EVALUATION OF THE CHEMOPREVENTIVE EFFECTS OF *BALANITE AEGYPTICA*AND *ZIZIPHUS SPINA-CHRISTY* SUPPLEMENTON N-METHYL-N-NITROSOUREA INDUCED COLON CARCINOGENESIS IN MALE WISTAR RATS

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Declaration

I declare that the work in this dissertation entitled "EVALUATION OF THE CHEMOPREVENTIVE EFFECTS OF *BALANITE AEGYPTICA*AND*ZIZIPHUS SPINA-CHRISTY* SUPPLIMENTON N-METHYL-N-NITROSOUREA INDUCED COLON CARCINOGENESIS IN WISTAR RATS" has been carried out by me under the supervision of Professor S.E Atawodi and Professor K.M Anigo 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 at this or any other Institution.

Labaran MOHAMMED	_____	_____
MSc/Sci/40725/12-13	Signature	Date

Certification

This dissertation entitled "EVALUATION OF THE CHEMOPREVENTIVE EFFECTS OF *Balanites aegyptica* (Desert date)AND *Ziziphus spina-christy* (Christ's thorn) SUPPLEMENTON N-METHYL-N-NITROSOUREA INDUCED COLON CARCINOGENESIS IN WISTAR RATS" By Labaran MOHAMMED SHUAIBU meets the regulations governing the award of Master's degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

I dedicate this work to Almighty Allah (S.W.T) the compassionate The Merciful Who Alone can do all things by his mighty power for being my source of inspiration and granting me the opportunity to round up my M.Sc. program and also to Alhaji Ahmed Rufa'i who lost his life as a result of car accident I got on my way to school (A.B.U Zaria) from Kano. May his soul rest in peace.

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ABBREVIATIONS

ANOVA -Analysis of Variance

CAT - Catalase

CEA - Carcinoembryonic antigen

DNA - Deoxyribonucleic acid

DOC - Deoxycholate

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EGCG - Epigallocatechin gallate

GTE - Green tea extract

Hb - Hemoglobin

HIV - Human immunodeficiency virus

HNPCC- Hereditary nonpolyposis colorectal cancer

MDA- Malondialdehyde

MMS - Methyl Methanesulfonate

MNNG -N-Methyl-N-nitro-N-Nitrosoguanidine

MNU - N-methyl N-Nitrosourea

RA - Rheumatoid Arthritis

RBC -Red blood cell

RNA -Ribonucleic acid

SOD - Superoxide Dismutase

WBC - White blood cell

Abstract

The colon carcinogenesis chemopreventive potential of different levels of *Balanites aegyptica* and *Ziziphus spina-christy* diets supplemented at 2.5%, 5% and 10% were examined on N-Methyl-N-Nitrosourea induced colon carcinogenesis in male wistar rats. Following ten (10) weeks of induction with N-Methyl-N-Nitrosourea (MNU) and concomitant feeding with supplemented diets, it was observed that groups on diet supplemented with *Balanites aegyptica*and *Ziziphus spina-christy*showed a significant ($p \leq 0.05$) decrease in carcinoembryonic antigen (CEA) levels in a dose-dependent manner. There was a significant ($p \leq 0.05$) increase in the body weight of the MNU treated with 10 % *Balanites aegyptica*and *Ziziphus spina-christy* supplemented diet group. The feeding rate among the treatment groups showed no significant ($p \leq 0.05$) difference. Furthermore, there was no significant ($p \leq 0.05$) difference in white blood cell, red blood cell counts, hemoglobin concentration and platelets. The MNU treated group that the diet was supplemented with the 10% *Balanites aegyptica*and *Ziziphus spina-christy* showed significant increase in superoxide dismutase and catalase activity, while the photomicrograph of the colon of MNU control showed hyperchromiticity, atrophy of gland and necrosis compared to normal mucosal features observed in the normal control with normal colon features. These findings suggest that *Balanites aegyptica*and *Ziziphus spina-christy* especially at 10% supplementation level were able to provide a chemopreventive effect against colon carcinogenesis, probably because of their fiber, polyphenol and flavonoid contents.

CHAPTER ONE

1.0 Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. The causes of cancer can involve external factors such as tobacco smoking, alcohol consumption and exposure to radiation, also genetically inherited factors such as genetic mutations and immune deficiencies (WHO 20014).

Estimated numbers of survivors for the 10 most prevalent cancer sites among males and females in the United States as of 2014 are 6,876,600 males and 7,607,230 females (American Cancer Society, 2014). Also in 2016, the Center for Disease Control listed cancer as the second leading cause of death behind heart disease. The loss of cellular regulation such as the mechanisms governing the control of growth and proliferation of cells often gives rise to cancer.

The National Cancer Institute suggests that there are five main categories of cancer viz-a-viz; carcinoma, sarcoma, leukemia, lymphoma and myeloma, and cancer of the central nervous system. Carcinoma is cancer arising from skin and other epithelial cells and tissue that encase organs in our body and is the most common type of malignant tumor. In contrast to carcinomas, sarcomas arise from our connective tissue such as bone, muscle and fat. Blood cancers such as leukemia arise from blood and bone marrow cells and promote the abnormal proliferation of blood progenitor cells such as leukocytes, (Zieve and Chen, 2009). Lymphoma and myeloma are also cancers targeting the immune system and thus mainly target lymphocytes and white blood cells. Lastly, cancers of the central nervous system target the brain and spinal cord and are thought to be some of the deadliest forms of this disease.

The development of cancer is suggested that initiation, promotion, and progression are the three main phases of tumor formation as reported by (Kufe *et al* 2003).

Cancer of the large bowel (colorectal cancer) includes all cancer originating from the cecum to the anus. Colorectal cancer can be subdivided into colon cancer, which ranges from caecum to the sigmoid (approximately 15 cm above the anal verge), and rectal cancer, that ranges from the recto-sigmoid to the anus. Rectal cancer constitutes approximately 25% of all colorectal cancers. In the year 2014, colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the US. The American Cancer Society estimates that 136,830 people were diagnosed with colorectal cancer and 50,310 people died from the disease. (Labianca *et al,* 2014). In the Netherlands, yearly approximately 8600 new cases of colorectal cancer are diagnosed and colorectal cancer accounts for 4500 deaths each year (Siesling *et al,* 2014).

High incidence rates are found in western world populations such as i.e. Western Europe, North America, and Australia. The lowest rates of colorectal cancer are found in the sub-Saharan Africa, South America and Asia, but are increasing in countries adopting western life-style and dietary habits according to the findings of (Miller andVainio, 2003).

Balanites aegyptica is a species of tree, classified either as a member of the *Zygophyllaceae* or the *Balanitaceae*. This tree is native to much of Africa and parts of the Middle East. (Aluka, 2009).*Balanitesaegyptiaca* is a multibranched, spiny shrub or tree which grows up to 10m in height. The leaves are alternate, two foliate, petioles are 3-6 mm long, leaflets are elliptic and have broadly pointed petioles up to 5 mm long. Fruit is an ovoid drupe, 2-5.6 cm long, found on a short thick stalk, and is faintly five grooved. The ripe fruit is brown or pale brown with a brittle coat enclosing a brown or brown-green sticky pulp and a hard stone seed. Seeds are found exalbuminous and embryo is with thick plano-convex, or two-lobed cotyledons and a superior radical.

Z spina-christi is a shrub sometimes tall tree reaching the height of 20m and a diameter of 60cm; it has very nutritious fruits that are usually eaten fresh or dry. (Adzu and Haruna, 2007)

Ziziphus spina-christi has significance to Jews, Christians and Muslims, and is by some traditions the tree from which Jesus' Crown of Thorns was made. (Zohary, 1972)

However, the scope of this present study is to monitor the effects of*Balanites aegyptica* and *Ziziphus spina-christi*on the activity of Carcino-embryonic antigen (CEA), Superoxide dismutase (SOD), Malondialdehyde (MDA), Catalase (CAT), colon histology and hematology in MNU induced colon cancer wista rats.

1.1 Statement of Research Problem

Colon cancer is estimated to be the second cause of cancer associated-mortality in many developed countries and one of the three leading causes of cancer-death in Nigerian (Ladabaum *et al*, 2003).

Management has not improved beyond surgery with or without adjuvant chemotherapy.

Besides, the costs are quite exorbitant as reported and unaffordable to poorest patients.

1.2 Justification for the Research

Ziziphus spina-christi and *Balanites aegyptica* are easy to cultivate, widely distributed and are being consumed as food throughout the year by many in Africa, Asia and Euroupe. According to Meda *et al*., (2013), Hector *et al*., (2013)*Ziziphus spina-christy*and *Balanites aegyptica* contains polyphenols and flavonoids which have been documented as having anticancer properties.

Most Nigerians cannot bear the cost of cancer screening or the orthodox treatment modalities,Colonoscopies have always been an unpleasant, painful, procedure that patients have

had to undergo, (Wineman, 2003).hence the need to sought a cheaper, easily accessible means of colon cancer management.

Recent urbanization/civilization has resulted in upsurge of confectionary food outlets in major cities resulting in many Nigerians changing their dietary habit from a fibre rich diet, to a highly Westernized diets which are high in fats and refined carbohydrates which increase the risk of colon cancer. Also the significantly lower incidences ofcolorectal cancers in the African countries as compared to Europe and Asiahas been mainly attributed to high fiber content in their diets. Like, *Ziziphus spina-christi* and *Balanites aegyptica* contains Flavanoid and polyphenols (Owen *et al,* 2000).

The majority of these cancers particularly colon cancer and deaths associated with them could be prevented by applying existing knowledge about cancer prevention, increasing the use of recommended screening tests, and ensuring that all patients receive timely, standard treatment. In the past decade, there has been unprecedented progress in reducing colorectal cancer incidence and death rates in the US, largely due to the prevention and early detection of colorectal cancer through screening. However, in 2014 only 59% of people age 50 or older, for whom screening were recommended, reported having received colorectal cancer testing consistent with current guidelines (Howlader *et al*., 2014).

The very challenging pursuits in the realm of pharmaceutical and medical sciences is the search for newer, more potent, safer, easily accessible and less expensive therapies which have the capacity to prevent colon cancer.

1.3 Null Hypothesis

*Ziziphus spina-christy*and *Balanites aegyptica*extracts can neither treat colon cancer nor help to prolong survival.

1.4 General Aim

- The main aim of this study was to investigate the potential of dietary inclusion of *Balanite aegyptica and Ziziphus spina-christy*in the prevention of colon carcinogenesis in Wistar rats.

1.5 Specific Objectives

1. To determine the effect of *Balanites aegyptica* and *Ziziphus spina-christy* on the levels of endogenous antioxidant activities (SOD, Catalase) and level of lipid peroxidation concentration in MNU induced colon carcinogenesis in rats.

2. To evaluate the effect of *Balanites aegyptica* and *Ziziphus spina-christy* on hematological parameters in MNU induced colon carcinogenesis in rats.

3. To investigate the effects of *Balanites aegyptica* and *Ziziphus spina-christy* on colon integrity and some biomarkers of cancer in MNU induced colon carcinogenesis in rats.

CHAPTER TWO

2.0 **LITERATURE REVIEW**

2.1 Colon cancer Development

Colon cancer is the second most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the United State, the leading cancer being lung cancer. The American Cancer Society estimates that 136,830 people were be diagnosed with colorectal cancer and 50,310 people die from the disease in 2014.In developing countries especially west Africa, the rate has not yet reached such magnitude. This suggests that there may be factors either anthropomorphic or environmental which may be responsible for this (Matanuska *et al.*,2015)

According to Abdulkareem, (2009) colon carcinoma or colon cancer is the commonest malignancy of the gastrointestinal tract worldwide. However previous studies had shown it to be a rare disease in Nigeria representing 3-6 % of all malignant tumours in most studies. It accounts for 10-50% of all GIT malignancies in Nigeria. Peak incidence is between the ages of 60-70years. When it occurs in the young, associated with polyposis syndrome or ulcerative colitis should be suspected. Contrary to previous report which showed it to be rare, recent report shows the incidence to be increasing; an 81% increase over a period of two decades was reported from Ibadan. A recent study from Lagos and Sagamu showed similar trend with an increase in annual frequency of this cancer from 14cases per annum to 32.3 cases per annum.

Recent urbanization/civilization has resulted in upsurge of confectionary food outlets in major cities resulting in many Nigerians changing their dietary habit from a fibre rich diet, which was common practice to a highly refined carbohydrate and fat diet. The age incidence of colon cancer in Nigeria is lower compared to developed countries; about 10 years' difference has been

reported in many studies. Peak age reported from Nigeria ranged between the ages of 42.9 years to 53 years with a mean of 46 years. 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 patients are below age 40years. Colon cancer in younger age has been shown to present a diagnostic and therapeutic problem and prognosis tend to be less favorable as reported by (Abdulkareem, 2009).

Colon cancer begins as a benign polyp, a growth of tissue that starts in the lining and grows into the center of the colon or rectum. The benign polyp can develop into an advanced adenoma with high-grade dysplasia eventually progressing to an invasive cancer, (Markowitz and Bertagnolli, 2009).

Colorectal cancer develops in the colon or the rectum, also known as the large intestine, the location of colon cancer is generally centralized in the large intestine whereas rectal cancer is located in the last six inches of the distal portion of the colon. While the exact known causes of colon cancer are yet to be established, known risk factors include smoking and alcohol consumption, age, genetics, personal history of colon polyps, family history of cancer, dietary components, and lack of exercise. Colon polyps and early colon cancers generally reside without symptoms until they are more advanced, thus this disease is often not diagnosed until symptoms are present. In almost all cases, adenomatous polyps are precursors to colon carcinomas and the sequence from adenoma to carcinoma takes several years and can be influenced by factors such as the environment and genetic alterations (Edwards *et al.*, 2014).

Colorectal cancer usually develops slowly, over a period of 10 to 20 years.Most begin as a noncancerous growth called a polyp that develops on the inner lining of the colon or rectum.The most common kind of polyp is called an adenomatous polyp or adenoma. Adenomas

arise from glandular cells, which produce mucus to lubricate the colorectum. An estimated one-third to one-half of all individuals will eventually develop one or more adenomas.Although all adenomas have the capacity to become cancerous, fewer than 10% are estimated to progress to invasive cancer.The likelihood that an adenoma will evolve into cancer increases as it becomes larger.Cancer that develops in glandular cells is called adenocarcinoma. Most colorectal cancers (approximately 96%) are adenocarcinomas (Pickhardt *et al.,*2015).

Once cancer forms in the inner lining of the large intestine, it can grow into the wall of the colon or rectum Cancer that has grown into the wall can also penetrate blood or lymph vessels. (Lymph vessels are thin channels that carry away cellular waste and fluid.) Cancer cells typically spread first into nearby lymph nodes, which are bean-shaped structures that help fight infections. Cancerous cells can also be carried in blood vessels to the liver or

lungs, or can spread into the pelvis and abdominal cavity to other organs and tissues, such as the peritoneum (membrane lining the abdomen) and ovary. The spread of cancer cells to distant parts of the body is called metastasis.

There are five distinct stages of colon cancer that are important in predicting curability and survival. Stage 0 is the earliest stage of colon cancer and includes the formation of polyps in the innermost lining or mucosa of the colon, this stage is also referred to as carcinoma *in situ,* (Zieve and Chen 2009). When the polyp has progressed through the inner lining of the colon to the sub-mucosa, the cancer is considered Stage 1. A large portion of colon and rectal cancers found at the two earliest stages are considered curable. Stage 2 involves the cancer in the colon invading into the surrounding tissue of the colon, the muscle layer, but not to the lymph nodes or surrounding organs. Cancer that has spread to the lymph nodes is considered a Stage 3 colon cancer and once it has invaded other organs in the body, it can be classified as a Stage 4

diagnosis and has the lowest rate of survival (Winawer *et al*., 2014). A diagram depicting the stages of colon cancer is captured below.

