COMPARATIVE CHEMOPREVENTIVE POTENTIALS OF THE FRUIT-PULPS OF *DIALIUM GUINEENSE* AND *DETARIUM MICROCARPUM* IN *N*- METHYL NITROSOUREA (MNU) INDUCED COLON CARCINOGENESIS IN RATS

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DECLARATION

I declare that the work in this Thesis entitled**COMPARATIVE CHEMOPREVENTIVE POTENTIALS OF THE FRUIT-PULPS OF *DIALIUM GUINEENSE* AND *DETARIUM MICROCARPUM* IN *N*- METHYL NITROSOUREA (MNU) INDUCED COLON CARCINOGENESIS IN RATS** was carried out by me in the Department of Biochemistry, Ahmadu Bello University, Zaria, under the Supervision of Prof. S. E. Atawodi, Prof. E.O.Onyike and Prof. D.A. Ameh. The information derived from the literature has been duly acknowledged in the text, and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Mubarak Labaran LIMAN _____ _____

Ph.D/Scie/16072/2011-2012 Signature Date

CERTIFICATION

This thesis entitled **COMPARATIVE CHEMOPREVENTIVE POTENTIALS OF THE FRUITS-PULPS OF *DIALIUM GUINEENSE* AND *DETARIUM MICROCARPUM* IN *N*- METHYL NITROSOUREA (MNU) INDUCED COLON CARCINOGENESIS IN RATS**by Mubarak Labaran LIMAN, meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved parents;
Hajiya Hafsa Kyauta Muhammad
and
Late Alh Labaran Muhammad Liman

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ABSTRACT

Dialium guineense (Wild) and*Detariummicrocarpum*Guill. &Perrare leguminous African plants used as dietary flavorings and thickeners and also are utilized in African ethno-medicine for the management of gastrointestinal ailments.The study is aimed at establishing the comparative potential of fruits-pulps of *D. guineense* and*D. microcarpum* in chemoprevention of colon carcinogenesis in rats.In an experimental *N*-methyl nitroso urea (MNU) induced colon carcinogenesis in rats, various concentrations of *D.microcarpum*and *D. guineense* fruit-pulpswere incorporated in diets of rats and evaluated for their chemo-preventive effects. After acclimatization, the rats were divided into 10 groups with 7 rats each and fed for 12 weeks with diets in which 2.5, 5.0 and 10.0% *D. microcarpum* or *D. guineense*portions were included by incorporation. The test groups were then intoxicated with MNU intrarectally at 72hourly interval for another 12 weeks while being fed with the experimental diets. Respective control groups, fed similar concentrations of the incorporated diet only and unincorporated diet with and without MNU intoxicationwere also included. All rats were sacrificed at the end of the experiment and whole bloodcollected for haematological analysis with serum separated for sandwich ELISA assay of carcinoembryonic antigen (CEA) and organ function assays. Liver, kidney and colon partswere collected and homogenized for antioxidant and tissue peroxidation assays. Sections of these tissues were subjected to histopathological studies with further immunohistochemical (IHC) staining of the colon using mutL homolog 1 (MLH1) antibody. A significant ($p<0.05$) elevation was observed in levels of CEA (138.37± 12.05 ng/ml) in the MNU positive control group when compared with the test groups on supplemented diets and the negative control group (63.12± 4.26 to 98.23± 8.73 ng/ml). The groups on 10% fruit-pulps incorporated diets particularly

showed significantly($p<0.05$)lower CEA levels (63.12 ± 4.26and 80.15 ± 5.80 ng/ml). Histopathological and IHC staining of the colon in the treated groups showed mild changes in tissues with moderate to high expression of the MLH1 antigen, when compared with the MNU control group which showed severe tissue damage and mild expression of the antigen.Taken together with the preceding findings on antioxidant and toxicity status, it was concluded that the inclusion of fruit-pulps of *D. microcarpum*and *D. guineense* in the experimental diet protected the blood and the organs of rats from induced MNU toxicity; prevented oxidative stress and other processes involved in colon carcinogenesis in rats; with *D. microcarpum*showing a higher protection therebyjustifying theirusage in foods and traditional medicine.

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LIST OF ABBREVIATIONS

ACF- Abberynt Crypt Foci.

ACS- American Cancer Society.

AIDS- Acquired Immune Deficiency Syndrome.

ALT- Alanine Amino Transferase.

AORTIC- African Organization for Research and Training in Cancer.

APC- Adenomatous Polyposis Coli.

AST- Aspartate Amino Transferase.

CAT- Catalase.

CEA- Carcinoembryonic Antigen.

CML- Chronic Myelogenous Leukemia.

COX- Cyclooxygenase.

DCC- Deleted Colon Cancer Gene.

FAP- Familial Adenomatous Polyposis.

GACP- Guidelines on Good Agricultural and Collection Practices.

G-CSF- Granulocyte Colony Stimulating Factor.

GLOBOCAN- Global Cancer.

HBG- Haemoglobin.

HIV- Human Immunodeficiency Virus.

HNPCC- Hereditary Non-Polyposis Colorectal Cancer.

IARC- International Agency for Cancer Research.

ICAM-Intercellular Adhesion Molecule.

IL- Interleukin.

K-ras- Kristen Rat Sarcoma.

MCH- Mean Corpuscular Haemoglobin.

MCHC- Mean Corpuscular Haemoglobin Concentration.

MCV- Mean Corpuscular Volume.

MDA- Malondialdehyde.

MLH1- Mutl Homolog 1.

MMR- Mismatch Repair Genes.

MNU- Methyl Nitrosourea.

MSH- Microsatellite Stable High.

MSI- Microsatellite Instability.

MSI-H- Microsatellite Instability High.

MSI-L- Microsatellite Instability Low.

NCI- National Cancer Institute.

NSAID- Non-Steroidal Anti-Inflammatory Drug.

PAH- Polycyclic Aromatic Hydrocarbons.

RBC- Red Blood Cells.

RNS- Reactive Nitrogen Species.

ROS- Reactive Oxygen Species.

SOD- Superoxide Dismutase.

TBA- Thiobarbituric acid.

TBARS- Thiobarbituric Reactive Substance.

TJ- Tight Junctions.

WBC- White Blood Cell.

WHO- World Health Organisation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Cancer is a public health concern in Africa and according to the International Agency for Research on Cancer (IARC, 2016), about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2012 in Africa. These numbers are projected to nearly double (1.28 million new cancer cases and 970,000 cancer deaths) by 2030 simply due to the aging and growth of the population with the potential to be higher because of the adoption of behaviours and lifestyles associated with economic development, such as smoking, unhealthy diet, and physical inactivity (AORTIC, 2011). Despite this growing burden, cancer continues to receive low public health priority in Africa, largely because of limited resources and other pressing public health problems, including communicable diseases such as acquired immune deficiency syndrome (AIDS)/human immunodeficiency virus (HIV) infection, malaria, cholera and tuberculosis that are seen as more challenging. It may also be in part due to a lack of awareness about the magnitude of the current and future cancer burden among policy makers, the general public and international private or public health agencies (Atawodi, 2012).

The American Cancer Society (ACS) estimates that in 2017 about 135,430 people will be diagnosed with colorectal cancer in the United States and about 50,260 people will die from the disease. In both men and women, colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death. Regrettably, African Americans (both men and women) are more likely than people of any other racial/ethnic group in the United States to develop colorectal cancer, and also to die due to it (ACS, 2017).

Reports on latest cancer statistics of 2012 from International Agency for Cancer research (IARC) by GLOBOCAN (2012) affirmed that of 14.1million new cancer cases, 8.3 million cancer deaths and 32.6 million people living with cancer within five years diagnostic period occurred in less developed regions comprising of many African countries. The reports further indicated that 8

million (57% of world total) of new cases, 5.3 million (65% of world total) of cancer deaths and 15.6 million (48% of world total) of those living under five years cancer diagnosis occurred in developing countries including Nigeria. The estimate for colorectal cancer in Nigeria stands at about 4,172 representing about 4.1% of total world's burden (GLOBOCAN, 2012). The majority of these cancers and deaths could be prevented by applying existing knowledge about cancer prevention and by increasing the use of established screening tests. Given the worst case scenario especially in developing countries of Africa and Nigeria in particular, the unregulated population increase has pushed food production and availability far below increasing population such that food for consumption is already getting scarce and costly, leaving consumers going for alternative cheaper and affordable, low quality, poorly stored and decaying products prone to mycotoxins which increases cancer susceptibility (Atawodi, 2015).

Plants materials have been used in the management of many ailments since ancient times. In Africa and other developing parts of the world, plants and plant resources play an essential role in nutrition and medicine. Although, the role of natural products as a source for remedies has been recognized since the beginning of mankind, only a few folk medicinal plants have been fully scientifically evaluated for their pharmacological activities. Atawodi *et al.*, (2009, 2010and 2015) reported extensively on the antioxidant and antimutagenic potentials of some African plants and the activities of these various plants were found in many instances to be related to indwelling polyphenolic compounds. The several chemicals of plant origin with therapeutic attributes still remain to be explored. Desert and tropical plants remain an important and promising field of exploration since appreciable concentration of phytotherapeutic components have been established to be present in many of them (Keay*et al*., 1958; Burkill, 1995).

Statement of Research Problem

Cancer is increasingly recognized as a critical public health problem worldwide with a higher mortality rate in the developing countries. Although, communicable diseases continue to burden African populations, it is becoming clear that non-communicable diseases also require the attention of those whose goal it is to ensure the health of Africans. Colon cancer is known to be more prevalent in societies that consume western style diets which are rich in fats and low in dietary fibres. There was a surge in urbanization of cities and towns in Nigeria and other developing countries with consequential socio-economic alteration (Nwosu, 2000).

The current lifestyle of humans almost everywhere in the world is in sharp contrast than earlier time, and as a consequence, humans suffer from a large number of chronic diseases (Green and Evan, 2002). In the past, infectious diseases killed our ancestors early, often younger than age 40, so they did not display the current epidemic of chronic diseases that arise in older age. Nowadays, people live longer because their medical status has been improved and therefore express symptoms of chronic diseases associated with senescence and lifestyle such as obesity, diabetes, hypertension, coronary heart disease, and cancer (Atawodi, 2012). Increases in life expectancy, changes in diet and lifestyle, and double burden of communicable diseases leading to wide use of modern drugs promise to increase the cancer burden in Africa over the coming years (AORTIC, 2011).

Colon cancer is the second most prevalent cancer in the world. The costs of detecting, managing and treating cancers with orthodox medicines are enormous and apparently unbearable by people

in developing African countries like Nigeria. WHO (1993) has advocated emphasis on preventive medicine and also encourages the development and use of traditional herbs to treat and manage diseases in developing countries.

Justification of Research

The lower incidence of colon cancers in societies that consume lesser of western style diets but more of diets based on plants (fruits and vegetables) and unrefined cereals has been attributed to relatively higher contents of dietary fibres and other bioactive phytochemicals in such plants based diets (Chun *et al*., 2007) . Colon cancer is among the cancers with high prevalence but yet is preventable through diet and life style changes.Physical inactivity and to a lesser extent, excess body weight, are consistent risk factors for colon cancer (Gill and Rowland, 2002). Exposure to tobaccoproducts early in life is associated with a higher risk of developing colorectal neoplasia (Kresty*et al*., 2001).

Development of cancer is strongly related to diet and environmental factors.Diet and nutritional factors are clearly important factors in colorectal cancer incidence. Experimental results for example, show a positive correlation between antitumor activity and antioxidant activity. These two activities were also correlated with the levels of polyphenols and flavonoids available in plant foods (Atawodi, 2012). Heat and radiation stresses are linked to an enhanced susceptibility to oxidation, successfully being resisted by the efficient antioxidative systems evolved in desert and tropical plants. It has been suggested that plant species living in extremely stressful environments may become an abundant natural resource of strong antioxidants. Also, water scarcity induces the production of therapeutic phytochemicals in desert plants. It is believed that

external desert conditions trigger biological pathways that might be effective in the production of bioactive substances with therapeutic potential (Atawodi, 2015).To increase our understanding of this diet-colon cancer link it is important to establish that diets and dietary constituents can prevent colon carcinogenesis and then understand the underlying mechanisms.

There are two diet-related strategies involved in cancer chemoprevention research; they are cancer prevention and dietary cancer prevention. Cancer prevention has been defined as the pharmacologic intervention with synthetic or naturally occurring chemicals or extracts to prevent, inhibit or reverse carcinogenesis, or prevent the development of invasive cancer (Schatzkin and Kelloff, 1995; Newman *et al*., 2000). Traditionally, beneficial effects of food-derived chemo-protective agents had been assessed either individually or as a few putative active constituents and often at pharmacological doses. Recently, whole food-based approaches for treatment and prevention of cancer have been explored due to the strong correlation between diets rich in colorful fruits and vegetables and lower incidence of certain cancers (Kresty *et al*., 2001). This type of food-based approach is defined as dietary cancer prevention, which also involves modifications in food consumption patterns necessary to decrease cancer development (Schatzkin and Kelloff, 1995).

Dialiumguineense and *Detariummicrocarpum* are tropical and semi-arid plants with significant culinary uses in a number of West African countries. Researchers have extensively reported the presence of antioxidative phytochemicals as well as antioxidant potentials of these plants (Abbreu*et al*., 1998; Akpata and Miachi 2001;Olugbuyiro*et al*., 2009; Gideon *et al*., 2013). Cytotoxic, hepatoprotective as well as antimicrobial potentials of the plants have also been

reported (Abbreu*et al*., 1999; Balogun*et al*., 2013). Therefore, investigation into the possible potential of these plants in chemo-prevention of colon cancer can never be overemphasized.

Research Questions/ Hypothesis

Null hypothesis

Dialium guineense and *Detarium microcarpum* fruits widely used as food and for medicinal purposes in Africa can neither prevent colon carcinogenesis nor reduce the effects associated with carcinogenesis and colon cancer development in experimental rats.

Aim of the Study

The study is aimed at establishing the comparative potential of fruits of *Dialium guineense* and *Detariummicrocarpum* in chemoprevention of experimental colon carcinogenesis in rats.

Specific Objectives

To evaluate the comparative biochemical effects of *Dialium guineense* and *Detariummicrocarpum* on organ function in experimental colon carcinogenesis.

To assess the comparative*in vivo* antioxidant potential of *Dialium guineense* and *Detariummicrocarpum* fruits in a colon carcinogenesis model.

To determine the comparative effects of *Dialium guineense* and *Detarium microcarpum* fruits onhaematological parameter of rats in a colon carcinogenesis model.

To determine the comparative effects of*Dialium guineense* and*Detarium microcarpum* fruits on thehistology of experimental colon carcinogenesis in rats.

To establish and compare the preventive effects of *Dialium guineense* and*Detarium microcarpum* on MNU induced carcinogenesis in rats.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Cancer

Cancer is defined as a malignant tumour and is amongst the public health problems worldwide. Tumours could be in two forms; benign or malignant. Benign tumours are those tumours that have no clear genetic basis whereas malignant tumours are those tumours that are genetically rooted. Hence, malignant tumours have tendency to metastasize and affect other cells or tissues (Lamprecht, 2003). The process of cancer development can be divided into different steps; it starts with DNA damage and mutations in the initiation phase, followed by growth of transformed cells in the promotion stage, leading to malignant growth and invasion in the progression stage.

Primarily, during development of colon cancer normal colonic epithelium transform into hyper-proliferative epithelium and then further into adenoma, carcinoma and eventually metastasis, through accumulation of genetic alterations, according to the model of Fearon and Vogelstein (1990). Crucial genes involved in this process include adenomatous polyposis coli (APC), K-ras, Deleted Colon Cancer gene (DCC) , p53, c-Myc, cyclooxygenase(cox-2), mismatch repair genes, cell adhesion genes(Evan and Vousden,2001). Accumulation of alterations during carcinogenesis leads to impairment of normal growth inhibition by increased cell growth and by inhibition of apoptosis, resulting in clonal expansion of tumour cells. Normally, the balance between cell proliferation and apoptosis in colonic mucosa is tightly regulated in order to maintain a constant cell number. The disturbance of the balance results in an escape from the normal homeostasis of cell number and favours the survival of the mutated and undifferentiated cells. Inhibition of

proliferation and increase in apoptosis of these aberrant cells are important mechanisms of prevention of colon cancer. Also, promotion of differentiation is an important preventive mechanism, since differentiation makes an escape from the tight control on cell growth less likely (Hall *et al*., 1994, Shanmugathasan andJothy, 2000)

Several forms of hereditary colon cancer are known. In Familial Adenomatous Polyposis (FAP) patients inherit a germ-line mutation in APC and develop many colon tumours. Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is related to microsatellite instability and a defect in DNA repair (Rumsby and Davie, 1995).

2.2 Chemical Carcinogenesis

A number of chemicals are involved in carcinogenesis, and are accordingly referred to as chemical carcinogens. That, is, chemical carcinogens are agents that are capable of inducing cancer in humans or animals. Most of the many hundreds of chemical carcinogens known have been recognized as such as a result of tests in rats or mice. A relatively small number are occupational carcinogens, having been found to cause cancer in persons exposed to them in the workplace. Sometimes occupational cancer has arisen from materials where the actual causativeagent remains unknown. Some chemicals used as drugs are also known to have caused human cancer. Many lists of human carcinogens have been published, and differ widely according to the strength of the evidence that is accepted (IARC, 2012). It should not be assumed that carcinogens recognized experimentally are necessarily less hazardous than accepted widely reported occupational carcinogens that happen to have been encountered in industrial processes, often under working condition.

Chemical carcinogenesis is a prolonged process with many stages, mostly very imperfectly understood, and a variety of other factors are known which potentiate or inhibit the development of cancer. Occupational cancer has commonly taken 20 or more years from first exposure to become apparent, and this time-lag contributes to the difficulties experienced in linking cause and effect. Nevertheless, identification of carcinogenic factors, coupled with changes in industrial practices and various legislative measures, have resulted in the virtualelimination of some former occupational cancers. Examples are cancer of the scrotum in chimney sweeps, cotton-spinners and tar workers, and bladder cancer in rubber workers. Asbestos dust, on the other hand, will present health problems for many years, though hopefully on a much smaller scale than when exposure to the most hazardous forms was very much greater (Armstrong and Doll,1975)

Occupational causes of cancer have been much simpler to identify when there has been a greatly increased risk of workers developing a particular form of cancer, or when the cancer caused has been one that is very uncommon in the general population. For example, past conditions in those parts of the chemical industry using certain aromatic amines led to a 30-fold increased risk of workers developing bladder cancer. Mesothelioma of the pleura and peritoneum, and haemangiosarcoma of the liver, are normally very rare forms of cancer but have arisen in workers exposed to asbestos dust and vinyl chloride respectively. The limitations of epidemiology are such, however, that it is very difficult to identify a cause of cancer where the cancer is one already common in the general population (especially lung cancer), and where the proportional increase in risk is not very large. Because of the long time-lag in chemical

carcinogenesis, it is particularly important to avoid exposure of young persons to potential carcinogenic agents. Special care is also needed to avoid exposure to carcinogens and other harmful chemicals of women who are pregnant, or may become pregnant. This is because the fetus is at high risk from harmful chemicals ingested by the mother (NCI, 2016)

Many, but not all, known chemical carcinogens fall into some fairly well-defined chemical classes, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines and nitro compounds, alkylating agents and N-nitroso amines and amides. The N-nitroso compounds do not include any accepted occupational carcinogens, but are outstanding in that most of the several hundred tested are potent experimental carcinogens. In the inorganic field, human cancers have been caused by occupational exposure to largely uncertain compounds of nickel, chromium and arsenic.

2.2.1 Carcinogenicity of N-nitroso compounds

N-nitroso compounds are formed by the interaction of a nitrogen-containing organic compound--such as an amine, amide, urea, guanidine, urethane or cyanamide and a nitrosating agent, such as a nitrogen oxide. These compounds can be divided into two categories; nitrosamines and nitrosamides- which differ in their chemical stability, the mechanism of their carcinogenicity and their mutagenicity. The nitrosamines are very stable once they are formed. They require chemical modification in an enzyme-catalysed reaction before they exhibit carcinogenic and mutagenic activity which can be local at site of application or diffused to other body parts (Marcejova and Britko, 2001). By contrast the nitrosamides can be hydrolysed, especially in neutral and alkaline solution. They exhibit carcinogenic and mutagenic activities without

modification and malignant tumours are produced at the site of their application (Morton *et al*, 2008).

The first report that nitrosamines cause cancer in laboratory animals wasby Magee and Barnes (1956) who showed that rats fed low levels (50 ppm) of dimethyl nitrosamine in their diet developed liver cancer. Since then more than 120 nitrosamines and nitrosamides have been examined for carcinogenic activity in animals. Although there is no direct evidence that N-nitroso compounds causes cancer in man, their carcinogenicity has been demonstrated in many other related animal species including: mice, rats, hamsters, fish, rabbits, guinea pigs, dogs and monkeys (Tarso*et al*, 2011, Douglass *et al*., 1978). About 80% of the N-nitroso compounds tested are carcinogenic to some degree. Their potency varies widely, from compounds where a single dose is sufficient to induce tumours to those where large doses given repeatedly produce no malignancy.

2.2.2 Mechanism of action of MNU

The nitrosamine, N-methyl-N-nitrosourea (MNU) is a direct acting carcinogen, inducing tumours in several species of animals and in a variety of organs, including the central nervous system, liver, prostate, colon, stomach, intestine, kidney, and skin (Marcejova and Britko, 2001). MNU is one of the most widely used chemicals in several studies of chemical carcinogen models. It has been predominately used in tumor induction and in the investigation of a variety of novel chemo-preventive and treatment agents. The works of several authors have established MNU as a potent carcinogen in the induction of diverse tumours on varying strains of rats and mice (Tarso *et al*,

2011, Yohizawa *et al*., 2000). Its effects on hamsters have been examined. The induction of mammary tumours in Sprague-Dawley rats has also been well reported.

N-methyl-N-nitrosourea (MNU), exerts its initiation action in carcinogenesis by methylating the nitrogenous bases of the DNA, thereby leading to K-ras mutation. Mutations in the K-ras gene are responsible for activation of the K-ras pathway which is implicated in colon carcinogenesis in humans and rats (Jancik *et al* 2010). Mutations of proto-oncogenes *ras* are most commonly found in colorectal carcinoma, appearing early in the process of carcinogenesis, already in the phase of hyperproliferating epithelium, leading to anaplastic/abberynt crypt foci (ACF), climaxing in adenocarcinomas and cancers. Functional studies in cell culture and mouse models support a critical role for K-ras mutation in colorectal cancer progression and maintenance (Jancik*et al*., 2010). In human tumours, as well as in tumours of chemically induced colon cancer in rats, increased expression of downstream targets of the K-ras pathway, has been found.

2.3 The Colon

Colon also called the large intestine is an organ of the digestive system that function to absorb water and store the waste-products of digestion until the body is ready to empty them out (Elias, 2012). Under normal condition, the colonic epithelium is renewed constantly by crypt proliferative cells which migrate upward along the crypt-villi axis as they terminally differentiate. The average time for these cells to proliferate is one day, and around 25% of the colonic epithelium is rejuvenated every day. Moreover, the crypt epithelial cells of the entire colon and rectum are replaced every three to four days (Lipkin, 1973).

Comment [U1]: Interchange paragraphs. Epithelial cell renewal days inconsistent

Comment [ML2]:

There are several types of cells in the colon; stem cells and daughter cells in the bottom of the crypt, and more specialized cells in the upper part of the crypt (Potten 1998; Ponz de Leon and Percesepe 2000). The stem cells are meant to differentiate into more specialized cells like goblet cells, absorptive cells and endocrine cells. Normally, proliferative cells are only found in thebottom of the crypt, whereas differentiation takes place in the middle of the crypts (Butler and Hewett, 1999). Cells on top of the crypt have a very short life span, and mutations in these cells are probably without impact for development of cancer (Potter 1999).

In a healthy colon there is a fine balance between cell growth and cell death, and the epithelial cells are normally renewed, however, in a situation of stress, cell division and proliferation can take over and disrupt the balance. This imbalance can contribute to a higher frequency of mutations, which is a risk factor for cancer development (Lipkin 1973).

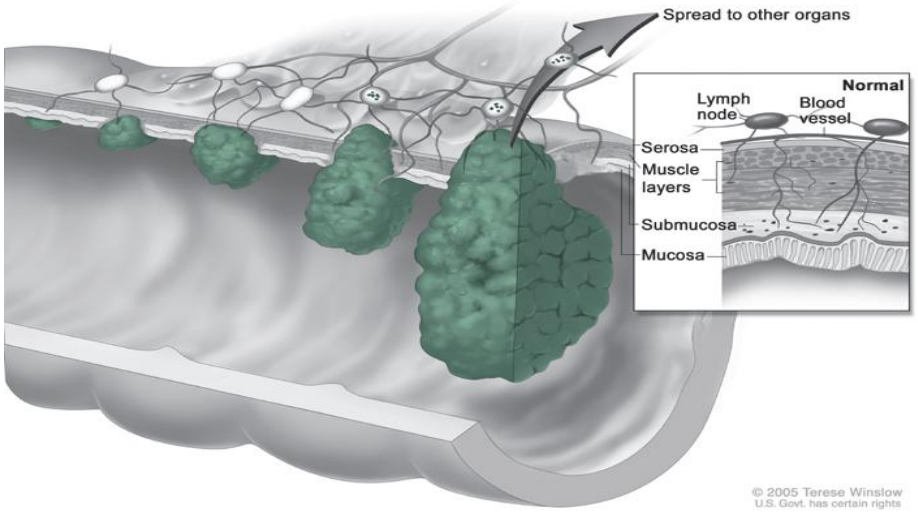

Figure 2.1. Diagrammatic Illustration ofthe Colon Showing Polyps Proliferation into Cancers. Source: Boyle and Langman, (2000)

2.3.1 Colon cancer etiology

Colorectal cancer usually results from multiple genetic and epigenetic changes leading to the transformation of colon epithelial cells into invasive adenocarcinomas (Ng & Yu, 2015). Therefore, genetics has been attributed as primary underlying factor colon cancer etiology either in polyposis ornon-polyposis syndromes. Polyposis syndrome is the familial adenomatous polyposis (FAP), which is associated with mutated gene or loss of FAP (also called the adenomatous polyposis coli (APC) gene (Grady, 2003). Non-polyposis colorectal cancer (HNPCC) syndrome which is also is hereditary is however, associated with induced germ-line mutations in DNA mismatch repair genes (Labianca*et al*., 2010).

