ASSESSMENT OF THE CHEMOPREVENTIVE EFFECT OF DIETARY INCLUSION WITH *CUCURBITA MAXIMA* LEAF ON N-METHYL-N-NITROSOUREA-INDUCED COLON CARCINOGENESIS IN WISTAR RATS

BY

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DECLARATION

I declare that the work in this dissertation entitled "**Assessment of the Chemopreventive Effect of Dietary Inclusion with *Cucurbita maxima* leaf on N-Methyl-N-Nitrosourea (MNU) Induced Colon Carcinogenesis in Albino Rats**"was performed by me in the Department of Biochemistry, Ahmadu Bello University Zaria under the supervision of Prof. S.E Atawodi, FASand Prof. I. A Umar. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project was previously presented for another degree or diploma at any university.

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CERTIFICATION

This dissertation entitled "**Assessment of the Chemopreventive Effect of Dietary Inclusion with *Cucurbita maxima* Leaf on N- Methyl-N-Nitrosourea (MNU) Induced Colon Carcinogenesis in Albino Wister Rat**" by Job Itanyi ONUCHE meets the regulations governing the award of the degree of M.Sc. Nutrition of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty for the gift of life and sound health during the period of this work.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACF Aberrant crypt foci

ANOVA Analysis of Variance

AST Aspartate aminotransferase

BHA ButylatedHydroxy Anisole

BHT ButylatedHydroxy Toluene

CAT Catalase

CEA Carcinoembrayonic antigen

DPPH 1,1-Diphenyl-2-picrylhydrazyl

DNA Deoxyribonucleic acid

ET AL And others

GLOBOCAN Global burden on cancer

GPxGlutathione Peroxidase

HBHaemoglobin

HDL High Density Lipoprotein

HNE Hydroxynonenal

LDL Low Density Lipoprotein

MDA Malondialdehyde

MLH1 Mult L Homologue 1

MMR Mismatch repair gene

MNU N-methyl N-nitrosourea

NADPH Nicotinamide Adenine dinucleotide Phosphate Reduced

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 Triacylglycerol

TBA Thiobarbituric Acid

WBC White blood cell count

WHOWorld Health Organization

% +NS Normal control

0%+MNU MNU control

2.5%+MNU MNU induced, fed with 2.5 percent of dietary inclusion of *Cucurbita maxima* leaf

5% +MNU MNU induced, fed with 5% percent of dietary inclusion of *Cucurbita maxima* leaf

10%+MNU MNU induced, fed with 10% percent of dietary inclusion of *Cucurbita maxima* leaf

10%+NS *Cucurbita maxima* leaf dietary inclusion control

ABSTRACT

There is growing attention on medicinal plants use as food for cancer inhibiting potential, so that chemo preventive dietary inclusion can be identified for human use. One of such plant is *Curcurbita maxima* commonly known as Giant pumpkin. The preventive effect of dietary inclusion at 2.5%, 5% and 10% with the leaf of *Cucurbita maxima* on colon carcinogenesis induced by N-methyl N-nitrosourea(MNU) for 12 weeks was assessed in male albino Wistar rats. The colon and liver of rats administered the *Cucurbita maxima* leaf included diet, showed significant($P \square 0.05$) increase in the activity of the endogenous antioxidant enzymes (CAT and SOD) in the treated groups when compared with the MNU control group. A dose-dependent decrease in oxidative stress was observed in the colon of treatment group with the highest percentage inclusion(10%) showing the lowest MDA value. A significant ($P \square 0.05$) increase in some hematological parameters like PCV, Red blood cell (RBC), heamglobin count (HB) were observed in the treated group when compared with the MNU control group. While an increase in white blood cell count (WBC) and neutrophils was observed in the MNU control group when compared with the normal control group. A significant ($P \square 0.05$) decrease in the level of Carcinoembrayonic antigen (CEA) was observed in the *Cucurbita maxima* leaf diet fed group when compared with the MNU control group. Immunohistochemical observation of the colon tissue showed mild to moderate expression of the DNA mismatch repair (MLH1) gene in the treated groups when compared with the MNU control group showing mild to no expression of the MLH1 gene. Histological observation of colon tissue showed a severe damage to the mucosa cell, with mucosa ulceration and sclerosis being observed in the MNU control group when compared with the treated groups and normal control that both showed a better architecture of the cells. The results therefore,shows the ability of *Cucurbita maxima* leaf dietary inclusion to improve endogenous antioxidant system maintain or improve heamatological parameter ,prevent oxidative stress, protect organ damage induced by MNU carcinogen, thus indicating the potential of *Cucurbita maxima* leaf to prevent the process of chemically induced colon carcinogenesis.

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Medicinal plants have been used throughout human history for treatment and management of different ailments (Aliyu *et al.,* 2008). The reason is because these plants have the ability to synthesize a wide variety of chemical compounds that have been found useful to man. these compounds perform important biological functions for the plant like defense against attack from predators such as insects, and fungi e.t.c with at least 12,000 of such compounds isolated so far; a number estimated to be less than 10% of the total available compounds (Tapsell *et al.,* 2006; Lai and Roy, 2004). This then justifies the chemopreventive and therapeutic effects of these plants on cancer and other diseases related to oxidative damage (Atawodi *et al.,*2010).These plants which are also referred to asherbs have been used to prevent and treat disease/disorderscommonly throughout non-industrialized societies because they are more affordable than purchasing expensive modern pharmaceutical drugs (Edgar *et al.,* 2002). A report made by World Health Organization (2000) puts it that, about 80% of the population of some Asian and African countries presently use herbal medicine for some aspect of primary healthcare, also according to a survey report released in May 2004 by the National Centre for Complementary and Alternative Medicine, herbal therapy, or use of natural products other than vitamins and minerals, was the most commonly used complementary alternative medicine therapy amounting to 18.9% in the United States (Barnes *et al.,* 2004). Furthermore, it is stated that these compounds found in these herbs are often responsible for mediating their therapeutic effects on the human body through processes that are identical to those already well understood mechanisms in conventional drugs (Tapsell *et al.,* 2006). The medicinal or therapeutic properties of these plants could be based on the antioxidant, antimicrobial , antipyretic and other physiological effects of the phytochemicals in

them (Ayo, 2014; Adesokan *et al*., 2008). It is interesting to note that one of the major challenge of modern medicine is in the treatment and management of colon cancer and also absence of a reliable colon cancer preventive drugs.Thereforeedible medicinal plants becomes key in the prevention, treatment and management of colon cancer (Bufalo *et al.*,2009). Many edible plants have been favored in the prevention and treatment of diverse ailment, One of such plant is *Cucurbita maxima* (Munteane*t al.,* 2013), Despite the wide investigation of plants for preventive and curative potency in different form of cancer.The menace of cancer is far from being over. Hence, there is a need to investigate more naturally abundant edible plants of medicinal potency in the fight against colon cancer until total and absolute protection from colorectal cancer is guaranteed. The focus of this investigation therefore, is to consider the possible preventive effect of the leaf of C*ucurbita maxima* formulated diet on colon carcinogenesis, using a carcinogen(MNU) in male albino wistar rats and thereafter enlisting it as one potent preventive plant against colon cancer if found/proved so.

1.2 Statement of Research Problem

The problem of cancer still persists despite great advancement in cancer research and in science. Cancer can still be seen as one of the most deadly diseases that are causing serious health problems, around the world, consequently there are still significant deficiencies and room for improvement (Sultana *et al.*,2014). According tothe National Cancer Institute, colorectal cancer is the commonest malignancy of the gastrointestinal tract worldwide, with about 140,000 cases each year in the United States, also incidence and death rates for colorectal cancer is increasing with age(SEER program, 2010). Colorectal cancer incidence and mortality rates are about 30% to 40% higher in men than in women (Murphy *et al.,* 2015). In Africa, the incidence is initially said to be

low, probably due to high young demographic population and high intake of fiber diet, which results in passage of bulky stool with short intestinal transit time, However, there is rising incidence in Africans especially in Nigeria from an average of 10-20 newly diagnosed colorectal cancer in a tertiary health institution per year to about 50 cases. This suddenrise might be due to urbanizations or movement of people from rural to urban settlement leading to high intake of food high in carcinogens (Archampong *et al*., 2013). Other interesting issue of note is that the treatment of colorectal cancer constitutes a great diagnostic and therapeutic challenge in resource-limited countries such as Nigeria, also late presentation of the disease, lack of an adequate screening program, lack of endoscopic facilities and lack of adjuvant therapy are among the hallmarks of the disease in most developing countries. Some of the major problems of colon cancer treatment are the unpredictable post operative survival rate and the reoccurrence. Also resistance to anticancer drugs have been observed, therefore, more research is required to explore the anticancer or preventive potential of edible medicinal plant (Chen, 2010) in order to forestall the disease in the first place.

1.3 Justification

Chemotherapy as well as conventional treatment for the cure of cancer such as colon cancer can cause adverse and toxic side effect, the cost of diagnosing and treatment of the disease can be a huge financial burden to the patient and family(Arun *et al.,* 2014).Therefore going by the popular saying that "prevention is better than cure" it can be appreciated that the alternative remedy to the harmful effects of these synthetic agents that are used in management of colon cancer and high cost of treatment and diagnosis is in the prevention of the diseases particularly with medicinal plants locally eaten as food)."Let food be your medicine and medicine be your food" is an

ideology Hippocrates launched more than 2400 years ago aimed at sensitizing the public on the need for inclusion of medicinal plant in their diet as a preventive measure against disease for a healthy life. One of such important medicinal plant, considering its proven health effects is *Cucurbita maxima (duch).*In recent times modern research have focused on evaluation of dietary plants for the prevention, management and treatment of several diseases, *Cucurbita maxima* is one of such plant that has been frequently used as functional food or medicine(Shahlah,*et al.*, 2013).

*Cucurbita maxima*can be found in many parts of Nigeria where the leaf is used among the Igbo, Igala, Idoma and Ibibio ethnic tribe as salad and vegetables in various delicacies, including yam porridge. The fruit and the leaf have traditionally been acclaimed to have some medicinal value by these ethnic groups. Substantial evidence from various experimental studies have also suggested that different parts of the plant including the leaf may contain anticancer constituent, Thus, considering the increasing incidence of colon cancer worldwide (Attarde *etal.*, 2010), and the need for preventing the disease through diet,it becomes important to investigate the potential of this plant to prevent colon cancer and thereafter enlisting it as one of the colon cancer preventive edible medicinal plant.

1.4 Nullhypothesis

*Cucurbita maxima*leaf dietary inclusion has no preventive effect on colon carcinogenesis.

1.5 Generalaim

The work is aimed at evaluating the preventive effect of dietary inclusion with the leaf of *Cucurbita maxima* on MNU induced colon carcinogenesis in male albino Wister rats.

1.6 Specific objective

The specific objective areto:-

i. Determine the effect of *Cucurbita maxima* leaf dietary inclusion on some haematological parameters in MNU induced colon carcinogenesis in albino Wistar rat

ii. Establish the possible role of *Cucurbita maxima* leaf dietary inclusion on the endogenous antioxidant status and level of oxidative stress in MNU induced colon cancer in rats.

iii. Determine the effect of *Cucurbita maxima* leaf dietary inclusion on some colon cancer biomarkers in carcinogenesis induced by MNU in albino rats.

CHAPTER TWO
LITERATURE REVIEW

2.1Phytochemicals

Plants produce chemical compounds as part of their normal metabolic activities. These compounds are referred to as phytochemicals and are divided into: (a) primary metabolites such as carbohydrates, proteins and fats, which are found in all plants and are responsible directly for their growth and development (b) secondary metabolites compounds which are found in a smaller range in plants, serving more specific functions such interaction with other organisms within their environment (Meskin, 2002). For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination. It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs; examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, quercetin from *moringa oleifera* leaf and digoxin from the foxglove (Eshak *et al.*, 2015).These plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs such as alkaloids (cocain, caffeine, nicotin atropine), polyphenols (anthocyanins, isoflavonone, tanins, phytoestrogens), glycosides (cyanoglycosides) and terpenes (carotenoids) (Springbob and Kutchan, 2009).

2.2 The Plant (*Cucurbita maxima)*

2.2.1 Scientific classification

Kingdom: Plantae
Division : Magnoliophyta
Order: Violales
Family: Cucurbitaceae
Genus: *Cucurbita*

Species:*maxima*
Botanical name: *Cucurbita maxima*



Plate 2-I: *Curcurbita maxima* Plant showing leaves and fruits.
Source: Snapshots taken from a Location in Ejule, Ofu LGA, Kogi State



Plate 2-II: Image of *Cucurbita maxima* showing leaves and flowers climbing a Tree.
Source: Snapshots taken from Location in Ejule, Ofu LGA of Kogi state

2.2.2 Habitat and distribution

Cucurbitamaxima belong to family cucurbiteaceae and also known as giant pumpkin in English and *Echi* in Igala tribe, kabawain Hausa andNdise by the Igbo tribe ofNigeria .It is a commonly grown vegetable and also cultivated worldwide for its consumable fruits and leaves(Moumita *et al.*, 2011).

2.2.3 Description

Cucurbita maxima is a large climbing herb, annual or perennial. Its aerial part consists of flexible succulent stem with trifoliate leaves. It is widely cultivated throughout the India and other warm regions of world for use as vegetable as well as medicine. (Rangarajan and Sathiyavani 2014).