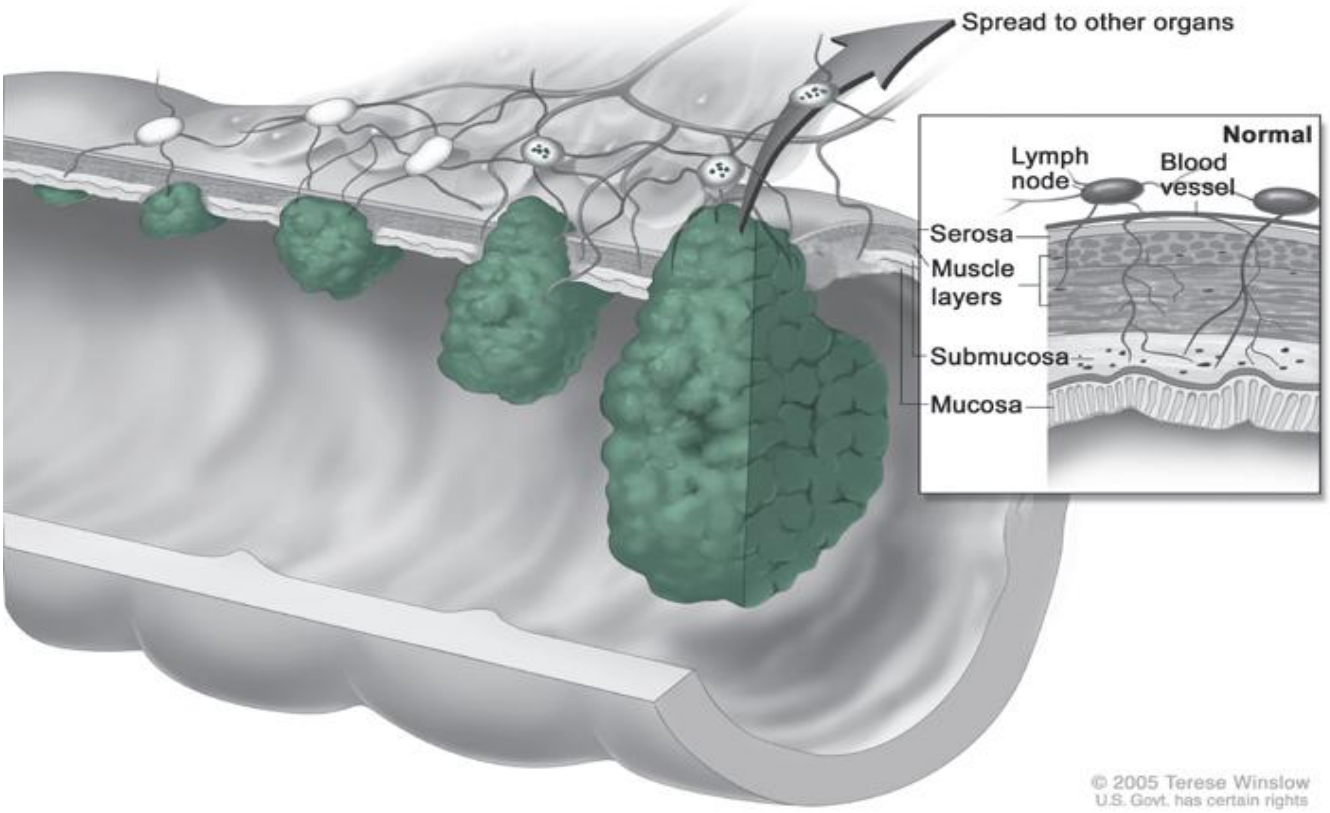


Figure 2.1 Formation of Colon cancer (Source American Cancer Society 2014)

2.1 Molecular Mechanisms Involved in Colon carcinogenesis

2.1.1 Free Radicals and Colon Cancer

When free radicals are produced in excessive and uncontrollable amounts, they and their derivative products may react with various cellular macromolecules, such as lipids, proteins, and DNA and may modulate gene expression. Free radicals were known to play a major role in gene mutations and oncogene overexpression. These are molecules that are unstable because they contain an unpaired electron. When a free radical molecule contacts the electrons of a stable molecule, the free radical molecule gains or loses electrons to achieve a stable paired-electron configuration. In the process, however, the electron balance of the stable molecule is disturbed, and the stable molecule becomes a free radical molecule. In this manner, free radicals initiate a chain reaction of destruction. Free radicals can damage DNA, protein, and fats. Indeed, free radical damage has been implicated as a major contributor to cancer, as well as to other degenerative diseases such as aging, cardiovascular disease, immune dysfunction, brain dysfunction, and cataracts. Free radicals can be produced by a variety of means. They can be produced by external factors such as radiation and cigarette smoke, and by internal events such as immune cell activity and cellular respiration (cellular "breathing" of oxygen) (Valko *et al*., 2015).

Human colonic contents are diverse mixture of bile, mucus, desquamated epithelial cells, various microorganisms and their fermentation products, undigested or unabsorbed food and its metabolic products, such as metals, salts, toxins, mutagens, carcinogens, and dissolved gases (like nitrogen, hydrogen, carbon dioxide, methane, oxygen). It is believed that intestinal mucosa is constantly challenged with diet- and bacterial-derived oxidants and carcinogens. Chronic

exposure of such challenging conditions may then lead to uncontrolled generation of free radicals, redox imbalance, and DNA damage, which can affect intestinal metabolic homeostasis with cancer as an endpoint (Guz *et al*., 2015).

Also in humans, up to 5 percent of oxygen taken in is converted to free radicals during cellular respiration (Reiter *et al*, 2005). During respiration, cells consume oxygen (O_2) and produce water (H_2O). Byproducts of this process include the superoxide radical (O_2–), which can lead to the production of the very damaging hydroxyl radical (OH). The hydroxyl radical is the most toxic of all the oxygen-based free radicals. Other important kinds of free radicals include the peroxyl and the alkoxyl radicals, both of which are involved in lipid peroxidation (oxidative damage to fats). In recent years, the term *reactive oxygen species* (ROS) has been adopted, since it includes the above-mentioned radicals plus hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). While not free radicals in themselves, these two can easily become free radicals in the body. The body maintains a variety of antioxidants as a multilevel defense against free radical damage. These include the enzymes superoxide dismutase, catalase; antioxidants synthesized in the body, such as glutathione, proteins, and uric acid; and antioxidants obtained from the diet, such as flavonoids, vitamins C and E, and beta-carotene.

Nevertheless, antioxidant defenses are not perfect, and DNA is damaged regularly. There may be as many as 10,000 oxidative hits to DNA per cell per day in humans (Ames *et al.*, 2003). The vast majority of these lesions are repaired by cellular enzymes. Those that are not repaired may progress toward neoplasia (the formation of cancer cells). Not only can free radicals initiate cancer, they can also facilitate cancer progression. And in fact, multiple human tumor cell lines have been reported to produce ROS (especially hydrogen peroxide) in vitro, (Szatrowski and Nathan, 2001). Under normal physiological conditions, few cells other than immune cells

produce hydrogen peroxide. Free radical production by tumor cells may help them mutate or display other malignant properties such as tissue invasion. For example, superoxide radicals have been reported to increase the invasive capacity of rat liver cancer cells in vitro as reported by (Shinkai *et al,* 2006). Generally, free radicals affect health negatively when they are overproduced or the body's antioxidant system is overwhelmed.

2.1.2 Cell Proliferation and Colon Cancer

In cancer, the concern majorly has to do with cell proliferation because a main reason cancer cells are so deadly is that they proliferate more during their lifetimes than do normal cells. The process of cellular proliferation takes place in a well-defined sequence of events called the cell cycle.

In brief, the cell cycle is a series of events whereby a cell divides and shares one-half of each chromosome with each offspring. Each offspring thus receives a double strand of DNA from each chromosome and uses it as a template to manufacturer a new, matching double strand; with a complete set of chromosomes, it is then ready to divide itself. The cycle, which takes about 24 hours, is divided into four phases (Ruch, 2004).

i. A gap phase (G1) immediately follows the completion of mitosis (cell division). During this phase, there is active gene transcription and synthesis of proteins, which serve to meet the needs of the growing new cell. Before leaving this phase, the cell is checked for integrity.
ii. A synthesis phase (S) follows G1, in which DNA is replicated. After mitosis, the cell only contains half the DNA needed to divide again. During DNA replication, the coiled DNA is relaxed by enzymes called topoisomerases, and the double strand is split up the middle. A new double strand is then synthesized (through the actions of the enzyme DNA

polymerase) to match the original double strand, all of which results in the needed pair of double strands (a complete chromosome).

iii. A second gap phase (G2) follows completion of DNA synthesis. This phase is a resting phase, at the end of which the cell is again checked for integrity.

iv. Lastly, in the mitotic phase (M), the cell divides. Here the chromosomes are split, and an identical set of double- stranded DNA goes to each daughter cell. Before leaving the mitosis phase, the cell is checked once more for integrity.

Thus the integrity of all dividing cells is checked at least three times during the cell cycle, at the ends of the G1, G2, and M phases. During these checkpoints, the DNA is examined to make sure it is undamaged. If it is damaged, an otherwise healthy cell will stop the cycle and try to repair the lesions. If the lesions are irreparable, the cell cycle will stop and the cell will commit suicide via apoptosis to avoid passing on damaged DNA. Apoptosis, or programmed cell death, is an orderly way for cells to die. Arrest at the G1 checkpoint, and possibly others, is mediated by the p53 protein, a transcription factor produced by the *p53* gene. The *p53* gene plays an extremely important role by inspecting and guarding DNA integrity. For this reason, the *p53* gene has been called the "guardian of DNA (Momand *et al*, 2000)

The homeostatic cycles of proliferation and apoptosis are fundamental mechanisms to maintain the intestinal mucosa integrity. However, dysregulation of cell proliferation and apoptosis results in hyperplasia and tumorigenesis. LPA is a potent inducer of proliferation of cancerous cells. Activation of LPA_1 promotes proliferation of DLD1 human colon cancer cells. In HCT116 or LS173T cells, LPA_2 and LPA_3, but not LPA_1, mediate LPA-induced cell proliferation, suggesting that there is a cell-line difference in the relative importance of each

LPA receptors in its ability to induce proliferation of human colon cancer cells (Sancho *et al*., 2016).

On the other hand, mutations in the *p53* gene, which are common in cancer cells, stop *p53* from performing its functions and allow cancer cells to continue to proliferate. In addition to the four cell-cycle phases described above, a fifth phase also exists that is not part of the cell cycle proper. It is an initial gap phase, in which no proliferative activity takes place. Most cells spend the majority of their time in this gap phase, and only enter the cell cycle if they are properly stimulated. This stimulation generally takes place in four sequential steps (Li *et al*, 2009):

a. One or more growth factors must bind to receptors on the cell surface.

b. The signal that is elicited by receptor binding must be transmitted to the cell's nucleus, the process of signal transduction.

c. "Early" genes must be activated to produce proteins. These genes include *fos*, *myc*, and *jun*

d. The fos, myc, and jun proteins, which are transcription factors, must bind to the DNA on other genes to initiate the synthesis of proteins such as cyclins that drive the cell cycle proper.

Cancer cells are no different from normal cells in their need to be stimulated before entering the cell cycle, except that stimulation is excessive in cancer cells.

2.1.3 Epigenetic Changes in DNA During Colon Carcinogenesis

It has become clear in recent years that some inheritable characteristics of cancer cells are not due to changes in DNA sequence (classical mutations) but rather to functional changes in the otherwise normal DNA (Rennie and Nelson, 2008). Unlike classical mutations, these "epigenetic" changes are reversible. This fact is quite important, since it suggests that some malignant characteristics may be normalized under the right circumstances. Epigenetic changes

and their reversal are still poorly understood, but their study may lead to therapies that cause cancer cells to revert to normal behavior. Metastasis, or the spread of tumor cells to distant locations, is one example of a process, in which epigenetic events may play a crucial role. If genetic makeup were the only determinant in the production of metastatic cells, one would expect an exponential increase in metastases from previous metastases. This is generally not the case, rather, it is likely that epigenetic changes play an important role in turning metastasis on and off (Safarians *et al.*, 2006).

Epigenetic alterations are much more frequent in colon cancer than genetic (mutational) alterations. As described by Vogelstein et al 2015 an average cancer of the colon has only 1 or 2 oncogene mutations and 1 to 5 tumor suppressor mutations (together designated "driver mutations"), with about 60 further "passenger" mutations. However, by comparison, epigenetic alterations in colon cancers are frequent and affect hundreds of genes. For instance, there are types of small RNAs called microRNAs that are about 22 nucleotides long. These microRNAs (or miRNAs) do not code for proteins, but they can "target" protein coding genes and reduce their expression. Expression of these miRNAs can be epigenetically altered. As one example, the epigenetic alteration consisting of CpG island methylation of the DNA sequence encoding miR-137 reduces its expression, and this is a frequent early epigenetic event in colorectal carcinogenesis, occurring in 81% of colon cancers and in 14% of the normal appearing colonic mucosa adjacent to the cancers. The altered adjacent tissues associated with these cancers are called field defects. Silencing of miR-137 can affect expression of about 500 genes, the targets of this miRNA (Vogelstein *et al*., 2014).

Epigenetic changes in DNA are characterized primarily by the attachment of a methyl (CH_3) group to a specific location in a cytosine base. Methylation of cytosine is actually the only

known non-aberrant modification to DNA, and it plays a role in determining which genes are activated for transcription. Thus, methylation acts as an intelligent switching system to control the production of proteins needed at different times. In this regard, it is more like a long-term switching system than a short-term one, since methylation patterns are passed from generation to generation. The greater the amount of cytosine methylation in a gene, the less it is expressed. Therefore, depending on the needs of the cell, some genes may be lightly methylated (hypo-methylated) and easily expressed, whereas others may be heavily methylated (hyper-methylated) and silenced. A moderate amount of methylation is normal for most genes. As an analogy, we can think of the methyl groups added to a gene as a series of "do not disturb" signs. If the gene contains enough of these signs, the cell knows that the gene should not be expressed. For example, one way the body avoids expressing genes that contain virally inserted bases is to attach extra "do not disturb" methyl groups to it (Zingg and Jones, 2007).

Because cytosine methylation is not perceived as a DNA error by the body (unlike mutations, which are errors the body tries to repair), cytosine methylation patterns are purposefully passed on to the daughter cells when a cell divides. Methylation patterns are passed on in the synthesis phase of the cell cycle when the daughter cell is using its double strand of DNA as a template to manufacture a matching double strand.