2.3.2 Colon carcinogenesis

Colorectal tumours appear as a consequence of loss of growth control. An imbalance in proliferation, differentiation and apoptosis results in an accumulation of hyperproliferative cells in the luminal surface (Stadler and Yeung, 1988). Such a hyperproliferative zone with undifferentiated cells in the upper crypt, constitutes a risk for progression into benign tumours (Figure 2.2).In 1988, Vogelstein *et al.* introduced a multistep genetic model of colorectal carcinogenesis (Vogelstein *et al*., 1988). According to this model, most carcinomas in the colon originate from pre-existing adenomas, which initially are induced by activation of proto-

oncogenes and/or inactivation of tumour suppressor genes, (whereof the last tend to predominate). At least four to five mutations in different genes are normally required for the formation of a malignant tumour (Stadler and Yeung, 1988).

Tumour suppressor genes can be inactivated by two different mechanisms; first by mutations and second, by loss of specific chromosomal regions. Familial adenomatous polyposis (FAP) is a hereditary syndrome caused by an inherited mutation in the adenomatous polyposis coli (APC) gene on chromosome 5q (Jen, *et al*., 1994). This mutation is believed to cause the hyperproliferative zones that proceed into polyps and adenomas in the colon, acting via modulation of cadherin activity and closure of tight junctions(TJs) (Troxell andChen, 1999). Alterations in the same region may also be important in the early carcinogenesis in patients without polyposis.

A second form of genetically predisposed colon cancer is hereditary non-polyposis colorectal cancer (HNPCC) (Lynch and Lynch 1994). Mutations in DNA mis-match genes are involved in this condition (Muller and Fishel, 2002). Another frequently observed alteration in the early stage is loss of methyl groups in DNA (DNA hypomethylation).This change may lead to aneuploidy with loss of gene alleles (Goelz*et al*., 1985). Another common feature in early carcinogenesis is mutations in the proto-oncogeneras. These adenomas constitute a risk, as they are likely to develop into larger and more dysplastic tumours through clonal expansion (Bos and Fearon, 1987).

Although the genetic changes often occur in the order as described above, the total accumulation of mutations and deletions seems to be more important for carcinomas to metastasize rather than their order with respect to each other (Fearon and Vogelstein 1990). Polyps and cancer are furthermore, more common in the left distal part of the colon, and affect men and women equally (Wiese and Thompson, 2003; Nasir and Kaiser,2004).

2.3.3 Inflammation and colon cancer

The incidence of colon cancer and inflammatory bowel disease (IBD) vary among the worldin a similar pattern. This variation is probably partly due to different diets, since developing countries such as Asia, Africa and South America have a lower incidence of cancer and IBD compared to Western countries (National Cancer Institute, 2016). A recent hypothesis suggests that a high risk diet (high in saturated fat and phosphate and low in calcium), such as is common in western countries, increases the permeability of tight junctions (TJs) between colonocytes and thereby exposes them to toxic agents in the lumen (Bruce and Giacca,2000). Various components of dietary origin can increase or decrease the leakage of TJs. Increased permeability makes it possible for growth factors and toxic agents to act on the exposed epithelium and induce inflammation. Growth factors and products of inflammation are also known to increase proliferation; the latter also induces mutations and apoptosis. Mutations of the APC gene can affect the function of cadherin and the closure of TJs (Troxell and Chen, 1999), and an abundance of growth factors can stimulate proliferation. The accessibility of calcium in the lumen markedly affects the function of the TJs. A decreased calcium level keeps the TJs open

and allows components/microbes to migrate from the lumen into the epithelial mucosa and vice versa (Ma and Hollander, 1992).

There are several lines of evidence that support an association between colonic inflammation and colon cancer. First, patients with IBD are at markedly increased risk of developing colon cancer (Gillen and Walmsley, 1994; Biasco and Brandi, 1995). Second, non-steroidal anti-inflammatory agents (NSAIDs) decrease the risk of colon cancer and reduce the formation of polyps in cancer patients (Giardiello and Hamilton, 1993; Phillips and Wallace, 2002). Third, infiltration of *Escherichia coli* has been reported in tumours and surrounding tissues (Swidsinski and Khilkin, 1998). Fourth, colon cancer patients have increased levels of inflammatory proteins in faeces (Kristinsson and Roseth, 1999)

2.3.4 Prevalence of colon cancer

The incidences of colorectal cancer vary widely around the world. In Europe and other developed countries, colon cancer is one of the major causes of cancer death (Silverberg 1985; Boyle and Langman 2000; Gill and Rowland 2002).Colorectal cancer is the third most common cancer in men (746,000 cases, 10.0% of the total) and the second in women (614,000 cases, 9.2% of the total) worldwide. Almost 55% of the cases occur in more developed regions. There is wide geographical variation in incidence across the world and the geographical patterns are very similar in men and women: incidence rates vary ten-fold in both sexes worldwide, the highest estimated rates being in Australia/New Zealand (44.8 and 32.2 per 100,000 in men and women respectively), and the lowest in Western Africa (4.5 and 3.8 per 100,000). Mortality is lower (694,000 deaths, 8.5% of the total) with more deaths (52%) in the

less developed regions of the world, reflecting a poorer survival in these regions. There is less variability in mortality rates worldwide (six-fold in men, four-fold in women), with the highest estimated mortality rates in both sexes in Central and Eastern Europe (20.3 per 100,000 for men, 11.7 per 100,000 for women), and the lowest in Western Africa (3.5 and 3.0, respectively for men and women). (GLOBOCAN, 2012) (Figure 2.2)

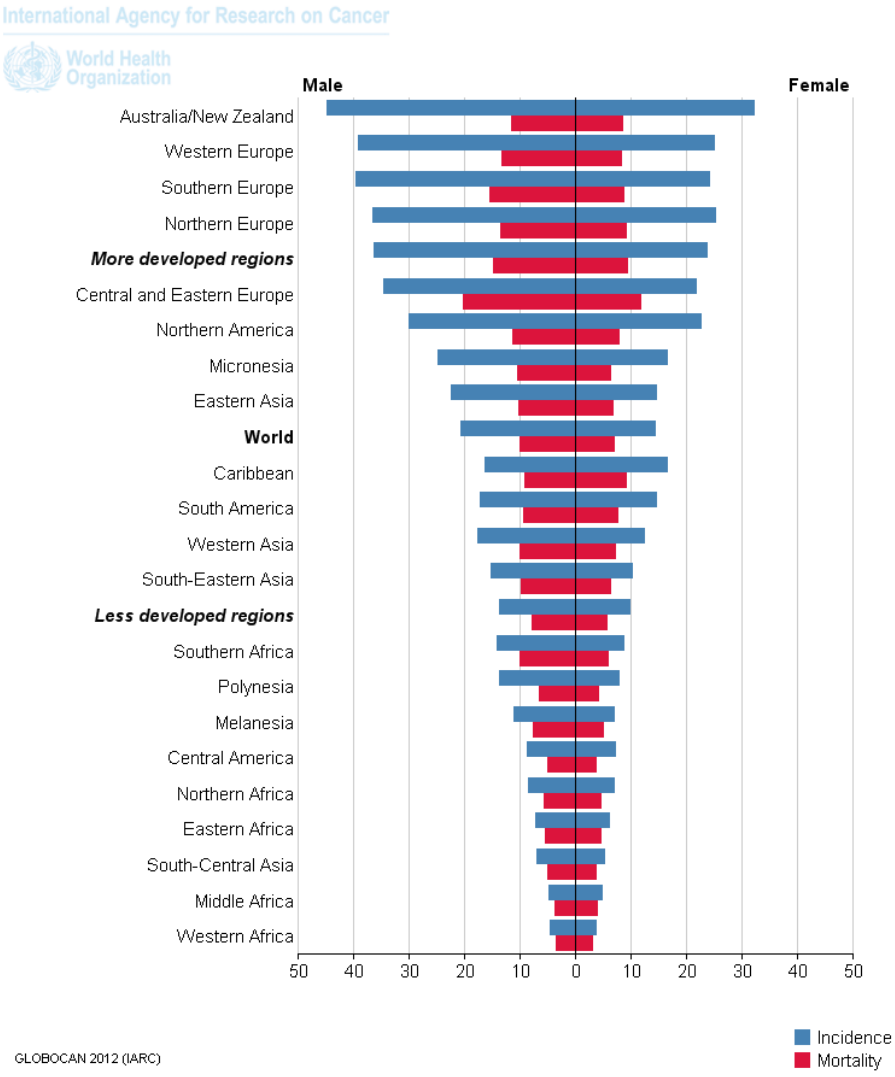

Figure 2.2: World Prevalence of Colorectal Cancer. (Source: GLOBOCAN- IARC, 2012).

2.3.5Biomarkers of colon cancer

2.3.5.1 Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen (CEA) was first described in 1965 by Gold and Freedman (1965), when they identified an antigen that was present in both fetal colon and colon adenocarcinoma but that appeared to be absent fromhealthy adult colon. Because the protein was detected in only cancer and embryonic tissue, it was given the name Carcinoembryonic antigen, or CEA. Subsequent work showed that CEA, or at least a CEA-like molecule, was also present in certain healthy tissues, although concentrations in tumours were on average 60-fold higher than in the nonmalignant tissues (Boucher*et al*., 1989).

In one of the earliest reports on CEA in serum, Thomson *et al*. (1969)found increased concentrations in 35 of 36 patients with colorectal cancer. In contrast, high values were not found in "normal" subjects, pregnant women, patients with non-gastrointestinal cancers, or in patients with miscellaneous benign gastrointestinal diseases. Although these findings were not confirmed, they nevertheless prompted widespread use of CEA as a marker for colorectal cancer. Thirty years after its initial detection in serum, CEA is one of the most widely used tumor markers worldwide and certainly the most frequently used marker for colorectal cancer.

The gene encoding CEA is now classified as a member of the immunoglobulin supergene family (Thomas, 1990; Thompson, 1991). This family includes genes coding for adhesion proteins such as intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 as well as the major histocompatibility antigens (Thompson*et al*., 1991). The human CEA gene family is clustered on chromosome 19q and comprises 29 genes. Of these, 18 are expressed, with

7 belonging to the CEA subgroup and 11 to the pregnancy- specific glycoprotein subgroup (Thompson*et al*., 1991). CEA exhibits considerable heterogeneity, which appears to be attributable to variations in its carbohydrate side chains. Most of the carbohydrate is composed of mannose, galactose, *N*-acetyl glucosamine, fucose, and sialic acid (Duffy, 2010).

In vitro experiments showed that CEA was capable of both homophilic (CEA binding to CEA) and heterophilic (CEA binding to non-CEA molecules) interactions (Thompson, 1991; Jessup and Thomas, 1989). Because alterations in cell adhesion are causally involved in cancer invasion and metastasis, it was further suggested that CEA may play a role in these processes (Jessup and Thomas, 1989). Evidence for a role in cancer dissemination was shown by Hostetter*et al*. (1990) who showed that after transplantation of colorectal tumours into nude mice, the number of liver metastases increased from 2% to 48% following injection of mice with CEA.

Serum carcinoembryonic antigen (CEA) is widely accepted as a clinically significant prognostic indicator of recurrence and therapeutic benefit in colorectal cancer (Duffy, 2010). Mann *et al*. (2004) demonstrated a benefit of low CEA levels (<200 ng/mL), reporting a 5-year survival of 48.9%, compared to 0% for those patients with a CEA ≥ 200 ng/mL. A meta-analysis showed that the low pre-operative CEA level (<50 ng/ml) was associated with a significantly better survival time following liver metastasis resection (Abbas *et al*., 2011). A further meta-analysis used a cut-off value of 200 ng/mL to stratify the two groups and also demonstrated an improved survival time with a lower CEA level (Kanas *et al*., 2012). Other literature also includes cut-off values of 20 ng/mL and 100 ng/mL, highlighting the variation of these values in different studies in man (Wang *et al*., 2010; Duffy, 2010;Mitsuyama*et al*. 2012).

2.3.5.2 Miss match repair proteins (MMR)

Mismatch repair (MMR) proteins are group of nuclear enzymes, which in all proliferating cells participate in repair of base-base mismatch, that occur during DNA replication. The proteins form complexes (heterodimers) that bind to areas of abnormal DNA and initiates its removal. Loss of MMR proteins leads to an accumulation of DNA replication errors in the proliferating cells, particularly in areas of the genome with short repetitive nucleotide sequences, a phenomenon known as microsatellite instability (MSI). Hence, MMR protein deficiency in cells is closely related to a high degree of MSI (MSI-H), in contrast to cells with a low degree of MSI (MSI-L) and cells that are MSI stable (MSS). In human, nine genes with MMR function have been identified, of which five have particular clinical interest because they may be mutated in families with hereditary non-polyposis colorectal cancer (HNPCC) (the relative frequency in parenthesis): MLH1 (49%), PMS1 (0.3%), PMS2 (2%), MSH2 (38%), and MSH6 (9%) (Surh and Chun, 2001). Persons carrying a mutation have normal MMR proteins, but in case of a DNA damage involving the non-mutated allele, the protein production stops (loss of heterozygosity). When forming complexes MLH1 heterodimerizes with PMS2, PMS1 or MLH3 (another MMR protein, for which a mutation has not been detected), while MSH2 heterodimerizes with MSH6. In case of MSH2 deficiency, MSH6 protein is also lost, possibly because of protein instability(Kanas *et al*., 2012).

Carriers of an MLH1 or MSH2 mutation has a more than 70% lifetime risk of developing a colorectal carcinoma and also markedly increased risk of developing endometrial carcinomas (50%), as well as an less increased risk of developing carcinomas of stomach, biliary tract, ovary

and urinary tract, as well as brain tumours and sebaceous skin tumours. The distribution of extra-intestinaltumours is somewhat dependent on the type of mutation.About 5% of colorectal carcinomas are associated with a germline mutation (Mann *et al*., 2004). In about 15% of colorectal carcinomas loss of MLH1 protein occurs without a mutation but because of a hypermethylation of the MLH1 promoter. MSI-H (MMR protein deficient) colorectal carcinomas are associated with significant survival advantage independently of grade and stage, compared to MSS colorectal carcinomas (which are characterized by chromosomal instability). MSI-H carcinomas usually display high prevalence of activated intraepithelial T-lymphocytes (which is suspected to increase tumour cell apoptosis and, hence, to impede the growth potential). They are more often localized in the right hemicolon, and show a slightly deviant morphologic spectrum, as there is an overrepresentation of mucinous adenocarcinomas and medullary carcinomas (inappropriately designated low differentiated (adeno-carcinomas). It appears that among colorectal carcinomas the response to chemotherapy is different in MSI-H and MSS cases, although the data are conflicting (Kanas *et al*., 2012)

Compared to molecular biological techniques, immunohistochemical analysis of MMR protein expression is much simpler and cheaper. Immunohistochemical analysis helps to pinpoint the affected gene and is readily accessible. Staining for MLH1, MSH2 or MSH6 in colorectal carcinomas can be carried out in patients and experimental animal's models. Tonsil, colon or intestine are often used as recommendable positive tissue control for MLH1 (Ramos-vera, 2005). Virtually all mantle zone B-cells must show at an at least weak to moderate nuclear staining reaction, while a moderate to strong nuclear staining reaction must be seen in the proliferating germinal center B-cells. Colon adenocarcinoma with loss of MLH1 expression is recommended

as negative tissue control for MLH1. No nuclear staining reaction should be seen in the neoplastic cells, whereas a nuclear staining reaction must be seen in stromal cells. A weak staining reaction of the nuclear membranes can be seen in scattered neoplastic cells in colon adenocarcinomas with loss of MLH1 expression (Ramos-vera, 2005).

2.3.5.3 Haematological Parameters

Hematology refers to the study of the characteristics, numbers and morphology of the cellular elements of the blood – the red cells (erythrocytes), white cells (leucocytes), and the platelets (thrombocytes) and the use of these results in the diagnosis and monitoring of disease ((NseAbasi, 2014). Blood act as a pathological reflector of the status of exposed animals to toxicant and other conditions hence can be used as an important tool for diagnosis of cancer of different sites.

Haematological parameters are those parameters that are related to the blood and blood forming organs. Haematological studies are useful in the diagnosis of many diseases since diseases progression in most instances results in progressive damage to blood tissues, immune systems as well as blood functions.Haematological components, concentration volume and activities are referred to as haematological parameters. They consist of red blood cells (RBC), white blood cells or leucocytes (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), lymphocytes, neutrophils, eosinophils, basophils etc. These parameters are valuable in monitoring the health status of blood bearing animals (Oguz*et al.*, 2014).

Current US guidelines grade anaemia based on haemoglobin levels as mild (100–lower limit of normal g/l), moderate (80–<100 g/l) and severe (<80 g/l), with anaemia defined as life-threatening when haemoglobin levels fall below 65 g/l (National Comprehensive Cancer Network [NCCN], 2010). Thrombocytopenia is chiefly associated with bleeding complications and is traditionally defined as a platelet count $<150.10^9$/L. Neutropenia is linked to an increased frequency of infections and fever and is generally defined as an absolute neutrophil count (ANC) of <1Æ5 · 109 cells/L (Richardson, *et al*., 2010).

It has been reported that RBC count and PCV values are influenced by stress, age, gender, season and genus (Özkan *et al*., 2012) ; PCV value under 30% and the decrease in PCV parallel to haemoglobin(HBG) are evaluated as anemia. The values of hematological parameters are highly influenced by disease, health and nutritional status of subjects. The values of hematological parameters are affected by a number of factors even in apparently healthy populations. These factors include age, sex, ethnic background, body build and social, nutritional and environmental factors, especially altitude. It has been shown in several studies that some of the hematological parameters exhibit considerable variations at different periods of life. At birth, the total HBG level, RBC count and PCV are shown to be higher than at any other period of life. The levels of these parameters then decrease during the next few months after birth, some more steeply than others, with the cells becoming hypochromic with the development of "physiologic" iron deficiency anemia. The hemoglobin content and the red cells then gradually rise to adult levels by the age of puberty.

Cancer is a pathological condition which arise due to malignant tumor as a result damaged genetic material**.** Increased WBC count can be found in patients with cancer either at the time of diagnosis or during follow-up period and can be secondary to infections, chronic steroid usage or bone marrow metastases. However, patients with some types of cancer may sometimes have leukocytosis distinct from these conditions, described as tumor-related leukocytosis, a condition which is caused mainly by unregulated autonomous production of hematopoietic cytokines such as Granulocyte colony stimulating factor (G-CSF), Interluikin-6 (IL-6). Previous studies showed leukocytosis to be secondary to the production of G-CSF by the tumor, and this was an epiphenomenon of the biologic aggressiveness of the tumor(Oguz*et al*., 2014).

Table 2.1: Application of Hematological Parameters in Diagnosis of Cancers.

Cancers and Malignant diseases	**Hematological abnormality**	**Hematological indicator**	**References**
Carcinoma of stomach Lymphoma, ovary, Breast other tumours Mucin-secreting carcinoma, Colorectal cancer Luekemia Gastrointestinal tumours with bleeding Mucin-secreting carcinoma, prostate cancer, colon cancer	*Pancytopenia* Marrow hypoplasia Leucoerythroblastic Metastases in marrow Megaloblastic, Folate deficiency B12 deficiency *Red cells* Anaemia of chronic disorders Most forms Iron deficiency anaemia Immune gaemolyticanaemia Microangiopathichaemolyticanaemia *White cells* Neutrophil leucocytosis. *Platelets and coagulation* Thrombocytosis Disseminated intravascular coagulation Activation of fibrinolysis. Prostate acquired inhibitors of coagulation Most forms	PCV Hb WBC ESR Lymphocytes Neutrophils Monocytes	Liqaa, 2014 Rabia*et al*., 2013 Oguz*et al*., 2014 Richardson *et al*., 2010

Haematotoxicity, such as anaemia, thrombocytopenia and neutropenia, and thromboembolic events are frequent complications of solid tumours and haematological malignancies, as well as the therapies used in their treatment (Richardson*et al*., 2010). For example, around 30–90% of patients with cancer have anaemia although the prevalence is affected by the definition of anaemia, cancer type, and disease stage. Anaemia is associated with a number of symptoms (e.g. fatigue, decreased exercise tolerance, and depression) that contribute to poorer patient quality of life (Richardson, *et al*., 2010) and has been linked to reduced survival.

In a study to evaluate the role of hematological parameters in the prognosis and diagnosis of gastric cancer (Rabia, *et al*., 2013), it was reported that some hematological parameters like HBG, RBC, Platelets, MCH, etc were significantly lower in the gastric cancer patients relative to apparently normal control. In another study on hematological parameters in breast cancers (Liqaa, 2014), significant differences ($p < 0.05$) were seen in the values for WBCs, RBCs, platelets count, PCV, lymphocyte and ESR ($p > 0.05$) among patients. The results reveal that mean PCV, RBCs and WBCs, platelet count, lymphocyte value were lower in patients compared to the controls while the mean ESR value were significantly higher than the control subjects. This study concluded that anemia; leucopenia and thrombocytopenia were common basic features to be considered in breast cancer patients.

2.3.6 Cancer therapy

The current standard of care for cancer is adjuvant chemotherapy that elicits a cell death response, typically by causing damage to the DNA or interfering with its replicative machinery (Chabner and Roberts, 2005) The three main treatment options for patients with colon cancer are

surgery, chemotherapy and radiation therapy. The best treatment option for the patient depends on their stage of cancer, whether the cancer has recurred, and the patient's general health condition (National Cancer Institute, 2016). Cancer therapeutic agents can generally be categorized as chemotherapy (standard), targeted therapy or natural products.

2.3.6.1 Chemotherapy

Standard chemotherapy is used for treatment of advanced stage cancer and is generally administered following surgical resection of the primary tumor mass (adjuvant chemotherapy). Aside from improvements to stability and toxicity, chemotherapy has not dramatically changed since the mid-1970s (Chatterjee *et al.*, 2011). For instance, the front-line of therapy for advanced colorectal carcinoma, one of the most fatal cancers, is 5-fluorouracil (5-FU), a pyrimidine analogue that inhibits DNA synthesis and has been used to treat cancer for over half a century (Goodwin and Asmis, 2009). Other drugs that have been used for decades against cancer include anti-metabolites like methotrexate, DNA alkylating agents such as cyclophosphamide or cisplatin, antimitotics like paclitaxel, and topoisomerase inhibitors like etoposide and camptothecins (Chabner and Roberts, 2005). While their long-track record implies superior utility in the fight against cancer, it belies the serious toxic effects associated with drugs that have such universally-vital targets and is always combined with agents that offer protection of normal cells (Blagosklonny, 2005).

2.3.6.2 Targeted therapy

Development of targeted therapy resulted from increased understanding of the complex molecular and genetic changes associated with cancer. The first breakthrough targeted therapy

was imatinib for the treatment of chronic myelogenous leukemia (CML) nearly a decade ago, which paved the way for new targeted molecules against other types of cancer (Chabner and Roberts, 2005). Soon after, monoclonal antibodies developed against oncogenic receptor tyrosine kinases (Herceptin, Cetuximab) and growth factors (Gefitinib) were approved for clinical use (Blagosklonny, 2005; Goodwin and Asmis, 2009). Hormone-dependent cancers can be effectively prevented and treated with endocrine therapy, such as tamoxifen for breast cancer. Tamoxifen is an estrogen receptor antagonist (Wang *et al,* 2010). Recent studies indicate that tamoxifen also inhibits components of the mitochondrial respiratory chain (MRC), leading to increased ROS production and apoptotic cell death (Parvez*et al,* 2008; Chatterjee *et al,* 2011). These targeted therapies are often used in combination with standard chemotherapy to enhance the cancer cell-killing effect (Goodman and Asmis, 2009; Fizazi*et al*, 2009). Unfortunately, tumours adapt to the selective pressures of targeted therapy, and gain resistance to treatment and metastatic capabilities (Don and Hogg, 2004; Letai, 2008; Fulda *et al*, 2010a).

2.3.6.3 Natural products

There has been a recent revival in the development of natural products as anticancer agents, based on their historical use in folk, Ayurvedic and traditional Chinese medicine (Deorukhkar*et al,* 2007; Fulda *et al*., 2010a). Plants and microorganisms are incredible sources of diverse phytochemicals, classified into various families such as alkaloids, flavonoids and isothiocyanates, with potential medicinal properties (Deorukhkar*et al,* 2007, Atawodi, 2012). In addition to the well-known natural products used as standard chemotherapy, such as paclitaxel (obtained from the bark of the Pacific Yew tree) or etoposide (derived from roots of the mayapple plant), new products that directly target the mitochondria to induce cell death are of

current interest (Chen *et al.*, 2012;Fulda *et al*., 2010a; Fulda *et al.*, 2010b). Exemplary models of a new class of mitochondrial-targeting anti-cancer agents, called *mitocans*, include the natural products betulinic acid, phenylethylisothiocyanate,α-tocopheryl succinate and polyphenols like resveratrol among others (Ralph and Neuzil, 2009). Due to the direct-targeted action of these agents on the mitochondria, they may bypass mechanisms of resistance to standard chemotherapy by tipping cancer cells over the "point-of-no-return" (Fulda *et al*, 2010a).

2.3.7 Colon cancer and diets

Among the factors that contribute to the development of colon cancer, diet is thought to be one of the most important, although the genetic predisposition also plays a significant role. Interestingly, factors of dietary origin in the colon can alter the expression of genes that are involved in the carcinogenesis process(Willett, 1998).Epidemiological studies have shown that people on a typical Western-style high-fat diet and a sedentary life style are at high risk for colon cancer (Armstrong and Doll, 1975). Furthermore, populations migrating from countries with a low risk of cancer to westernized countries, show an increased risk for colon cancer (Potter and Slattery, 1993). A diet low in saturated fat, and high in fruit and vegetables and fiber is believed to decrease the risk of colon cancer (Howe and Benito, 1992). In line with this, vegetarians show a lower risk of developing colon cancer compared to omnivores (Frentzel-Beyme and Chang-Claude 1994). Taken together, these findings strongly indicates that diet plays an important role in the aetiology of colon cancer, although it is still not clear which dietary factors are most important. Therefore, it is generally believed that diet and dietary factors are important in

different types of cancer, especially cancers of the gastro-intestinal tract (MacLennan and Macrae, 1995; Alberts and Martinez, 2000; Schatzkin and Lanza, 2000).

