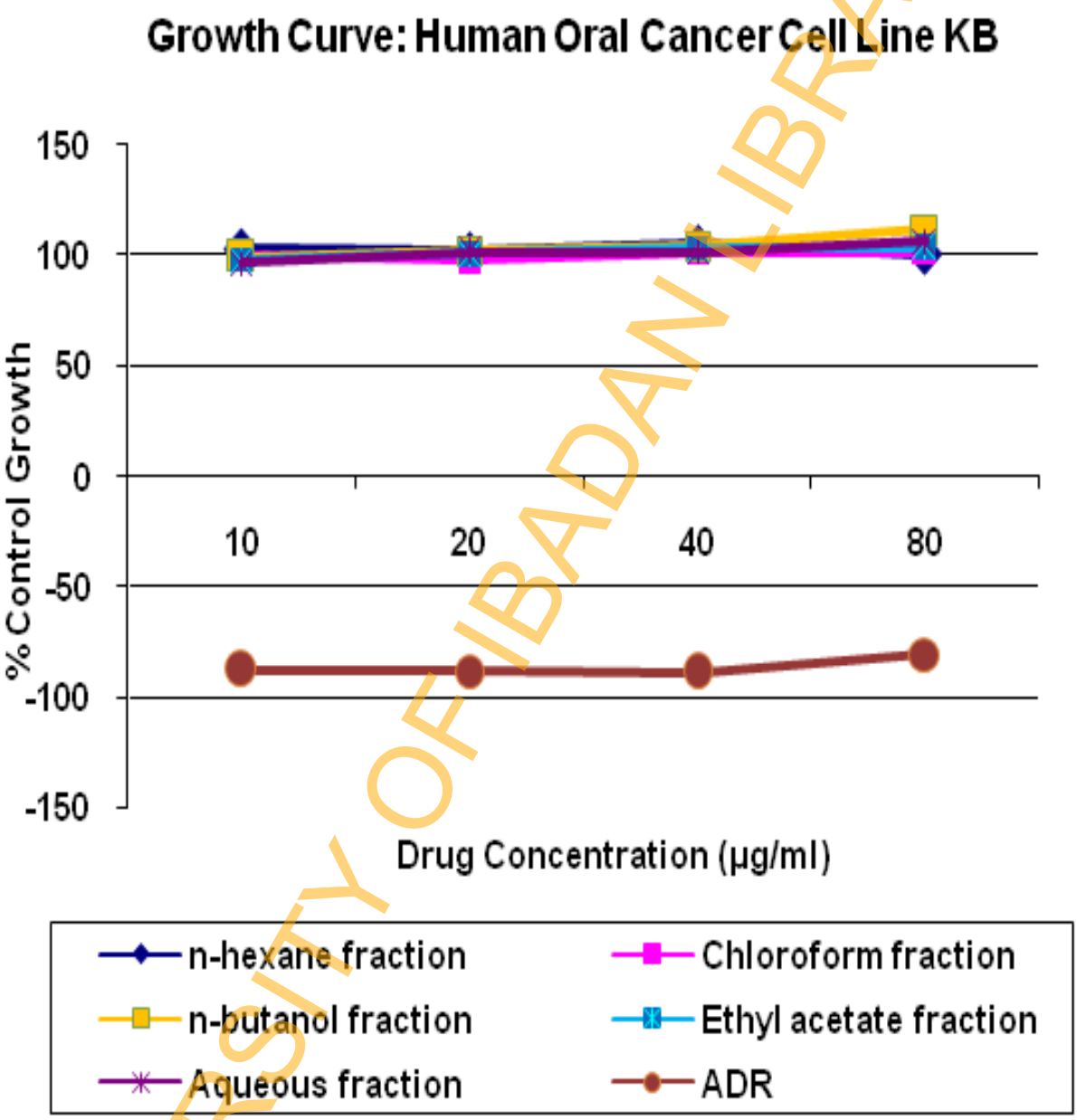


Figure 4.13 Growth curve showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human oral cancer cell line KB. KB cells were treated with Adriamicin (ADR) or with 80 µg/ml of fractions for 48 hours, the cell viability was assessed by SRB assay.

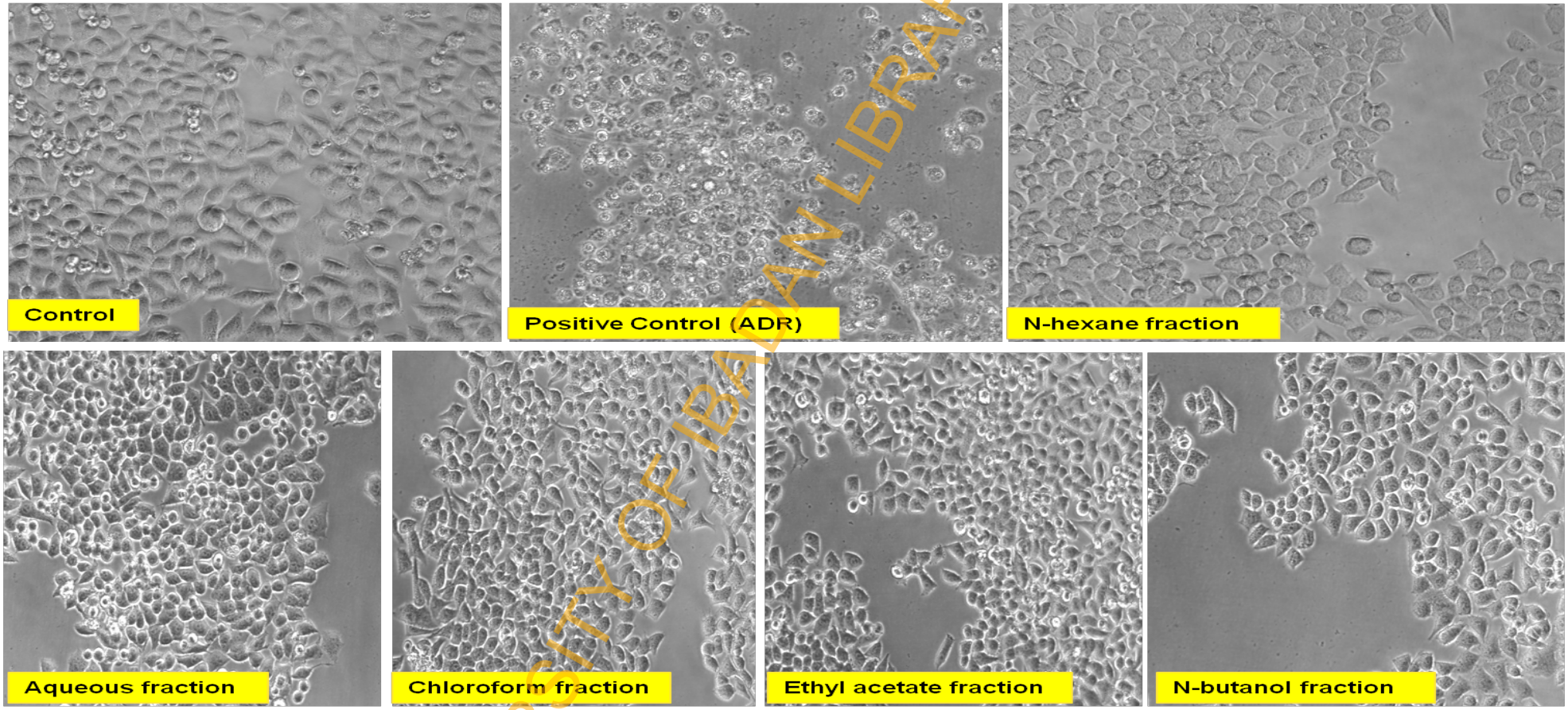


Plate 4.3: Phase-contrast micrographs showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human oral cancer cell line KB. KB cells were treated with Adriamicin (ADR) or with 80 µg/ml of fractions for 48 hours, the cell viability was assessed by SRB assay.

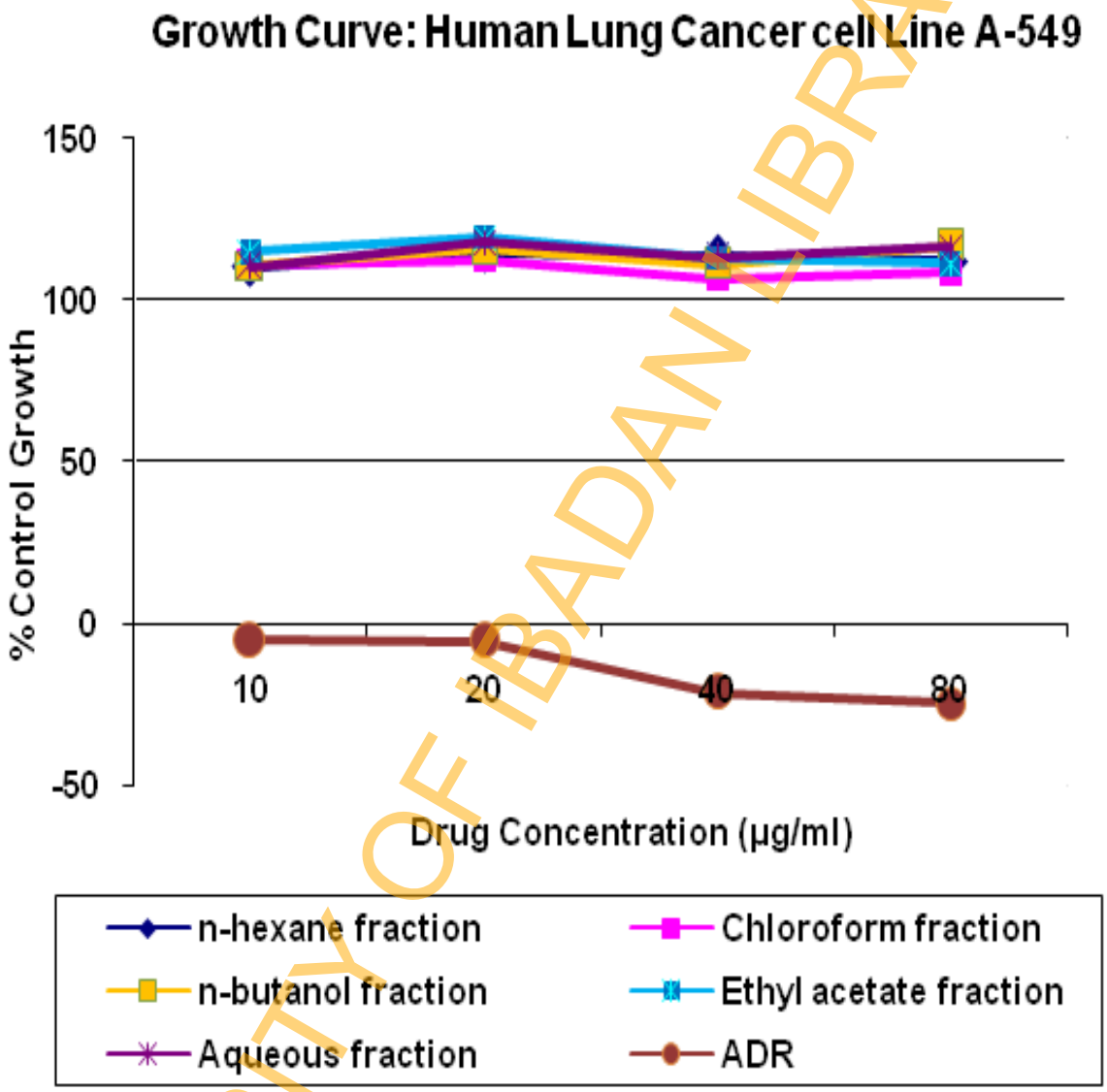


Figure 4.14 Growth curve showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human lung cancer cell line A-549. A-549 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

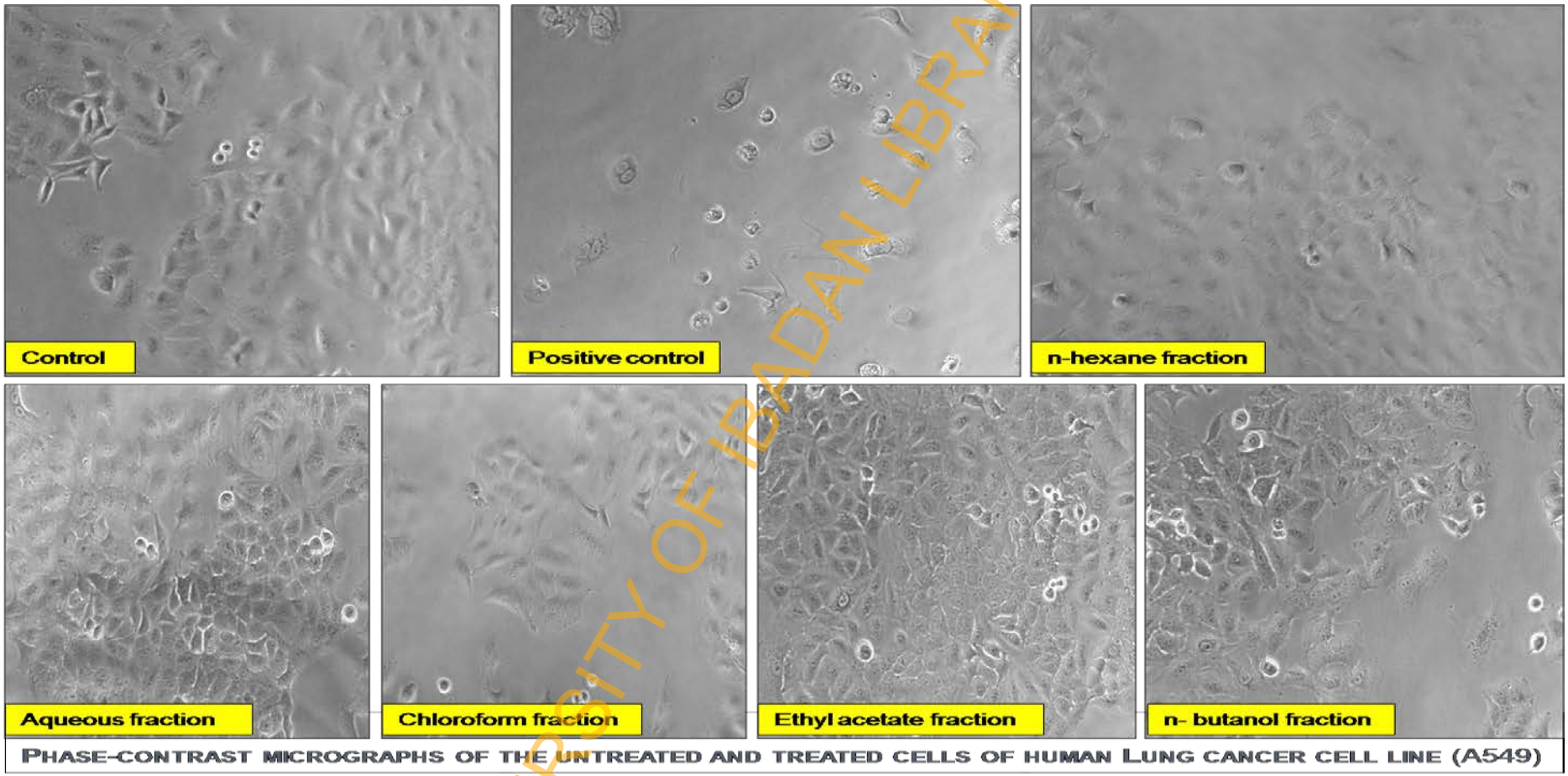


Plate 4.4: Phase-contrast micrographs showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human lung cancer cell line A-549. A-549 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

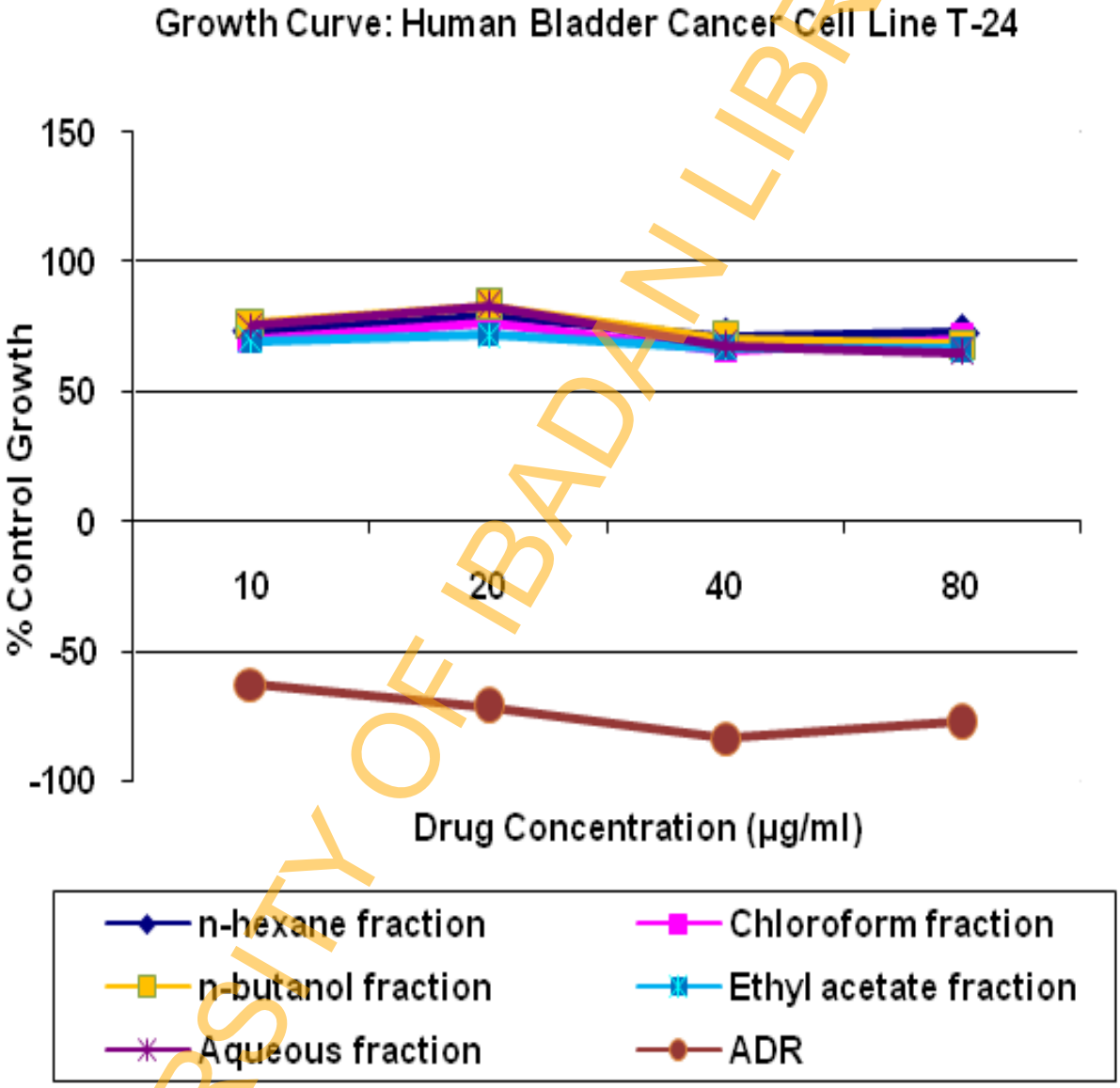


Figure 4.15: Growth curve showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human bladder cancer cell line T-24. T-24 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