In addition to epigenetic alteration of expression of miRNAs, other common types of epigenetic alterations in cancers that change gene expression levels include direct hypermethylation or hypomethlyation of CpG islands of protein-encoding genes and alterations in histones and chromosomal architecture that influence gene expression. As an example, 147 hypermethylations and 27 hypomethylations of protein coding genes were frequently associated with colorectal cancers. Of the hypermethylated genes, 10 were hypermethylated in 100% of

colon cancers, and many others were hypermethylated in more than 50% of colon cancers.[1] In addition, 11 hypermethylations and 96 hypomethyaltions of miRNAs were also associated with colorectal cancers. Recent evidence indicates that early epigenetic reductions of DNA repair enzyme expression likely lead to the genomic and epigenomic instability characteristic of cancer (Baldassarre *et al.*,2015)

Specific enzymes recognize the methyl groups on the template DNA and add matching methyl groups to the new strands as they are being formed. Abnormalities in cytosine methylation are very common in cancer cells, and they occur early in the carcinogenic process. Most genes in cancer cells tend to be hypo-methylated and therefore overexpressed. This is particularly true of the many oncogenes that facilitate carcinogenesis. However, a smaller number of genes tend to be hyper-methylated and therefore silenced. This is particularly true of the small number of tumor suppressor genes. One example is silencing the *p53* gene that inspects and protects DNA. Hyper-methylation and silencing can occur in other genes also. For example, silencing of the gene controlling the production of transforming growth factor-beta (TGF-beta) can occur in cancer (Kang *et al*, 2009). Like the proteins made from tumor suppressor genes, TGF-beta is a protein that represses cell proliferation. Therefore, silencing the TGF-beta gene can lead to increased cancer cell proliferation. As another example, hyper-methylation and silencing of the gene that makes the estrogen receptor protein can occur in prostate cancer (Li *et al* 2000). Estrogen inhibits prostate cancer growth, and a lack of estrogen receptors removes this inhibition. Lastly, hyper-methylation and silencing of the genes that make cell-to-cell adhesion proteins (especially the gene controlling the adhesive protein E-cadherin) are also common in many types of cancer as reported by (Tykco, 2000). Reduced cell-to-cell communication, can facilitate cancer progression. Epigenetic changes and mutations work together to give cancer

cells a growth advantage. In fact, epigenetic changes can lead to an increased rate of classical mutations in at least three ways.

First, hypo-methylation of oncogenes or hyper-methylation of tumor suppressor genes make later mutations more likely. For example, hyper-methylation and silencing of the *p53* gene allow mutations to occur with greater frequency, since DNA is not repaired and mutated cells are not forced to die.

Second, when a methyl group is added to cytosine, the combination is easily converted to other bases besides cytosine, thereby producing base substitution mutations (Gonzalgo and Jones, 2007).

Third, the hyper-methylation of genes interferes with their repair if they have been otherwise damaged (Wachsman, 2007). It was classical mutations and epigenetic changes are closely linked. This suggests that by reducing abnormal methylation, mutations may also be reduced. Antioxidants may help reduce abnormal methylation, since oxidative damage to DNA alters cytosine methylation patterns, (Weitzman *et al*, 2004).

2.1.4Mutations During Colon cancer

Mutations play a critical role in both carcinogenesis and cancer progression, since mutations are the force that initiates a cancer cell, as well as the driving force for their survival and adaptation once formed. Two of the most important consequences of mutations are overexpression of certain genes called oncogenes (onco = cancer), which facilitate carcinogenesis and cancer progression, and under-expression of other genes called tumor suppressor genes, which inhibit carcinogenesis and cancer progression (Loeb and Loeb, 2000).

In healthy cells, the normal expression of oncogenes assists in various normal cell activities, including proliferation. However, when overexpressed, that is, when too many of their proteins are produced or they are produced at the wrong times, oncogenes transform healthy cells into cancer cells or assist in cancer progression. Overexpression of oncogenes can be caused by exposure to carcinogens, radiation, or certain viruses. Overexpression can also be caused by free radicals, such as those produced during chronic inflammation according to (Ernst 2009). In contrast, tumor suppressor genes are those genes that, when normally expressed, inhibit carcinogenesis. Thus, *p53* is a tumor suppressor gene. When they are under-expressed or when they mutate to nonfunctional forms, as is common in cancer, carcinogenesis and progression are facilitated.

Selected Oncogenes and Tumor Suppressor Genes

Oncogenes Action

a. B-cell lymphoma-2Protects cancer cells by inhibiting apoptosis (programmed cell death) and by reducing damage from free radicals (Steinman, 2005).
b. c-mycAffects cell proliferation, differentiation, and apoptosis. Normal expression of the gene is needed to initiate cell division as well as to prevent differentiation. Members of this gene family also include n-*myc* and l-*myc*.
c. fos and junEncode proteins that act as transcription factors to facilitate proliferation and other activities. Their proteins help initiate entry of a cell into the cell cycle. In their normal form, these genes are transiently expressed following the stimulation of a cell by growth factors. When overexpressed, they promote uncontrolled proliferation.

d. Receptor tyrosine-protein kinase erbB-2 also known as *c-erb-2* Overexpressed in approximately 33% of breast cancers and associated with a poor prognosis. It produces proteins that can facilitate signal transduction and act in other ways to assist cancer cells.
e. Mouse double minute 2 (MDM2) homologMainly functions to inhibit *p53* activity. In normal cells, the MDM2 protein binds to the p53 protein and maintains p53 at low levels by increasing its destruction. When normal cells are under stress and the activity of the p53 protein is needed, the ability of MDM2 to bind to p53 is blocked or altered in a way that prevents MDM2-mediated degradation. As a result, p53 levels rise, causing apoptosis. In cancer cells, this oncogene is overexpressed, causing chronically low p53 levels and protection from apoptosis (Momand *et al.*, 2000).
f. rasOncogenic forms of *ras* induce several changes in tumor cells, including changes in form and structure and changes in gene expression. The result is excessive DNA synthesis and an increase in chromosomal abnormalities. *Ras* oncogenes are thought to stimulate the production of enzymes (specifically, collagenases) that facilitate tumor invasion. Ras proteins may also protect cancer cells by inhibiting apoptosis, particularly by increasing the expression of *MDM2* genes.

Suppressor Genes Action

Apoptosis regulatorBaxPromotes cell death by competing with *Bcl-2*. While *Bax* acts as an inducer of apoptosis, the formation of *Bcl-2/Bax* conjugates evokes a survival signal for cells. The *p53* gene controls, in part, both *Bcl-2* and *Bax* gene expression.

Genes that produce connexin proteins (e.g., *Cx32* and *Cx43*) Form gap junctions (or portals) between neighboring cells. Communication through gap junctions not only inhibits

carcinogenesis, but restoration of communication also normalizes malignant behavior of cancer cells.

p53Called the "guardian of DNA." Whereas the wild-type (normal) *p53* gene suppresses tumor growth by initiating DNA repair and inducing death of irreparable cells, the mutant *p53* gene acts as an oncogene to allow proliferation of cells with DNA damage. Changes in the *p53* gene are one of the most common mutations found in human cancers.

The root cause of oncogene overexpression and tumor suppressor gene under-expression is gene mutations. In addition, mutations can lead to abnormal production or activity of transcription factors, abnormal signal transduction, or other events that exacerbate oncogene overexpression and tumor suppressor gene under-expression. Base sequence changes are limited only by their toxicity to the cell. If the sequence change is not toxic and the cell does not repair it, the cell survives and the changes are passed on to subsequent generations. Sequence changes are relatively common, and most are repaired by the ever vigilant DNA repair system (Rennie and Nelson, 2008). If the repair system malfunctions, as is the case in some inherited disorders, the risk of developing cancer increases. Even when fully functional, however, the repair system is not perfect, and a small probability exists that damaged DNA will escape repair during any one cell division. Often this is a desirable fault, since slight changes in the DNA are required for evolution and adaptation of the species. Indeed, most species are programmed for a certain low level of ongoing mutations. If the damage is more than slight, however, or if it is in DNA sequences found in oncogenes or tumor suppressor genes, cancer may be initiated. The sequence changes of classical mutations can be cause by a number of events. Some occur spontaneously, and some are due to external causes.

One sequence change that can occur is the transformation of one base into another. Since the structures of the bases are quite similar, some bases are easily converted to others by simply adding or removing a few atoms (in particular, an amino group NH_2) as observed by (Gonzalgo and Jones, 2007).

Other sequence changes are caused by external carcinogens, which alter a base in some way. For example, ultraviolet light can fuse two bases together; alkylating chemicals can add extra small molecules to a base (usually a methyl group, CH_3); free radicals can add an oxygen group to a base; and still other carcinogenic chemicals can add large molecules to a base. If the alteration does not cause the cell to die, the altered base is prone to being mismatched during DNA replication, resulting in a base sequence change. Sequence changes can also be caused by base deletions or additions. Deletions can be large or small and can occur when a base is cleaved from DNA by a misplaced enzyme, or they can be the result of errors during DNA synthesis. Additions can be caused by viruses, which insert new DNA (Gonzalgo and Jones, 2007).

2.1.5Mutator Phenotype Theory and Colon Cancer

The maintenance of a species, and perhaps of an individual, requires that DNA replication be exceptionally accurate. Information contained in DNA must be accurately copied and transmitted to each daughter cell. The fact that the DNA in cancer cells is different from the DNA in normal cells indicates that carcinogenesis involves substantial errors in DNA replication, deficits in DNA repair, and alterations in chromosomal segregation (*Loeb et al., 2014).*

Whether due to classic mutations or epigenetic changes, ongoing genetic alterations are crucial for the continued survival of cancer cells. Cancer cells live in a changing, hostile environment; among other things, immune cells and cancer treatments try to destroy them, the

body in general tries to inhibit their activity in its attempts to maintain homeostasis, and since they overcrowd themselves, they must compete for nutrients and oxygen (Loeb, 2008). Only the strongest cells survive, and as conditions change, cancer cells must also change. The mutator phenotype theory proposes that surviving cancer cells exhibit two characteristics. First and most obvious, they contain specific mutations that allow them to survive the present adverse environment. Second and less obvious, they contain mutations that allow them to mutate easily. In other words, they contain mutations in genes that normally function to maintain genetic stability.

Thus they have the characteristic, or phenotype, of genetic instability, (Loeb, 2008, Loeb, 2001 and Christians *et al,* 2005). The mutator phenotype theory helps explain why cancer cells have a much higher rate of gene mutations than normal cells. Proponents of this theory predict that as analytical techniques improve, perhaps thousands of gene mutations will be discovered in cancer cells, (Jackson and Loeb, 2008). Indeed, no single genetic change is found in 100 percent of any single type of cancer, but a bewildering number of gene mutations have so far been discovered.

According to the mutator phenotype theory, an early event in cancer progression is mutations in the genes that control genetic stability. These include mutations in the *p53* gene, in DNA polymerase enzymes (which catalyze the synthesis of DNA strands during replication), in genes that encode for DNA repair enzymes, and in genes that control chromosome segregation during mitosis. These genetic changes then allow a higher rate of random mutations, some fatal to cancer cells and some that promote their survival. Examples of the latter are mutations that lead to the overexpression of oncogenes. As tumors encounter new obstacles to expansion, a high mutation rate helps assure their survival. In fact, obstacles to expansion such as drug therapy and

competition for nutrients and oxygen may actually increase the mutation rate as tumors attempt to adapt (Ellison and Rubin, 2002)(Jackson and Loeb, 2008). Some genetic changes that occur in cancer cells are epigenetic changes, and as these may be reversible. Aside from reversal of epigenetic changes, it may also be possible to reduce the rate of classical mutations and thereby inhibit the progression of tumors (Loeb, 2008). DNA damage and Mutations require two events: DNA damage and lack of DNA repair. One source of DNA damage that may play a primary role in supporting high mutation rates is reactive oxygen species (ROS). Because of inflammation and other factors, cancer cells normally exist in an environment that is rich in ROS. Indeed, chronic inflammatory diseases, which produce high levels of ROS, have been associated with genetic instability and a high incidence of cancer (Loeb, 2008,Ness and Cottreau, 2009) (Tamatani *et al.,* 2009).

In addition, chronic inflammation has been associated with increased cancer recurrence after surgery (Irani *et al.*, 2009). Some investigators have attempted to explain the involvement of ROS in ongoing mutations and cancer progression through what is called the persistent oxidative stress theory.

Consequences of a Mutator Phenotype

The hypothesis that cancers express a mutator phenotype and that this phenotype drives the proliferation of cancers and generates variants for clonal selection (Fig. below) have both theoretical and practical consequences. Among the theoretical implications are:

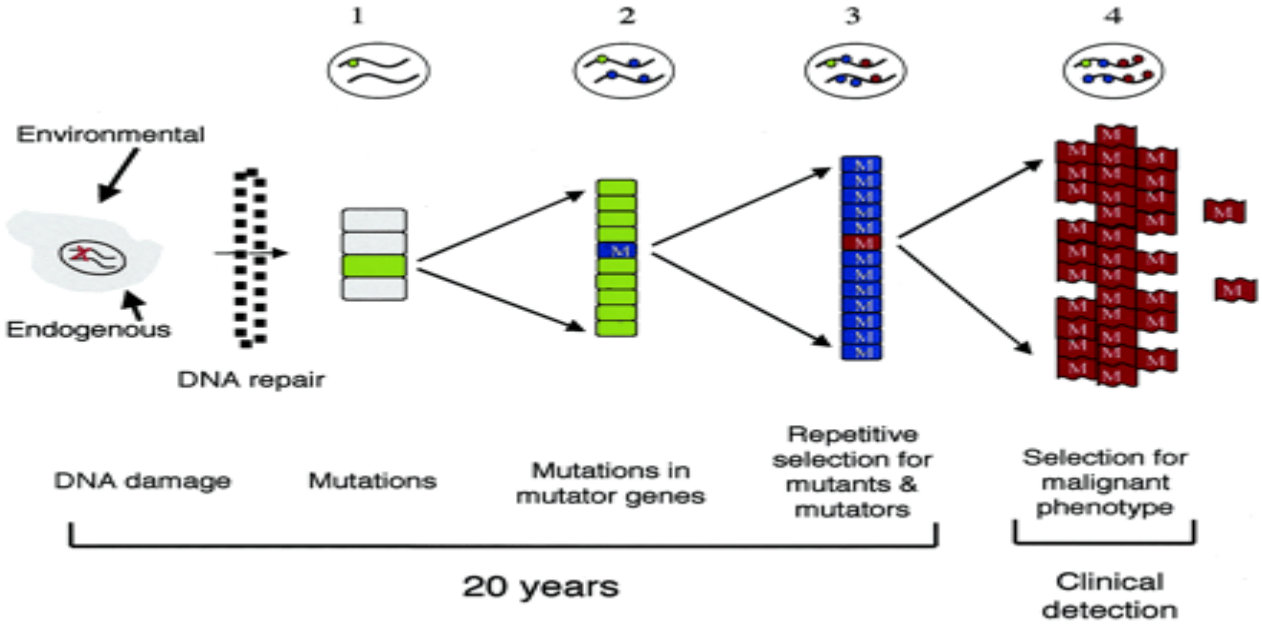


Fig. 1. Mutation accumulation during tumor progression. 1, random mutations result when DNA damage exceeds the cell's capacity for error-free DNA repair. 2, these random mutations can result in clonal expansion and mutations in mutator genes (M). 3, repetitive rounds of selection for mutants yield additional coselection mutants in mutator genes. 4, from this population of mutant cancer cells, there is selection for cells that escape the host's regulatory mechanisms for the control of cell replication, invasion, and metastasis.