Many scientists are currently investigating the phytonutrients present in different fruits, as well as the interaction among plant constituents. A study done by Harris and colleagues (2001) compared the effects of freeze-dried whole black raspberries to the effects of single compounds on the inhibition of colon cancer in rats. In the study, a 10% freeze-dried black raspberry diet that contained 167, 200, 72 and 21 mg/kg of calcium, ellagic acid, sitosterol and ferulic acid, respectively was found to inhibit tumor multiplicity in the rats by 71%. Similar studies had to use individual doses of 250, 500 and 2,000 mg/kg, respectively of these compounds in order to be as effective as the 10% freeze-dried black raspberry diet at inhibiting the colon cancer. These finding are very positive because they suggest that fruits that contain low doses of multiple compounds could be used in place of large doses of single compounds to provide chemoprotective properties, thereby reducing the possibility of reaching toxic levels oflarge doses of single compounds.

The National Cancer Institute (NCI, 2016) has identified more than 1000 different phytochemicals that possess cancer-preventive activity, and many of these can be found in common foods; soybeans, ginger, grapes, green tea, citrus fruits, tomatoes and cruciferous vegetables are some examples (Surh and Chun, 2003). The dominating group of phytochemicals is polyphenols, whereof phenolic acids and flavonoids are the most important subgroups. Resveratrol, quercetin, catechin, genistein, hesperetin,

anthocyanidin are some examples of important dietary polyphenols (Scalbert and Williamson, 2000).

Studies have also shown that faeces and faecal water have a remarkable antioxidant capacity (Jenner, *et al*., 2005). Some ofthe antioxidant capacity in faeces was correlated with the intake of beverages rich in polyphenols (especially coffee and wine) (Garsetti and Pellegrini, 2000). Only a minor part of the ingested polyphenols are found in plasma, and the concentration rarely exceeds 1μM, even if the intake is 1000 times' larger (Hollman and de Vries, 1995; Scalbert and Williamson, 2000). Most of the polyphenols are poorly absorbed due to glycosylation of the phenolic ring and high molecular weight and the elimination rate in plasma is very fast. The vast majority of the polyphenols are conjugated in the liver and excreted with the bile into the intestinal lumen (Scalbert and Williamson 2000). The highest concentrations of polyphenols are consequently found in the colon, where the gut microflora can convert them into more simple compounds such as phenolic acids. These phenols are very likely to exert a local direct effect in the gastrointestinal tract (Garsetti and Pellegrini, 2000; Halliwell and de Vries, 2005). Polyphenols are reducing agents and can together with vitamin C and vitamin E protect the gastrointestinal cells from oxidative damage (Scalbert and Williamson 2000). A high availability of phenols in the lumen may also upregulate toxin metabolizing and antioxidant enzymes. They can also together with phytate from fiber, chelate catalytic iron and thereby decrease the production of harmful hydroxyl radicals (Garsetti and Pellegrini, 2000).

Other nutrients that may act locally as antioxidants are β-carotene, and selenium. Vitamin E exists in many different isoforms, but α-tocopherol is the only biologically active form. The

other forms of tocopherols andtocotrienols are excreted into the lumen via the enterohepatic shunt, which will give rise to a high local concentration. These may like the phenols, act locally in the lumen as oxygen scavengers (Halliwell and de Vries, 2005).

2.4Oxidative Stress

Oxidation is a necessary and important function in all living things and in the process reactive species are always generated. The generally accepted hypothesis is that in any biological system an important balance must be maintained between the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and their removal (Mazaih*et al*., 2009).Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body by normal metabolic action.The reduction of molecular oxygen to water by the addition of four electrons is the major source of energy for most aerobic organisms. As charges are transferred to oxygen for the release of energy, new forms of free oxygen species are formed and these are called reactive oxygen species (ROS).

Neither molecular oxygen; because of its two unpaired electrons of similar spin, nor water reacts very readily with biologic molecules. However, partially reduced and more reactive oxygen molecules can be formed under a variety of circumstances and these are highly reactive. While these reactive species have beneficial uses which include protecting the cellular environment against pathogens invasion, their presence in excess can be harmful to cell since they are capable of causing peroxidation of the phospholipids in cell membranes. However, in normal biological systems, their action is opposed by a balanced system of antioxidant defences including

antioxidant compounds and enzymes. Upsetting of this balance causes oxidative stress, which can lead to cell injury and death.

Activated oxygen species were now increasingly recognized to be the mediator of the cell injury in diseases and have been implicated in the pathogenesis of certain human diseases such as aging, cancer, atherosclerosis and diabetes (Atawodi *et al.,* 2004). Other processes that generate ROS include inflammation, strenuous exercise, detoxification, exposure to some chemicals and radiation, cigarette smoke and alcohol. Current researches into free radicals have confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancer and neurodegenerative diseases. Therefore, much attention has been focused on the use of natural antioxidants to inhibit lipid peroxidation, or to protect the damage caused by free radicals.

2.5 Antioxidants

Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves. Generally, antioxidants are antidotes for oxidative stress, and hence, nutritional antioxidants like ascorbic acid and tocopherols are known to complement the action of endogenous antioxidant enzymes. Similarly, polyphenols which are large family of natural compoundsthat are widely distributed in plant foods have been reported to have profound antioxidant abilities, because their considerable diversities make them different from other antioxidants. They include flavonoids, tannins, lignins and stilbenes among the majorclasses.

Their antioxidant abilities contribute to the beneficial health effects of many vegetables and fruits (Atawodi *et al*., 2012).

2.5.1 Roles of antioxidants in oxidative stress induced-diseases

In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress. Reactive oxygen and nitrogen species (ROS and RNS, respectively) are endogenous intermediates constantly produced in the human body and essential parts of its functions. They are components in signaling cascade involved in cellular functions such as proliferation, inflammation and adhesion. Violation of the necessary balance of cellular oxido/redox status toward a more oxidative stress, results in pathological manifestations (Plaa, 1976; Lieber and Dicarli, 1976). The extensive list of disorders and pathogenesis in which radicals and oxidants have been implicated is still growing (Kamiyama*et al*., 1993). As research on the role and involvement of ROS and RNS advances, more and more biological functions are being found to be associated with these species (Atawodi and Onaolapo, 2010).

An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions. ROS/RNS also cause DNA damage which is associated with the development of cancer, cardiovascular diseases, cataract, neurological disorders and lung disease (Recknegel*et al*, 1982). Aging is also thought to occur as a result of a constant exposure of the organs to ROS/RNS, with a cumulative damage, through the entire life, along with a gradually decreasing repair capacity and increasing degenerative changes in the organs (Newberne*et al*, 1982). Antioxidants could maintain the necessary oxido/redox balance

within these organs and are proven to be a promising antidote against oxidative stress in many human disorders (Atawodi and Onaolapo, 2010).

2.5.2 Mechanism of action of antioxidants

Two principal mechanisms of action have been proposed for antioxidants (Lieber and Dicarli, 1976). The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical). This they do by donating hydrogen atom rapidly to a lipid radical, forming a new radical, more stable than the initial one. The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalysts. This they achieve by reacting with the metabolites that produce the free radicals thereby limiting the levels of the free radicals.

2.5.3 Naturally occurring endogenous antioxidants

Naturally occurring antioxidants, of high or low molecular weight, can differ in their composition, their physical and chemical properties and in their mechanism and site of action. They can be divided into the following categories:

2.5.3.1 Enzymes

Enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, attenuate the generation of reactive oxygen species by removing potential oxidants or by transforming ROS/RNS into relatively stable compounds.

Superoxide dismutase (SOD) **:**

SOD which was discovered in the late 60s catalyses the transformation of the superoxide radical (O_2^-) into hydrogen peroxide, which can then be further transformed by the enzyme catalase into

water and molecular oxygen. While superoxide anion is itself reactive, though not particularly to large extent, it can reduce transition metal ions, such as iron and get converted to one of the most reactive peroxides (Vellosa*et al.*, 2007). Thus, elimination of superoxide can attenuate the formation of the harmful peroxides and hydroxyl radical.

$$O_2^{\cdot} + O_2^{\cdot} + 2H^{+} \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Catalase**:**

The role of superoxide dismutase is the dismutation of superoxide anion (O_2^{-}) into oxygen and hydrogen peroxide (H_2O_2). Hydrogen peroxide is however a highly reactive and potent oxidant, as such there is need for it to be converted to a less active form. Catalase converts hydrogen peroxide further into oxygen and water, thus complementing the antioxidant activities of SOD.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Glutathione peroxidase**:**

Glutathione peroxidase (GPx) reduces lipid peroxides (ROOH), formed by the oxidation of polyunsaturated fatty acid (PUFA), to a stable, non-toxic molecule-hydroxyl fatty acid (ROH). Together with phospholipases GPx can also convert phospholipid hydroperoxides (PL-OOH) into phospholipid hydroxide (PL-OH), thereby protecting the cell from potentially harmful hydroperoxides (Recknagel*et al.*, 1982).

2.5.3.2 High molecular weight proteins

High molecular weight proteins such as albumin, ceruloplasmin, transferrin and haptoglobin, which are all present in plasma, bind to redox active metals and can limit the production of metal-catalysed free radicals. Albumin and ceruloplasmin can bind copper ions, and transferrin

binds free iron. Haptoglobin binds heme-containing proteins and can thus clear them from the circulation. Both free and heme-associated proteins have pro-oxidant properties due to their reaction with H_2O_2 to form ferryl species which can easily initiate lipid peroxidation.

2.5.3.3 Low molecular weight antioxidants

These are subdivided into lipid-soluble antioxidants (tocopherol, carotenoids, quinones, and some phenolics) and water-soluble antioxidants (ascorbic acid, uric acid and some polyphenols). They delay or inhibit cellular damage mainly through their free radical scavenging property.

Tocopherols (Vitamin E) :

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, α-tocopherol has been most studied as it has the highest bioavailability (Manach*et al*., 2004)). α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Trenerry, 2008). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol. However, the roles and importance of the various forms of vitamin E are presently unclear. Nonetheless, γ-tocopherol is known to be a nucleophile that may react with electrophilic mutagens, and tocotrienols may be important in protecting neurons from damage (Trenerry, 2008).

Ascorbic acid (Vitamin C):

Ascorbic acid is an essential nutritional antioxidant. Purified ascorbic acid is a crystalline compound with an empirical formula of $C_6H_8O_6$ and a molecular weight of 176.1. It is a keto-lactone with structural formula similar to that of carbohydrates. Levine (1995) reported that insects, invertebrates and fish lack the ability to synthesize ascorbic acid. The biosynthetic capacity was found to start in the amphibians and reptiles in which the synthesis was localized in the kidney while the synthetic capacity disappeared in the guinea pig, the flying mammals, Indian fruit bats, monkeys and man. The failure of primates to synthesize ascorbic acid has been found to be due to a common defect; which is the absence of the terminal enzyme in ascorbic acid biosynthesis, L-gulonolactone oxidase. This enzyme catalyses the oxidation of L-gulonolactone to L-2-ketogulonolactone which spontaneously isomerizes to form ascorbic acid. It is the loss of this single enzyme (the gene for the enzyme is defective) that renders ascorbic acid an essentialvitamin. Ascorbic acid is a reducing agent and can reduce and thereby neutralize reactive oxygen species such as hydrogen peroxide. Ascorbic acid is an important water soluble antioxidant vitamin in humans.

Phenolics:

These are polyhydroxy alcohols or their derivatives. Their function in counteracting oxidative stress is due to the ability of their hydroxyl groups to stabilize and quench singlet radicals. They are among the most widely distributed natural antioxidants found in plants (Atawodi*et al.*, 2009).

2.5.4 Plants as sources of natural antioxidants

Plant extracts containing low molecular mass compounds have been successively used in phytotherapy since ancient times. It has been demonstrated that many naturally occurring phytochemicals possess notable activity as radical scavengers and lipid peroxidation inhibitors

(Atawodi, 2005). In addition to plant extracts, numerous naturally occurring compounds are useful as antioxidant, ranging from alpha tocopherol and beta carotene to other plant antioxidants such phenolic compounds (tannins, flavonoids, anthrocyanins, chalcones, xanthones, xanthones, lignans, depsides, and depsidoneetc), terpenes (sesquiterpenes and diterpineses), alkaloids, organic sulphur compounds etc. Numerous of experiments have been carried out concerning the antioxidant activity of several plant extracts and powders. The results of these experiments reveal that, the activity is due to several secondary metabolites especially phenolic compound *e.g.*: flavonoids, tannins etc. The polyphenolic compounds generally present a tonifying action because of their natural antioxidant properties. Other action stressed is the stimulation of protein synthesis and the promotion of ammonia elimination. More specifically, the compounds cause a stabilization of cell membrane components in cell organelles such as lysosomes as well as stabilization of the plasmatic membrane of erythrocytes, mastocytes, fibrocytes, hepatocytes and other similar cells(Ojo & Ladeji, 2005).

2.6 Polyphenols

Polyphenols are a large family of natural compounds widely distributed in plant, their chemistry generally characterized by the presence of more than one phenol unit or building block per molecule. Polyphenols occur in most plant foods and their antioxidant capacities contribute to the beneficial health effects of vegetables and fruits. However, their considerable diversities make them different from other antioxidants. Several thousands of natural polyphenols have been identified in plants, many of them in plant foods (Shahidi and Naczk 1995).

2.6.1 Classification of polyphenols

Over 8,000 different phenolic compounds belonging to four major groups namely flavonoids, phenolic acids, coumarins and tannins have been isolated from different natural products (Shahidi and Naczk, 1995). These molecules are among the secondary metabolites of plants. Secondary metabolites are generally involved in defence against ultraviolet radiation or aggression by pathogens (Claudine *et al.,* 2004). These compounds may be classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. Distinctions are thus essentially made between the phenolic acids, flavonoids, stilbenes, and lignans. In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another.

The flavonoids, which share a common structure consisting of two aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols(e.g: quercetin, kaempferol, myricetin, isorhamnetin), flavones (e.g: luteolin, apigenin), isoflavones (eg: daidzein, genistein, glycitein, biochanin A, formononetin), flavanones (e.g: eriodictyol, hesperetin, naringenin), anthocyanidins (e.g: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin), and flavanols (e.g: catechins, epicatechins, theaflavins, thearubigins and proanthocyanidins) (Chun *etal*, 2007).

Stilbenes are structurally consisting of double bonds in between two separate phenyl groups. Chemically hydroxylated derivatives of stilbenes referred to as stilbeniods are common polyphenols compounds found in plants. Examples are resveratrol and pterostilbene. The lignans

are also a group of chemical compounds found in plants. Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals and also act as antioxidants. Some examples of lignans are pinoresinol, podophyllotoxin, and steganacin.Tannins are generally polyphenolic compounds of high enough molecular weight to form complexes with proteins. They are further classified to hydrolysable tannins and condensed tannins (Osawa*et al*., 1994).

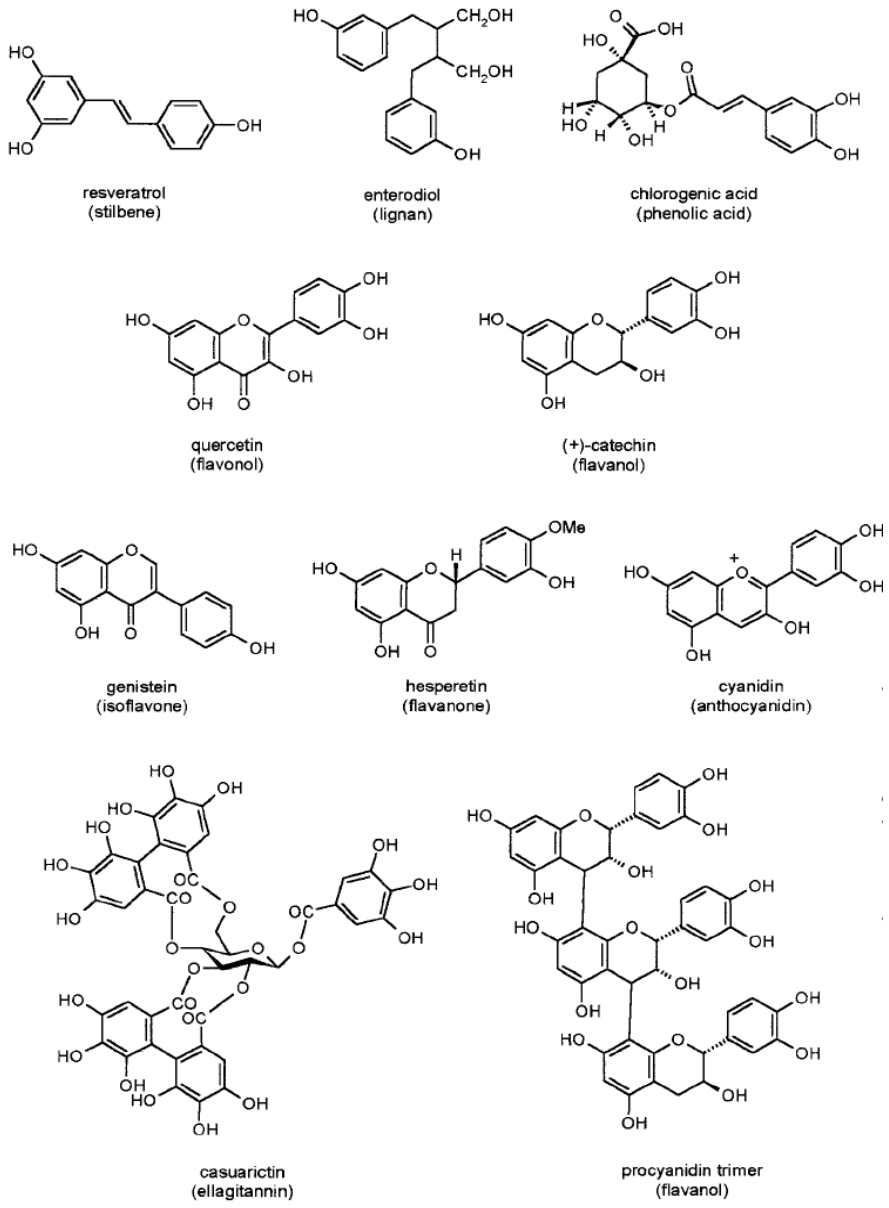

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Figure 2.3: Chemical Structure of Some Polyphenols (Scalbert and Garry (2000).

2.6.2 Polyphenols in plants foods

Phenolic molecules in plants are too diversified, often characteristic of a plant species or even of a particular organ or tissue of that plant. It is therefore impossible to know precisely the nature of all of the polyphenols that we ingest. As such it is more desirable to know the main classes of the polyphenols consumed; the main foods or herbs that contain them and their average to precise contents in these plants. The main classes of polyphenols are defined according to the nature of their carbon skeleton namely phenolic acids, flavonoids, tannins and the less common stilbenes and lignans.

The most frequently encountered of the phenolic acids are caffeic acid and to a lesser extent, ferulic acid (Clifford and Scalbert, 2000). Ferulic acid is associated with dietary fibre and is linked through ester bonds to hemicelluloses. One of the main food sources of ferulic acid is wheat bran (5 mg/g) (Kroon *et al.*1997). Caffeic acid is also found however, usually in the form of esters. The most frequently encountered caffeoyl ester is chlorogenic acid, which is present in many fruits and vegetables and in coffee. One cup of instant coffee (200 ml) for instance contains 50–150 mg of chlorogenic acid (Clifford and Scalbert, 2000).

Other phenolic acid derivatives are hydrolysable tannins. The hydrolysable phenolic acids are either gallic acid as in gallotannins (in mango fruit) or other phenolic acids derived from the oxidation of galloyl residues in ellagitannins (in blackberry, raspberry, strawberry, wine and brandy aged in oak barrels), their occurrence is much more limited than that of condensed tannins. (Clifford and Scalbert, 2000).

Flavonoids are the most abundant polyphenols in our diets. Theycan be divided into several classes according to the degreeof oxidation of the oxygen heterocycle: flavones, flavonols,isoflavones, anthocyanins, flavanols, proanthocyanidins andflavanones.The occurrence of some of these flavonoids is restricted few foodstuffs. The main source of isoflavones is soy, whichcontains ~1 mg of genistein and daidzein per gram dry beans. These two isoflavones have received considerable attentiondue to their estrogenic properties and their suggested role inthe prevention of breast cancer and osteoporosis (Adlercreutzand Mazur, 1997). Citrus fruits are the main food source of flavanones and the most widely consumed is hesperidin (Clifford and Scalbert, 2000).

Other flavonoid types are common to various food sources. Quercetin, one of themain flavonol in our diet, is present in many fruits and vegetables aswell as in beverages. It is particularly abundant in onions (0.3 mg/g fresh weight) and tea (10–25 mg/L)(Hertog*et al*., 1993). Flavonesare less common and were identified in sweet red pepper (luteolin)and celery (apigenin) (Hertog*et al*., 1992). The main flavanolsare catechins. They are very abundant in green tea, red wine and chocolate (Scalbert and Garry, 2000).

Proanthocyanidins are polymeric and are usually present in associationwith flavanols and catechins. Common sources are fruits such as apple, pear and grape, beverages such as red wine and tea, as wellas chocolate (Scalbert and Garry, 2000). Anthocyaninsare pigments of red fruits such as cherries, plums, strawberries, raspberries,blackberries, grapes, red currants and black currants. Theircontents vary from 0.15 (strawberries) to 4.5 mg/g (cherries)in fresh fruit and the average content in red winesis 26 mg/L (Clifford and Scalbert, 2000).

Stilbenes are not widespread in food plants. Nevertheless, oneof them, resveratrol, which was revealed during the screeningof medicinal plants, has recently received great attention forits anticarcinogenic properties and presencein wine. However, it's very low concentration in wine (0.3–2mg/L in red wines) makes the attributionof protective effects to this molecule unlikely (Scalbert and Garry, 2000).

Lignans have been identified in human plasma and urine (Adlercreutzand Mazur, 1997). Their dietary origin is established, but not much literature was available about theirprecursor in foods. The only foods that containconsiderable quantities of lignans are flaxseed and flaxseedoil (Scalbert and Garry, 2000). When fed to humans or animals, theyare metabolized by the gut microflora into the "mammalian lignans". Lignans are recognized as phytoestrogens due to their estrogenagonist and antagonist properties.

Other dietary polyphenols are not well-defined chemical entities andresult from the oxidative polymerization of flavonoids and phenolic acids.This may occur during ripening or food processing (grinding, fermentation,storage, cooking etc.). These ill-defined phenoliccompounds are the main polyphenols in black tea and wine, particularlyaged wine (Scalbert and Garry, 2000)

2.6.3 Roles of polyphenols as antioxidants

Polyphenols are receiving increasing interest from consumer, food manufacturers and researchers for several reasons. Epidemiological studies have suggested associations between the consumption of polyphenols-rich foods or beverages and the prevention of diseases. Fruit and

vegetable consumption prevents cancers (Atawodi *et al*., 2010a). It may also prevent stroke, whereas wine consumption might prevent coronary heart disease (Ghosh *et al*., 2006). The consumption of tea may protect against cancers and coronary heart diseases (Atawodi, 2012), and that of soy may protect against breast cancer and osteoporosis (Adlercreutz and Mazur, 1997). The reason is linked to the fundamental chemical nature and antioxidant potentials of polyphenols.

Polyphenols are reducing agents, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids, they protect the body's tissues against oxidative stress. Commonly referred to as antioxidants, they may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, inflammation and others. The antioxidant effects of many plants are mainly due to the presence of phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Shahidi and Naczk, 1995). Although, there are some reports on the antioxidant effects of *Detarium microcarpum and Dialium guineense*,their roles in colon carcinogenesis have not been previously reported.

Detarium microcarpum

2.7.1 Description, origin and distribution of *Detarium microcarpum*

*Detarium microcarpum*Guill. (Fabacea) is an African leguminous medicinal plant found in the forests (Mabberley, 1987) .*Detarium* belongs to the tribe *Detarieae* and is related to *Copaifera*. It is confined to Africa and comprises 3 species: *Detarium microcarpum*, *Detarium senegalense*J.F.Gmel. and*Detarium macrocarpum* Harms, which are morphologically very similar, but ecologically differentiated. *Detarium microcarpum* is typically a species of the dry

savanna, *D. senegalense* is more riparian and also occurs in dry forest while*D.microcarpum* occurs in humid forest.

Figure 2.4: The Tree and Fruits of *Detarium microcarpum.*

Detarium microcarpum is not genetically endangered, but in regions where population pressure is high it is overexploited for wood and is also frequently cut down on agricultural land. This can lead to its local disappearance. *Detarium microcarpum* is distributed in the semi-arid sub-Saharan Africa, from Senegal to Cameroon, extending eastwards to the Sudan. It has an irregular distribution and can be locally very common. The species is often left when farmland is cleared. Typically, it is found in high rainfall savannah areas, dry forests and fallow land, on sandy or iron rich hard soils. It also occurs in open savannah of Northern Nigeria, parts of Chad and Niger republic as a more stunted tree with smaller fruit and is called "*Taura"* in Hausa.

2.7.2 Cultivation and horticultural practices of *Detarium microcarpum*

Detarium microcarpum grows on dry soil in wooded savanna and open woodland, and is locally very common. Typically, it is found in high rainfall savannah areas, dry forests and fallow land, on sandy or iron rich hard soils. It also occurs in open savannah as a more stunted tree with smaller fruits. It is most common in regions with an annual rainfall of 600–1000 mm. It is mainly found on shallow, stony and lateritic soils, and on hills.

Detarium microcarpum regenerates well from shoots produced by the trunk or roots. Shoots from the trunk are much more vigorous than seedlings and can reach a height of 1.5–2 m in 1–2 years. In Cameroon the average seedling height after 3 years is 0.6 m, and the seedling may reach 1.5 m in 4 years. *Detarium microcarpum* flowers during the rainy season, from July–September (–November), and bears fruit from September–January (–May). It sheds its leaves in

November and produces new ones in March. The main flowering period of a tree is up to 8 days only, and flowers are pollinated by insects, especially in the mornings (Burkill, 1995).

Traditionally in Africa the fruits of *D. microcarpum* are collected manually by hand plucking. The fruits of *Detarium microcarpum* can be kept for 1–3 years in jute bags. The leaves and bark are used fresh or dried for future use. As WHO recommended, however, for all medicinal plants, care must be taken in collection of useable part. The guidelines on good agricultural and collection practices (GACP) for medicinal plants should be followed (WHO 2003). *D. microcarpum* should not be collected in or near areas where high levels of pesticides or other possible contaminants are used or found, such as roadsides, drainage ditches, mine tailings, garbage dumps and industrial facilities which may produce toxic emissions . After collection, the plant material should be subjected to appropriate preliminary processing, including elimination of undesirable materials and contaminants. The plant material should be protected from insects, rodents, birds and other pests, and from livestock and domestic animal.