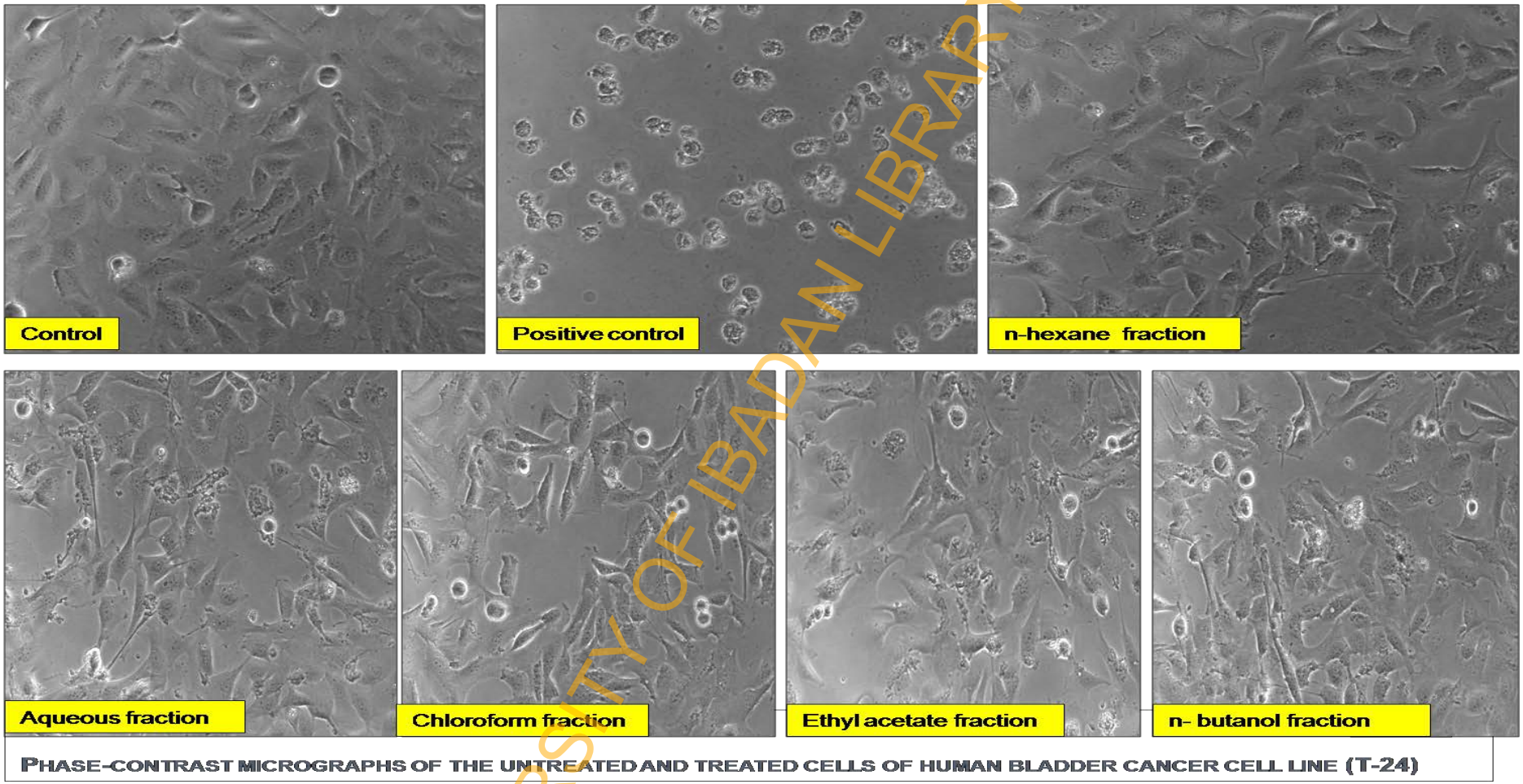


Plate 4.5: Phase-contrast micrographs showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human bladder cancer cell line T-24. T-24 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

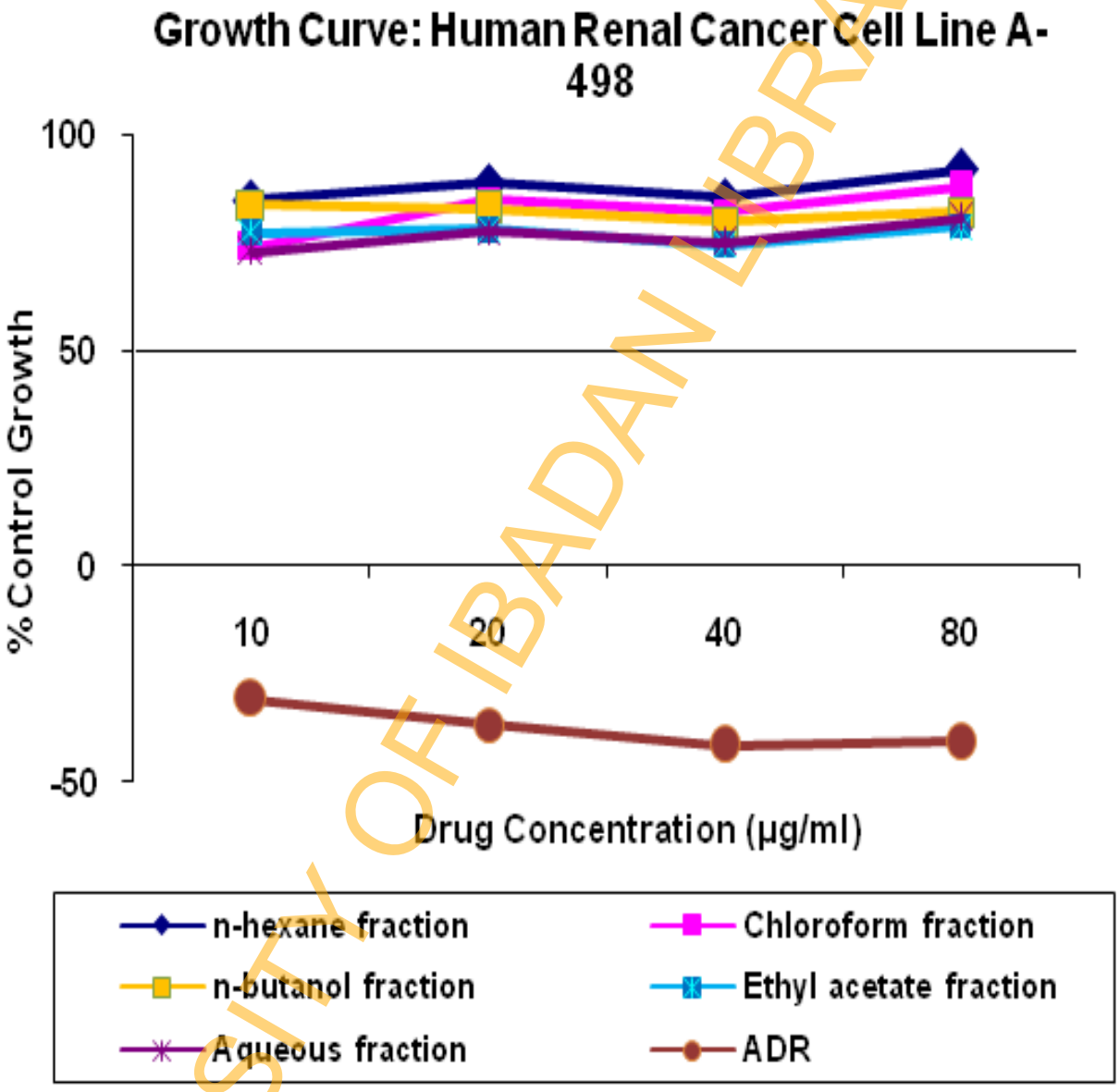


Figure 4.16: Growth curve showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human renal cancer cell line A-498. A-498 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

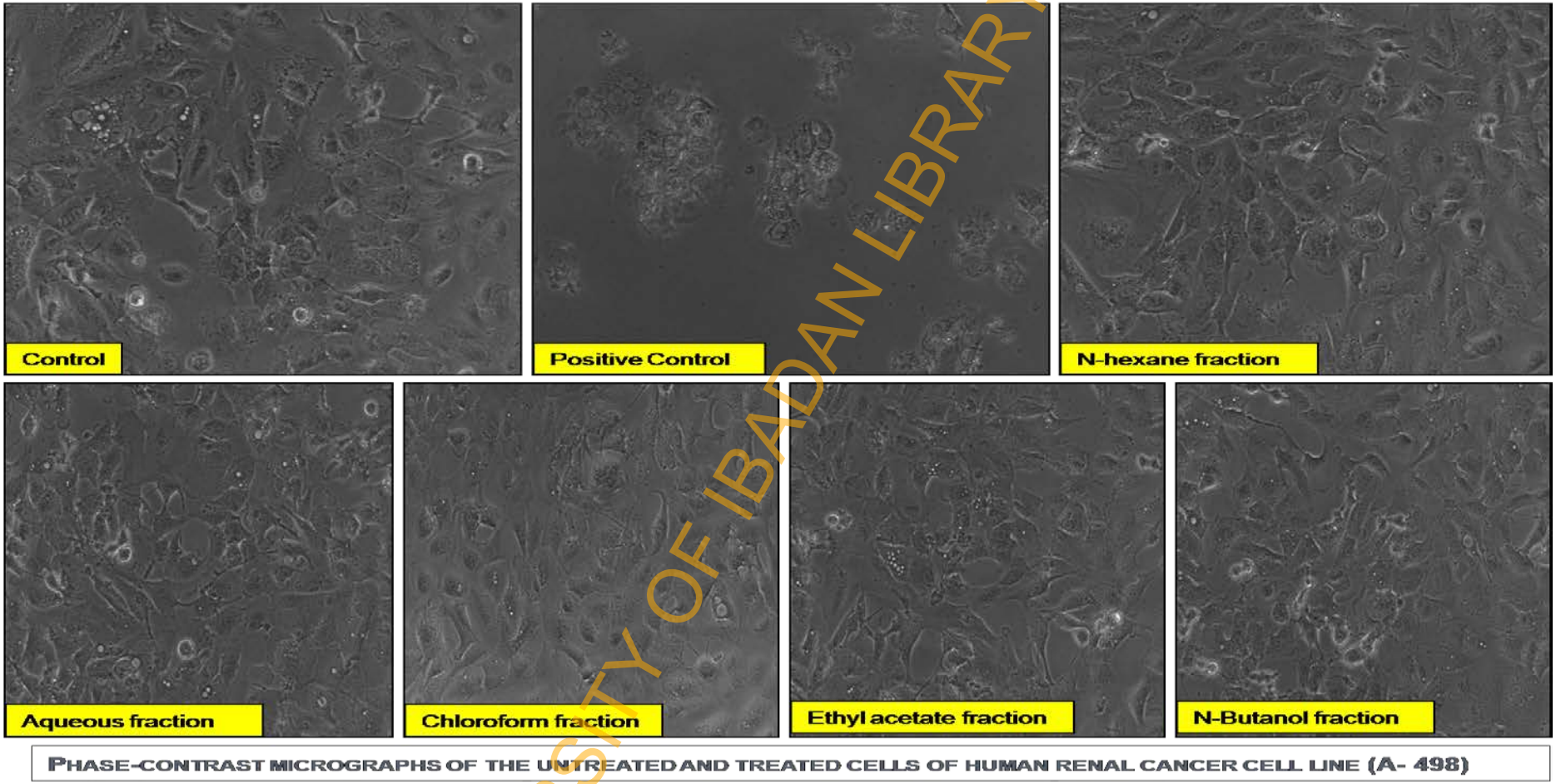


Plate 4.6 Phase-contrast micrographs showing the effect of the fractions of aqueous extract of *Adansonia digitata* on the viability of human renal cancer cell line A-498. A-498 cells were treated with Adriamicin (ADR) or with 80 µg/ml of the fractions for 48 hours, the cell viability was assessed by SRB assay.

Experiment 5. Effects of fractions of aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD) on the cell cycle distribution of breast (MCF-7) and Lung (A-549) cancer cell lines.

Introduction: The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. Some anticancer agents exert cytotoxicity by inducing DNA double strand breaks (DSBs) leading to cell cycle arrest and cell death (Ulakaya *et al.*, 2011; Tanaka *et al.*, 2013). To investigate the effects of the various fractions of AEFAD on breast and lung cancer cell growth, their effects on the cell cycle profile was therefore tested

Principle:

The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content. Samples are analyzed at rates below 1000 cells per second in order to yield a good signal of discrimination between singlets or doublets.

Procedure:

MCF-7 and A-549 cells were seeded at density of 88 x 10^4 cells/well in 6-well plates. After 24h cells were treated with various fractions of AEFAD. After 24 hrs treatment and incubation at 37 ˚C and 5 % CO_2, cells were washed with PBS harvested with trypsin and centrifuged. Cells pellet were resuspended in propidium iodide (PI) buffer (0.2 % BSA and 0.1 % sodium azide in PBS) and kept at 4^{o}C in dark until analysis. 1 µg/mL PI was added and Cell viability analyzed by flow cytometry on a FACSCalibur. PI was

excited at 488 nm, and fluorescence analyzed at 610 nm. A total of 10,000 events in each sample were acquired. Using CellquestPro software, the percentages of live and dead cells were determined.

Results:

Results (table 4.5) indicate that AEFAD fractions did not show any significant ($p<0.05$) G0/G1 phase arrest on MCF-7 cells as compared with control. However, there is increase in apoptosis at 50µg/ml of AF, EAF, NBF and a significant 7 fold increase in apoptosis at 50µg/ml NHF. Sub- G1 peaks, generally accepted to represent dead cells (Rao *et al.*, 2011), were present in breast cancer cell line treated with NBF which corroborates the apoptotic activities of NBF on KB in the previous experiment.

Result presented in tables 4.6 show the effect of the fractions on cell cycle distribution of A-549 cells, the results indicate that there was a slight G0/G1 phase arrest at 50 and 100 µg/ml in the aqueous fraction treated group, though it was not concentration dependent, also there was G0/G1 arrest in the NHF treated group at both G0/G1.

Conclusion: NHF at 50µg/ml had a profound apoptotic effect on breast cancer cell line and also arrested the lung cancer cell line at the G0/G1 phase.

Table 4.5: Cell cycle Analysis on MCF-7 of different pulp fractions at 24 hours

AF= Aqueous fraction, EAF= Ethyl acetate fraction, NHF= n-hexane fraction and NBF= n-butanol fraction.

Cell line/ Fraction	Drug(µg/ml)	G0-G1%	S%	G2-M%	% Apopt.
MCF-7	Cntr.	74.47	18.86	6.67	1.11
MCF-7 AF	50	73.27	19.45	7.28	2.61
MCF-7 AF	100	71.81	21.67	6.52	1.14
MCF-7	Cntr.	74.47	18.86	6.67	1.11
MCF-7 EAF	50	69.66	20.70	9.64	5.60
MCF-7 EAF	100	71 .90	18.75	9.34	4.45
MCF-7	Cntr.	74.47	18.86	6.67	1.11
MCF-7 NHF	50	69.60	21 .10	9.30	7.14
MCF-7 NHF	100	66.73	29.44	3.83	4.75
MCF-7 NBF	Contr.	74.66	25.34	0.00	17.24
MCF-7 NBF	50	75.58	24.42	0.00	21.17
MCF-7 NBF	**100**	61 .98	12.27	25.74	18.46

Table 4.6: Cell cycle Analysis on A549 of different pulp fractions at 24 hours

AF= Aqueous fraction, EAF= Ethyl acetate fraction, NHF= n-hexane fraction and NBF= n-butanol fraction.

Cell line/ Fraction	Drug (µg/ml)	G0-G1%	S%	G2-M%	% Apopt.
A-549 AF	**Ctr.**	60.39	30.65	8.96	2.36
A-549 AF	**50**	63.16	29.35	7.50	1.74
A-549 AF	**100**	63.05	29.40	7.55	2.51
A-549 AF	**Ctr.**	60.39	30.65	8.96	2.36
A-549 EAF	**50**	63.58	36.42	0.00	1.38
A-549 EAF	**100**	61.95	37.91	0.13	1.91
A-549 AF	**Ctr.**	60.39	30.65	8.96	2.36
A-549 NHF	**50**	62.72	26.73	10.55	1.64
A-549 NHF	**100**	67.83	25.57	6.60	2.12
A-549 AF	**Ctr.**	60.39	30.65	8.96	2.36
A-549 NBF	**50**	54.20	28.99	16.20	1.52
A-549 NBF	**100**	57.98	29.38	12.64	1.96

Experiment 6. Cytotoxicity studies of graded doses of methanol extract of the stem bark of *Adansonia digitata* (MESBAD) on MCF-7 cell line

Introduction: The search for potent anticancer agents from plant origin is a necessity, because of the expensive nature of anticancer drugs and toxicity associated with synthetic drugs which has made it difficult for people from low income countries to readily obtain these drugs. Researches towards the understanding of the mechanism underlying carcinogenesis and ways of treatment with bioactive agents from medicinal plants have recently received greater attention. Although there are some reports on the medicinal uses of the stem bark of *Adansonia digitata*, there is very little information about the anticancer potential of the stem bark of this very important forest tree. The aim of this experiment therefore, was to assess the effect of duration of exposure of the various concentration of the MESBAD on MCF-7 breast cancer cell lines.

Principle: MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

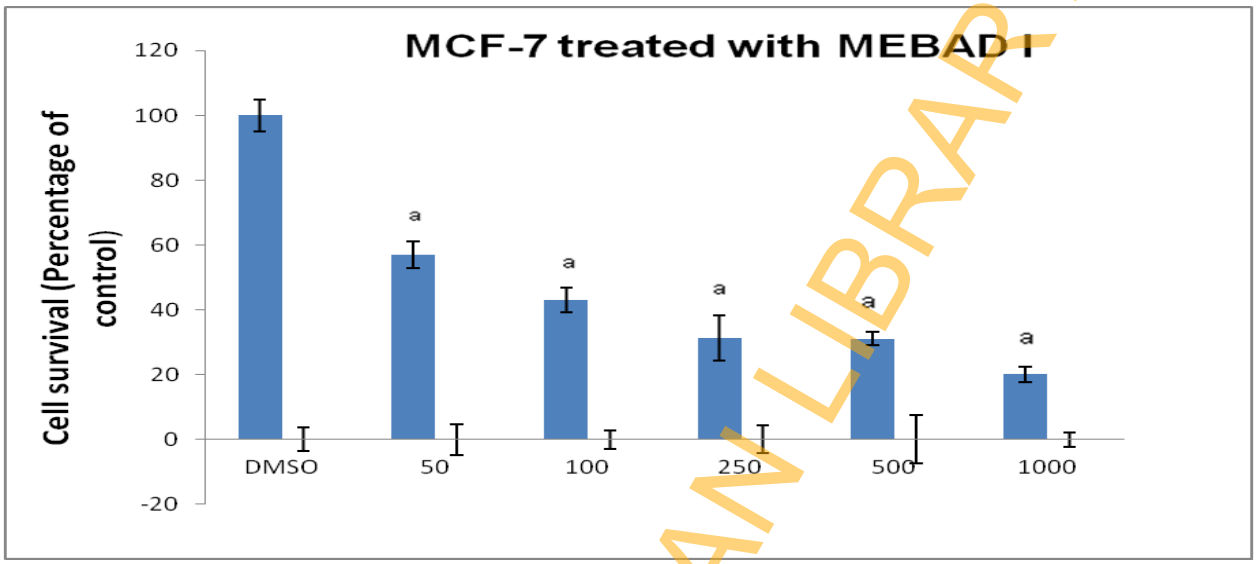
Procedure: The cell lines were cultured at appropriate densities in suitable media for 24hours, after 24hours, they were treated with DMSO and different concentrations (50, 100, 250, 500 and 1000 µg/ml) of the MESBAD for 48hours. At the end of the experiment, cell viability was determined. Cytotoxicity of the fractions was determined by MTT assay as described by Mosmann (1983) with little modifications. **MTT (**3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay**.**

The human breast cancer cell line (MCF-7) was obtained from the University of Cape Town Medical School and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week. The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95 % air and 100 % relative humidity. After 24 hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addition, the plates were incubated for an additional 48hrs at 37 °C, 5 % CO_2, 95 % air and 100% relative humidity. The medium without samples served as control and triplicates were maintained for all concentrations. After 48 hrs of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline PBS) was added to each well and incubated at 37 °C for 4hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then the absorbance was measured at 570 nm using micro plate reader. The percentage cell inhibition was thereafter determined. Nonlinear regression graph was plotted between percentage cell inhibition and Log10 concentration and IC50 was determined using GraphPad Prism software.

Results: The results of cytotoxicity are represented in figure 4.17 a and b, the MESBAD show a dose dependent cytotoxic effect on MCF-7 cell line, after three consecutive experiments.

