PREVENTIVE EFFECT OF DIETARY *CUCURBITA MAXIMA* DUCH FRUIT INCLUSION ON N- METHYL-N-NITROSOUREA (MNU) INDUCED COLON CARCINOGENESIS IN MALE WISTAR RATS

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DEPARTMENT OF BIOCHEMISTRY
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DECEMBER 2017

DECLARATION

I declare that the work in this Dissertation entitled **"Preventive Effect of Dietary *Cucurbita maxima* Fruit (Duch) Inclusion on N-Methyl-N-Nitrosourea (MNU) Induced Colon Carcinogenesis in Wistar Rats"** has been performed by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other Institution.

IBRAHIM ISIAKA	Signature	Date

CERTIFICATION

This Dissertation entitled **"Preventive Effect of Dietary *Cucurbita maxima* Fruit (Duch) Inclusion on N- Methyl-N-Nitrosourea (MNU) Induced Colon Carcinogenesis in Wistar Rats"** by **IBRAHIM ISIAKA** meets the regulations governing the award of the degree of Master of Science in Nutrition of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The preventive effect of dietary C*ucurbita maxima* fruit inclusion at 2.5%, 5% and 10% on colon carcinogenesis initiated with N-methyl-N-nitrosourea (MNU) induction for twelve (12) weeks was evaluated. Group A= Normal saline with standard feed, Group B= MNU with standard feed, Group C= MNU with dietary inclusion of 2.5% *Cucurbita maxima* powder, Group D= MNU with dietary inclusion of 5% *Cucurbita maxima powder,* Group E= MNU with dietary inclusion of 10% *Cucurbita maxima* powder, Group F= Dietary inclusion control normal saline with 10% Cucurbita maxima powder. After twelve (12) weeks of treatment, the colon and liver of rats undergoing MNU intoxication but fed *Cucurbita maxima* fruit included diets showed a significant ($p \leq 0.05$) increase in the activity of endogenous antioxidant enzymes (catalase and superoxide dismutase) when compared with MNU control group. Also a dose-dependent decrease in the extent of lipid peroxidation was observed in the colon and liver of dietary *Cucurbita maxima* fruit included treated groups. A significant ($p \leq 0.05$) increase in white blood cell, platelet level, neutrophils and lymphocyte counts in MNU control group was observed when compared to other groups fed with dietary *Cucurbita maxima* fruit inclusion, while a significant ($p \leq 0.05$) decrease in packed cell volume, haemoglobin count and red blood cell were observed in MNU control group compared to other groups fed with dietary *Cucurbia maxima* fruit inclusion. Histological observation of colon and liver tissue sections showed severe focal area of inflammatory cells in the MNU control group while normal control group and other groups that received various dietary *Cucurbita maxima* fruit inclusion showed milder to no tissue damage. A statistically significant ($p \leq 0.05$) decrease in carcino-embrayonic antigen (CEA) levels was observsed in all dietary *Cucurbita maxima* fruit inclusion groups compared to MNU control group. Immunohistochemical observations of the colon tissue sections showed no expression of

Mut L Homolog gene in MNU control group while moderate to mild expression of *Mut L Homolog* gene in normal control group and other groups treated with 2.5%, 5% and 10% of dietary *Cucurbita maxima* fruit inclusion. These results indicate potential of dietary *Cucurbita maxima* fruit inclusion to antagonizing the process of chemically induced colon carcinogenesis by enhancement of endogenous antioxidants systems and prevention of oxidative stress.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABC	Avidin Biotin Complex
ACF	Aberrant Crypt Foci
ANOVA	Analysis of Variance
AORTIC	African Organization for Research and Training in Cancer
APC	Adenomatous Polyposis Coli
AST	Aspartate Aminotransferase
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
CAT	Catalase
CEA	Carcinoembrayonic antigen
CIN	Chromosomal Instability
CRC	Colorectal Cancer
DPPH	1, 1-Diphenyl-2-picrylhydrazyl
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EVF	Erythrocyte Volume Fraction
GLOBOCAN	Global Burden on Cancer
GSHPx	Glutathione Peroxidase
Hb	Haemoglobin
HNE	Hydroxynonenal
Ht or Hct	Haematocrit

IBD	Inflammatory bowel diseases
MDA	Malondialdehyde
MSI	Microsatellite instability
MLH1	*Mult L homologue* 1
MMR	Mismatch Repair Gene
MNU	N-methyl nitrosourea
NADPH	Nicotinamide Adenine dinucleotide Phosphate Reduced
NCI	National Cancer Institute
PCV	Packed cell volume
PUFAs	Polyunsaturated Fatty Acids
RBC	Red blood cell count
RNS	Reactive nitrogen specie
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TAG	Triacylglyerol
TBA	Thiobarbituric Acid
WBC	White Blood Cell
WHO	World health organization

CHAPTER ONE

2.0 INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. The development of most cancers requires multiple steps that occur over many years. Certain types of cancer can be prevented by eliminating exposure to tobacco and other factors that initiate or accelerate this process. Other potential malignancies can be detected before cells become cancerous or at an early stage, when the disease is most treatable. Cancer is treated with surgery, radiation, chemotherapy, hormones, and immunotherapy. Worldwide, one in eight deaths is due to cancer, cancer causes more deaths than AIDS, tuberculosis, and malaria combined. When countries are grouped according to economic development, cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries (following heart diseases). According to recent World Health Organization (WHO) projections, cancer will have replaced ischemic heart disease as the overall leading cause of death worldwide in 2010 (WHO, 2007).

According to estimates from the International Agency for Research on Cancer (IARC), there were 12.7 million new cancer cases in 2008 worldwide, of which 5.6 million occurred in economically developed countries and 7.1 million in economically developing countries. The corresponding estimates for total cancer deaths in 2008 were 7.6 million (about 21,000 cancer deaths a day), 2.8 million in economically developed countries and 4.8 million in economically developing countries. By 2030, the global burden is expected to grow to 21.4 million new cancer cases and 13.2 million cancer deaths simply due to the growth and aging of the population, as well as reductions in childhood mortality and deaths from infectious diseases in developing countries Ferlay *et al*., (2010).

The estimated future burden could be much larger than given above due to the adoption of western lifestyles, such as smoking, poor diet, physical inactivity, and reproductive factors, in economically developing countries. Cancers related to these factors, such as lung, breast, and colorectal cancers, are increasing in economically transitioning countries. Rates of cancers common in Western countries will continue to rise in developing countries if preventive measures are not widely applied. In economically developed countries, the three most commonly diagnosed cancers were prostate, lung and bronchus, and colorectal among men, and breast, colorectal, and lung among women. In economically developing countries, the three most commonly diagnosed cancers were lung, stomach, and liver in men, and breast, cervix uteri, and lung in women. In both economically developed and developing countries, the three most common cancer sites were also the three leading causes of cancer death.

Globally, colorectal cancer (CRC) is the second most common cancer in women (614, 000 cases per year) and the third most common in men (746, 000 cases per year). The incidence rates are higher in more developed countries (737, 000 cases per year) than in less developed ones (624, 000 cases per year). However, mortality is higher in the latter (52% of total deaths), which indicates poor survival. In 2015, the GLOBOCAN online analysis tool has predicted 61, 228 new CRC cases for Asia. Accordingly, 25, 816 of these cases are associated with people who are less than 65 years old Fearon *et al.*, (2011).

1.1 Statement of Research Problem

Cancer is still one of the most deadly diseases that cause serious health problems, physical disabilities, mortalities, and morbidities around the world. It is the second leading cause of death all over the world. Although great advancement has been made in the treatment of cancer progression, there is still significant deficiencies and room for improvement Sabira Sultana *et al.*, (2015). Colorectal carcinoma is the commonest malignancy of the gastrointestinal tract worldwide, with about 140,000 cases each year in the United States, according to the National Cancer Institute. Incidence and death rates for colorectal cancer increase with age. This means that about 90% of new cases and 93% of deaths occur in people 50 and older (SEER program 1969-2010). It is observed that the median age of colon cancer diagnosis is put at 69 in men and 73 in women, and that of rectal cancer diagnosis is put at 63 in men and 65 in women. Overall, colorectal cancer incidence and mortality rates are about 30% to 40% higher in men than in women (Murphy *et al.,* 2010). The reasons for this likely reflect complex interactions between gender-related differences in exposure to hormones and risk factors. However, the incidence is rising in Africans from an average of 10-20 newly diagnosed colorectal cancer in a tertiary health institution per year to about 50 Archampong *et al.,* (1994). Others are the complications that accompany resection and the damages caused by radiotherapy recently; resistance to anticancer drugs has been observed therefore, more research is required to explore the anticancer or preventive potential of dietary supplementation with medicinal plants (Vinay *et al.,* 2011).

1.2 Justification

Cancer is the second leading cause of death worldwide. Conventional cancer therapies cause serious side effects and at best, merely extend the patient's lifespan by a few years. The demand to utilize alternative concepts or approaches to the treatment of cancer is therefore escalating. However, the potential treatment of cancer is still under investigation. In fact the plants occupy a good place in the treatment Srijita-Dutta *et al.*, (2015).

The medicinal plants and their products, particularly vegetables have antioxidant activity leading to anticancer effect. Many doctors recommend that people who wish to reduce the risk of cancer must eat vegetables every day in their diet Srijita-Dutta *et al.*, (2015). The vegetables contain many phytochemicals having antioxidant activity. The antioxidant protects the cells from damage caused by free oxygen radicals. Here in this study, we will give a better therapeutic approach to cancer by the maximum use of vegetable (*Cucurbita maxima* fruit.) against colon cancer.

The fruit of *Cucurbita maxima* can be found in some part of Nigeria and it is used among the Hausa, igala and ibo ethnic tribes as day to day food and vegetables in various delicacies. Substantial evidence from various experimental studies has also suggested the anticancer potential of the different parts of the plant including the fruits on different types of cancer. Thus, considering the increasing incidence of colon cancer worldwide and the need for approaching and solving the problem of colon cancer through diet (dietary inclusion), there is therefore the need to investigate its potential (*Cucurbita spp*.) to prevent colon cancer Attarde *et al*., (2010).

1.3 General Aim

The aim of this work is to evaluate the preventive effect of dietary *Cucurbita maxima* fruit inclusion on MNU induced colon carcinogenesis in albino Wister rats.

1.4 Specific Objectives

i. To determine the effect of *Cucurbita maxima* fruit dietary inclusion on haematological parameters in MNU induced colon carcinogenesis in Albino wistar rats.

ii. To establish the possible role of *Cucurbita maxima* fruit included diet on the antioxidant status in MNU induced colon carcinogenesis in Albino wistar rats.

iii. To evaluate the effect of included diet of *Cucurbita maxima* fruit on some markers of MNU induced colon carcinogenesis in Albino wistar rats.

1.5 Null Hypothesis

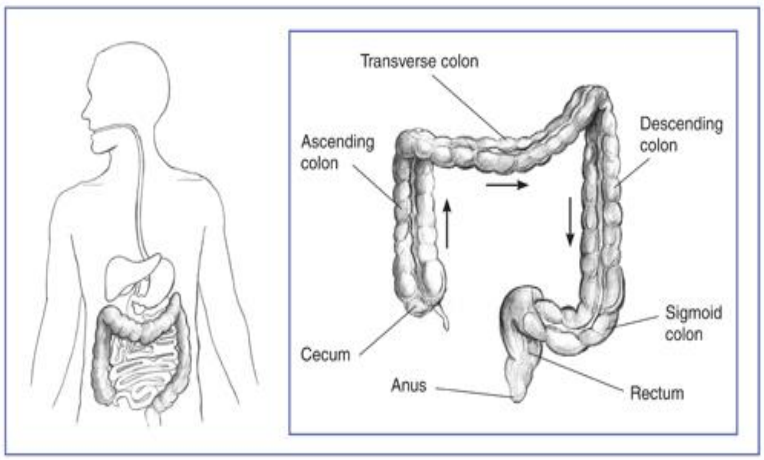
Dietary *Cucurbita maxima* fruit inclusion has no preventive effect on colon carcinogenesis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Human Colon

The colon is an organ shaped like a question mark **(?)** that forms part of the large intestine (Figure1) (NCCN, 2014). It is approximately 1.5 metres long and is divided into four segments based on the vascular supply to each segment. The right colon, otherwise known as the ascending colon, receives its blood supply from the right colic and the ileocolic artery. The most proximal portion of the right colon is termed the caecum and is identified by the appendix, which a small appendage attached to three longitudinal bands of muscle called the taeniae coli. The transverse colon connects the right colon to the left colon. Its blood supply is derived from the middle colic artery and, like the right colic artery, it is derived from the superior mesenteric artery, a branch of the aorta. The juncture at which the right and transverse colon join is termed the hepatic flexure and the juncture at which the transverse colon and left colon join is termed the splenic flexure. Flexures are points at which the large bowel is tethered and supported in order to prevent excessive twisting that could lead to a condition called volvulus Mobarhan *et al.*, (2012). Volvulus is a serious problem that can result in diminished blood supply to the bowel, with subsequent necrosis and perforation. The transverse colon also serves as a tethering point for the greater omentum, which is a large fatty apron of tissue that essentially covers the entire abdomen, separating the small and large bowel from the anterior abdominal wall. The purpose of the omentum is to ‘police’ the abdomen, identifying holes or perforations of the bowel wall and sealing them. In addition, it seems to be an adhesive target for neoplastic cells, which otherwise float free in the abdomen. The left colon connects the transverse colon to the sigmoid colon, which is named for its S-shaped appearance in the abdomen. The left colon and sigmoid colon are supplied by the left colic and sigmoidal arteries, branches of the inferior mesenteric artery Mobarhan *et al.*, (2012).



Source: Encyclopedia of Life Sciences 2001

FIG. 2.1: Anatomy of the large intestine showing the four sections: caecum and ascending, transverse, descending and sigmoid. The rectum forms the most distal portion of the large intestine.

2.2 Colon Cancer

According to Encyclopedia of life sciences 2001, Colon cancer is a disease of the large intestine which begins at a structure called the caecum, located in the right lower quadrant of the abdomen, and continues through all portions of the abdomen to its junction with the rectum, located in the deep pelvis.

2.3 Incidence of Colon Cancer

Colorectal cancer is the third most common cancer in men and the second in women Landis *et al.,* (*2011*). Worldwide, an estimated 1.2 million cases of colorectal cancer occurred in 2008. The highest incidence rates were in North America, Australia, New Zealand, Europe, and Japan. Rates were low in Africa and South Central Asia. Rates were substantially higher in men than in women, Cummings *et al.,* (*2011*).

The incidence of colorectal cancer is increasing in certain countries where risk was historically low (Japan), (Cummings *et al.,* 2011). In high-risk/high-income countries, trends are either gradually increasing (Finland, Norway), stabilizing (France, Australia), or declining (United States) with time. The greatest increases in the incidence of colorectal cancer are in Asia (Japan, Kuwait, Israel) and Eastern Europe (Czech Republic, Slovakia, Slovenia). In fact, rates among males in the Czech Republic, Slovakia, and Japan have not only exceeded the peak rates observed in long-standing developed countries such as the United States, Canada, and Australia but they continue to increase, Cummings (2011). The decrease in colorectal cancer incidence in the United States partially reflects the increase in detection and removal of precancerous lesions; the increase in several Asian and Eastern European countries may reflect changes in risk factors for colorectal cancer that are associated with westernization such as elevated obesity and smoking prevalence. In contrast to incidence trends, decreasing colorectal cancer mortality rates have been observed in a large number of countries worldwide and are most likely attributed to colorectal cancer screening and/or improved treatments (GLOBOCAN, 2008). However, increases in mortality rates are still occurring in countries that have more limited resources, including Mexico and Brazil in South America and Romania and Russia in Eastern Europe.

About 608,700 deaths from colorectal cancer occurred in 2008 worldwide, accounting for 8% of all cancer deaths, Cummings *et al.*, (2011).

Although inadequate research opportunities and data collection systems raise concerns of possible under-reporting, available data suggest a lower burden of CRC in Africa. While the overwhelming majority of the population in Africa is of indigenous African origin (black), countries with substantial other racial groups, such as in South Africa, report significant population (racial) differences in CRC burden. The lowest burden of disease have been noted among blacks, and especially those who have maintained their traditional lifestyle including diet, an intermediate burden among Indians and those of mixed ancestry (coloreds) and the highest burden among persons of European origin (whites). This contrasts the findings in the United States where African Americans suffer a disproportionally higher burden of CRC when compared to other population groups Cummings *et al.,* (2011).