2.7.3 Nutrient composition and uses of *Detarium microcarpum*

The seeds are processed into flour and used traditionally in Nigeria as a flavoring agent and as a soup thickener. It has been reported that the seeds of *D. microcarpum*contains a large amount of water-soluble, non-starch polysaccharide, xyloglucan which suggests that this plant has considerable potential in food, drugs and chemical industries (Wang *et al*.,1997). Oibiokpa*et al*., (2014) reported crude protein content obtained in *D. microcarpum* fruit pulp as 4.68% while the crude fat content was 2.23%.The fruit pulp also contained 4.47% moisture, 4.47% ash, 11.06% crude fibre and 65.38% total carbohydrates. The mineral composition of the fruit pulp indicate

that potassium was the most abundant (908.10 mg/100 g) and cadmium was the least abundant (0.03 mg/100 mg). Vitamin analysis revealed that the fruit is rich in vitamin C (55.10 mg/100 g). The fruit was also discovered to contain 12.44 mg/100 g, vitamin E, 4.20 mg/100 g vitamin B2 and 0.17 mg/100 g folic acid (Oibiokpa*et al.*, 2014).The seed gum contains D-galactose as a major monosaccharide, as well as D-mannose and D-glucose. The seeds yield 7.5% oil, with linoleic acid being the predominant fatty acid. The gum content (water-soluble polysaccharides) is high. The hulled seed flour contains per 100 g: water 3.5–6.5 g, crude fibre 3 g, crude fat 13–15 g, crude protein 13.5–27 g, carbohydrate 39 g, Ca 500 mg, Mg 500 mg, Fe 100 mg (Akpata and Miachi 2001).

2.7.4 Medicinal uses of*Detarium microcarpum*

The roots, stems, bark, leaves and fruits are all used to treat ailments e.g. tuberculosis, meningitis, itching and diarrhoea. The fruits and leaves are used traditionally in the treatment of dysentery and syphilis. (Iwu, 1993; IkhiriandIlagouma 1995) and the root water extract is used for leprosy (Collier, 2001). The fruit is edible and rich in vitamin C and the leaves and seeds are also used in cooking. The bark, leaves and roots of *Detarium microcarpum* are widely used throughout its distribution area because of their diuretic and astringent properties. They are prepared as infusions or decoctions to treat rheumatism, venereal diseases, urogenital infections, hemorrhoids, caries, biliousness, stomach-ache, intestinal worms and diarrhoea including dysentery (Burkill, 1995). They are also used against malaria, leprosy and impotence. A decoction of the powdered bark is widely taken to alleviate pain, e.g. headache, sore throat, back pain and painful menstruation. The fresh bark or leaves are applied to wounds, to prevent and cure infections. In Mali the bark is also used to treat measles, hypertension, itch and tiredness,

while a decoction of the leaves or roots is taken against paralysis, meningitis, tiredness, cramps and difficult delivery. The powdered seeds are applied to skin infections and inflammations, whereas the fruit is eaten to cure meningitis and malaria. In Burkina Faso the fruit pulp is used for treating skin infections. A preparation of the fruits is taken against dizziness in Niger and Togo (Keay *et al.*, 1958). In Senegal a mixture of the leaves of *Detarium microcarpum*, *Sclerocaryabirrea* (A. Rich.) Hochst. and*Acacia macrostachya*Rchb. ex DC. pounded in milk is considered very efficient for snakebites. In Benin a decoction of the leaves is taken to treat fainting and convulsions.

In West Africa the roots are part of a medico-magical treatment for mental conditions, and for protection against bad spirits. In veterinary medicine the leaves and roots are used to treat diarrhoea in cattle in southern Mali, and in Benin to treat constipation. In Niger cattle are made to inhale the smoke of the leaves to treat fever (Abbreu *et al.*, 1998). Incorporation of 0.5% gum in wheat flour increased the water absorption and the mixing tolerance index of the dough significantly. Oil-water emulsions stabilised by the seed flour or gum tolerate freezing and thawing better than commercial salad dressing, egg powder and gum tragacanth emulsions. Addition of the seed polysaccharide to fruit products (mango, orange, pineapple, tomato) improved their stability in storage (at 26°C) for at least 2 months and was well acceptable to consumers.

2.7.5 Chemical compositions of *Detarium microcarpum*

The commonly known phytochemical compounds in the genus Detarium are flavanes (Mahmood *et al*., 1993), however, a wide range of chemical constituents have been isolated from the plant *D. microcarpum*. These include diterpenes and water soluble polysaccharides, proteins and

coumarins(Peter *et al*., 2012). From the CH_2Cl_2 extract of the fruit pulp of *D. microcarpum*, Cavin *et al*., (2006) isolated 4 clerodane diterpenes 3,4-expoxy cherodan-13E-en-15-oic acid, 5α,8α–(2-oxokolavenic acid, 3,4-dihydroxyclerodan-13Z-en-15-oic acid, as well as 2-oxokolavenic acid and copalic acid. From the bark extract of *D. microcarpum* Mahmood et al., (1993) isolated two tetranorditerpenes-the derodane diterpenes catechin and cis-2- oxokolavenic acid, as well as the diterpene copalic acid and coumarins. Also from the bark extract, Abreu *et al*. (1998) isolated sitosterol, β-sitosterol, lupol, stigmasterol, camposterol, γ-quinide, (-)-bomesitol, D-pinitol, myoinositol, sucrose, D-glucose and D-fructose. The seed gum was reported to contain D-galactose as a major monosaccharide, as well as D-mannose and D-glucose (Peter *et al*., 2012).

2.7.6 Biological activities of *Detarium microcarpum*

Olugbuyiro (2009) reported on anti-viral activity and cytotoxicity of *D. microcarpum*column fractions in the Huh-7 Replicon assay. The active fraction MTH- 1700 (6.155g) which was eluted with EtOAc-MeOH (75:25) demonstrated good inhibitory and selective potency (83.87%) against Hepatitis C Virus in a dose dependent manner compared with control (86.76%). The ethanol extract of the bark showed antimicrobial action against *Pseudomonas aeruginosa*, *Citrobacterfreundii*, *Klebsiellapneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Listeria monocytogenes* (Abbreu *et al.,* 1998). The extract showed moderate antitumour activity against breast cancer cells. The flavanes present in a methanol extract of *Detarium microcarpum* showed strong inhibitory effects on HIV-1 or HIV-2 infection (Abbreu *et al*, 1999). A bark extract showed significant molluscicidal activity against *Lymnaeanatalensis*. It has been found to contain 2 tetranorditerpenes, the clerodanediterpenes catechin and cis-2-oxokolavenic acid

(0.5%), the diterpenecopalic acid (1.7%) and coumarin (1%). A methanol extract of the leaves exhibited strong feeding deterrent activity against the termite *Reticulitermessperatus*.

2.8*Dialium guineense*

2.8.1 Description and distribution of *Dialium guineense*

Dialium guineense (Wild) belongs to the family of Fabacea, commonly called black velvet or velvet tamarind. It is a tree of an average height of 30 m with densely leafy crown, smoothgreyish bark. Leaves are hairy and the flowers are usually whitish while the fruits are less circular and flattened. The pulp of the fruit is edible and sweet to sour.

Dialium guineense can be found in West African countries such as Ghana, Sierra Leone, Senegal, and Nigeria. *Dialium guineense* is commonly known as "Awin" among the Yoruba in the Western part of Nigeria, as "*Icheku*" among the Igbo in the Eastern part of Nigeria, and as "*Tsamiyarbiri*" among the Hausas in the Northern part of Nigeria (Akinpelu*et al*., 2011).

Figure 2.5: The Tree and Fruits of *Dialium guineense.*

2.8.2 Cultivation andhorticultural practices of *Dialium guineense*

The tree possesses densely, hairy leafy crown, smooth greyish bark and whitish flowers which bears densely velvet black fruits that are more or less circular and flattened enclosing dry, brownish, sweet acidic edible pulp (Hutchinson and Daniel, 1958). In Nigeria, the tree flowers from September to October, and fruits from October to January (Keay, 1989). Fruits are usually harvested by hand plucking. A well-established velvet tamarind plant bears one set of fruit per year and yields up to 200kg of fruits annually. The leaves and bark are used fresh or dried for future use. As WHO recommended, however, for all medicinal plants, care must be taken in collection of useable part. The guidelines on good agricultural and collection practices (GACP) for medicinal plants should be followed (WHO 2003). *D. guineense* should not be collected in or near areas where high levels of pesticides or other possible contaminants are used or found, such as roadsides, drainage ditches, mine tailings, garbage dumps and industrial facilities which may produce toxic emissions. After collection, the plant material should be subjected to appropriate preliminary processing, including elimination of undesirable materials and contaminants. The plant material should be protected from insects, rodents, birds and other pests, and from livestock and domestic animals

2.8.3 Nutrient composition and uses of *Dialium guineense*

The pulp of the fruit is edible and sweet, fairly low levels of ascorbic acid and tannin are present. It is a fairly good source of protein and minerals used traditionally as beverage flavoring, soup and porridge thickeners or simply chewed as a delicacy.Ogugbenle and Ebadan (2013) reported the ash moisture, crude fat, crude fibre, crude protein and

carbohydrate of the Velvet Tamarind (*Dialium guineense)*fruit-pulp were: 4.63%, 8.22%, 5.80%, 7.15%, 24.3% and 49.9% respectively. Furthermore, they reported the water and oil absorption capacities were: 238% and 162% which makes the pulp exhibit a high water retention capacity, the least gelation concentration was 17.0% while the foaming capacity and stability were: 43.5% and 62.2% respectively. The *in-vitro* protein digestibility was reported as 66%. Glutamic acid was the most concentrated amino acid with the value of 14.8 mg/g crude protein while histidine (2.12 mg/g crude protein) was the least concentrated amino acid. The percentage total essential amino acid with histidine was 60.8%. The calculated isoelectric point was 0.54 while the predicted protein efficiency ratio (P-PER) was 2.62 (Ogungbenle and Ebadan, 2013)

2.8.4 Medicinal uses of *Dialium guineense*

The bark and leaves of *D. guineense* have medicinal properties and are used against several diseases. The fruits of the plant are chewed among some women in southeast Nigeria to improve lactation and check genital infection (Nwosu, 2000). *D. guineense* is used as chewing stick (indigenous tooth brush) among Nigerian populace (Akinpelu*et al*., 2011). *D. guineense* leaves and stem bark are used as folklore remedies for the treatment of infections such as diarrhoea, severe cough, bronchitis, wound, stomach aches, malaria fever, jaundice, antiulcer and hemorrhoids (Bero*et al*., 2009).

2.8.5 Chemical compositions of *Dialium guineense*

Okwu and Ekeke (2003) reported in their studies that the plant contains saponin which is presumed to add to the cleaning effect of teeth and at the same time prevent caries and

plaques on the teeth of the users. Researchers have reported the presence of some phytochemicals such as alkaloids, tannins, flavonoids and phenolic compounds (Aliyu *et al*., 2009; Amaeze *et al*., 2011; Omoregie and Osagie, 2012).

2.8.6 Biological activities of *Dialium guineense*

Some of the scientifically validated activities of the plant leaves and stem bark include its analgesic and antibacterial activities (Ezeja *et al*., 2011), antioxidant, antimicrobial and antiulcer properties activities (Gideon *et al*., 2013).Significant antioxidant and molluscidal activities of *D. guineense* exhibited have also been reported (Akinpelu *et al*., 2011; Amaeze *et al*., 2011). Earlier researchers have linked the antioxidant and antimicrobial potentials to the content and richness of some phytochemicals such as alkaloids, tannins, flavonoids and phenolic compounds (Atawodi *et al*., 2010a; Aliyu*et al*., 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/Reagents

The chemicals/reagents N-methyl-N-nitrosourea (MNU), sodium dihydrogen phosphate (monobasic), disodium hydrogen phosphate (dibasic), sodium hydrogen carbonate, sodium carbonate, sodium citrate, methanol, hydrogen peroxide, glacial acetic acid were supplied by Sigma-Aldrich Chemicals, Missouri, USA while chloroform, sodium hydroxide, xylene, ethanol, thiobarbituric acid, trichloroacetic acid were from Merck and Co, Darmstadt, Germany.

3.1.2 Assay Kits

Rat carcinoembryonic antigen (CEA) ELISA kit was obtained from USCN Life Science Inc (Cloud-Clone Corp, USA), Immunohistochemistry assay kits was from Bio-Vison Incorporated, 155 S. Milpitas Boulevard, Milpitas, CA 95035 USA) while aminotransferases assay kits and kidney function assay kits were from Sigma-Aldrich Chemicals, Missouri USA.

3.1.3 Apparatus

Equipment used include UV spectrophotometer (6305 model, Jenway Instruments, 50/60Hz, U.K.), pH meter (3150 model, Jenway Instruments, UK.), Weighing Balance (GF-2000, A&D Instrument Ltd, Japan), water bath (Grant NL-420S series, Grant instruments Ltd,

England), temperature regulated centrifuge (TGL – 16G. B. Bran Scientific & Instrument England), Microplate reader (GM-3000, model 200711012; B. Bran Scientific Company England), Hematocrit Auto-Analyser (Abacus junior, Hematology analyser, DIATRON GmbH. Wien Australia) and Electrical Homogenizer (RQ-127A/D – Rajendra Elec. Ind. Ltd. India).

3.2 Methods

3.2.1 Collection and authentication of plant samples

Dried fruit-pulp of *Detarium microcarpum* and *Dialium guineense* were collected from Ringim Local Government Area of Jigawa State, Nigeria, and identified at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria where samples of *D. microcarpum* and *D. guineense* were deposited with voucher numbers 901451 and 1855 respectively.

3.2.2 Preparation of plant samples

Each plant sample was further dried in the laboratory at room temperature, pounded using laboratory mortar and pestle and then sieved using 2mm gap sieve (meshed utensil) to ensure homogeneity. The sieved samples were stored in air-tight opaque glass container until required for use.

3.2.3 Experimental animals

Seventy (70) male wistar albino rats (weighing 80 – 150 g) were obtained from the Animal House of the Physiology Department, Ahmadu Bello University Zaria and transferred to the animal-holding facility in the Department of Biochemistry, where the animals were maintained

throughout the period of the study. The rats were weighed and divided randomly into 10 groups of 7 animals each. The animals were fed and given water *ad libitum* and pre-conditioned for a period of two weeks.

3.2.4 Experimental diet Formulation and feeding

Poultry growers pellet feed (Vital feeds, Grand Cereals, Jos, Plateau State, Nigeria) was used as feed in the entire study. The feed composition was crude protein 20%, fat 9%, fibre 9%, calcium1% and phosphorous 0.45%. The pelletized feed was similarly grounded and sieved to the same particulate size with the grounded fruit pulps. The feed was then mixed with grounded fruit-pulps of *D. microcarpum* and *D. guineense* according to the percentage of inclusion of 2.5%, 5% and 10%. Through mixing was done until the entire feed and fruit sample become homogeneous. The normal control and MNU control groups were given normal feed without inclusion. In the initial phase of the experiment feeding lasted for twelve (12) weeks after which MNU induction began. The feeding with respective diets continued all through the induction period (second phase) which lasted for another twelve (12) weeks.

Table 3.1 Experimental Animal Groupings and Diet Formulation

Animal Group	% inclusion of Fruit-pulp in diet	MNU	Normal Saline
1	0.0	-	-
2	0.0	+	-
3	2.5% *D. guineense*	+	-
4	5.0% *D. guineense*	+	-
5	10.0% *D. guineense*	+	-

6	10.0% *D. guineense*	-	+
7	2.5% *D. microcarpum*	+	-
8	5.0% *D. microcarpum*	+	-
9	10.0% *D. microcarpum*	+	-
10	10.0% *D. microcarpum*	-	+

3.2.5 Induction Protocol of Colon Carcinogenesis

Rats in each test and MNU control group were administered with 0.2ml of 1% *N*-methyl-nitrosourea (MNU) intrarectallywith the aid of a cannula (8cm), 72 hourly for ten (10) weeks, while the rats in each control group were simultaneously administered normal saline for the same period of time.

3.2.6 Animal sacrifice

At the end of the induction period, all the animals were sacrificed by cutting the jugular vein after asphyxiation in mild chloroform anesthesia chamber. The blood, serum, colon and major organs of the animals were then collected for biochemical assay and other investigations.

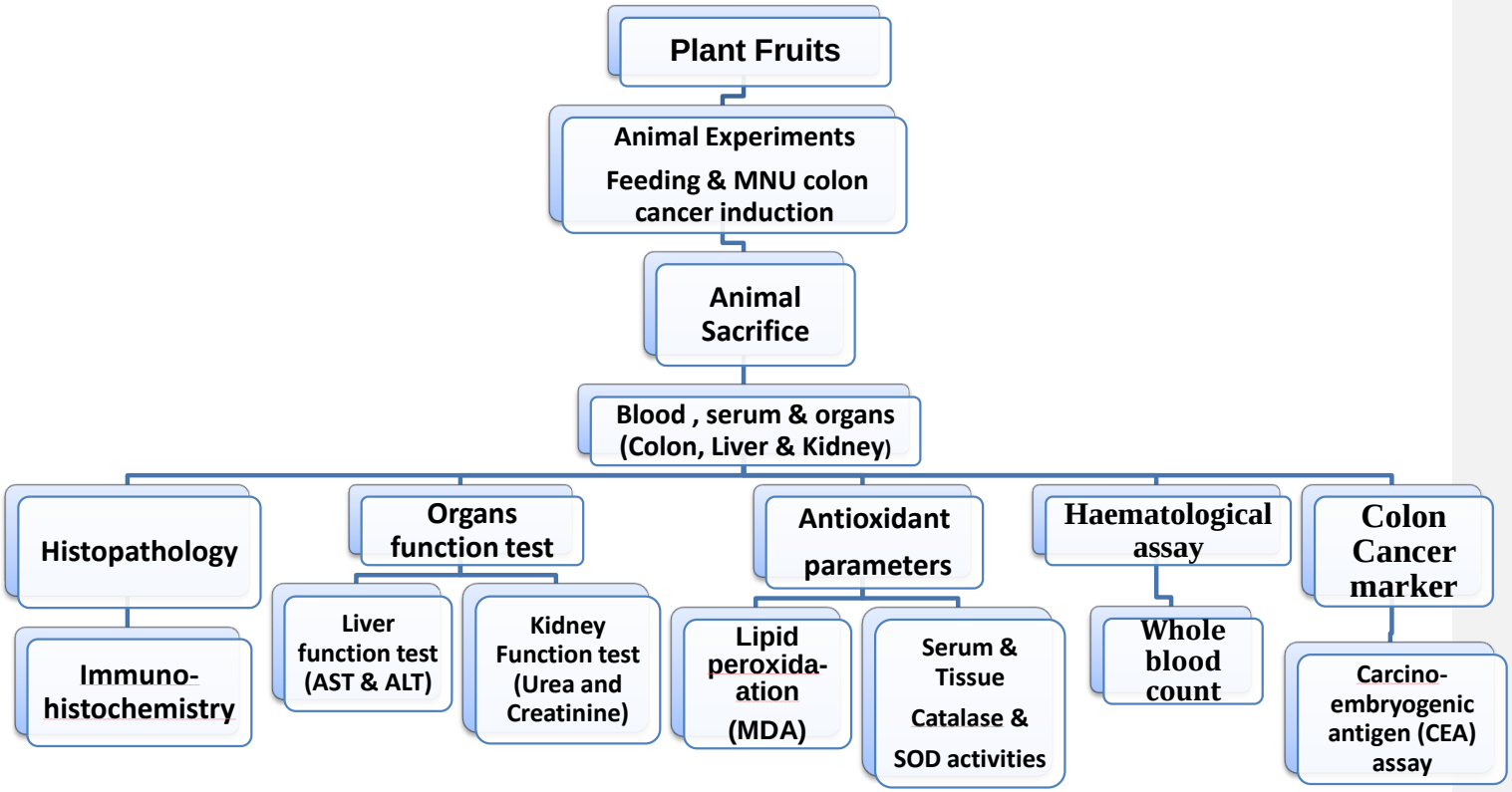


Figure 3.1 Flow Chart of Experimental Design for Evaluation of Preventive Effects of *Detarium microcarpum* and *Dialium guineense* Fruits in MNU-Induced Colon Carcinogenesis Model.

3.3 Organ Function Test

3.3.1 Liver function test: determination of aspartate aminotransferase (AST) activity

Principle: AST determination (using the Randox laboratories Ltd. assay kit) was based on description of Reitman and Frankel (1957). AST, also known as SGOT (Serum Glutamate-oxaloacetate) catalyses the reaction:

α-Ketoglutarate + L-Aspartate $\xrightarrow{\text{AST}}$ L-Glutamate + Oxaloacetate

Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine, (pH 7.4) to give the corresponding hydrazone, which gives brown colour in alkaline medium which can be measured colorimetrically.

Procedure: Buffered phosphate, aspartate and α-ketoglutarate Substrate (0.5ml) were added to 0.5ml of serum, mixed properly and incubated at 37^{o}C for 30minutes. Solution of 2, 4-dinitrophenylhydrazine (0.5ml) was then added, mixed well and allowed to stand at room temperature for 20minutes. Then, 2.5ml of NaOH was added, mixed and absorbance was read against blank at 546nm. AST activity was calculated as: AST (U/I), from the standard calibration curve that was prepared.

3.3.2 Liver function test: determination of alanine aminotransferase (ALT) activity

Principle: ALT determination (using the Randox laboratories Ltd. assay kit) was based on method of Reitman and Frankel (1957). ALT also known as SGPT (Serum Glutamate-pyruvate transaminase), catalyses the reaction:

α-Ketoglutarate + L-Alanine $\xrightarrow{\text{ALT}}$ L-Glutamate + Pyruvate

Pyruvate so formed is coupled with 2, 4-Dinitrophenyl hydrazine, (pH 7.4) to give the corresponding hydrazone, which gives brown colour in alkaline medium which can be measured colorimetrically.

Procedure: Buffered phosphate, alanine and α-ketoglutarate substrate (0.5ml) were added to 0.5ml of serum, mixed properly and incubated at 37oC for 30 minutes. Solution of 2, 4-dinitrophenylhydrazine (0.5ml) was then added, mixed well and allowed to stand at room temperature for 20 minutes. Then, 2.5ml of NaOH was added, mixed and absorbance was read against blank at 546nm. ALT activity was calculated as: ALT (U/I), from the standard calibration curve that was prepared.

3.3.3 Kidney function test: determination of creatinine concentration.

This was determined by method described by use of kit (Randox laboratories Ltd.) (Stephen *et. al*., 2007).

Principle: This method is based on picric acid forming a red colored complex with creatinine. The intensity of the color, measured at 525nm is directly proportional to creatinine concentration in the sample.

Procedure: Exactly 0.2 ml of serum and creatinine standard were added into separate clean tests tube containing 2.0 ml of creatinine working reagent which is made up of picric acid

and sodium hydroxide. They were mixed and absorbance A1 was read after 30seconds, at exactly 2 minutes later, absorbance A2 was also read at 510nm.

Concentration of creatinine (mg/dl) in the serum =

$$\frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times \text{concentration of standard (mg/dl)}$$

3.3.4 Kidney Function Test: determination of Blood Urea Concentration

Urea was determined using the method described by Stephen *et al*., (2007) using assay kit (Randox laboratories Ltd).

Principle: Serum urea is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction as shown in the equation below:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenol (blue coloured compound)}$$

Procedure: Exactly 100µl of reagent 1 which is made up of EDTA, sodium nitroprusside and urease was added in a clean test tube containing 10µl of serum. They were mixed and incubated at 37^{o}C for 10 minutes and 2.5ml of reagent 2 which is made up of phenol (diluted) and 2.5ml of reagent 3 which is made up of sodium hypochlorite (diluted) and sodium hydroxide were added, mixed immediately and incubated at 37oC for 15 minutes. Absorbance was taken at 540nm. Concentration of urea was calculated as follows:

$$\text{Urea concentration (mg/dl)} = \frac{\text{Sample absorbance} \times \text{standard concentration (mg/dl)}}{\text{Standard absorbance}}$$

3.4 Antioxidant Assays (*In Vivo*)

3.4.1 Thiobarbituric acid reactive substances (TBARS) assay

Thiobarbituric acid reactive substances are low-molecular weight by products produced at the decomposition of lipids. Lipid peroxidation was determined as thiobarbituric acid reactive substance (TBARS) according to the method of Okhawa *et al.,* (1979) as described by Atawodi *et al.,* (2011) using trichloroacetic acid (TCA) and thiobarbituric acid (TBA).

Principle: Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde (MDA), a product which react with thiobarbituric acid. The assay is dependent on the tendency of MDA to react with thiobarbituric acid to form Thiobarbituric Acid Reacting Substances (TBARS) (Li-Fu *et al.,* 2012). The product of the reaction is a coloured complex which absorbs light at 535nm and can hence be measured photometrically. The colour intensity of the product is directly proportional to the TBARS- MDA concentration in the sample.

Procedure: Exactly 1ml of trichloroacetic acid (15%) was measured into a test tube, followed by 1ml of thiobarbituric acid and 50µl of the tissue homogenate. The mixture was incubated at 80^{o}C for 30 minutes in a waterbath and allowed to cool for 2 minutes followed by centrifugation at 3000 rpm for 10 minutes. Absorbance of the clear supernatant was read spectrophotometrically at 535nm. The same method was used in determination of TBARS concentration of the kidney and colon homogenates. Concentrations of MDA were expressed in nmol/mg protein as follows:

$$\text{Concentration (nmol/mg protein)} = \frac{\text{Sample Absorbance}}{1.56\times 10^{-5}\times \text{protein concentration (mg)}}$$

3.4.2 Assay for catalase activity

Catalase assay was used to quantitatively measure catalase activity in the samples using Abei's method (1974). Principle: The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. Increasing levels of catalase activity in the samples causes a decrease in H_2O_2 concentration.

$2H_2O_2 \longrightarrow 2H_2O + O_2$ (gas)

Procedure: Exactly 10µl of serum was added to test tube containing 2.80cm^3 of 50mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1cm^3 of fresh 30mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240nm at 5 minutes using spectrophotometer. Molar extinction coefficient (E) of 0.041 $mM^{-1}cm^{-1}$was used to calculate the catalase activity thus:

Catalase Activity = Absorbance × E

3.4.3 Superoxide dismutase (SOD) activity determination

Superoxide dismutase activity in the samples was determined according to the method of Martin *et al*. (1987).

Principle: Superoxide Dismutase activity assay was based on the SOD-mediated decrease in the rate of auto-oxidation of hematoxylin in aqueous alkaline solution, which yields a chromophore with maximum absorbance at 560nm.