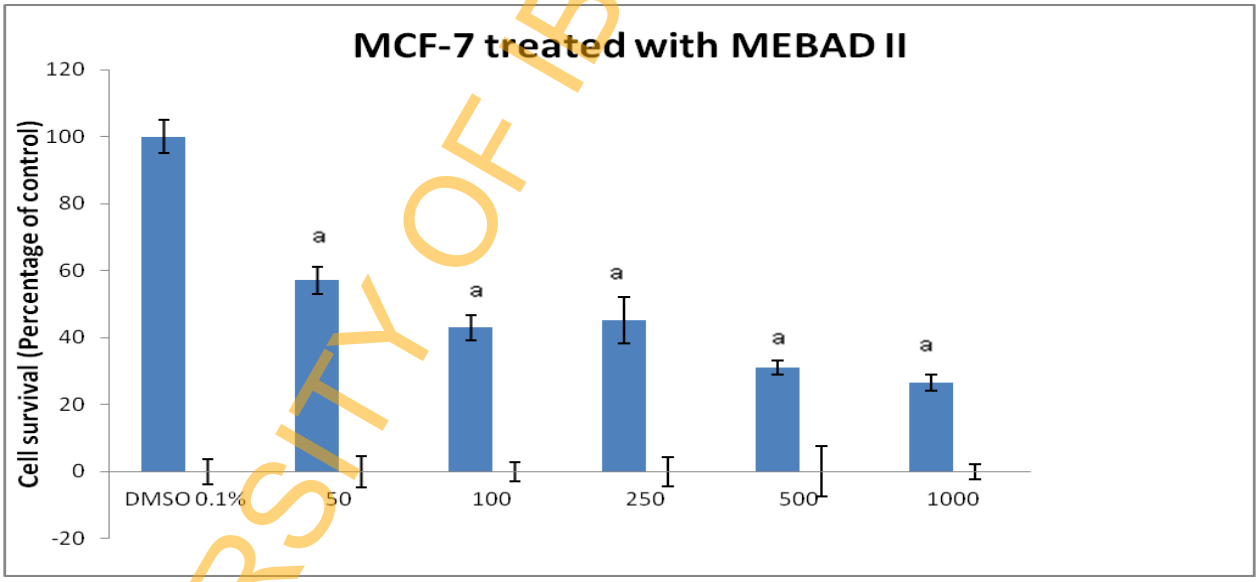
Conclusion: This experiment revealed that MESBAD possess anticancer activity on MCF-7 cell line. Cytotoxicity of MESBAD is concentration dependent, therefore, the higher the concentration, the higher the toxicity induced by MESBAD.

a



MESBAD Concentration (µg/ml)

b



MESBAD Concentration (µg/ml)

Figure 4.17 a and b Results of MTT assay using MESBAD on MCF-7 cells. [a] mean difference is significant ($p < 0.05$) when compared with the DMSO group. **IC50 = 100 µg/ml**

Experiment 7. Long term antiproliferative effect of graded doses of methanol extract of the stem bark of *Adansonia digitata* (MEBAD) on MCF-7 breast cancer cells

Introduction: The menace of cancer is global, in Nigeria; about 100,000 new cases are reported each year with breast cancer taking the lead (Adebamowo and Ajayi, 2000). Breast cancer is the second leading cause of death worldwide after cardiovascular diseases. MESBAD had been suggested to have anticancer activities, and current research also support this suggestion with the short term cytotoxicity studies earlier reported in this thesis, the current experiment sought to investigate the long term antiproliferative effect of MESBAD on MCF-7 cells. The aim of this experiment is therefore to assess the long term antiproliferative effect of MESBAD on breast cancer cell line (MCF-7) using the clonogenic assay.

Principle: The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. A cell that retains its ability to synthesize proteins and DNA and go through one or two mitoses, but is unable to divide and produce a large number of progeny is considered dead. This is very commonly referred to as loss of reproductive integrity or reproductive death and is the end point measured with cells in culture. On the other hand, a cell that is not reproductively dead and has retained the capacity to divide and proliferate indefinitely can produce a large clone or a large colony of cells and is then referred to as "clonogenic." The ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce; this is what is determined by the clonogenic assay

Procedure

Clonogenic assay is a useful tool to test whether a given anticancer agent can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells that can often only be determined microscopically. MCF-7 cells were cultured according to the requirements, the medium was removed, and cells rinsed with 10 ml PBS, 4 ml 0.25% trypsin was added to the cells and incubated at 37 °C for 1-5 min, 10 ml medium with 10% FBS was thereafter added, and cells were detached by pipetting up and down. Cells were counted using a hemocytometer. The desired seeding concentration was prepared, and then seeded into dishes or 6-well plates.

Cells were harvested and plated after treatment; up to 50 x 10^4 cells were plated. Serial dilutions were made with different numbers of cells and incubated in a CO_2 incubator at 37 °C for 1-3 weeks until cells in control plates have formed colonies with substantially good size (50 cells per colony is the minimum for scoring).

In order to fix and stain, the medium was removed, and cells were then rinsed with 10 ml PBS. PBS was removed and 2-3 ml of fixation solution was added and left in the dishes/plates at room temperature (RT) for 5 min, the fixing solution was thereafter removed and 0.5% crystal violet solution was added and incubated at RT for 2 h. 10 ml medium with 10% FBS was then added, and cells were detached by pipetting. The crystal violet was removed carefully and the dishes/plates were immersed in tap water to rinse off crystal violet. The dishes/plates were thereafter air-dried on a table cloth at RT for up to a few days. The number of colonies was then counted with a stereomicroscope.

Results:

The long term antiproliferative effect of MESBAD on MCF-7 breast cancer cells are presented in plate 4.7 and 4.8 respectively. MESBAD caused a clear reduction in the proliferation of MCF-7 cells when compared with the control that was treated with DMSO, the antiproliferative activity is dose dependent as seen in the number of colonies present in the cells treated with ½ IC50 and IC50 (plate 4.7). In order to ascertain the activity noticed, multiple of the IC50 was used to check the possibility of not having cells at all. The result present a similar pattern as the first with the cells treated with IC50x4 almost not having colonies anymore (plate 4.8).

Conclusion: Methanol extract of the stem bark of *Adansonia digitata* shows a dose dependent long term antiproliferative effect on MCF-7 breast cancer cells.

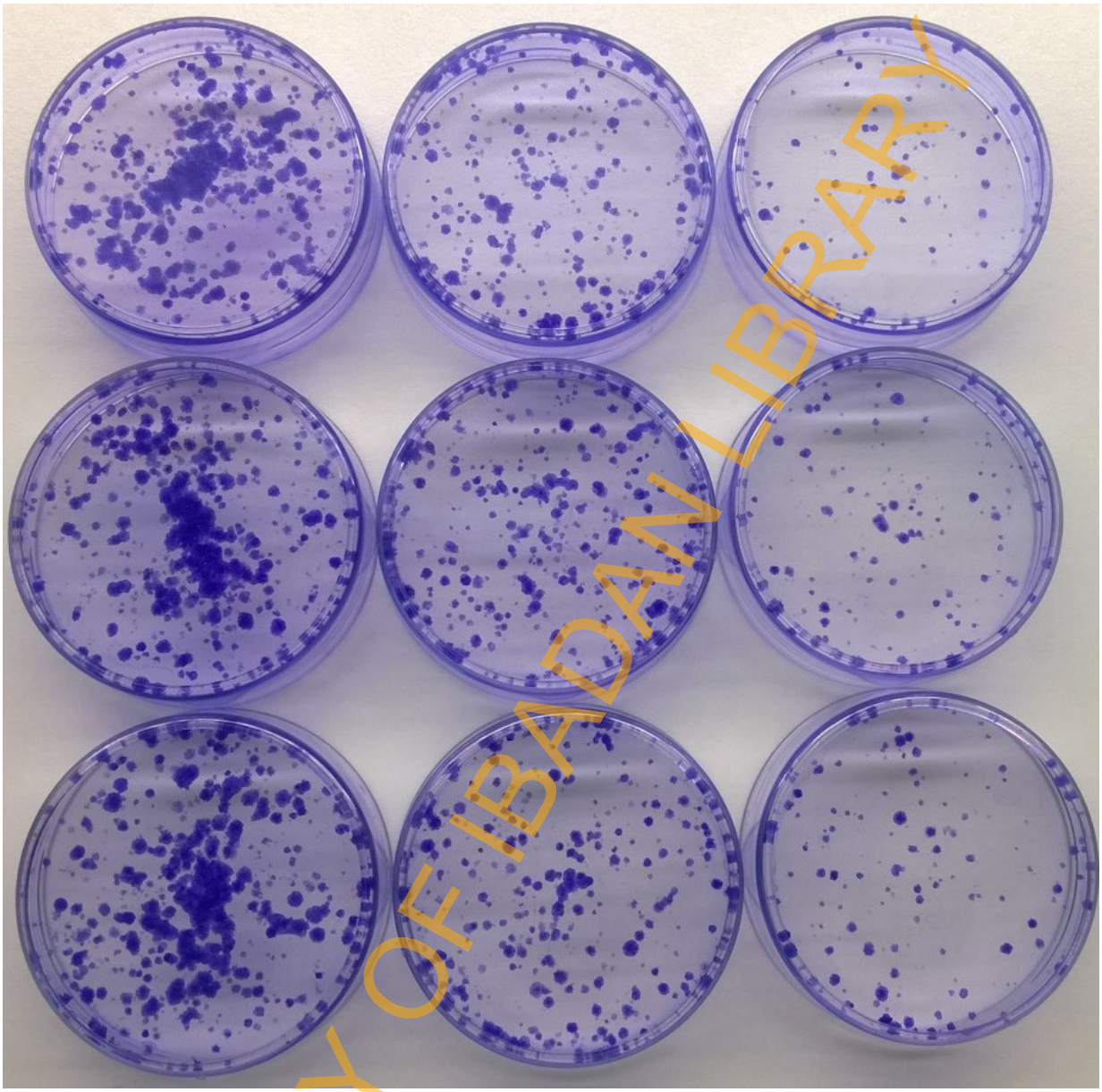
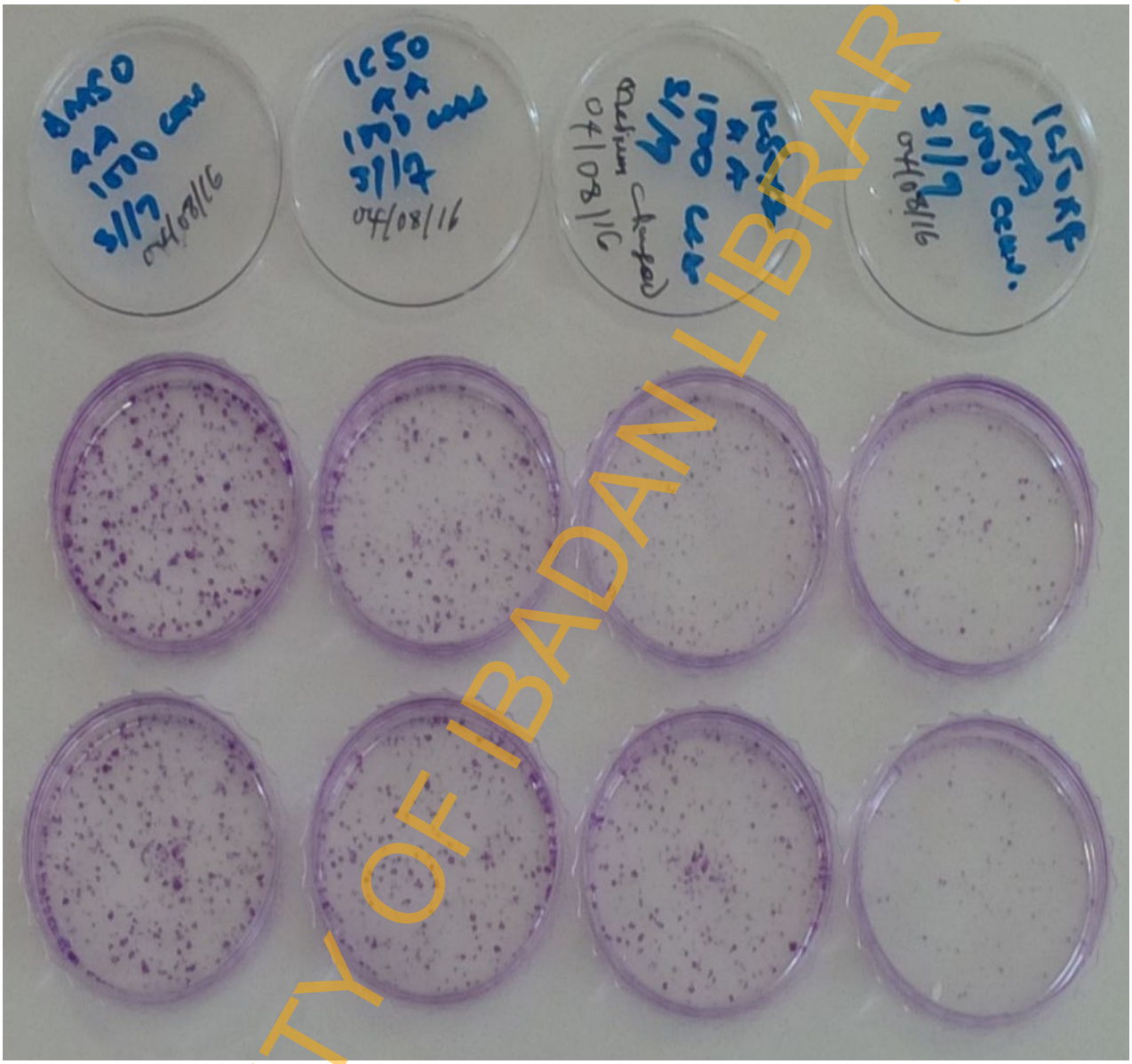


Plate 4.7: Results of the long term effect of MESBAD on MCF-7 cells survival and proliferation at ½ IC50 and IC50 of MESBAD



0.1% DMSO (400µg/ml) IC_{50} (100µg/ml) IC_{50}x2 (200µg/ml) IC_{50}x4

Plate 4.8: Results of the long term effect of MESBAD on MCF-7 cells survival and proliferation at IC50 and IC50x2 and IC50x4 of MESBAD

Experiment 8. Apoptotic protein expression pattern of methanol extract of the stem bark of *Adansonia digitata* (MESBAD) treated breast cancer cells (MCF-7).

Introduction: In higher organisms, cells belong to a community of organised system, where their number is tightly regulated either by cell division or by cell death. The cells that are no longer needed are programmed to commit suicide and die, that programmed cell death is reffered to as apoptosis. In a wide variety of physiological processes in adult tissues and during fetal development, apoptosis plays critical roles (Reed, 2000).

p53, also known as TP53 or tumor protein is a gene that codes for a protein that regulates the cell cycle and hence functions as a tumor suppression. It is very important for cells in multicellular organisms to suppress cancer. $p21^{Cip1}$ (alternatively $p21^{Waf1}$), also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, is a cyclin-dependent kinase inhibitor that inhibits the complexes of CDK2 and CDK1. This protein is encoded by the CDKN1A gene located on chromosome 6 (6p21.2) in humans.

There is a relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. In many cell types, p53-mediated growth inhibition is dependent on induction of p21, which is an inhibitor of cyclin-dependent kinases that are required for cell cycle progression. In this experiment, the expression pattern of both p53 and p21 were assessed by western blotting technique.

Procedure for Western blot analysis

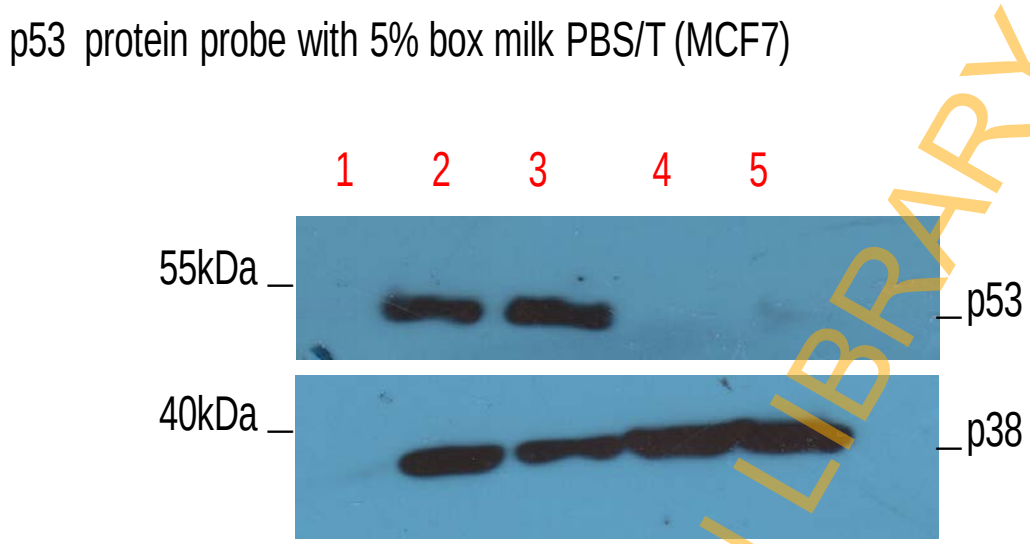
MCF-7 cells were washed twice with ice-cold PBS and collected by scraping with a 1 ml plunger. Whole cell extracts were prepared using 2X Laemmli sample buffer, boiled for 10 min and stored at -20°C. The protein concentration for each cell extract was determined using the BCA Protein Assay kit (Pierce, USA), with bovine serum albumin

as the standard. Equal amounts of protein were loaded in each lane and resolved on 6-15 % SDS-PAGE gels and then transferred electrophorectically to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). Membranes were blocked for 1 hour at room temperature with PBS containing 5% non-fat dry milk and probed with appropriate primary antibodies at 4°C with shaking. Membranes were washed in PBS containing 0.1% Tween 20 (PBS/T) and incubated with goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA, USA) in blocking solution at room temperature with shaking for 1 hour. Membranes were again washed in PBS/T and visualised by enhanced chemiluminescence (Pierce, USA). The following dilutions of these antibodies and dilutions were used: 1:1000 rabbit polyclonal anti-PARP1/2 (sc-7150), mouse monoclonal anti-p53 (sc-126), rabbit polyclonal anti-p21 (sc-756), rabbit polyclonal anti-p21 (sc-756), 1:5000 rabbit polyclonal anti-p38 (M0800) (Sigma, St. Louis, MO, USA).

Results:

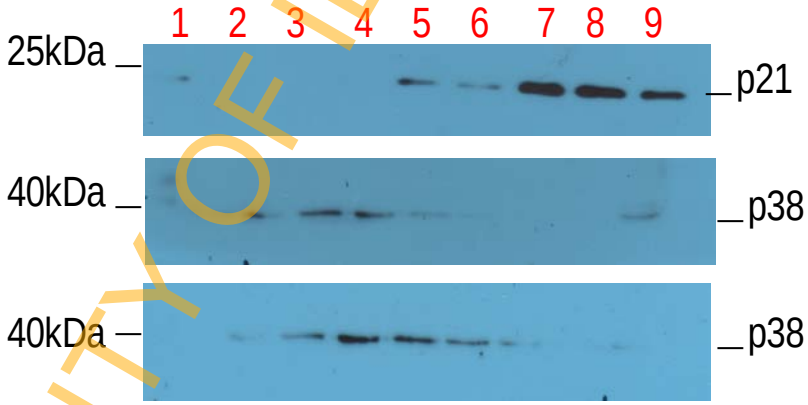
The expression pattern of PARP, LC3β, p53 and p21 were checked, in order to predict the mechanism of apoptosis of MESBAD. PARP cleavage was not achieved and LC3β was not expressed. p53 and p21 were however expressed (Plates 4.9 and 4.10). p53 levels were markedly increased at 24 and 48 hrs in the IC50x2 treated cells (Plate 4.9), while their p21 levels were also markedly increased at 24 hours and 48hrs of ½ of the IC50 and also at IC50 both at 24 and 48hrs treatment (Plate 4.10) suggesting a p53 independent induction of p21 in these cells. Together these results suggest that methanol extract of the stem bark of *Adansonia digitata* induced DNA damage.

Conclusion: The results revealed that the anticancer activity of MESBAD is p53 dependent at IC50 x 2. MESBAD up regulate the expression of p21 which is an early apoptotic response and also up regulate the expression of p53 in the 48h treated group while the PARP is not cleaved, suggesting that the anticancer activity of MESBAD may not be via the apoptotic pathway only.



1 = Treated with 0.1% DMSO. **2** = IC_{50} x2 (24h)
3 = IC_{50} x 2 (48h) **4**= IC_{50} x 4 (24h). **5** = IC_{50} x 4(48h)

Plate 4.9: Results of western blot analysis for apoptotic marker (p53)



1 = Treated with 0.1% DMSO **2** = IC_{50} x2 (24h)
3 = IC_{50} x 2 (48h) **4** = IC_{50} x 4 (24h). **5** = IC_{50} x 4(48h). **6** = 1/2 IC_{50} (24h). **7** = 1/2 IC_{50} (48h)
8 = IC_{50} (24h). **9** = IC_{50} (48h)

Plate 4.10: Results of western blot analysis for apoptotic marker (p21)**.**

CHAPTER FIVE

5.0: DISCUSSION

Cancer is a leading cause of death worldwide, deaths from cancer has continued to rise. Continuous exposure of humans to chemicals such as arsenic, through long-term ingestion of contaminated water and its attendant health problems has been widely reported (Chakraborti *et al.,* 2002; Arteel *et al.,* 2008; Jayanthika *et al*., 2001; Parvez *et al.,* 2006), also the cytotoxic effects on normal cells following administration of cyclophosphamide in the treatment of certain form of cancer has also been reported in literature (Baumann and Preiss, 2001; Hales, 1982; Zarei and Shivanandappa, 2013). Patients undergoing treatment with cyclophosphamide may also be exposed to *Adansonia digitata*. As a result, patients can be concomitantly exposed to both the drug and the medicinal plant and even sodium arsenite.