Tumor Progression Is Genetically Irreversible

The concept of a mutator phenotype implies that a large number of mutations are produced randomly throughout the genome of cancer cells; only a few of which result in clonal proliferation. This heterogeneity of mutated genes militates against any possibility of reverting cancer cells to wild-type normal cells. Suppression of these mutations can occur by other mutations or epigenetic silencing and therefore reduce the expression of the cancer phenotype, but this would not re-establish the normal genome. Thus, the classical experiments in which tumor cells reverted to normal cells may be at the level of their phenotype but not their genotype. One cannot eliminate the possibility that epigenetic mechanisms drive tumor progression. In particular, the silencing of DNA repair may facilitate the production of the multiple mutations found in tumors. Silencing of gene expression may be a requirement for cancer cells to tolerate the introduction of aneuploidy. Although strong arguments can be invoked for the role of epigenetic mechanisms in carcinogenesis, we still lack sufficient knowledge of these mechanisms to design critical experiments to validate these concepts (Meyn 2013)

The Types of DNA Damage and Mutations Found in aTumor Are Unlikely to Yield Clues about Initiating Eventsin Carcinogenesis

For solid tumors, it takes 20 years or more from the time of exposure to a chemical carcinogen until the clinical detection of a tumor. Thus, it seems improbable that DNA lesions initiating the carcinogenic process would still be present in tumor cells many generations later, unless ongoing DNA damage by the same agent is required for tumor formation. Moreover, the mutations found in a tumor may not result from the most frequent chemical lesions in DNA.

Most DNA-damaging agents cause a variety of chemical alterations in DNA; the most frequent lesions may not be the most mutagenic.Despite the above argument, there are reports that exposure to a specific agent produces a specific mutation. This could indicate that repetitive exposure to a specific agent could be the driving force in clonal selection as might occur in UV-induced skin cancers, aflatoxin-induced liver cancers, and perhaps smoking-associated lung cancers. Exposure to UV induces skin cancers, and the mutations detected in the p53 gene are found opposite pyrimidine dimers and involved T→C or CC→TT tandem substitutions that are characteristic of UV damage. Areas with aflatoxin contamination have increased incidence of primary hepatomas, and p53 mutations occur predominately at codon 249 and involve G→T substitutions. A less compelling case is provided by the G→A transversions found in the p53 gene in lung cancers. Although a similar substitution is found upon exposure of human epithelial cells to benzo[a]pyrene in tissue culture, G→A transitions are also the result of errors by DNA polymerases. The major question is whether these mutations are diagnostic of specific adducts produced by the carcinogens, or do they reflect selection of mutations in a random population that promote clonal proliferation Tlsty *et al*.,(2014)

Benign Cancers Contain Multiple Mutations

The arguments suggesting that the acquisition of a mutator phenotype is an early event in carcinogenesis imply that early tumors such as colon adenomas and uterine hyperplasia and leiomyomas should contain microsatellite and chromosomal aberrations. Evidence supports this assertion. With new techniques it should soon be possible to recreate the archeology of mutated genes that promote clonal expansions as tumors progress (Dreher and Junod, 2006)

Late in Tumorigenesis, Selection May Be against aMutator Phenotype

A mutator phenotype may be an early event that drives tumor progression, yet it may no longer be present by the time a tumor is clinically apparent. Although many mutations in cancer cells are neutral or advantageous, some will be deleterious. Thus, there is likely to be a critical threshold, after which selection will be against a mutator phenotype. As a result, tumors may no longer exhibit a mutator phenotype but will never the less reveal its history, random mutations throughout their genomes.

Tumors Contain Cells with Preexisting Drug-resistant Mutations

If one considers the likelihood that a mutator phenotype is an early event in carcinogenesis and the 20 years it takes for a cancer to become clinically apparent, it seems probable that each tumor, consisting of millions of cells, contains one or more cells that have already accumulated mutations rendering them drug or antibody resistant. The preexistence of these mutations was first demonstrated by Tlsty *et al*.,(2014) ; tumor cell lines, but not normal cell lines, rapidly amplified genes, rendering them resistant to chemotherapeutic agents. Similarly, it is unlikely that there will be antigens that occur in all tumors of a given type and render these tumors susceptible to immunotherapy. The current success of immunotherapy is primarily based on overexpression of normal antigens by specific tumors. Instead, the concept of a mutator phenotype suggests that immunity may have to be tailor made to individual tumors.

2.1.6 Persistent Oxidative Stress Theory and Colon Cancer

There is growing support for the concept that reactive oxygen species (ROS), which are known to be implicated in a range of diseases, may be important progenitors in carcinogenesis.

When free radicals are produced in excessive and uncontrollable amounts, they and their derivative products may react with various cellular macromolecules, such as lipids, proteins, and DNA and may modulate gene expression (Valko *et al*., 2016)

The persistent oxidative stress theory proposes that the chronically elevated levels of ROS to which cancer cells are exposed contribute to their survival and progression, (Toyokuni *et al.,* 2005), (Clutton *et al.,* 2006), (Dreher and Junod, 2006); (Kondo *et al.,* 2009). If extreme enough, oxidative conditions do stress cancer cell populations, killing a percentage of the cells.

However, it is now well established that mild levels of ROS can stimulate cell proliferation and cancer progression, (Burdon *et al.,* 2005), (Arora-kurungati *et al.,* 2009), (del Bello *et al.,* 2009). For example, a recent study on patients with colorectal cancer reported that carcinoma cells, but not corresponding normal cells or benign tumors, were oxidatively stressed (as measured by oxidative modifications to DNA bases). This study also reported that cancer cell proliferation rates were positively correlated to oxidative stress levels, (Kondo *et al.,* 2009). Other studies have also reported that cancer cells exhibit more DNA damage than adjacent normal cells and that cancer patients show higher levels of ROS production and DNA damage than healthy subjects, (Toyokuni *et al.,* 2005). Clearly, one way by which oxidative conditions can facilitate cancer progression is by increasing the rate of classical mutations.

Colon cancer originates from the epithelial cells that line the bowel. These cells divide rapidly and have a high metabolic rate, which has been found as a potential factor that may be responsible for increased oxidation of DNA. Study on primary rat colonocytes has shown that cells from lower crypt sections are more sensitive towards hydrogen peroxide damage than differentiated cells at the surface of the crypt. Since proliferating cells (stem cells and their

dividing daughter cells) in the colon are based in the lower part of the crypt, this may show that proliferating cells are putative target cells of colon carcinogenesis. Stem or progenitor cells have been shown to be very sensitive to the redox environment. Their self-renewal and differentiation depend largely on redox environment in the gut mucosa. Proliferating cells are also exceptionally sensitive against DNA damage because the DNA is present as single strand in the S-phase of the cell cycle and serves as template for the complement strand in daughter cells. DNA damage in single strand could lead to varying mutations in the DNA of daughter cells, which could not be repaired (Foksinski*et al*., 2015)

DNA damage can result in either cell cycle arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with colon carcinogenesis. However, recent evidence has suggested that the generation of ROS may play important role in all phases of carcinogenesis, that is, the initiation, promotion, and progression stages (Valko 2016).

2.1.7 Transcription Factors and Colon Cancer

Colorectal cancer is one of the commonest of solid malignancy in the world. Activating transcription factor 3 (ATF3), a homolog of the mouse TI-241 and rat LFR-1, is a stress responsive gene that has been widely indicated in different malignancies. However, the role of ATF3 in colon cancer is paradoxical with both a suggested pro- and anti-tumorigenic role. These three transcription factors (p53 protein, NF-B, and AP-1) play a vital role in both healthy and cancerous cells. Cancerous cells gain a survival advantage by producing insufficient or nonfunctional p53 proteins and by producing excessive amounts of NF-B and AP-1 (Zhang *et al*., 2016).

The *p53* gene acts as the guardian of DNA, and in the event of DNA damage it performs three crucial functions. First, it halts the cell cycle, for example, by increasing the expression of the *p21* gene. Second, it initiates DNA repair. Third, if the DNA cannot be repaired, it initiates apoptosis, for example, by increasing the expression of the *Bax* gene. To perform these diverse functions, the p53 protein acts as a transcription factor, binding to and initiating the expression of multiple genes. Because of its role in protecting DNA and halting the cell cycle, a functional *p53* gene protects against cancer. Not surprisingly then, mutations of the *p53* gene are very common in cancer cells. The mutated *p53* gene is unable to perform its normal functions, thereby allowing cancer cells to proliferate and increasing the possibility of additional mutations by allowing cells with a mutator phenotype to survive (Ranelletti *et al,* 2000). Moreover, the mutated *p53* gene can produce abnormal proteins that can facilitate cancer progression, (Lanyi *et al* 2008). Mutations in the *p53* gene are associated with increased tumor grade, stage, and proliferation, as well as poor prognosis (Kahlenberb *et al.,* 2000, Blaszyk *et al.,* 2000, Ireland *et al.,* 2000, Geisler *et al.,* 2000), (Symonds *et al.,* 2004). In addition to the outright mutations of the *p53* gene in cancer cells and, subsequently, the production of abnormal p53 proteins, the p53 protein can also be made nonfunctional after its production.

The functionality of the p53 protein is regulated by *ras* activity via the effects of ras proteins on *MDM2* expression and by both the redox status of the cell and trace metal concentrations in the cell, (Meplan *et al,* 2000.,)(Verhaegh *et al.,* 2007)(Meplan *et al.,* 2009). The p53 protein contains ten different cysteine residues, and although disulfide bonds do not actually form between these cysteine residues, a similar type of bond does form, based on the insertion of zinc atoms between the sulfur atoms. These bonds, which help to maintain the active three-dimensional structure of the protein, are redox-sensitive and are altered by oxidants.

Indeed, nitric oxide and other free radicals that bind to cysteine can change the structure of the p53 protein, making it inactive (Sun and Oberley, 2006). Cancer cells tend to be in a pro-oxidant state, and the excess oxidants in such a state may thus help reduce p53 activity.

Nuclear factor-kappa B (NF-B) and activator protein- 1 (AP-1) are transcription factors that control a wide range of cellular activities. Both transcription factors are particularly important to the functioning of immune cells. In general, NF-B can be thought of as a transcription factor whose activity leads to immune activity, inflammation, and cell proliferation, (Biswas *et al.,* 2000, Kim *et al.,* 2000). Like the p53 protein, it binds to and helps regulate several different genes. NF-B helps regulate the genes that produce tumor necrosis factor (TNF), collagenases (enzymes that degrade collagen), cell adhesion molecules (VCAM and ICAM), and immune-stimulating proteins (interleukins 1, 2, 6, and 8, Conner and Grisham 2006, Allison 2007, Winyard and Blake, 2007).

Allison (2007) reported that AP-1 can be thought of as a transcription factor whose activity leads primarily to cell proliferation. Actually, AP-1 is made up of a pair of fos and jun proteins. These proteins stimulate the expression of genes that drive the cell cycle. Thus the actions of fos and jun proteins are mediated, at least in part, through AP-1 activity. In cancer cells, the amount and activity of AP-1 and NF-B are commonly excessive, providing the cells with a proliferation and survival advantage. For example, one study observed excessive activation of NF-B in 93 percent of childhood acute lymphoblastic leukemia patients (Kordes *et al.,* 2000).

Another study observed that the basal expression of NF-B was fourfold higher in metastatic melanoma cells compared to normal cells and that oxidative stress (created by hydrogen peroxide) stimulated a greater expression of NF-B in melanoma cells compared to

normal cells (Meyskens *et al*, 2009). By producing excessive amounts of NF-B and AP-1 or by excessively activating them, or both, cancers cells profit in three ways. First, their proliferation is stimulated. Second, apoptosis is reduced. Third, the inflammation that NF-B causes is of general use to cancer cells. Inflammation can facilitate angiogenesis. Indeed, preliminary experiments have reported that excessive NF-B activity in cancer cells is correlated to invasiveness, metastasis, and poor prognosis (Bours *et al.,* 2004)(Nakshatri *et al.,* 2007).

In similar ways, ROS can also sensitize other kinases that act inside the cell, such as PKC (Gopalakrishna and Jaken, 2000). Activation of the surface receptor (or internal kinases) can be blocked by kinase inhibitors. Likewise, ROS signals originating from activated receptors (or internal kinases or ras proteins) can be blocked by antioxidants. If not blocked, the signals stimulate NF-B and/or AP-1 activity, which in turn stimulate gene transcription. Gene transcription leads to additional inflammation, proliferation, and cell migration. It can be see then that redox status is linked with signal transduction via the protein phosphorylation pathways in at least two ways. First, surface receptor activity and the activity of internal kinases are sensitive to ROS. Second, activation of these kinases, and ras proteins, produce ROS. These internally produced ROS, in conjunction with ROS that enter the cell from outside, affect the activity of transcription factors (Lelkes *et al.,*2008) (Vepa *et al.,* 2009).

It will be noted, however, that the role of ROS in the activation of NF- B and AP-1 is still being debated. It is possible the effects may be limited to specific cell types and conditions, and some effects certainly occur through poorly understood mechanisms, (Renard and Raes, 2009). Quite likely, ROS are not the driving force behind NF-B and AP-1 activation. Rather, their role is probably a supportive one (Bowie and O'neil 2000). If the concentrations of ROS are too

high, they will not participate in signal transduction they will only damage the cell, inducing apoptosis or necrosis.

Snail1 as a transcription factor has been shown to be crucial for cellular movement during cancer progression and metastasis. Therefore, in colon cancer patients, enhanced levels of Snail1 are usually associated with poor clinical outcome, probably due to down-regulation of E-cadherin expression. There is evidence showing that the E-cadherin gene (*CDH1*) is also inactivated in diffuse type cancers such as breast lobular carcinoma and diffuse gastric carcinoma. In these cases, neoplastic cells have lost their epithelial properties and display a highly invasive pattern. The question arises regarding which factors are responsible for E-cadherin silencing during EMT. The continuous expression of this epithelial marker during developmental EMT in Snail1-deficient mouse embryos clearly supports the idea that transcriptional repression of E-cadherin is mostly related to Snail1 activity (Kroepil *et al.,* 2015)

The first stage of colorectal cancer is adenoma. During cancerogenesis this lesion progresses to adenocarcinoma and then to metastatic cancer. Adenoma is also the first stage of colorectal cancerogenesis exhibiting Snail1 expression, which is inversely correlated with expression of E-cadherin. This finding is in agreement with the statement that Snail1 acts as a transcriptional repressor of *CDH1*. It is well demonstrated that repression of *CDH1* is a consequence of molecular reaction cascade, during which a major role is played by interactions with regions containing the consensus core sequence (CAGGTC), a motif that is a subset of E-box (CANNTG). The expression of Snail1 in adenoma tissue suggests that the EMT process promoting cancer metastasis is already active in precancerous lesions. Studies with the use of transgenic mouse models such as HER-2 and PyMT have revealed that tumour cells may disseminate in morphologically benign hyperplastic lesions (Wu *et al*., 2016)

2.1.8 Growth Factors and Signal Transduction Involved in Colon Cancer

Growth factors and signal transduction play crucial roles in stimulating cell proliferation and in maintaining a cell's life. Briefly, growth factors are soluble extracellular proteins that bind to receptors on the outside of the cell. This binding elicits a chemical signal that is transferred to the cell's nucleus through a series of steps called signal transduction. The result is the activation of transcription factors and the initiation of gene expression. In addition to growth factors, cell-to-cell contact can also generate signals at the cell's surface that are again transferred to the nucleus via signal transduction. In normal cells, all these processes are tightly regulated. In cancer cells, however, the regulations fail, and growth factor activity and excessive signal transduction increase "do proliferate" and "do not die" signals, while decreasing "do die" signals. These changes in signals allow the cell to live a long life with many cell divisions, (Dumont *et al.*, 2006).