Procedure: Exactly 920µl of phosphate buffer pH 7.8 was added into a clean test tube containing 40µl of sample; they were mixed and incubated for 2 minutes at 250C. Exactly 40µl of hematoxylin (1:50) was added to start the auto-oxidation reaction, mixed quickly and

theabsorbance was measured at 560nm every 10 seconds for 5 minutes. The SOD concentration was calculated as percentage inhibition of the rate of autoxidation of hematoxylin as follows:

Average rates=ri,

$r_i = Rate_i/Rate_{blank}$

Where;$Rate_i$is the average auto-oxidation rate of sample, and $Rate_{blank}$ is the average auto-oxidation rate of the blank solution. Percentage of SOD inhibition of reaction rate is 100%-r_i

The SOD concentration in the sample added to the reaction mixture is given by:

$$C_i \text{ (SOD U/mL)} = 125*(100\%-r_i)$$

3.5 Haematological Assay

Whole blood samples were collected from all the experimental rats into tri-potassium ethylenediamine tetra-acetic acid (K_3EDTA) anticoagulant bottle, mixed by gentle inversion for complete blood count (CBC) analysis using haematology auto analyser (Sysmex). The automated analysis was done following the manufacturer's operational guidelines.

3.6 Carcinogenesis Biomarker Assays

3.6.1 Determination of carcinoembryonic antigen (CEA)

The level of Carcinoembryonic antigen was determined via enzyme-linked immunosorbent assay (ELISA) using a CEA kit according to manufacturer’s instruction manual.

Principle: The sandwich‘method of ELISA was employed in the assay. The method is so named because the microtiter plate wells have been pre-coated with a CEA compatible biotin-conjugated antibody to which the CEA in the samples bind to form an antibody-antigen complex which then binds a secondary antibody conjugated with an enzyme, avidin-conjugated horseradish peroxidase. The enzyme catalyses the cleavage of hydrogen peroxide in the detection reagent to give a characteristic colouration whose intensity is measured spectrophotometrically at 450nm. Optical density of the product is assumed to be proportional to the amount of CEA antigen bound.

Procedure: All reagents, samples and standards were prepared as described in the kit manual. Exactly 100µl of sample/standard was added to each well and incubated for 2 hours at 37^{o}C. The plate was aspirated and exactly 100µl of prepared detection reagent A was added and incubated for 1 hour at 37^{o}C. The plate was aspirated and washed 3 times with distilled water (fill to the brim of the titer plate). Exactly 100µl of prepared detection reagent B was added and incubated for 30 minutes at 37^{o}C. The plate was aspirated and washed 5 times, and 90µl of substrate solution was added and incubated for 25 minutes at 37^{o}C. Finally, 50µl of stop solution was added and absorbance was immediately read at 450nm.

3.6.2 Histology of organs

Histopathological investigation of the organs (colon, liver and kidney) was carried out according to the method described by Lamb, (1981).

Procedure: The organ pieces (1-2 micrometers thick) were fixed in 10% buffered neutral formalin for 24 h and washed in running water for 24 h. Samples were dehydrated in an autotechnicon and then cleared in benzene. Absolute embedding was removed by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the moulds, followed by microtome. The specimens were then stained with Hematoxylin and Eosin (H&E). The H&E stained specimens were examined microscopically at x 40 and x 75 magnification to histopathologically classify the organ as described by Lamb, (1981).

3.6.3 Immunohistochemistry assay (IHC)

The method used is the Avidin Biotin Complex (ABC) method also referred to as the avidin biotin immunoperoxidase method. Exactly 2 microns thick of formalin fixed and paraffin embedded tissues were cut for the IHC. Antigenic sites were retrieved using citric acid (pH 6.0) solution in pressure cooker at 100^{o}C for 25 minutes. Peroxidases, protein and biotin blocks were done using hydrogen peroxide, avidin and biotin respectively. *MLH1* specific primary antibody was diluted (1.100) and incubated on sections at 6^{o}C for 60 minutes. These were followed by the biotylinated secondary antibody, streptavidine and diaminobenzidine (DAB)/substrate reaction as described by Ramos-vara (2005).

Pre-Analytical Procedure: The processed tissue were sectioned at 2microns on the rotary microtome and placed on the hot plate at 70^{o}C for 1hour. Sections were rehydrated by passing them on 2 changes of xylene, then 3 changes of descending grades of alcohol (molecular grade) and to water. Antigen retrieval was performed on the sections by heating

them in a citric acid solution of pH 6.0 using the Microwave at power 100^{o}C for 15 minutes. The sections were equilibrated gradually with cold water to displace the hot citric acid for at least 5 minutes for the section to cool.

Peroxidase blocking was done on the sections by simply covering section with 3% hydrogen peroxide (H_2O_2) for 15min. Sections were washed with phosphate buffered solution (PBS) and protein blocking were performed using avidin (100µl to cover the slide) for 15 minutes. Sections were washed with PBS (about 100µl of pH 7.2) and endogenous biotin in tissue was blocked using biotin for 15 minutes, and then incubated with the respective diluted primary antibody (*MLH1*) for 60 minutes.

Excess antibodies were washed off with PBS and a secondary antibody (LINK) was applied on the sections for 15 minutes. Slid sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15 minutes. A working DAB solution is made up by mixing 1 drop (20 microns) of the DAB chromogen to 1ml of the diaminobenzidine (DAB) substrate. This working solution was applied on sections after washing off the HRP with PBS for at least 5 minutes. Excess DAB solution and precipitate were washed off with water and sections were counterstained with hematoxylin solution for at least 2 minutes and blued briefly. Sections were dehydrated in alcohol, cleared in xylene, coverslipped and mounted for visualization.

Post-Analytical Procedure: In the post-analytical process, the stains were interpreted by a pathologist in context with positive and negative tissue controls, using bright field

microscopy (magnification at x40) and the results were reported. The percentage expressions of the MLH1 miss match repair protein were also determined.

3.7 Determination of Protein Concentration

The Biuret reagent was prepared by adding 3 g of $CuSO4•5H_2O$ and 9 g of sodium potassium citrate to 500 mL of 0.2 N NaOH solution, followed by the addition of 5 g of KI. The resulting solution was then brought to a total volume of 1 L with 0.2 N NaOH. Protein level from the serum and organs homogenates were determined using biuret reagent.

Protein standards and the sample were prepared with saline solution (8.5 g/L). Exactly 3.0 mL of Biuret reagent was added to each standard and sample, the solution was mixed well and incubated at room temperature for 30 minutes and measured at 540 nm against blank. A linear fit was applied to the standard results to obtain the standard curve. The resulting calibration curve exhibits a linear relationship with a correlation coefficient (R2) of 0.9996 and $y = 196.4x + 0.196$. The unknown sample was measured using the calibration curve; the concentration of protein in the sample was calculated.

3.8 Statistical Analysis

The results obtained were analyzed using analysis of variance (ANOVA) to get the grouped mean, and Duncan multiple range test was used to test for significant difference between the grouped means at 95% confidence level ($p \leq 0.05$). A statistical package for social sciences (SPSS version 16.0) was used to analyze the data.

CHAPTER FOUR

4.0 RESULTS

4.1 Effects of Dietary Inclusion of *D. microcarpum* and *D. guineense* on Organ's Functions.

4.1.1 Liver function assay.

The level of AST and ALT were significantly ($p<0.05$) higher in the MNU control than the normal control and sample control groups. The groups given the higher percentage (5% and 10%) of the sample showed significantly ($p<0.05$) lower concentration of these enzymes than the groups given the lower percentages (2.5%) of the sample (Figures 4.1 and 4. 2). Comparing the test groups, it can be observed that the groups fed the *D. microcarpum* incorporated fruit-pulps diet have relatively lower levels of the liver function enzymes that those fed with the *D. guineense* fruit-pulp incorporated diet and this difference was particularly statistically ($p<0.05$) prominent in the groups that were on the 10% incorporated diet.

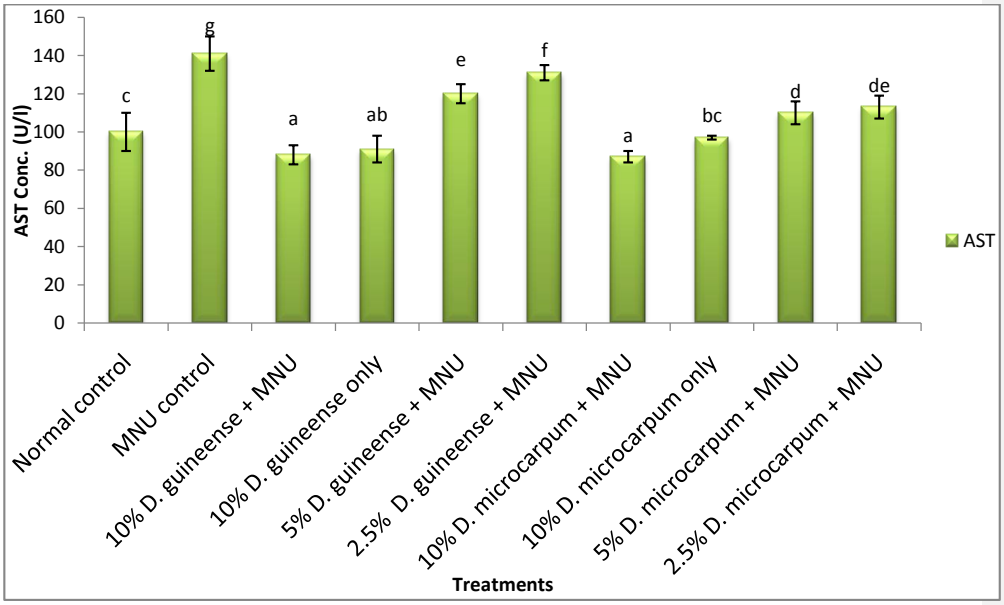


Figure 4.1: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Serum Aspartate Aminotransferase Activity (U/L) Following 12 Weeks MNU Administration in Rats.

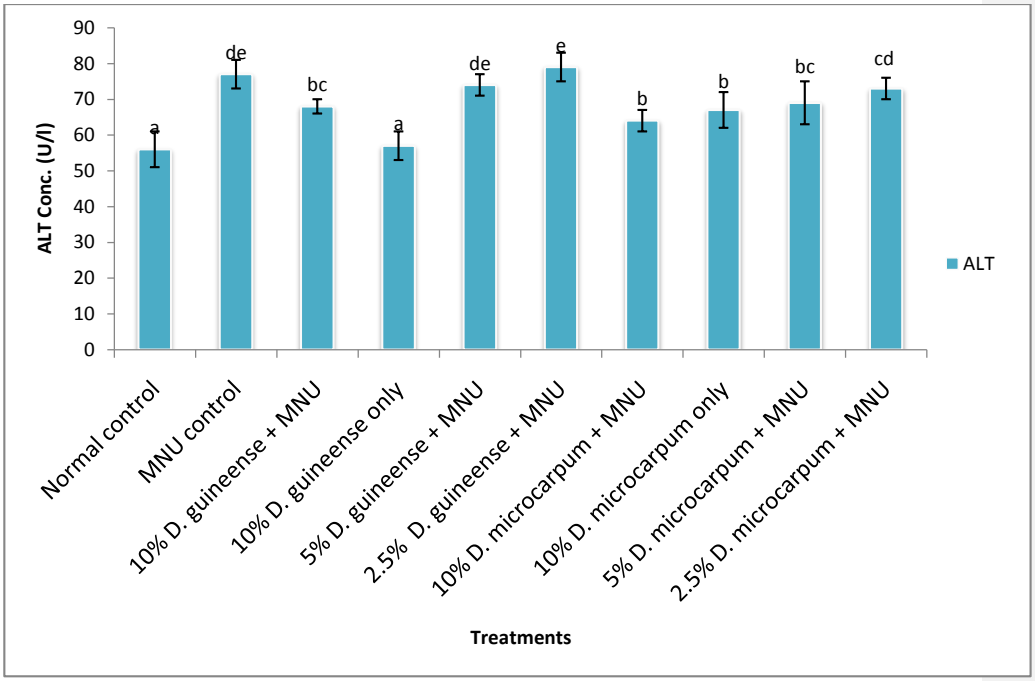


Figure 4.2: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Serum Alanine Aminotransferase Activity (U/I) Following 12 Weeks MNU Administration in Rats.

4.1.2 Kidney function assay

Serum urea and creatinine concentration were determined and kidney function markers. Results of the kidney function test also showed (Table 4.1) high concentration of creatinine and urea in the MNU control which was significantly ($p<0.05$) higher than groups fed with incorporated fruit-pulp diets. There were however, no significant difference ($p>0.05$) between the respective *D. microcarpum* and *D. guineense* diet groups except the groups given the lowest percentage (2.5%) of incorporated diets of *D. guineense* which was observed to have significantly ($p<0.05$) higher concentration of urea (Table 4.1).

Table 4.1: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Serum Urea and Creatinine Concentration (Mg/dl) Following 12 Weeks MNU Administration in Rats.

Treatments	Urea (mg/dl)	Creatinine (mg/dl)
Normal control	73.0±7.0^{a}	1.7±0.6^{ab}
MNU control	125.0±7.0^{d}	3.1±0.6^{c}
10% *D. guineense* + MNU	83.0±1.1^{b}	2.3±0.7^{bc}
10% *D. guineense* only	79.0±9.0^{ab}	2.0±0.7^{ab}
5% *D. guineense* + MNU	77.1±9.1^{ab}	2.0±0.7^{ab}
2.5% *D. guineense* + MNU	105.2±8.1^{c}	2.6±0.6^{bc}
10% *D. microcarpum* + MNU	71.0±8.2^{a}	1.4±0.0^{a}
10% *D. microcarpum* only	78.3±3.0^{ab}	1.7±0.6^{ab}
5% *D. microcarpum* + MNU	79.4±6.1^{ab}	1.7±0.6^{ab}
2.5% *D. microcarpum* + MNU	77.0±7.2^{ab}	2.3±0.7^{bc}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$).

4.2 Effects of Dietary Inclusion of *D. microcarpum* and *D. guineense* on *In Vivo* Antioxidant Parameters in Rats

4.2.1 Organ's thiobarbituric acid reactive substances (TBARS) assay.

Results obtained from TBARS assay following 12 weeks MNU administration in rats shows (Table 4.2) higher concentrations of malondialdehyde (MDA) in the MNU control group which was significantly ($p<0.05$) different than all other groups in all the organs and tissue. On the other hand, no significant difference ($p>0.05$) were observed when the normal control group and fruit-pulps diet fed groups were compared. Furthermore, there were no significant difference ($p>0.05$) when the respective corresponding test groups of *D. microcarpum* and *D. guineense* fruit-pulp were compared among themselves (Table 4.2).

Table 4.2: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Organ's Malondialdehyde Concentration (n Mol/Mg Protein) Following 12 Weeks MNU Administration in Rats.

Treatments	Liver (n mol/mg protein)	Kidney (n mol/mg protein)	Colon (n mol/mg protein)
Normal control	228±24^{a}	168±16^{a}	96±3^{bc}
MNU control	381±18^{d}	228±79^{c}	157±23^{f}
10% *D. guineense* + MNU	276±27^{b}	198±24^{ab}	79±17^{ab}
10% *D. guineense* only	228±16^{a}	135±14^{a}	59±6^{ab}
5% *D. guineense* + MNU	258±45^{ab}	244±55^{bc}	101±27^{bcd}
2.5% *D. guineense* + MNU	345±18^{c}	256±21^{bc}	122±18de
10% *D. microcarpum* + MNU	276±43^{b}	179±12^{a}	81±18^{ab}
10% *D. microcarpum* only	221±8^{a}	158±83^{a}	64±7^{ab}
5% *D. microcarpum* + MNU	250±15^{ab}	251±35^{bc}	113±14^{cde}
2.5% *D. microcarpum* + MNU	345±26^{c}	276±35^{c}	131±24^{e}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$)

4.2.2 Superoxide dismutase (SOD) activity

Table 4.3 shows the activities of SOD in the liver, kidney, colon and serum. While there were no significant ($p>0.05$) difference in the SOD activities between the MNU and normal control groups in the liver and serum, there were significantly lower activities of SOD in the kidney and colon in the normal control group when compared to the MNU control (Table 4.3). There were no observable significant difference ($p>0.05$) in the SOD enzyme activity in the liver and serum when the test groups and the normal control group are compared, however, differences ($p>0.05$) are observed in some of the test groups of the kidney and colon when compared with the MNU control groups. Similarly, no statistical difference exist ($p>0.05$) across the test groups in all the organs when respective treatment groups of *D. microcarpum* and *D. guineense* were compared.

Table 4.3: Effect of Dietary Inclusion of *D. guineense a*nd *D. microcarpum* Fruit-Pulps on SOD Activities (U/mL) Following 12 Weeks MNU Administration in Rats.

Treatments	Liver (U/mL)	Serum (U/mL)	Kidney (U/mL)	Colon (U/mL)
Normal control	23.39±3.27^{ab}	39.47±4.0^{bc}	23.39±6.12^{e}	20.47±3.27^{ef}
MNU control	17.54±4.0^{a}	20.476.12^{ab}	16.08±8.01^{ab}	11.7±3.06^{ab}
10% *D. guineense* + MNU	16.32±3.54^{b}	31.43±7.12^{c}	29.24±5.17^{cde}	18.27±3.65^{cde}
10% *D. guineense* only	19.01±4.0^{ab}	37.28±4.0^{ab}	19.01±4.0^{de}	25.58±7.31^{f}
5% *D. guineense* + MNU	17.54±4.0^{a}	25.58±5.17^{a}	15.35±5.42^{bc}	13.89±4.0^{abcd}
2.5% *D. guineense* + MNU	17.54±4.0^{a}	25.58±8.17^{ab}	18.27±3.65^{bc}	12.43±4.17^{abc}
10% *D. microcarpum* + MNU	19.01±4.81^{ab}	37.28±4.0^{ab}	18.27±5.17^{de}	17.54±4.0^{bcde}
10% *D. microcarpum* only	20.47±3.27^{ab}	38.01±6.12^{ab}	12.66±5.42^{e}	21.93±5.17^{ef}
5% *D. microcarpum* + MNU	19.01±3.54^{ab}	29.24±8.95^{ab}	19.01±4.0^{cd}	19.01±4.0^{de}
2.5% *D. microcarpum* + MNU	18.27±3.17^{a}	14.62±0.0^{ab}	17.54±4.0^{a}	10.23±4.0^{a}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$).

4.3.2 Catalase activity

The assessment of the catalase activities in organs, tissue and serum as shown in Table 4.4 reveals low activity of this enzyme in the liver, kidney, colon and serum of the MNU control group which were significantly ($P<0.05$) lower than the normal control, treatment control and the group given the higher percentage of *D. microcarpum* fruit-pulps. Furthermore, the groups treated with *D. Microcarpum* fruit-pulps incorporated diet showed significantly ($P<0.05$) higher activities of the enzyme in the liver and serum than the corresponding groups treated with *D. guineense* fruit-pulp incorporated diet (Table 4.4).

Table 4.4: Effect of Dietary Inclusion of *D. guineense* and*D. microcarpum* Fruit-pulps on Catalase Activities (µMol/Min/Mg Protein) Following 12 Weeks MNU Administration in Rats.

Treatments	Liver (Umol/min/mg protein)	Serum (Umol/min/mg protein)	Kidney (Umol/min/mg protein)	Colon (Umol/min/mg protein)
Normal control	87.78 ± 10.15^{ef}	115.99 ± 8.87^{e}	67.08 ± 8.72^{d}	59.19 ± 8.56^{d}
MNU control	36.92 ± 6.06^{ab}	65.68 ± 6.3^{a}	44.53 ± 5.91^{abc}	31.04 ± 3.47^{a}
10% *D. guineense* + MNU	46.21 ± 7.8^{b}	87.37 ± 8.97^{c}	40.7 ± 6.19^{a}	52.77 ± 5.55^{cd}
10% *D. guineense* only	64.61 ± 10.9^{c}	98.05 ± 10.39^{d}	52.74 ± 6.13^{c}	56.95 ± 4.54^{cd}
5% *D. guineense* + MNU	38.5 ± 4.29^{ab}	74.27 ± 2.51^{ab}	43.28 ± 4.5^{ab}	54.32 ± 7.35^{cd}
2.5% *D. guineense* + MNU	33.7 ± 6.49^{a}	73.77 ± 4.43^{ab}	41.62 ± 4.9^{a}	44.58 ± 7.47^{b}
10% *D. microcarpum* + MNU	83.91 ± 8.43^{de}	118.45 ± 4.13^{e}	64.9 ± 8.41^{d}	53.4 ± 7.25^{cd}
10% *D. microcarpum* only	94.26 ± 2.26^{f}	100.76 ± 1.54^{d}	68.78 ± 8.34^{d}	55.46 ± 6.82^{cd}
5% *D. microcarpum* + MNU	76.73 ± 5.6^{d}	88.82 ± 7.71^{c}	50.94 ± 4.04^{bc}	48.4 ± 3.91^{bc}
2.5% *D. microcarpum* +	66.19 ± 8.19^{c}	75.16 ± 3.97^{b}	46.58 ± 1.57^{abc}	43.48 ± 2.67^{b}

MNU

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$)

4.4 Haematological Parameters (Full Blood Count)

Effects of dietary inclusion of *D. guineense* and *D. Microcarpum* fruit-pulps were checked on rats haematological parameters. There were no significant difference ($P>0.05$) between the MNU and the normal control groups in the levels of white blood cell, neutrophil and lymphocyte (Table 4.5). There were however, significant difference ($P<0.05$) in the levels of monocyte (Table 4.5) PCV (Figure 4.3), HGB (Figure 4.4) and RBC (Figure 4.5) between the normal control and MNU control groups. While there were no significant difference ($P>0.05$) in haematological parameters between the corresponding fruit-pulp incorporated diet groups of *D. guineense* and *D. microcarpum*, there were generally significantly ($P<0.05$) higher levels PCV, HGB and RBC in these groups when compared with the MNU control group (Figures 4. 3 – 4.5).

Table 4.5: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-Pulps on White Blood Cell, Neutrophil, Lymphocyte and Monocyte Levels Following 12 Weeks MNU Administration in Rats.

Treatments	WBC (x109/l)	NUET. (%)	LYM (%)	MONO. (%)
Normal control	8.6 ± 3.6^{ab}	61 ± 8.4^{ab}	33.8 ± 5.4^{ab}	16.6 ± 1.1^{e}
MNU control	7.6 ± 1.1^{a}	52.6 ± 9.0^{ab}	46.6 ± 9.7^{b}	12 ± 0.7^{a}
10% *D. guineense* + MNU	12.8 ± 5.4^{bc}	62 ± 11.6^{ab}	38 ± 11.6^{ab}	15.2 ± 0.5^{de}
10% *D. guineense* only	7.9 ± 2.7^{ab}	56.2 ± 9.6^{ab}	43 ± 10.1^{ab}	14.8 ± 1.6^{cd}
5% *D. guineense* + MNU	10.6 ± 3.1^{abc}	56.6 ± 9.4^{ab}	40 ± 6.2^{ab}	14 ± 1.2^{bcd}
2.5% *D. guineense* + MNU	7.4 ± 2.0^{a}	60.2 ± 6.5^{ab}	36.8 ± 6.8^{ab}	13.2 ± 0.5^{abc}
10% *D. microcarpum* + MNU	10.5 ± 2.3^{abc}	57.6 ± 8.6^{ab}	39.4 ± 7.4^{ab}	14.2 ± 1.3^{bcd}
10% *D. microcarpum* only	12.9 ± 3.0^{bc}	66.8 ± 10.5^{b}	29.4 ± 7.7^{a}	14.8 ± 2.2^{cd}
5% *D. microcarpum* + MNU	7.7 ± 2.5^{a}	55.6 ± 11.6^{ab}	42 ± 13.0^{ab}	15.2 ± 0.8^{de}
2.5% *D. microcarpum* + MNU	13.9 ± 6.2^{c}	49.6 ± 10.4^{a}	47.8 ± 17.8^{b}	12.8 ± 1.3^{ab}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$).

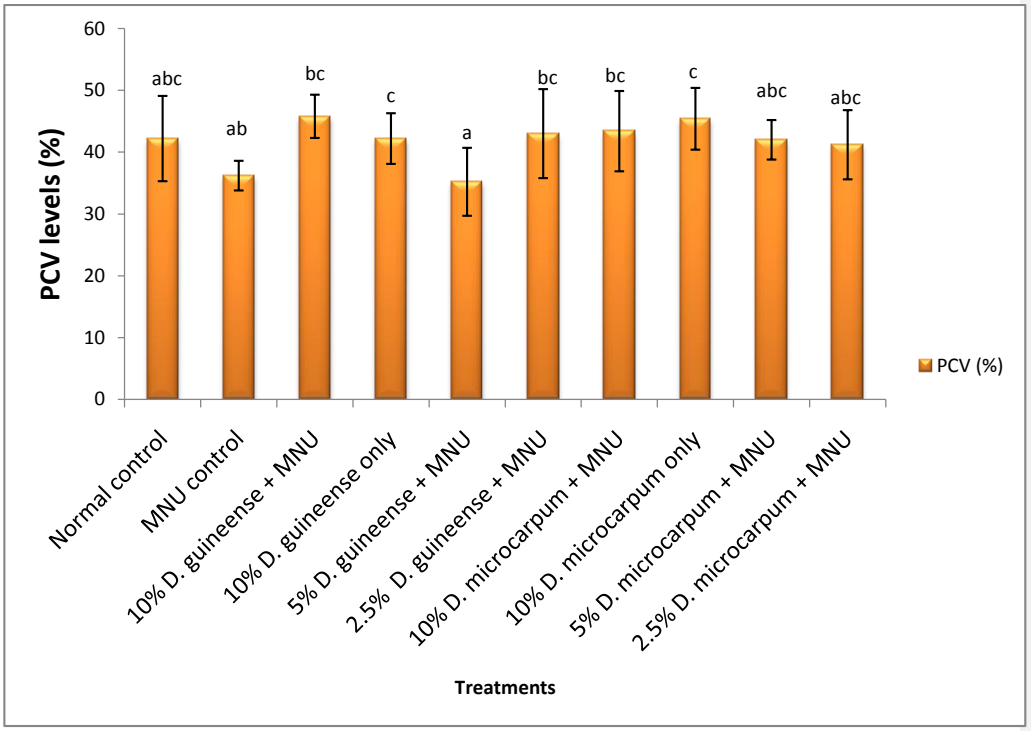


Figure 4.3: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Blood Packed Cell Volume PCV (%) Following 12 Weeks MNU Administration in Rats.