Carcinogenesis is frequently associated with DNA damage, changes in ploidy of cells and chromosomal aberrations which can all result from exposure to arsenic (Sinha *et al.*, 2005). Arsenic can act as co-mutagen due to its ability to inhibit the activities of thiol containing enzymes (Mazumder, 2005), such as DNA ligase (Chattopadhyay *et al.*, 2001), resulting in defective DNA replication, repair, recombination and joining of single- and double-stranded DNA breaks (Sunderman, 1984). Thus, counteraction of these toxicities and cytogenetic abnormalities with nutraceuticals and other dietary species could be a useful strategy towards solving the problem and by extension the menace of cancer.

Adansonia digitata also called baobab tree in both English and French is characteristic of the sahelian region, it belongs to the malvaceae family (De Caluwe *et al*., 2010). The plant is a very massive tree with a very large trunk (up to 10m diameter) which can grow up to 25m in height and may live for hundreds of years. Baobab tree has multi-purpose

uses and every part of the plant is reported to be useful (Igboeli *et al.*, 1997; Gebauer *et al.*, 2002). Previous reports revealed that the leaves, the seeds and the pulp from baobab are rich in nutrients (Becker, 1983; Glew *et al.*, 1997; Diop *et al.*, 2005; Nkafamiya *et al.*, 2007; Chadare *et al.*, 2009). The medicinal use of the plant include: anti-pyretic, analgesic, anti-inflammatory, antidote to poison etc. In the present study, we investigated the cytoremediatory effect of the aqueous extract of the fruit pulp of *Adansonia digitata* and methanol extract of the stem bark, on sodium arsenite and cyclophosphamide induced toxicities in rats and also their cytotoxic potentials on cancer cell lines.

The serum enzymes activities of γGT, AST, and ALT are used in the diagnosis of hepatic injuries and assessment of liver function and integrity. Elevation in the level of these enzymes is an indication of hepatic lesion or toxicity (Cornelius, 1979; Ideo *et al.*, 1972) and oxidative stress in the hepatocytes (Dinari *et al*, 1979).

Observation made in this study showed that administration of SA or CP resulted in significant ($p<0.05$) increase in the mean serum AST and ALT, and more than fourfold increase in γGT activities as compared with negative control group.This observation is consistent with earlier observations on the hepatotoxicity of SA (Mallick *et al*, 2003, Odunola *et al*, 2011) and of CP (Chakraborty *et al.*, 2009). Simultaneous treatment of AEFAD with SA or CP reduced the induction of γGT activities by about 3 fold and 2 fold, when compared with SA or CP treated groups, respectively. This further confirmed the hepatoprotective effect of the fruit pulp of *Adansonia digitata* earlier reported by Al-Qarawi *et al.*, (2003). Histological analyses of the liver samples corroborate the observations made with the enzyme markers of hepatotoxicity. There were no visible lesions in the control group. Diffuse portal and sinusoidal congestion were observed in SA or CP treated groups. There is very mild portal congestion in the 200mg/kg AEFAD group; there is cellular infiltration by mononuclear cells in the 400mg/kg AEFAD group.

There is a moderate portal and central venous congestion, also a mild periportal cellular infiltration by mononuclear cells in the groups administered both SA and AEFAD at 200mg/kg. There is severe portal congestion, with moderate periportal hepatic necrosis, mild fibroplasia and cellular infiltration by mononuclear cells in the SA with 400mg/kg AEFAD group. There is moderate portal congestion, and mild periportal hepatic necrosis and cellular infiltration by mononuclear cells in the group co administered with CP and AEFAD at 200mg/kg and finally, there is moderate portal congestion, and mild periportal hepatic necrosis and cellular infiltration by mononuclear cells in the group co administered with CP and AEFAD at 400mg/kg.

Studies have earlier implicated SA and CP to be genotoxic (Odunola, 2003, IARC, 1987). The effects of AEFAD on their genotoxicity were assessed using the bone marrow micronucleus induction assay, a predictive index for evaluating the clastogenic potential of environmental and occupational chemical exposure. Findings from the present study reaffirmed SA and CP clastogenicity, treatment with SA or CP alone induced formation of significant higher frequency of micronucleated polychromatic erythrocytes (mPCEs) in the bone marrow cells of rats when compared with the negative control. Environmental contaminants and clastogens are known to effect the formation of micronuclei by inducing chromosomal breaks and interfering with spindle formation (Eastmond and Tucker, 1989, Gudi *et al.*, 1990). In groups treated with AEFAD alone, there was no significant difference in the mean frequency of mPCES scored in the bone marrow cells when compared with the negative control. Treatment with AEFAD and SA or AEFAD and CP produced reduction in the frequency of mPCEs scored in the bone marrow cells of rats which compare well with the control group, administered distilled water only. This shows that AEFAD protect against SA and CP induced clastogenicity in rats.

The lipid peroxidation levels measured by malondialdehyde production, significantly increased in the SA or CP treated groups when compared with the negative control. This suggests increased generation of reactive oxygen species in the toxicant groups. Other scientists have reported the characteristic induction of lipid peroxidation by SA and CP in the liver, lung and serum of mice and rats with the observation linked with generation of reactive oxygen species (Yamanaka *et al*, 1990; El-Dermerdash *et al*, 2009; Morakinyo *et al*, 2010; Patel and Block, 1985; Venkatesan and Chandrakasan, 1995; Kaya *et al*., 1999; Lear *et al*., 1992; Matthew and Kuttan, 1997; Premkumar *et al*., 2001). The malondialdehyde level observed in the groups treated with extract and SA or CP was appreciably lower when compared with the SA or CP treated groups.

In addition, treatment with SA or CP produced increased hepatocellular proliferation when compared with the control, suggesting that the toxicants are able to promote hepatocellular proliferation. The induction of increased liver cell populations in the exposed group may be an adaptation by the rats to get rid of the toxicants. AEFAD ameliorated the increased cellular proliferation, producing significant reduction in the number of cells scored per mm^2 when compared with the intoxicated groups. The administration of the extract and exposure to toxicants did not have effect on the values of some hematological parameters examined, but exposure to CP caused a reduction in PCV level. There was also an increase in the WBC count of the SA treated group as compared with control; SA may therefore be eliciting immune response. The increased WBC count was not restored even with the administration of AEFAD. Other parameters were neither quantitatively nor qualitatively affected and there were no inclusions in the red cells or white cells as observed from the cell morphology. The hemoglobin analysis is not in agreement with the earlier report by Chakraborty *et al*. (2009), who observed a decrease in the hemoglobin level in cyclophosphamide treated rats.

The results suggest that AEFAD has the capacity to ameliorate sodium arsenite- and cyclophosphamide-induced toxicities in male wistar rats. Moderate consumption of AEFAD may play an important role in human hepatoprotection and resistance to liver damage. AEFAD supplementation may possibly influence the response to chemotherapy and ameliorate/reduce development of adverse effects that result from the treatment of cancer with cyclophosphamide in patients.

The antioxidant activities and cytotoxic effect of AEFAD and its fractions were also investigated *in vitro*. DPPH, ABTS, nitrite scavenging capacity and reducing antioxidant power assay are widely used to determine the antioxidant capacity of plant extracts due to their simplicity, stability, and reproducibility. In this study, the DPPH, ABTS, nitrite scavenging capacity and reducing antioxidant power assay provided comparable results for the antioxidant capacity measured in AEFAD and its fractions.

The DPPH radical scavenging capacity of all the fractions from AEFAD increased, the IC50 value of NBF has the closest IC50 (43.44 µg/ml) to that of the standard (27.22 µg/ml), while the n-hexane fraction has the highest IC50 value (140.72 µg/ml). Therefore, NBF showed the highest DPPH radical scavenging activity. For the ABTS assay, the NBF fraction also demonstrated the highest scavenging activity of all the fractions tested with IC50 value of (38.28 µg/ml), as compared to the standard (7.41 µg/ml), followed by the EAF, CAQ and CRF. For the nitrite scavenging capacity, Ethyl acetate fraction gave the IC50 value (66.05 µg/ml) closest to the standard (35.52 µg/ml). In the reducing power assay the more antioxidant compounds convert the oxidation form of iron (Fe+3) in ferric chloride to ferrous (Fe+2) in the results of this research, the n-butanol fraction showed the highest antioxidant content when compared with other solvent fractions. Fruit pulp of *Adansonia digitata* extract and its fractions exhibited good antioxidant activities against various oxidative systems *in vitro*.

In order to evaluate the cytotoxic effects of AEFAD fractions, cytotoxicity studies were carried out with A-549 Human Lung carcinoma cell line, MCF-7 Human Breast cancer cell line, KB Human Oral Cancer Cell Line, T-24 Human Bladder Cancer Cell Line and A-498 Human Renal Cancer Cell Lines. Elsaid, (2013) previously reported the antitumor effects of the extracts of seeds and fruit pulp of *Adansonia digitata* on Ehrlich Ascites Carcinoma. The cell lines were exposed to various sample concentrations (10, 20, 40, or 80 µg/mL). In the present study, cytotoxicity studies results using Sulphurhodamin B (SRB) assay clearly demonstrated that AEFAD and its fractions did not induce significant cytotoxic effects on the various cancer cell lines studied at the various concentrations tested. However, the results of the phase contrast micrographs showed that the CRF, EAF and NBF (at 80µg/mL) inhibited some level of cell proliferation on the KB cell line, these were exhibited by some characteristic apoptotic morphological changes which are similar to the ones observed in the positive control. These results suggest that CRF, EAF and NBF of AEFAD may possess antiproliferative effects on KB cell line at higher concentration and may be subsequently used in the treatment of oral cancer.

The spectrum profile of GC-MS confirmed the presence of 7 major components, which are Pentadecanoic acid, Hexadecanoic Acid, 11-Octadecenoic acid, Octadecanoic acid, Oleic acid, Nonadecanoic acid, 3,11-Tetradecadiene-1-ol. The antitumor effects of fatty acids such as hexadecanoic acid obtained from plant extracts had earlier been reported by Lai *et al.*, (2008).

The antioxidant and some observable antiproliferative activities of AEFAD fractions may be the result of the synergistic effects of various compounds present in the extract which suggests that AEFAD can be used as a source of antioxidant and possibly anticancer

agent at a higher dose and could be of significance in the food industry and for the control of various human and animal diseases.

The quantitative measurement of nuclear DNA content of a cell at high speed by flow cytometry, employing the use of a fluorescent dye that binds stoichiometrically to the DNA has been used as an index for the measurement of cytotoxicity. Some anticancer agents exert cytotoxicity by inducing DNA double strand breaks (DSBs) leading to cell cycle arrest and cell death (Ulakaya *et al.*, 2011; Tanaka *et al.*, 2013). To investigate the effects of the various fractions of AEFAD on breast and lung cancer cell growth, their effects on the cell cycle profile were investigated.

The study indicates that AEFAD fractions did not show significant G0/G1 phase arrest as compared with control. However, there is increase in apoptosis at 50µg/ml of AF, EAF, NBF and a significant 7 fold increase in apoptosis at 50µg/ml NHF. Sub- G1 peaks, generally accepted to represent dead cells (Rao *et al.*, 2011), were present in breast cancer cell lines treated with NBF which corroborates its antiproliferative activity observed on KB.

Also, the effects of the fractions of AEFAD on A-549 cell cycle distribution was also examined, the results indicate that there was a G0/G1 phase arrest at 50 and 100 µg/ml in the aqueous fraction treated cells, but this was not significant when compared with the control and also not concentration dependent, there was G0/G1 arrest in the NHF treated group at both 50 and 100 µg/ml. NHF at 50µg/ml had a profound apoptotic effect on breast cancer cell line and also arrested the lung cancer cell line at the G0/G1 phase.

The hepatoprotective and anticancer potentials of the methanol extract of the stem bark of *Adansonia digitata* (MESBAD) were also investigated. The various health benefits

offered by plants are tremendous and these have been in a way linked to the several phytochemicals which they possess, since secondary metabolites and other constituents in plants have been reported to be responsible for their medicinal properties (Hill, 1952). The MESBAD was therefore subjected to the phytochemical screening test using Trease and Evans, and Harbourne methods for the determination of flavonoids, alkaloids, tannins, anthraquinoines, saponins e.t.c. In this study, various phytochemicals and bioactive compounds present in MESBAD were analyzed. The outcome of this analysis revealed the presence of alkaloids in abundance, also saponins, flavonoids and polyphenols in an appreciable amount. Some of these phytochemicals have been found in many plants and had been reportedly used for medicinal purposes. The concentration of some phytochemicals in MESBAD is in this order:- Alkaloids > Saponins > Flavonoids > Total Phenol > Tanins.

The liver and body weights of the treated rats were taken and compared, to establish the direct effect of the treatments on the liver and body weights, there was no significant effect on the liver and relative liver weights of the treatment groups across board, when compared with the control group, which was administered distilled water only and the group administered the toxicant, SA. Serum AST activity in Group 6 was not higher when compared with the negative control but γGT activities was higher, while ALP activity was significantly higher when compared with the negative control, this shows the hepatotoxicity of sodium arsenite. Administration of the extract alone at both 300 and 400 mg/kg body weight did not produce any significant increase in the activities of the enzymes except in the AST activities in the group administered 400mg/kg MEBAD. Co administration of the MESBAD with SA significantly reduced the elevated activities of AST and ALP, this portrays the hepatoprotective properties of MESBAD. The histological studies also support the liver function enzyme assays by presenting a clearer

description of the hepatotoxicity of SA and the hepatoprotective role of MESBAD. The antigenotoxic potential of MESBAD was also explored by conducting the micronucleus induction assay in the rat bone marrow cells. Administration of SA significantly increased the frequency of mPCEs in the bone marrow of rats by about 6 folds when compared with the control while co-administration of the extract and SA at 300 mg/kg body weight gave a sharp decrease in the frequency of mPCEs by about 3 folds, when compared with SA only group, suggesting the anticlastogenic potential of MESBAD. Therefore, MESBAD has the potential to mitigate or remediate toxicities resulting from SA intoxication in rats, and it may offer protection in circumstances of co-exposure and cases of arsenicosis. The phytochemical constituents may be responsible for the hepatoprotective and antigenotoxic/anticlastogenic activities noticed in this experiment.

The traditional use of the of the stem bark of *Adansonia digitata* include among other uses, the treatment of cancer, and to date there is no scientific data to validate its use. The cytotoxic potential of MESBAD was therefore investigated on MCF-7 breast cancer cells. The MESBAD shows a dose dependent cytotoxic effect on MCF-7 cell line, after three consecutive experiments. The higher the concentration of MESBAD, the higher the cytotoxic effect and *vice versa*, as shown by the intensity of the purple formazan formed after the addition of the MTT salt to the cells. This experiment revealed that MESBAD possess anticancer activity on MCF-7 cell line. Cytotoxicity of MESBAD is concentration dependent, the higher the concentration, the higher the toxicity induced by MESBAD.

In order to further establish the short term cytotoxic effect of MESBAD noticed, clonogenic cell survival assay was employed to evaluate its long term antiproliferative potential on MCF-7 cells. The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large

colony or a clone. The ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce; this is what is determined by the clonogenic assay. MESBAD caused a clear reduction in the proliferation of MCF-7 cells when compared with the control that was treated with DMSO, the antiproliferative activity is dose dependent as seen in the number of colonies present in the cells treated with ½ IC50 and IC50. In order to be sure of the reproducibility of the observed effect, multiples of the IC50 was used to check the possibility of complete disappearance of colonies. The result presented a similar pattern as the first with the cells treated with IC50x4 having just scanty colonies. MESBAD shows a dose dependent long term antiproliferative effect on MCF-7 breast cancer cells.

The mechanism by which MESBAD exert its cytotoxic activity was therefore the next point of call for investigation. The expression pattern of certain apoptotic markers (PARP, LC3β, p53 and p21) were examined. PARP cleavage is one of the major characteristics of apoptosis, but in the study, PARP was not achieved and LC3β was not expressed, p53 and p21 were however expressed. There was a robust p53 response in MCF7 cells, which in general correlated with an increase in levels of the cell cycle regulator p21, p53 levels were markedly increased at 24 and 48hrs in the IC50x2 treated cells while their p21 levels were also markedly increased at 24 hours and 48hrs of ½ of the IC50 and also at IC50 both at 24 and 48hrs treatment, suggesting a p53 dependent induction of p21 in these cells. These suggest that MESBAD induced DNA damage and therefore bring about a p53 dependent apoptosis. The results suggest that the anticancer activity of MESBAD is p53 dependent at IC50 x 2, owing to its up regulation. MESBAD up regulate the expression of p21 which is an early apoptotic response and also up regulate the expression of p53 in the 48h treated group suggesting that the anticancer activity of MESBAD may not be entirely via the apoptotic pathway.

CHAPTER SIX

6.0 SUMMARY OF FINDINGS / CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE.

6.1 Summary / Conclusion

Aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD) remediate sodium arsenite- and cyclophosphamide-induced toxicities in male wistar rats. Consumption of AEFAD may play an important role in human hepatoprotection and resistance to genotoxicity.

AEFAD antioxidant supplementation may influence the response to chemotherapy and ameliorate/reduce development of adverse effects that result from the treatment of cancer with cyclophosphamide in patients.

N-hexane fraction of AEFAD induced apoptosis at the sub G0 phase of the cell cycle in MCF-7 breast cancer cells. Also, the aqueous and n-hexane fractions of AEFAD induced cell cycle arrest at the G0/G1 phase of the A-549 lung cancer cells.

Methanol extract of the stem bark of *Adansonia digitata* (MESBAD) is hepatoprotective, and anticlastogenic, moderate consumption of MESBAD will play an important role in human hepatoprotection and resistance to genotoxicity.

Methanol extract of the stem bark of *Adansonia digitata* also possess both short term and long term anticancer properties against MCF-7 breast cancer cell line, the mechanism of anticancer activity is due to the up regulation of p53 and p21 genes.

6.2 Contributions to Knowledge

Scientific evidence emanating from this thesis shows the following:

1. Aqueous extract of the fruit pulp and methanol extract of the stem bark of *Adansonia digitata* are antigenotoxic on sodium arsenite induced genotoxicity.

2. Aqueous extract of the fruit pulp and methanol extract of the stem bark of *Adansonia digitata* are hepatoprotective

3. Aqueous extract of the fruit pulp of *Adansonia digitata* reduced the cytotoxic effects resulting from the administration of cyclophosphamide.

4. The N-butanol fraction of the aqueous extract of the fruit pulp of *Adansonia digitata* possesses the highest antioxidant content.

5. Methanol extract of the stem bark of *Adansonia digitata* up regulate the expression of p53 and p21 in MCF-7 cell line

6. Methanol extract of the stem bark of *Adansonia digitata* has a dose dependent antiproliferative effects on MCF-7 breast cancer cell line

7. The anticancer activity of methanol extract of the stem bark of *Adansonia digitata* (MESBAD) is p53 dependent.

REFERENCES

Adam-vizi, V. and Seregi, M. 1982. Receptor dependent stimulatory effect of nor-adrenaline on Na+, K+-ATPase in rat brain homogenate: Role of lipid peroxidant. *Biochemical Pharmacology* 31: 2231-2236.

Adebamowo, C. A. and Ajayi, O. O. 2000. Breast Cancer in Nigeria. *West African Journal of Medicine* 19: 179-194.

Adegoke, A. M., Gbadegesin M. A., Otitoju, A. P. and Odunola, O. A. 2015. Hepatotoxicity and Genotoxicity of Sodium Arsenite and Cyclophosphamide in Rats: Protective Effects of Aqueous Extract of *Adansonia digitata* L. fruit Pulp. *British Journal of Medicine & Medical Research* 8(11): 963-974.

Aitken, R. J. 1994. A free radical theory of male infertility. *Reproduction Fertility and Development* 6: 19– 23.

Aitken, R. J. 1995. Free radicals, lipid peroxidation and sperm function. *Reproduction Fertility and Development.* 7: 659 -68.

Aitken, R. J. and Fisher, H. 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioassays* 16: 259–267.

Ajose, F. O. 2007. Some Nigerian plants of dermatologic importance. *International Journal of Dermatology* 46 (suppl 1): 48-55.