Pumpkin is the common name for the genus Cucurbita of the family Cucurbitaceae (gourd family) which includes the pumpkins and squashes, the pumpkin varies much in form, being sometimes nearly globular, but more generally oblong or ovoid in shape. It is smooth and very variable in color. The seed is cooling and of the nature of the Melon. An annual creeper with stems up to 30 feet (9 m) long, furnished with large claspers. The leaves are large and rough like Melons. The flowers are large and fruit also very large and containing white flattish seeds (Rangarajan and Sathiyavani 2014).

2.2.4 Traditional uses of *Cucurbita maxima*

This plant has been traditionally used in many countries such as India, China, Brazil, Yugoslavia and America as antidiabetic and antihyperlipidemic **(**Sharma *et al.*,2013**).** antitumor, antihypertensive, anti-inflammatory, immunomodulatory and antibacterial agents**(**Sonu and Ramana2013; Prajapti *etal.*, 2006).

2.2.5 Evidence from experimental analysis of *Cucurbita maxima*

2.2.5.1 Nutritional composition and culinary uses

Cucurbita maxima have been found to contain moisture, lipids, protein, ash, non-polar lipids, glycosides and phospholipids. The major fatty acid present in the lipids is linolenic (Prajapti *et al.*,2006). According to Okon *et al.*, (2014), the leaves was reported to contain ash content of 12.91%, fibre 11.21%, protein content of 14.21%, lipid 6.31%, carbohydrate 69.22%, moisture content of 74.41% and caloric value of 348.98 Kcal. The leaf was shown to be a very good source of mineral with P, Ca, Mg, K, Fe, Cu and Zn and also contain vitamin A and C (Okon *et al.*, 2014). Other parts of the plant have also been shown to contain Vitamin A, beta-carotene, Thiamine (Vitamin B1), Riboflavin(VitaminB2), Niacin (Vitamin B3) Pantothenic acid (B5) VitaminB6, Folate(Vitamin B9) Vitamin C, Vitamin E, Calcium, Iron, Magnesium, Phosphorus, Potassium, Sodium, and Zinc (Lewis*et al.*, 2005). In Nigeria, it is a traditional vegetable crop grown mainly for its leaves, fruit and seed and consumed either by boiling the fruit, using the leaves as salad component or by roasting or baking the seed (Facciola, 1990; Aruah *et al.*,2011).

2.2.5.2 Pharmacological and therapeutic effect

The seed of *Cucurbita maxima* have pharmacological activities such as antibacterial, anti-inflammatory and antioxidant effects. The most critical health benefit attributed to *Cucurbita maxima* seed oil is preventing the growth and reducing the size of the prostate (Abd EL-Aziz and Abdul EL-kalek 2011) considering its proved health effects, *Cucurbita maxima* can be also included among the other medicinal plants with known pharmacological activities such as: antitumor (Hartwell, 1967; Saha *et al*., 2011), antiobesity (Das *et al*., 2010),antigenotoxic(Villasefior and Lemon, 1996), remedy for carbuncles (Al-Rawi and Chakravarty, 1964), uses in cataplasms (Al-Rawi and Chakravarty, 1964;Pitier, 1926), tonic (Al-

Rawi and Chakravarty, 2015), and warts cure (Hartwell,1967). The leaves have been shown to posses antioxidant and α amylase inhibition activity(Shahlah *et al* 2013).**In vivo methanol extract of the aerial part of *Cucurbita maxima*studied for anticancer activity against Ehrlich ascities carcinoma cells in mice. Oral administration of the methanol extract of the *Cucurbita maxima*at a dose of 200mg/kg and 400mg/kg produced cytotoxicity against the Ehrlich ascities carcinoma cells in mice in dose dependent manner (Saha *et al*., 2011).** Also evidence from methanolic extractof leaves of*Cucurbita*. *maxima* showed strong antioxidant(DPPH scavenging) and α-amylase inhibition (IC50 125 µg/ml and IC50 2.1 mg/ml),respectively(Shahlah *etal.,* 2013).

2.2.5.3 Phytochemical content

Preliminary phytochemical evaluation on different extracts of the seed of Cucurbita maximareveals the presence of proteins, carbohydrates, flavonoids, saponins and tannin (Ashok*et al.,* 2013) while The leaf reveal the presence of cardiac glycosides, flavanoids, alkaloids, steriods saponins and tannins with chloroform extraction with absence of saponins and steroids with aquous or ethanol extraction (Ikwuemeand Mohammed,2016)

2.3 Crude Fiber

Crude fibers are called roughages, found in the cell wall of plant food such as grains, beans, vegetables and fruits. They are of two categories namely: the soluble which dissolve in water and theinsoluble whichdoes not but add to the feeling of fullness and slow the release of sugar from food into the blood (Tran, 2001).sources of soluble fiber are oat bran, oatmeal, apples, strawberry dried beans giant pumpkin etc, while sources of insoluble are fruit pulp, grains peels, crunchy vegetables, whole wheat, wheat bran, barley grains e.t.c.

2.3.1 Fiber and cancer prevention

Fiber are important dietary substances in the fight against cancer especially colon cancer, because increase intake of fiber can reduce the risk of colorectal cancer, this is possible because of the tendency of fiber to add bulk to the digestive system thereby shortening the amount of time it takes for waste to travel through the colon(Fusch *et al*.,1999). Food waste might contain some carcinogens which might directly or indirectly get into food therefore shortening the time the carcinogen spends in the colon will help reduce the risk of initiation of cells by the carcinogens, furthermore, fibers(i.e from *cucurbita maxima* leafs)can be broken down in the lower intestine by some bacteria to butyrate which can inhibit tumors growth and development of colon and rectum therefore are enlisted as antitumor substance(Avivi *et al*.,2000)

2.4 Colon and its Function

The colon and rectum are parts of the digestive system, which is also called the gastrointestinal system, this is illustrated in figure 2.3 below, The first part of the digestive system (the stomach and small intestine) processes food for energy while the last part (the colon and rectum) absorbs fluid to form solid waste (fecal matter or stool) that then passes out of the body, when food is chewed and swallowed, it travels through the esophagus to the stomach where it is partly broken down and then sent to the small intestine(small bowel). The smallintestine is narrower than the large intestine (colon and rectum), but it is actually the longest segment of the digestive system (about 20 feet). The small intestine continues breaking down the food and absorbs most of the nutrients. Most of the large intestine is made up of the colon, a muscular tube about 5 feet long. The colon absorbs water and salt from the food matter and serves as a storage place for waste matter (American Cancer Society 2014).The colon has four sections: The first section is called the ascending colon. It starts with a small pouch (the cecum) where the small bowel attaches to the

colon and extends upward on the right side of the abdomen, the cecum is also where the appendix attaches to the colon. the second section is called the transverse colonwhich goes across the body from the right to the left side in the upper abdomen. The third section, called the descending colon, continues downward on the left side. The fourth and last section is known as the sigmoid colonbecause of its "S" or "sigmoid" shape. The waste matter that is left after going through the colon is called feces or stool. It goes into the rectum,, where it is stored until it passes out ofthe body through the anus.

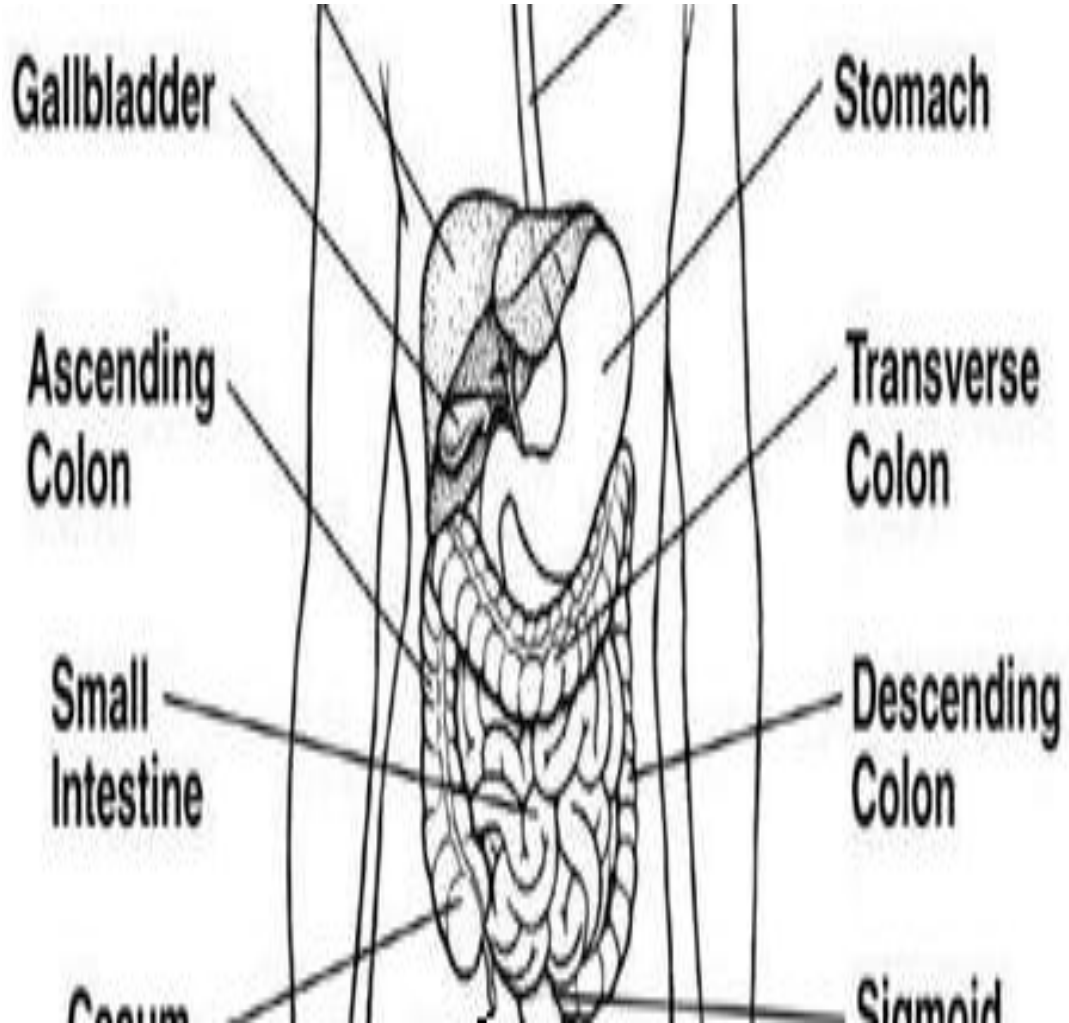


Figure 2.1 The digestive System Showing the Position of the Colon and Rectum.
Source: The America Cancer Society (2015).

2.4.1 Mechanism of colon cancer

Cancersare life threatening and dreadful disease condition represented by the abnormal proliferation of cellswhich can invade the adjacent tissues leading to destruction of these tissues (Gennari *et al.,* 2007).They can spreads to distant organs through blood stream and lymphatic vessels affecting important genes i.e. Oncogenes and the DNA mismatched repair gene etc (Knudson, 2001). Colon cancer is a term used for cancer that starts in the colon, and is usually different from rectal cancer and colorectal cancer which is the cancer of the rectum and rectum/colon respectively, they are usually form on the mucosal lining of the bowel wall which is composed of multiple layers, that includes the mucosa, the submucosa, the muscularis propria (containing circular and smooth muscle layers) and the serosa (Timothy, 2001).

Colon cancer can starts from different cells on the colon such as the glands(adenocarcinomas), hormone producing cells(Carcinoid tumors), immune cells or lymph nodes(lymphoma) etc which can eventually grow into the wall of the colon ,when cancer cells are in the wall of the colon, they can then grow into blood vessels or lymph vessel; a tiny channels that carry away waste and fluid (American cancer society, 2016).This then drains into nearby lymph nodes, which are bean-shaped structures containing immune cells that help fight against infections. Once cancer cells spread into blood or lymph vessels, they can travel to nearby lymph nodes or to distant parts of the

body, such as the liver kidney, lungs etc. Theseprocess is known as metastasis, but it should be noted that where ever the cancer cells metastasize to, they are still referred to as colon cancer (American Cancer Society, 2016). Several risk factors have been associated with colon cancer such as lifestyle, diet, physical inactivity, obesity smoking alcohol age, family history and inherited syndrome (Carrie, 2015).