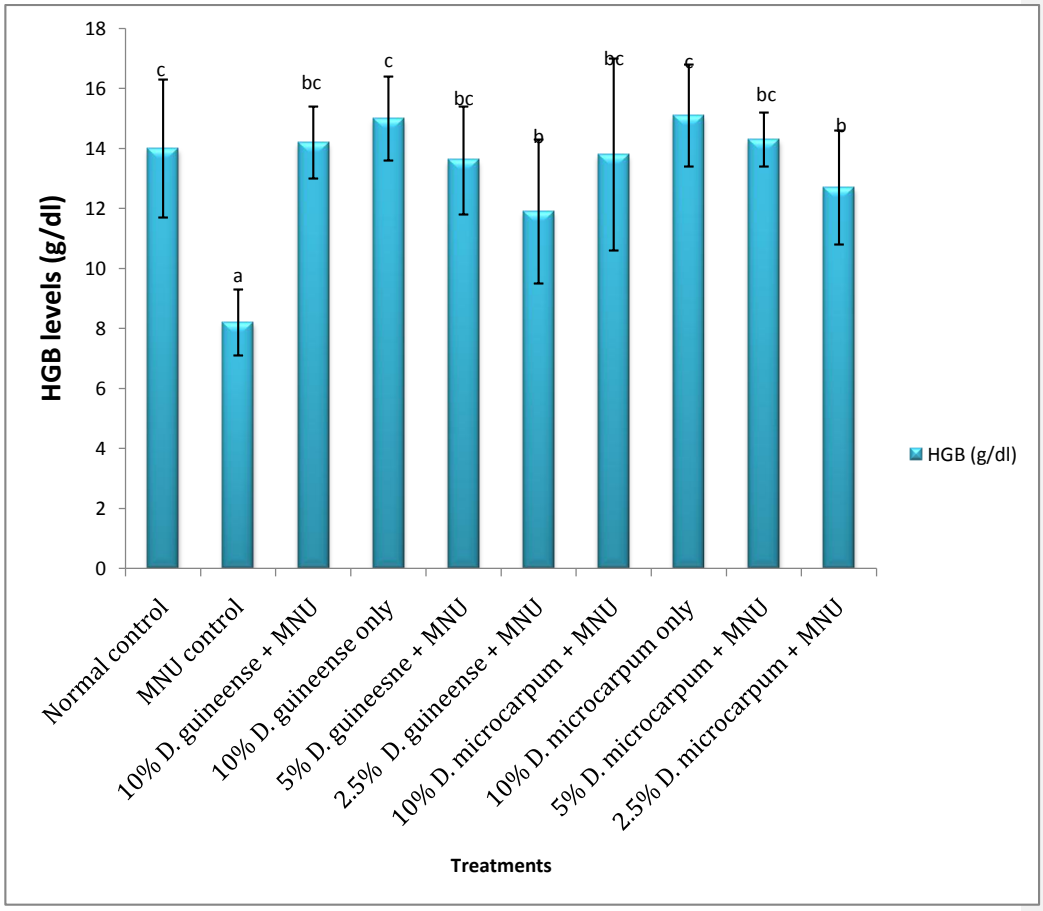


Figure 4.4: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Blood Haemoglobin HGB (%) Following 12 Weeks MNU Administration in Rats.

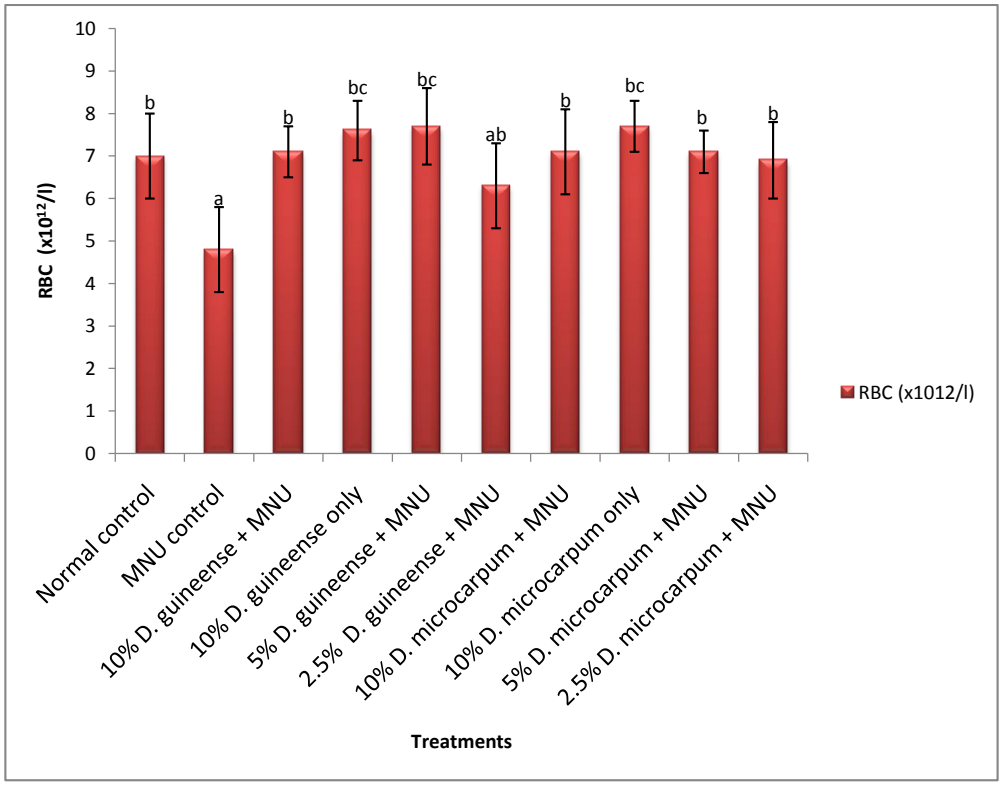
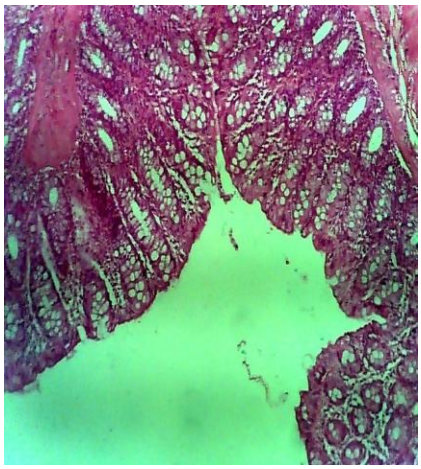


Figure 4.5: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Red Blood Cell (RBC) Counts ($X10^{12}$/L) Following 12 Weeks MNU Administration in Rats.

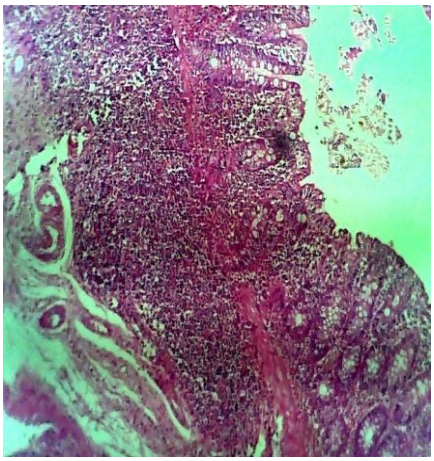
4.5 Histology of Organs and Tissue

4.5.1 Histology of the colon

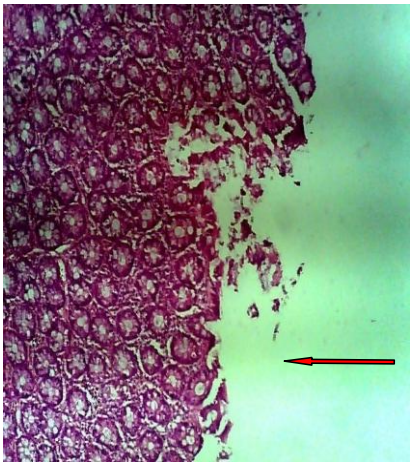
Photomicrographs of histological section of the colon following 12 weeks MNU administration in rats which were fed diets having incorporated *D. microcarpum* and *D. guineense* fruit-pulps, indicates mucosal epithelial hyperplasia and sub-mucosal inflammation in the MNU control group (Plate 4.1b), mucosal ulceration with sub-mucosal inflammation in the 2.5% *D. microcarpum* + MNU group (Plate 4.1c) , mild ulceration in the 2.5% *D. guineense* + MNU group (Plate 4.1d) and ulceration with moderate inflammation in the 5% *D. guineense* + MNU group ((Plate 4.2e). While there was mild ulceration and hyperplasia in MNU control group (Plate 4.1b), there were no significant observable findings in the normal control group (Plate 4.1a), fruit-pulps only control (Plates 4.3i and 4.3j) and intoxicated groups consuming diets with the highest percentage (10%) of fruit-pulps inclusion (Plate 4.2g and 4.2h). Comparatively, the colon architecture was generally preserved better in the *D. microcarpum* treated groups when compared with the *D. guineense* groups particularly in the 5% and 10% incorporated groups.



4.1a: Normal control showing normal mucosa (x40)(H&E)

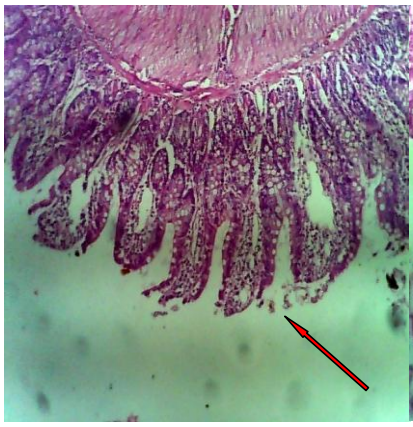


4.1b: MNU control indicating hyperplasia in mucosal epithelialand sub-mucosal inflammation (x40)(H&E)

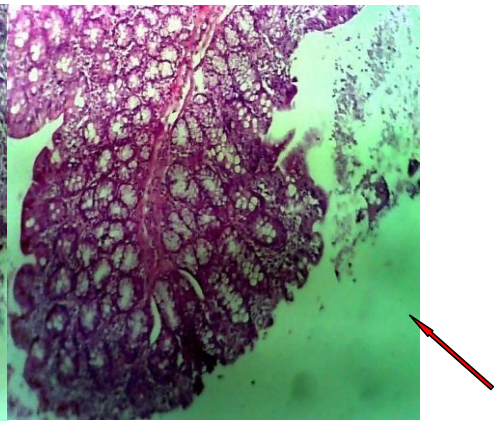


4.1c: 2.5% D. *microcarpum* + MNU showing surface mucosal ulceration with sub-mucosal inflammation (x40) (H&E)

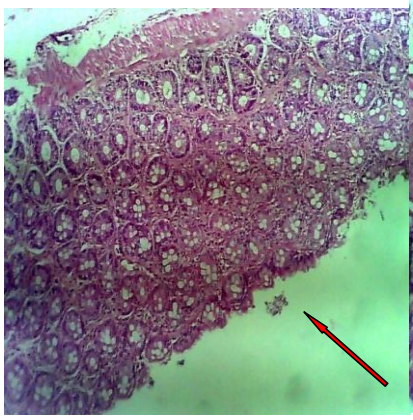
4.1d: 2.5% D. *guineense* + MNU showing mild ulceration (x40)(H&E)



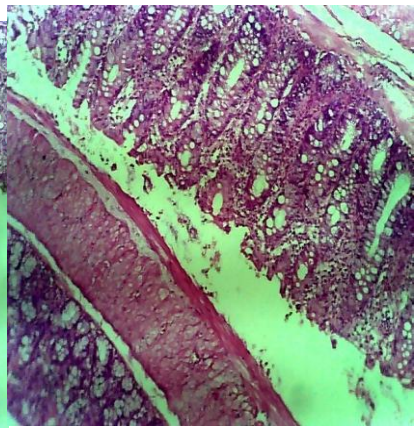
4.1e: 5% *D. microcarpum* + MNU showing essentially normal colon epithelia with focal mild ulceration (x40) (H&E).



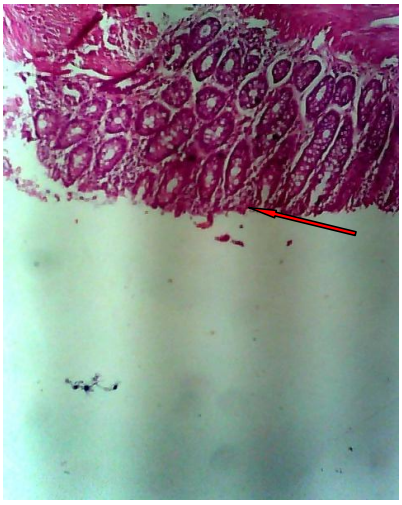
4.1f: 5% *D. guineense* + MNUshowing surface ulceration with moderate inflammation (x40) (H&E).



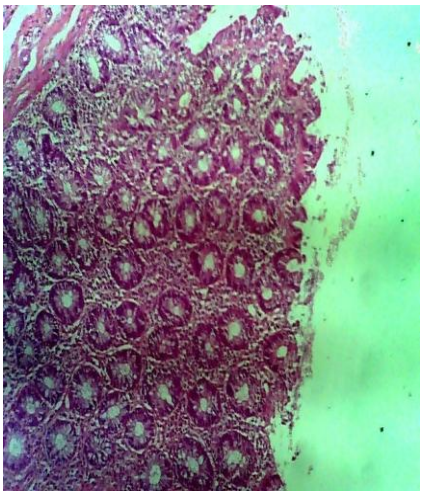
4.1g: 10% *D. microcarpum* + MNU showing normal mucosal epithelia (x40) (H&E).



4.1h: 10% *D. guineense*+ MNU showing relatively normal surface epithelia (x40) (H&E).



4.1i: 10% *D. microcarpum* only control with normal mucosal epithelia (x40) (H&E).

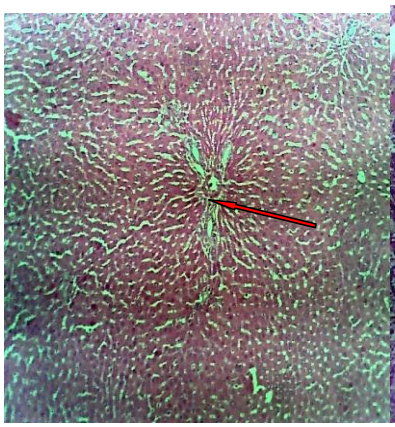


4.1j: 10% *D. guineense* only control having normal epithelia with mild ulceration (x40) (H&E).

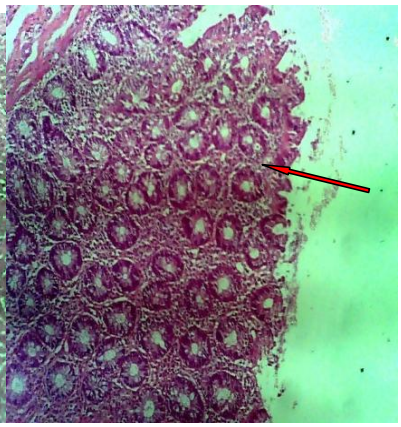
Plates 4.1: Photomicrographs of Section of Rat's Colon Fed Diets Inclusive of Fruit-pulps of *D. microcarpum* and *D. guineense* with 12weeks MNU Administration.

4.5.1 Histology of the liver

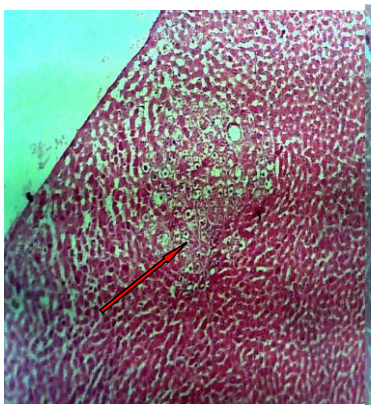
Photomicrographs of histological section of the liver shows apparently normal liver with intact lobules & central vein in the normal control (Plate 4.2a), fruit-pulps controls (Plates 4.2i and 4.2j) as well as all the test groups except the group given the lowest percentage of *D. Microcarpum* + MNU which shows normal liver but with mild cytoplasmic clearing (Plate 4.2d). The MNU control group on the other hand shows destruction / moderate cytoplasmic clearing (Plate 4.2b). There was generally no observable difference between test groups of *D. microcarpum* and *D. guineense* particularly at the 5% and 10% dietary incorporation levels (Plates 4.2e, 4.2f, 4.2g and 4.2h).



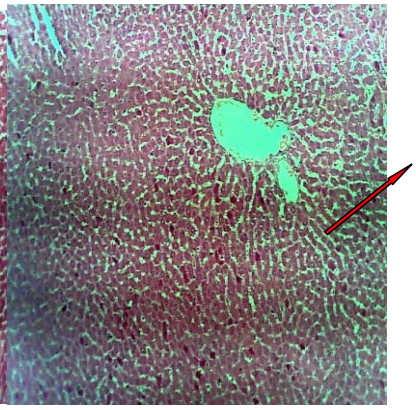
4.2a: Normal control - Normal liver with lobules & central vein intact (x20) (H&E).



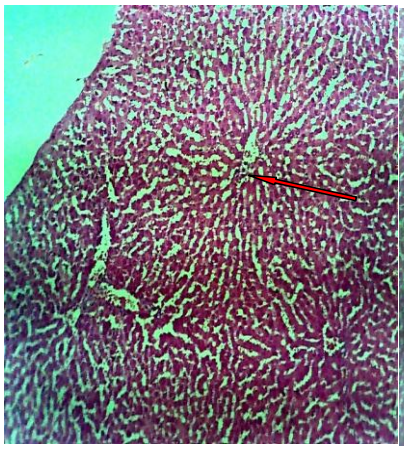
4.2b: MNU control showing destruction/ moderate cytoplasmic clearing (x40)(H&E).



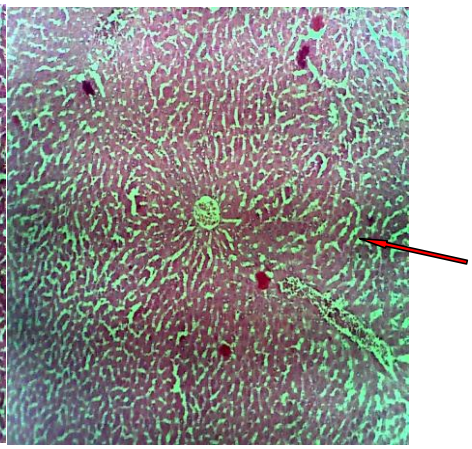
4.2c: 2.5% D. *microcarpum* + MNU-Normal but with mild cytoplasmic clearing (x20) (H&E).



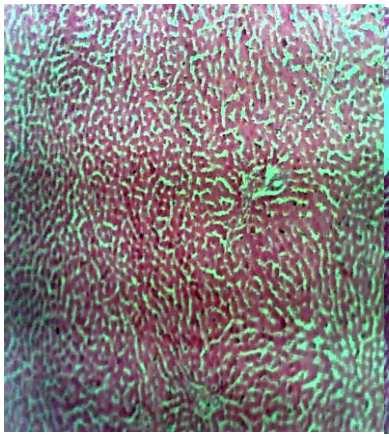
4.2d: 2.5% *D. guineense* + MNU showingpredominantly normal liver (x20**)** (H&E).



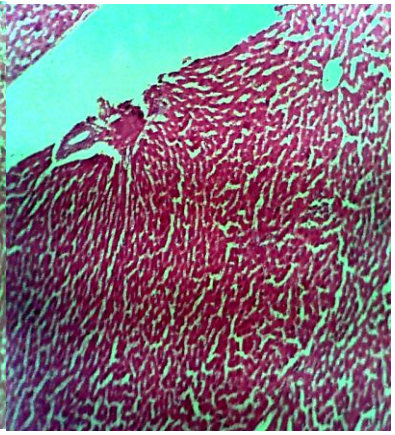
4.2e: 5% *D. microcarpum* + MNU – Normal liver architecture (x20) (H&E).



4.2f: 5% *D. guineense +* MNU -Normal liver (x20) (H&E).



4.2g: 10% *D. microcarpum+* MNU normal liver (x20) (H&E).



4.2h: 10% *D. guineense+* MNU showing normal liver (x20) (H&E).

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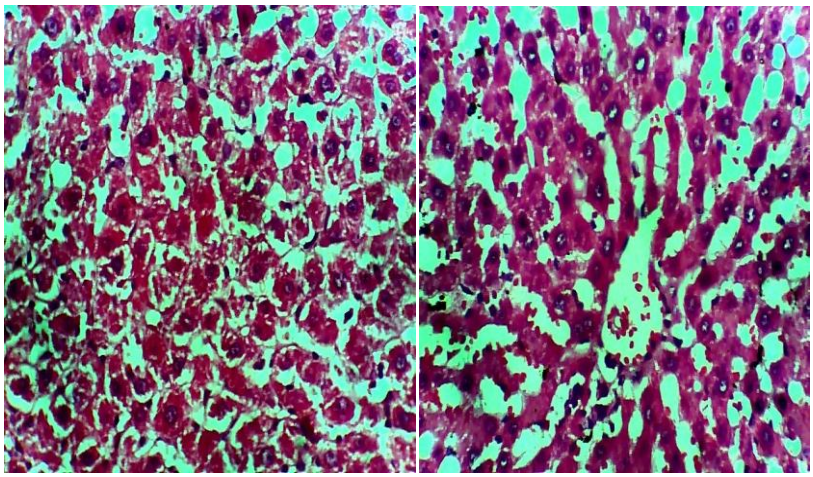


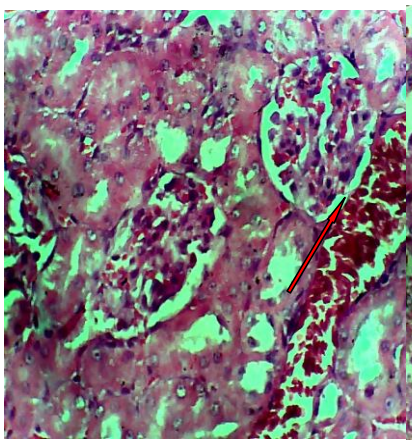
4.2i: 10% *D. microcarpum* only control showing normal liver (x40) (H&E).

4.2j: 10% *D. guineense* only showing normal liver (x40) (H&E).

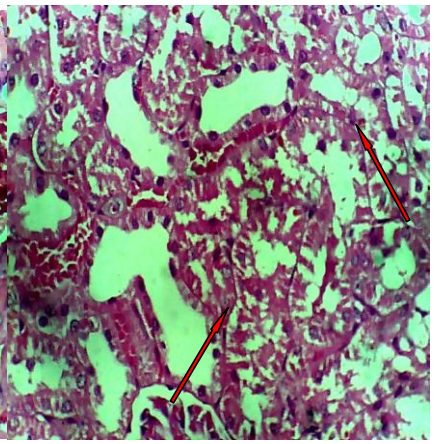
Plate 4.2: Photomicrographs of Section of Rat's Liver Fed Diets Inclusive of Fruit-pulps of *D. microcarpum* and *D. guineense* with 12weeks MNU Administration.

4.5.3 Histology of the kidney

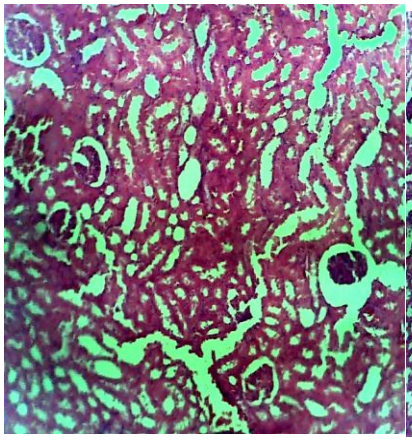
Photomicrographs of histological section of the kidney following 12 weeks MNU administration in rats showing non-significant observable difference between the groups on normal diet and all the other respective treatment groups except the MNU control group which indicates necrosis of glomeruli and tubular distension (Plate 4.3b). Generally, no differences in the kidney were observed when the *D. microcarpum* and *D. guineense* treatment and control groups are compared.



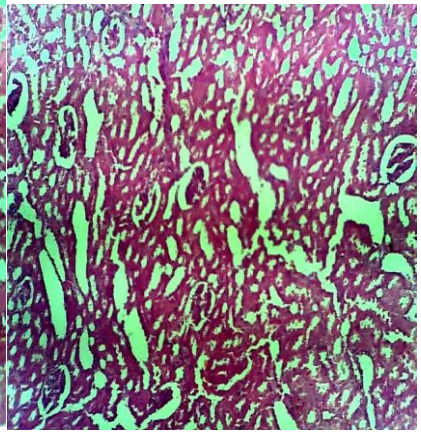
4.3a: Normal control showing normal glomeruli in kidney (x75) (H&E).



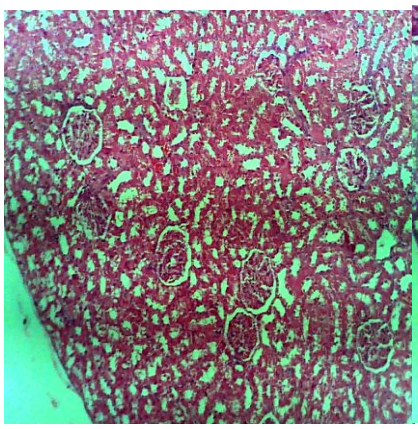
4.3b: MNU control showing necrosis of glomeruli & tubular distension (x75) (H&E).



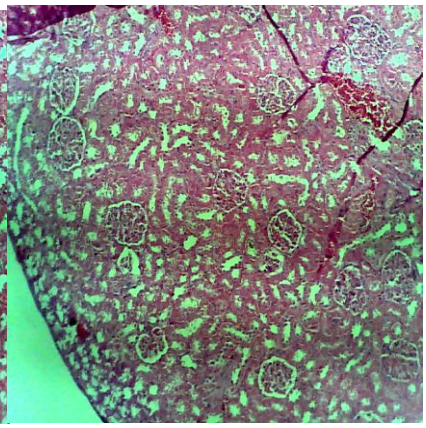
4.3c: 5% *D. microcarpum* + MNU-Normal kidney (x40) (H&E).



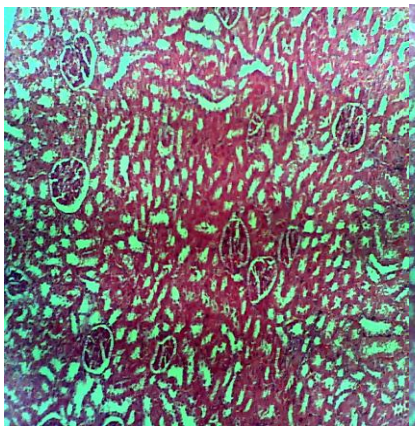
4.3d: 5% *D. guineense* + MNU Normal kidney (x40) (H&E).