All cells in the body at every moment are subject to the dynamic balance that exists between pro-life and pro-death signals. Two very basic pro-life signals are "do not die" and "do proliferate"; their counterpart pro-death signals are "do die" and "do not proliferate." All of these signals exist more or less simultaneously, and their relative strengths decide a cell's fate. Of these signals, "do die" and "do not proliferate" seem to be the defaults. That is, if cells are not told to live, they will undergo apoptosis, and if they are not told to proliferate, they will not enter the cell cycle. The signals that tell a cell to live or to proliferate generally come from outside the cell, commonly from contact with growth factors and from contact, or lack of it, with other cells and tissues. To illustrate how these signals, occur in normal tissues, consider the liver tissue of a healthy animal. The liver cells are in close contact with one another, both through actions of cell adhesion molecules on the surface of the cells and through gap junctions (portals) between cells. The liver cells do not undergo apoptosis, since contact with neighboring cells continually

generates “do not die” signals. Also, they do not enter the cell cycle because there are few “do proliferate” signals. Now imagine that the animal receives an injury that slices away a small section of its liver. The injured area soon becomes inflamed and steeped in growth factors, (Korutla and Kumar, 2004). These factors, produced by immune cells, released from the blood, or derived from other sources, cause various types of cells to proliferate. For example, blood vessel cells proliferate to replace those damaged in the injury, and liver cells are also stimulated to proliferate by these growth factors. In addition, the reduced cell-to-cell contact at the edge of the healthy liver tissue signals that new liver cells are needed. In response to the growth factors and signals from reduced cell-to-cell contact, stem cells or other poorly differentiated cells in the liver enter the cell cycle and proliferate. The entire repair process and all the cell proliferation that goes on in it is wondrously orchestrated so that once tissue repair is complete, no new tissue is produced, (Liang *et al.,* 2009) stated previously that cell-to-cell contact is needed to generate “do not die” signals, but then research shows that liver cells are stimulated to proliferate by lack of cell-to-cell contact. This apparent contradiction can be explained by considering that the growth factors present at the wound site provide signals that both stimulate proliferation and mimic the “do not die” signals normally originating from cell-to-cell contact.

In addition, the liver cells at the edge of the injury are only partially separated from other liver cells. The situation with cancer cells is both like and unlike the example of injured liver tissue. It is similar in that cancer cells, like liver cells, are stimulated to proliferate and not to undergo apoptosis. It is different in that the proliferation of cancer cells continues indefinitely. Moreover, the sources of “do proliferate” and “do not die” signals are also somewhat different. In both cases, inflammation results in the production or release of growth factors, but in cancer, the cells themselves also produce their own growth factors. In addition, cancer cells can get the

most out of any available growth factors by producing excessive amounts of both the growth factor receptors and the proteins needed in signal transduction. Cancer cells differ from injured liver cells in other ways. For one thing, cancer cells are generally less differentiated than liver cells. Since poorly differentiated cells are prone to proliferate, a relatively large population of cancer cells can enter the cell cycle in response to growth factor activity and signal transduction. In addition, oncogenes within cancer cells can directly produce proteins such as fos and jun that initiate the cell cycle, and ones such as cyclins that drive the cell cycle proper, (Loda *et al.*, 2007). Lastly, Yin *et al*, (2006). states that other gene derangements in cancer cells can lead to overproduction of proteins that protect against apoptosis (such as Bcl-2) and can lead to malfunctions or underproduction of proteins that induce apoptosis (such as p53 and Bax).

All the above factors allow cancer cells to override the complex controls that normally govern proliferation and survival. An analogy can be drawn between cancerous. and injured tissues; the environments of both allow increased cell proliferation, but the former acts as a wound that does not heal, and so its cell population expands without the limits inherent in the final stages of normal healing, (Nordenberg *et al.*, 2006).

Philip and Harris (2005) observed that receptor proteins span the width of the plasma membrane and are therefore able to transfer the signal from growth factors outside the cell to structures within the cell. Once inside the cell, the signal generated at the receptor is transferred to the cell's nucleus by kinase enzymes and other proteins. In many cases, the receptor itself is composed partly of a kinase enzyme. Kinases and other proteins relay the signal from the receptor to the nucleus in much the same way a baton is relayed from start to finish by different

runners in a track race. This relay process is called signal transduction. Researchers commonly use a different analogy and speak of the relay process as a signal cascade, referring to the way water flows over a series of falls. One feature common to the enzymes involved in signal transduction is that when the signal comes to them, they are energized by the attachment of phosphorus atoms. It is as if they receive a shock of energy, and this shock allows them to pass on phosphorus atoms to other carrier proteins, which then carry the signal to the next enzymes. The process of attaching phosphorus atoms to enzymes or other proteins is called protein phosphorylation. Phosphorus atoms are held tightly together by atomic bonds, and when a group of phosphorus atoms is split apart, a large amount of energy is released, (Srivastava, 2005). The phosphorus groups needed for protein phosphorylation are delivered to the enzyme by ATP, the primary energy source in a cell provides a short description of several of the most important growth factors in cancer; the descriptions include the type of receptor protein each growth factor uses. The cellular receptors for most growth factors are protein tyrosine kinases (PTKs). PTKs, but note here that receptor PTKs can be produced by oncogenes and that an entire family of receptor PTKs exists, each with a slightly different function. By producing excessive amounts of PTKs or hypersensitive PTKs oncogenes can allow a cancer cell to become easily stimulated. In addition, oncogenes can also produce growth factors themselves or proteins that act as growth factors. In these ways, they help cancer cells proliferate even in environments that do not favor proliferation.

The growth factors and their receptor growth factor comments and receptor type, (Tannock and Hill, 2002) (Smith, 2007).

i. Epidermal Growth Factor (EGF)

The EGF receptor is overexpressed in many types of human cancers. It can be activated by at least five different growth factors, including EGF and TGF-alpha. EGF receptor binding can produce several effects, including increased cell proliferation, cell motility, invasion, and metastasis, receptor type (PTK)**.**

ii. Fibroblast Growth Factors (FGF)

FGFs such as basic FGF (bFGF) can be overproduced by many types of tumor cells. They are heparan-binding growth factors, which allows them to be stored in the extracellular matrix, the connective tissue surrounding cells and tissues. FGFs stimulate proliferation of many cell types and are involved in angiogenesis, receptor type (PTK).

iii. Insulin-Like Growth Factors (IGF)

IGFs stimulate cell proliferation and share many properties with insulin, except they do not stimulate glucose utilization. They can be overproduced by many types of tumor cells. IGF receptors occur on a variety of human tumors, including breast cancers, receptor type (PTK).

iv. Platelet-Derived Growth Factor (PDGF)

PDGF stimulates the proliferation of epithelial cells and other cells. It is released by platelets to stimulate wound healing and can also be produced by tumor cells,

Receptor type (PTK), **(**Smith, 2007).

v. Transforming Growth Factor-alpha (TGF-alpha)

Like epidermal growth factor, TGF-alpha binds to EGF receptors. It is produced by macrophages, brain cells, and other cells. It induces angiogenesis in vivo, receptor type (PTK).

vi. Transforming Growth Factor-beta (TGF-beta)

TGF-beta is a multifunctional protein that controls proliferation, differentiation, and other cell activities. It can either increase or decrease proliferation, depending on the cell type and conditions. In the early stages of transformation, TGF-beta inhibits proliferation of several cell types; however, advanced cancers can become resistant to its growth-inhibitory actions. In these cancers, TGF-beta can promote invasion and metastasis, partly through its immunosuppressive effects comprised of three distinct proteins.

vii. Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor is also known as vascular permeability factor. It induces endothelial proliferation and vascular permeability and plays an important role in angiogenesis. It is related to PDGF and is produced by epithelial cells, macrophages, and smooth muscle cells, receptor type (PTK). Receptor PTKs are receptor-enzyme units that span the plasma membrane; in technical terms, they consist of an extracellular receptor domain, a trans-membrane domain, and an intracellular tyrosine kinase domain. The intracellular portion consists of the kinase enzyme with a small attached tail. Both the tail and the enzyme itself can receive phosphorus groups from ATP, (Blobe *et al.*, 2004) which energize them. PTKs can also exist within the cell, in which case their structure is different, but here the focus is on receptor PTKs. PTKs derive their name from the high concentration of the amino acid tyrosine in their intracellular portion. Most other PTKs consist of only a single chain, but the process of activation is the same. Briefly, the extracellular portion of the receptor PTK is activated by binding to a growth factor. Binding activates the intracellular portion, where ATP gives up phosphorus groups to the enzyme, thereby producing ADP. The phosphorus signal is carried to another intracellular target, such as another kinase, by a carrier molecule. A wide range of pro-cancer events rely on abnormally high activity of protein tyrosine kinases for signal transduction. These include proliferation, cell

migration, and angiogenesis, as well as avoidance of apoptosis and differentiation. Indeed, the PTK activity of squamous cell carcinomas, for example, can be from six-fold to eightfold higher than that in adjacent normal tissues, (Rydell *et al.*, 2001).

Like protein tyrosine kinase, protein kinase C (PKC) helps relay chemical signals through the cell, and a wide range of pro-cancer events rely on abnormally high PKC activity for signal transduction. Unlike PTK, PKC resides totally within the cell and is activated by other membrane receptors such as PTK. PKC is found in almost all tissues, with the highest activity in the brain, (Niles, 2004). PKC is a family of at least 12 related lipid-dependent enzymes, many of which are overexpressed in cancer cells. PKC becomes activated through a series of steps that are initiated when a stimulating agent, such as a growth factor, contacts the appropriate receptor on the cell surface. In short, the signal generated by receptor binding activates a kinase such as a PTK. This in turn activates both cytosolic and membrane PKC via compounds referred to as "second messengers." In addition, second messengers stimulate the movement of cytosolic PKC to the plasma membrane, where it can be phosphorylated. Like phosphorylation of PTK, phosphorylation of PKC allows high-energy phosphorus molecules to be transferred to other carrier molecules, thereby sending along the signal that originated at the surface receptor, (Lin *et al.*, 2007).

During cytosolic PKC activation, phosphorylation of the kinase receptor activates the enzyme phospholipase A2 (PLA2). This activated enzyme causes the release of arachidonic acid (AA) from the lipid bilayer. The free arachidonic acid stimulates the phosphorylation of cytosolic PKC. During membrane PKC activation, phosphorylation of the kinase receptor activates the enzyme phospholipase C (PLC). PLC in turn, cleaves phosphatidyl inositol biphosphate (PIP2), a membrane bound compound, into two parts—its lipid tail, diacylglycerol

(DAG), which remains in the membrane, and its non-lipid portion, inositol triphosphate (IP3), which moves in the cell. IP3 in turn stimulates the release of calcium ions that stimulate the movement of cytosolic PKC to the plasma membrane. PKC at the plasma membrane is then stimulated to undergo phosphorylation by DAG in conjunction with the phospholipid molecules in the membrane itself. Since they help relay the original signal, arachidonic acid, DAG, IP3, and calcium are all referred to as second messengers of PKC. PKC activity is commonly increased in cancer cells and plays a role in many cellular processes, (Davidson and Kennedy, 2006), (Alessandro *et al.*, 2006) (Rose and Hatala, 2004) (Philip and Harris, 2005). It is needed for angiogenesis and metastasis of some cell lines; highly metastatic lines contain more membrane-bound PKC than do weakly metastatic ones. PKC may also play a role in the activity of telomerase, an enzyme that has been associated with excessive life spans of cancer cells.

In addition to PTK and PKC, other kinases are involved in signal transduction and, when overactive, can facilitate cancer cell proliferation. In some cases, PTK or PKC activation occurs first, then the signal later passes through these kinases.

Cyclin-dependent kinases are not involved with transferring a signal from surface receptors to the nucleus, but they do help control the signals that drive the cell cycle. The cell cycle is regulated in part through the cyclic production and destruction of a family of proteins called cyclins, (Liu *et al.*, 2009). They derive their name from their oscillatory nature. Liu *et al* (2009) observed that these proteins help to instruct the cell to begin the mitosis phase, and they instruct it to move through other cell cycle transitions as well. Once produced, cyclins become activated through binding with specific kinases called cyclin-dependent kinases (Cdks). Without activation by Cdks, cyclins are nonfunctional and cannot drive cell proliferation. In healthy cells, the ability of Cdks to activate cyclins is held in check by a family of cyclin-dependent kinase

inhibitors. Primary Cdk inhibitors include the protein p21 and the closely related p27, (Gartel and Tyner, 2008) (Johnson and Walker, 2009), a p21 is of particular interest, since its production is induced by the p53 protein. Induction of p21 protein is one way that p53 halts the cell cycle in injured cells. Induction of p21 stops cell proliferation but still allows DNA repair to proceed. p21 can also be induced independently of the p53 protein. Among other beneficial effects, the p27 protein seems to be involved in mediating the growth-inhibitory effects of TGF-beta. Not surprisingly, mice lacking the *p27* gene are prone to developing cancer. Furthermore, loss of *p27* activity has been associated with poor prognosis for prostate and colorectal cancer patients, (Loda *et al.*, 2007); (Macri and Loda, 2008). Cancer cells commonly under-express p21 or p27 genes or both.

Like PTK and PKC, ras proteins also play a role in signal transduction and are commonly overproduced in cancer cells. Oncogenic forms of the *ras* gene have been observed in 20 to 30 percent of human cancers. Their appearance is most frequent in pancreatic cancer (greater than 80 percent of patients) and colon cancer (greater than 50 percent) as reported by Singh *et al* (2006). Ras proteins play a primary role in the MAPK signal transduction cascade. Briefly, phosphorylation of a surface receptor such as a receptor PTK stimulates the membrane-bound ras protein. Activation of ras, in turn, stimulates the phosphorylation of the kinase series raf, MEK, and MAPK. MAPK activation plays a central role in initiating cell proliferation, as it can directly interact with nuclear transcription factors. The ras protein also plays a role in initiating a number of other signal transduction cascades, including activation of PI kinase and activation of PKC, (Pincus *et al,* 2000). Since the ras protein is overexpressed in a large number of cancers and it

plays an important role in multiple signal transduction cascades, inhibition of ras action is now considered an important goal in cancer treatment, (Rowinsky *et al.,* 2009).

Moreover, inhibition of ras action is important because the ras protein, via stimulation of the MAPK signal transduction cascade, induces the expression of the *MDM2* gene, whose protein serves to inhibit the activity of the p53 protein. In this way, ras activity reduces the ability of the p53 protein to induce apoptosis in cancer cells (Ries *et al.,* 2000). When ras proteins are first produced (by expression of the *ras* gene), they are not functional. They become functional only when they are joined with a lipid tail and that tail becomes anchored in the plasma membrane. Given the way ras proteins work, there are three conceivable ways their activity can be inhibited. The first is to inhibit either the upstream signaling that activates them or the downstream signaling that occurs after ras activation, or both. For example, PTK and/or PKC activation can lead to downstream ras activation.