Colorectal carcinoma (CRC) is relatively uncommon in Nigeria, compared to the developed countries. In 2008, colorectal cancer was the third commonest cancer in males and the second in females worldwide. However almost 60% of these cases occurred in developed countries and only 2% of new cases and 3% of deaths due to colorectal cancer occurred in Africa (Globocan 2008). Recent studies of CRC indicate an increasing trend, which has been attributed to the adoption of sedentary western lifestyle and diet, migration patterns and the improved availability of requisite specialized medical services Duduyemi *et al.,* (2013).

2.4 Causes and Mechanism of Colon Carcinogenesis

Carcinogenesis is the formation of a cancer, whereby normal cells are transformed into cancer cells. The process is characterized by changes at the cellular, genetic, and epigenetic levels and abnormal cell division, in some cancers forming a malignant mass Lim *et al.,* (2006). Cell division is a physiological process that occurs in almost all tissues and under many circumstances. Under normal circumstances, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained by regulation of both processes to ensure the integrity of tissues and organs. According to the prevailing accepted theory of carcinogenesis Tariq *et al.,* (2016), the somatic mutation theory, mutations and epimutations in DNA that lead to cancer (only certain mutations and epimutations can lead to cancer and the majority of potential mutations and epimutations will have no such effect) disrupt these orderly processes by disrupting the programming regulating the processes, upsetting the normal balance between proliferation and cell death. This results in uncontrolled cell division and the evolution of those cells by natural selection in the body. The uncontrolled and often rapid proliferation of cells can lead to benign tumors; some types of these may turn into malignant tumors (cancer). Benign tumors do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active, for instance, producing a hormone. Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening Tariq *et al.,* (2016).

More than one mutation is necessary for carcinogenesis. In fact, a series of several mutations to certain classes of genes is usually required before a normal cell will transform into a cancer cell Dow *et al.,* (2015). On average, for example, 15 “driver mutations” and 60 “passenger” mutations are found in colon cancers, Wood *et al.,* (2007). Mutations in those certain types of genes that play vital roles in cell division, apoptosis (cell death), and mutations and epimutations in DNA repair genes will cause a cell to lose control of its cell proliferation, Weisenberger *et al.,* (2010).

Oncovirinae, viruses that contain an oncogene, are categorized as oncogenic because they trigger the growth of tumorous tissues in the host. This process is also referred to as viral transformation. Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes that regulate cell growth and differentiation must be altered, Crose (2008). Genetic and epigenetic changes can occur at many levels, from gain or loss of entire chromosomes, to a mutation affecting a single DNA nucleotide, or to silencing or activating microRNA that controls expression of 100 to 500 genes, Lim *et al.,* (2005)*;* Balaguer *et al.,* (2010). There are two broad categories of genes that are affected by these changes; Oncogenes may be normal genes that are expressed at inappropriately high levels, or altered genes that have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes that inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell, Fearon *et al.,* (2009).

Damage of DNA is considered to be the primary cause of cancer Bonadon, (2008); Bernstein *et al.,* (2011). More than 60,000 new naturally occurring DNA damages arise, on average, per human cell, per day, due to endogenous cellular processes. Additional DNA damages can arise from exposure to exogenous agents. As one example of an exogenous carcinogenic agent, tobacco smoke causes increased DNA damage, and these DNA damages likely cause the increase of lung cancer due to smoking, Cunningham *et al.,* (2011). In other examples, UV light from solar radiation causes DNA damage that is important in melanoma, Przybylowska *et al.,* (2013) H. pylori infection produces high levels of reactive oxygen species that damage DNA and contributes to gastric cancer, Dow *et al.,* (2015), and the Aspergillus metabolite, aflatoxin, is a DNA damaging agent that is causative in liver cancer, Bernstein *et al.,* (2011). DNA damages can also be caused by endogenous (naturally occurring) agents. Katsurano *et al*., (2011) indicated that macrophages and neutrophils in an inflamed colonic epithelium are the source of reactive oxygen species causing the DNA damages that initiate colonic tumorigenesis, Katsurano *et al.,* (2011) and bile acids, at high levels in the colons of humans eating a high fat diet, also cause DNA damage and contribute to colon cancer Bernstein *et al.,* (2011).

A deficiency in DNA repair would cause more DNA damages to accumulate, and increase the risk for cancer. For example, individuals with an inherited impairment in any of 34 DNA repair genes are at increased risk of cancer with some defects causing up to 100% lifetime chance of cancer, Tariq *et al.,* (2016).

Colorectal cancer (CRCs) can arise from one or a combination of three different mechanisms, namely chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI). According to Ferlay *et al.,* (2015), the classical CIN pathway begins with the acquisition of mutations in the adenomatous polyposis coli (*APC*), followed by the mutational activation of oncogene *KRAS* and the inactivation of the tumor suppressor gene, *TP53*. Aneuploidy and loss of heterozygosity (LOH) are the major players in CIN tumors, which not only constitute most of the sporadic tumors (85%) but also involve familial adenomatous polyposis cases associated with germline mutations in the *APC* gene. The CIMP pathway is characterized by promoter hypermethylation of various tumor suppressor genes, most importantly *MGMT* and *MLH1*. This hypermethylation is often associated with *BRAF* mutation and microsatellite instability Weisenberger *et al.,* (2010). The MSI pathway involves the inactivation of genetic alterations in short repeated sequences. This activation occurs in CRCs in DNA mismatch repair (MMR) genes, and is a hallmark condition in familial Lynch syndrome (LS), which also appears in ~15% of the sporadic CRC cases. In addition, the hypermethylation of the MMR genes may lead to MSI. This mechanism is often associated with the CIMP pathway Weisenberger *et al.,* (2010). MSI tumors are often associated with proximal colon and poor differentiation but better prognosis. Moreover, the three mechanisms often overlap in CRC.

In order for cells to start dividing uncontrollably, genes that regulate cell growth must be dysregulated Bernstein *et al.,* (2011). Proto oncogenes are genes that promote cell growth and mitosis, whereas tumor suppressor genes discourage cell growth, or temporarily halt cell division to carry out DNA repair. Typically, a series of several mutations to these genes is required before a normal cell transforms into a cancer cell. This concept is sometimes termed “oncoevolution.” Mutations to these genes provide the signals for tumor cells to start dividing uncontrollably. But the uncontrolled cell division that characterizes cancer also requires that the dividing cell duplicates all its cellular components to create two daughter cells. The activation of anaerobic glycolysis (the Warburg effect), which is not necessarily induced by mutations in proto-oncogenes and tumor suppressor genes, Tariq *et al.,* (2016) provides most of the building blocks required to duplicate the cellular components of a dividing cell and, therefore, is also essential for carcinogenesis Lopez *et al.,* (2010).

2.5 Some Diagnostic (Biomarkers) in Colon Carcinogenesis

2.5.1 Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) is a glycoprotein, which is present in normal mucosal cells but increased amounts are associated with adenocarcinoma, especially colorectal cancer. CEA therefore has a role as a tumor marker. Sensitivity and specificity are low, however, so it is of more use for monitoring than for screening or diagnosis. CEA levels are useful in assessing prognosis (with other factors), detecting recurrence (especially for disease that cannot be evaluated by other means) and monitoring treatment in people with colorectal cancer. CEA is particularly recommended for postoperative follow-up of patients with stage II and III colorectal cancer if further surgery or chemotherapy is an option, Sturgeon *et al.,* (2009).

Carcinoembryonic antigen (CEA) is one of the most widely used tumor markers worldwide. Its main application is mostly in gastrointestinal cancers, especially in colorectal malignancy.

Serial CEA measurements can detect recurrent colorectal cancer with a sensitivity of 80%, a specificity of 70%, and can provide a lead time of 5 months. CEA is the most frequent indicator of recurrence in asymptomatic patients and currently is the most cost-effective test for the preclinical detection of resectable disease. Overall, however, little evidence is available that monitoring of all patients with diagnosed colorectal cancer leads to enhanced patient outcome or quality of life.

2.5.2 Normal range of CEA level

Individual laboratory normal ranges vary but CEA level is usually deemed to be normal at 2.5-5 μg/L. increasing levels of CEA suggestive of active disease may be more clinically helpful than absolute level, Sturgeon *et al.,* (2008). Levels exceeding 10 μg/L are rarely due to benign disease or the moderate elevation that may occur due to smoking, Fakih *et al.,* (2006).

2.5.3 Sensitivity of CEA

Sensitivity in early-stage colorectal cancer is very low and increases with the stage of the disease. Studies reporting sensitivity vary in the cut off for what constitutes a normal CEA level so results are varied. Using a cutoff point of 5μg/L, the proportions of patients with increased values are 3%, 25%, 45% and 65% for patients with Dukes' A, B, C and D disease respectively. Around 72% of cases with unresectable or metastatic disease have elevated CEA levels Sturgeon *et al.,* (2008).

2.5.4 Monitoring treatment:

The major role for CEA levels is in following patients for relapse after intended curative treatment of colorectal cancer. CEA levels typically return to normal within four to six weeks after successful surgical resection. The CEA level can also be used to assess the response to chemotherapy. It is recommended that people with stage II or III colorectal cancer have CEA surveillance every three months for at least three years after treatment. National Institute for Health and Care Excellence (NICE) guidelines recommend at least every six months, although most other authorities recommend every three months, Sturgeon *et al.,* (2008). Clinical trials have shown improvement in survival after five years in patients who underwent CEA monitoring as part of post-treatment management, Sturgeon *et al.,* (2008); Jeffery *et al.,* (2007). However, normal levels do not necessarily indicate that recurrence has not occurred, Hara *et al.,* (2008).

Currently, the most useful application of CEA is in the detection of liver metastasis from colorectal cancers. Because of the relative success of surgery in resecting hepatic metastases, serial determinations of the marker are recommended for detecting cancer spread to the liver. There are plans that in the future, preoperative concentrations of CEA may be included with the standard staging procedures for assessing prognosis.

According to the 2001 American Association for Clinical Chemistry, Carcinoembryonic antigen (CEA) was first described in 1965 by Gold and Freedman, when they identified an antigen that was present in both fetal colon and colon adenocarcinoma but that appeared to be absent from healthy 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 tumors were on average 60-fold higher than in the nonmalignant tissues, Boucher *et al.,* (1989). In one of the first reports on CEA in serum. Thomson *et al.,* (1991) 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 no gastrointestinal cancers, or in patients with miscellaneous benign gastrointestinal diseases. Although these findings were not confirmed, they nevertheless prompted widespread use of carcinoembryonic antigen as a marker for colorectal cancer. Thirty years after its initial detection in serum, carcinoembryonic antigen is one of the most widely used tumor markers worldwide and certainly the most frequently used marker in colorectal cancer.

2.5.5 Carcinoembryonic Antigen as a Marker for Colorectal Cancer screening

In screening for colorectal cancer, the aim should be to detect disease at either Dukes' A or B stage. Malignancy detected at more advanced stages is unlikely to be more treatable than that detected through the usual course of events. Using an upper limit of normal of 2.5 mg/L, (Fletcher *et al.*,1986) calculated that carcinoembryonic antigen has a sensitivity of 36% and a specificity of 87% in screening for Dukes' A and B colorectal cancer. These findings, combined with the low heterogeneity, which appears to be attributable to variations in its carbohydrate side chains, Thomas *et al.*, (1990). Most of the carbohydrate is composed of mannose, galactose, *N*acetylglucosamine, fucose, and sialic acid, Thomas *et al.*, (1990).

Carcinoembryonic Antigen is a member of the immunoglobulin superfamily. Two types of immunoglobulin domains are found: an N-terminal domain of 108 amino acids homologous to the immunoglobulin variable domain (IgV-like) and six domains homologous to the immunoglobulin constant domain of the C-2 set (IgC2-like), Thompson *et al*., (1991; Hammerstron *et al.*, (1999).

Carcinoembryonic antigen is attached to the cell membrane by a glycosyl phosphatidylinositol anchor and probably is released as a soluble form by a phospholipase C or phospholipase D, Thompson *et al.*, (1991). Structural similarity of CEA to certain immunoglobulin-related proteins, such as ICAM-1 and ICAM-2, initially suggested that CEA might act as an adhesion molecule. In vitro experiments showed that CEA was capable of both homophilic (CEA binding to CEA) and heterophilic (CEA binding to non-CEA molecules) interactions. 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 *et al.*, (1989). Evidence for a role in cancer dissemination was obtained recently by Hostetter *et al.*, (1990), who showed that after transplantation of colorectal tumors into nude mice, the number of liver metastases increased from 2% to 48% following injection of mice with CEA. There is, however, no direct evidence that CEA is causally involved in cancer dissemination.

Although *in vitro* data implicate CEA in cell adhesions, its localization to the apical surface of mature enterocytes in healthy human colon is difficult to reconcile with this role. In the healthy colon, CEA has been found to bind certain strains of *Escherichia coli*. According to Thompson *et al.*, (1991), this binding may facilitate bacterial colonization of the intestine. On the other hand, suggested that CEA may play a role in protecting the colon from microbial infection, possibly by binding and trapping infectious microorganisms.

2.6 Immunohistochemistry

Immunohistochemistry (IHC), or immunocytochemistry, is a method for localizing specific antigens in tissues or cells based on antigen-antibody recognition; it seeks to exploit the specificity provided by the binding of an antibody with its antigen at a light microscopic level. IHC has a long history, extending more than half a century from 1940, when Coons developed an immunofluorescence technique to detect corresponding antigens in frozen tissue sections. However, only since the early 1990s has the method found general application in surgical pathology, Gown (2002). A series of technical developments led eventually to the wide range of IHC applications in use today. The enzymatic label (horseradish peroxidase), developed by Avrameas and by Nakane and colleagues (1966), allowed visualization of the labeled antibody by light microscopy in the presence of a suitable colorogenic substrate system. In Oxford, Taylor and Burns developed the first successful demonstration of antigens in routinely processed formalin-fixed paraffin-embedded (FFPE) tissues, Coppola *et al.*, (2011*).* A critical issue in the early development of immunoperoxidase techniques was related to the need to achieve greater sensitivity. Greater sensitivity would facilitate staining of FFPE tissues—from a simple one-step direct conjugate method to multiple-step detection techniques such as the peroxidase antiperoxidase (PAP), avidin-biotin conjugate (ABC), and biotinstreptavidin (B-SA) methods—and would eventually lead to amplification methods (such as tyramide) and highly sensitive " polymer-based" labeling systems, Ager *et al.*, (2015).

2.6.1 Basic Principles of Immunohistochemistry

Surgical pathologists have long recognized their fallibility, although they have not always publicized it, Gown (2002). They have, however, sought more certain means of validating morphologic judgments. A variety of "special stains" were developed to facilitate cell recognition and diagnosis; most of these early stains were based on chemical reactions of cell and tissue components in frozen sections (histochemistry). In certain circumstances, these histochemical stains proved to be of critical value in specific cell identification. More often, they served merely to highlight or emphasize cellular or histologic features that supported a particular interpretation without providing truly specific confirmation. When the new field of immunohistochemistry was created by combining immunology with histochemistry, a wide variety of truly specific special stains were generated. The aims of IHC are akin to those of histochemistry. Indeed, IHC builds on the foundations of histochemistry; it does not replace histochemistry but rather serves as a valuable adjunct that greatly extends the variety of tissue components that can be demonstrated specifically within tissue sections or other cell preparations. As emphasized by pioneers in this field of functional morphology, “the object of all staining is to recognize microchemically the existence and distribution of substances which we have been made aware of macrochemically, Nakane *et al*, (1966).

The basic critical principle of IHC, as with any other special staining method, is a sharp visual localization of target components in the cell and tissue, based on a satisfactory signal-to-noise ratio. Amplifying the signal while reducing non-specific background staining (noise) has been a major strategy to achieve a satisfactory result that is useful in daily practice. After more than two decades, advances in IHC have provided a feasible approach to performing immunostaining on routinely processed tissues, such that this method is now “routine” for the performance of IHC “special stains” in surgical pathology laboratories using FFPE tissues. However, demands for improved reproducibility and for quantification have led to a growing recognition that IHC has the potential to be more than just a special stain. If properly controlled in all aspects of its performance, IHC can provide a tissue-based immunoassay with the reproducibility and quantitative characteristics of an ELISA (enzyme-linked immunosorbent assay) test, which not only detects the presence of an “analyte” (protein or antigen) but also provides an accurate and reliable measure of its relative or real amount.