2.4.2 Causes and symptoms of colon cancer

It is often impossible to evaluate the specific cause for specific colon cancer, However there are certain factors known to increase the risk of cancer such as tobacco use, alcohol, environmental pollutant, infectious agents, customs, habit, life styles and diet (Lrigaray *et al*., 2007).Early colorectal cancer often has no symptoms, which is why screening is so important but as the tumor grows, it may obstruct the intestine and may produce the symptoms like bleeding from the rectum, presence of blood in the stool, dark or black stools, cramping or discomfort in the lower abdomen, urge to have a bowel movement when the bowel is empty etc,other possible symptoms may include: a change in the shape of the stool (e.g., more narrow than usual), constipation or diarrhea that lasts for more than a few days, Decreased appetite and unintentional weight loss. In some cases, blood loss from the cancer can lead to anemia (low number of red blood cells), causing symptoms such as weakness and excessive fatigue. Timely evaluation of symptoms consistent with colorectal cancer is essential, even for adults younger than age 50, among whom colorectal cancer incidence is rare, but increasing, and for whom screening is not recommended.(Colorectal Cancer Facts & Figures 2014; 2015;2016**).**

2.4.3 Global and Regional incidence of Colon Cancer

Apart from skin cancers, colorectal cancer is the third most common cancer diagnosed in both men and women in the United States with about 93,090 new cases yearly,Colon cancer also is the third leading cause of cancer-related deaths in the united states when men and women are considered separately, and the second leading cause when both sexes are combined with an expected to cases of about 49,700 deaths during 2015(American Cancer Society, 2015). It is of note that colon cancer is now a serious health issue of both developed and developing countries, in 2012, there were an estimated 14.1 million cases around the world, of these 7.4 million cases were in men and 6.4 million women. This number is expected to increase to 24 million by 2035 (Ferlay, 2013). Furthermore there has been increase in colon cancer incidence and mortality are in countries of Eastern Europe, Asia and South America with contrast, decrease or stabilizing incidence and mortality rates in countries like USA, Australia, New Zealand and several Western European countries (Center *et al.,* 2009). Reasons might be due to early detection and prevention through polypectomy, improvements in preoperative care, as well as chemotherapy and radiotherapy (Murphy *et al.*, 2015).

In Africa the incidence of colon cancer is relatively low when compared to the developed countries with incidence about three times higher in developed countries than in Africa i.e incidence rates range from 30 or more per 100 000 people in North America, parts of Europe, Australia, New Zealand, and Japan to less than 5 per 100 000 in much of Africa and parts of Asia (Ferlay *et al,* 2010). Southern African countries have the highest incidence with West Africa and middle African having the lowest incidence rate in Africa (Ahmedin *et al.,* 2011).

In Nigeria, it was previously reported that colon cancer is a rare disease representing about 3-6% of all malignant tumors, this low incidence was attributed to fiber rich diet of the population and lack of heritable polyposis syndrome (WHO 2015). Contrary to this, recent reports have shown

increase incidence of about 81% over a period of two decades from Ibadan. A study from Lagos and Sagamu showed similar trend with an increase in annual frequency of this cancer from 14 cases per annum to 32.3cases per annum. It also accounts for 10-50% of all GIT malignancies in Nigeria with peak incidence of 60-70years and occurring in the young having polyposis syndrome or ulcerative colitis (Abdulkareem, 2009). Colon cancer is responsible for about 8% of estimated cases of cancer both in men and women with about 8% and 9% death from cancer in men and women respectively in west African countries like Nigeria (Siegel e*t al.*, 2015). This rise is attributed to recent urbanization/civilization resulting to upsurge of confectionary food outlets in major cities in Nigeria thereby making them change their dietary habit from a fiber rich diet, which was common practice to a highly refined carbohydrate and fat diet. There has also been an increase in the proportion of young patients with colon cancer. Reports from various parts of Nigeria showed that 35-42% of colon cancer cases are below age 40yrs, this phenomenon has shown to be a diagnostic and therapeutic problem and prognosis is therefore essential. Generally colon cancer is more common in males than females with average male to female ratio of 1.5:1 in Nigeria (Abdulkareeem , 2009).

A further look at the incidence of colon cancer within the country have shown a significant differences between the various ethnic groups, this might be attributed to food stuffs or diet, exposure and adoption to western lifestyle which are peculiar to these various ethnic groups(Irabor, 2012). A case study is seen in the work of Irabor (2012).who found out that from 2002 to 2010, a total number of 500 colorectal cancer cases were operated on at the University Teaching Hospital Ibadan, giving anannual rate of about 63 patients. Colon cancer cases were 244 (48.8%), rectosigmoid locations were 34(6.8%) and rectal cancer cases were 222(44.4%). Out of these patients, 73% were Yoruba dominant and indigenous ethnic group in Ibadan of the

Southwest region, 13.5% were Igbo ethnic group of south east region, 15.2% were from the South south region while 1.5% were Hausa from the Northern region. This was attributed to difference diet or food in these various regions. Foods commonly consumed by people living in the south eastern region are rich in crude fiber when compared to that of the south west and northern part of the country.

2.4.4 Plants possessing preventive potency against colon carcinogenesis

Continuous and extensive research in the field of science have revealed some plants substance that have shown promising potential against colon carcinogenesis. These includesButyrate from germinated barley which was shown to have a preventive effect on colon cancer *in vivo* and had the potency to attenuate mucosal inflammation and to increase fecal butyrate production in colitis (Osamu *et al.,* 2008). Squalene significantly, suppresses colon ACF(aberrant crypt foci) formation and crypt multiplicity thusstrengthens the hypothesis that squalenepossesses chemopreventiveactivity against colon carcinogenesis(Chinthalapally *et al.*, 1998).The aerial parts of *Artemisia princeps* is effective at preventing inflammation-associated colon cancer (Kyung *etal.,* 2015).*Moringa olefera* is also a promising plant with virtually all its parts showing a very strong antioxidant status and free radical scavenging potiential which is a basis for preventing colon carcinogenesis (Atawodi *et al.,* 2010 ; Siddhuraju*et al.,* 2003). Aqueous infusion of the leaf of *Camellia sinensis* (green tea) contain (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and have been shown to posses numerous anticancer activities on colon cancer and other form of cancers(Sabira *et al.,* 2014).Evidence from the work of Simsek *et al* (2013) shows a positive anticancer effect of the plant*Cynara syriaca*on colorectal cancer cells. Also *Withania somnifera*(L.) family (Solanaceae) is a medicinal plant having antitumor, anti-

aging, anti-stress, anti-oxidant and immunomodulatory properties. Invitro cytotoxicity of the ethanol extracts of root, stem and leaves of this plant was evaluated against five human cancer cell including colon cancer with a promising result obtained(Yadav *et al.,* 2010). Furthermore methanolic extract of *Polygonum multiflorum* shows that the plant contain the compound emodin and questin which strongly inhibit the growth of colon cancer(Choi *et al*., 2007)

2.5 Mechanism of N-methyl-n-nitrosoruea(MNU) carcinogenicity and toxicity

N-1-Nitrosourea (MNU) is a highly reliable alkylating agent that exhibits its toxicity by transferring its methyl group to nucleotide bases in nucleic acids, which can lead to AT:GC transition mutations, They are alkylnitrosamide compound that induced colon cancer similar to neoplastic lesions observed within the human colon (Rosenberg *et al.,* 2009) . It is a direct alkylating /genotoxic agent which do not require metabolic and biochemical activation and thus are potent topical carcinogens widely use in successfully inducing colon tumor,withexperiment evidence abound that this carcinogen has been successfully used in inducing cancers in rodents, mice and rats(Wood *et al* 2007). The mechanism of action of direct alkylating agent (MNU) is the conversion of the parent molecule into an active carbonium cation and subsequently alkylation of cellular macromolecule such as DNA(Saminathan 2014). 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, 2014). A clear example of MNU toxicity is seen when cells are exposed to this carcinogen leading to inactivation of genes required for repair of base-base mismatches in DNA, referred to as mismatch repair (MMR) genes (e.g MLH1, MLH2, etc). This damaged or deactivated genes then lacks the ability to repair DNA mutation which then accumulates prompting cells to replicate uncontrollably. This MMR deficiency can be detected during Immunohistochemical analysis, other examples of MNU carcinogenicity in animal models

are reported from successful induction of squamous cell carcinomas of the fore-stomach, sarcomas and gliomas of the brain, adenocarcinomas of the pancreas, mammary carcinomas, leukemia, and lymphomas (Injac *et al*., 2009).

*2.*6Carcinoembryonic Antigen (CEA)

Carcinoembryonic Antigen is a glycoprotein of MW approx. 180 kD which are normally present at only very low concentrations in adult plasma but its concentration can be increased during the process of carcinogenesis or in the presence of tumours in gastric, bronchial, uterine, ovarian cancers, and particularly colorectal cancers. Carcinoembryonic antigen (CEA) is still a widely known tumor markers of colon cancer which levels are used to determine the extent and outcome of carcinogenesis induced by carcinogens and for preoperative and postoperative assessment of colon cancer (Hyeon *et al.*, 2013).

2.6.1 Advantages and limitation of the CEA biomarker

Carcinoembryonic Antigens in combination with other cancer biomarkers like immunohistochemistry of MLH1 gene(DNA mismatch repair gene), hitopathological damages to cells and cytokines can be used as diagnostic screening index for carcinogenesis and of colon cancer (Ahmet *et al.,* 2012). Smokers also have elevated CEA when compared to non smokers and this when not taking into consideration during screening might lead to false positive and misleading result (Jung *et al.*, 2013).

2.7 Immunohistochemistry and DNA Mismatch repair gene

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 (Lee, 2011). 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” under the microscope. The

mismatch repair gene are of different types such MLH1, MSH2,PMS1, PMS2 GTBP/MSH6 which can be loss or damaged during carcinogenesis or cancer leading to inability of this genes to produce specific proteins that recognize and repair errors that occur during DNA replication, thus mutations accumulates in other gene involve in death and growth control of cells (Boland *et al.*, 2008).

2.8 Oxidative Stress

The cumulative production of ROS/RNS through either endogenous or exogenous process is termed oxidative stress and is common for many types of cancer cell that are linked with altered redox regulation of cellular signaling pathways(Valko*et al.*,2006). Oxidative stress is a phenomenon associated with pathogenesis of several diseases including atherosclerosis, ulcerative colitis, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, cancer, diabetes mellitus, inflammatory diseases, as well as psychological diseases or aging processes (Rana *et al.*, 2014; Hamouda *et al.*, 2011; Subash *et al.*, 2010; Polidori *et al.*, 2007; Matritim *et al.*, 2003). Oxidative stress can also be defined as an imbalance between production of free radicals or reactive metabolites (oxidants) and their elimination by protective mechanisms, referred to as antioxidative systems (Rana *et al.*, 2014; Patil *et al.*, 2007). This imbalance leads to damage of important biomolecules and organs with potential impact on the whole organism. Oxidative and antioxidative processes are associated with electron transfer influencing the redox state of cells and the organism (Salwa and Abass, 2011).

2.8.1 Free radicals

Free radicals are species (atoms, molecules, ions) that have one or more unpaired electrons in their outer orbital that make them very unstable and quite reactive with other molecules by pairing up

their electron(s) to generate a more stable compound (Salwa *et al.,* 2011). Reactive oxygen species (ROS) formed during physiological and pathological conditions in the body are extremely reactive and react with proteins, lipids, carbohydrates and nucleic acids (Carmen *et al.,* 2012). Free radicals are derived from oxygen, nitrogen and sulphur to form reactive oxygen species, reactive nitrogen species and reactive sulphur species respectively. The nitrogen derived free radicals include nitric oxide (NO), peroxy nitrite anion (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3); the thiol derived free radicals include sulphite (SO_3^{2-}), disulfide S oxide (DSSO) and sulfenyl (RS.) radicals (Lu *et al*., 2010). When the body is overloaded with free radicals and cannot be gradually destroyed, their accumulation in the body generates a phenomenon called oxidative stress (Carmen *et al.,* 2012).

2.8.2 Mechanisms of oxidative damage by free radicals

The mechanism is by homolytic cleavage of covalent bond of normal molecule, with each fragment retaining one of paired electrons by loss of single electron from normal molecule or by addition of single electron to normal molecule **(**Manibusa *et al.,* 2007; Weber *et al.,* 2003; Recknagel *et al.,* 1989),any of which can lead to oxidative damage to lipids protein and DNA molecule

2.8.3 Oxidative damage to lipids by free radicals

All of the most important classes of biomolecules may be attacked by free radicals but lipids are probably the most sensitive. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs) which are readily attacked by oxidising radicals (Vasudevan *et al.,* 2011). The oxidative destruction of PUFAs is known as lipid peroxidation which involves pulling out of hydrogen

atom(s) from the vicinity of polyunsaturated fatty acid double bond which is particularly damaging because it proceeds as a self-perpetuating chain-reaction.

2.8.4 Oxidative damage to proteins by free radicals

Oxidative attack on proteins results in site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross linked reaction products, altered electrical charges and increased susceptibility to proteolysis (Carmen *et al.,* 2012; Lobo *et al.,* 2010).

2.8.5 Oxidative damage to DNA by free radicals

Activated oxygen species and agents that generate oxygen free radicals, such as ionizing radiations, induce numerous lesions in DNA that causes deletion, mutations and other lethal genetic effects (Craft *et al.,* 2012; Lu *et al.,* 2010). Characterization of this damage to DNA has indicated that both sugar and base moieties are susceptible to oxidation, causing base degradation, single strand breakage and cross links to proteins (Lu *et al.,* 2010).

2.9 Antioxidant System

Antioxidants are exogenous or endogenous compounds which either prevent or delay the generation of toxic oxidants or intercept those that are already generated to inactivate them, thereby blocking the chain of propagation reaction by these oxidants (Halliwell, 2010). They can also initiate repair processes (e.g damaged DNA repaired by sulphaoxide reductase) which remove damaged macromolecules to prevent their accumulation that may further hinders cellular process and viability (Subash *et al.,* 2010). They have ability to protect human body cells from the damages caused by unstable free radicals (highly reactive chemicals that play part in generating oxidative stress in biological system) by stabilizing them via electron donation.