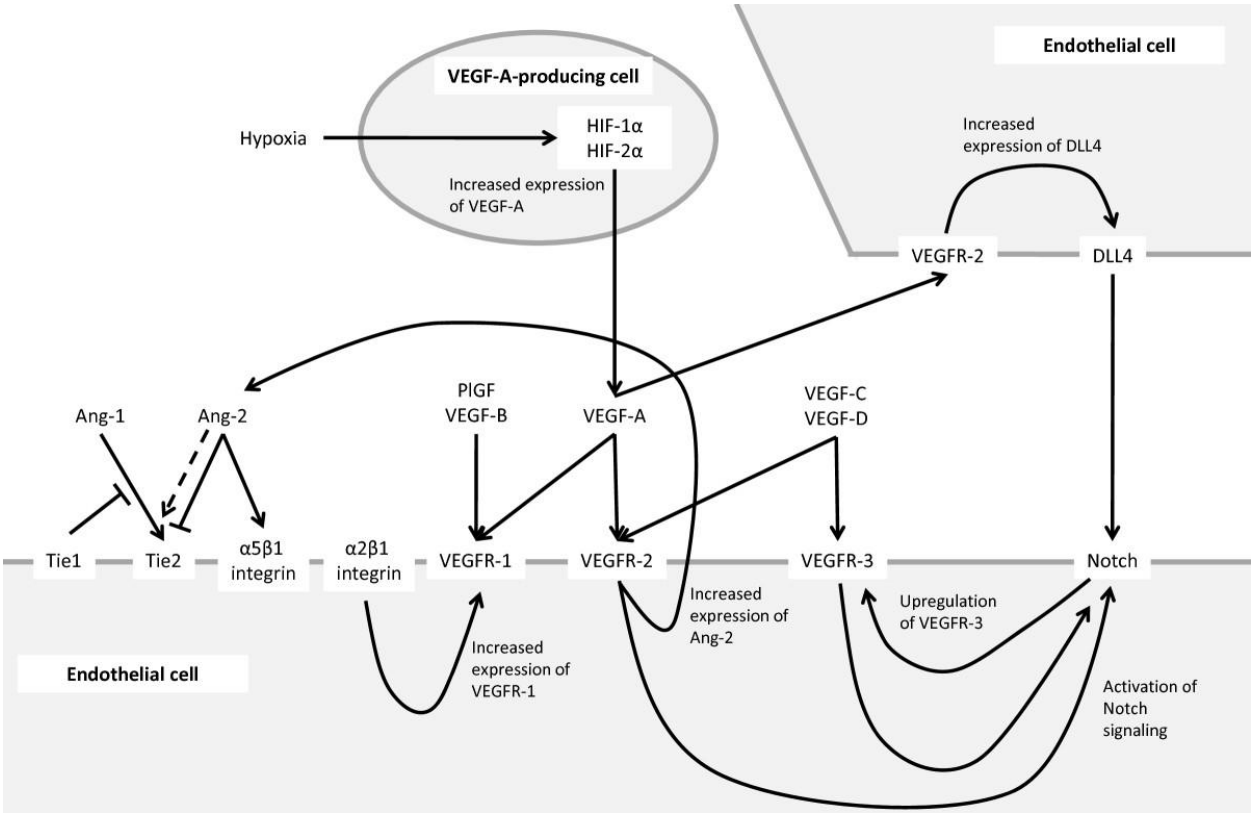
2.1.9Angiogenesis and Colon Cancer

Carcinogenesis is a multistep process reflecting a series of genetic and epigenetic alterations in normal human cells that drives their progressive transformation into highly malignant derivatives. The successful growth of metastatic cells depends on the interactions and the properties of the cancer cells and the potential target organs, proposed as the "seed and soil" hypothesis by Paget. Angiogenesis being the growth of new blood vessels, is needed anywhere new tissue is growing. Thus it does not only occur in benign and malignant tumors but also in wound healing, ovulation, menstruation, and pregnancy. Abnormal angiogenesis also takes place in other diseases, including rheumatoid arthritis, psoriasis, and atherosclerosis (Li *et al*., 2015).

Angiogenesis is a highly regulated, complex process orchestrated by a number of intersecting pathways, including vascular endothelial growth factor (VEGF), angiopoietins,

Notch, and integrins. In normal tissues, there is a balance between proangiogenic and antiangiogenic factors. During physiologic angiogenesis in adults, there is a temporary shift to proangiogenic factors; this is held in check by inhibitory mechanisms. In contrast to normal angiogenesis, tumor angiogenesis is outside of the control of normal physiologic inhibition, and there is an imbalance of proangiogenic and antiangiogenic factors. The diversity of pathways and molecules involved in angiogenesis may offer avenues for therapeutic intervention. This article aims to provide an overview of tumor angiogenesis, focusing on the role of VEGF. It will also review preclinical and clinical evidence for the use of antiangiogenic agents in the treatment of colorectal and other cancers (Folkman *2013).*

Levels of VEGF-A have been shown to be increased in many cancers, including colorectal, prostate, and breast. Downstream signaling activated by VEGF-A binding to VEGFR-2 includes the PLC-gamma/PKC/Ras/Raf/MEK/MAPK signaling pathway. Other VEGF family members, along with other signaling mediators, affect and overlap with the function of VEGF-A. For instance, PlGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) can modulate the effects of VEGF-A on angiogenesis, and VEGF-A can form heterodimers with PlGF or VEGF-B. Because of alternative splice variants, an enormous diversity of heterodimerization with different functional properties may be formed, which presents a challenge to assessing the functional consequences. In addition, VEGF-A has overlapping function with VEGF-C and VEGF-D. VEGF-A-mediated signaling also intersects with angiopoietin (Ang)/Tie, Notch/delta-like ligand (Dll)4, hypoxia-inducible factor (HIF)-1α and HIF-2α, and integrin pathways (Figure below) (Kut C 2015).



Selected crosstalk between VEGF and other signaling pathways. VEGF signaling intersects with a number of other signaling pathways, including Ang/Tie, Notch/Dll4, integrins, and hypoxia to orchestrate tumor angiogenesis. Ang = angiopoietin; Dll4 = delta-like ligand 4; HIF = hypoxia-inducible factor; PlGF = placenta growth factor; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor. Dashed arrow indicates Ang-2 can serve as a partial Tie-2 agonist under certain conditions.

Researchers believe that if angiogenesis can be inhibited in cancer patients, tumor growth will be inhibited or even reversed (Kerbel, 2000). Although inhibition of tumor angiogenesis is a very promising anticancer therapy, it is not without challenges. The same factors and environments that drive angiogenesis during cancer also drive angiogenesis during wound healing and other normal conditions in which it occurs. Since wound healing and other normal angiogenic processes are so vital for survival, the body employs redundant mechanisms to assure that angiogenesis occurs when needed.

Although it has been known for over a hundred years that tumors contain an abnormally dense blood vessel network, it was not until the 1960s that investigators realized tumors induce their own blood supply. In the 1970s investigators found that the growth of solid tumors is dependent upon the growth of new blood vessels. The blood vessels formed during angiogenesis supply the tumor with oxygen and nutrients and provide access to the circulation for metastasizing cells. Growth in experimental tumors is slow and gradual before angiogenesis but explosive afterward. The proliferation rate of individual cells may be similar before and after angiogenesis, but the high rate of tumor growth after angiogenesis appears to be due to a severe reduction of apoptosis (Lu and Tanigawa, 2007).

In tumors with active angiogenesis, cells do not die when they should, and therefore more cells are alive to proliferate. Cancer cells die by apoptosis unless exposed to growth factors or similar stimuli. Angiogenesis occurs in and produces an environment steeped in growth factors. Angiogenesis is a complex process in which existing mature blood vessels generate sprouts, and these sprouts develop into complete new vessels. During angiogenesis, vascular cells proliferate at abnormally rapid rates. Whereas under normal circumstances capillary cells divide approximately once every seven (7) years, capillary cells in experimental tumors may divide once every 7 to 10 days (Scott and Harris, 2004). Denekamp (2003) and Paper (2008) reported that angiogenesis within tumors or wounds involves at least four steps;

i. Cancer cells (or adjacent tissues) secrete angiogenic factors.

ii. The basement membrane surrounding a mature capillary vessel dissolves, and a bud begins to grow. The basement membrane is a layer of specialized connective tissue that encircles capillaries and serves as the connection point between the extracellular matrix (ECM), the ground substance surrounding cells and tissues and holding them in place, and the capillary itself. The basement membrane also provides structural support to the capillary.

iii. Vascular (endothelial) cells proliferate and migrate from the bud toward the angiogenic stimulus—often that means toward a low-oxygen (hypoxic) environment.

iv. The sprout eventually forms a hollow tube (lumen) and joins its end with another sprout to form a new capillary vessel. Further budding from the newly formed capillary follows as the process repeats itself.

Angiogenesis is a relatively rapid process; buds can form within 48 hours of exposure to an angiogenic factor (Folkman, 2005). In spite of active angiogenesis, the blood supply in a solid

tumor is relatively limited compared to that of normal tissue. Tumor angiogenesis results in chaotic vessel growth, and the new vessels are surrounded by poorly developed basement membranes. Because of their abnormal basement membranes, the vessels tend to be thin-walled and leaky. Some sprouts may not fuse with others, and they become dead-end sacs. These factors, in conjunction with a lack of tumor lymph vessels, lead to the creation of pressure gradients within tumors. Pressure gradients, in turn, compress the vessels and further restrict or occlude blood flow. A lack of circulation appears to be a primary cause of the central necrosis found in many large tumors. Although this process does destroy some cancer cells, the increased pressure also limits the uptake of treatment agents and immune cells into the tumor, (Jain, 2000).

2.1.10 Cell Adhesion Molecules and Colon Cancer

Cell adhesion molecules are specialized proteins located on the outside of the plasma membrane (Agrez and Bates, 2004). Due to recent advances in laboratory techniques, research on CAMs has flourished and the pivotal role they play is understood more completely. Through interactions with the ECM and other cells, CAMs regulate proliferation, architecture, cell migration, differentiation, apoptosis, angiogenesis, and invasion (Juliano and Verner 2003, Roth 2004, Agrez and Bates, 2004, Pignetelli and Vessey, 2004, Brooks 2006). CAMs can be thought of as the fingers of a cell, but instead of 10 fingers, cells have many hundreds, each lasting only for a few hours. Depending on the type of CAM, they generally have three functions.

First, they grasp molecules on other cells or on the matrix. In some cases, this grasping helps a cell to move and in others, helps it stay in place.

Second, they send signals to the nucleus telling it what they feel.

Third, they, or associated proteins, receive signals back from the nucleus that alters CAM behavior.

It may seem then that signal transduction mediates messages both to and from CAMs, and compounds that affect signal transduction, such as PTK and PKC inhibitors, can greatly affect CAM expression and activity. In addition to sending signals to alter CAM behavior, the nucleus can also increase or decrease the production of different signal transduction, such as PTK and PKC inhibitors, can greatly affect CAM expression and activity. In addition to sending signals to alter CAM behavior, the nucleus can also increase or decrease the production of different CAMs, depending on the signals it receives.

Four families of CAMs discussed are: integrins, cadherins, selectins, and the immunoglobulin superfamily of adhesion molecules (Pignetelli and Vessey 2004). The first three families of CAMs we discuss are integrins, selectins, and the immunoglobulin superfamily of adhesion molecules. Because all of these play a role in immune cell migration, it is useful to outline how these CAMs allow leukocytes (immune cells) to attach to and migrate through a vessel wall during an immune response. After floating in the bloodstream and arriving at a target location such as an infected area, leukocytes must attach to the inner vascular wall, then slip through the vascular tissue (Pignetelli and Vessey, 2004). This three-part process consists of;

(a) transient interactions between selectins and integrins on the leukocytes and vascular cells, which pull leukocytes from the circulation and initiate their rolling along the vascular wall;

(b) interactions with chemotactic proteins secreted by vascular cells, which cause leukocytes to creep along the vascular wall toward the site of infection; and cadherins, selectins, and the immunoglobulin superfamily of adhesion molecules. Although each of these CAMs plays complex roles, in general the cadherins hold tissues tightly together and the other three help in

cell migration, especially that of immune cells. One other CAM family, the CD44 surface molecule, also helps cells to migrate.

(c) interactions between the immunoglobulin superfamily of adhesion molecules (ICAMs, VCAMs, PECAMs) and integrins that induce the cells to arrest (stop at a particular place on the cell wall), spread, and finally migrate through the vascular wall (Sheski *et al.*, 2009).

The process is of interest not only because it is important in the immune response, but also because it is likely that blood-borne tumor cells bind, creep, and stop at a metastatic site in a similar fashion. In other words, integrins, selectins, and the immunoglobulin superfamily of adhesion molecules all likely play a role in cancer metastasis. The exact role they play in cancer is complex; but in general, these CAMs tend to be overexpressed in cancer cells, and thus cancer metastasis.

Integrins bind to a number of ECM proteins, including collagen, laminin, and fibronectin, and they are receptors for certain selectins and immunoglobulin superfamily adhesion molecules, including intercellular adhesion molecules (ICAMs). Like other CAMs, the intracellular root of integrins is attached to the cell's internal (actin) cytoskeleton. Through these connections, integrins and other CAMs can affect signal transduction and cellular structure. Integrins are the most ubiquitous and versatile of all adhesion receptors (Pignatelli and Stamp, 2005). Of all the CAMs, they are the ones most responsible for anchoring cells to the ECM. Their expression and regulation is controlled in a complex, dynamic fashion involving feedback from cell-cell, cell matrix, and cell-growth factor interactions. At different stages in a cell's life and/or in response to changes in their microenvironment, various integrins are expressed to allow necessary growth, architectural changes, movement, and function. Quantitative and qualitative changes in integrin expression have been observed in a large number of human tumors. Numerous integrins can be

affected, and the effects may be complex. In general, however, integrins tend to be overexpressed in cancer cells, a situation that leads to increased arrest of the cells on the vascular lining during metastasis (Miloszewska *et al.*,2008) (Carrieras *et al.*, 2009).

The selectin family consists of three members, named for the cells on which they were first discovered. L-selectin is expressed on leukocytes and attaches to activated endothelial cells. E-selectin is produced by endothelial cells and attaches to leukocytes. P-selectin is preformed, then stored in platelets and some endothelial cells; it attaches to the same cells as E-selectin. The junctions produced by selectin binding are relatively weak. Hence E-selectin plays a role in leukocyte rolling. When overexpressed in cancer, selectins, like integrins, tend to help metastatic cancer cells arrest at locations in the vasculature, where they can then begin new colonies. Each selectin molecule contains strings of proteins called lectins that are devoid of sugar chains. These proteins bind sugars (carbohydrates) on adjacent cells in a "lock and key" fashion. They can also bind with integrins in a similar fashion (Trikha *et al.*, 2006).

The immunoglobulin superfamily of cell adhesion molecules contains the evolutionary precursors of the immune system, (Akimoto *et al.,* 2008). The most studied groups of immunoglobulin adhesion molecules are N-CAMs (found in nervous tissue), intercellular cell adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), and platelet endothelial cell adhesion molecules (PECAMs). All of these play a role in assisting immune cells and cancer cells to arrest at a target location on the vascular wall.

In contrast to the three families of CAMs discussed above that play a role in immune cell migration, cadherins play a role in maintaining the structure of tissues (Akimoto *et al,* 2008). Cadherins are a family of calcium-dependent adhesion molecules. They bind to other cadherins on adjacent cells in a zipperlike fashion, forming a tight bond between cells, the tightest formed

by any cell adhesion molecule. Cadherins are named for the tissues in which they were first found.

The various types include E-cadherin (epithelial cells), N-cadherin (nerve cells), and P-cadherin (placental cells). E-cadherin deserves special mention here because its expression is closely related to the degree of cellular differentiation (Bracke *et al.,* 2006). Cancer cells, being relatively poorly differentiated, commonly display a decreased number of E-cadherin molecules or a decrease or malfunction in catenin molecules, which are the proteins that attach cadherins to the intracellular actin cytoskeleton. Reduced cadherin expression or function allows cancer cells to detach from adjacent cells. Not surprisingly then, E-cadherin activity appears to act reliably and consistently to suppress invasion of cancer cells. Moreover, under-expression of E-cadherin has been associated with poor prognosis, decreased differentiation, and increased tumor invasion and metastasis in a wide range of human tumors (Heimann *et al.*, 2000) (Bracke *et al.,* 2006).