2.6.2 Principles of Immunohistochemistry

Immunohistochemistry is been widely used in diagnosing carcinogenesis and cancer because it takes advantage of the principle of the antigen-antibody affinity reaction through its ability to identify and localize proteins of interest via detection with labeled conjugates Rekardson (2014). This antigens have corresponding antibodies produced by the body to form a complex compounds which are usually picked up by immunochemical dyes or stains giving a distinctive color (brownish color in the case of the MLH1 gene) known as “expression.

2.7 Animal Models in Colon Carcinogenesis

2.7.1 Rodent Colon Tumor Models (History and Overview)

The study of experimental colon carcinogenesis in rodents has had a remarkably long history, dating back almost 80 years. Perhaps the earliest published study of Lorenz *et al.* (1941) demonstrated tumorigenesis in the forestomach and intestine of mice following feeding with the polycyclic aromatic hydrocarbon, methylcholanthrene. Lisco *et al.* (1947) reported that feeding radioactive yttrium to rats induced a high proportion of colon tumors. Walpole *et al.* (1952) reported that white rats given injections of 4-aminodiphenyl and 3,2-dimethyl-4-aminodiphenyl developed colon tumors. However, the most commonly used model for sporadic colorectal cancer (CRC) takes advantage of the organotropism of the colon carcinogens, 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM). Identification of these carcinogens arose initially from an early population study of Guamanians in which hydrazines were reported to be possible colon carcinogens with the hydrazine source being consumed cycad flour, Laqueur *et al.*, (1963). When large quantities of cycad flour were fed to rats, adenocarcinomas in the colon arose in some of the animals. The carcinogen in cycad flour was subsequently found to be cycasin, a form of methylazoxymethanol (MAM), Laqueur *et al*., (1964). DMH, a metabolic precursor of MAM, was used in several early studies to induce tumors in rats, Thumherr *et al.*, (1975). Repetitive treatment with this methylating agent was reported to produce colon tumors in rodents that exhibit many of the pathological features associated with the human disease, Ward, (1974). Thus, DMH has provided cancer researchers with a reproducible experimental system for studying ‘sporadic’ (nonfamilial) forms of CRC, LaMont, (1978*)*. However, AOM offers advantages over DMH, including enhanced potency and greater stability in dosing solution, Neufert *et al*., (2007). Since then, several thousand studies using AOM have been published Toth *et al.,* (1976). We have used this mouse model extensively for 15 years and have compiled considerable data on sensitivity and lesion characteristics across a number of mouse lines, Flynn and Kralj-Hans *et al*., (2007).

The occurrence of spontaneously occurring cancers within the gastrointestinal tract of rodents is not as rare as might be expected. For example, Rowlatt *et al.*, (1969) described the occurrence of a wide range of tumors of the intestinal tract in aged C57BL/6 mice, although the majority of the lesions were located in the small intestine. Miwa *et al.* (1976) reported 28 cases of spontaneous colon tumors in male wistar rats. Vandenberghe *et al.* (1985) in a study of Campylobacter-like bacteria reported 17 naturally occurring primary endophytic adenonocarcinomas in the ascending colon of Wistar rats. An examination of animal husbandry also demonstrated the presence of adenocarcinomas in the ascending colon of ACI rats foster bred by an Osaka female rat, perhaps depending on milk factor and humidity, Miyamoto *et al*., (1991)*.* Newmark *et al.,* (2001) found that maintaining mice on a semipurified diet, referred to as the new Western diet, designed to recapitulate a human Western diet, was capable of inducing colon tumors in 42% of 18-month-old C57BL/6 mice. Richie *et al*., (2007) showed an induction of colon tumors in p53 knockout mice by causing glutathione depletion with buthionine sulfoximine treatment.

In summary, there are a number of advantages to studying the pathogenesis of carcinogen-induced colon cancer in rodent models. The models are highly reproducible, they can be readily tested on animals with different genetic backgrounds and the pathogenesis recapitulates human CRC, at least at the early stages. Historically, the majority of colon carcinogenesis studies have been carried out in rats, Madera *et al.* (1983). However, the high frequency of tumors generated within the distal colon of mice, as well as the histogenesis of multiple adenomas with subsequent development of adenocarcinomas, also validate the importance of this species for studying the pathogenesis of colon cancer, Nambiar *et al*., (2003). An advantage of murine models is the availability of extensive genetic information on individual mouse lines, the existence of recombinant inbred mouse panels and the ever-increasing number of transgenic, knockout and knock-in genetic models that are available for study.

2.8 Role of MNU in Colon Carcinogenesis Studies

N-methyl-N-nitrosourea (MNU), are among the primary causes of cancer. Verma *et al.*, (2013) MNU an alkylating agent was reported to induce retinal degeneration in mice, rats and other animals following systemic administration, providing a simple model of retinal degenerative diseases, Neufert *et al.*, (*2007).* It exhibits its toxicity by transferring its methyl group to nucleo bases in nucleic acids which can lead to AT: GC transition mutation, Swanson *et al.* (1996). The molecular mechanism of MNU involves specific G-35 point mutation in codon 12 which results in substitution of normal glycine with an aspartic acid, Saminathan *et al*., (2014). A clear example of MNU toxicity is seen when cells are exposed to the carcinogen that leading to inactivation of genes required for repair of base-base mismatches in DNA, referred to as mismatch repair (MMR) genes (MLH1, MLH2, etc). This damaged or deactivated genes then lacks the ability to repair DNA mutation which then accumulates and prompts cells to replicate uncontrollably, This MMR deficiency can be detected during Immunohistochemical analysis. Exposure to MNU can result in skin and eye irritation headache, nausea, and vomiting. MNU is reasonably anticipated to be a carcinogen based on sufficient evidence of carcinogenicity in experimental animals.

Various cancers which were successfully induced in animal models include: squamous cell carcinomas of the fore-stomach, sarcomas and gliomas of the brain, adenocarcinomas of the pancreas, mammary carcinomas, leukemia, and lymphomas, Perse *et al*., (2009).

2.9 Haematological Parameters in Cancer/Colon Carcinogenesis and their Application as Possible Cancer Biomarker.

Haematology refers to the study of the 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, Merck Manual, (2012). Haemtological studies are useful in the diagnosis of many diseases as well as investigation of the extent of damage to blood, Onyeyili *et al.* (1992)*;* Togun *et al.,* (2007). Haematological studies are of ecological and physiological interest in helping to understand the relationship of blood characteristics to the environment, Ovuru and Ekweozor (2004) and so could be useful in the selection of animals that are genetically resistant to certain diseases and environmental conditions, Mmereole (2008); Isaac *et al.* (2013). Haematological parameters are good indicators of the physiological status of animals, Khan and Zafar, (2005). Haematological parameters are those parameters that are related to the blood and blood forming organs, Waugh *et al.* (2001)*;* Bamishaiye *et al*. (2009). Blood act as a pathological reflector of the status of exposed animals to toxicant and other conditions, Olafedehan *et al.,* (2010). As reported by Isaac *et al.* (2013), animals with good blood composition are likely to show good performance.

Laboratory tests on the blood are vital tools that help detect any deviation from normal in the animal or human body, Ogunbajo *et al.* (2009). The examination of blood gives the opportunity to investigate the presence of several metabolites and other constituents in the body of animals and it plays a vital role in the physiological, nutrition and pathological status of an organism, Aderemi (2004); Doyle (2006). According to Olafedehan *et al.* (2010), examining blood for their constituents can provide important information for the diagnosis and prognosis of diseases in animals. Blood constituents change in relation to the physiological conditions of health (Togun *et al.,* 2007). These changes are of value in assessing response of animals to various physiological situations, Khan and Zafar (2005). According to Afolabi (2010), changes in haematological parameters are often used to determine various status of the body and to determine stresses due to environmental, nutritional and/or pathological factors.

2.9.1 Haematological Components and their Functions

Blood which is a vital special circulatory tissue is composed of cells suspended in a fluid intercellular substance (plasma) with the major function of maintaining homeostasis Soetan *et al.,* (2013). Haematological components, which consist of red blood cells, white blood cells or leucocytes, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration are valuable in monitoring feed toxicity especially with feed constituents that affect the blood as well as the health status of farm animals, Oyawoye and Ogunkunle (2004). Red blood cells (erythrocytes) serve as a carrier of haemoglobin. It is this haemoglobin that reacts with oxygen carried in the blood to form oxyhaemoglobin during respiration Chineke *et al.*, (2006). According to Soetan *et al.*, (2013) red blood cell is involved in the transport of oxygen and carbon dioxide in the body. Thus, a reduced red blood cell count implies a reduction in the level of oxygen that would be carried to the tissues as well as the level of carbon dioxide returned to the lungs, Ugwuene (2011); Soetan *et al.*, (2013).

The major functions of the white blood cell and its differentials are to fight infections, defend the body by phagocytocis against invasion by foreign organisms and to produce or at least transport and distribute antibodies in immune response. Thus, animals with low white blood cells are exposed to high risk of disease infection, while those with high counts are capable of generating antibodies in the process of phagocytocis and have high degree of resistance to diseases, Soetan *et al.*, (2013) and enhance adaptability to local environmental and disease prevalent conditions, Kabir *et al.*, (2011); Okunlola *et al.* (2012).

Blood platelets are implicated in blood clotting. Low platelet concentration suggests that the process of clot-formation (blood clotting) will be prolonged resulting in excessive loss of blood in the case of injury. Packed Cell Volume (PCV) which is also known as haematocrit (Ht or Hct) or erythrocyte volume fraction (EVF), is the percentage (%) of red blood cells in blood (Purves *et al.* 2003). According to Soetan *et al.* (2013) packed cell volume is involved in the transport of oxygen and absorbed nutrients. Increased packed cell volume shows a better transportation and thus results in an increased primary and secondary polycythemia. Haemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates, Maton *et al.*, (1993) with the exception of the fish family, channichthyldae, Sidell and Brien, (2006) as well as tissues of invertebrates. Haemoglobin has the physiological function of transporting oxygen to tissues of the animal for oxidation of ingested food so as to release energy for the other body functions as well as transport carbon dioxide out of the body of animals, Ugwuene (2011).

2.10 Oxidative Stress and Cancer / Colon Carcinogenesis.

Oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the oxidants potentially leading to damage has been suggested to be that which cause aging and various diseases in humans. In modern western medicine, the balance between anti-oxidation and oxidation is believed to be a critical concept of maintaining a healthy biological system. Oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, cancer, Parkinson's disease, immune dysfunction and ageing, Souri *et al.*, (2008). Antioxidants can be effective in the prevention of free radical formation by scavenging or promotion of their decomposition and thus, suppress such disorders. Hence, there is growing interest in natural antioxidants from herbal and food plant sources, Atawodi (2010); Ghanbari *et al.,* (2012) as epidemiological and laboratory studies have strongly supported their therapeutic and protective efficacies in biological systems, Souri *et al.,* (2008); Atawodi (2011). Besides, vegetables, spices and herbs are rich in natural antioxidants that protects against oxidative stress and thus play important roles in the therapy and chemoprevention of diseases that have their etiology and pathophysiology in reactive oxygen species, Atawodi *et al.,* (2010); Manzoor *et al.,* (2012).

Oxidative DNA damage is a major source of the mutation load in living organisms, with more than one hundred oxidative DNA adducts (purine, pyrimidine, and the deoxyribose backbone) having been identified, Lu *et al.,* (2001); von Sonntag (1987); Dizdaroglu (1992); Demple and Harrison (1994). The estimated frequency of oxidative DNA damage in human cells is 104 lesions/cell/day, Fraga et al. (1990); Lu *et al.,* (2001). Being highly reactive, the hydroxyl radical is the predominant ROS that targets DNA, Lu *et al.,* (2001). Hydrogen peroxide, a precursor to hydroxyl radical, is less reactive and more readily diffusible and thus more likely to be involved in the formation of oxidized bases through Fenton and Haber-Weiss reactions, Guyton and Kensler (1993); Barber and Harris (1994). ROS induced DNA damage can result in single- or double-strand breakage, base modifications, deoxyribose modification, and DNA cross-linking. Cell death, DNA mutation replication errors, and genomic instability can occur if the oxidative DNA damage is not repaired prior to DNA replication, Marnett (2000); J. P. Cooke (2003); Klaunig and Kamendulis (2004); Valko *et al.,* (2006).

In traditional Chinese medicine for more than 2000 years, a general recommendation to their consumption is to increase the intake of foods rich in antioxidant compounds (e,g polyphenols, carotenoids) due to their well-known healthy effects. As a consequence, these evidences accelerated the search for antioxidants principles, which led to the identification of natural resources and isolation of active antioxidant molecules. Many plants have been identified as having potential antioxidant activities and their consumption recommended, Kitts, *et al.,* (2000); Lee *et al.,* (2003).

Bioactive phenols are very interesting as antioxidants because of their ability to act as efficient free radical Abbreviations: BHT, Butylated hydroxytoluene; DPPH, 1,1- diphenyl-2-picrilhydrazyl; AML, acute myeloid leukemia, Langley-Evans, (2000). In the last two decades the number of publications on the potential health benefits of polyphenols, have increased enormously, Tiwari, (2001); Katalinic *et al.,* (2006).

2.11 Antioxidants

Antioxidants are believed to play a very important role in the body defense system against ROS Boxin et al. (2002), Vivek and Surendra (2006). In another term antioxidant is “any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate, Halliwell and Gutteridge (1995). Halliwell (2007) reported that an antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule. Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers, and helps in converting the radicals to less reactive species. A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc

Natural products of plant origin are major source of vitamin C, vitamin E, and beta –carotene and have been proposed as potential source of natural antioxidants with strong activity (Block *et al.,* 1992). This activity is mainly due to the presence of phenolic compounds such as flavonoids, phenols, flavonols and proanthocyanidins, Bose *et al.,* (2012). Antioxidants are chemical compounds which contain monohydroxy/polyhydroxy phenol; they just work to slow down the lipid peroxidation, German *et al.,* (2013). These compounds have low activation energy to donate hydrogen atom and therefore, cannot initiate the second free radicals. The free radical electrons are stable and thus, slow down the oxidation. Cells contain many antioxidant systems to prevent injury. Prevention of excessive ROS and repair of cellular damage is essential for cell's life, Guttridge *et al.,* (2011).

Polyphenols are the major plant compounds with high level of antioxidant activity. This activity could be due to their ability to absorb, neutralize and to quench free radicals, Bose *et al.,* (2012). Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation, Bose *et al.,* (2012). Antioxidant is one of the most essential ingredients of today's therapy since they reduce in vivo oxidative damages. Plants are the good resources for natural antioxidants. DPPH radicals scavenging method is widely used to investigate the total antioxidant activities in plants. Antioxidant substances block the action of free radicals which have been implicated in the pathogenesis of many diseases including atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease and in the aging process.

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced cell damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase (SOD) – metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity, Anuj *et al.,* (2016).

Superoxide dismutase (SODs) are a group of key enzymes functioning as the first line of antioxidant defense by virtue of the ability to convert highly reactive superoxide radicals (dismutation) into hydrogen peroxide and molecular oxygen, Wang *et al.,* (2016).

Catalase is a tetrameric porphyrin-containing enzyme that is located mainly in peroxisomes. It catalyzes the conversion of H2O2 to water and molecular oxygen in two steps, Valko *et al.,* (2006); Chaudiere *et al.,* (1999), and Aslani *et al.,* (2016).

Catalase-Fe (III) + H2O2→compound I

Compound I + H2O2→catalase-Fe (III) + 2H2O + O2

Ligand induced ROS is generated in nonphagocytic cells, Fanburg *et al.,* (2015). NADPH oxidase is activated in vascular smooth muscle cells by Angiotensin II and 5-HT neurotransmitter. This in turn, generates hydrogen peroxides and superoxide anions. ROS activated by Ang-II stimulate the p38 Mitogen Activated Protein Kinase (MAPK) pathway and cause cell hypertrophy. ROS activated by 5-HT stimulate ERK (extra cellular signal regulated kinase), MAPK pathway and cause cell proliferation.

2.1.2 Inflammation

Inflammation is a protective strategy evolved in higher organisms in response to detrimental insults such as microbial infection, tissue injury and other noxious conditions. It is an essential immune response by the host that enables the removal of harmful stimuli as well as the healing of damaged tissue. Acute inflammation has therefore been considered as a part of innate immunity, the first line of host defense against foreign invaders and danger molecules. Mankind has known the classical symptoms of inflammation for hundreds of years, which include redness, pain, swelling and heat, Medzhito**v** (2008).