2.9.1 Classification of human antioxidants according to the source

According to the source, antioxidants could be classified into: (a) endogenous (b) exogenous

2.9.1.1 Endogenous antioxidants

The endogenous group includes metallo-enzymes superoxide dismutase (zinc, manganese, and copper), glutathione peroxidase (selenium), catalase and proteins like albumin, transferrin, ceruloplasmin, metallothionein and haptoglobin, others are Coenzyme Q, Ferritin, Uric acid Alpha-lipoic acid Bilirubin, Methallothioneine, Melatonin etc(Rizzo*etal.,* 2010).

2.9.1.2 Exogenous antioxidants

The most essential form of exogenous antioxidants are dietary phytochemicals(such as polyphenols, quinones, flavonoids, catechins, coumarins, terpenoids) and the smaller molecules like ascorbic acid (Vitamin C), alpha-tocopherol, beta-carotene, Vitamin-E and their supplements (Prochazkova *et al.,* 2011).

2.9.2 Classification of human antioxidants according to their functions

By functions, antioxidant can be classified into: first line defence antioxidants, second line defence antioxidants and third line defence antioxidants(Pradedova *et al* 2011).

2.9.2.1 First line defence antioxidants

Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), Glutathione reductase (GR) and some minerals like Selenium, Manganese, Copper and Zinc come under first line defence antioxidants (Volkova *et al* 2012).

Catalase (CAT): is an antioxidant enzyme widely distributed in all animal tissues. The enzyme is known to protect the system from highly reactive hydroxyl radicals through hydrogen peroxide decomposition. Depletion of this enzyme may enhance the cellular damage caused by assimilation of superoxide and hydrogen peroxide (Eshek*et al.,* 2016; Abiodun *et al.,* 2014). Among the

antioxidant enzymes, catalases are ubiquitous haeme enzymes that are found in aerobic organisms, ranging from bacteria to higher plants and animals (Khalid, 2007). Catalase has one of the highest turnover numbers of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second (Mates *et al*., 1999).

The reaction of catalase in the decomposition of living tissue is shown below: $2H_2O_2 \rightarrow 2H_2O + O_2$. The presence of catalase in a microbial or tissue sample can be tested by adding a volume of hydrogen peroxide and observing the reaction. The formation of bubbles, oxygen, indicates a positive result. This easy assay, which can be seen with the naked eye, without the aid of instruments, is possible because catalase has a very high specific activity, which produces a detectable response.

The complete biochemical mechanism of catalase is not currently known, (Boon *et al*., 2007) but the reaction is believed to occur in two stages:

H_2O_2 + Fe(III)-E → H_2O + O = Fe(IV) - E(.+)

H_2O_2 + O = Fe(IV) - E (.+) → H_2O + Fe(III) - E + O_2

Here Fe(III)-E represents the iron center of the heme group attached to the enzyme. Fe(IV)-E(.+) is a mesomeric form of Fe(V)-E, meaning the iron is not completely oxidized to +V, but receives some "supporting electrons" from the heme ligand. This heme has to be drawn then as a radical cation (+), as hydrogen peroxide enters the active site, it interacts with the amino acids Asn147 (asparagine at position 147) and His74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly formed water molecule and Fe(IV) = O. Fe(IV) = O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen (Boon *et al*., 2016).

Glutathione peroxidas (GPxs): are members of the family of antioxidant enzymes that scavenge hydrogen peroxidase in the presence of reduced glutathione, and seven isoforms having different substrate specificities and tissue distribution have been identified. Glutathione peroxidase is a selenium dependent enzyme that contains a selenium atom incorporated within the selenocysteine residue (Khalid, 2007) which utilizes the reducing equivalents of glutathione to reduce hydrogen peroxide and it may be the main mechanism for protection against the deleterious effects of hydrogen peroxide.

Superoxide dismutase (SOD): is a group of metallo-enzymes that play a critical role in the first line of defence against oxidative stress caused by free radicals in many organisms. SOD protects cells and cell components against reactive oxygen species (ROS) by catalysing the conversion of oxygen radicals to hydrogen peroxide and molecular oxygen, and thus provides a protective role against oxidative stress (Abiodun*et al.,* 2014; Khalid*et al.,* 2013).

2.9.2.2 Second line defence antioxidants

Reduced glutathione (GSH), Vitamin C, Vitamin E, uric acid, carotenoids, albumin, bilurubin, Vitamin A and flavonoids come under second line defence antioxidants (Carocho and Ferreira 2013).

2.9.2.3 Third line defence antioxidants

Lipase, proteases, DNA repair enzymes, transferases and methionine sulphoxide reductase come under third line defence antioxidants(Lobo*et al.,* 2010).

CHAPTER THREE

3.0 MATERIALSANDMETHODS

3.1 Materials

3.1.1Chemicals/Reagents

The carcinogen used was procured from Sigma Chemical Co, England. Carcinoembryonic antigen assay kit was procured from Diagnostic Automation Inc CA. Others are formal-saline, chloroform, graded alcohol, xylene, paraffin, eosin, hematoxylin, hydrogen peroxide, obtained from the department of Histopathology, Ahmadu Bello University Teaching Hospital (ABUTH). All other reagents and chemicals are of analytical grade.

3.1. 2 Equipment

Equipments are blender, dissecting set, centrifuge, homogenizer, syringe, cannula, microscope, blender, dissecting kits, refrigerator, electrical weigh balance, water trough, cages, feeders, microtome machine, spectrophotometer, ELISA machine e.t.c

3.1.3 Plant Sample collection

Fresh leaves of *Cucurbita maxima* were collected from Ejule in Ofu LGA of Kogi state and taken to the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, for identification and authentication with a voucher number 1077. It was then rinsed rapidly in tap water, dried and ground to powder using laboratory pestle and mortar and was then kept in an air tight container away from light till use

3.1.4 Experimental diets

Grower mash Vital Feed brand produced by UAC company in Jos, Plateau State, of Nigeria was purchased and used in the entire experiment. The feed served as normal diet and was also used as basal diet along with *Curcurbita maxima* to the formulated dietary inclusion of (0% ,2.5%, 5% and 10%). for the treated group.

3.1.5 Experimental animals

Forty two (42) apparently healthy male Albino rats of the Wistar strain weighing 80-100g were purchased from the Animal Unit of the National Veterinary Research Institute (NVRI) Vom, Plateau state, Nigeria. The animals were housed in standard cages with wooden shavings as their beddings at room temperature at the animal house, Department of Biochemistry, Ahmadu Bello University Zaria and allowed free access to water and fed standard diet *ad libitum* for two weeks to acclimatize. Ethical clearance was obtained from the Bioethical Committee of Ahmadu Bello University Zaria

3.2 Methods

3.2.1 Experimental design to assess the chemopreventive effect of *Cucurbita maxima* leaf dietary inclusion on colon carcinogenesis

3.2.1.1Animal grouping

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 supplementation or standard diet for six (6) weeks and subsequently induced with normal saline and MNU for Twelve(12) weeks. The groups are as follows

Group 1: Norma saline, fed with standard feed.

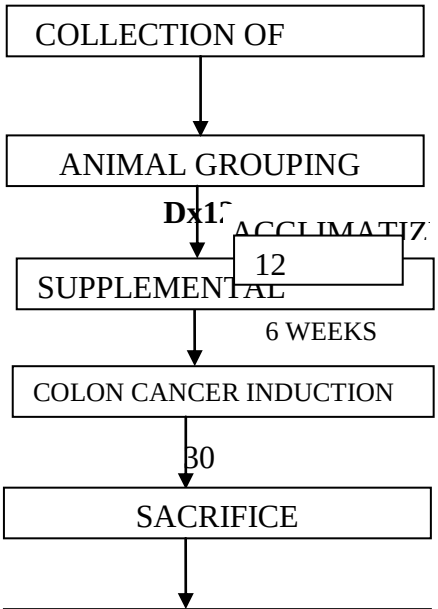
Group 2: MNU induced, fed with standard feed and water *ad libitum*

Group 3: MNU induced, fed with 2.5% *Cucurbita maxima* leaf dietary inclusion and water *ad libitum*

Group 4: MNU induced, fed with 5% *Cucurbita maxima* leaf dietary inclusion and water *ad libitum*

Group 5: MNU induced, fed with 10% Cucurbita maxima leaf dietary inclusion and water *ad libitum*.

Group 6:: Normal Saline, fed, with 10% *cucurbita maxima* leaf dietary and water *ad libitum*



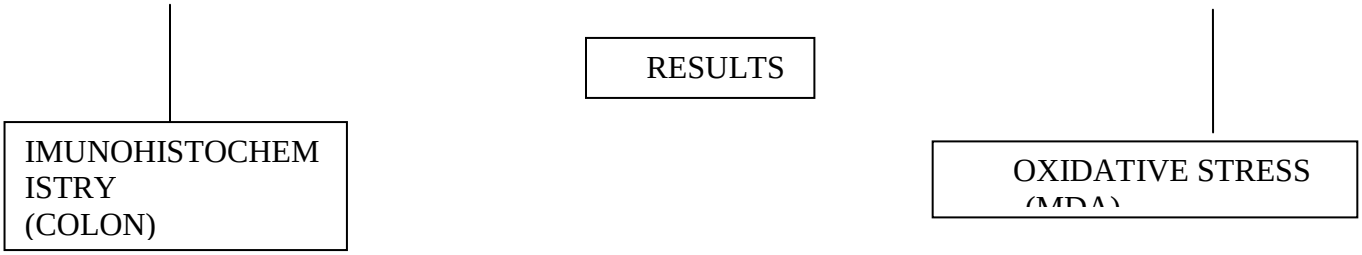


Figure 3.1 Experimental flow chart

3.2.2 Recordsoffoodconsumption and body weight

The food consumption record of animals in each group were maintained weekly. The dietswas weighed prior to the feeding; the left over feeds were also weighed and subtracted from the known mass of food given to the rats. This is to obtain accurate weight of the food consumed. The animals were weighed prior to the commencement of the study and divided into the various experimental groups and weighing continued weekly, changes in body weight of the rats was noted individually to know the difference between the control and experimental groups.

3.2.4 Preparation of MNU and Induction of colon carcinogenesis

All experimental rats received 200µl intrarectal instillation of aqueous solution containing 2.0mg of N-methyl N- nitrosourea. .the administration was performed every 72 hours until the end of the experiment(12 weeks).

3.2.5 Organs/tissue collection

The animals were sacrificed by decapitation at the end of twelveweek after the last MNU injection. The rats were dissected and the colon, liver and kidney were removed and fixed quickly in 10% W/V formal-saline for subsequent analyses.

The rat colon, liver and the kidney were dissected out quickly, washed immediately with ice-cold normal saline to remove blood and weighed (absolute weight) using a digital weighing scale (Halim *et al.,* 2011) and refrigerated.

3.2.6 Tissue homogenization

The colon, liver and kidney (100 mg tissue/mL buffer) were crushed using mortar and pestle and homogenized in 50 mM phosphate buffer (pH 7.4) to obtain a 10% w/v solution .The homogenate were then centrifuged at 10,000rpm for 10 minutes and the supernatant collected with Pasteur pipette was then used for subsequent analysis.

3.2.7 Collection of blood serum

Blood samples were gently collected into a plain tubes and allowed to stand for at least four(4) hours, after which the supernatant serum were gently removed into a new plain enpendiov tube and thereafter stored in the refrigerator for subsequent analysis.

3.2.8Hematological analysis

The whole blood of sacrificed animals were collected into EDTA bottles for analysis of hematological 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 hemoglobin concentration (MCHC). The determination of hematological parameters was done within two hours of sample collection using Automated Hematologicalanalyzer.

3.2.9 Lipid peroxidation by measuring the malondialdehyde (MDA) level

The concentration of thiobarbituric acid reactive substances (TBARS) in the tissue homogenate were 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.1.0ml of thiobarbituric acid (TBA) was added to 1.0ml of trichloro acetic acid (TCA), 50µL of the supernatant of tissue homogenate was added to the mixture and shaken thoroughly. The reaction mixture was then incubated in a boiling water bath at 80ºC for 30 minute, after the 30 minute of incubation, the resulting solution is cooled in ice water bath for 10 minute and centrifuged at 3000rpm for 10 minute and the supernatant collected and the absorbance read at 535 nm against the reagent blank. The blank preparation was performed according to the procedure above except that the sample was replaced with distilled water.

3.2.10 Total protein determination (Lowry's method)

Total protein determination of the colon, kidney liver and serum were determined according to Lowry's method (1999), this was done by preparing two reagent namely: solution A and B, as described in the appendix . The Solution were mixed in the ratio of 50:1 respectively to produce a third solution called solution C (Lowry's reagent) and was kept at 4^0C. Thereafter, 2µL, 5µL and 10Lµ of the sample was added to various test tubes and volume adjusted by a known dilution to 200uL. 2.5ml ofthe freshly prepared solution C was then added to the test tubes and kept for 15min after which 100uL of Folin solution (1N) was added shaken and was allowed to incubate for 30mins and absorbance taken at 750nm. For the standard preparation 0.01g of BSA was added to10mL distilled water shaken thoroughly and stored. Seven test tubes were set and 2mL of the BSA solution was then added to the first test tube to serve as stock and serial dilution was carried out by adding 2mL of distilled water, shaken well and continued up to the last test tube. 200Lµwas taken out of each test tube unto another 7 test tube and 2.5mL of the lowry's reagent addedand shaken properly. 100µL of Folin reagent was then added, shaken and incubated for 30min, then absorbance taken at 750nm. A standard curve of absorbance against concentration was obtained from the reading which was used to calculate the concentration of total protein in the sample by extrapolating known absorbance of sample to the standard of known concentration and absorbance to obtain the unknown concentration of the sample.