The last form of cell-to-cell communication discussed is intercellular communication through gap junctions. Such communication almost universally maintains tissue health and inhibits cancer progression (Holder *et al.,* 2003). Gap junctions, which are portal structures between adjacent cells, allow cells to exchange ions and small molecules directly, including ions and molecules used in signal transduction. For example, calcium ions, which act as second messengers during PKC activation, can be exchanged through gap junctions. Indeed, waves of ions can pass among cells of a tissue through gap junctions. This wave can actually be seen visually, since gap junctions will also transfer a dye between cells of a tissue. Gap junctions can also transfer toxins, thus allowing a toxin to be distributed over many cells; such spreading and dilution of a toxin can help prevent cell death. Gap junctions themselves are comprised of proteins called connexins which can be seen as short rods that, when arranged in a circle, form a

tube. The tube in one cell then links up with the tube in an adjacent cell, thereby forming the gap junction. When cells become cancerous, they detach from neighboring cells. This occurs in part through down-regulation of connexin genes. In fact, many if not all tumor-promoting agents reduce gap junction communication (Yamasaki *et al.,* 2009).

Therefore, in recent years connexin genes have become viewed as a family of tumor suppressor genes. Connexin genes are usually not mutated in cancer cells but are generally silenced via hypermethylation and/or their protein products are altered after production so as to make them nonfunctional (Yamasaki *et al.,* 2009). Connexin proteins can be rendered nonfunctional through abnormal protein phosphorylation, including abnormal PKC activity, (Casen-Cummings *et al.,*2008). Lack of cell adhesion molecules and, especially, E-cadherin can also render connexin proteins nonfunctional (Holder *et al,* 2003, Yamasaki *et al.,* 2009).

2.1.11 The Extracellular Matrix (ECM) and Colon Cancer

The ECM not only provides a barrier to tumor invasion, it also governs the behavior of the cells it surrounds. The makeup of the ECM varies in gross or subtle ways in different body tissues. These differences partly govern not only the architecture of the tissue but also the function and proliferation of the tissue's cells. The production and maintenance of the ECM is a dynamic process, and ECM GAGs are renewed and replaced rapidly, usually within a month's time. Since the source of GAG synthesis is the cells themselves, a complex, two-way regulatory loop exists. Cells affect ECM formation and ECM formation affects cells. Because of this intimate relationship, cells cannot be adequately discussed apart from their immediate environment. For example, most normal cells undergo apoptosis when they become detached from the ECM (Kerbal, 2000).

Recall that apoptosis may be an ever-present default pathway and that cell survival is maintained only so long as cells receive the appropriate anti-apoptotic survival signals; these signals can be produced partly through cell-matrix interactions. In contrast, most cancer cells do not undergo apoptosis when they become detached from the ECM; instead, they exhibit an increased tendency to proliferate. In part, prevention of apoptosis may be due to the ability of cancer cells to self-stimulate the signal transduction pathways that would normally be activated through cell-matrix (or cell-cell) interactions. Cancer cells can in fact produce matrix components that favor their migration and proliferation. In particular, a number of cancer cell lines do not assemble a functioning matrix. Although they do produce the proteoglycan components, the components tend to be in an under-sulfated form (Brewer *et al.,* 2000) (Majewski *et al.*,2005) (Majewski *et al.*,2006) (Zhou *et al.,* 2008).

Such altered proteoglycans are less capable of gluing collagen and other protein fibers to one another, of maintaining cell attachment, and of binding growth factors. These characteristics facilitate increased invasion and proliferation. In addition to producing chemically altered matrix components, some cancer cells produce an abnormally large volume of matrix components. This is especially true with cancer cells that have a high ability to invade and metastasize (Joseph *et al.,* 2006,Kudelka *et al.,* 2007, Joseph and Isaacs 2008, Bhargava *et al.,* 2009). Matrix synthesis may occur within the cancer cell itself or within fibroblasts stimulated by the cancer cell (Folkman and Hochberg, 2003). In either case, insulin and other growth factors may provide the stimulatory signals for excessive matrix synthesis (Lu and Tanigawa, 2007). Not surprisingly, elevated levels of hyaluronic acid (a GAG extracellular matrix component) have been observed in the blood of cancer patients. For example, one study reported that patients with lymphoma exhibited increased plasma levels of hyaluronic acid, and the highest levels were found in

patients with relapsing or resistant disease (Scott and Harris, 2004). Increased plasma levels of hyaluronic acid have also been found in lung cancer patients (Denekamp, 2003). Therefore, hyaluronic acid, especially its low-molecular-weight fragments, has been proposed as a plasma diagnostic marker to measure cancer progression (Jain, 2000, Scott and Harris, 2004, Paper, 2008).

Hyaluronic acid synthesis though needed to repair the damaged ECM during wound healing, it is much more likely, however, that it facilitates cancer progression (Paper, 2008). For one thing, the excess hyaluronic acid produced during cancer is probably not of the normal type, or it is produced in relative imbalance to other ECM components such as collagen and fibronectin. This type of imbalance (high GAG production and low collagen production) occurs in the dysfunctional matrix produced around varicose veins, a condition that has some similarities to cancer invasion. Even if the excess hyaluronic acid produced by cancer cells is of a normal type, it may still facilitate tumor progression. For example, normal hyaluronic acid facilitated invasion of brain cancer cells in vitro (Jain, 2000). It is not clear exactly how this occurs, but hyaluronic acid is produced on the leading edge of invading tumors and could assist tumor cell migration by opening up spaces in the ECM for tumor cell movement (recall that hyaluronic acid has a sponge-like quality). In addition, hyaluronic acid could further promote cell migration by providing a path tumor cells can "walk" on. Furthermore, hyaluronic acid forms a "halo" around invading tumor cells, which could protect them from immune recognition and attack (Folkman, 2005).

Lastly, the production of hyaluronic acid could indirectly facilitate angiogenesis. As the newly formed hyaluronic acid is overcome by the leading edge of the invading tumor mass, it is digested by tumor-generated hyaluronidases, which produce hyaluronic acid fragments. These

fragments can act as angiogenic factors, not unlike the fibrin breakdown products, (Folkman, 2003, Folkman, 2007).

It was also observed that cancer cells can alter their relationship with the ECM to their own benefit. They can produce abnormal ECM components or produce an excessive volume or abnormal mix of ECM components, or any of these.

As discussed previously, the strength and structure of the ECM is provided by collagen fibers linked together by GAG chains (Ho, 2005). To break free of the ECM, cancer cells (or cancer-stimulated immune cells) produce enzymes that degrade these ECM components. These enzymes include glycosidases, which degrade GAG chains, and proteases, which digest protein structures. Many different types of proteases and glycosidases may be involved in cancer invasion, but only two primary ones are discussed here: collagenases, proteases that digest the protein collagen, and hyaluronidases, a family of glycosidases that digest hyaluronic acid. Proteases and glycosidases appear to mediate at least five processes that influence cancer progression. Proteases can stimulate inflammation, cell proliferation, invasion, metastasis, and angiogenesis. However, the inhibitors of these enzymes could impede all these processes (Folkman, 2003):

The inflammatory response, including that associated with cancer, involves the release of histamine, free radicals, eicosanoids, and proteases such as collagenases. It appears that each individual product of inflammation affects the release of others; for example, some proteases may cause stimulated macrophages to produce two to six times more free radicals, (Haddow, 2002). Similarly, various proteases increase PGE2 synthesis, and a variety of protease inhibitors inhibit PGE2 synthesis.

Kim *et al* (2003) reported that some protease stimulates cell proliferation. Invasion requires a delicate balance between conditions that stabilize the ECM (enzyme inhibition and

matrix synthesis) and conditions that destabilize it (enzyme production and abnormal matrix synthesis). When the ECM is highly stable, it is difficult for tumor cells to break loose and invade. Similarly, when the ECM is highly unstable, it is difficult for tumor cells to use the ECM as a track for movement (Haddow ,2002).

For successful metastasis, cancer cells must generally penetrate the basement membrane surrounding capillaries to enter and exit from the circulation. Because the basement membrane is a collagen-rich form of the ECM, collagenases are required for this process (Sleeman *et al.*, 2006).

Proteases may facilitate angiogenesis by inducing inflammation and increases in capillary permeability, and by freeing other angiogenic factors stored in the extracellular matrix. In addition, glycosidases produce hyaluronic acid fragments, and these may directly stimulate angiogenesis (Sleeman *et al.*, 2006).

Hyaluronidase is capable of forming channels through the ECM. It follows that the hyaluronidase produced by cancer cells helps them invade surrounding tissues, (Honda *et al*, 2001). In much the same way, the hyaluronidase in snake and bee venoms allows the rapid spread of their poisons through the extracellular matrix. Moreover, injections of hyaluronidase have been shown useful in enhancing the antitumor effects of locally applied chemotherapy drugs, by helping the drugs spread to tumor tissues (Spruss *et al.*, 2005, Muckenschnabel *et al.*, 2006, Smith *et al.*, 2007). Hyaluronic acid is actually degraded, or digested, in a two-step process. In step one, it is converted to medium- length sugar chains by hyaluronidase. In step two, these chains are further shortened by the enzymes beta-glucuronidase and *N*-acetyl-beta-glucosaminidase. *N*acetyl-beta-glucosaminidase activity is elevated in the plasma of patients with a variety of cancers, including those of the breast, stomach, liver, pancreas, and

colorectum(Severini and Aliberti, 2004, Severini *et al.,* 2005). It is also excessively secreted by human ovarian cancer cells in vitro and increases their ability to degrade ECM components, (Woynarowska *et al,* 2002, Niedbala *et al,* 2005). Elevated beta-glucuronidase levels have been observed in the urine of patients with bladder and kidney cancers (Ho and Kuo, 2005, Ho, 2005). It appears to be less sensitive than *N*-acetyl-beta-glucosaminidase as a diagnostic marker for cancer.

Like hyaluronidase and its assistant enzymes, the enzyme elastase is also involved in matrix destruction and invasion. It is produced by a variety of cells, including macrophages and tumor cells. Elastase degrades components of the extracellular matrix, including elastin, collagen, and other proteoglycans; it also degrades fibronectin.

It is however clear that production of hyaluronidase or collagenase by tumor cells or other adjacent cells can lead to matrix digestion (West and Kumar, 2009) which subsequently allows tumor cell invasion. One more aspect of this process is the mechanism by which tumor cells "walk" during invasion. It is useful to consider the close analogy between the migration of immune cells toward an infection site and the movement of cancer cells away from the central tumor. In both cases, the cells rely on the attraction of their surface adhesion proteins to other proteins in the extracellular matrix. Immune cells and cancer cells "walk" to their intended targets by gripping and then internalizing proteins of the ECM (Sherman *et al.,* 2004, Sy *et al.,* 2006).

The "legs" of immune cells, fibroblasts, and some cancer cells appear to be RHAMM proteins (RHAMM = receptor for hyaluronic acid mediated motility) and variants of the CD44 family of proteins. CD44 proteins are surface receptors for both hyaluronic acid and collagen, and they mediate attachment, cellular uptake, and degradation of these proteins. RHAMM

proteins are receptors for hyaluronic acid. CD44 and RHAMM proteins facilitate the uptake of hyaluronic acid and collagen into the cell, which are then degraded by enzymes. In a number of human breast cancer cell lines, high CD44 expression has been associated with high invasive capacity (Culty *et al.,* 2004). Sherman *et al* (2004) reported that elevated levels of soluble CD44 occurred in a high percentage of patients with metastatic disease, and removal of the primary tumor reduced these levels. Accordingly, other human studies have reported that levels of soluble CD44 variants may act as an indicator of tumor burden and metastasis, (Braumuller *et al.,* 2000, Guo *et al,* 2004) . Resting immune cells produce normal CD44 proteins, which do not facilitate movement, whereas stimulated cells produce CD44 variants that do. Cancer cells appear to mimic stimulated immune cells by also expressing CD44 variants, thereby allowing increased migration, (Braumuller *et al.,* 2000). The expression of CD44 variants may also play a role in metastasis. The basement membrane surrounding capillaries contains hyaluronic acid, and CD44 variants on blood-borne tumor cells help the cells attach to the vasculature, (Sleeman *et al.,* 2006). In one study, intravenous injection of CD44 inhibitors (CD44 antibodies) reduced the proliferation and metastatic potential of CD44-expressing melanoma cells in mice, (Guo *et al.,* 2004). In addition to CD44 activity, cell migration is dependent on a number of other events, such as the presence of chemo-attractant compounds (such as leukotrienes), growth factor signaling, and the interactions of other cell adhesion molecules (CAMs) with matrix components.

2.1.12 Metastasis and Colon Cancer

Most cancers do metastasize. Metastatic cells travel through the blood and lymphatic circulation systems, and the metastatic colonies they form are often more life threatening than the primary tumor. In fact, the growth of a tumor at its primary location is generally not a cause of death, except in a limited number of cancers such as those of the brain, liver, and lungs (Honn

et al., 2007). Brain cancer, for example, is one of the few cancers that rarely metastasize (Calabresi and Schein, 2003). The metastatic process is inherently inefficient. In a study of patients with kidney cancer, between 10 million and 1 billion cancer cells were released from their tumors into the bloodstream per day. In spite of this enormous release, 20 percent of the patients showed no evidence of new tumor development, even after 30 months (Janeway and Travers, 2007).

In animal tumors, only 0.001 percent of the released tumor cells develop into metastatic colonies (Janeway and Travers, 2007, Kobayashi, 2009). The reasons for the inefficiency of the metastatic process are uncertain, but since at least five different sequential steps are required for its success, interruption of any one of them could derail the whole process. The last step in this process seems particularly sensitive to inhibition. A metastatic colony develops according to the steps listed below. Like many aspects of cancer, metastasis is a complicated process, and numerous factors can affect each step.

i. Cells detach from the primary tumor and invade the basement membrane surrounding blood vessels to gain access to the bloodstream, a process called intravasation.

ii. Once they are circulating in the bloodstream, the migrating cells evade attack by the immune system and survive other adverse conditions.

iii. The migrating cells adhere to the wall of a blood vessel at the metastatic site- the process of cell arrest.

iv. The arrested cells exit the blood vessel and, by invading through the basement membrane, enter the tissues. This process is called extravasation.

v. The tumor cells proliferate and the new tumor induces angiogenesis

.

Study by Woodruff (2006) suggests that at least in some cases, the majority of traveling metastatic cells come to rest in a target capillary bed and that most of these successfully exit the circulation (step four above). After this point, however, only a small fraction begins to proliferate and form a new tumor. The majority remains dormant, neither proliferating nor undergoing apoptosis.

Immune cells can destroy cancer cells within a tumor and also cancer cells that are metastasizing T cells appear to play the largest role in the destruction of tumor cells, and macrophages and NK cells do the same in the destruction of metastasizing cells, (Nakamura and Matsunaga, 2008).

Although T cells are important in destroying cancer cells within a tumor, to be effective they require adequate antigen presentation. For this reason, T cells rely on antigen-presenting cells such as macrophages and dendritic cells (Ohtsubo *et al.,* 2009). Since tumor cells often disrupt the mechanics of antigen presentation, they are able to evade T-cell recognition.

Natural killer (NK) cells are large, specialized, non-T, non-B lymphocytes. NK cells were discovered in the mid-1970s and constitute up to 15 percent of the total lymphocyte population in healthy subjects (Kobayashi, 2009). They are capable of killing a broad range of human solid tumor, leukemic, and virus-infected cells. Depressed NK cell activity and depressed NK cell populations appear to be associated with the development and progression of cancer, as well as AIDS, chronic fatigue syndrome, psychiatric depression, various immunodeficiency syndromes, and certain autoimmune diseases. NK cells may present the first line of defense against metastatic spread of tumors. In animal experiments, low NK cell populations have been

associated with greater survival of injected cancer cells and increased development of lung metastasis. Correction of the NK cell population restored resistance, (Kobayashi, 2009, Woodruff, 2006, Kim *et al.*, 2000, Dithmar *et al.*, 2009).