Albores, A., Cebrian, M. E., Bach, P. H. and Connelly, J. C. 1989. Sodium arsenite-induced alteration in bilirubin excretion and heme metabolism. *Journal of Biochemical Toxicology* 4: 73-78.

Aller, M. A., Ortega, L., Sánchez-Patán, F., Anchuelo, R., Cruz, A., Losada, M. and Arias, J. 2008. Microsurgical Extrahepatic Cholestasis in the Rat: A Histopathological Liver Study. *The Open Pathology Journal* 2:71-77.

Al-Qarawi, A. A., Al-Damegh, M. A. and El-Mougy, S. A. 2003. Hepatoprotective Influence of *Adansonia digitata* Pulp. *Journal of Herbs, Spices & Medicinal Plants* 10(3): 1-6.

Amadi, B. A., Agomuo, E. N. and Ibegbulem, C. O. 2004. Research Methods in Biochemistry. *Supreme Publishers, Owerri*. 90-115.

American Cancer Society, 2013: Cancer Facts & Figures. Atlanta:1–60.

American Cancer Society, 2016: Cancer Facts & Figures. Atlanta:1–66.

Ames, B. N., Gunney, E. G., Miller, J. A. and Bartsch, H. 1972. Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* (*PNAS)* 69: 3128-3132.

Arteel, G. E., Guo, L., Schlierf, T., Beier, J. I., Kaiser, J. P., Chen, T. S., Liu, M., Conklin, D. J., Miller, H. L., von Montfort, C. and States, J.C. 2008. Subhepatotoxic exposure to arsenic enhances lipopolysaccharide-induced liver injury in mice. *Toxicology and Applied pharmacology* 226 (2): 128–139.

ASHP (American Society of Health-System Pharmacists). 2016. Cyclophosphamide. Archived from the original on 2 January 2017. Retrieved 8 December, 2016.

ATSDR (Agency for Toxic Substances and Disease Registry). 2007. Toxicological Profile for arsenic. Update. Atlanta, GA: U.S. *Department of Health and Human Services, Public Health Service.*

Bagwell, C. B. 1993. Theoretical aspects of data analysis. In: *Clinical Flow Cytometry Principles and Application.* Ed Bauer, K. D., Duque, R. E and Shankey, T. V. 41-61.

Baker, A., Payne, C. M., Briehl, M. M. and Powis, G. 1997. Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis *in vitro* and *in vivo*. *Cancer Research* 57: 5162-5167.

Bao, J. S., Cai, Y., Sun, M., Wang, G. and Corke, H. 2005. Anthocyanins, flavonols, and free radi-cal scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. *Journal of Agricultural and Food Chemistry* 53: 2327-2332.

Bauer, K. B. and Jacobberger, J. W. 1994. Analysis of intracellular proteins. In: *Methods in Cell Biology*: *Flow Cytometry* 41, 2nd edition, eds. Darzynkiewicz Z., Crissman H. A., Robinson J. P. 351-376.

Baumann, F. and Preiss, R. 2001. Cyclophosphamide and related anticancer drugs. *Journal of Chromatography B: Biomedical Sciences and Applications* 764: 173-192.

Becker, B. 1983. The contribution of wild plants to human nutrition in the Ferlo (Northern Senegal). *Agroforestry Systems* 1: 257-267.

Bishop, J. 1995. Cancer: the rice of the genetic paradigm: *Genes and Development* 9(11): 1309-1315.

Blois, M. S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 181: 1199-1200.

Blomhoff , R., Carlsen, M., Halvorsen, B., Holte, K., Bohn, S., Dragland, S., Sampson, L., Willey, C., Senoo, H., Umezono, Y., Sanada, C., Barikmo, I., Berhe, N., Willett, W., Phillips, K. and Jacobs, D. 2010. The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. *Nutrition Journal* 9 (1): 1-11.

Bosch, C. H., Sié, K., Asafa, B. A. 2004. *Adansonia digitata* L. Record from Protabase. Grubben, G. J. H. and Denton, O. A. (Editors). PROTA (*Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale)*, Wageningen, Netherlands.

Bukowski, R. 1999. The need for cytoprotection. *European Journal of Cancer* 32A: S2-S4.

Cai, Y., Wu, M. H., Ludeman, S. M., Grdina, D. J., Dolan, M. E. 1999. Role of O^6-alkylguanine-DNA alkyltransferase in protecting against cyclophosphamide-induced toxicity and mutagenicity. *Cancer Research* 59: 3059–3063

Calesnick, B. Wase A. Overby L. R. 1966. Availability during human consumption of the arsenic in tissues of chicks fed arsanilic- As acid. *Toxicology and Applied Pharmacology* 9(1): 27–30.

Campbell, F. Aug. 15, 2008. Arsenic-loving Bacteria Rewrite Photosynthesis rules. *Chemistry World.* Retrieved Nov. 12, 2012, from https://www.chemistryworld.com/news/arsenic-loving-bacteria-rewrite photosynthesis-rules/3000398.article

Celis, J. E. 1998. Cell biology: A laboratory handbook, 2nd edition, *Academic Press, San Diego, California* 1.

Chabner, B. A., Ryan, D. P., Paz-Ares, L., Garcia-Carbonero, R., Calabresi, P. 2001. Antineoplastic agents. In Goodman & Gilman's. *The Pharmacological Basis of Therapeutics* 10th ed.. Eds. Hardman, J. G and Limbird, L. E. New York: McGraw Hill 1389-1459.

Chadare, F. J., Linnemann, A. R., Hounhouigan, J. D., Nout, M. J. R., Van Boekel, M. A. J. S. 2009. Baobab Food Products: A Review on their Composition and Nutritional Value. *Critical Reviews in Food Science and Nutrition* 49: 254-274.

Chakraborti, D., Rahman, M. M., Paul, K., Sengupta, M. K., Chowdhury, U. K., Lodh, D., Chanda, C. R., Saha, K. C., Mukherjee, S. C. 2002. Arsenic calamity in India and Bangladesh sub-continent-whom to blame? *Talanta* 58: 3-22.

Chakraborty, P., HossainSk, U., Murmu, N., Kumar Das, J., Pal, S. and Bhattacharya, S. 2009. Modulation of Cyclophosphamide-Induced Cellular Toxicity by DiphenylmethylSelenocyanate *In vivo*, an Enzymatic Study. *Journal of Cancer Molecules* 4(6): 183-189.

Chang, T. K., Weber, G. F., Crespi, C. L., Waxman, D. J. 1993. Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Research* 53: 5629-5637.

Chayen, J., Altman, F. P., Butcher, R. G. 1973. The effect of certain drugs on the production and possible utilization of reducing equivalents outside the mitochondria. In: Dikstein S (ed) *Fundamentals of cell pharmacology*. Charles C. T., Springfield, Ill: 196–230.

Chen, C. S., Lin, J. T., Goss, K. A., He, Y. A., Halpert, J. R., Waxman, D. J. 2004. Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics. *Molecular Pharmacology* 65: 1278-1285.

Cohen, S. M., Garland, E. M., St John, M., Okamura, T. and Smith, R. A. 1992. Acrolein initiates rat urinary bladder carcinogenesis. *Cancer Research* 52: 3577–3581.

Colvin, O. M. 1999. An overview of cyclophosphamide development and clinical applications. *Current Pharmaceutical Design* 5: 555-560.

Connor, A. M., Luby, J. J., Tong, C. B. S. 2002. Variability in antioxidant activity in blueberry and correlations among different antioxidant activity assays. *Journal of American Society for Horticultural Science* 127: 238-244.

Cornelius, C. E. 1979. Biochemical evaluation of hepatic function in dogs. *Journal of the American Animal Hospital Association* 5: 25–29.

COX, P. J. 1979. Cyclophosphamide cystitis-Identification of acrolein as the causative agent. *Biochemical Pharmacology* 28: 2045-2049.

Crocitto, L. E, Simpson, J. F, Wilson, T. G. 1996. Bladder augmentation in the prevention of cyclophosphamideinduced haemorrhagic cystitis in the rat model. *British Journal of Urology* 78: 530–533.

Das, U. B., Mallick, M., Debnath, J. M., Ghosh, D. 2002. Protective effect of ascorbic acid on cyclophosphamide-induced testicular gametogenic and androgenic disorder in male rats. *Asian Journal of Andrology* 4: 201-207.

De Caluwé, E., Halamová, K., Van Damme, P. 2010. *Adansonia digitata* L. – A review of traditional uses, phytochemistry and pharmacology. *Afrika focus* 23: 11-51

De Caluwé, E., Halamová, K., Van Damme, P. 2010. *Adansonia digitata* L. – A review of traditional uses, phytochemistry and pharmacology. *Afrika focus* 23: 11-51.

Decker, E. A. 1998. Antioxidant mechanisms. In: Akoh, C. C., Min, D. B. editors. Food lipids, chemistry, nutrition, and biotechnology. New York: Marcel D. P. 397–401.

Decker, E. A., Chan, W. K. M., Livisay, S. A., Butterfield, D. A., Faustman, C. 1995. Interactions between carnosine and the different redox states of myoglobin. *Journal of Food Science* 60: 1201-1204.

DeLeve, L. D. 1996. Cellular target of cyclophosphamide toxicity in the murine liver: role of glutathione and site of metabolic activation. *Hepatology* 24: 830-837.

Dhar, R. K., Biswas, B. K., Samanta, G., Mandal, B. K., Chakraborti, D., Roy, S., Jafar, J., Islam, A., Ara, G., Kabir, S., Khan, A. W., Ahmed, S. A., Hadi, A. A. (1997). Groundwater arsenic calamity in Bangladesh. *Current Science* 73(1): 48-59.

Dinari, G., Cohen, M. I. McNamara, H. and Kochen, J. A. 1979. Effect of lead exposure on the activity of some hepatic enzymes in rat. *Pediatric Research* (5Ptl*):* 644-646.

Diop, A. G., Sakho, M., Dornier., M., Cisse, M., Reynes, M. 2005. Le baobab africain (*Adansonia digitata L.*): principales caractéristques et utilisations. *Fruits* 61: 55-69.

Dirven, H. A., Venekamp, J. C., van Ommen, B. and van Bladeren, P. J. 1994. The interaction of glutathione with 4-hydroxycyclophosphamide and phosphoramide mustard, studied by 31P nuclear magnetic resonance spectroscopy. *Chemico-Biological Interactions* 93: 185-96.

Done, A. K., Peart, A. J. 1971. Acute toxicities of arsenical herbicides. *Clinical Toxicology* 4: 343–355.

Eastmond, D. A. Tucker, J. D. 1989. Identification of aneuploidy inducing agents using Cytokinesis blocked human lymphocytes and antikinetochore antibody. *Environmental and Molecular Mutagenesis* 13: 34-43.

Ejikeme, C. M., Ezeonu, C. S. and Eboatu, A. N. 2014. Determination of physical and phytochemical constituents of some tropical timbers indigenous to Niger Delta Area of Nigeria. *European Scientific Journal* 10(18): 247–270.

El- Demerdash, F. M., Yousef, M. I., Radwan, F.M.E. 2009. Ameliorating effect of curcumin on sodium arsenite induced oxidative damage and lipid peroxidation in different rat organs. *Food and Chemical Toxicology* 47: 249-254.

Ellenhorn, M. J. 1997. Ellenhorn's medical toxicology. *Diagnosis and treatment of human poisoning*. Baltimore, MD: Williams & Wilkins: 1538-1542.

Elmore, S. 2007. Apoptosis: A review of programmed cell death. *Toxicologic Pathology* 35: 495–516.

Elsaid, F. G. 2013. The Effect of Seeds and Fruit Pulp of *Adansonia digitata L*. (Baobab) on Ehrlich Ascites Carcinoma. *Food and Nutrition Sciences* 4: 38-46.

Etkin, N. L. and Ross, P. J. 1982. Food as medicines and medicines as food. *Social Science & Medicine 16: 1559-1573.*

Fasola, T. R., Adeyemo, F. A., Adeniji J. A. and Okonko, I. O. 2011. Antiviral Potentials of Enantia chlorantha Extracts on Yellow Fever Virus. *Nature and Science* 9(9): 99-105.

Fenselau, C., Kan, M. N., Rao, S. S., Myles, A., Friedman, O. M. and Colvin, M. 1977. Identification of aldophosphamide as a metabolite of cyclophosphamide *in vitro* and *in vivo* in humans. *Cancer Research* 37: 2538-2543.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C. D., Parkin, D. GLOBOCAN 2008. Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10. Lyon, France: *International Agency for Research on Cancer;* Year. Available at: http://globocan.iarc.fr. 2010.

Forni, A. M., Koss, L. G., Geller, W. 1964. Cytological study of the effect of cyclophosphamide on the epitheliumof the urinary bladder in man. *Cancer* 17: 1348–1355.

Fraiser, L. H., Kanekel, S., Kehrer, J. P. (1991). Cyclophosphamide toxicity; characterizing and avoiding the problem. *Drugs* 42: 781-795.

Freshney, R. I. 2000. Culture of Animal Cells: A Manual of Basic Techniques, 4th edn, New York, NY, USA*:* John Wiley & Sons. 486.

Friedman, H. S., Pegg, A. E., Johnson, S. P., Loktionova, N. A., Dolan, M. E., Modrich, P., Moschel, R. C., Struck, R., Brent, T. P., Ludeman, S., Bullock, N., Kilborn, C., Keir, S., Dong, Q., Bigner, D. D. and Colvin, O. M. 1999. Modulation of cyclophosphamide activity by O^6-alkylguanine-DNA alkyltransferase. *Cancer Chemotherapy and Pharmacology* 43: 80–85.

Gamcsik, M. P., Dolan, M. E., Andersson, B. S., Murray, D. 1999. Mechanisms of resistance to the toxicity of cyclophosphamide. *Current Pharmaceutical Design* 5: 587-605.

Gbadegesin, M. A., Adegoke, A. M., Ewere, E. G. and Odunola O. A. 2014. Hepatoprotective and anticlastogenic effects of ethanol extract of Irvingia gabonensis (IG) leaves in sodium arsenite-induced toxicity in male Wistar rats. *Nigerian Journal of Physiological Sciences* 29: 29-36.

Gbadegesin, M. A., Odunola, O. A., Akinwumi, K. A. and Osifeso O. O. 2009. Comparative hepatotoxicity and clastogenicity of sodium arsenite and three petroleum products in experimental Swiss Albino Mice: The modulatory effects of Aloe vera gel. *Food and Chemical Toxicology*. 47(10): 2454-2457

Gebauer, J., El-Siddig, K., Ebert, G. 2002. Baobab (Adansonia digitata L.): A Review on a Multipurpose Tree with Promising Future in the Sudan. *Gartenbauwissenschaft* 67: 155-160.

Germolec, D. R., Spalding, J., Yu, H. S., Chen, G. S., Simeonova, P. P., Humble, M. C., Bruccoleri, A., Boorman, G. A., Foley, J. F., Yoshida, T., Luster, M. I. 1998. Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. *American Journal of Pathology* 153(6): 1775-1785.

Glew, R. H., VanderJagt, D. J., Lockett, C., Grivetti, L. E., Smith, G.C., Pastuszyn, A., Millson, M. 1997. Amino Acid, Fatty Acid and Mineral Composition of 24 Indigenous Plants of Burkina Faso. *Journal of Food Composition and Analysis* 10: 205-217.

Green, D. R. 2011. Means to an End: Apoptosis and Other Cell Death Mechanisms. Cold Spring Harbour (New York): *Cold Spring Harbour Laboratory Press.* 220.

Greene, M. H., Harris, E. L., Gershenson, D. M., Malkasian, G. D., Melton, L. J., Dembo, A. J., Bennett, J. M., Moloney, W. C. and Boice, J. D. 1986. Melphalan may be a more potent leukemogen than cyclophosphamide. *Annals of Internal Medicine* 105: 360–367.

Griveau, J. F., Dumont, E., Renard, P., Callegari, J. P. and Le Lannou, D. 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *Journal of Reproduction and Fertility* 103: 17-26.

Gruenwald, J. 2009. Novel botanical ingredients for beverages. *Clinics in Dermatology* 27(2): 210-216.

Gruenwald, J. and Galizia, M. 2005. *Adansonia digitata L.* Market Brief in the European Union for selected natural ingredients derived from native species; The United Nations Conference on Trade and Development (UNCTAD).

Gudi, R. D., Sandhu, S. S. and Athwal, S. R. 1990. Kinetochore identification in Micronuclei in mouse bone marrow erythrocytes. An assay for detection of aneuploidy-inducing agent. *Mutation Research* 234: 263-268.

Guha Mazumder, D. N., Haque, R., Ghosh, N., De, B. K., Santra, A., Chakraborty, D. and Smith, A. H. 1998. Arsenic levels in drinking water and the prevalence of skin lesions in West Bengal, India. *International Journal of Epidemiology* 27: 871-7.

Gullen, N. M., Wolf, L. R., St. Clair, D. 1995. Pediatric Arsenic Ingestion. *American Journal of Emergency* 13: 432-435.

Gulubova, M., Vlaykova, T., Kalinova, K., Vodenicharov, A., Vasilev, I. and Velev, V. 2005. Liver tryptase-positive mast cells and fibrosis in children with hepatic echinococcosis. *Journal of Indian Association of Pediatric Surgery* 10(4): 237-243.

Haas, J. F., Kittelmann, B., Mehnert, W. H., Staneczek, W., Möhner, M., Kaldor, J. M. and Day, N. E. 1987. Risk of leukaemia in ovarian tumour and breast cancer patients following treatment by cyclophosphamide. *British Journal of Cancer* 55: 213–218.

Hales, B. E. 1982. Comparison of the mutagenicity and teratogenicity of cyclophosphamide and its active metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard and acrolein. *Cancer Research* 42: 3016-3021.

Halliwel, B. 1990. How to characterize a biological antioxidant. *Free radical Research Communications* 9:1-32

Halliwell, B., Murcia, M. A., Chirico, S. And Aruoma, O. I. 1995. Free radicals and antioxi-dants in food and *in vivo*: what they do and how they work. *Critical Reviews in Food Science and Nutrition* 35: 7–20.

Handa, S. S., Khanuja, S. P. S, Longo G. and Rakesh D. D. 2008. Extraction Technologies for Medicinal and Aromatic Plants. International Centre for Science and High Technology Trieste. 21-25.

Haque, R., Bin-Hafeez, B., Ahmad, I., Parvez, S., Pandey, S. and Raisuddin, S. 2001. Protective effect of Emblica officinalis Gaertn in cyclophosphamide treated mice. *Human Experimental Toxicology* 20: 643-650.

Harborne, J. B. 1973. Methods of plant analysis. In: *Phytochemical Methods.* Chapman and Hall, London.

Heddle, J. A. and Salmone, M. F. 1981. The micronuclei assay: *In vivo*. In Stich, H.F. and San, R.H.C. eds., *Topics in environmental physiology and medicine*. Short term tests for chemical carcinogens. Springer-Verlag, New York Heidelberg, Berlin: 243-249.

Heddle, J.A. 1973. A rapid *in vivo* test for chromosomal damage. *Mutation Research* 18: 187-190.

Hill, A. F. 1952. Economic Botany. A textbook of useful plants and plant products. 2nd edn. McGraw-Hill Book Company Inc, New York USA.

Hohorst, H. J., Draeger, U., Peter, G. and Voelcker, G. 1976. The problem of oncostatic specificity of cyclophosphamide (NSC-26271): Studies on reactions that control the alkylating and cytotoxic activity. *Cancer Treatment Reports* 60: 309-315.

Huang, Z., Roy, P. and Waxman, D. J. 2000. Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide. *Biochemical Pharmacology* 59: 961-72.

IARC (International Agency for Research on Cancer) Acrylamide. 1994. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, some industrial chemicals.* 60: 389–433

IARC (International Agency for Research on Cancer). 1975. Cyclophosphamide. In Some Aziridines, N-, S-, and O-Mustards and Selenium. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* 9*:* 135-156.

IARC (International Agency for Research on Cancer). 1980. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Metals and Metallic Compounds. 23: 39–141.

IARC (International Agency for Research on Cancer). 1981. Some antineoplastic and immunosuppressive agents. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* 26: 1–411.

IARC (International Agency for Research on Cancer). 1987. Overall evaluations of carcinogenicity: An updating of IARC Monographs volumes 1 to 42. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* 7: 1–440.

IARC (International Agency for Research on Cancer). 1995. Dry cleaning, some chlorinated solvents and other industrial chemicals. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* 63: 1–551.

Ideo, G. A., Morganti, A. and Dioguardi, N. 1972. Gamma-glutamyl transpeptidase: A clinical and experimental study. *Digestion* 5: 326–336.