3.2.11 Estimation of endogenous antioxidants

3.2.11.1 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 is determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample.

Heamatoxylin $\xrightarrow{\text{Auto-oxidation}}$ H_2O +Heamatin (active form of heamatoxylin)

To 920µL of phosphate buffer (0.05M, pH7.8) was added 40µL of sample. A reagent test was be prepared by replacing the sample with 40µL of sample dilution buffer (0:85% NaCl). The mixture was incubated for 2 minutes at 25^0 C before the addition of 40µL of heamatoxylin. Following the addition of 40µL of heamatoxylin, absorbance of the sample test and reagent test were read at 560nm immediately and after 5minutes against the sample blank which is distilled water. Enzyme activity was calculated.

3.2.11.2 Catalase activity assay

Catalase activity was determined according to Aebi (1974) by monitoring the decomposition of hydrogen peroxide (H_2O_2). The principle is based on the assumption that the enzyme catalase tend to facilitate the breakdown of hydrogen peroxide into water and oxygen, therefore the rate of consumption of the hydrogen peroxide in the sample medium is directly proportional to the activity of the enzyme. 10microLitre of supernatant of tissue homogenate was added to 2800 microLitre of 50mM potassium phosphate buffer (pH 7.0), 100microLitre of 30mM H_2O_2 was added to initiate the reaction. The absorbance of the reaction mixture was taken at 240nm for 5 minute and H_2O_2 consumption was calculated using extinction coefficient, 39.4 mM-1cm-1 and the following formula: Concentration =dA÷E(Mm). U/ml =Conc÷3, U/ml/mg protein= (U/ml)÷ Total protein

where U is the enzyme activity, dA is change in absorbance taken for 5 minute, E is the molar extinction coefficient.

3.2.12 Carcinoembryonic antigen(CEA) assay

Carcinoembryonic antigen is one of the most widely used tumor markers worldwide and certainly the most frequently used marker in colorectal cancer. The concentration of CEA was determined by enzyme-linked immunosorbent assay (ELISA) test which is 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 is 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 are washed with water to remove unbound labelled antibodies. A solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is stopped with the addition of Stop Solution changing the colour to yellow. The concentration of CEA is directly proportional to the colour intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm. The CEA concentration of sample was obtained using the following formulae. Concentration =Absorbance-Blank x standard x dilution factor.

3.2.13 Histological analysis of the organs

The organ tissues were rapidly fixed for 18-24 hours in 10 % formal saline and dehydrated in ascending grades of alcohol (70%, 80%, 90%, 95%, and absolute 100 %) 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 tissue was then 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 (Boano *et al.,* 1999).

3.2.14 Immunohistochemical analysis.

This analysis is based on the principle that the DNA mismatch repair gene protein 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 under microscope when stained with immunochemical reagent dye (eosin and hematoxylin). Inactive gene complex are expected not to be counterstained by the dye thereby retaining the original color of the dye referred to as nor mild expression, while the active MLH1 gene will be counterstained producing a brownish color known as expression or moderate expressions when viewed under the microscope.Samples with brown color on the mucosa cells were taking to be positive active MLHI gene expression with deeper brown color indicating better expressions vice versa, while absence of the brown color were taking as absence of MLHI activity with faint or no color indicating poor expression(Goldstein *et al*., 2007). The method used is the Avidin Biotin Complex (ABC) method also referred to as the avidin biotin Immunoperoxidase method. Exactly 2 microns thick of formalin fixed and paraffin embedded tissues were cut for the IHC. Antigenic sites were retrieved using citric acid (pH 6.0) solution in pressure cooker at 100^0C 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).

3.3 Statistical analyses

The statistical significance between the control and other groups of experimental animals were determined by one-way ANOVA followed by Duncan and Bonfereni multiple comparisons test .Statistical test will be performed at $p \leq 0.05$ level of significance. The results were set as mean ± SD.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of *Cucurbita maxima* Diet Inclusion on some Hematological Parametersof the Experimental Animals

The hematological parameters of treatment groups respectively administerd MNU intra-rectally and orally fed with *Curcurbita maxima* leaf diet inclusion is seen in Table 4.1 bellow with the result showing significantly($p<0.05$) lower PCV(42.65±1.52), hemoglobin(12.16±0.91), red blood cell(7.10±0.26) and higher white blood cell(19.53±0.89) in the MNU control group when compared to the normal control and the treatment groups .The group taking the highest percentage inclusion(10%+MNU) did not show any statistical significant ($p<0.05$) difference in PCV, hemoglobin and total red blood cell level when compared to the normal control while other percentages of inclusion groups (2.5% and 5%) showed lower values of PCV when compared with the normal control group The dietary inclusion control group showed a significantly ($p<0.05$) higher PCV (55.80±1.02), and hemoglobin(18.63±0.35) values when compared to normal control values of 49.90 ±1.00 and 16.30±01.00, respectively.

Table 4.1 : Hematological Parameters of Experimental Animals Administered MNU and Fed with*Curcurbita maxima* Leaf Dietary Inclusionfor 12 weeks

n=6; values are in mean± standard deviation; values with different superscript down the columns are significantly different at P<0.05

TREATMENT	PCV (%)	HGB(g/dl)	WBC($X10^{9}/L$)	RBC($x10^{12}/L$)	NEUTROPHIL(%)	LYMPHOCYTES(%)
0%+NS	49.00±1.00^{a}	16.30±1.00^{b}	9.46±2.20^{a}	8.60±0.10^{a}	23.67±1.15^{a}	58.33±4.24^{a}
0%+MNU	42.65±1.52^{b}	14.16±0.91^{a}	19.53±0.89^{b}	5.10±0.26^{b}	35.00±2.0^{c}	72.33±4.04^{b}
2.5+MNU	45.26±1.15^{c}	15.20±0.84^{c}	16.0 ±3.00^{bc}	7.10±0.20^{a}	29.33±1.15^{b}	79.67±4.08^{b}
5%+MNU	49.67±0.57^{c}	16.20±0.67^{c}	16.53±1.28^{c}	8.26±0.57^{a}	29.00±1.73^{b}	73.33±4.16^{b}
10%+MNU	51.33±0.86^{a}	17.30±0.02^{b}	7.13±0.57^{d}	8.4±0.23^{a}	25.00±2.64^{b}	62.00±3.46^{c}
10%+NS	55.80±1.02^{d}	18.63±0.35^{d}	9.06±0.90^{a}	8.77±0.25^{a}	21.33±5.50^{a}	53.3±3.46^{a}

4.2.Effect of *Cucurbita maxima*leaf dietary inclusion on MDA Concentrations in exprimental animals

The level of lipid peroxidationin the liver and colon of the experimental animals administered MNU carcinogenintra-rectallyfor 12 weeks with corresponding percentages of dietary inclusion is shown in TSable 4.2 below. The result shows the MNU control grouphaving the highest level of oxidative stress($p<0.05$) in the colon(78.51±6.88) andliver (14.62±1.57) when compared to the normal control and dietary inclusion groups. It could also be seen that when dose of *Cucurbita* maxima diet was increased, There is a slight lower level of MDA in the dietary inclusion control group of the liver(10.71±0.40), colon(52.08±7.63) when compared to the normal control groups. The resultsalso shows a dose-dependent decrease in level of peroxidation in the colon of the experimental animals when the percentage of *Cucurbita maxima*wereincreased in the diet 2.5%(71.65±12.22), 5.0% (67.94±4.08) and10% (66.47±3.89).

Table4. 2:MDA Concentrations in Experimental Administered MNU Intra-rectally, fed withdifferent Percentages of*Curcurbita maxima* Leaf DietaryInclusion for 12 Weeks

	MDA Concentration (ɳ M)	
	LIVER	**COLON**
0%+NS	11.83±0.86[a]	56.30±5.82[a]
0%+MNU		

	14.62±1.57[b]	78.51±6.88[b]
2.5%+MNU	11.85±0.18[a]	71.65±12.22[b]
5%+MNU	11.53±1.29[a]	67.94±4.08[c]
10%+MNU	11.98±0.58[a]	66.47±3.89[c]
10%+NS	10.711±0.40[a]	52.08±7.63[a]

n=6; values are in mean±standard deviation; values with different superscript down the columns are significantly different at P<0.0

4. 3. Effect of *Curcurbita maxima*leaf dietary inclusionon Superoxide dismutase (SOD) activity in the Liver and Colon of experimental animals

The superoxide dismutase (SOD) activity in tissue homogenate of the experimental animalsadministered MNU carcinogen and fed with *Cucurbita maxima*leaf dietaryinclusionis shown as follows in figure 4.1. The result shows significantly ($p<0.05$) lower SOD activity in the colon and liver of MNU control group when compared to the control groups and treated groups. There is significant difference between the SOD activity of the group fed the highest percentage inclusion anddiet control of *Cucurbita maxima* leaf in the liver and colon.Butcompared with the normal control, there is no statistical ($p<0.05$) difference. Greater SOD activity is seen upon increase in *Cucurbita maxima* leaf supplementation in the liver and colon.

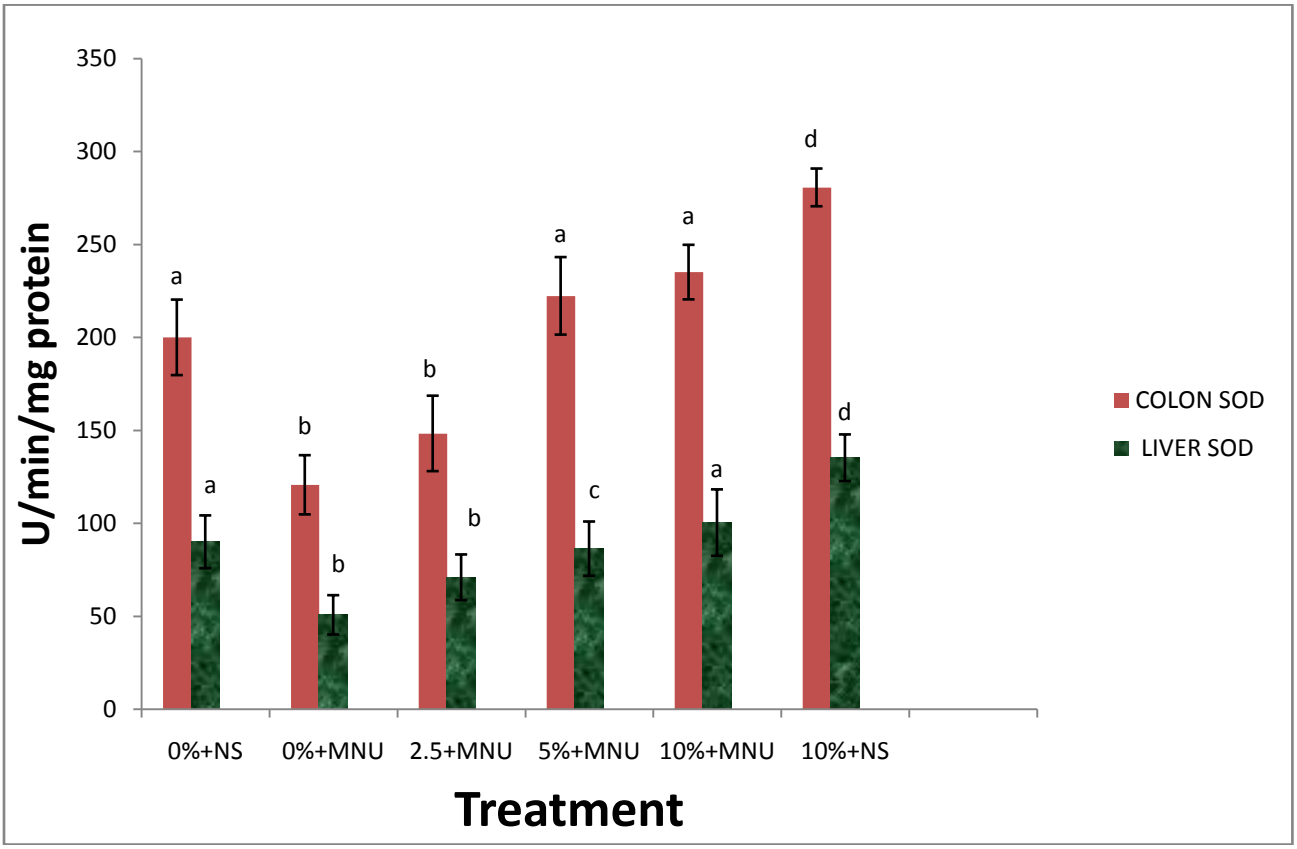


Figure 4. 1: SOD activity in tissue homogenate of experimental animals administered MNU, intra-rectally, fed with Dietiary inclusion of *Cucurbita maxima* leaf for 12 weeks

n=6; values are in mean± standard deviation; values are setat significantly different at P<0.05 with different subscript indicating statistical difference

4.4. Effect of *Cucurbita maxima* leaf dietary inclusion on Catalase activity in the Colon and Liver cells of Experimental animals

Figure 4.2 shows the catalaseactivity in the tissue homogenate of the liver, and colon in the experimental groups administered MNU, fed with *Cucurbita maxima* supplemented diet. The result shows significantly($p<0.05$) higher catalase activity in the colon of the group taken the dietary inclusion without MNU induction when compared to the normal control. The MNU control group shows a significantly ($p<0.05$) lower catalase activity in the colon and liver when compared with the normal control and various treatment groups.increase percentage of the cucurbita maxima leaf dietary inclusion shows an increased(dose-dependent)level of SOD activity in the colon homogenate of the experimental animal.