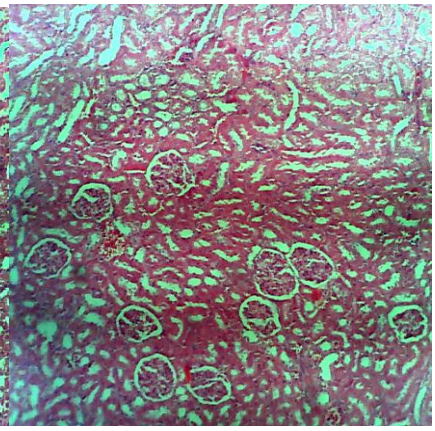
4.3e: 5% *D. microcarpum +* MNU-Normal kidney (x40) (H&E).



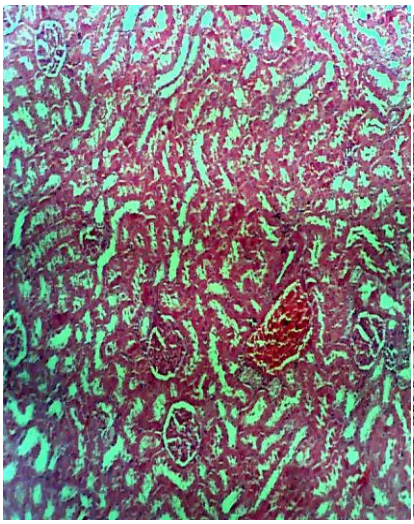
4.3f: 5% *D. guineense +* MNU Normal kidney (x40) (H&E).



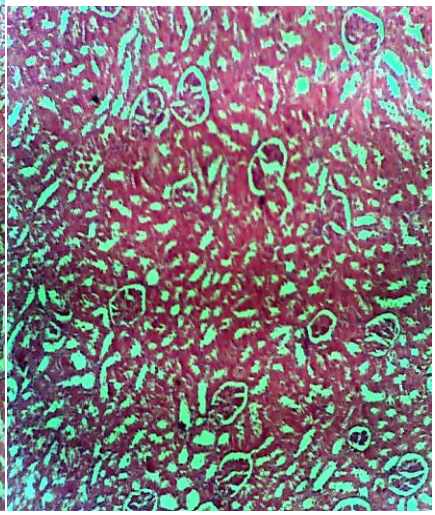
4.3g: 10% *D. microcarpum+* MNU - Normal kidney (x40) (H&E).



4.3h: 10% *D. guineense +* MNU Normal kidney (x40) (H&E).



4.3i: 10 % *D. microcarpum* only controls- Normal kidney (x40)



4.3j: 10% *D. guineense* only control showing normal kidney (x40).

Plate 4.3: Photomicrographs of Section of Rat's Kidney Fed Diets Inclusive of Fruit-pulps of *D. microcarpum* and *D. guineense* with 12weeks MNU Administration.

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4.6 Carcinoembryonic Antigen Assay

The CEA determination as shown in figure 6 indicates significantly ($p<0.05$) high concentration of the CEA antigen in the MNU control than all other groups. On the other hand, there were no significant difference ($P>0.05$) between these other groups and the normal dietary control group except the 5% and 10% *D. microcarpum* dietary incorporated group which has statistically significantly ($p<0.05$) lower levels of CEA than the normal control. Comparatively, the levels of CEA in the 5% and 10% of *D. microcarpum* dietary incorporated group was significantly ($p<0.05$) lower than the levels of CEA in *D. guineense* respective groups.

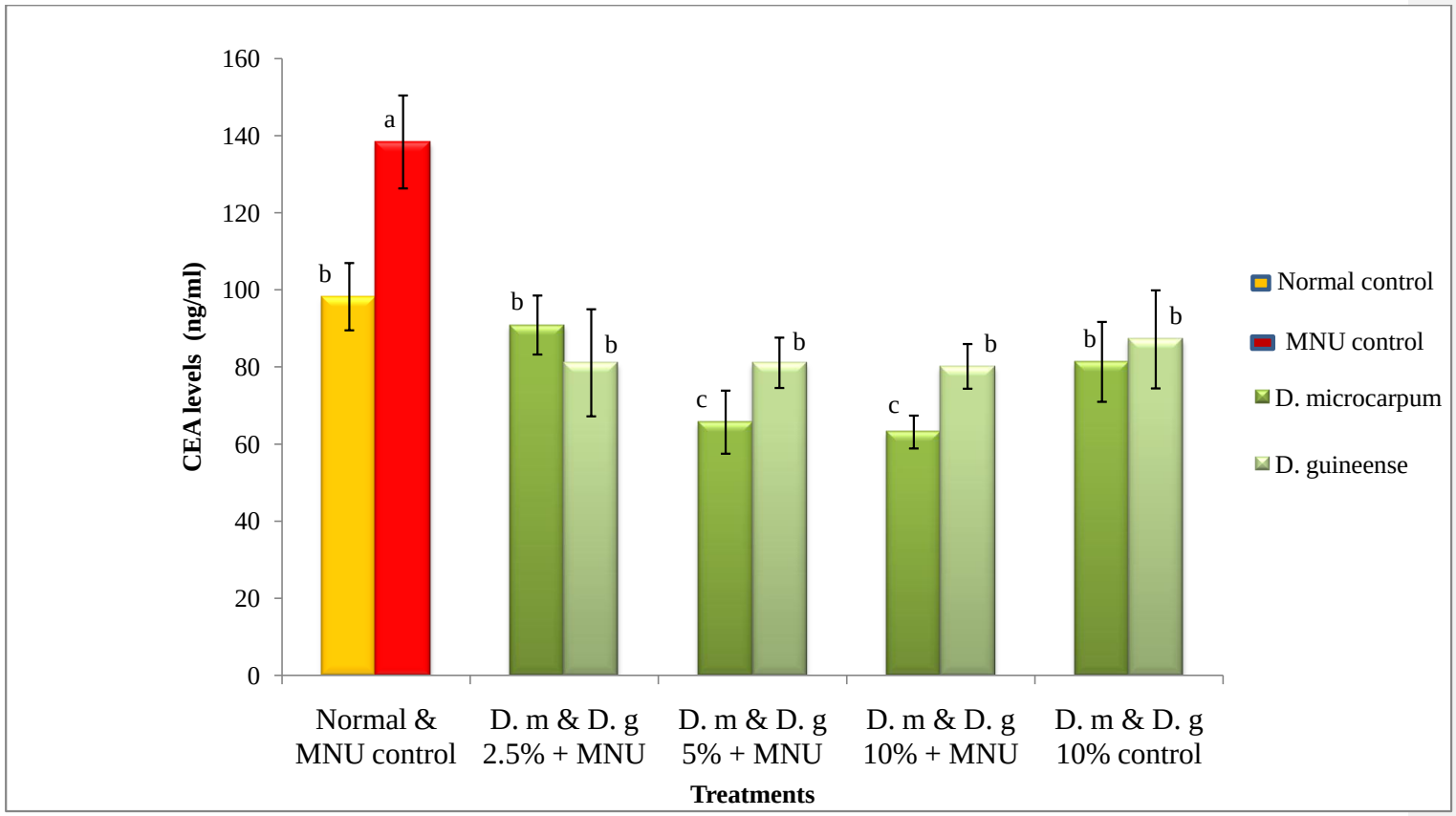
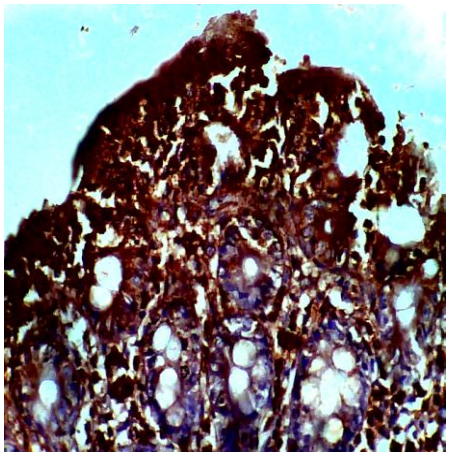


Figure 4.6: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Carcinoembryonic Antigen Level (ηg/ml) Following 12 Weeks MNU Administration in Rats.

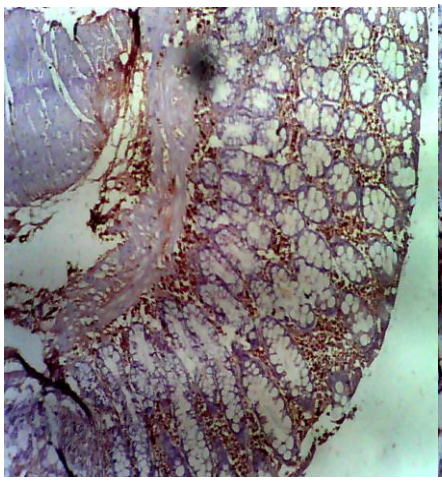
4.7 Immunohistochemical Assessment

Further examination of the colon with immunohistochemical (IHC) staining reveals a mild expression of MutL homolog 1 (MLH1) miss match repair antigen in the MNU control group (Plate 4.4b) and the groups fed with 2.5% fruit-pulps incorporated diets (Plates 4.4c and 4.4d). The untreated normal group showed observable high expression of the MLH1 antigen (Plate 4.4a). Similarly, the test groups fed with 10% *D. microcarpum* with and without MNU intoxication showed high expression of the MLH1 protein (Plates 4.4g and 4.4i) while the 5% incorporated group showed moderate expression of MLH1 (Plate 4.4e). Other test groups incorporated with *D. guineense* showed moderate expression of the MLH1 antigen following IHC staining (Plates 4.4f, 4.4h and 4.4j). In comparison, the expression of MLH1 antigen was generally higher in the *D. microcarpum* groups than it were in the *D. guineense* groups particularly in the 10% dietary incorporation levels (Plates 4.4g and 4.4h).



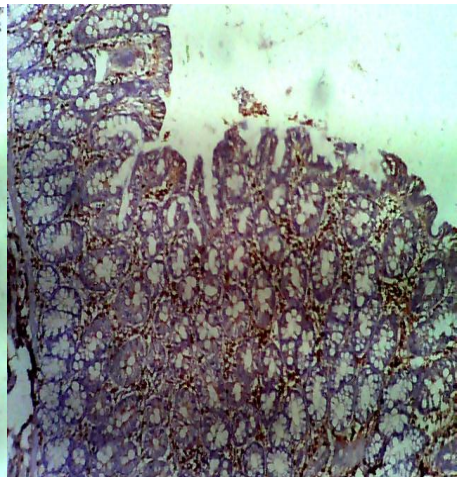
4.4a: Normal control = High expression of *MLH1.*

4.4b: MNU control = Mild expression of *MLH1.*



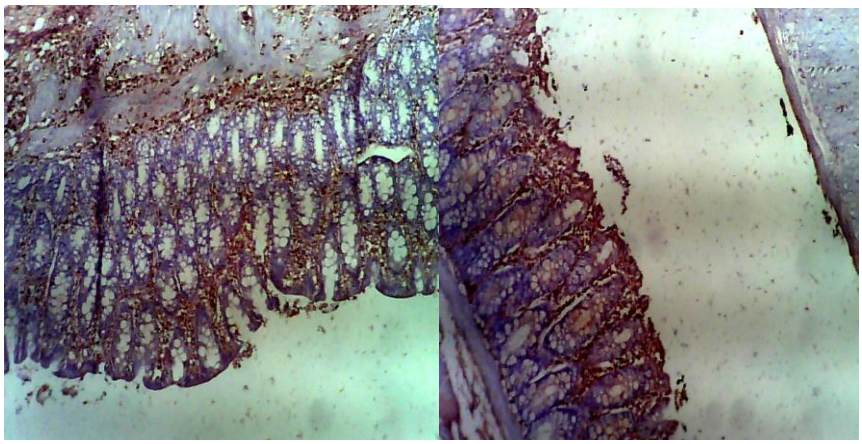
4.4c: 2.5% *D. microcarpum+* MNU= Mild expression of the *MLH1.*

(x40) (H)



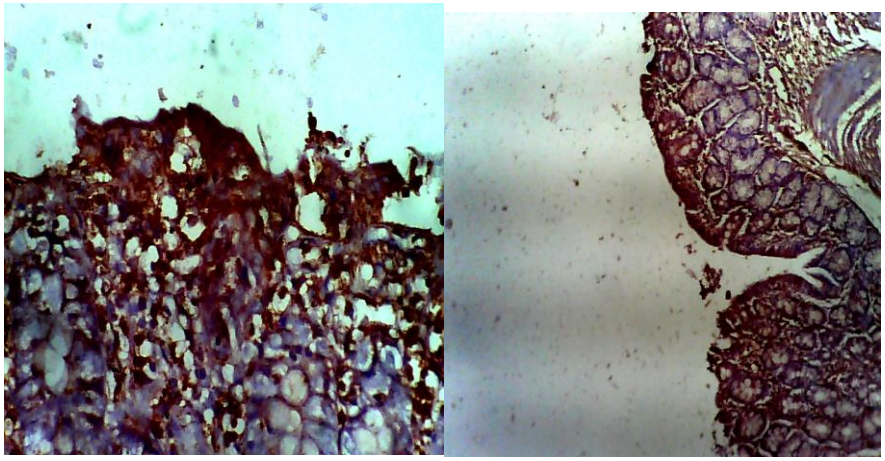
4.4d: 2.5% *D. guineense+* MNU= Mild expression of *MLH1.*

(x40) (H)



4.4e: 5% *D. microcarpum+* MNU= Moderate expression of the *MLH1.* (x40) (H)

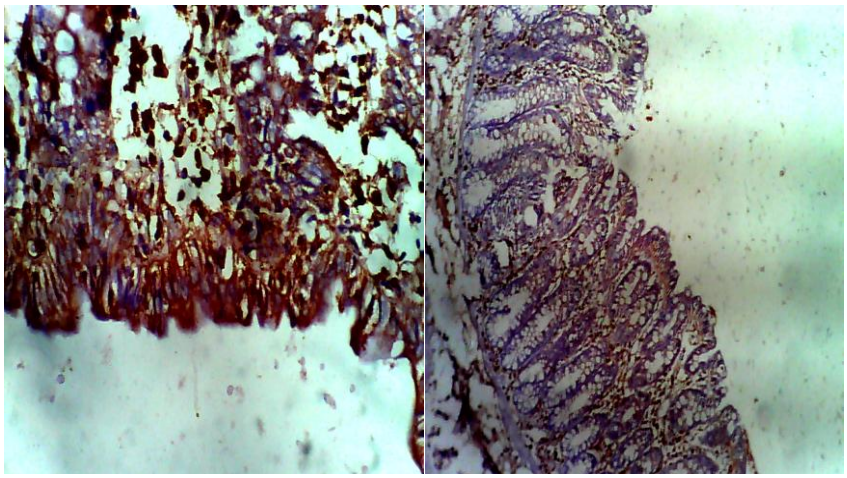
4.4f: 5% *D. guineense+* MNU= Moderate expression of the *MLH1.* (x40) (H)



4.4g: 10% *D. microcarpum+* MNU= High expression of the *MLH1.*

(x40) (H)

4.4h: 10% *D. guineense+* MNU= Moderate expression of the *MLH1.* (x40) (H)



4.4i: 10% *D. microcarpum* only = High expression of *MLH1*

4.4j: 10% *D. guineense* only = Moderate expression of *MLH1*

Plates 4.4: Immunohistochemical Staining of the Colon Section Showing Expression of *MLH1* Miss Match Repair Protein in Rats Fed Diets Inclusive of *D. microcarpum* and *D. guineense* Fruit-pulps with 12weeks MNU Administration.

CHAPTER FIVE

5.0 DISCUSSION

Primary prevention of cancers involves the identification and avoidance of environmental or lifestyle factors related to carcinogenesis while secondary prevention entails the use of chemopreventive agents either natural or synthetic, as components of diets or medications to protect the cellular system from developing cancer (Ahmed *et al*., 2013). Cancer chemoprevention involves the use of pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis, thereby preventing the development of invasive cancer (Schatzkin and Kelloff, 1995). Recently, whole food-based approaches for treatment and prevention of cancer have been explored due to the strong correlation between diets rich in colourful fruit-pulps and vegetables and lower incidence of certain cancers (Kresty *et al*., 2001). This type of food-based approach is known as dietary cancer prevention, which also involves modifications in food consumption patterns necessary to decrease cancer development (Schatzkin and Kelloff, 1995).

Approaches aimed at reduction of colorectal cancer risk by preventing its development or halting its process in early stages is considered an ideal strategy to reduce the overall cancer burden. Epidemiological and experimental evidence indicating that dietary intake is an important etiological factor in colorectal neoplasia is convincing (Gill and Rowland, 2002; Alberts and Martinez, 2000). Study designs used to test a number hypotheses include: ecological studies; where patterns of consumption and cancer incidence or mortality rates are compared among different populations, case-control studies; where reported past diet as

recalled by individuals with cancer is compared against recall among those without the disease and, prospective studies; where diet is assessed among cancer-free individuals and correlated with subsequent cancer occurrence or mortality (Haenzel and Kurihara, 1968; Gill and Rowland, 1993). In this study, a retroprospective evaluation of preventive potential of diets inclusive of varying concentrations of the fruit-pulps of *Detarium microcarpum* and *Dialium guineense* in MNU induced carcinogenesis in rats was carried out.

The Globocan report (2012) indicate a higher burden of colon cancer in individuals from Western cultures and suggest that dietary factors may have contributed to the causation and development of most of this colorectal cancers. Concurrently, people of African descent or Black Africans were reported to be more genetically predisposed to colon cancer (ACS, 2011). Paradoxically, the African continent particularly the Western African region is among the regions in the world with least incidence of colon cancers (GlOBOCAN, 2012). This might not be surprising considering the fact that as an agrarian society, dietary cultures in Africa necessitates intake of unrefined foods with fresh vegetables and an array of natural fruits. In addition, western Africa is blessed with enormous biodiversity resources, due to the tropical and sub-tropical climate. It is known that plants produces antioxidant chemicals as secondary metabolites through evolution as a natural means of surviving in an aggressive environment (Bouquet, 1969). Africa's tropical climate receives an unfair share of strong ultraviolet rays of the tropical sunlight, and has myriad of pathogenic microbes, including several species of bacteria, fungi and viruses, suggesting that African plants could accumulate chemo-preventive substances more than plants from the other hemispheres (Atawodi, 2005).

High consumption of fruit and vegetables has been shown to be associated with a decreased risk of colorectal neoplasia (WHO, 1993). Results of most published studies have shown an inverse association between intake of vegetables and colon cancer, while data for fruit consumption are less compelling. Foods high in fiber have also been shown to be inversely associated with colon cancer risk in most instances. Mechanisms responsible for the protective effect of fruit and vegetables include inhibition of nitrosamine formation, provision of substrate for formation of anti-neoplastic agents, dilution and binding of carcinogens, alteration of hormone metabolism, antioxidant effects, and the induction of detoxification enzymes by cruciferous vegetables (Steinmetz and Potter, 1991). Tropical and semi-arid fruits that could be consumed and utilized in dry forms have a longer shelf life than conventional fresh fruits. Therefore, they could be stored over a longer period of time and transported across long distances with ease. *Detarium microcarpum* and *Dialium guineense* are tropical and semi-arid plants that have culinary and medicinal uses across western Africa (Akpata and Miachi, 2001; Ezeaja *et al*., 2011). They are mostly consumed in dry form and have a long shelf life raging from three to five years when properly stored (Ikhiri and Ilagouma, 1995). Understanding the attributable health benefits of such fruit-pulps and promoting their routine utilization in diet will go a long way in improving the health status of fellow Africans.

The liver is the organ with the utmost potential for detoxification of xenobiotics and is known to be assaulted upon exposure of the organism to toxic xenobiotics. ALT and AST are indices of toxicological activities assaulting the liver, other organs and muscles. Their levels

are known to be elevated in the serum of patients with cardiovascular disease, liver disease and muscle disease (Recknagel *et al.*, 1982, Liman and Atawodi, 2014). In line with the above, significant ($p<0.05$) elevation in the levels of ALT and AST (Figures 4.1 and 4.2) observed in the MNU treated control when compared to the untreated normal control implies that possible damages to liver or muscles had occurred as a result of MNU induction. However, the treated but fruit-pulps incorporated diet groups shows significant ($p<0.05$) decrease when compared to the MNU treated control, which is an indication of the protective effects of the fruit-pulps incorporated diets against MNU induced organ damages. The levels of AST particularly in the 10% incorporated diet group were significantly lower than the levels in the untreated control group, suggesting a strong protective activity of the fruit-pulps. In addition the observed low levels of these enzymes activity in the groups treated with only 10% incorporated diet without MNU intoxication is a clear indication that these fruit-pulps are safe and signify no toxic effects to the liver or muscles. Comparatively, this observed organ protective effects of the fruit-pulps against MNU toxicity is more elaborate in the *D. microcarpum* incorporated groups (Figures 4.1 and 4.2) that it were in the *D. guineense* groups.

The kidney is also a target organ for xenobiotics next to the liver. Various toxins that are potentially toxic to the liver are known to also assault the kidney (Liman and Atawodi, 2014). The key function of the kidney is the excretion and elimination of wastes as well as recovery of water and valuable solutes by ultra-filtration. Urea and creatinine are metabolites that are excreted by the kidney. Impairment of the kidney functions leads to decreased clearance rate of urea and creatinine which conversely results in their elevated levels within

the serum (Atanghwo *et al*., 2008). The study reveals a significantly ($p<0.05$) elevated levels of the urea and creatinine in the MNU treated groups as compared to the untreated normal control, the positive control and the test groups (Table 4.1)

The inhibition of elevation of AST, ALT urea and creatinine levels by the fruit incorporated diets after MNU intoxication clearly illustrates the organ-protective potentials of these fruit-pulps with respect to objective one of the research. This is corroborated by similar findings on effects of other foods and medicinal plant with antioxidant potentials on these biochemical parameters (Atawodi *et al*., 2013, Aliyu *et al*., 1995, Waim *et al*., 2005, Usha *et al*., 2007). Comparatively, both fruit-pulps showed similar organ protective abilities as no significant ($p>0.05$) differences were observed in the levels of AST, ALT urea and creatinine across the respective test groups of *D. microcarpum* and *D. guineense*. This means that incorporation of *D. microcarpum* and *D. guineense* fruit-pulps in routine diets might provide individuals with ability to resist liver and kidney damages caused by toxicants with similar mode of action as MNU. The practical implication of this is possible protection of the local population that consume these plants from liver and kidney diseases caused by accidental ingestion or inhalation of toxic chemicals capable of causing liver or kidney damages, like those found in pesticides, fertilisers, food additives, automobile exhaust etc. By extension, such protective potentials could be beneficial in neutralising the effects of toxic environmental pollutants with carcinogenic effects.

The N-methyl-N-nitrosourea (MNU) is a direct acting carcinogen, inducing tumors in several species of animals and in a variety of organs, including the central nervous system, liver, prostate, colon, stomach, intestine, kidney, and skin (Marcejova and Britko, 2001). MNU

exerts its initiation action in carcinogenesis by methylating the nitrogenous bases of the DNA, leading to K-ras mutation (Ng *et al*., 2015). Furthermore, carcinogens like MNU are capable of causing oxidative stress through their mechanism of action which can lead to peroxidative degradation of cellular membranes and endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to formation of lipid peroxide, which in turn gives thiobarbituric reactive substances (TBARS) like malondialdehyde (MDA) (Hanna *et al*., 2009; Atawodi *et al*., 2013). The level of TBA-MDA complex was measured as an oxidative marker in the rat's liver, kidney and colon homogenates. The MDA levels in the three organs were generally elevated in the MNU only treated groups when compared to the untreated normal control, implying that there was peroxidation of the cellular phospholipids by the MNU induction. Conversely rats fed with fruit-pulps incorporated diets showed significant ($p<0.05$) decrease in the MDA levels which suggests inhibition of MNU-induced lipid peroxidation in rat organs (Table 4.2). In addition no significant ($p>0.05$) differences were observed particularly between the groups fed with 10% fruit-pulps incorporated diets and the untreated normal control fed unincorporated diets, implying that the fruit-pulps inclusion was responsible for the prevention of the lipid peroxidation in the treated groups. Generally, both fruit-pulps showed similar potential to inhibit lipid peroxidation over the experimental period since no significant ($p>0.05$) observable difference exist across the fruit-pulp incorporated diet test groups when the respective groups of *D. microcarpum* and *D. guineense* were compared. Similar findings on modulation of MDA in animals have been reported by other researchers regarding effects of plant extracts with antioxidative potentials. These finding have also been corroborated by similar research findings on induction of lipid peroxidation

by toxic chemicals like carbon tetrachloride and its modulation by antioxidants (Liman and Atawodi, 2014; Atawodi *et al*., 2013, Hanaa *et al*., 2009).

Cellular antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase are normal defence systems against oxidative stress. At the hallmark of oxidative stress, antioxidant defense enzymes like catalase and superoxide dismutase (SOD) get overwhelmed and subsequently, their activities become lowered (Luengthanaphol *et al*., 2004). The increase in superoxide dismutase activity resulted in the accumulation of hydrogen peroxide, which stimulates increase in catalase activity (Martin *et al*., 1987). Catalase and SOD are ubiquitous enzymes and as such their activities in the serum and organs are important indices for evaluating oxidative stress in animals.

It was observed that there was generally significant ($p<0.05$) difference in these enzymes activities between the MNU treated control when compared with the untreated normal control and the incorporated diets test groups. Generally, while the activities of the catalase and SOD are lowered in the MNU only treated group, depicting the prevalence of oxidative stress, the activities of these enzymes were elevated to normal or near normal particularly in the groups on the 5% and 10% fruit-pulps incorporated diets (Tables 4.3 and 4.4). The non-significant ($p>0.05$) difference between the fruit-pulps incorporated groups and the untreated normal control suggest that the fruit-pulps were able to maintain or restore the enzymes activities back to normal values, thereby counteracting MNU induced oxidative stress. Interestingly, these restorative effects on antioxidant enzyme is seen to vary with percentage inclusion level of the fruit-pulps in the diets. Groups that were incorporated with 10% fruit-

pulps of *D. microcarpum* showed significantly ($p<0.05$) higher activities when compared with those on 2.5% fruit-pulps dietary inclusion in most instances. Comparatively, the two fruit-pulps showed similar effects in their abilities of modulation of catalase and SOD activities. The stimulation of antioxidant enzymes activities which potentiates antioxidant defence system by natural antioxidant in diets has been widely reported and is in agreement with the findings of this study (Dastmalchi *et al*, 2007; Clifford and Scalbert, 2000; Atawodi *et al*., 2011).