As a protective strategy for the host, one of the main aims of inflammation is to reinstate cellular homeostasis in response to any damaging condition. The mechanism underlying the initiation of inflammation is therefore tightly connected to the physiological state of homeostasis. And so, inflammation is considered as an 'adaptive response' to any harmful effect threatening the integrity of the cellular homeostasis. It is quite plausible to understand that such an adaptive response operates at the expense of normal cellular functions, Medzhitov (2010). As a result, the longer this response persists in, the host will encounter the more damaging consequences. In contrary to its beneficial role as a safeguard for cellular physiology, the inflammatory response is required to be least enduring in order to avoid any escalation of its unfavorable circumstances.

2.1.2.1 Molecular mechanism underlying inflammation

Inflammatory stimuli are first recognized by the host cells through specific transmembrane receptors, called pattern recognition receptors (PRRs), which are expressed by cells of both innate and adaptive immune systems. PRRs are germline-encoded receptors, which are responsible for sensing the presence of infecting microorganisms as well as the incidence of any cellular damage (Akira et al., 2006). They do so by recognizing structures conserved in microbes, called pathogen-associated molecular patterns (PAMPs), as well as endogenous molecules derived from internal injuries, called danger-associated molecular patterns (DAMPs). To date, a number of PRRs have been identified with the selective ability to detect PAMPs, DAMPs or both and these include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-1-like receptors (RLRs) and NOD-like receptors (NLRs). The interactions of these receptors with the appropriate stimuli result in transmitting signals to nucleus where the activation of a selective set of genes takes place via both transcriptional and posttranscriptional mechanisms, Akira *et al.*, (2006); Medzhitov (2007).

2.1.2.2 Inflammation and cancer

Over the last decade it has become evident that inflammation plays a critical role in promoting cancer, in particular the tumorigenesis, a process of tumor formation. In addition to cancer cells, various types of immune cells are commonly found within tumors. Interestingly, an inflammatory microenvironment is also more frequently found as an essential part of all tumors, Mantovanietal (2008); de Visseretal (2006). It has been demonstrated that the inflammatory response triggered by infection is also associated with increased cancer risk, de Martel and Franceschi (2009). A recent study has revealed that lung tumorigenesis caused by tobacco smoke is in fact initiated by IKKbeta and JNK1-mediated chronic inflammation, suggesting that the pathways of both tumorigenesis and inflammation are closely linked, Takahashietal (2010). It is believed that apart from the tumor-promoting inflammatory response, an active anti-tumor immunity is also present in most tumor microenvironments. It is thus suggested that the progression of tumorigenesis is dictated by the battle between tumor-promoting inflammation and anti-tumor immunity. Obviously, in established tumors, antitumor immunity is profoundly dominated by tumor-promoting inflammation, Smyth *et al.,* (2006); Lin and Karin (2007). Studies show that inflammatory microenvironments not only promote cancer cell growth but also increase mutation rates, possibly by producing reactive oxygen species and nitrogen intermediates which can cause DNA damage and genomic instability, Grivennikov *et al.,* (2010). The role of inflammation in genomic instability is supported by the observation that activation-induced cytidine deaminase (AID), an enzyme which triggers genomic instability and is usually overexpressed in many cancers, is induced by inflammatory cytokines Okazaki *et al.,* (2007). Apart from genomic instability, environmental factors such as carcinogens, infectious microbes, tobacco smoke and inhaled pollutants have been considered to play critical roles in inflammation induced cancers¸ Aggarwal *et al.,* (2009). Therefore, not all chronic inflammatory diseases are connected to cancer risk. For example, rheumatoid arthritis does not promote cancer whereas inflammatory bowel disease and chronic hepatitis do due to the exposure to dietary and environmental carcinogens. The connection between inflammation and cancer does not operate in one direction only as numerous studies showed that DNA damage can also lead to inflammation. Using the model of hepatocellular carcinoma induced by the carcinogen diethyl nitrosamine (DEN), several reports demonstrated that the DNA damage-induced necrotic cell death lead to inflammation, Maeda *et al.,* (2005); Sakurai *et al.,* (2008). Cancer-associated oncoproteins such as Ras, Myc and RET can also lead to inflammation by activating signaling pathways involved in the production of pro inflammatory cytokines and chemokines, Mantovani *et al.,* (2008). Last of all, tumor-associated inflammation can also be instigated by the modern cancer therapy itself such as radiotherapy and chemotherapy. These therapies are usually associated with a substantial amount of necrotic death of not only cancer cells but also surrounding normal cells, which in turn activates the inflammatory response, Zong and Thompson (2006). Therefore, based on the literature, it can be concluded that inflammatory response is an integrated part of cancer biology, right from the beginning of tumorigenesis toward the application of therapeutic interventions.

2.12 *Cucurbita Species*

2.12.1 Description of plant:

Taxonomical Position of *Cucurbita maximus*

Division:	Spermatophyta
Sub-Division:	Angiospermae
Class:	Dicotyledonae
Sub-Class:	Polypetalae
Series:	Caliciflorae
Order:	Passiflorales
Family:	Cucurbitaceae
Genus:	*Cucurbita*
Species:	*maximus* **Source:** International Journal of Phytopharmacy

Pumpkin leaves are orbicular in outline and not pointed or much lobed, with deep sinus at base. In general, pumpkin stems are more rigid, prickly, and angular (with an approximate five-degree angle) than squash stems, which are generally softer, more rounded, and more flared where joined to the fruit.

The pumpkin varies greatly in shape, ranging from oblate to oblong. The rind is smooth and usually lightly ribbed. Although pumpkins are usually orange or yellow, some fruits are dark green, pale green, orange-yellow, white, red and gray. Leaves are nearly orbicular in outline and not pointed or much lobed but with deep sinus at base. Corolla prevailingly with soft abtuse more or less crinkly revolute or hanging lobes, the tube with parallel sides or bulging at base, peduncle short, spongy, nearly cylindrical, not expanded at attachment to fruit, (Lirtika *et al.*,2011).

Plate 2.1: Picture of the fruit sizes, shapes and color for *Cucurbita maxima* which were collected from Ochi Ogbonicha in Ofu LGA Kogi State, Nigeria.

Cucurbita maxima is a guard like squash belonging to the genus *cucurbita* and the family curcubitaceae. Other species in this genus include *Curcubita mixta*, *Curcubita pepo* and

Curcubita moschota Singh (2012). It is known by various names: for instance, it is known as Giant pumpkin or winter squash in English, Echi in Igala Language, Kabewa in Hausa, ebeshe in Nupe, Ogbokolo, Okoro, Anya in Igbo and Elegede, Isi, in Yoruba. It is collectively ranked among the 10 leading vegetable crops worldwide. China and India are the world leading producers. Other major producers are U.S.A, Egypt, Mexico, Ukraine, Cuba, Italy, Iran and Turkey (FAOSTAT, 2007). Squashes (*Cucurbita* spp.) are members of the economically important *Cucurbitaceae*. There are three economically important *Cucurbita* species, namely *Cucurbita pepo* L., *Cucurbita maxima* Duch and *Cucurbita moschata* Duch, which have different climatic adaptations and are widely distributed in agricultural regions worldwide, Paris and Brown (2011); Wu *et al.,* (2007).

2.13 Origin and Distribution

The *Cucurbita* genus is of American origin. *Cucurbita maxima* are the most diverse *Cucurbita* species after *Cucurbita pepo* and are found throughout tropical and temperate regions. Phenotypic diversity within populations of *Cucurbita* is high and includes variation in shape, size and colour of fruits; number and size of seeds; quality, color and thickness of fruit flesh and precocity in fruit production, among other traits, Whitaker and Robinson, (1986); Hernandez *et al*., (2012). The centre of diversity, *Cucurbita maxima*, lies in South American temperate zones, where landraces exhibit an array of interesting traits. Many landraces of this species are also found in North America, Australia and different countries of Africa **(**Zambia and Nigeria**)**; Asia (China, India, and Iran) and Europe (Spain and Turkey), Ferriol and Pico (2008). Turkey is one of the important diversity centers for cultivated cucurbits because of their adaptation to diverse ecological conditions as a result of natural selection and also farmers' selections, Sari *et al*., (2008). Pumpkins are native to North and Central America, Whitaker (1975), where some

Cucurbita varieties were cultivated before Europeans reached that area. Nowadays, pumpkins are cultivated in almost all areas with an appropriate climate in the world.

2.14 Culinary Uses

The fruits are the most valuable part of *Cucurbita* plants; some of them own a high nutritional value and a high level of carotenoids and for this reason, in many countries, *Cucurbita* fruits are important sources of provitamin A in human nutrition, being consumed usually boiled or baked, according to the local traditions, Muntean *et al.,* (2013). Almost all parts of *Cucurbita maxima* plants are edible and healthful. The fruits (pumpkins or squashes) are well known; they are large, weighing 8 to 10 kg, sometimes up to 20 kg. The orange and yellow color of their flesh is related with the contained carotenoids – phytochemicals that may reduce risk of some cancers, heart attacks and some aging processes, Muntean *et al.,* (2013). The flowers are also loaded with carotenoids and can be eaten raw in salads or cooked. The leaves contain lot of iron and other minerals and vitamins; smaller new leaves are good ingredients in salads, while stems can be chopped and boiled with other vegetables, providing fibers. The seeds can be dried and eaten raw or baked; they contain valuable nutrients oils, proteins and vitamins, Muntean *et al.,* (2013).

2.15 Medicinal Uses

Pumpkin is a creeper and is extensively found very frequently on the roofs of houses all over India. The parts which are used are as follows, seed, pulp, and fruit stalk. The fruits of pumpkin are large weighing 8 to 10 kg, sometimes up to 20kg.the medium sized fruit of “Arka Chandan”, an old variety from Indian institute of horticulture research, Hessaraghatta, weighs 2 to 3 kg and has orange color flesh.

Pumpkin seeds contain many valuable functional components and have been traditionally used for herbal, therapeutic as well as clinical application. Pumpkin seeds have been used as safe deworming and diuretic agents, and the seed oil as a nervine tonic, Das *et al.,* (2010). Pumpkin seed oil has a strong antioxidant property, and has been recognized for several health benefits such as prevention of the growth and reduction of the size of prostate, reduction of bladder and urethral pressure and improving bladder compliance, alleviation of diabetes by promoting hypoglycemic activity, and lowering level of gastric, breast, lung, and colorectal cancer. Pumpkin is cultivated throughout India and in most warm region of the world, Attarde *et al*., (2010).

2.16 Chemical Composition

The chemical constituent such as 24 β ethyl 5 α cholesta-7, 22, 25- trien-3 β ol, 24 β-ethyl-5α-cholesta-7, 25-dien-3 β ol, avenasterol, spinasterol, 24 dihydrospinasterol, 24-methyl lathosterol and 25-dehydrofungisterol has been obtained from seeds, Das *et al.,* (2010). 25-dehydroporiferasterol, clerosterol, isofucosterol, stigmasterol, sitosterol, campesterol, and codisterol, isolated from seeds. 13 C-NMR of chondrillasterol studied, isolation of two new carotenoidscucubitaxanthins A and B and their structure elucidation.

Similar to other cucurbitaceous plant it contains saponin, fixed oil, resin, protein, sugar and starch. The fresh vegetable contains 89.50 % moisture and the completely dried material contains ethereal extract of albuminoids 6.12% (containing nitrogen 0.98%), soluble carbohydrates 77.33%, woody fifer 8.55%, and ash 7.00%. The seeds collected from Pakistan yields an oil (39.1%) having the following physic chemical constituents, the 3 fatty acid composition of oil is as follows, myristic, palmitic, stearic, oleic and linoleic acid 30.3%. seeds contains sterol glycosides and sterols fatty acid esters which showed antitumor activity in mouse.

The alcoholic and etheral extracts of pumpkin seeds were found to be active against the nematode Fasciolopis buski, in vitro and in vivo test systems, Kirtika *et al.*, (2009). The therapeutic efficacy of the seeds in the treatment of nematodiasis in calves below 6 month of age was found to be 40.6% by the oral route. Chemical composition of the leaves is as follows, moisture 85.9%, lipid 1.75%, protein 4.58% and ash 1.82%.

Composition of lipid portion is as follows, nonpolar lipids 45.1%, glycolipids 24.4%, and phospholipids 30.5%, Kirtika *et al.*, (2009). The major fatty acid present in the lipid was linolenic. According to Kirtika *et al.*, (2009). The leaves contain (mg/100g) calcium 36.38%, magnesium 38.80, iron 2.04%, zinc 0.76%, copper 0.42%. An esterase was purified from the fruits.

2.17 Known Pharmacological Activities

2.17.1 Anticancer Activity- Anticancer activity of methanol extract of *Cucurbita maxima* against Ehrlich as-cites carcinoma.

Cancer is a pathological state involving uncontrolled proliferation of tumor cells. The study was carried out to investigate the antitumor potential of MECM (methanol extract of *Cucurbita maximus*) against EAC (Ehrlich Ascites Carcinoma) bearing mice. EAC is a very rapidly growing carcinoma with very aggressive behavior. It is able to grow in almost all strains of mice. The Ehrlich Ascitic tumor implantation induces *per se* a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascitic fluid formation and accumulation. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells. MECM treatment significantly reduced tumor volume probably by lowering the ascitic nutritional fluid volume. Further, the packed cell volume and the number of viable EAC tumor cells in peritoneum were significantly lower in the mice treated with MECM when compared to the tumor control group. These results could indicate either a direct cytotoxic effect of MECM on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition, Saha, *et al.*, (2011a).

2.17.2 Antiobesity Activity - Potentiative Activity Of Cucurbita Maxima Seed Extract With Beta Vulgaris & Smilax Regelii Root Extract To Reduce Extra Fats From The Body.

The seed extract of *Cucurbita maxima* is effective for reducing fats individually as well as its combination effort through different mechanism. *Cucurbita maxima* seed sample is rich of manganese having tendency to produce thyroxine, Das *et al.*, (2010).

2.17.3 Antidiabetic Activity – Antidiabetic activity of *Cucurbita maxima* aerial parts

Streptozocin, a glucose analogue is a potent diabetogenic agent and widely used for inducing diabetes in a variety of animals by the selective degeneration and necrosis of pancreatic cells, Saha *et al.*, (2011a). The study on the Antidiabetic activity of *Cucurbita maxiam* indicates that MECM significantly reduced the elevated fasting blood glucose level with to those of diabetic control animals, Saha, *et al*., (2011b).

2.17.4 Evaluation of Hepatoprotective Activity of *Cucurbita maxima* Aerial Parts.

The study on hepatoprotective activity of Cucurbita maxima was performed to investigate the hepatoprotective activity of methanol extract of *Cucurbita maxima Duchesne* (*Cucurbitaceae*) (MECM) against carbon-tetrachloride (CCl4) induced hepatotoxicity. Liver damage induced by CCl4 is a commonly widely used model for the screening of hepatoprotective drugs. CCl4 is biotransformed by the cytochrome P- 450 system to produce the trichloromethyl free radical (CCl3‡), and this further reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical (CCl3OO‡). This free radical in turn covalently binds to cell membranes and organelle to elicit lipid peroxidation, also disturbs Ca2+ homeostasis, and finally results in cell death. Estimating the activities of serum marker enzymes, like SGOT, SGPT and ALP can make assessment of liver function. When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol, are released in to the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage, Saha *et al.*, (2011e).

2.17.5 Diuretic Activity - Diuretic activity of seeds of *Cucurbita maxima* duchesne in wistar rats.

The seeds of *Cucurbita maxima* Duchesne are used traditionally as diuretics reported by Jose *et al.,* 2008. The concentration of Na+ and K+ in urine was determined by flame photometer. The volume of urine and Na+ and K+ concentration of test group was compared with the control group. The results revealed that the aqueous extract of seeds of *Cucurbita maxima* showed significant increase in urine volume when compared to control group. But the excretion of Na+ and K+ in urine was not significantly increased in drug treated group when compared to control group, Jose *et al*., (2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

The carcinogen, N-Methyl-N-Nitrosourea (MNU) was procured from BDH Chemical Ltd, Poole, England. Carcinoembryonic antigen assay kit was procured from Diagnostic Automation Inc. CA. Other chemicals used are formal-saline, graded alcohol, xylene, paraffin, eosin, hematoxylin, were obtained from the department of Histopathology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika. All other reagents and chemicals were of analytical grade.

3.1.2 Feeds Used

Grower mash feed produced by UAC livestock’s company was used in combination with the Powdered *Cucurbita maxima* fruit.