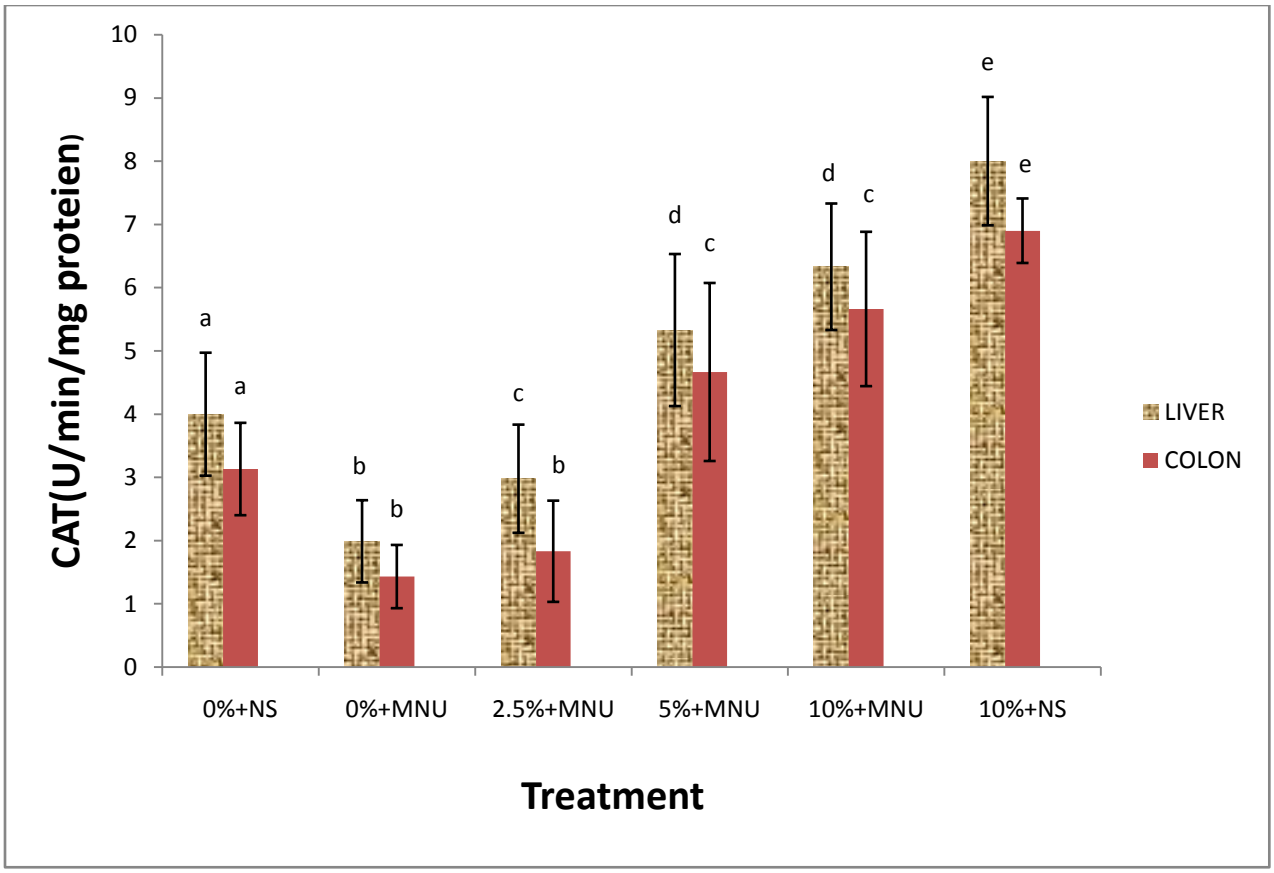


Figure 4:2 Catalase activity in tissue homogenate of experimental animals administerd MNU intra-rectally, fed with Corresponding percentage of Dietary inclusion of *Curcurbita maxima* leaf for 12 Weeks

n=6 values are in mean± standard deviation; values are set in significantly different at P<0.05

4.5. Effect of *Cucurbita maxima* Leaf Dietary inclusion on Carcinoembryonic antigen(CEA) Level in the Serum

The effect of *Cucurbita maxima* leaf dietary inclusion on the CEA level of the experimental animals in various groups administerd MNU intra-rectally for 12 weeks is seen in figure 4.3 below .The result shows a significant ($p<0.05$) high CEA level in the MNU control group when compared with the treatment groups, the normal control group and dietary control group. There is a dose-dependent decrease in CEA value in colon of the treatment groups when the percentage of inclusion of

Cucurbita maxima leaf were increased from 2.5% to 10% and no significant($p<0.05$) differences between the normal control and dietary control group when compared statistically.

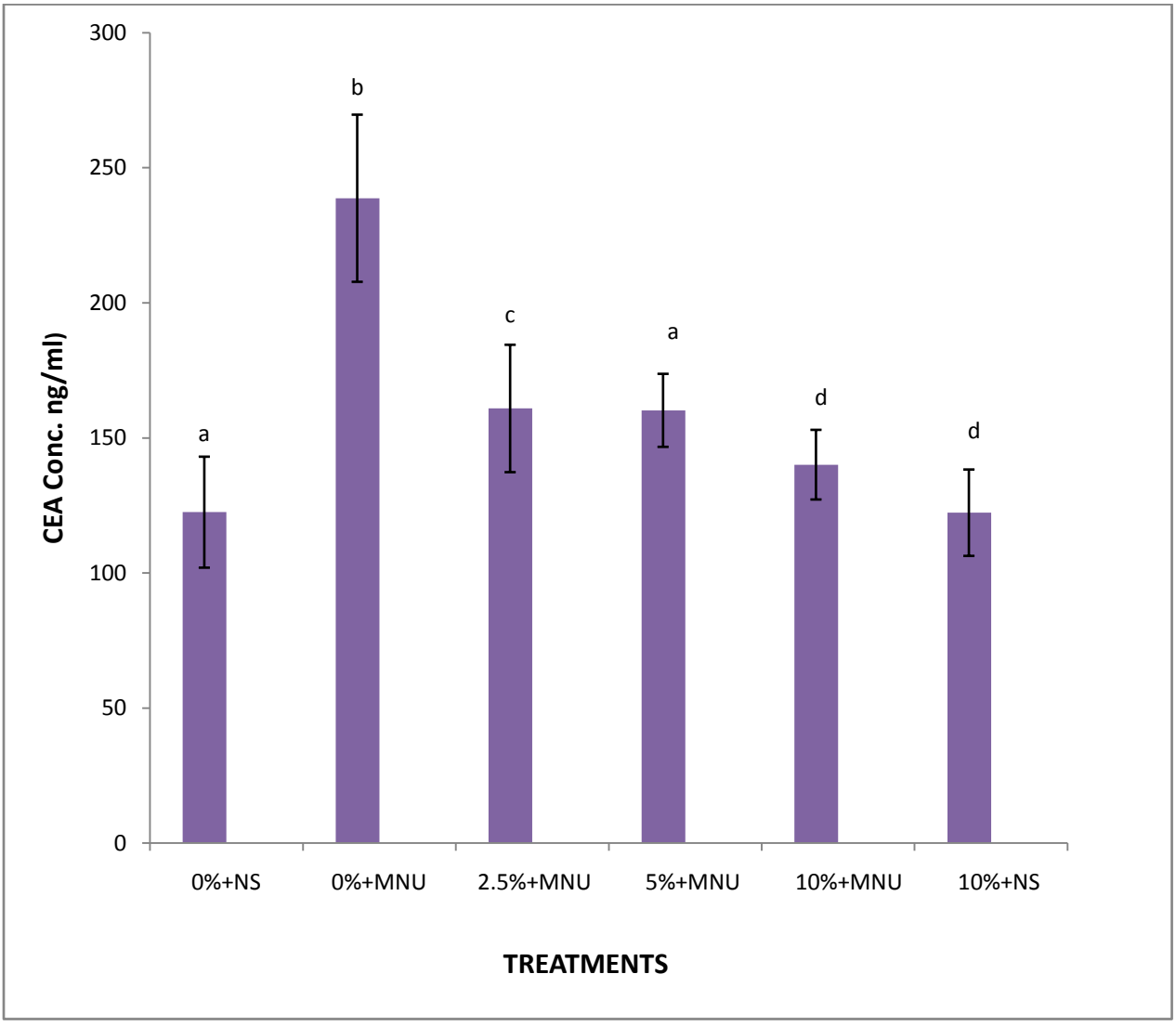
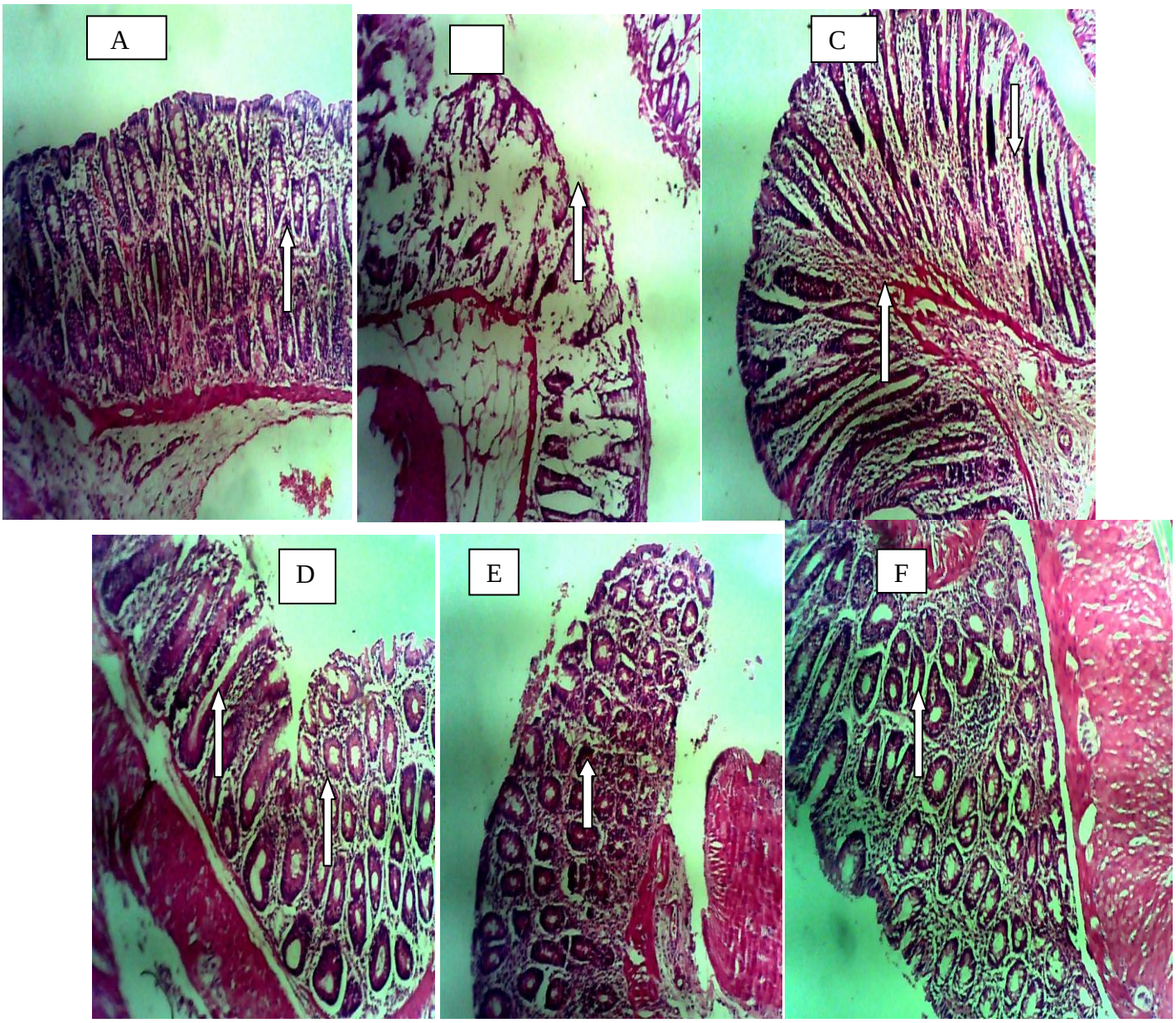


Figure4. 3: Carcinoembryonic antigen concentration in serum of animals administered MNU intra-rectally and fed with *Cucurbita maxima* leaf dietary inclusion for 12 weeks