Igboeli, L. C., Addy, E.O.H. and Salami, L. I. 1997. Effects of some processing techniques on the antinutrient contents of baobab seeds (*Adansonia digitata*). *Bioresource Technology* 59: 29-31.

Ivankovic, S., Eisenbrand, G. and Preussmann, R. 1979. Lung carcinoma induction in BD rats after a single intratracheal instillation of an arsenic-containing pesticide mixture formerly used in vineyards. *International Journal of Cancer* 24:786-788.

Jackson, S. 2015. Baobab: The Tree of Life – An Ethnopharmacological Review. *The Journal of the American Botanical Council* 108: 42-53.

Jacobson-Kram, D. and Montalbano, D. 1985. The reproductive effects assessment group's report on the mutagenicity of inorganic arsenic. *Environmental Mutagenesis* 7: 787-804.

Jane, N.C. 1986. Schalm's Veterinary Haematology (Jain, N.C., ed) 4th edition, Lea and Ferbiger, Philadelphia.

Jayanthika, B. W., Gandolfi, A. J., Alan, P. and Lantz, R.C. 2001. Sodium Arsenite Enhances AP-1 and NFkB DNA Binding and Induces Stress Protein Expression in Precision-Cut Rat Lung Slices. *Toxicological Sciences* 61: 283–294.

Joqueviel, C., Martino, R., Gilard, V., Malet-Martino, M., Canal, P. and Niemeyer, U. 1998. Urinary excretion of cyclophosphamide in humans, determined by phosphorus-31 nuclear magnetic resonance spectroscopy. *Drug Metabolism & Disposition* 26: 418-28.

Kaur, C. and Kapoor, H. C. 2001. Antioxidants in fruits and vegetables – the millennium‟s health. *International Journal of Food Science and Technology* 36 (7): 703-725.

Kaur, S., Singh, G. and Kaur, K. 2014. Cancer stem cells: An insight and future perspective. *Journal of Cancer Research and Therapeutics* 10: 846-52.

Kaya, H., Oral, B., Ozguner, F., Tahan, V., Babar, Y. and Delibas, N. 1999. The effect of melatonin application on lipid peroxidation during cyclophosphamide therapy in female rats. *ZentralblGynakol* 121: 499-502.

Keay, R. W. J. 1989. Trees of Nigeria. Clarendon Press, Oxford; 137.

Keepers, Y. P., Pizao, P. E., Peters, G. J., van Ark-Otte, J., Winograd, B., and Pinedo, H. M. 1991. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *European Journal of Cancer and Clinical Oncology* 27(7): 897-900.

Kehrer, J. P. and Biswal S. S. 2000. The molecular effects of acrolein. *Toxicological Sciences* 57: 6-15.

Kerharo, T. and Adam, J. G 1974 . la pharmacopia senegalaise traditionanelle. Plantes medicates et toxiques Editions Vigot Frerez, Paris.

Khan, A. W., Ahmad, S. A., Sayed, M. H. S. U., Hadi, S. A., Khan, M. H., Jalil, M. A., Ahmed, R. and Faruquee, M. H. 1997. Arsenic contamination in groundwater and its effect on human health with particular reference to Bangladesh. *Journal of Preventive and Social Medicine* 16(1): 65–73.

Khan, M. A., Travis, L. B., Lynch, C. F., Soini, Y., Hruszkewycz, A. M., Delgado, R. M., Holowaty, E. J., van Leeuwen, F. E., Glimelius, B., Stovall, M., Boice, J. D., Jr, Tarone, R. E. and Bennett, W. P. 1998. p53 mutations in cyclophosphamide-associated bladder cancer. *Cancer Epidemiology, Biomarkers and Prevention* 7: 397–403.

Kinlen, L. J. 1985. Incidence of cancer in rheumatoid arthritis and other disorders after immunosuppressive treatment. *The American Journal of Medicine* 78: 1A 44–49.

Korsmoyer, S. J. 1992. Bcl-2: Antidote to programme cell death. *Cancer Surveys* 12: 105.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor: II. General properties. *The Journal of General Physiology* 20: 291–310.

Lai, C. S., Mas, R. H., Nair, N. K., Maji, M. I., Mansor, S. M. and Navaratnam, V. 2008. Typhonium flagelliforme inhibits cancer cell growth *in vitro* and induces apoptosis: an evaluation by the bioactivity guided approach. *Journal of Ethnopharmacology* 118:14–20.

Lamson, D. W. and Brignall, M. S. 1999. Antioxidants in cancer therapy; their actions and interactions with oncologic therapies. *Alternative Medicine Review.* 4(5): 304-329

Lear, L. and Nation, R. L., 1992. Stupans I. Effects of cyclophosphamide and adriamycin on rat hepatic microsomal glucuronidation and lipid peroxidation. *Biochemical Pharmacology* 44: 747-753.

Lee, T. C. and Ho, I. C. 1995. Modulation of cellular anti-oxidant defense activities by sodium arsenite in human fibroblast. *Archives of Toxicology* 69: 498-504.

Lelieveld, P. and van Putten, L. M. 1976. Biologic activity of two derivatives and six possible metabolites of cyclophosphamide (NSC-26271). *Cancer Treatment Reports* 60: 373-9.

LFR (Leatherhead Food Research) /Phytotrade Africa summary report, 2009. Nutritional evaluation of baobab dried fruit pulp and its potential health benefits. London, UK.

Liedberg, C. F., Rausing, A. and Langeland, P. 1970. Cyclophosphamide hemorrhagic cystitis. *Scandinavian Journal of Urology* 4: 183–190.

Lindgren, A., Danielsson, B. R. G., Dencker, L. and Vahter, M., 1984. Embryotoxicity of arsenite and arsenate: distribution in pregnant mice and monkeys and effects on embryonic cells *in vitro*. *Acta Pharmacologica et Toxicologica* 54: 311–320.

Ludeman, S. M. 1999. The chemistry of the metabolites of cyclophosphamide. *Current Pharmaceutical Design* 5: 627-43.

Mabuchi, K., Lilienfeld, A. M. and Snell, L. M. 1979. Lung cancer among pesticide workers exposed to inorganic arsenicals. *Archives of Environmental Health* 34:312-320.

Mallick, P., Mallick, J. C., Guha, B. and Khuda-Bukhsh, A. P. 2003. Ameliorating effect of micodoses of potentizes homeopathic drug, arsenic album, on arsenic-induced toxicity in mice. *BMC Complementary and Alternative Medicine* 3: 7.

Martin, J. L., Machado, A. F., Loza, M. A., Mao, G. E., Lee, G. S., Hovland Jr. D. N., Cantor, R. M. and Collins, M. D. 2003. Effect of arsenite, maternal age, and embryonic sex on spina bifida, exencephaly and resorption rates in the splotch mouse. *Birth Defects Research* (Part A) 67: 231–239.

Mates, J. M., Perez-Gomez, C. and De Castro, I. N. 1999. Antioxidant enzymes and human diseases. *Clinical Biochemistry* 32: 595-603.

Mathew, S. and Kuttan, G. 1997. Antioxidant activity of Tinosporacordifolia and its usefulness in the amelioration of cyclophosphamide induced toxicity. *Journal of Experimental & Clinical Cancer Research* 16: 407-411.

Mazumder, D. N. 2005. Effect of chronic intake of arsenic-contaminated water on liver. *Toxicology and Applied Pharmacology* 206: 169-175.

McCarroll, N., Keshava, N., Cimino, M., Chu, M., Dearfield, K., Keshava, C., Kligerman, A., Owen, R., Protzel, A., Putzrath, R. and Schoeny, R. 2008. An evaluation of the mode of action framework for mutagenic carcinogens case study: Cyclophosphamide. *Environmental and Molecular Mutagenesis* 49: 117–131.

Merian, E., 1984. Introduction on environmental chemistry and global cycles of chromium, nickel, cobalt, beryllium, arsenic, cadmium and selenium and their derivatives. *Toxicological and Environmental Chemistry* 8: 9–38.

Michaud, D. S. 2007. Chronic inflammation and bladder cancer. *Urologic Oncology* 25: 260–268.

Milton, A. H. and Rahman, M. 1999. Environmental pollution and skin involvement pattern of chronic arsenicosis in Bangladesh. *Journal of Occupational Health* 41: 207–208.

Morakinyo, A. O., Achema, P. U., Adegoke, O. A. 2010. Effect of Zingiber Officinale (Ginger) on Sodium Arsenite Induced Reproductive Toxicity in Male Rats. *African Journal of Biomedical Research* 13: 39 – 45.

Moreb, J. S., Gabr, A., Vartikar, G. R., Gowda, S., Zucali, J. R. and Mohuczy, D. 2005. Retinoic acid down-regulates aldehyde dehydrogenase and increases cytotoxicity of 4-hydroperoxycyclophosphamide and acetaldehyde. *Journal of Pharmacology and Experimental Therapeutics* 312: 339-45.

Mortensen, A., Skibsted, L. H., and Truscott, T. G. 2001. The interaction of dietary carotenoids with radical species. *Archives of Biochemistry and Biophysics* 385: 13–19.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: 55-63.

Munro, A. and Bassir, O. 1969 Oxalate in Nigerian Vegetables. *West African Journal of Biological and Applied Chemistry* 12(1):4-8.

Nagasubramanian, R., Hansen, R. J., Delaney, S. M., Cherian, M. M., Samson, L. D., Kogan, S. C., and Dolan, M. E. 2008. Survival and tumorigenesis in O^6-methylguanine DNA methyltransferase-deficient mice following cyclophosphamide exposure. *Mutagenesis* 23(5): 341–346.

Natarajan, A. T. 1984. Origin and significance of chromosomal alterations. In: G. Obe (ed), *Mutations in man*. Springer Verlag, Berlin: 156-176.

Nkafamiya, I. I., Osemeahon, S.A., Dahiru, D. and Umaru, H. A. 2007. Studies on the chemical composition and physicochemical properties of the seeds of baobab (*Adansonia digitata*). *African Journal of Biotechnology* 6: 756-759.

Nunez R. 2001. DNA Measurement and Cell Cycle Analysis by Flow Cytometry. *Current Issues in Molecular Biology* 3(3): 67-70.

O'Neil, M. J. editor, 2006. The Merck Index, 14th ed. Whitehouse Station, NJ: Merck & *Co., Inc*. 1554.

Odunola, O. A. 2003. Comparative effects of some local food condiments on sodium arsenite induced clastogenicity. *African Journal of Medicine and Medical Sciences* 32: 75-80.

Odunola, O. A., Kazeem, A. K. and Ibegbu, D. M. 2011. The Influence of Garlic and Spondias mombin on Sodium Arsenite induced Clastogenicity and Hepatotoxicity in Rats. *Pacific Journal of Science and Technology* 12(2): 401-409.

Oyaizu, M. 1986. Studies on product of browning reaction prepared from glucose amine. *The Japanese Journal of Nutrition and Dietetics* 44: 307-315.

Parvez, F., Chen, Y., Maria, A., Hussain, A. I., Hassina, M., Dhar, R., van Geen, A., Graziano, J. H. and Ahsan, H. 2006. Prevalence of arsenic exposure from drinking water and awareness of its health risks in a Bangladeshi population; results from a large population-based study. *Environmental Health Perspectives* 114(3): 355-359.

Pastore, A., Federici, G., Bertini, E. and Piemonte, F. 2003. Analysis of glutathione: Implication in redox and detoxification. *Clinica Chimica Acta* 333:19-39.

Patel, J. M. and Block, E. R. 1985. Cyclophosphamide-induced depression of the antioxidant defense mechanisms of the lungs. *Experimental Lung Research* 8: 153-165.

Petres, J., Baron, D. and Hagedorn, M. 1977. Effects of arsenic cell metabolism and cell proliferation: Cytogenic and biochemical studies. Environental Health Perspectives 19: 223-227.

Povirk, L. F and Shuker, D. E. 1994. DNA damage and mutagenesis induced by nitrogen mustards. *Mutation Research* 318: 205–226.

Premkumar, K., Pachiappan, A., Abraham, S. K., Santhiya, S. T., Gopinath, P. M. and Ramesh, A. 2001. Effect of Spirulina fusiformis on cyclophosphamide and mitomycin-C induced genotoxicity and oxidative stress in mice. *Fitoterapia* 72: 906-911.

Prentice, A., Laskey, M. A., Shaw, J., Hudson, G. J., Day, K. C., Jarjou, L. M. A., Dibba B. and Paul, A. A.1993. The calcium and phosphorus intakes of rural Gambian women during pregnancy and lactation. *British Journal of Nutrition* 69(03): 885-896.

Preston, R. J., Dean, B. J., Galloway, S., Hoden, H., Mcfee, A.F. and Shelby, M. 1987. Mammalian *in vivo* cytogenetic assays: Analysis of chromosome aberrations in bone marrow cells. *Mutation Research* 189: 157-65.

Rabinovitch, P. S. 1993. DNA analysis guidelines. *In: Practical Considerations for DNA Content and Cell Cycle Analysis.* Ed Bauer, K. D., Duque, R. E. and Shankey, T.V. (Nunez, 2001). 117-142.

Ramadan, A., Harraz, F. M. and El-Mougy, S. A. 1994. Anti-inflammatory, analgesic and antipyretic effects of the fruit pulp of *Adansonia digitata*. *Fitoterapia* 65(5) 418-422.

Ramu, K., Fraiser, L. H., Mamiya, B., Ahmed, T. and Kehrer, J. P. 1995. Acrolein mercapturates: synthesis, characterization, and assessment of their role in the bladder toxicity of cyclophosphamide. *Chemical Research in Toxicology* 8: 515-524.

Rao, Y. K., Wu, A. T. H., Geethangili, M., Huang, M. T., Chao, W. J., Wu, C. H., Deng, W. P., Yeh, C. T. and Tzeng, Y. M. 2011: Identification of antrocin from Antrodia camphorata as a selective and novel class of small molecule inhibitor of Akt/mTOR signaling in metastatic breast cancer MDA-MB-231 cells. *Chemical Research in Toxicology* 24:238–45.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans C. 1999: Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* 26(9):1231-1237.

Rec. Gscc. (DGKC) (1972). Optimised standard colorimetric methods. *Journal of Clinical Chemistry and Clinical Biochemistry* 10:182.

Reed, W., Hannisdal, E., Boehler, P. J., Gundersen, S. Host, H. and Marthin, J. 2000. The prognostic value of p53 and c-erb B-2 immunostaining is overrated for patients with lymph node negative breast carcino-ma: a multivariate analysis of prognostic factors in 613 patients with a follow-up of 14-30 years. *Cancer* 88:804-813.

Reitman, S. and Frankel, S. 1957. A colorimetric method for the determination of serum level of glutamate-oxaloacetate and pyruvate transaminases. *American Journal of Clinical Pathology* 28:56–63

Ren, S., Yang, J. S., Kalhorn, T. F. and Slattery, J. T. 1997. Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethyl cyclophosphamide in human liver microsomes. *Cancer Research* 57: 4229-4235.

Reynolds, J. G., Naylor, D. V. and Fendorf, S. E. 1999. Arsenic sorption in phosphate amended soils during flooding and subsequent aeration. *Soil Science Society of America Journal* 63:1149–1156.

Rice–Evans, C., Omorphos, S. C. and Baysal, E. 1986. Sickle cell membranes and oxidative damage. *Biochemical Journal* 237: 265-269.

Rosengard, B. R. and Cochrane, D. E. 1983. Complement-mediated cytolysis: a quick, simple method for determining levels of immunoglobulin E bound to mast cells. *Journal of Histochemistry and Cytochemistry* 31(3): 441-444.

Ross, J. S. 1996. DNA Content analysis. *In: DNA Ploidy and Cell Cycle Analysis in Pathology*. Igaku-Shoin, NY.

Roy, P., Yu, L. J., Crespi, C. L. and Waxman, D. J. 1999. Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. *Drug Metabolism & Disposition* 27: 655-666.

Russo, I. H. and Russo, J. 1998. Role of hormones in mammary cancer initiation and progression. *Journal of Mammary Gland Biology and Neoplasia* 3: 49–61

Sai, K., Hayashi, M., Takagi, A., Hasegava, R., Sofun, T. and Kurokawa, Y. 1992. Effects of antioxidants on induction of micronuclei in rate peripheral blood reticulocytes by potassium bromated. *Mutation Research* 269:113-118.

Sakata, T., Smith, R. A., Garland, E. M. and Cohen, S. M. 1989. Rat urinary bladder epithelial lesions induced by acrolein. *Journal of Environmental Pathology, Toxicology and Oncology* 9: 159–169.

Salamon, M., Heddle, J., Stuart, E. and katz, M. 1980. Towards an improved micronucleus test. Studies on 3 model agents, Mitous-ycin C, cycolophasphamide and dimethyl benzanthracene. *Mutation Research/Environmental Mutagenesis and Related Subjects* 74: 347-356.

Samarasinghe, B. 2013. Hallmarks of Cancer 3: Evading Apoptosis. (Blog) Scientific American. Available at: https://blogs.scientificamerican.com/guest-blog/hallmarks-of-cancer-3-evading-apoptosis/. Accessed: 04/07/2016.

Schalm, O. W. 1965. Veterinary Hematology, 2nd ed., Lea and Febiger, philadephia, P.A.

Schmid, W. 1975. The micronucleus test. *Mutational Research* 31: 9-15.

Shankey, T. V., Rabinovitch, P. S., Bagwell, B., Bauer, K. D., Duque, R. E., Hedley, D. W., Mayall, B. H., Wheeless, L. and Cox, C. 1999. Guidelines for Implementation of Clinical DNA Cytometry. 14(5) 472-477.

Sidibe, M. and Williams, J. T. 2002. Baobab, *Adansonia digitata*. International Centre for Underutilised crops, Southampton, UK.

Siegel, R. L., Miller, K. D. and Jemal, A. 2016. *CA: A Cancer Journal for Clinicians* 66:7–30 Cancer Statistics.

Singh, A., Kaur, M., Choudhary, A., and Kumar, B. 2015. Effect of Butea monosperma leaf extracts on cyclophosphamide induced clastogenicity and oxidative stress in mice. *Pharmacognosy Research* 7(1), 85–91.

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* 299: 152–178.

Sinha, D., Dey, S., Bhattacharya, R. K. and Roy, M. 2007. *In vitro* mitigation of arsenic toxicity by tea polyphenols in human lymphocytes. *Journal of Environmental Pathology, Toxicology and Oncology* 26. 207-220.

Skehan, P. 1995. Assays of cell growth and cytotoxicity. In: Studzinski (Ed.), *Cell Growth and Apoptosis. A Practical Approach.* Oxford University Press, New York. 169.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., Mc Mohan, J., Vistica, D., Warren, J. T., Bokesh, H., Kenny, S and Boyd, M. 1990. SRB Assay- New calorimetric cytotoxicity assay for anticancer drug screening. *Journal of the National Cancer Institute* 82: 1107-1111.

Sladek, N. E., Dockham, P. A. and Lee, M. O. 1991. Human and mouse hepatic aldehyde dehydrogenases important in the biotransformation of cyclophosphamide and the retinoids. *Advances in Experimental Medicine and Biology* 284: 97-104.

Sladek, T. L. and Jacobberger, J. W. 1993. Flow cytometric titration of retroviral expression vectors: Comparison of methods for analysis of immunofluorescence histograms derived from cells expressing low antigen levels. *Cytometry*. 14: 23-31.

Slater, T. F., Sawyer, B. and Strauli, U. D. 1963. Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. *Biochimica et Biophysica Acta* 77: 383-393.

Smith, A. H. 1997. Report and action plan for arsenic in drinking water focusing on health, Bangladesh. Assignment Report, WHO Project BAN CWS 001, March (*available on the Internet at http:// socrates.berkeley.edu/˜asrg/*).

Smith, A. H. 1998. Technical report and review of action plan for arsenic in drinking water in Bangladesh focusing on health. Assignment Report, WHO Project BAN CWS 001/D. (*available on the Internet at http://socrates.berkeley.edu/ asrg/*).

Smith, R. E., Bryant, J., DeCillis, A. and Anderson, S. 2003. Acute Myeloid Leukemia and Myelodysplastic Syndrome After Doxorubicin-Cyclophosphamide Adjuvant Therapy for Operable Breast Cancer: The National Surgical Adjuvant Breast and Bowel Project Experience. *Journal of Clinical Oncology* 21(7): 1195-1204

Sramkoski, R. M., Wormsley, S. W., Bolton, W. E., Crumpler, D. C. and Jacobberger, J. W. 1999. Simultaneous detection of cyclin B1, p105, and DNA content provides complete cell cycle phase fraction analysis of cells that endoreduplicate. *Cytometry* 35: 274-283.