3.1.3 Animals used

A total of 42 Wister rats were used for the experiment

3.2 Apparatus and Equipment

Based on the experimental design, the equipment used are listed below:

Microplate reader (GM-3000, model 200711012; B. Bran Scientific Company England), pH meter (Jenway 3150 model, UK.),UV spectrophotometer (Jenway 6305 model, 50/60Hz, U.K.), face mask, cooler, blender, pH meter (Jenway 3150 model, UK.), dissecting set, centrifuge, homogenizer, syringe, cannula, microscope, hand glove, electrical weighing balance (GF-2000, A&D Instrument Ltd, Japan), water trough, cages, feeders, microtome machine, micropipette, dissecting tape, masking tape, pins, ice blocks, tissue papers, cotton wools, nylon bag, permanent marker, dissecting set.

3.2 Methods

3.3 Sample Collection and Preparation

Fresh fruits of *Cucurbita maxima* weighing between (1kg to 2.5kg) were collected from Ochi Ogbonicha village in Ofu LGA of Kogi State Nigeria and taken to the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, for identification, authentication and a voucher specimen was deposited with number 1077. The fruits were washed in clean water, cut to remove seeds and sliced into cubes of approximately 2 mm, and later dried under morning sun (8 am to 10 am) for approximately 7 days and pounded to fine texture. The powder was kept at room temperature in an air tight container till used, and away from sunlight.

3.4 Preparation of Experimental Diets

Grower mash feed produced by UAC Livestock Company was procured from Samaru major market, Zaria Kaduna State and was used in the overall experiment. The feed served as normal diet. The powdered *Cucurbita maxima* fruit was added to the normal feed and mixed evenly to produce dietary inclusion of 0%, 2.5%, 5% and 10%. The procedure for mixing the normal feed and powdered Cucurbita maxima fruit is done by addition of percentage of fruit powder to the normal feed with addition of little distilled water (by sprinkling) to form a complete dough.

3.5 Feeding and Acclimatization of Experimental Animals

A total of forty two (42) healthy male Wistar rats weighing 80-100g were purchased from the Animal Unit of the National Veterinary Research Institute (NVRI) Vom, Jos Plateau state, Nigeria. The animals were housed in standard cages with wooden shavings as their beddings at room temperature at the Department of Biochemistry Animal House, Ahmadu Bello University Zaria and allowed free access to water and fed standard diet *ad libitum.* The animals were given two weeks of acclimatization period.

3.6. Experimental Grouping / Design

At the end of the acclimatization period, all animals were weighed and randomly divided into six (6) groups of seven animals each (n=7). The experimental animals were fed according to the group's percentage of dietary inclusion or standard diet for six (6) weeks and subsequently given MNU for period of 12 weeks and or normal saline as described below. The groups are as follows:

Group A: Normal saline + standard feed.

Group B: MNU Control: MNU + Standard feed.

Group C: MNU + dietary inclusion of 2.5% *Cucurbita maxima* powder.

Group D: MNU + dietary inclusion of 5% *Cucurbita maxima* powder.

Group E: MNU + dietary inclusion of 10% *Cucurbita maxima* powder.

Group F: Dietary inclusion control normal saline + 10% *Cucurbita maxima* powder.

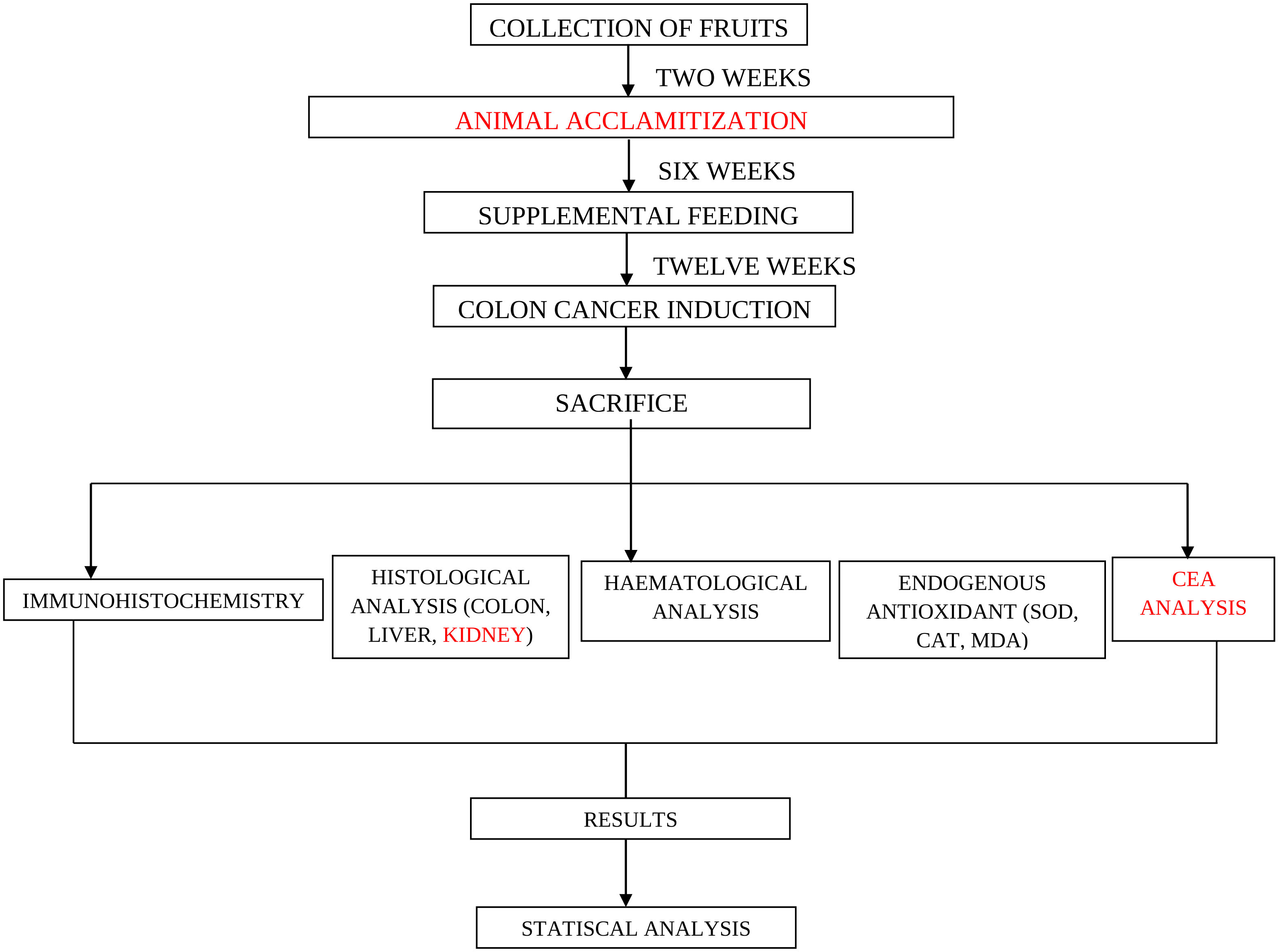


Figure 3.1: Experimental Design for the Dietary Inclusion for Prevention of Colon Carcinogenesis in Male Wistar Rats.

3.6 Induction of Colon Cancer

After six (6) weeks of supplemental feeding (inclusion), the rats in the experimental groups received an intra-rectal instillation of freshly prepared MNU solution (2.0mg) using a feeding tube with a cannula while the rats in the control group also received an intra-rectal instillation of freshly prepared normal saline (0.87%. Nacl).

3.7 Collection of Blood, Organs and Tissues

The animals were sacrificed by decapitation one week after the end of the twelve weeks of the last MNU administration and the blood was collected in heparinized test tubes. The tubes for serum samples were allowed to stand at room temperature for 30 minutes, the clot was removed by centrifugation at 2000 x g for 10 minutes, the supernatant was collected using Pasteur pipette into polypropylene tubes and stored at -20ºc for subesequent analysis. The animals were dissected and a portion of the colon, spleen, lung, liver, and kidney were removed and fixed quickly in 10% formalin for subsequent analyses.

The colon, liver and kidney (100 mg tissue/ml buffer) were crushed using mortar and pestle and homogenized in 50mM phosphate buffer (pH 7.4); the homogenate was centrifuged at 10,000 rpm for 10 minutes and the supernatant collected with Pasteur pipette which was used for analysis. The supernatant of each organ were collected separately in a clean label plastic tube and stored at -20ºc until required analysis.

3.8 Lipid peroxidation by measuring the malondialdehyde (MDA) level

The concentration of thiobarbituric acid reactive substances (TBARS) in the tissue homogenate was estimated by the method of, Fraga *et al*., (1988). The formation of malondialdehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation.

Exactly 1ml of the tissue homogenate was treated with 2.0 ml of thiobarbituric acid–trichloro acetic acid–hydrochloric acid reagent and mixed thoroughly. The mixture was incubated in a boiling water bath for 15 min, cooled and centrifuged at 3000rpm for 10 min. The absorbance of the supernatant were read at 535 nm against the reagent blank.

Malondialdehyde concentration was determined using the formula below:

$$\text{Concentration (nmol/ml)} = \frac{\textbf{Absorbance of the sample}}{\textbf{E x 1.0x10}^{-9}\textbf{ x1000}}$$

Where E is molar extinction coefficient = 1.56×10^5 nM/L

3.8.1 Estimation of Stress Status / Free Radicals Generation

3.8.2 Superoxide Dismutase (SOD) activity

The enzyme superoxide dismutase (SOD) decomposes superoxide anion into hydrogen peroxide and oxygen at a high reaction rate. SOD activity assay method is based on monitoring the auto-oxidation rate of haematoxylin as originally described by Martin *et al.*, (1987). In the presence of SOD enzyme, the rate of auto oxidation is inhibited and the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample.

Hematoxylin $\xrightarrow{\text{Auto-oxidation}}$ H_20+Hematin (active form of hematoxylin)

The procedure involved adding 920 μL of phosphate buffer (pH 7.8), which was added into clean test tube containing 40 μL of sample, mixed and incubated for 2 min at 25ºC, before 40 μL of hematoxylin was added, mixed quickly and the absorbance was measured at 560nm.

Superoxide dismutase Activity was calculated using the formula below:

$$\text{Unit of Activity (U/min/mg protein)} = \frac{\mathbf{R\ x100\ (100\text{-}R)}}{\mathbf{50\ x\ PC}}$$

Where R = $\underline{(Abs_B - Abs_S)}$, PC= Protein concentration, Abs_B = Absorbance of Blank,

Abs_S= Abs_BAbsorbance of Sample.

3.8.3 Catalase assay:

Catalase (CAT) activity was measured using the procedure reported by Abei (1984). Briefly, the method is as follows: 10 μL of serum was added to test tube containing 2.8 mL of 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 ml of fresh 30 mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240 nm for 5 min. on a spectrophotometer (Jenway 640 UV/Vis). A molar extinction coefficient of 0.0411 mM^{-l} cm^{-l} was used to calculate catalase activity, using the formula below:

$$\text{Unit of Activity (U/min/mg protein)} = \frac{\mathbf{Change\ in\ Absorbance}}{\mathbf{E\ x\ Abs_B\ x\ PC}}$$

Where A = Absorbance of Blank, E = Molar extinction coefficient PC= Protein concentration

The reaction was measured at 240 nm for 3 min and H_2O_2 consumption was calculated using extinction coefficient, 39.4 mM^{-l} cm^{-l}.

3.9 Haematological Analysis

The whole blood of sacrificed animals was collected into EDTA bottles and analyzed for haematological parameters such as hemoglobin concentration (Hb), white blood cell count (WBC), red blood cell count (RBC), packed cell volume (PCV), platelet (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC). The determination of hematological parameters was done within two hours of sample collection using Automated Hematological analyzer.

3.10 Histological Analysis of the Colon and other Organs

The colon tissue was rapidly fixed for 18-24 hours in 10 % formal saline and dehydrated in ascending grades of alcohol (70%, 80%, 90%, 95%, and 100 % respectively) for 2 hours each. The tissue was cleared in xylene and subsequently transferred through a pure paraffin in an oven for 1 hour and then into a second pot of melted paraffin for additional 2 hours (infiltration). The tissues were immersed in a mould containing molten paraffin wax, which is allowed to solidify (embedding) while the tissue is inside and ready for sectioning. Sections of 3 μm in thickness were prepared according to standard micro techniques onto glass slide and stained with hematoxylin and eosin. Photomicrographs under light microscope at high power magnifications (× 250) were examined for histopathological changes.

3.11 Carcinoembryonic Antigen Assay

The concentration of CEA was determined by enzyme-linked immunosorbent assay (ELISA) test which was based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a monoclonal antibody directed against a distinct antigenic determinant on the intact CEA molecule was used for solid phase immobilization (on the microtiter wells). A goat anti-CEA antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the CEA molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 1 hour incubation at room temperature, the wells were washed with water to remove unbound labelled antibodies. The color development was stopped with the addition of Stop Solution changing the color to yellow. The concentration of CEA is directly proportional to the color intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm.

3.12 Immunohistochemical Analysis

This analysis is based on the principle that the any damaged DNA mismatch repair gene(MLH1) by the MNU carcinogen present in the tissue samples will serve as an antigen that binds to the MLH1 antibody to form a complex. This complex subsequently produces a color that can be seen using microscope. The method used is the Avidin Biotin Complex (ABC) method also referred to as the avidin biotin Immunoperoxidase method, Gold *et al* (1965). 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 100^0C for 25 minutes. Peroxidases, protein and biotin blocks were done using hydrogen peroxide, avidine and biotin respectively. *MLH1* antibody was diluted (1.100) and incubated on sections at 60^0C for 60 minutes. These were followed by the biotylinated secondary antibody, streptavidine and DAB/substrate reaction as described by Goldstein *et al*., (2007); Wolff *et al*., (2007).

3.13 Statistical Analysis

Data was analyzed using SPSS version 20.0. The statistical significance between the control and other groups of experimental animals was determined by one-way ANOVA followed by Duncan test for multiple comparisons. Statistical test was performed at $p \leq 0.05$ level of significance, Day *et al* (1989). The results were set as Mean ± SD.

CHAPTER FOUR

4.0 RESULTS

Cucurbita maxima fruit inclusion on packed cell volume (PCV), neutrophil, lymphocyte revealed that the MNU control group showed a lower percentage level of packed cell volume (PCV) which was significantly ($p \leq 0.05$) different from the normal control group and the groups that received various percentage of dietary *Cucurbita maxima* fruit inclusion. The MNU control group showed higher percentage level of neutrophil and lymphocyte density which was significantly ($p \leq 0.05$) difference from the normal control group and the group on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively (Figure 4.1).

Effect of dietary *Cucurbita maxima* fruit inclusion on haemoglobin and platelet concentration in the blood serum of different experimental group revealed that the MNU control group showed lower level of Haemoglobin and Platelet concentration which was significantly ($p \leq 0.05$) different from the normal control group and the group on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively (Figure 4.2).