The combined findings of restoration of higher antioxidant enzymes levels and lowered MDA and related compounds, clearly illustrates that the fruit-pulps incorporated diets exhibited strong antioxidant potential in MNU induced toxicity model. The confirmation of the antioxidant potentials of these plant fruit-pulps *in vivo* is an important finding in this research (objective two) as these plants might be an accessible and acceptable source of natural antioxidants for routine nutritional use or nutraceutical utilization. This in particularly important since most of the regions where these plants are abundant (including Northern Nigeria) are faced with a high prevalence rate of diseases associated with oxidative stress and the economic situation prevailing therein renders it difficult for most of the sufferers to access orthodox chemotherapy (AORTIC, 2011). Identification of locally available plants with antioxidant abilities and preventive potentials like this and recommending its utilization can help in reducing the burden of oxidative stress related diseases like cancer.

The values of hematological parameters are highly influenced by disease, health and nutritional status of subjects. The decreased PCV, RBC and HBG levels observed in the

MNU treated control group agrees with known phenomena that toxicity can lead to destruction of hepatocytes and erythrocytes (Usha *et al.,* 2007). Pre-treatments with the fruit-pulps incorporated diets however alleviates this situation and prevented the elevation PCV, HBG and RBC levels from normal levels (Figure 4.3, 4.4 and 4.5). On the other hand, there were no significant difference ($P>0.05$) in the levels of white blood cell, neutrophil and lymphocyte between the MNU control, the normal control group and fruit-pulps incorporated groups (Table 4.1). Haematotoxicity, such as anemia, thrombocytopenia, neutropenia, and thromboembolic events are frequent complications of solid tumors and hematological malignancies, and the therapies used to treat them (Richardson, 2010). For example, around 30–90% of patients with cancer have anemia although the prevalence is affected by the definition of anemia, cancer type, and disease stage. In a study by Liqaa (2014) on hematological parameters in breast cancer, significant differences ($p < 0.05$) were seen in the values for WBCs, RBCs, platelets count, PCV, lymphocyte and ESR among patients The fact that differences in this study were only observed in respect to erythrocytes and not observed with respect to white blood cells might be explained from the point of carcinogenesis initiation to cancer development. While fully developed cancers can have profound influence on red blood cells and white blood cells components, during initiation of cancer (carcinogenesis) observable impacts are mostly seen with respect to erythrocytes, hence the implication that leads to anemia (Rabia *et al.,* 2013). Since many degenerative diseases including cancer progress with concomitant damages to erythrocytes, the finding here implies that routine consumption of these plant fruit-pulps, might protect individuals against destruction of erythrocytes due to carcinogen exposure (Objective three).

Histological investigation of colon tissue sections of untreated control group showed normal histological structure of the mucosa, submucosa and muscularis layers (Plate 4.1a). While colonic tissue sections of MNU induced colon cancer group showed hyperplasia and associated with pleomorphism and inflammation in the lining epithelial cells of the glandular structure (Plate 4.1b), examination of colon tissue sections of MNU-induced rats that were on incorporated diets treated showed few inflammatory cells infiltration and mild ulceration. These effects were more pronounced in the groups that were on 5% and 10% incorporated diets (Plate 4.1e, 4.1f, 4.1g and 4.1h) implying that the dose of inclusion was important in effecting the protective activities. Similar observations were reported by Ahmed *et al*., (2013) who investigated the curative effects of treatment with *Boswellia serrata* extract after MNU induction using 5-fluorouracil as a standard drug for the control group. Their results indicated a marked improvement in the histological feature of the colon tissues of the groups treated with *Boswellia serrata* extract or 5-fluorouracil. The influence of incorporated diets in protection of the colonic tissue thereby preventing the onset of carcinoma might be attributable to the strong antioxidant potential of the fruit-pulps due to presence of polyphenols (Claudine *et al*., 2004). Polyphenols and other phytochemicals with antioxidant activities have been reported to have anti-inflammatory properties, antiproliferative and apoptotic effects (Atawodi, 2012).

Histological examination of liver tissue section shows normal liver with intact lobules & central vein in the normal control (Plate 4.2a). On the other hand liver of the MNU control group shows moderate destruction of the liver architecture and a noticed cytoplasmic clearing (Plate 4.2b). The test groups that were fed incorporated diets however showed apparently

normal liver except the group on lowest percentage supplementation with *D. microcarpum* which indicate mild cytoplasmic clearing (Plate 4.2c). Similar observations were seen after the histopathological examination of the kidney. The kidney tissue and the glomeruli were all apparently normal in all the groups except the MNU control group which shows necrosis of glomeruli and some degree of tubular distension around the glomerulus (Plate 4.3b).

Taken together, the observable damages to the histology of the colon, liver and the kidney of the MNU control groups confirms the toxic impacts of MNU on these organs. Interestingly, the animals fed with incorporated diets of the fruit-pulps of *D. microcarpum* and *D. guineense* were able to resist the toxic impacts of MNU on these organs (Objective four). This might be due to the known fact that consumption of diet that is rich in antioxidant could boost the antioxidant status of organisms, hence putting them is a better position to resist assaults by toxic xenobiotics (Chun *et al*., 2007). These findings were in agreement with reported findings of other researchers who investigated the organ protective effects of certain foods and medicinal plants in chemical toxicity model (Ahmed *et al*., 2013, Liman and Atawodi, 2013)

Several researchers have established MNU as a potent carcinogen in the induction of diverse tumors (breast, colon and prostate cancers) on varying strains of rats and mice (Yohizawa *et al*., 2000; Tarso *et al*, 2011). Carcinoembryonic antigen (CEA) is one of the most widely used tumor marker of gastrointestinal cancers particularly colorectal cancers. Serum CEA is widely accepted as a clinically significant prognostic and diagnostic indicator in colorectal cancer. A study has shown that a low CEA level (<100 ng/mL) measured at metastasis diagnosis is a significant prognostic indicator of overall survival in patients with colorectal

liver metastases (McNally *et al*., 2015). There was an observed significant ($p < 0.05$) elevation of CEA levels in MNU control group when compared with the untreated normal group and the other test groups fed fruit incorporated diets. This is an important predictive indicator that MNU toxicity has climaxed to the initiation of carcinogenesis. The prognosis that elevated CEA levels are indicators of carcinogenesis is in agreement with study report by McNally and co-workers (2015). The result on CEA levels showed no significant variation ($P>0.05$) when the untreated normal groups are compared with most of the test groups that were fed incorporated diets. However, the 5% and 10% *D. microcarpum* incorporated dietary group showed significantly ($p < 0.05$) lower levels of CEA than the untreated normal control implying a higher preventive activity of the plant when compared to *D. guineense* that showed no significant ($P>0.05$) variation in CEA levels with normal control at this inclusion dose. These finding are in agreement with finding of other researchers (Duffy, 2010; Grady, 2003, McNally *et al*., 2015) that reported high CEA levels in experimental animals with induced cancers. Furthermore the observed CEA levels of the animals in the normal control and groups fed incorporated diets were below the desired threshold CEA levels (<100 ng/mL) indicated for overall survival in cancer metastases. This might explains why there were no recorded mortalities in the animals of the normal group and the test groups fed incorporated diets while on the contrary two animals died in the MNU control group at the eighteenth and twentieth week of the experiment.

Immunohistochemistry (IHC) is the process of using antibodies to detect proteins (antigens) in cells within a tissue section (for instance colon, liver, pancreas or the heart). This tool is used to localize specific antigens in tissue sections with labelled antibodies based on antigen-

antibody interactions. The immune reactive products can be visualized by a marker including fluorescent dyes, enzymes in general; radioactive elements or colloidal gold. In this study the IHC assay exploits the MutL homolog 1 (MLH1) miss match repair protein. Mismatch repair (MMR) proteins are group of nuclear enzymes, which in all proliferating cells participate in repair of base-base mismatch, that occur during DNA replication. The proteins form complexes (heterodimers) that bind to areas of abnormal DNA and initiates its removal. Loss of MMR proteins leads to an accumulation of DNA replication errors in the proliferating cells, particularly in areas of the genome with short repetitive nucleotide sequences, a phenomenon known as microsatellite instability (MSI). Hence, MMR protein deficiency in cells is closely related to a high degree of MSI (MSI-H) which results in mutation that will initiate carcinogenesis, in contrast to cells with a low degree of MSI (MSI-L) and cells that are MSI stable (MSS) which are usually normal (Muller and Fishel, 2002). Carriers of an MLH1 or MSH2 mutation has a more than 70% lifetime risk of developing a colorectal carcinoma and also markedly increased risk of developing endometrial carcinomas (50%), as well as a less increased risk of developing carcinomas of stomach, biliary tract, ovary and urinary tract, as well as brain tumors and sebaceous skin tumors (Jen and Powell, 1994).

The observed mild expression of the MLH1 protein in the MNU control group (Plate 4.4.b) is an illustration of loss of MMR protein which demonstrate a high degree of microsatellite instability (MSI-H) due to mutation; a hallmark in the initiation of carcinogenesis. Similar observations of mild expression were seen in the group fed with low dose (2.5%) of incorporated diets (Plates 4.4c and 4.4d). However, as the fruit-pulps incorporation dose increases through 5% to 10% a marked moderate to high expressions of the MLH1 protein

were observed (Plates 4.4e, 4.4f, 4.4g and 4.4f). Similarly, such high expression of MLH1 were noted in the untreated normal control and dietary fruit-pulp only control (Plates 4.4a, 4.4i and 4.4j). This implies that groups of rats fed with 5% and 10% fruit-pulp incorporated diets were able to resist the carcinogenic effects of MNU that arose from damages to MMR protein leading to microsatellite instability. These findings were of significant importance since the colon microsatellite profile provides useful prognostic information on colon carcinogenesis (Giardiello and Hamilton 1993). MMR defects determined by IHC are used clinically not only to establish early colorectal carcinogenesis but as a tool to differentiate between sporadic and hereditary mutations. For example while MLH1 and MLH2 loss leads to hereditary non-polyposis colorectal cancer (HNPCC) or sporadic colorectal cancer, loss of adenomatous polyposis coli (APC) gene is responsible for the familial adenomatous polyposis (FAP) or Lynch syndrome (Grady, 2003).

The results from the IHC staining have clearly established that a sporadic mutation was induced by MNU which consequently initiates the process of carcinogenesis in the MNU control group. However, in the fruit-pulp incorporated diets groups the findings indicate that carcinogenesis induction were being prevented in a dose dependent fashion. The preventive effects of the dietary inclusion of the fruit-pulps has been established (Objective five). These observations were in agreement with similar finding reported by various studies which demonstrate the loss of MMR activity as an early detectable stage in the initiation of colorectal carcinogenesis (Ahmed *et al*., 2013). Comparatively, the modulation of MLH1 expression was more obvious in the *D. microcarpum* fruit-pulp test group than the *D. guineense* test group. This can be clearly seen when respective groups of the two fruit-pulps

on higher dietary incorporation levels (5% and 10%) were particularly compared. While at the 5% inclusion level only moderate expression of the MLH1 antigen was observed for both respective groups (Plates 4.4e and 4.4f), at the 10% dietary inclusion level there was an observed higher expression of MLH1 in the *D. microcarpum* group (Plate 4.4g) in respect the *D. guineense* group which still showed only moderate expression of the antigen (Plate 4.4h). Similar high expression of the antigen was seen in the normal control group (Plate 4.4a), suggesting that *D. microcarpum* fruit-pulps were better able to modulate the MLH1 antigen to normal levels.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In summary the study has established the following contributions to the advancement of knowledge in:

i. that the incorporation of fruit-pulps *D.guineense* and *D.microcarpum* fruits into the diets of rats protected their colon, liver, kidney from MNU toxicity.

ii. that the incorporation of the fruit-pulps of *D. guineense and D. microcarpum* into the diets of rats positively challenged oxidative stress in colon carcinogenesis model, due to MNU toxicity.

iii. that the incorporation of fruit-pulp of *D. guineense and D. microcarpum* into the diets of rats enhanced the levels of haematological parameters particularly PCV, haemoglobin and RBC of rats undergoing experimental colon carcinogenesis.

iv. It was demonstrated that dietary inclusion of these fruits can prevent the onset of the process of colon carcinogenesis.

6.2 Conclusion

The study has demonstrated that the incorporation of fruit-pulps of *D. microcarpum* and *D. guineense* in the diet protected the blood and the organs of the rats from induced MNU toxicity; prevented oxidative stress and other processes involved in colon carcinogenesis in rats, with *D. microcarpum* showing a better protection.

The findings of this work indicate that the fruits of *D. microcarpum* and *D. guineense* have the ability to prevent the initiation and proliferation of chemically induced carcinogenesis in rats, thus justifying their usage in foods and traditional medicine in West Africa. In the overall, the study indicate the colon cancer preventive potential of these tropical African fruits with *D. microcarpum* showing a better preventive potential

6.3 Recommendations

i. Improved culinary utilization and routine consumption of these fruits as dietary components is hereby recommended as a means of preventing incidence of colon cancer.

ii. Further chemical and pharmacological investigations are required however, for isolation, characterization, and structure elucidation of the bioactive constituents of these fruits.

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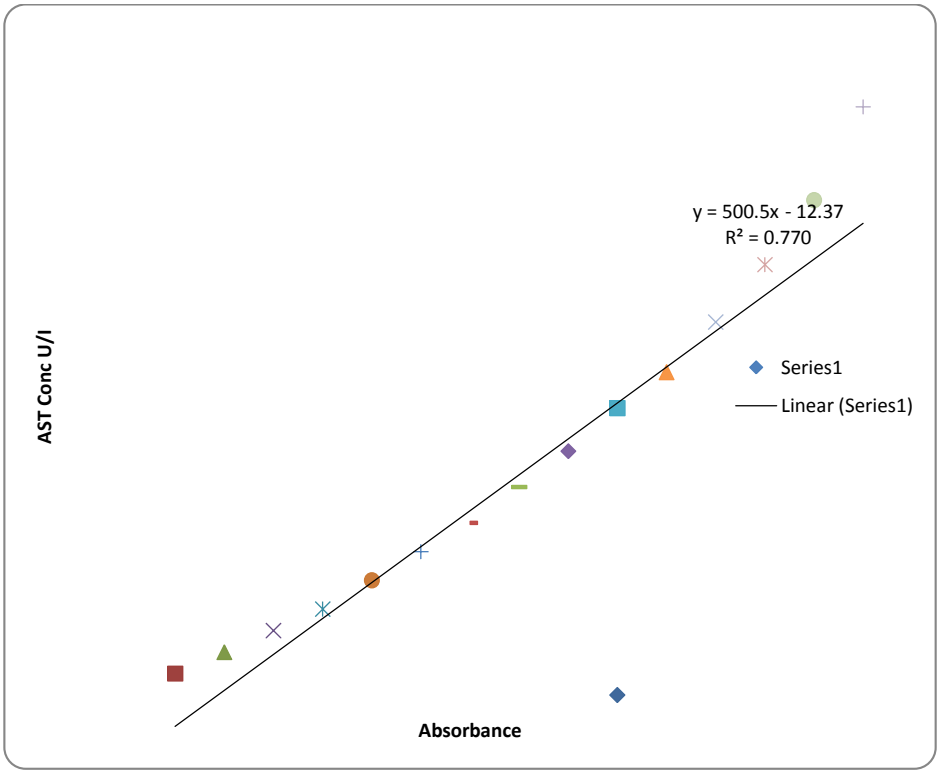
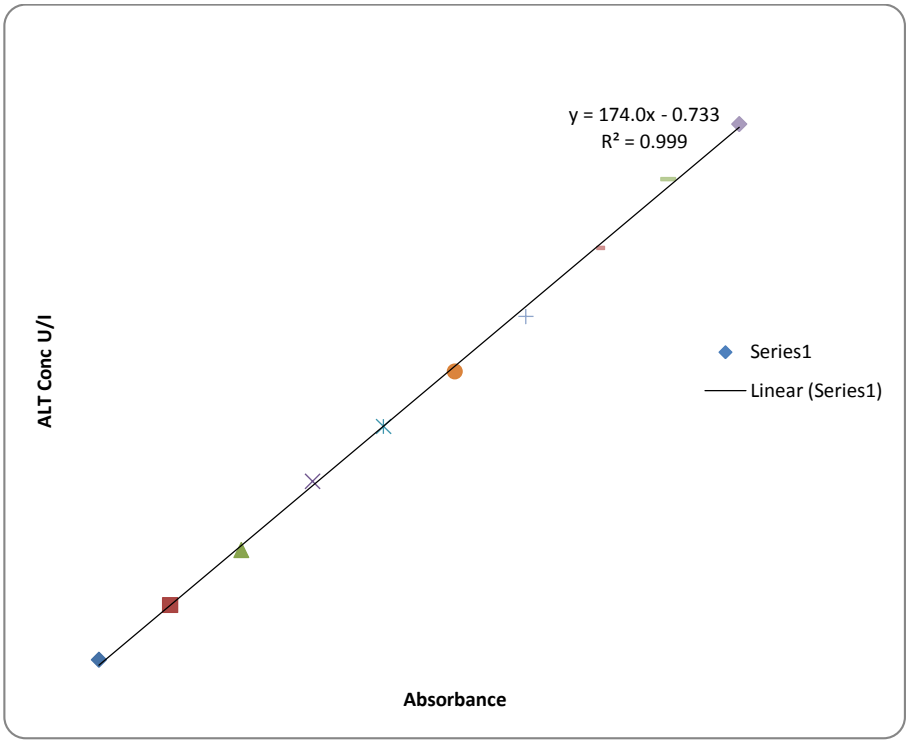
APPENDIX



Appendix I: Aspartate aminotransferase (AST) standard curve.

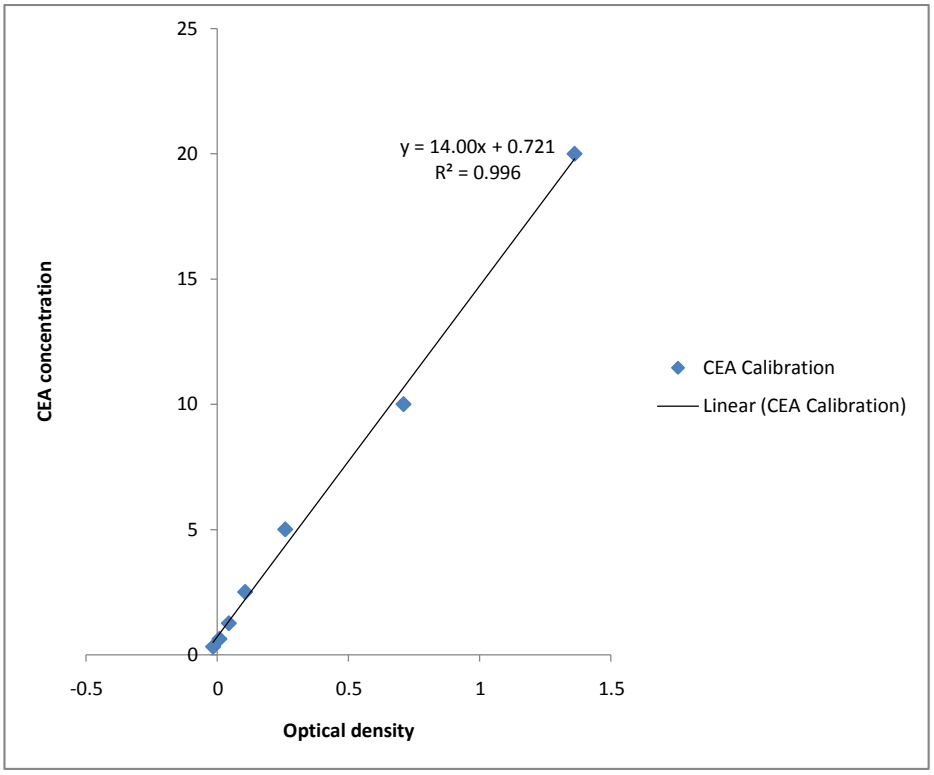


Appendix II: Alanine aminotransferase (AST) standard curve.

Appendix III: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Serum AST and ALT Concentration (Mg/dl) Following 12 Weeks MNU Administration in Rats.

Treatments	AST (U/I)	ALT (U/I)
Normal control	100 ± 10^{c}	56 ± 5^{a}
MNU control	141 ± 9^{g}	77 ± 4^{de}
10% *D. guineense* + MNU	88 ± 5^{a}	68 ± 2^{bc}
10% *D. guineense* only	91 ± 7^{ab}	57 ± 4^{a}
5% *D. guineense* + MNU	120 ± 5^{e}	74 ± 79^{de}
2.5% *D. guineense* + MNU	131 ± 4^{f}	79 ± 4^{e}
10% *D. microcarpum* + MNU	87 ± 3^{a}	64 ± 3^{b}
10% *D. microcarpum* only	97 ± 1^{bc}	67 ± 5^{b}
5% *D. microcarpum* + MNU	110 ± 6^{d}	69 ± 6^{bc}
2.5% *D. microcarpum* + MNU	113 ± 6^{de}	73 ± 3^{cd}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$).



Appendix IV: Carcinoembryonic antigen (CEA) standard curve.

Appendix V: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Serum CEA Concentration (ng/ml) Following 12 Weeks MNU Administration in Rats.

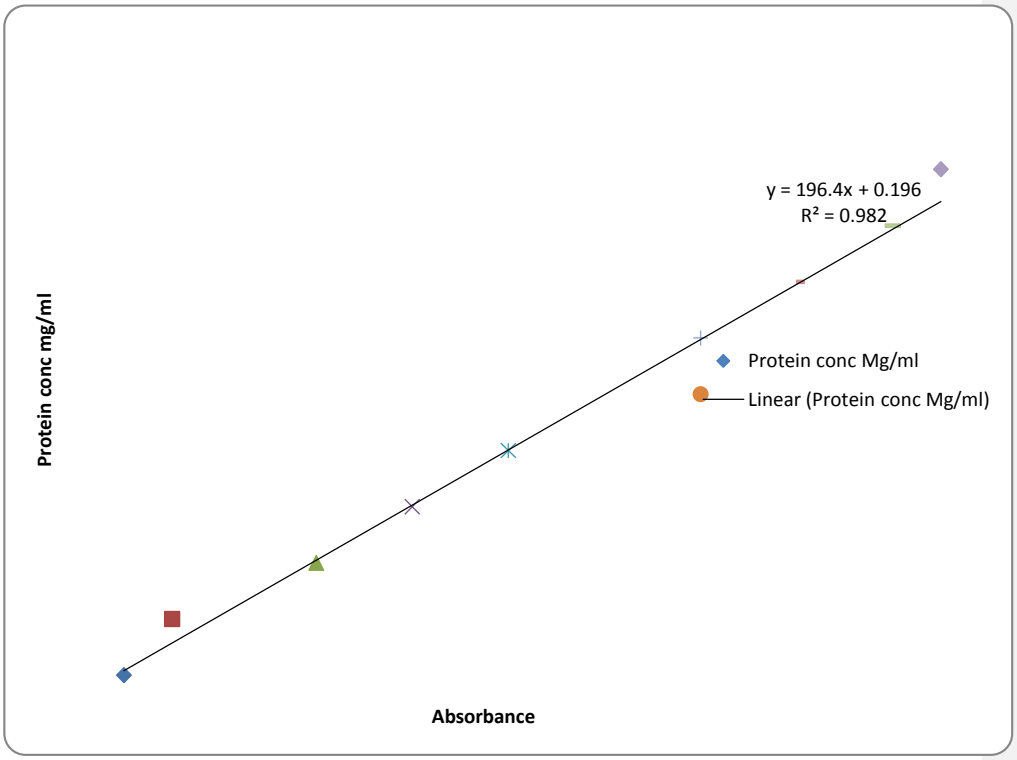
Treatments	CEA (ng/ml)
Normal control	98.23±8.73[a]
MNU control	138.37±12.05[b]
10% *D. guineense* + MNU	90.88±7.65[c]
10% *D. guineense* only	65.68±8.18[c]
5% *D. guineense* + MNU	63.12±4.26[b]
2.5% *D. guineense* + MNU	81.32±10.36[b]
10% *D. microcarpum* + MNU	91.58±13.89[b]
10% *D. microcarpum* only	81.08±6.53[b]
5% *D. microcarpum* + MNU	80.15±5.8[b]
2.5% *D. microcarpum* + MNU	87.15±12.72[b]

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$)

Appendix VI: Effect of Dietary Inclusion of *D. guineense* and *D. microcarpum* Fruit-pulps on Blood PCV. HBG and RBC levels Following 12 Weeks MNU Administration in Rats.

Treatment	PCV (%)	HGB (g/dl)	RBC ($x10^{12}$/l)
Normal control	42.2 ± 6.9^{abc}	14.0 ± 2.3^{c}	7.0 ± 1.0^{b}
MNU control	30.2 ± 2.4^{ab}	8.2 ± 1.1^{a}	4.8 ± 1.0^{a}
2.5% *D. guineense* + MNU	45.8 ± 3.5^{bc}	14.2 ± 1.2^{bc}	7.1 ± 0.6^{b}
5% *D. guineense* + MNU	42.2 ± 4.1^{c}	15.0 ± 1.4^{c}	7.6 ± 0.7^{bc}
10% *D. guineense* + MNU	35.2 ± 5.5^{a}	13.6 ± 1.8^{bc}	7.7 ± 0.9^{bc}
10% *D. guineense only*	43.0 ± 7.2^{bc}	14.2 ± 2.4^{b}	6.3 ± 1.0^{ab}
2.5% *D. microcarpum* + MNU	43.4 ± 6.5^{bc}	11.9 ± 3.2^{bc}	7.1 ± 1.0^{b}
5% *D. microcarpum* + MNU	45.4 ± 5.0^{c}	15.1 ± 1.7^{c}	7.7 ± 0.6^{bc}
10% *D. microcarpum* + MNU	42.2 ± 3.2^{abc}	14.3 ± 0.9^{bc}	7.1 ± 0.5^{b}
10% *D. microcarpum only*	41.2 ± 5.6^{abc}	12.7 ± 1.9^{b}	6.9 ± 0.9^{b}

Values are Mean±SD. Values with different superscript down the column are significantly different ($p<0.05$)



Appendix VII: Protein (Albumin) Standard Curve.