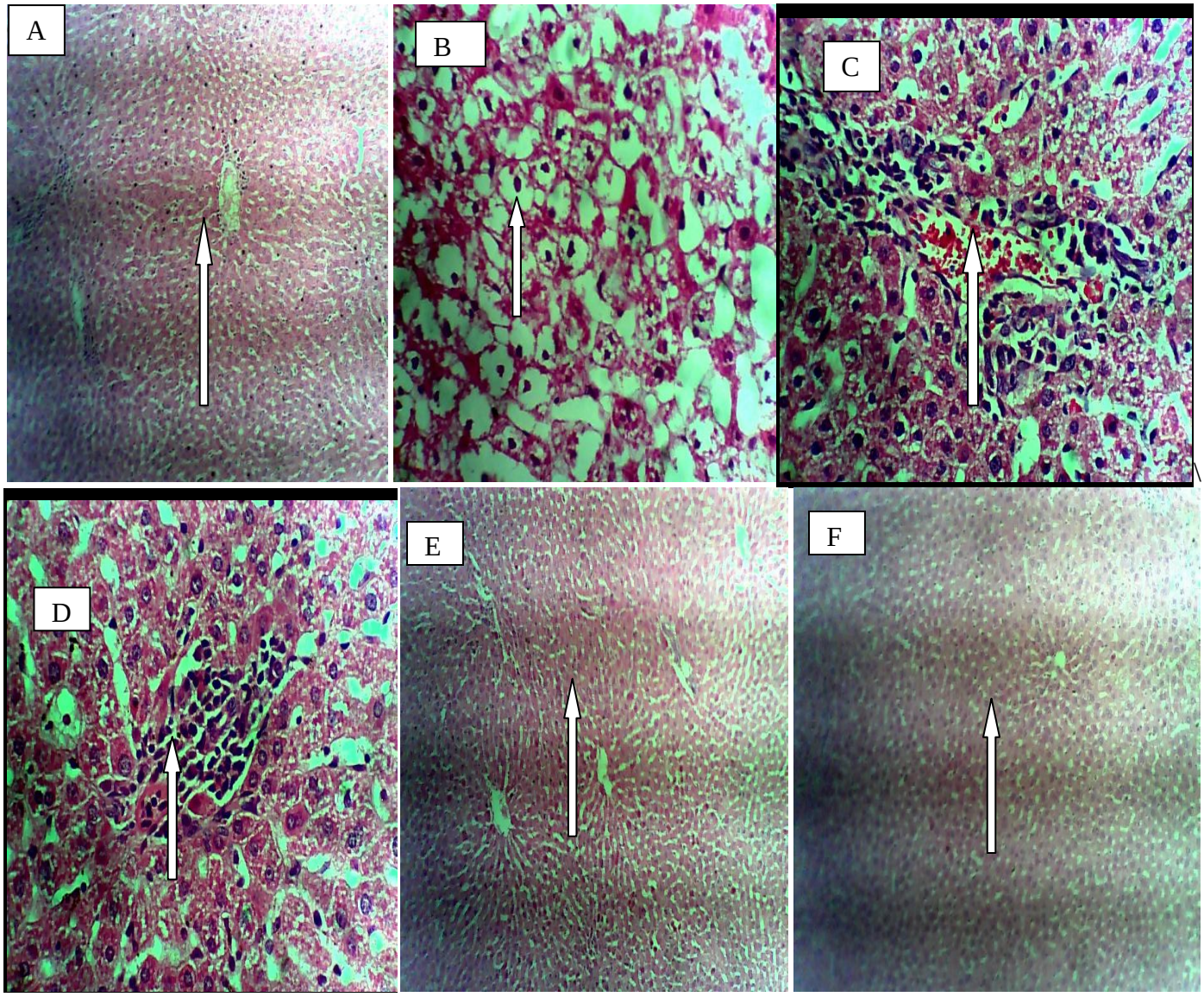
4.6. Effect of *Cucurbita maxima* leaf dietary inclusion on the histology ofthe colon and livertissue ofthe experimental animals.

The histological section of the colon, and liver in various treatment groups administered MNU carcinogen intra-rectally, fed with dietary inclusion of*Cucurbita maxima* leaf for 12 weeks are shown in plate 4-I, and II below, with the MNU control group (B) showing a colon with severe damage to the mucosa cells when compared to the colon tissue of the normal control group (A). A repaired colon cells is seen in the groups administered the various percentages of the dietary inclusion of *cucurbita maxima* leafs which is dose- dependent with 10%+MNU group showing a better preventive damage when compared to the other supplemented groups. The dietary control groups and normal control groups shows an essentially normal colon cell architecture.The livercells of the MNU control group shows mild damages to the hepatocytes (cytoplasmic clearing and focal inflammation within and between the cells respectively) when compared to the normal control and other group which are showing an essentially normal liver cell in the all the percentages of the dietary inclusion groups.



Magnification:(x10). Stains:Eosin and Hematoxylene

Plate 4-I: Representaive photomicrographs in colon tissue section of rats administered MNU intra-rectally and fed with corresponding *Cucurbita maxima* leaf inclusion diet for 12weeks (A) Normal control group and (B)MNU control group showing a severe ulceration specifically deeper mucosal ulceration(C)) 2.5% inclusion with *Cucurbita maxima* leaf induced with MNU showing moderate sclerosis of the mucosa glands, (D) 5.0% inclusion with *Cucurbita maxima* leaf induced with MNU shows mild ulceration and moderate inflammation within the cells. (E) 10% inclusion with*Cucurbita maxima* leaf induced with MNU showing mild ulceration and moderate inflammation within the mucosa glands. (F) 10 % inclusion with *cucurbita maxima* leaf without MNU showing essentially normal colon with mucosa gland intact.



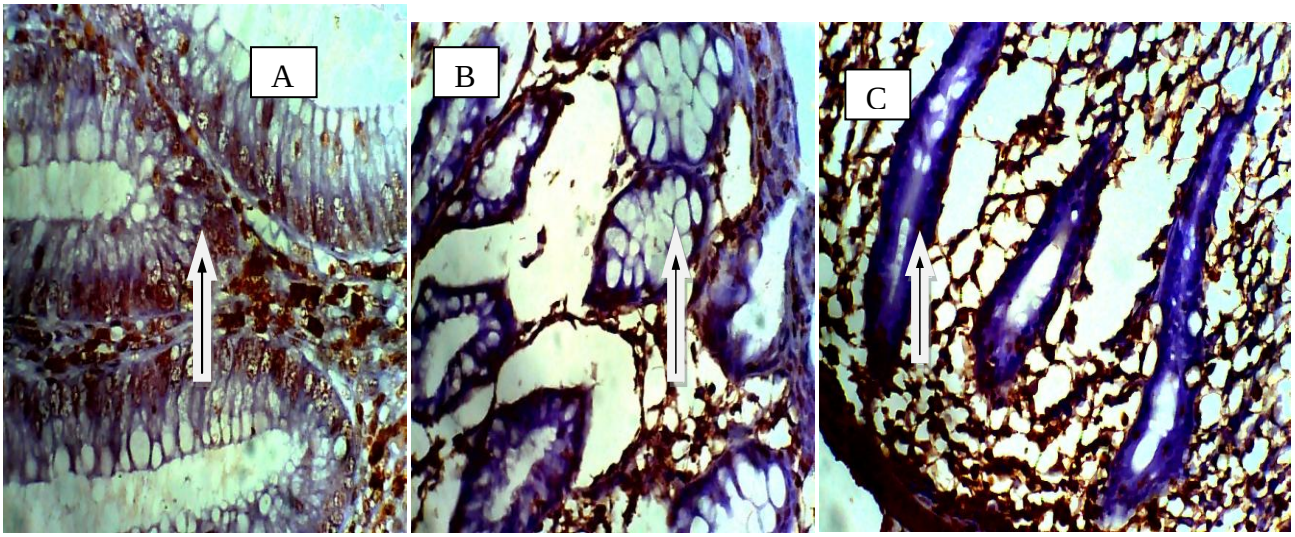
Magnification: (x10)
STAIN:(H&E)

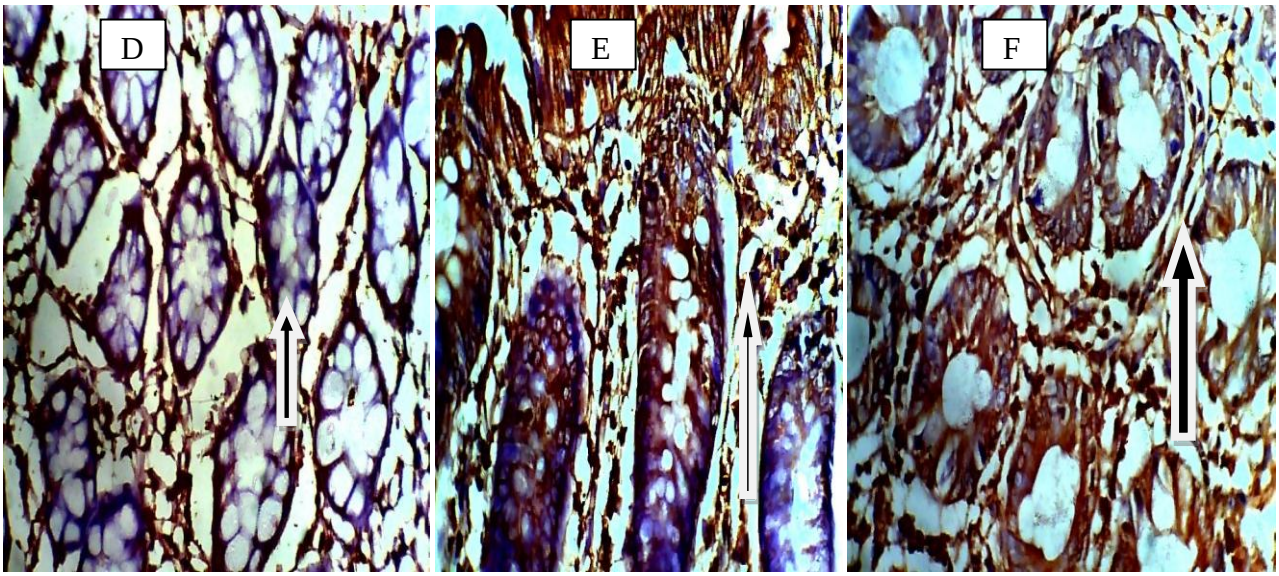
Plate 4-II: Representaive photomicrographs inliver tissue section of rats administered MNU fed with Cucurbita maxima leaf dietary inclusion for 12weeks. (A) Normal control group showing essentially normal liver cells.(B)MNU control shows liver with mild cytoplasmic clearing of the hepatocytes and focal inflammation.(C)2.5% inclusion with *Cucurbita maxima* leaf induced with MNU showing moderate to mild inflammations within the hepatic cells (D) 5% inclusion with *Cucurbita maxima* leaf induced with MNU showing essentially mild inflammation within cells and normal parenchymal architecture of liver with cental vein (E) 10% dietary inclusion with MNU induction showing a normal liver cells (F) 10% inclusion with *Cucurbita maxima* leaf without induction with normal liver cells architecture.

4.7.Effect of *Cucurbita Maxima* Leaf Dietary Inclusion on DNA Mismatch Repair (MLH1)Gene protein in the Colon cells of the experimental Animals

The effect of *Cucurbita maxima* leaf dietary inclusion on the MLH1 gene (DNA mismarch repair gene) expression following admistration of the MNU carcinogen for 12 weeks in albino rat is seen in plate 4-

III below. The results shows mild to no expresion (deep blue colouration of mucosa cells when stained) B in the MNU control groups when compared to the normal control group (A) having moderate expression(brown colouration on mucosa cells when stained). the 2.5% (C),5% (D) and 10% (E) dietary inclusion group with *Cucurbita maxima* leaf also indicates a dose –dependent mild to moderate level of expressions of theMLH1 gene protein when compared





(Magnification: X40)

STAIN:(H&E)

Plate 4-III:Photomicrograph immunochemistry of the Colon in experimental animals intra-rectally administered MNU, fed with dietary inclusions of *Cucurbita maxima* leaf for 12 weeks.