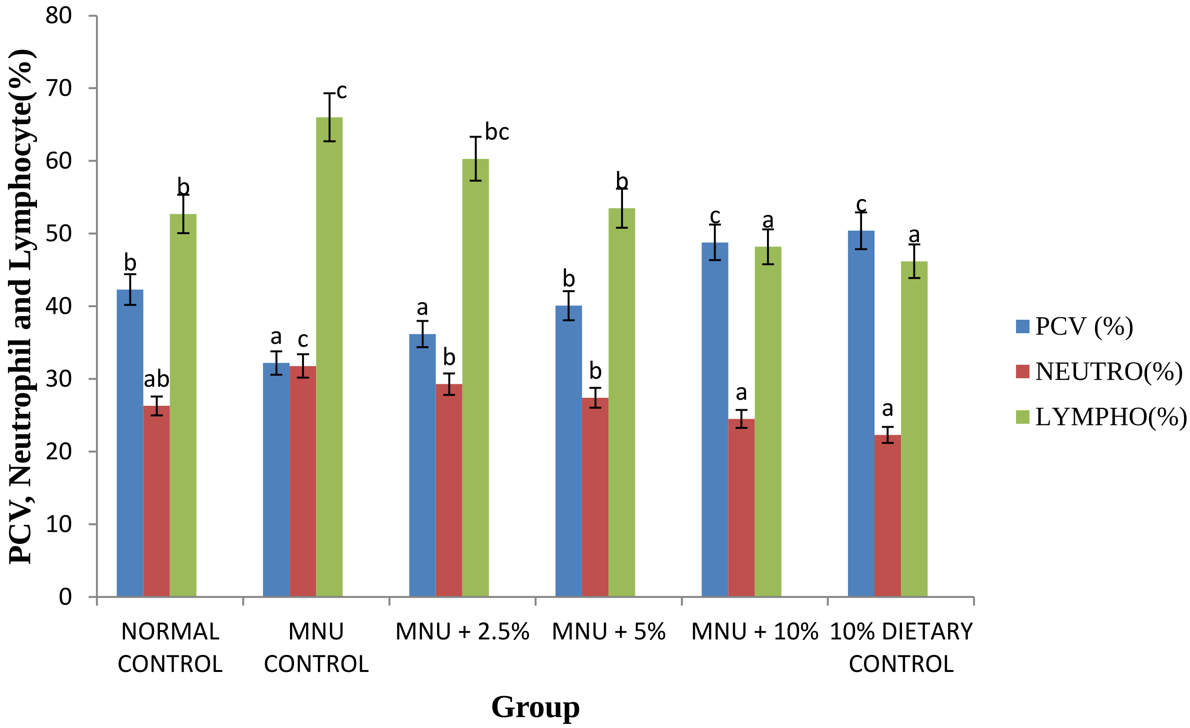


Figure 4.1: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Packed Cell Volume, Neutrophil and Lymphocyte Values following 12 Weeks MNU Administered in Different Experimental Groups. Values are Mean±SD. Values having different letters across the column are significantly (p<0.05) different

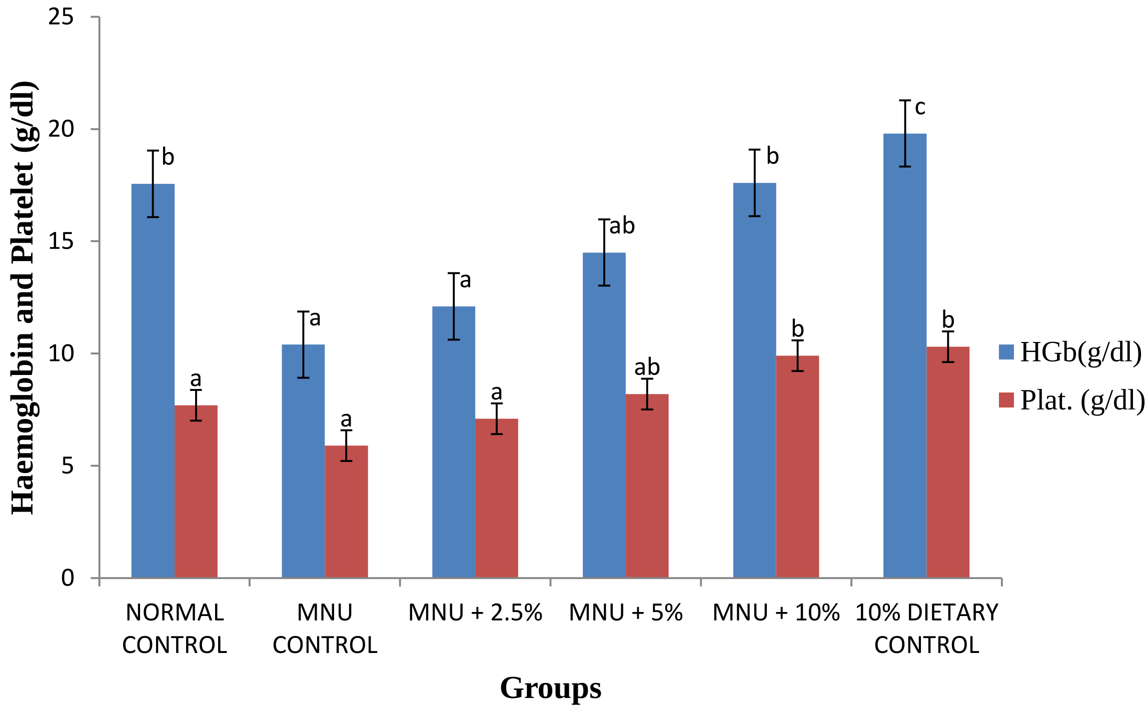


Figure 4.2: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Haemoglobin and Platelet Concentration following 12 Weeks MNU Administered in Different Experimental Groups. Values are Mean±SD

Effect of dietary *Cucurbita maxima* fruit inclusion on white blood cell and red blood cell counts in the blood of different experimental group revealed that the MNU control group showed higher level of white blood cell counts which was significant ($p \leq 0.05$) difference from the normal control group and the group that received various percentage of dietary *Cucurbita maxima* fruit inclusion. The MNU control group showed lower level of red blood cell counts which was significant ($p \leq 0.05$) difference from the normal control group and the group on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively (Figure 4.3)

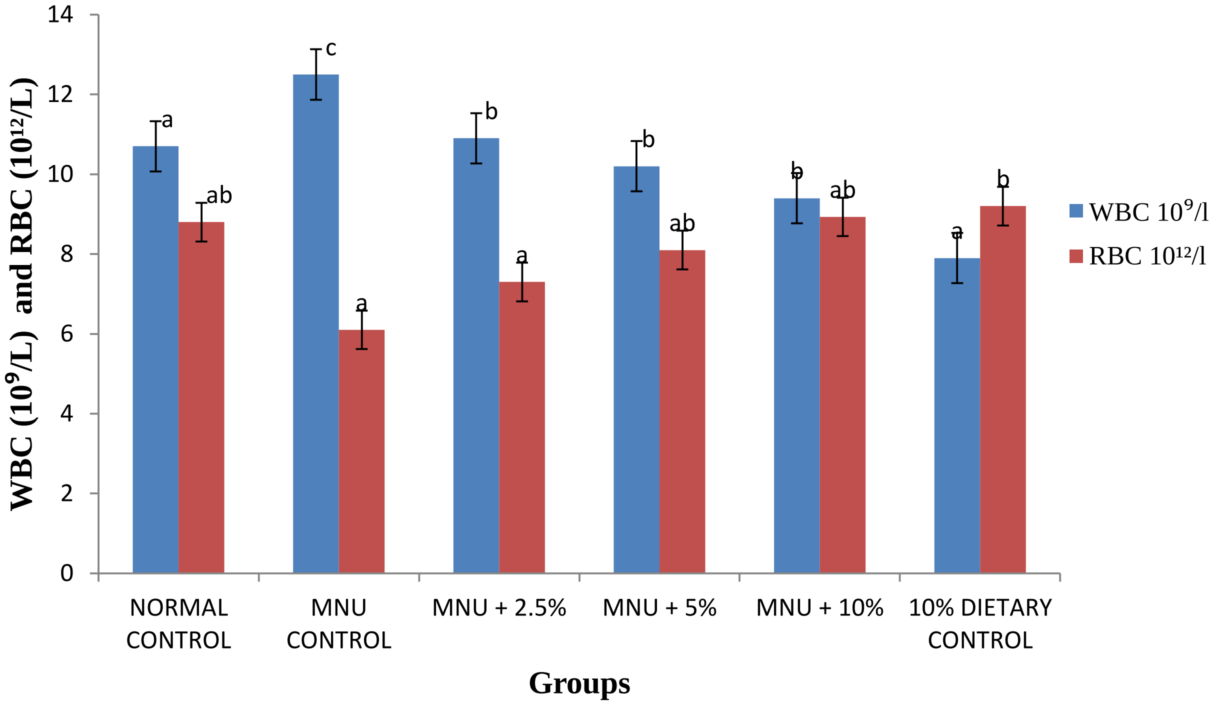


Figure 4.3: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on White Blood Cell and Red Blood Cell following 12 Weeks MNU Administered in Different Experimental Groups

Values are Mean±SD

Table 4.1 displays the result of Oxidative Stress-related Biochemical Parameters effects on Lipid Peroxidation- LPO. There is a significantly increase in MDA level of the MNU treated control group which was significantly ($p \leq 0.05$) higher than the normal control group and other groups on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively.

Assessment of the antioxidant enzyme catalase in the liver and colon homogenate, revealed a lower activity for MNU control group which was significant differences ($p \leq 0.05$) from the normal control group the other groups on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively (Table 4.2).

Assessment of the antioxidant enzyme Superoxide dismutase in the liver and colon homogenate, revealed a lower activity for MNU control group which was significant differences ($p \leq 0.05$) from the normal control group the other groups on different percentages of dietary *Cucurbita maxima* fruit inclusion (2.5%, 5% and 10%) in both colon and liver respectively (Table 4.3).

Table 4.1: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Melon aldehyde levels following 12 Weeks Intrarectal MNU Instillation

GROUP TREATMENT	MDA levels (nmol /mg protein)	
	Colon	liver
A Normal Control	113.50±0.9[c]	56.00±4.4[c]
B MNU Control	236.81±10.7[d]	96.97±1.5[d]
C MNU+2.5% of *C. maxima* fruit	116.73±1.1[c]	51.96±3.9[c]
D MNU+5% of *C. maxima* fruit	60.25±2.6[b]	29.59±3.2[b]
E MNU+10% of *C. maxima* fruit	28.59±1.22[a]	28.51±2.8[b]
F 10% Dietary Control	20.73±1.6[a]	16.69±1.4[a]

Values are Means±SD; Values having different letters down the column are significantly different ($p \leq 0.05$)

Table 4.2: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Catalase (CAT) Activity following 12 Weeks Intrarectal MNU Instillation

GROUP TREATMENT	CAT levels (U/mg protein)	
	Colon	**liver**
A Normal Control	**29.92±0.9[d]**	**54.57±7.59[c]**
B MNU Control	**11.50±1.8.0[a]**	**31.76±3.4[a]**
C MNU+2.5% of *C. maxima* fruit	**16.76±3.7[ab]**	**41.51±2.2[b]**
D MNU+5% of *C. maxima* fruit	**21.38±2.0[bc]**	**53.89±2.7[c]**
E MNU+10% of *C. maxima* fruit	**27.97±1.3[cd]**	**62.36±2.1[c]**
F 10% Dieatry Control	**37.63±10.3[e]**	**71.38±6.8[d]**

Values are Means±SD; Values having different letters down the column are significantly ($p \leq 0.05$) different

Table 4.3: Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Superoxide dismutase Activity following 12 Weeks Intrarectal MNU Instillation

GROUP TREATMENT	SOD levels (U/ml/mg protein)	
	Colon	liver
A Normal Control	264.37±1.8[c]	69.51±1.0[b]
B MNU Control	212.89±4.5[a]	68.44±6.4[a]
C MNU+2.5% of *C. maxima* fruit	240.05±7.2[b]	68.31±2.6[b]
D MNU+5% of *C. maxima* fruit	292.81±12.0[d]	73.78±4.2[b]
E MNU+10% of *C. maxima* fruit	316.67±3.7[e]	81.89±1.2[c]
F 10% Dietary Control	275.48±3.5[e]	88.21±0.8[d]

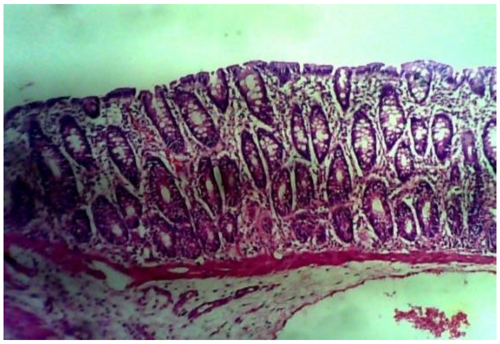
Values are Means±SD; Values having different letters down the column are significantly different ($p \leq 0.05$)

The Photomicrograph of histological section of the colon following 12 weeks MNU administration in rats showing essentially normal colon in the normal control group and the groups that are treated with 10% of dietary *Cucurbita maxima* fruit inclusion with MNU and normal saline respectively, but there is focal inflammation in the MNU control group and the groups treated with 2.5% and 5% dietary *Cucurbita maxima* fruit inclusion (Plate 4.1).

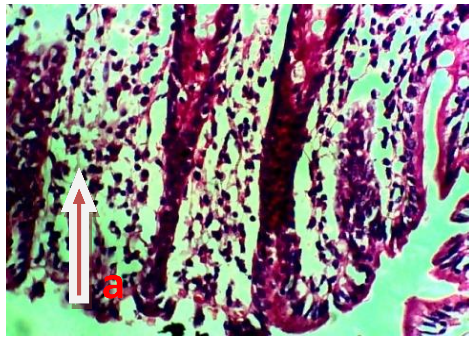
The Photomicrograph of histological section of the liver following 12 weeks of MNU administration in rats showed essentially normal liver in the normal control group and the groups that are treated with higher concentration of MNU (5% and 10%) of dietary *Cucurbita maxima* fruit inclusion with MNU and normal saline respectively, but there is focal inflammation in the MNU control group and the group treated with 2.5% dietary *Cucurbita maxima* fruit inclusion (Plate 4.2).

Effect of dietary *Cucurbita maxima* fruit inclusion on carcinoembryonic antigen (CEA) in the blood serum of different experimental group revealed that the MNU control group showed higher level of CEA concentration which was significantly ($p \leq 0.05$) different from the normal control group and the group that received 2.5%, 5% and 10% of dietary *Cucurbita maxima* fruit inclusion (Figure 4.4).

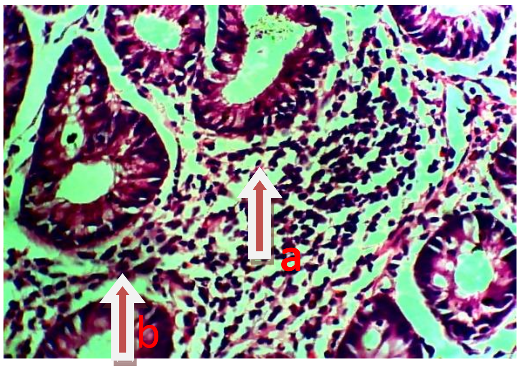
The Photomicrograph of immunohistochemistry section of the colon following 12 weeks MNU administration in rats showed that there is moderate to mild expression of MLH1 antibody in the normal control group and the groups treated with 10% dietary *Cucurbita maxima* fruit inclusion, but there is no expression of MLH1 antibody in the MNU control group and the groups that received 2.5% and 5% of dietary *Cucurbita maxima* fruit inclusion (plate 4.3).



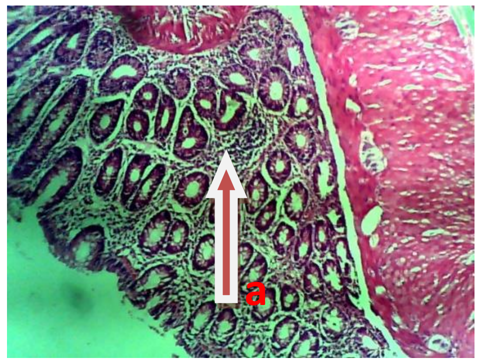
1a: Normal control group showing essentially normal colon. (X40).



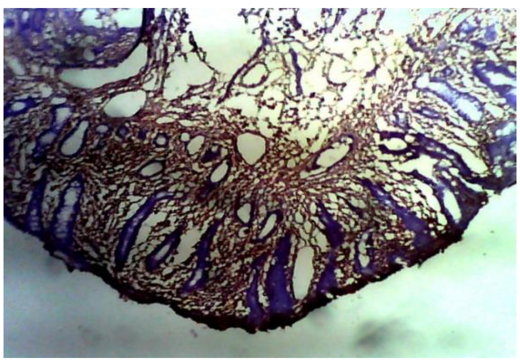
1b: MNU control group showing essentially normal colon but with mild (a)interglandular inflamation. (X40).



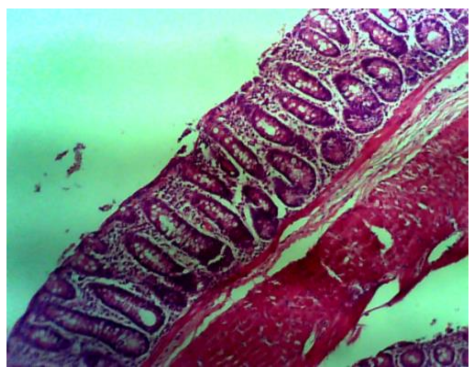
1c: MNU + 2.5% showing essentially normal colon but with (a) deeper mucosal inflammation and (b) mild ulceration. (x40)



1d: MNU + 5% showing essentially normal colon but with (a) moderate mucosal inflammation. (x40)

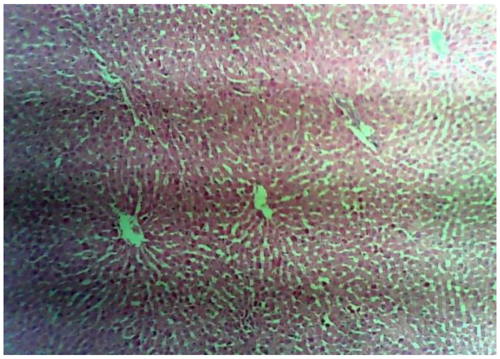


1e: Normal control group showing essentially normal colon. (X40).