Sunderman, F. W. 1984. Recent advances in metal carcinogenesis. *Annals of Clinical & Laboratory Science* 14: 93-122.

Suriyavathana, M., Usha, V. and Shanthanayaki, M. 2010. Studies on phytochemical analysis and antioxidant activity of selected medicinal plants from Kolli hills. *Journal of Pharmacy Research* 2: 260-262.

Szasz, G. 1969. A kinetic photometric method for serum gamma glutamyl transferase. *Clinical Chemistry* 124: 124-136.

Szinicz, L. and Forth, W. 1988. Effect of As_2O_3 on gluconeogenesis. *Archives of Toxicology* 61: 444-449.

Takhar, P. and Mahant, S. *In vitro* methods for Nanotoxicity Assessment: Advantages and Applications. MM College of Pharmacy, MM University, Mullana-Ambala.

Tanaka, M., Kataoka, H., Yano, S., Ohi, H., Kawamoto, K., Shibahara, T., Mizoshita, T., Mori, Y., Tanida, S., Kamiya, T. and Joh, T. 2013. Anti-cancer effects of newly developed chemotherapeutic agent, glycoconjugated palladium (II) complex, against cisplatin-resistant gastric cancer cells. *BMC Cancer* 13:237.

Tee, C., Gentile, J. M., Baguley, B. C., Pearson, A. E., Gregory, T. and Ferguson, L. R. 1997. *In vivo* effects of chlorophyllin on the antitumour agent cyclophosphamide. *International Journal of Cancer* 70: 84–89.

Testoni, M. L., Bolzan, A. D. and Bianchi, N.O. 1997. Effects of antioxidants on streptonigrin-induced DNA damage and clastogenesis in CHO cells. *Mutation Research* 373(2): 201-206.

Tietz, N. W., Burtis, C. A., Ducan, P., Ervin, K., Petitclera, C. J., Rinker, A. D., Shney, D. and Zygowicz, E. R. (1983). A reference method for measurement of alkaline phosphatase activity in human serum. *Clinical Chemistry* 29: 751-761.

Tseng, W. P. 1977. Effect of dose-response relationships of skin cancer and Blackfoot disease with arsenic. *Environmental Health Perspective* 19: 109-119.

Udobi, C. E. and Onaolapo, J. A. 2009. Phytochemical analysis and antibacterial evaluation of the leaf stem bark and root of the African locust bean (Parkiabiglobosa). *Journal of Medicinal Plants Research* 3(5): 338-344

Ulukaya, E., Ari, F., Dimas, K., Sarimahmut, M., Guney, E., Sakellaridis, N. and Yilmaz, V. T. 2011: Cell death-inducing effect of novel palladium (II) and platinum (II) complexes on non-small cell lung cancer cells *in vitro*. *Journal of Cancer Research and Clinical Oncology* 137: 1425–34.

Valagussa, P., Moliterni, A., Terenziani, M., Zambetti, M. and Bonadonna, G. 1994. Second malignancies following CMF-based adjuvant chemotherapy in resectable breast cancer. *Annals of Oncology* 5: 803–808.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J. and Telser, J. 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and Cellular Biochemistry* 266: 37–56.

Van Buren, J. P. and Robinson, W. B. 1981: Formation of Complexes between Protein and Tannic Acid. *Journal of Agric Food Chemistry* 17: 772 –777.

VandeCreek, L., Rogers, E. and Lester, J. 1999. Use of alternative therapies among breast cancer outpatients compared with the general population. *Alternative Therapies In Health And Medicine* 5:71-76.

Venkatesan, N. and Chandrakasan, G. 1995. Modulation of cyclophosphamideinduced early lung injury by curcumin, an anti-inflammatory antioxidant. *Molecular and Cellular Biochemistry* 142: 79-87.

Vertuani, S., Braccioli, E., Buzzoni, V. and Manfre- dini, V. S. 2002. Antioxidant Capacity of *Adansonia digitata* Fruit Pulp and Leaves. *Acta Phytotherapeutica* 5(2): 2-7.

Vichai, V. And Kirtikara, K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols* 1: 1112 - 1116

Vimalanathan, S. and Hudson, J. B. 2009. Multiple inflammatory and antiviral activities in *Adansonia digitata* (Baobab) leaves, fruits and seeds. *Journal of Medicinal Plants Research* 3(8): 576-582.

von Eitzen, U., Meier-Tackmann, D., Agarwal, D. P. and Goedde, H. W. 1994. Detoxification of cyclophosphamide by human aldehyde dehydrogenase isozymes. *Cancer Letters* 76: 45-9.

Von Le De Bur, M. and Schmidt, W. 1973. The micronucleus test: methodological aspects. *Mutation Research* 19: 109-117.

Weinberg, R. A. 1989. Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. *Cancer Research* 49(14): 3713–3721.

Wickens, G. E. 1982. The Baobab – Africa's upside-down tree. Kew Bulletin 37: 173-209.

Wickens, G. E. The Baobabs: Pachycauls of Africa. *Springer* Madagascar and Australia.

Wilmer, J. L., Erexson, G. L. and Kligerman, A. D. 1990. Effect of acrolein on phosphoramide mustard-induced sister chromatid exchanges in cultured human lymphocytes. *Cancer Research* 50: 4635–4638.

Winski, S. L. and Carter, D. E. 1995. Interaction of the rat blood cell sulhydryls with arsenate and arsenite. *Journal of Toxicology and Environmental Health* 46: 379–97.

Yamanaka, K., Hoshno, M., Okanato, M., Sawamura, R., Hasegawa, A. and Okada, S. 1990. Induction of DNA damage by dimethylarsine, a metabolite of inorganic arsenic is for the major part likely due to its peroxyl radical. *Biochemical and Biophysical Research Communications* 168: 58-64.

Yazzie, D., VanderJagt, D. J., Pastuszyn, A., Okolo, A. and Glew, R. H. 1994. The amino acid and mineral content of baobab (*Adansonia digitata L.*) leaves. *Journal of Food Composition and Analysis* 7(3): 189-193.

Yule, S. M., Boddy, A. V., Cole, M., Price, L., Wyllie, R., Tasso, M. J., Pearson, A. D. J. and Idle, J. R. 1995. Cyclophosphamide metabolism in children. *Cancer Research* 55: 803-809.

Yule, S. M., Price, L., Mc Mahon, A. D., Pearson, A. D. and Boddy, A. V. 2004. Cyclophosphamide metabolism in children with non-Hodgkin's lymphoma. *Clinical Cancer Research* 10: 455-60.

Zarei, M. and Shivanandappa, T. 2013. Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of Decalepis hamiltonii in mice. *Food and Chemical Toxicology* 57: 179-184.

Zhang, J., Tian, Q. and Zhou, S. 2006. Clinical Pharmacology of Cyclophosphamide and Ifosfamide. *Current Drug Therapy* 1: 55-84.

APPENDIX I

LIST OF PUBLICATIONS AND ABSTRACTS IN PROCEEDINGS

- **Adegoke A.M.,** Gbadegesin M.A., Otitoju A.P. and Odunola O.A. (2015). Hepatotoxicity and Genotoxicity of Sodium Arsenite and Cyclophosphamide in Rats: Protective Effects of Aqueous Extract of *Adansonia digitata L.* Fruit Pulp. *British Journal of Medicine and Medical Research Vol.8. No.11: 963 – 974*

✓ **Adegoke A.M.,** Gbadegesin M.A.and Odunola O.A. (2017). Methanol Extract of *Adansonia digitata* Leaf Protect Against Sodium Arsenite-induced Toxicities in Male Wistar Rats. *Pharmacognosy Research. Vol. 9(1): pp. 7–11.*

✓ **Adegoke A.M**., Gbadegesin M.A. and Odunola O.A (2014). *Adansonia digitata* stem bark extract mitigates sodium arsenite induced-toxicity in rats. Proceedings of the Fourth Unibadan Conference of Biomedical Research. Theme 'Biotechnology for Health and Sustainable Development', Conference Centre, University of Ibadan, Ibadan, Nigeria, 1-4 July 2014.

✓ **Adegoke A.M.,** Gbadegesin M.A and Odunola O.A (2014). Modifying role of Aqueous extract of *Adansonia digitata* fruit pulp on cyclophosphamide induced-toxicities. Proceedings of the 52nd Annual Scientific Conference, Australasian Association of Clinical Biochemists (AACB) Adelaide 27 – 29 October, 2014.

✓ Odunola O.A., **Adegoke A.M.,** Gbadegesin M.A and Otitoloju A.P. (2014). The effects of *Adansonia digitata* fruit pulp extract on sodium arsenite-induced toxicities in rats. Proceedings of the 53rd Annual Meeting and ToxExpo of the Society of Toxicology (SOT) Phoenix Arizona, USA. March 23rd – 27th, 2014.

✓ **Adegoke A.M**, Gbadegesin M.A and Odunola O.A (2014). Potential use of methanol extract of *Adansonia digitata* as a nutraceutical. 2nd International Symposium on Natural Products. Cape Town, South Africa, 23 - 25 September, 2014.

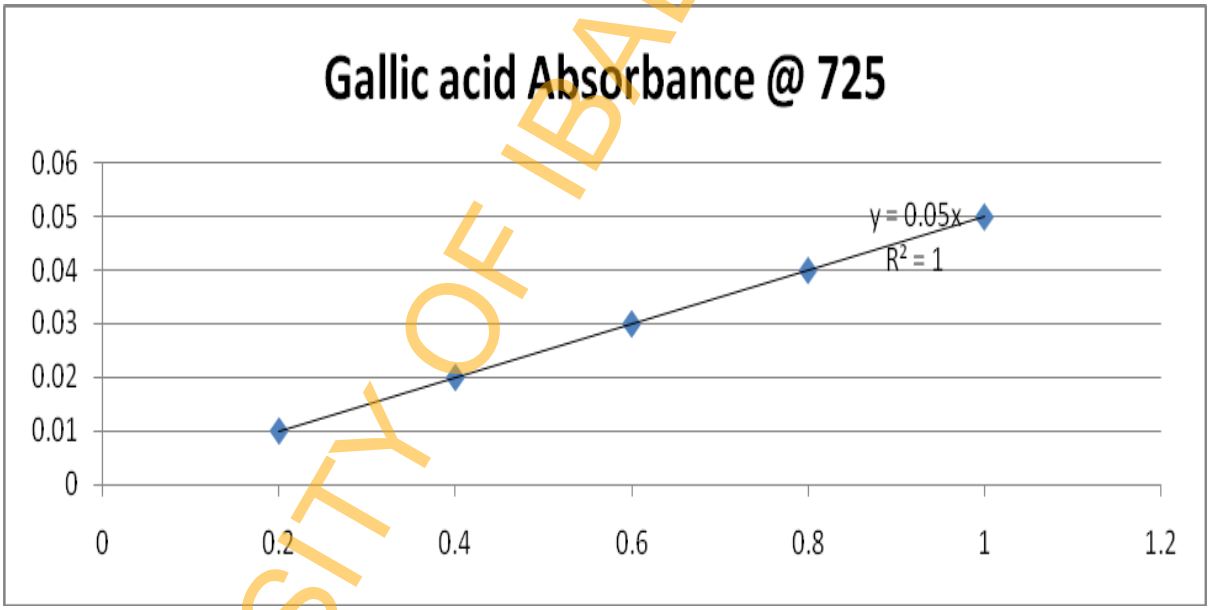
APPENDIX II

LIPID PEROXIDATION ASSAY CALCULATIONS AND STANDARD CURVE FOR GALLIC ACID

Lipid Peroxidation Assay Calculation

Lipid peroxidation was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$

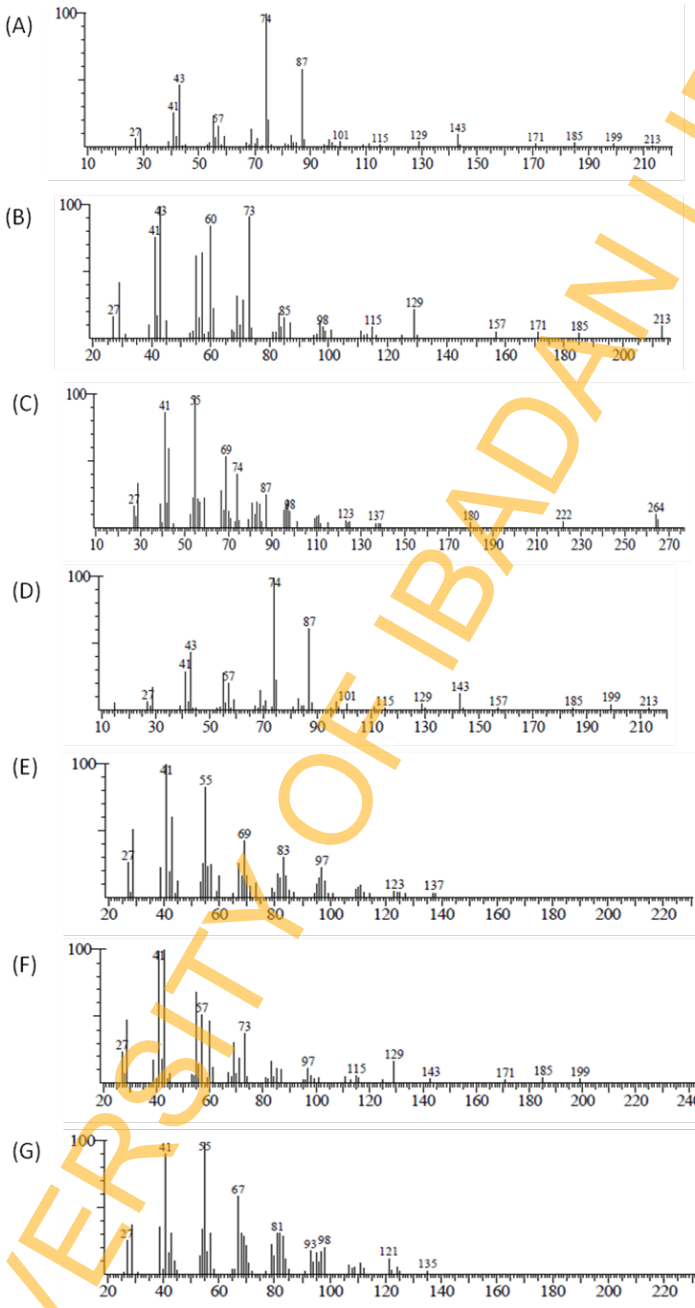
$$MDA = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample}}$$



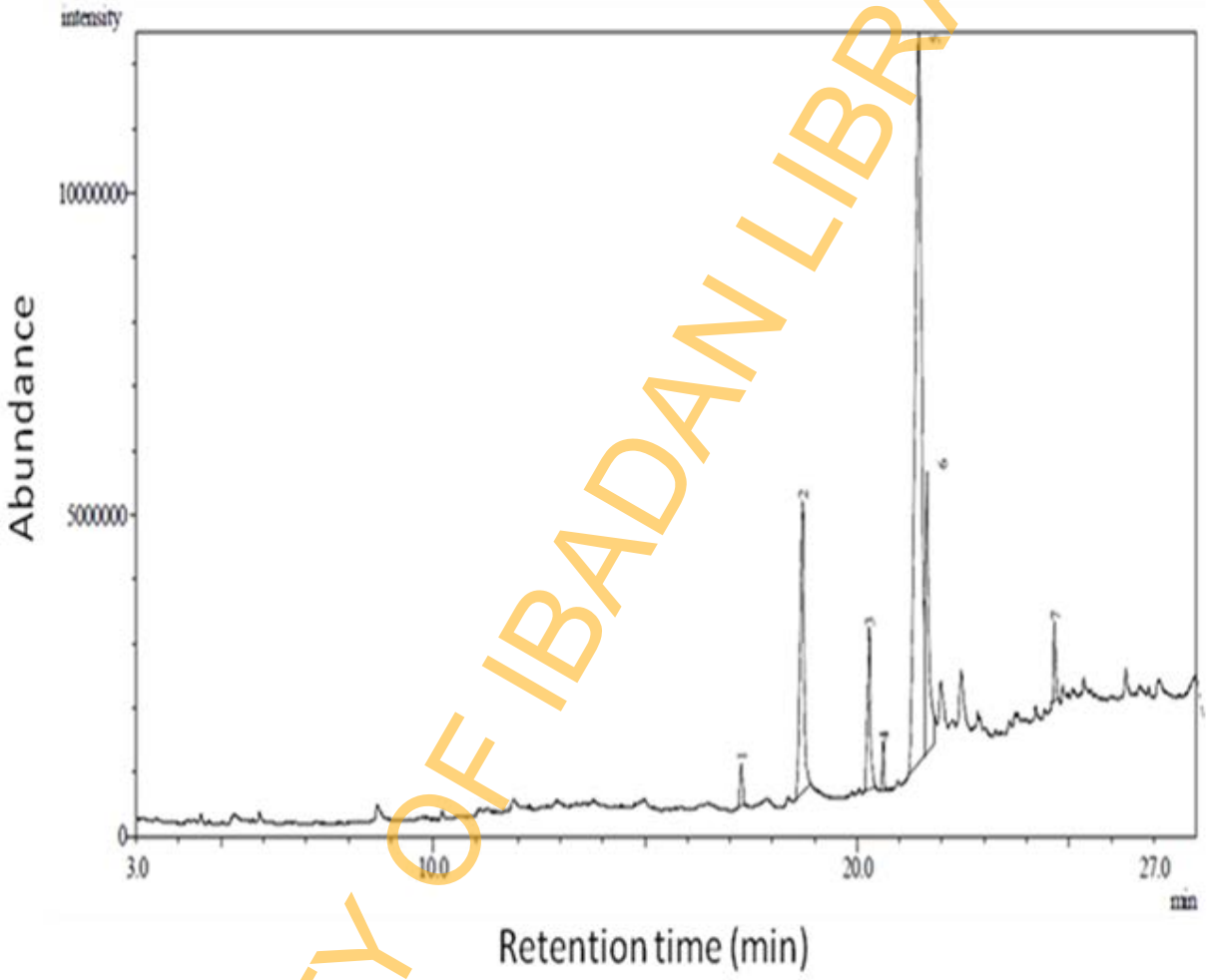
Standard curve for gallic acid

APPENDIX III

MS SPECTRUM AND GC/MS CHROMATOGRAM OF THE AQUEOUS EXTRACT OF THE FRUIT PULP OF *ADANSONIA DIGITATA* LINN.



MS Spectrum of the aqueous extract of the fruit pulp of *Adansonia digitata* Linn. The X-axis and Y-axis of the chromatogram show mass/charge (m/z) and abundance, respectively. **(A)** Pentadecanoic acid; **(B)** n-Hexadecanoic acid **(C)** 11-Octadecenoic acid **(D)** Octadecanoic acid; **(E)** Oleic Acid; **(F)** Nonadecanoic acid; **(G)** 3,11-Tetradecadien-1-ol.



The GC/MS Chromatogram of the Aqueous Extract of *the* fruit pulp of *Adansonia digitata* (AEFAD).

APPENDIX IV

FLOW CYTOMETRY AND WESTERN BLOT ANALYSIS REAGENTS

Flow Cytometry

Propidium Iodide solution

2 mM MgCl2

10 mM Pipes buffer

0.1 M NaCl

0.1% Triton X-100

0.01 mg/ml Propidium iodide

Western Blot analysis

2X Laemmli sample buffer

4% SDS

20% glycerol

0.004% bromphenol blue

0.125 M Tris HCl, pH 6.8

10% β-mercaptoethanol

RIPA

150 mM NaCl

1% Triton X-100

0.1% SDS

20 mM Tris (pH 7.5)

1% deoxycholate

Sodium Dodecyl Sulphate (SDS)-polyacrylamide gels

Resolving gel:

Acryl-bisacryl-amide mix (30:08) (percentage depending on size of protein of interest)

0.375 M Tris (pH 8.8)

0.1% SDS

0.1% TEMED

0.1% Ammonium persulphate

Stacking gel:

5% Acryl-bisacryl-amide mix (30:08)

0.192 M Tris (pH6.8)

0.1% SDS

0.1% TEMED

0.1% Ammonium persulphate

Acryl-bisacryl-amide mix (30:08):

29 g acrylamide

1 g N,N`-methylenebisacrylamide

Make up to 100 ml, heating at 37°C to dissolve chemicals. Store at 4°C, protected from light

Running buffer:

1 g SDS

3.03 g Tris

14.41 g Glycine

Make up to 1 liter

Transfer buffer:

2.9 g Glycine

5.8 g Tris

0.37 g SDS

200 ml isopropanol

Make up to 1 liter and store at 4°C.