CHAPTER FIVE

5.0 DISCUSSION

Phytochemical analysis of the plant *Curcurbita maxima* from previous work shows that the plant contains Vitamin A, betacarotene, thiamine (Vitamin B1), riboflavin (Vitamin B2), niacin(Vitamin B3) pantothenic acid (B5) Vitamin B6, folate,Vitamin C, Vitamin E, Calcium, Iron, magnesium, phosphorus, potassium, sodium, zinc, significantly iron and Vitamins like B6, B9 which are vital for the formation of RBC and HB(Yongabi *et al*.,2014).This might give an explanation as to why the group of animals fed with the highest percentage of Cucurbita maxima leaf inclusion only, shows a significant ($p<0.05$) increase in PCV, HB and RBC level (Table 4.1) when compared to the normal control which is evident also in the work of Yongabi *et al* (2001) showing thatthe leaves of *Cucurbita maxima* plant is antianeamic by possessing the ability to boost PCV, RBC and Hemoglobin count in albino wistar rats. Cancer disease has been seen to be associated with anemia

(Peter and Stephen,2013). Therefore, the significantly low PCV, HB and RBC count and high WBC in the MNU control group when compared with the normal control and the group fed with the various percentages of the*Cucurbita maxima* leaf dietary supplement might be attributed to the fact that the continuous exposure of the animals to the carcinogens might have generated free radicals leading to oxidative stress and possibly hemolytic or destruction of the erythrocytes(Hisham *et al.*, 2011).Furthermore exposure of the colon to the carcinogen might have lead to inflammation and in response to this condition the body mobilize its immune response therefore increasing the WBC(Table 4.1). The level of oxidative stress and lipid peroxidation can be estimated by determining the activity of endogenous antioxidants enzymes (SOD and CAT) and thiobarbiturate acid reactive substances (Malondialdehyde MDA, 4-hydroxynonenal HNE) (Khalid *et al.,* 2013; Wilson *et al.,* 2011). The significant increase ($P \leq 0.05$) in the levels of Malondialdehyde (MDA) in the colon and liver cells (Table4.2) of the MNU control group when compared with the normal control and other experimental group, may be as a result of an enhanced membrane lipid peroxidation by free radicals generated and the failure of the antioxidant defense mechanisms in the system to reduce the excessive formation or detrimental effects of such free radicals probably because the endogenous antioxidants have been overwhelmed (Kim *et al.,* 2010). It might probably be due to the continuous exposure of this group to the carcinogen.The low level of oxidative stress and lipid peroxidation upon increase inclusion with the leaf of*Cucurbita maxima*even slightly more than the normal control (figure 4.1) may be due to the high free radical scavenging potential of the plant leaf which is in agreement with the work of (Moumita,*et al.*,2011).

The significant decreased ($P0 \leq .05$) activities of SOD and CAT specifically in the colon and the liver tissues of MNU control group when compared with the normal control (Figure 4.1and 4.2) may be due to high concentration of these free radicals generated by the MNU carcinogen during the 12

weeks of induction.This may have led to decrease level or inactivation (adaptive response) of these endogenous antioxidant enzymes (Showkat *et al*., 2010). However treatment with dietary inclusion with the leaf of *Cucurbita maxima* in the group fed with the highest percentageshowed restored levels of SOD and CAT activities back to normal. Thus, this result suggests that *Cucurbita maxima*leafpossess free radical scavenging activity due to the presence of antioxidant phytochemicals such as phenolic acids, flavonoids and ascorbic acid which may have exerted beneficial action against pathophysiological alterations caused by the presence of superoxide and hydroxide free radicals as well as hydrogen peroxide thereby preventing damage to the colon. The SOD and catalase activities is found to be significantly more pronounced in the colon than the other organ probably because it is the direct point of induction of the carcinogen or the route of administration. The group fed with the dietary inclusion without MNU induction also showed better SOD and CAT activity when compared with the normal control and other groups; this might be due to the high antioxidant composition of the plant (Kim *et al*., 2010),Thereby enhancing the antioxidant status of the animals during the twelve (12) weeks of supplementation.

Carcinoembryonic antigen is a glycoprotein of MW approximately180 kD which are 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 cancer(Nozoe*et al.,* 2001). They are well known biomarker used to determine the extent of preoperative colon cancer,and nalysis for CEA concentration in serum of experimental animal showed a significantly high CEA value in the MNU control group when compared with the normal control groups and dietary inclusion groups. This might be to due to occurrence of carcinogenesis which is in agreement with the work of Robert *etal*.,(2000) suggesting over expression of CEA where ever there is cancer cells. The low dose-dependent CEA values upon increase percentage of inclusion with the leaf of *Cucurbita maxima*

in the diet of the experimental animalswhen compared to the MNU group might be due to high antioxidant boosting ability of the plant preventing oxidative stress thereby preventing carcinogenesis. Also experimental evidence shows that *Cucurbita maxima* leaf contain significant amount of crude fiber which might help to reduce contact between the colon cells and carcinogen thus preventing initiation of cancer. The crude fiber in the leaf of *Cucurbita maxima* might be converted to butyrate which is an inhibitor to tumor growth(Avivi *et al.,* 2000), possibly explaining the reason for the low CEA value especially in the dietary control group.

The histopathological changes in the colon of the MNU-induced control group showed that MNU caused severe damage to the mucosa cells. But a better colon cells architecture is seen following the administration of various percentages of the dietary inclusion of *Cucurbita maxima* leafs with 10%+MNU inclusion group showing the a better damage prevention when compared to the other inclusion groups. This might be attributed to the ability of the various supplemented diet owing to their endogenous antioxidant content,to prevent damage to normal cellular structure such as lipid, protein etc (Peter *et al.*,2013). This was further substantiated by the supplement control groups showing an essentially normal colon with the mucosa intact glands.According to Kaur *etal*., (2011), the abnormalities associated with mismatch repair gene is usually seen in about 95% of hereditary non polyposis colorectal cancer (HNPCC). Immunohistochemistry is beenwidely used in diagnoses ofcarcinogenesisin colorectal 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(Lee 2011). These specific proteinsproduced by the Mult homolog 1 (MLH1) genes are conjugated by their corresponding antibodies to form a complex compounds which are usually picked up by immunochemical dyes a giving and counterstained by hematoxylene stain giving a brown or blue

coloration of the mucosa cells indicatingthe presence or absence of the MLH1 gene respectivelywhen viewed under the microscope The distinct blue color on the mucosa cells of the MNU control group(D) and the 2.5 % dietary inclusion group when compared with the normal control (A) might possibly be due to damaged or mutated MLH1 gene which is also in agreement with the work of(Kaur *etal.*, 2011). Also defect in the DNA mismatch repair gene like the MLH1 gene can lead to itsinability 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 (Boland et al., 2008). The brown coloration seen in the 5% (F) and 10% (C) dietary inclusion group might be as result of the preventive measure of the plant against damage to the DNA mismatch repair gene (ie MLH1) by the MNU carcinogen or free radicals generated, this is evident in the free radical scavenging potential of*Cucurbita maxima* leafs(Moumita, *et al.*, 2011).

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CHAPTER SIX

6.0 RESULT SUMMARY AND CONCLUSION

6.1 Summary

A. The study show that, *Curcurbita maxima* leaf dietary inclusion exhibited anti-anemic effect as evident in maintaining the hematological indices like PCV, Hb RBC, WBC e.t.c from haemolysis in the rat induced with the MNU carcinogen, thus the plant has the ability to boost hematological parameters

B. Dietary inclusion with the leaf of *Cucurbita maxima* was able to significantly ($P<0.05$)prevent lipid peroxidation or oxidative stress in both the colon and liver of the albino ratsagainst MNU carcinogen toxicity, this prevention is dose –dependent with higher prevention observed in the 10% dietary inclusion of *Cucurbita maxima*.

C. The dietary inclusion with the leaf of *Cucurbita maxima* was able to restore and maintain the endogenous antioxidant (SOD and CAT) activity in the colon and liver of the albino rats.

D. Dietary inclusion with the leaf of C*ucurbita maxima* significantly kept the concentration of the carcinoenbroyoninic (CEA)antigen level low in the serum of albino rats administered MNU and alongside fed with the experimental diet.

E. Better architectural structure is seen in the glands and mucosa cells of the colon in albino rats fed on dietary inclusion of *Curcurbita maxima* leaf both with and without induction with the MNU carcinogen

F. *Cucurbita maxima* leaf dietary inclusion showed a better protection against damages to the DNA mismatch repair gene (i.e MLH1) by the MNU carcinogen

6.2 Conclusion

The results from the present study showed that dietary inclusion with *Cucurbita maxima* leaf can possibly provide a preventive effect against colon carcinogenesis generated by N-methly-N-nitrosourea(MNU), and therefore should be encouraged for inclusion in the daily diet, thus be enlisted as one edible indigenous plant relevant to the fight against colon cancer.

6.3 Recommendation

Elaborate and further experimental design to evaluate the chemopreventive effect of dietary inclusion of*Cucurbita maxima* leaf on other types of cancers is recommended. Also studies can be undertaken to evaluate the ameliorative effect of *Cucurbita maxima* leaf inclusion diet on already grown colon

cancer tumors(e. g effect of inclusion diet on tumor growth). The mechanism by which *Cucurbita maxima* exert its effect can be a subject of further work.

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APPENDIX 1.0

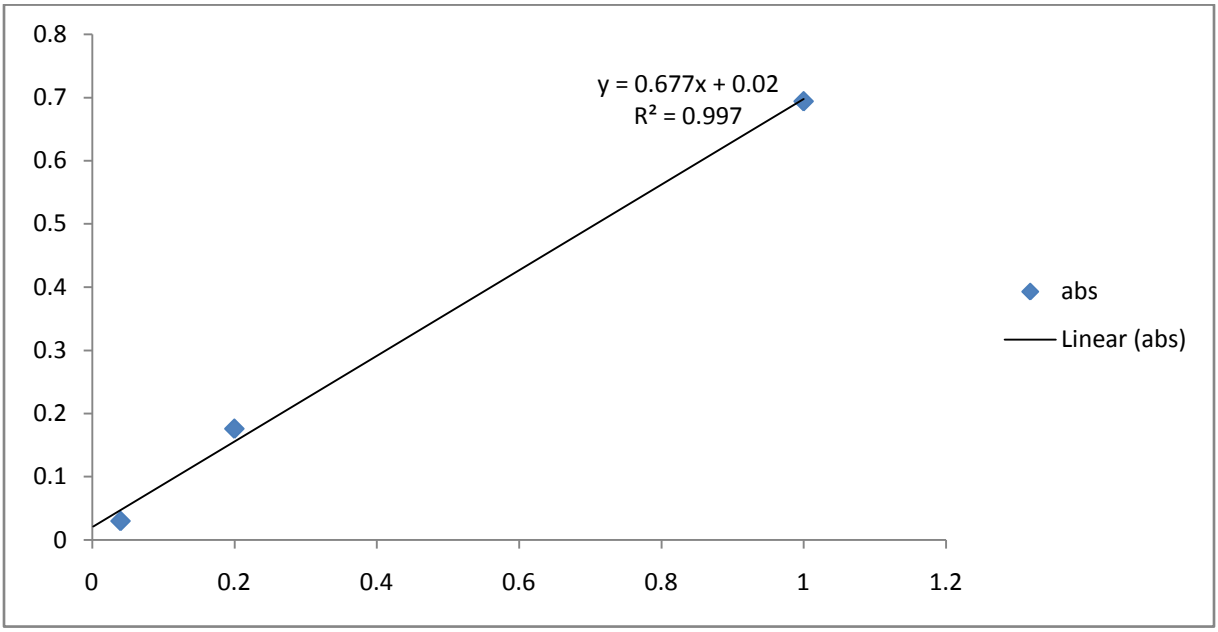


Figure 1: Standard curve for protein determination

APPENDIX 2.0

Table showing effect of *Cucurbita maxima* leaf diet inclusion on CEA Concentrations in Rats Administerd MNU carcinogen intra-rectally, for 12 Weeks

CEA Concentration (n g/ml)	
TREATMENTS	CEA
0%+NS	122.50±20.55[a]
0%+MNU	238.77±38.95[b]
2.5%+MNU	160.91±20.59[c]
5%+MNU	160.21±13.52[c]
10%+MNU	140.09±20.90[d]
10%+NS	122.3± 26.00[a]

n=6 values are in mean± standard deviation; values with different superscript down the columns are significantly different at $P<0.05$

APPENDIX 3.0

SOD Activity in Tissue Homogenate of Treatment groups of Animals administerd MNU and fed with *Cucurbita maxima* leaf Dietary inclusion for 12 weeks.

SOD Specific activity(n mol/ml/mg protein)		
TREATMENT	COLON ($x10^{-3}$)	LIVER ($X10^{-3}$)
0%+NS	237.00±17.32[a]	40.00± 0.35[a]
0%+MNU	145.60±11.93[b]	20.7 ± 0.57 d
2.5+MNU	141.00±5.29[b]	25.00 ± 0.03[d]
5%+MNU	222.33± 3.78[a]	53.30±4.61[c]

10%+MNU	235±18.68[a]	46.33±2.88[b]
10%+NS	251.67± 18.14[c]	50.00±10.00[bc]

APPENDIX 4.0

Catalase Activity in Tissue Homogenate of Experimental Animals induced by MNU Carcinogen, fed with Dietary inclusion of *Cucurbita maxima* for 12 weeks

Catalase activity (n mol/ml/mg protein)

TREATMENT	**LIVER ($x10^{-3}$)**	**COLON($X10^{-2}$)**
0%+NS	4.00±.0.77[a]	3.133±0.41[a]
0%+MNU	1.99±0.80[b]	1.433±0.11[b]
2.5+MNU	3.98±0.25[c]	1.833±0.75[b]
5%+MNU	5.33±0.48[d]	4.667±1.45[c]
10%+MNU	6.33±0.55[d]	4.267±0.15[c]
10%+NS	8.00±1.00[e]	6.933±0.70[d]

APPENDIX 5.0

Description for calculating 100g of the different percentages of *cucubita maxima* leaf dietary inclusion used as experimental diet.

Formulation	powdered leaf (g)	weight of normal feed(g)	Total(g)
%	0	100	100
2.5%	2.5	97.5	100
5%	5	95	100
10%	10	90	100