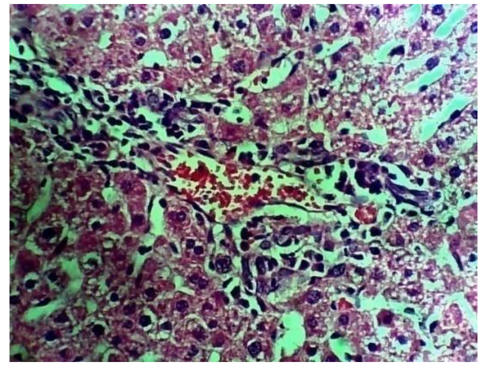


1f: Normal control group showing essentially normal colon. (X40).

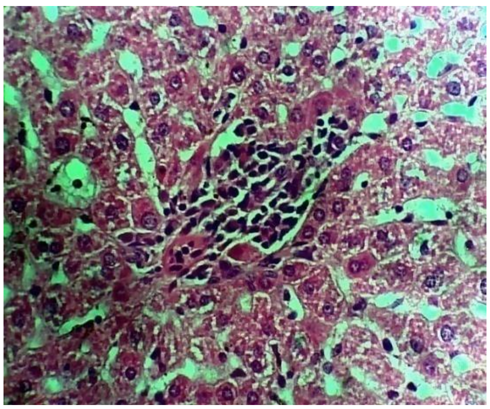
Plates 4.1: Photomicrogragh of Histopathological Section of the Colon following 12 Weeks MNU Administration in Rats Fed with different Level of Dietary *Cucurbita maxima* Fruit Inclusion Diet



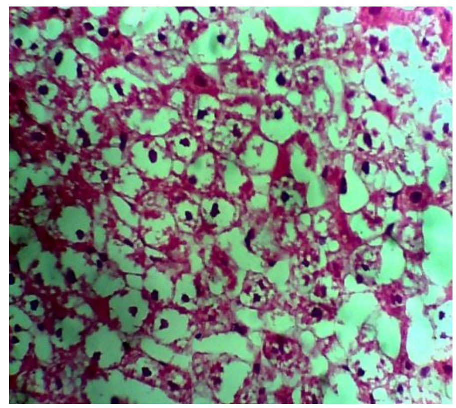
2a: Normal control group showing essentially normal liver (X40).



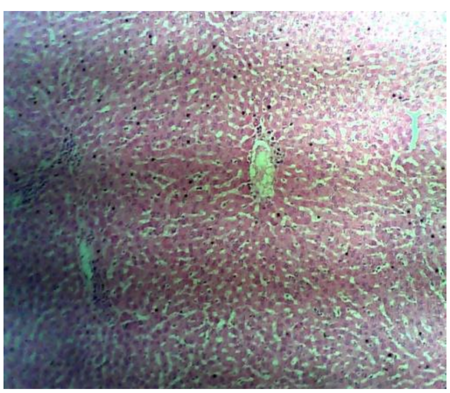
2b: Normal control group showing essentially normal colon. (X40).



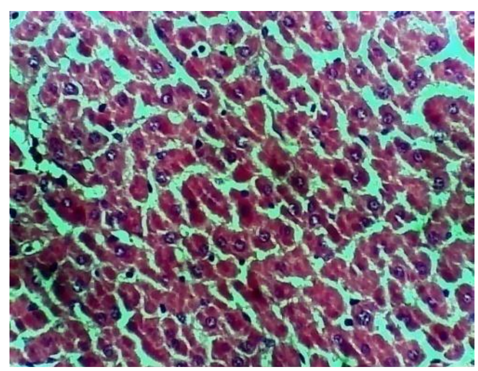
2c: Normal control group showing essentially normal colon. (X40).



2d: Normal control group showing essentially normal colon. (X40).



2e: Normal control group showing essentially normal colon. (X40).



2f: Normal control group showing essentially normal colon. (X40).

Plates 4.2: Photomicrograph of Histopathological Section of the Liver following 12 Weeks MNU Administration in Rats Fed with different Level of Dietary *Cucurbita maxima* Fruit Inclusion Diet.

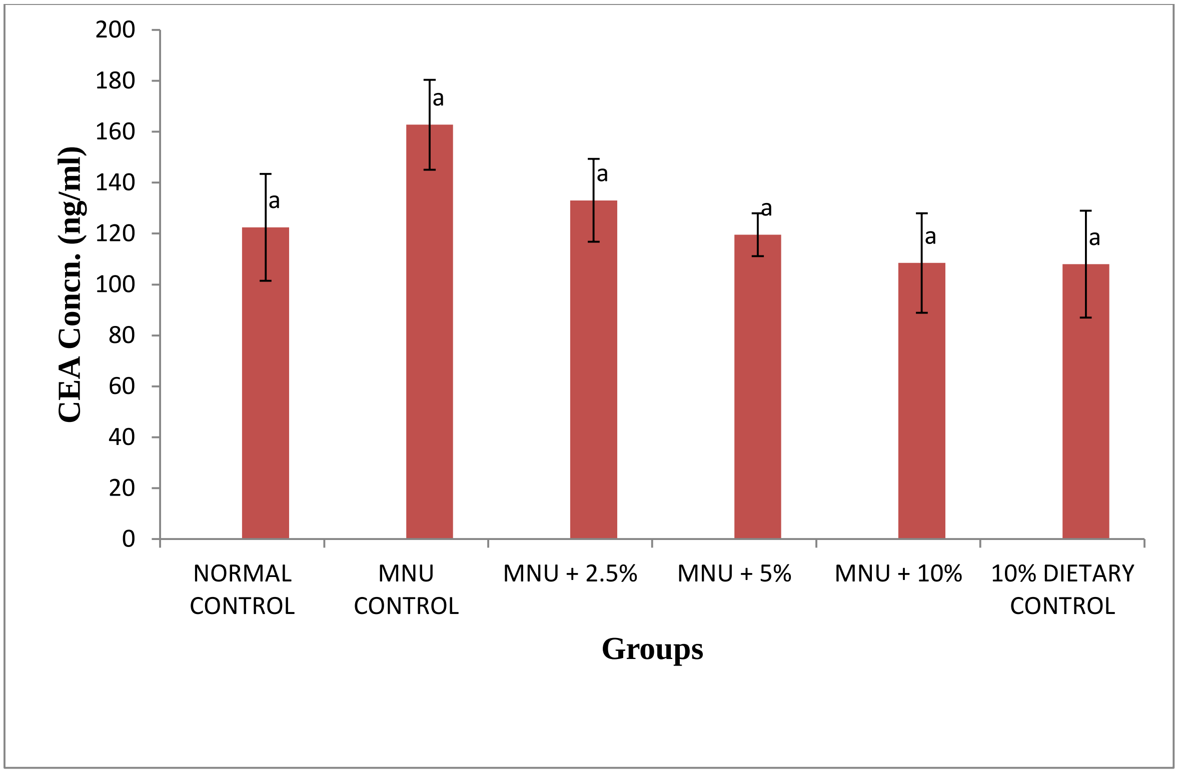
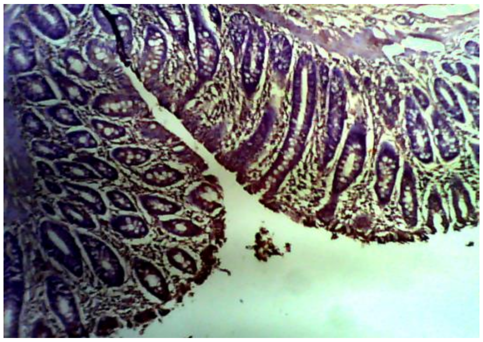
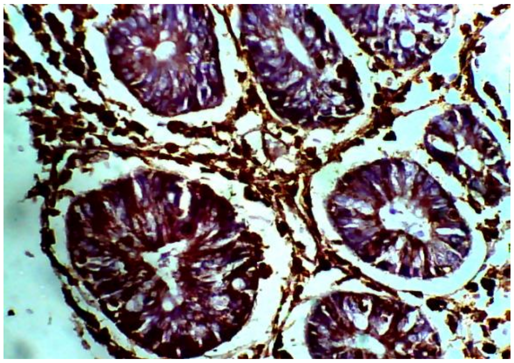


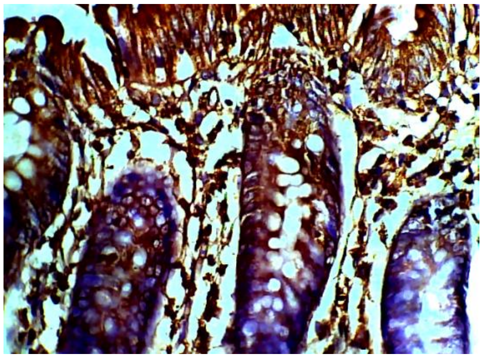
Figure 4.7: Carcinoembryonic antigen Concentration of the Serum following Intrarectal Administration of MNU in Rats Fed with Different Levels of Dietary *C. maxima* Fruit Inclusion.



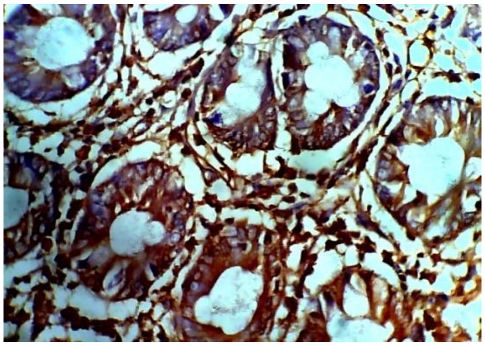
3a: Normal control group showing no expression of the antibody (MLH1).



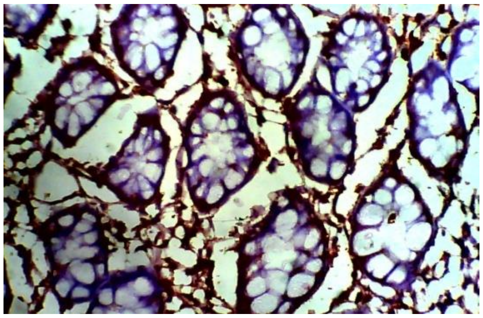
3b: MNU control group showing moderate expression of the antibody (MLH1). (x40)



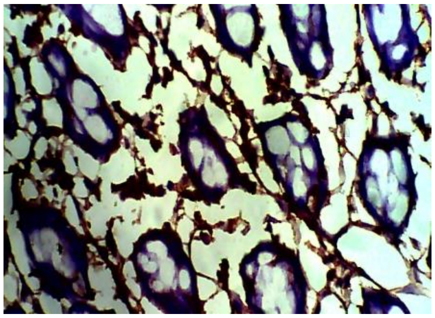
3c: MNU+2.5 showing moderate expression of the antibody (MLH1). (x40)



3d: MNU+5% showing mild expression of the antibody (MLH1). (x40)



3e: MNU+10% showing mild expression of the antibody (MLH1). (x40)



3f: Dietary control group showing no expression of the antibody (MLH1). (x40)

Plates 4.3: Photomicrogragh of Immunohistochemistry Section of the Colon, following 12 Weeks MNU Administration in Rats Fed with Different Level of Dietary *C. maxima* Fruit Inclusion.

CHAPTER FIVE

5.0 DISCUSSION

Dietary inclusion of *Cucurbita maxima* fruit showed result of hematological indices of rats fed with various diet mixtures. The significant ($P \leq 0.05$) decrease in the packed cell volume (PCV) and hemoglobin (Hb) concentration of the MNU control group indicating anemia, which may be due to damage to red blood cell, Mani *et al.,* (2014), which in this research is connected with MNU toxicity. Thus, low PCV level might suggest oxidative stress-related RBC damage in the MNU treated control group, while the increase level of PCV in the *Cucurbita maxima* powdered treated groups with percentage inclusion of 2.5%, 5% and 10%, suggest the potency of the dietary *Cucurbita maxima* fruit inclusion with its phenolic contents, Sopan *et al.,* (2014) to alleviate oxidative stress-related damage to red blood cell membrane. The significant ($p \leq 0.05$) increase in WBC in MNU control group compared to the normal control group which is not significantly ($p \geq 0.05$) different from other groups that received 2.5% ,5% and 10% dietary inclusion of *Cucurbita maxima*, suggested that the plant has potency in boosting the immune system, Sapon *et al.,* (2014) (Figure 4.3).

Oxidative stress has been suggested to contribute to the pathogenesis of carcinogenicity and lipid peroxidation which is one of the characteristic features of cancer, Halliwell (1994). The reactive intermediates, produced by oxidative stress, can alter the membrane bilayers and cause the lipid peroxidation of polyunsaturated fatty acids (PUFA) leading to the formation of lipoperoxyl radical (LOO^{-}) which in turn, reacts with the lipid to yield a lipid radical and a lipid hydroperoxide (LOOH). Lipid hydroperoxide LOOHs are unstable and they generate new peroxyl and alkoxyl radicals and decompose into secondary products, Halliwel and Chirico, (1993). Malondialdehyde (MDA) is found during oxidative degeneration as a product of free oxygen radicals which is accepted as an indicator of lipid peroxidation MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in non-diseased organ, Nielsen *et al.*, (1994).

In the present study, N-methyl-N-nitrosourea (MNU) intoxication resulted in lipid peroxidation (LPO), which produced MDA as by product that caused damage to the cell membranes (Moller *et al.,* 1998; Siddhartha *et al.,* 2011). The groups that were fed various percentages of *Cucurbita maxima* fruit powder (2.5%, 5% and 10%) indicated significant decrease levels of MDA in the Colon and Liver respectively which suggest that the lipid peroxidation of membranes caused by MNU intoxication was prevented by inclusion of the *Cucurbita maxima* fruit powder and thus implying that this fruit contain antioxidant compounds. This antioxidant activity could be due to compounds such as phenolic acid and phenolic diterpenes, Sapon *et al.,* (2014), that were capable of scavenging free radicals from the tissues of the treated rats.

According to Gacche *et al*., (2010), 2,2–diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *Cucurbita maxima* fruit is 41%, inhibition of LPO is 40.3%, Vitamin C is 15.36mg, Total phenols is 13.30mg and FICA 22.3% respectively. A redox balance between pro-oxidants and antioxidants is essential for the normal cellular functioning, Nordberg and Amer, (2001); Rahal *et al.,* (2014). Impairment in the ratio of oxidants and antioxidants initiates in the patho-physiological events that culminate in molecular and cellular damage to macromolecules and vital organs, Valko *et al*., (2006).

The antioxidants SOD and CAT play an important role in reducing cellular stress. SOD scavenges the superoxide radical by converting it to hydrogen peroxide and molecular oxygen, Robinson (1998), while CAT brings about the reduction of hydrogen peroxide and protects higher tissues from highly reactive hydroxyl radicals Brioukhanov and Netrusov, (2004).

In the present study, the activity of CAT was significantly depleted in MNU control group rats. Dietary *Cucurbita maxima* fruit inclusion significantly ($p \leq 0.05$) increased the CAT activity in the group that received various percentages of 2.5%, 5% and 10% and also in the 10% dietary control group rats when compared to MNU control group rats. The SOD activity was significantly decreased in rats exposed to only MNU, dietary *Cucurbita maxima* fruit inclusion significantly ($p \leq 0.05$) increased the SOD activity in the groups which took the 2.5%, 5% and 10% respectively and also in 10% inclusion control group. Dietary *Cucurbita maxima* fruit has excellent antioxidant activity which may protect individuals from oxygen free radicals and lipid peroxidation induced damage, Rahal *et al.,* (2014)

According to Thompson and Adlakha (1991), MNU rat model have several advantages such as reliability of tumour induction organs site specificity, tumour of ductal origin and predominantly carcinomatous histopathologic characterization and the ability to examine tumour initiation and promotion processes. The histopathological findings of the colon and liver following 12 weeks MNU administration in rats in (Plate 4.1 and Plate 4.2) showing essentially normal colon and liver in the normal control group and the groups that were treated with 5% and 10% dietary inclusion of *Cucurbita maxima* fruit, strongly suggest high anti-oxidative effect of the *Cucurbita maxima* fruit, which may be as a result of it high contents of antioxidant compounds such as polyphenols and antioxidant vitamins, Muntean *et al.,* (2013). Inflammation observed in the

MNU control group and the groups treated with 2.5% dietary inclusion of *Cucurbita maxima* fruit, revealed that MNU caused severe damage to the .mucosal cells, which is connected with MNU toxicity and that higher percent inclusion is necessary for significant effect.