Stripping buffer

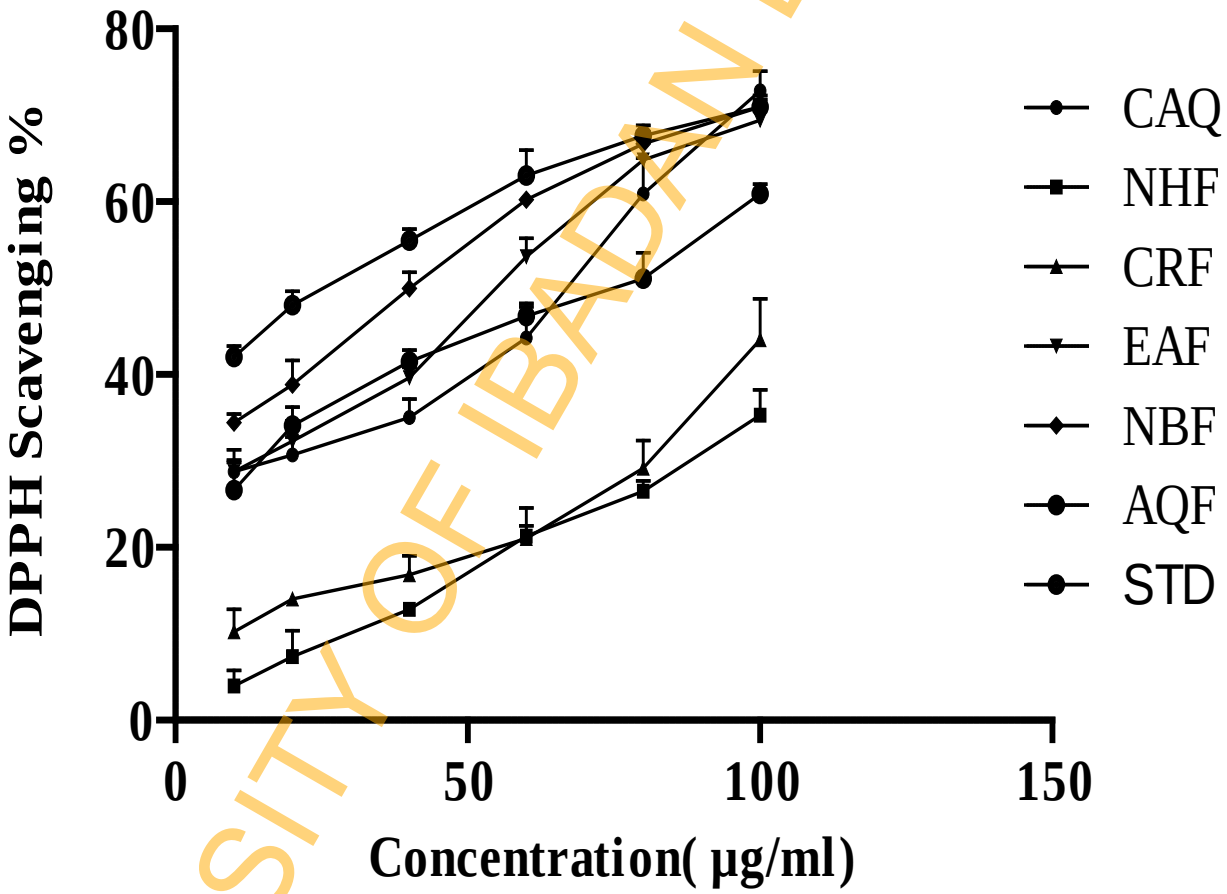
62.5 mM Tris-HCl (pH6.7)

2% SDS

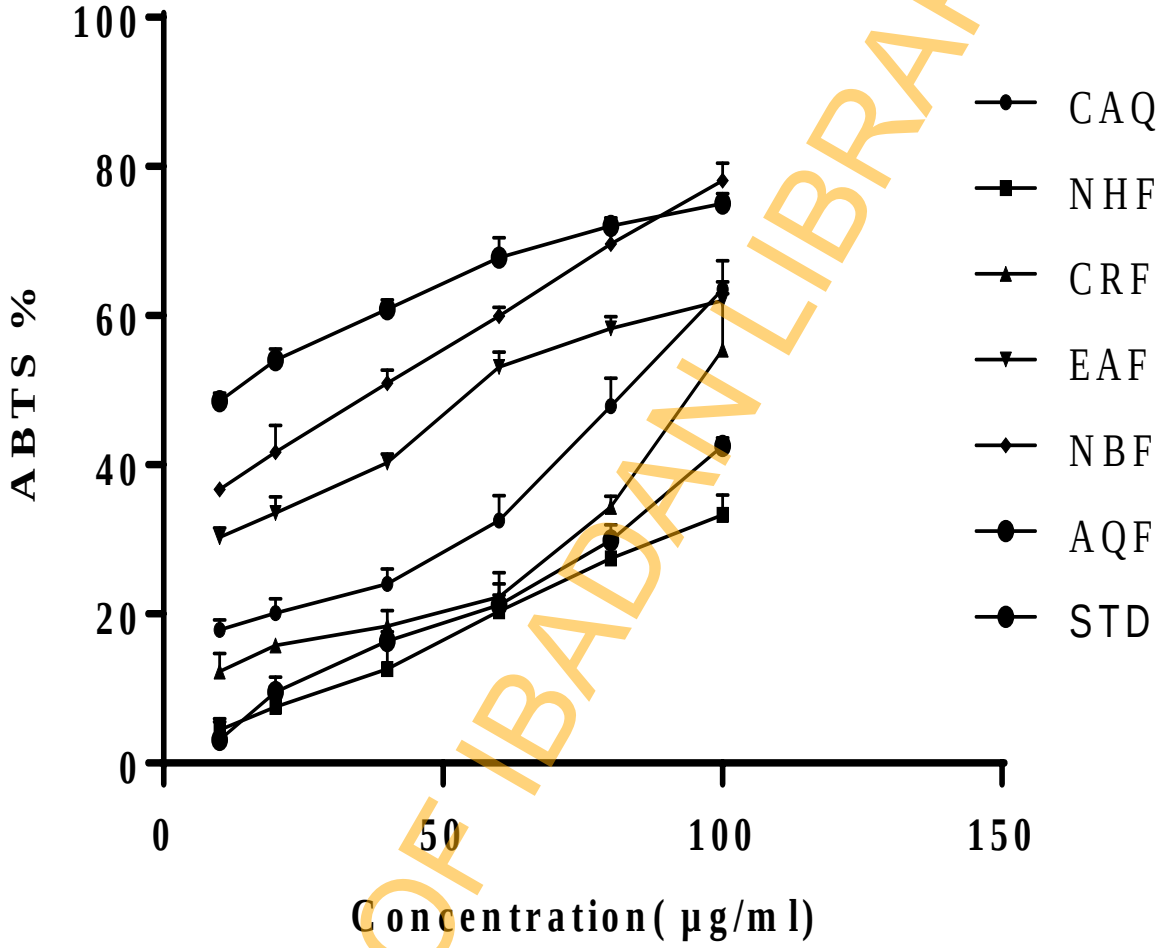
100 mM β-mercaptoethanol

APPENDIX V

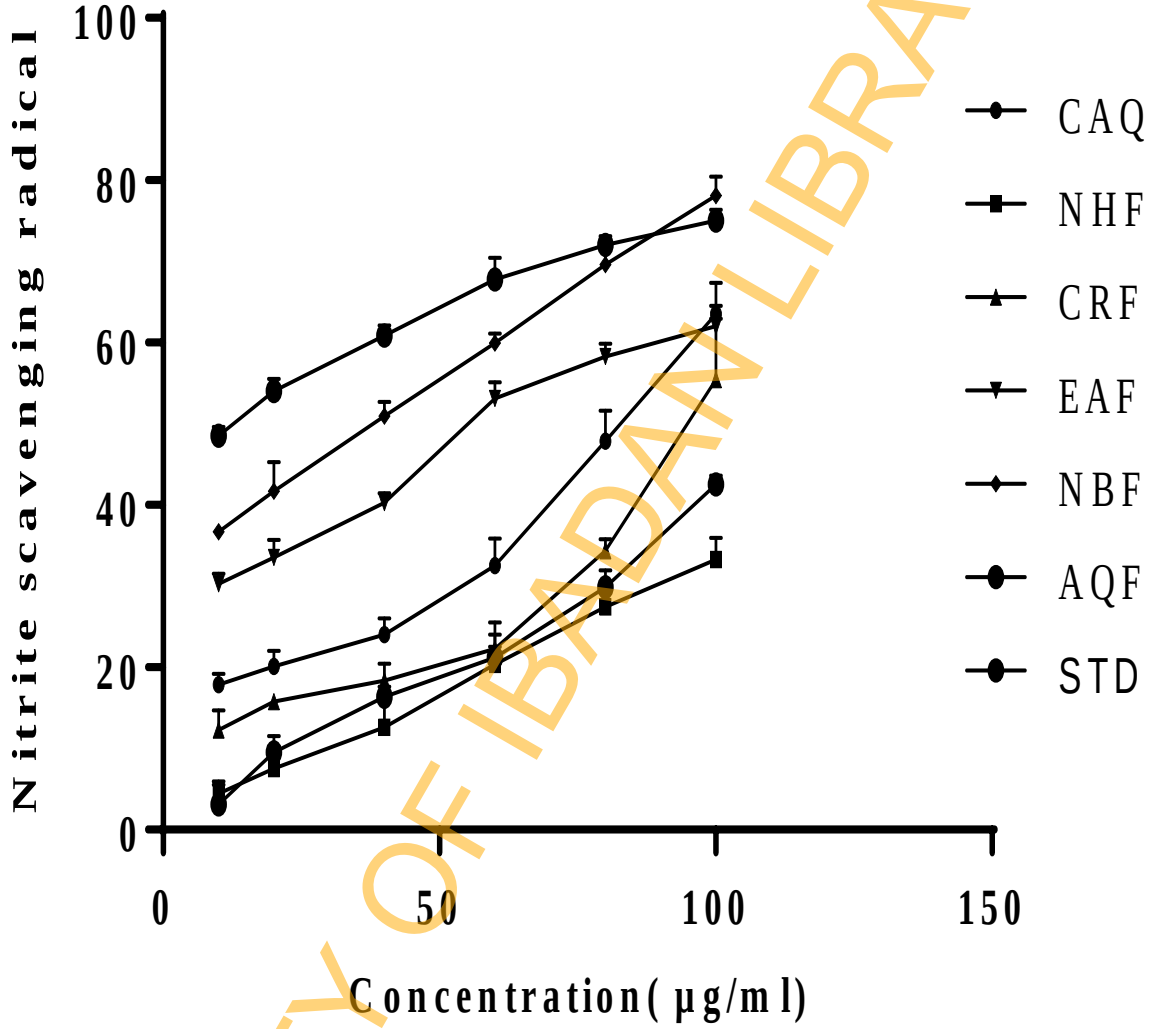
DPPH, ABTS AND NITRITE SCAVENGING ACTIVITIES OF THE CRUDE AND VARIOUS SOLVENT FRACTIONS OF THE AQUEOUS EXTRACT OF THE FRUIT PULP OF *ADANSONIA DIGITATA* (AEFAD).



DPPH scavenging activities of the crude and various solvent fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD). Each value represents mean ± S.D of 3 determinations. CAQ= Crude aqueous extract, NHF= n-hexane fraction, CRF= Chloroform fraction, EAF= Ethyl acetate fraction, NBF= n-butanol fraction, AQF= Aqueous fraction, STD= Standard (ascorbic acid).



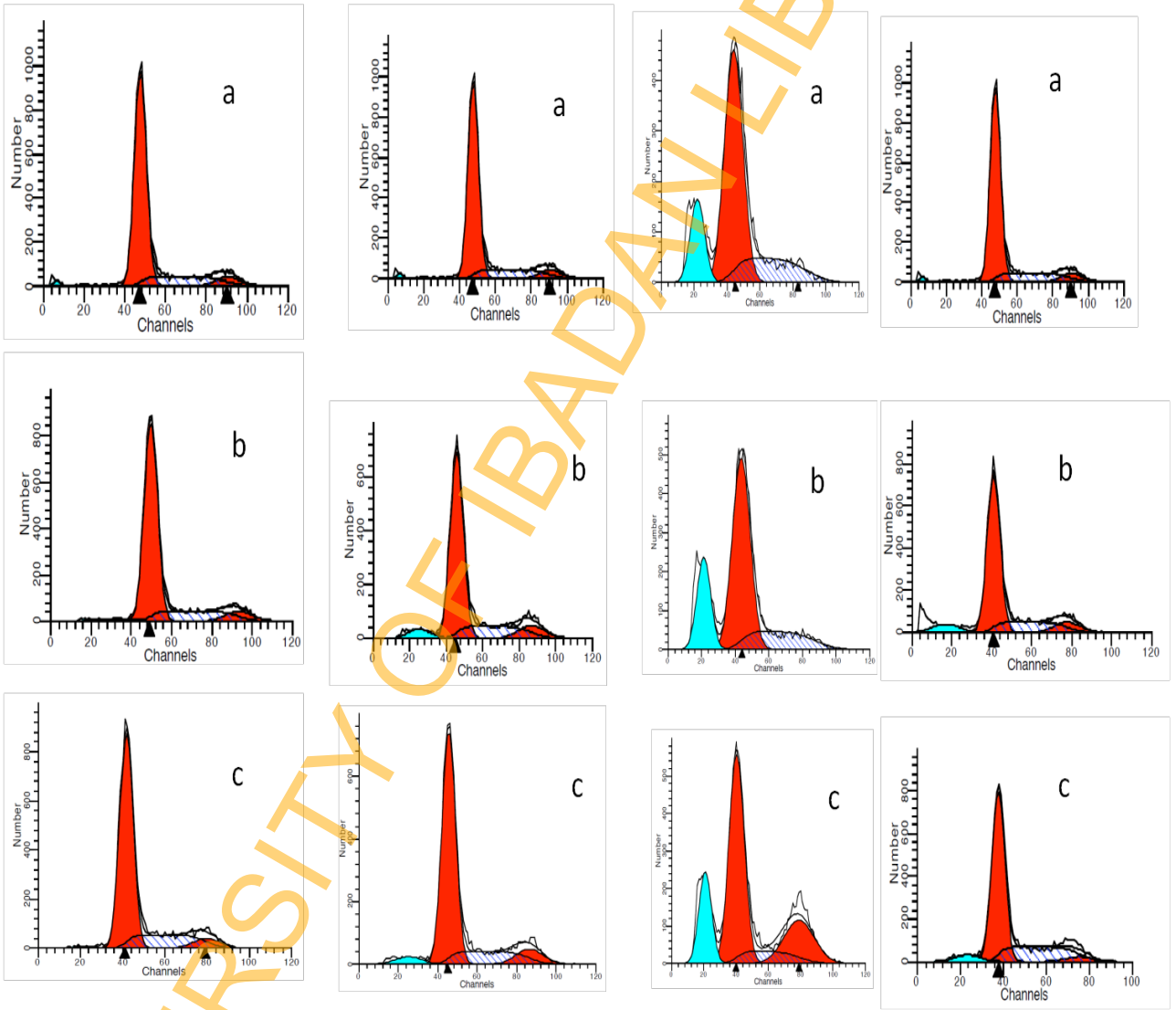
ABTS scavenging activities of the crude and various solvent fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD). Each value represents mean ± S.D of 3 determinations. CAQ= Crude aqueous extract, NHF= n-hexane fraction, CRF= Chloroform fraction, EAF= Ethyl acetate fraction, NBF= n-butanol fraction, AQF= Aqueous fraction, STD= Standard (ascorbic acid).



Nitrite scavenging properties of the crude and various solvent fractions of the aqueous extract of the fruit pulp of *Adansonia digitata* (AEFAD). Each value represents mean ± S.D of 3 determinations. CAQ= Crude aqueous extract, NHF= n-hexane fraction, CRF= Chloroform fraction, EAF= Ethyl acetate fraction, NBF= n-butanol fraction, AQF= Aqueous fraction, STD= Standard (ascorbic acid).

APPENDIX VI

CELL CYCLE PHASE DISTRIBUTION OF MCF-7 AND A549 CELL LINES AFTER TREATMENT WITH FRACTIONS OF AEFAD



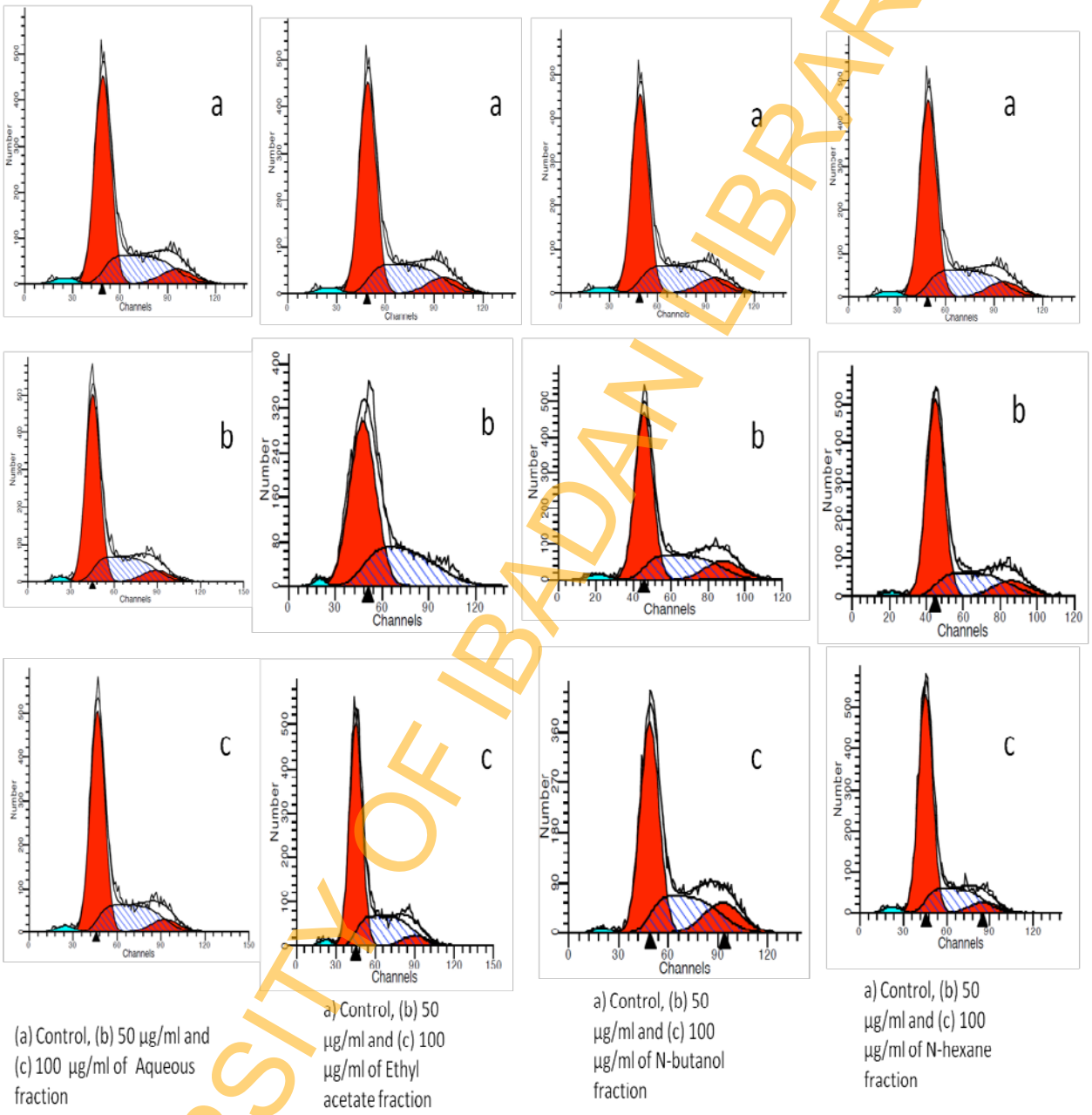
a) Control, (b) 50 µg/ml and (c) 100 µg/ml of aqueous fraction

a) Control, (b) 50 µg/ml and (c) 100 µg/ml of ethyl acetate fraction

a) Control, (b) 50 µg/ml and (c) 100 µg/ml of N-butanol fraction

a) Control, (b) 50 µg/ml and (c) 100 µg/ml of N-hexane fraction

Cell cycle phase distribution of MCF-7 cell line, after treatment with fractions of AEFAD.



Cell cycle phase distribution of A-549 cell line, after treatment with fractions of AEFAD.