Immunohistochemistry has been widely used in diagnosing carcinogenesis because it takes advantage of the principle of the antigen-antibody affinity reaction through its ability to identify and localize proteins of interest via detection with labelled conjugates Mann (1902). These antigens produced in disease condition have corresponding antibodies produced by the body to form a complex compounds which are usually picked up by immunochemical dyes or stains giving a distinctive colour (brownish colour in the case of the *Mut L homoloq* 1 gene) as "expression" under the microscope. The distinct moderate to mild expression of *Mut L-homoloq* 1 antibody in the normal control group and the groups treated with 5% and 10% dietary inclusion of *Cucurbita maxima* fruit in (Plate 4.3) strongly suggest high anti-oxidative effect of the *Cucurbita maxima* fruit, Sapon *et al.,* (2014), which may be as a result of its high contents of antioxidant compounds including polyphenols and antioxidant vitamins. In contrast there was expression of *Mut L homoloq* 1 antibody in the MNU control group and the groups treated with 2.5% and 5% dietary inclusion of *Cucurbita maxima* fruit, due to damaged or mutated *Mut L-homoloq* 1 gene Sapon *et al.,* (2014). According to Thompson *et al*. (1995) and Welsch *et al*., (1985), defect in the DNA mismatch repair gene like the *Mut L homoloq* 1 gene can lead to its inability to recognize and repair errors that occur during DNA replication. This loss of DNA mismatch repair gene activity accelerates the rate of accumulation of mutations in other gene leading to carcinogenesis, Sapon *et al.,* (2014); Kim *et al.,* (2004).

CEA is a glycoprotein which is normally present at only very low concentrations in adult plasma but its concentration can be increased during the process of carcinogenesis or in the presences of tumors particularly colorectal cancers, Herbert *et al.,* (1980). They are well known biomarker use to determine the extent of preoperative colon cancer, Hajime *et al.,* (2012). The significant ($p \leq 0.05$) increase in the level of CEA concentration of MNU control group may be as a result of MNU toxicity. According to Sukumar *et al.,* (1983) MNU toxicity can lead to inflammation and damage to normal mucosal cells, while a significant ($p \leq 0.05$) decrease in the level of CEA concentration in the groups treated with 2.5%, 5% and 10% of dietary *Cucurbita maxima* fruit inclusion strongly suggests the potency of the extract to prevent oxidative-stress related damage to cell membrane do to their ability to absorb, neutralize and to quench free radicals, which may be as a result of it high contents of antioxidant compounds such as polyphenols and flavonoids, Muntean *et al.,* (2013).

CHAPTER SIX

6.1 SUMMARY

This study established that;

(i) *Cucurbita maxima* fruit significantly ($P \leq 0.05$) protected haematological indices (Packed cell volume, Haemoglobin, Red blood cell and White blood cell from MNU-induced toxicity.

(ii) *Cucurbita maxima* fruit significantly ($P \leq 0.05$) prevented protein damage in the organs (liver and colon) from MNU-induced damage and damage to the DNA mismatch repair gene (*Mut L homoloq* 1) by the MNU carcinogen.

(iii) *Cucurbita maxima* fruit prevented oxidative stress in colon carcinogenesis by increasing the activities of catalase and SOD, and also by decreasing the concentration of MDA in the organs (liver and colon).

6.2 CONCLUSION

Cucurbita maxima fruit has potential to prevent colon carcinogenesis and protect against MNU-induced oxidative stress, organs damage and its adverse effects on haematological parameters.

6.3 RECOMMENDATION

From this study it is recommended that people should be encouraged to increase their consumption of *Cucurbita maxima* fruit because it has high antioxidants which play vital roles in prevention of cancer and other diseases.

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APPENDICES 1.0

Effect of *Cucurbita maxima* fruit on Percentage Packed Cell Volume (PCV), Neutrophil and Lymphocyte following 12 Weeks MNU Administration in the Blood of Different Experimental Group.

Group	Treatment	PCV(%)	NEUTRO(%)	LYMPHO(%)

i	**Normal Control**	**42.30±2.2^{b}**	**26.30±6.2^{ab}**	**52.70±4.5^{b}**
ii	**MNU Control**	**32.20±4.2^{a}**	**31.80±5.3^{c}**	**66.00±3.5^{c}**
iii	**MNU+2.5% of *C. maxima* fruit**	**36.20±2.4^{a}**	**29.30±2.4^{b}**	**60.30±2.4^{bc}**
iv	**MNU+5% of *C. maxima* fruit**	**40.10±3.1^{b}**	**27.40±3.4^{b}**	**53.50±3.5^{b}**
v	**MNU+10% of *C. maxima* fruit**	**48.80±2.3^{c}**	**24.50±3.1^{a}**	**48.20±1.2^{a}**
vi	**10% of *C. maxima* fruit Control**	**50.40±1.3^{c}**	**22.30±2.5^{a}**	**46.20±3.2^{a}**

Values are Mean±SD; Values having different letters across the column are significantly different ($p \leq 0.05$).

APPENDICES 2.0

Effect of *Cucurbita maxima* Fruit on Haemoglobin and Platelet Concentration in the Blood Serum following 12 Weeks MNU Administration in Different Experimental Group.

Group	Treatment	Hb(g/dl)	TP (g/dl)

i	**Normal Control**	**17.56±1.5[b]**	**7.70±0.5[a]**
ii	**MNU Control**	**10.40±1.3[a]**	**5.90±0.8[a]**
iii	**MNU+2.5% of *C. maxima* fruit**	**12.10±1.2[a]**	**7.10±0.2[a]**
iv	**MNU+5% of *C. maxima* fruit**	**14.50±1.2[ab]**	**8.20±1.0[ab]**
v	**MNU+10% of *C. maxima* fruit**	**17.60±0.5[b]**	**9.90±0.6[b]**
vi	**10% of *C. maxima* fruit Control**	**19.80±0.4[c]**	**10.30±0.7[b]**

Values are Mean±SD; Values having different letters across the column are significantly different ($p \leq 0.05$).

APPENDICES 3.0

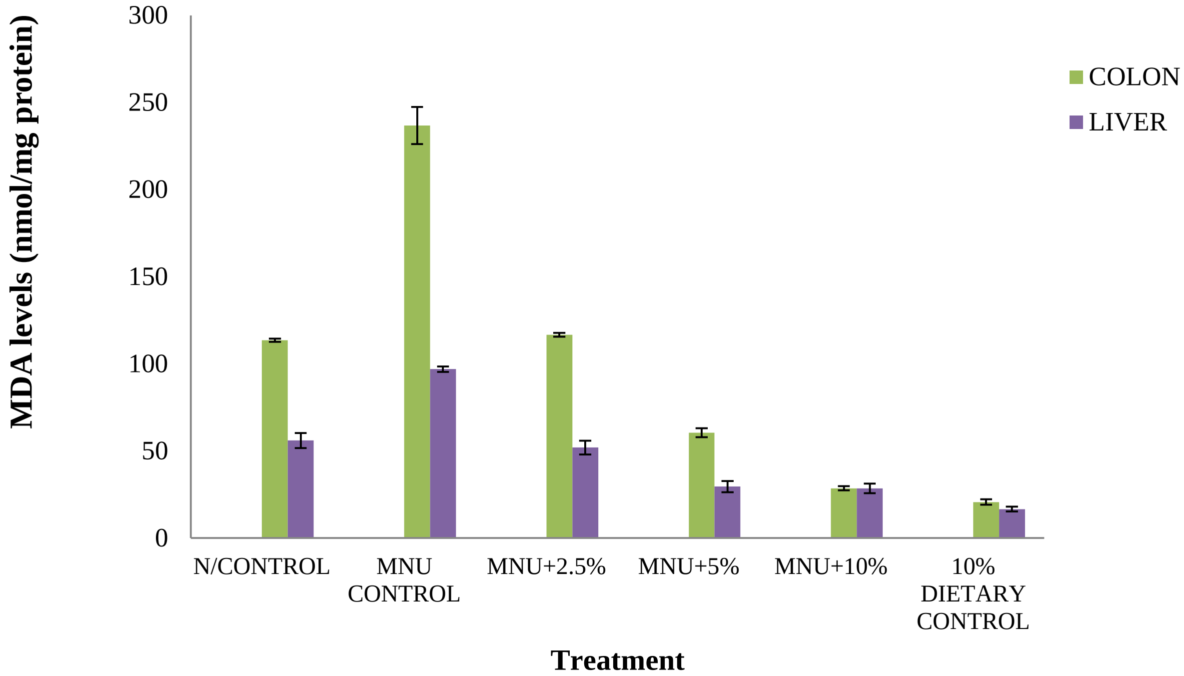
Effect of *Cucurbita maxima* Fruit on White Blood Cell and Red Blood Cell in the Blood following 12 Weeks MNU Administration in Different Experimental Group.

	Group Treatment	WBC (10^9/l)	RBC (10^{12}/l)
i	**Normal Control**	**10.70±2.5[a]**	**8.80±0.3[ab]**
ii	**MNU Control**	**12.50±2.1[c]**	**6.10±0.5[a]**
iii	**MNU+2.5% of *C. maxima* fruit**	**10.90±3.5[b]**	**7.30±1.0[a]**
iv	**MNU+5% of *C. maxima* fruit**	**10.20±3.2[b]**	**8.10±0.6[ab]**

v	**MNU+10% of *C. maxima* fruit**	**9.40±0.6^{b}**	**8.93±0.5^{ab}**
iv	**10% of *C. maxima* fruit Control**	**7.90±1.3^{a}**	**9.20±0.8^{b}**

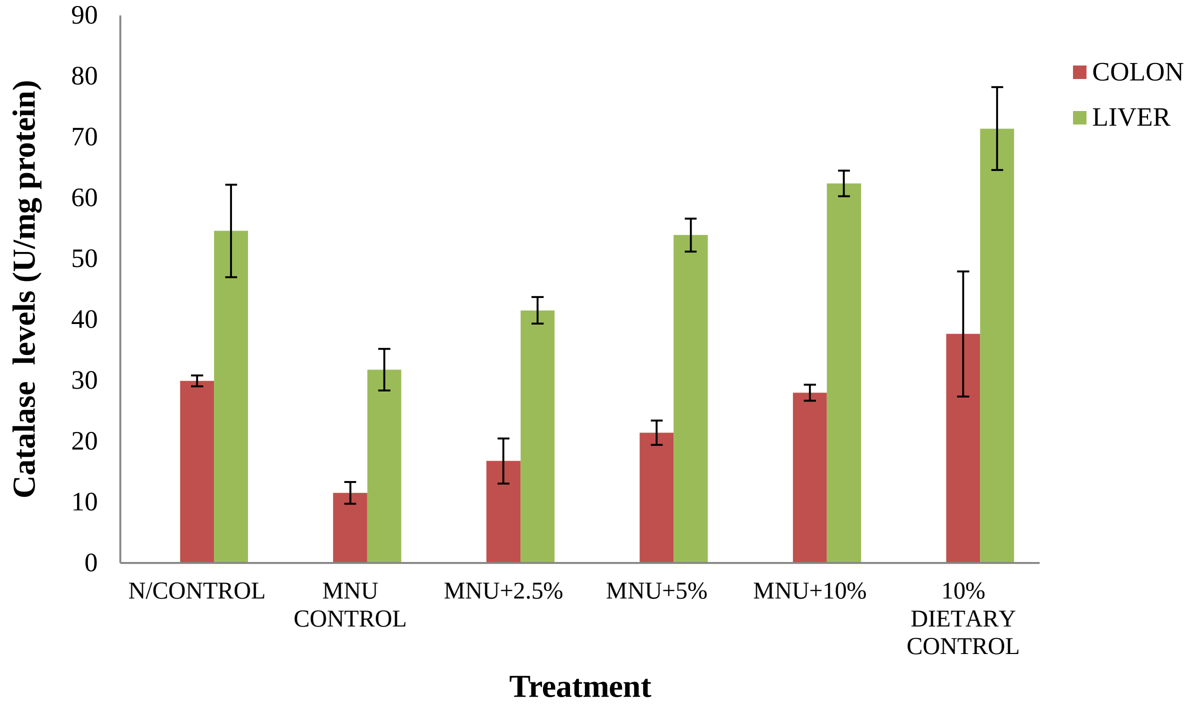
Values are Mean±SD; Values having different letters across the column are significantly different ($p \leq 0.05$).

APPENDICES 4.0



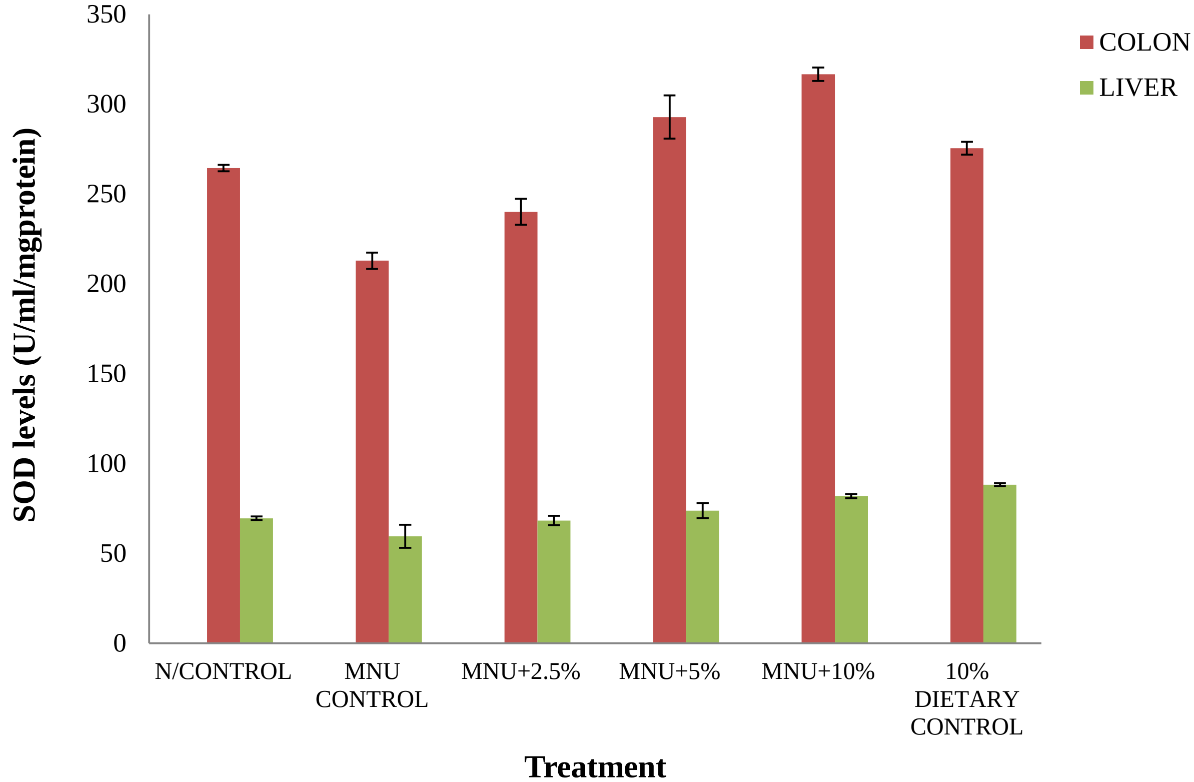
Effect of Dietary *Cucurbita maxima* Fruit Inclusion on MDA Level following 12 Weeks MNU Administration in Rats. Values are Mean ±SD

APPENDICES 5.0



Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Catalase Activity following 12 Weeks MNU Administration in Rats. Values are Mean ±SD

APPENDICES 6.0



Effect of Dietary *Cucurbita maxima* Fruit Inclusion on Superoxide dismutase Activity following 12 Weeks MNU Administration in Rats.

APPENDICES 7.0

Carcinoembryonic Antigen Concentration of the Serum following 12 Weeks Intrarectal Administration of MNU in Rats Fed with Different Level of Dietary Inclusion of *C. Maxima* Fruit.

Group	Treatment	CEA Concentration (ng/ml)
i	NORMAL Control	122.50±21.00[a]
ii	MNU Control	162.77± 17.65[a]
iii	MNU+2.5% of *C. maxima* fruit	133.06±16.26[a]
iv	MNU+5% of *C. maxima* fruit	119.56±8.37[a]
v	MNU+10% of *C. maxima* fruit	108.46±19.58[a]
vi	10% of *C. maxima* fruit Control	107.97±20.96[a]

Values are Mean±SD; Values having different letters across the column are Significantly different ($p \leq 0.05$).