In human studies, patients with solid tumors commonly have diminished NK cell activity, but the association between low NK cell activity and metastatic spread is not so strong as in animals. This lack of correlation, however, may be due in part to the fact that most clinical studies of immunostimulants that affect NK cell activity were conducted in patients with advanced cancers.

Like NK cells, macrophages do not recognize foreign antigens. In addition, they do not recognize self-proteins (Wahl and Kleinman, 2008). Their activation mechanisms are poorly understood but may involve receptors for certain carbohydrates, complement, and other proteins. Although bacterial products are among the most potent activators, macrophages can also be activated by contact with tumor cells. Macrophages destroy foreign substances by phagocytosis (ingestion); by secretion of proteases, hydrogen peroxide, or other enzymes or radical species; or by secretion of certain cytokines, such as tumor necrosis factor. Macrophages may play an important role in the immune response to cancer. Holleb *et al* (2001) observed that treatments that suppress the function of macrophages have been associated with increased tumor incidence and increased metastasis. Tumor-associated macrophages, however, have also been reported to promote angiogenesis and tumor growth.

According to Calabresi and Schein (2003), the immune system must recognize a cancer cell as foreign before it can be destroyed. If the immune system is able to recognize a substance as foreign, that substance is referred to as being antigenic. One might expect the immune system to have trouble recognizing tumors as foreign, but in fact most tumor cells appear to be strongly

antigenic. Unfortunately, recognition of a foreign substance does not necessarily ensure that an immune reaction will take place. Although most human tumor cells are apparently strongly antigenic, they are only weakly immunogenic. (The ability of a foreign substance to evoke an immunologic reaction in a host is called its immunogenicity.) Therefore, rejection of the tumor is difficult, even with a fully functioning immune system. Recent investigations have reported that a few cancers, such as melanoma and kidney cancer, are more strongly immunogenic, but even these often escape destruction by the immune system. The low immunogenicity of most tumors may be due to their ability to successfully evade immune attack. Immune evasion can occur through one or more of the following mechanisms (Becker *et al.*, 2001, Holland *et al.*, 2003, Calabresi and Schein, 2003).Modulation of surface proteins:Tumor cells can evade immune cell recognition by reducing their expression of MHC molecules, adhesion molecules, or co-stimulatory molecules.

Antigenic modulation:Antigenic modulation occurs when the tumor cell loses its surface antigens upon exposure to specific antibodies. In addition, contact between surface antigen and antibody may induce some cancer cells to absorb and degrade the antigen-antibody complex, leaving fewer ways for immune cells to recognize the cancer cell as foreign.

Induced immunosuppression**:**Cancer patients are frequently immunosuppressed, a condition that can sometimes be corrected by removing the bulk of the tumor. There are a variety of ways tumor cells can induce local or systemic immunosuppression. One method is through the excessive production and shedding of antigens; the shed antigens combine with antibodies to form antigen-antibody complexes. This initially stimulates the immune system but in excess eventually overwhelms and suppresses it. Circulating immune complexes are elevated in many forms of cancer and have been suggested as potential tumor markers (Aziz *et al.*, 2002). Tumors,

or the immune cells they attract, can also locally produce immunosuppressive substances such as PGE2, TGF-beta, IL-10, and others, (Wojtowicz-Praga, 2007, Wahl and Kleimann, 2008). In addition, unspecified substances produced by tumors can impair the function of the T-cell receptor complex (Kiessling *et al.,* 2006).

Shedding of surface receptors:Some tumor cells shed their receptors for TNF. As a result, TNF is unable to bind to the cell and destroy it. The shed receptors can also bind with TNF, thus preventing it from reaching other tumor cells. Whatever the exact cause, patients with advanced cancers of any type frequently exhibit nonspecific defects in both innate and adaptive immunity. According to a recent study, they may also experience specific defects in immunity, such as lack of responsiveness of CD8 T cells to the tumor antigens present (Lee *et al.,* 2009). This finding supports the hypothesis that the immune system is able to recognize and respond to cancer cell antigens, even though this ability can be undermined.

2.2 Colon cancer Risk Factors

A risk factor is anything that affects your chance of getting a disease such as cancer. Different cancers have different risk factors. Some risk factors, like smoking, can be changed. Others, like a person's age or family history, can't be changed. But having a risk factor, or even many, does not mean that you will get the disease. And some people who get the disease may not have any known risk factors. Researchers have found several risk factors that might increase a person's chance of developing colorectal polyps or colorectal cancer.

Although the exact origin of colon cancer is unknown, there are several risk factors involved in the incidence of this disease. Some of the most significant risk-factors demonstrated to be involved in the incidence of colon cancer are described (Liu *et al*., 2015).

2.2.1 Inflammatory Bowel Disease

According to Zieve and Chen (2009), Colitis and Crohn's disease, which are two major subsets of Inflammatory bowel disease (IBD), are considered increased risk factors for colon cancers. Colitis is recognized as inflammation of the colon, more specifically the large intestine and rectum, and can be caused by a variety of diseases and infections. Ulcerative colitis, a variation of colitis, is similar to the characteristics of colitis but with the presence of ulcers and open sores in the colon. Zieve and Chen (2009) also reported that Crohn's disease, often considered chronic inflammation, is observed as a constant relapse of inflammation in the colon and mainly occurs in the intestines but can affect any area of the gastrointestinal tract, (Zieve and Chen, 2009). Crohn's disease is an autoimmune disease thought to possibly have an inheritable link.

The risk of colon cancer increases with the duration of IBD and the degree of inflammation of the bowel, (Xie and Itzkowitz, 2008). Population based studies have suggested that the increase for colon cancer in patients with IBD is approximately 2-3 fold higher than in patients without inflammatory bowel disease, (Xie and Itzkowitz, 2008). IBD patients with a family history of colon cancer also have a 2-fold higher risk of acquiring this disease, (Xie and Itzkowitz, 2008). The age of onset of colon cancer often occurs earlier in patients with IBD as well, therefore earlier screening for colon cancer is recommended to patients with IBD to detect early mucosal dysplasia, (Xie and Itzkowitz, 2008).

High protein intake, specifically animal protein via meat and fish consumption has been significantly associated with inflammatory bowel disease as well as ulcerative colitis. There was no such association related to plant proteins in the prospective study consisting of over 65,000 patients over a 10-year period. Animal proteins have greater amounts of sulphur containing

amino acids such as methionine, which the colonic microflora accrues into hydrogen sulfide, resulting in the pathogenesis of IBD and ulcerative colitis. Such bacteria-derived cell poisons in the form of hydrogen sulfide are specifically elaborated by animal or meat and fish intake, and not by plant foods (Magee, 2015).

2.2.2 Hereditary Non-Polyposis Colon Cancer

Lynch syndrome (HNPCC or hereditary nonpolyposis colorectal cancer) is an autosomal dominant genetic condition that has a high risk of colon cancer as well as other cancers including endometrial cancer (second most common), ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. The increased risk for these cancers is due to inherited mutations that impair DNA mismatch repair. It is a type of cancer syndrome (Kastrinos *et al*., 2015)

Hereditary non-polyposis colon cancer (HNPCC) is also referred to as Lynch Syndrome. As mentioned previously, family members with a history of colon cancer have an increased risk for developing the disease, even more so than those with a history of FAP (Baba, 1997). Lynch syndrome is diagnosed when there are multiple cases of colon cancer on the same side of the family. It is thought that mutations in mismatch repair genes on chromosomes 2p and 3p are held responsible for this form of colon cancer as these mutations appear in almost all colon tumors from patients diagnosed with HNPCC (Baba, 1997). As HNPCC is an autosomal dominant mutation, only a mutation in one copy of the gene is required. Generally, 50% of children from a gene carrier will be at an increased risk for developing this disease, and 100% of gene carriers will develop this form of colon cancer (Mecklin *et al.*, 1994).

Individuals with HNPCC have about an 80% lifetime risk for colon cancer. Two-thirds of these cancers occur in the proximal colon. The mean age of colorectal cancer diagnosis is 44 for members of families that meet the Amsterdam criteria. Also, women with HNPCC have an 80% lifetime risk of endometrial cancer. The average age of diagnosis of endometrial cancer is about 46 years. Among women with HNPCC who have both colon and endometrial cancer, about half present first with endometrial cancer, making endometrial cancer the most common sentinel cancer in Lynch syndrome. In HNPCC, the mean age of diagnosis of gastric cancer is 56 years of age with intestinal-type adenocarcinoma being the most commonly reported pathology. HNPCC-associated ovarian cancers have an average age of diagnosis of 42.5 years-old; approximately 30% are diagnosed before age 40. Other HNPCC-related cancers have been reported with specific features: the urinary tract cancers are transitional carcinoma of the ureter[13] and renal pelvis; small bowel cancers occur most commonly in the duodenum and jejunum; the central nervous system tumor most often seen is glioblastoma

In the United States, about 160,000 new cases of colorectal cancer are diagnosed each year. Hereditary nonpolyposis colorectal cancer is responsible for approximately 2 percent to 7 percent of all diagnosed cases of colorectal cancer. The average age of diagnosis of cancer in patients with this syndrome is 44 years old, as compared to 64 years old in people without the syndrome (Lee *et al.*,2015*).*

2.2.3 Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is an inherited condition in which numerous adenomatouspolyps form mainly in the epithelium of the large intestine. While these polyps start out benign, malignant transformation into colon cancer occurs when they are left untreated. Three variants are known to exist, FAP and attenuated FAP (originally called hereditary flat

adenoma syndrome) are caused by APC gene defects on chromosome 5 while autosomal recessive FAP (or MYH-associated polyposis) is caused by defects in the *MUTYH* gene on chromosome 1. Of the three, FAP itself is the most severe and most common; although for all three, the resulting colonic polyps and cancers are confined to the colon wall and removal can greatly reduce the spread of cancer (Soravia *et al.*, 2015)

Familial adenomatous polyposis (FAP) affects approximately one out of thirteen individuals and is a genetically inherited disease of the colon that is diagnosed when a patient develops over 100 benign colon/rectum polyps, (Zieve and Chen, 2009). Polyps can begin developing from the early teen-years through late adulthood. If this disease goes untreated, it is almost certain that the patient will develop colon cancer. Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene are thought to be the general cause of FAP, thus genetic testing in families with a known risk of the disease is often recommended. Because APC is located on chromosome 5 and not on the sex chromosomes, it can be inherited maternally or paternally. Attenuated familial adenomatous polyposis (AFAP) is another form of FAP where patients develop fewer than 100 polyps in the colon/rectum. The polyps that do form often arise on the right side of the colon whereas in the classic form of the disease they occur on all sides of the colon, (Nilbert *et al,* 2008). Health problems associated with AFAP are similar to those of FAP but with an increased risk for cancer.

The incidence of the mutation is between 1 in 10,000 and 1 in 15,000 births. By age 35 years, 95% of individuals with FAP (>100 adenomas) have polyps. Without colectomy, colon cancer is virtually inevitable. The mean age of colon cancer in untreated individuals is 39 years (range 34–43 years).Attentuated FAP arises when APC is defective but still somewhat functional. As a result, it retains part of its ability to suppress polyps. Therefore, attenuated FAP

manifests as colorectal cancer unusually late (age 40–70, average=55), and typically with few, or at least far fewer polyps (typically 30), than the more usual version of FAP, at an age when FAP is no longer considered much of a likelihood or risk according to usual FAP epidemiology (Amos-Landgraf 2015)

2.3 Colon cancer Biomarker

Any measurable specific molecular alteration of a cancer cell either at the DNA, RNA, protein, or metabolite level can be referred to as a cancer biomarker. The expression of a distinct gene can enable its identification in a tissue in which none of the surrounding non-cancerous cells express the specific marker. It is difficult to distinguish related disease subtypes that have different clinical outcomes. There is therefore a need for more exact molecular biomarkers for use in clinical practice. Recently, the discovery of cancer biomarkers has become a major focus of cancer research and there are thousands of publications on cancer biomarkers. The ideal biomarkers for cancer have applications in determining predisposition, early detection, assessment of prognosis, and drug response. The biomarker that serves as a target for drug development would have an additional advantage. Desirable characteristics of molecular markers for cancer are postulated, but no biomarker meets these ideal characteristics. Hence, there is an urgent need for cancer biomarkers with more accurate diagnostic capability, particularly for early-stage cancer. (Hundt 2015)

2.3.1 Carcinoembryonic Antigen (CEA)

CEA stands for Carcinoembryonic antigen. CEA is a type of protein molecule that can be found in many different cells of the body, but is typically associated with certain tumors and the developing fetus, (Medicinenet 2014). CEA is most frequently tested in blood. It can also be

tested in body fluids and in biopsy tissue. The normal range for CEA in an adult non-smoker is < 2.5 µg/L and for a smoker < 5.0µg/L. The best use of CEA is as a tumor marker, especially for cancers of the gastrointestinal tract. When the CEA level is abnormally high before surgery or other treatment, it is expected to fall to normal following successful surgery to remove all of the cancer. A rising CEA level indicates progression or recurrence of the cancer. In addition, levels >20 ng/ml before therapy may be associated with cancer which has already spread (metastatic disease).

2.3.2 Catalase

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (such as bacteria, plants, and animals). It catalyzes the decomposition of hydrogen peroxide to water and oxygen. **(**Chelikani 2004) It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second. **(**Goodsell 2004)

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. **(**Boon 2007) It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7, **(**Aebi 1984) and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species.

The reaction of catalase in the decomposition of hydrogen peroxide in living tissue:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

2.3.3 Malondialdehyde (MDA)

Malondialdehyde is the organic compound with the formula CH2(CHO)2. The structure of this species is more complex than this formula suggests. This reactive species occurs naturally and is a marker for oxidative stress.Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-products (ALE), in analogy to advanced glycation end-products (AGE). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism. (Del Rio 2005)

Malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts, the primary one being M1G, which is mutagenic. (Cardiovasc 2005) The guanidine group of arginine residues condense with malondialdehyde to give 2-aminopyrimidines Human ALDH1A1 aldehyde dehydrogenase is capable of oxidizing malondialdehyde.

2.3.4 Superoxide dismutase (SOD)

Superoxide dismutase is an enzyme that alternately catalyzes the dismutation (or partitioning) of the superoxide (O2−) radical into either ordinary molecular oxygen (O2) or hydrogen peroxide (H2O2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage. Hydrogen peroxide is also damaging, but less so,[citation needed] and is degraded by other enzymes such as catalase. (Hayyan 2016) Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. One

exception is Lactobacillus plantarum and related lactobacilli, which use a different mechanism to prevent damage from reactive (O_2^-).

SOD enzymes deal with the superoxide radical by alternately adding or removing an electron from the superoxide molecules it encounters, thus changing the O_2^- into one of two less damaging species: either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). This SOD-catalyzed dismutation of superoxide may be written, for Cu,Zn SOD, with the following half-reactions :

$$Cu^{2+}\text{-SOD} + O_2^- \rightarrow Cu^{+}\text{-SOD} + O_2$$

$$Cu^{+}\text{-SOD} + O_2^- + 2H^+ \rightarrow Cu^{2+}\text{-SOD} + H_2O_2$$

2.4 Screening and Detection of Colon cancer

Screening and detection of colon cancer includes; colonoscopies, barium enemas, and CT colonographies, (Zieve and Chen, 2009). It is possible to detect polyps in the colon at an early stage utilizing these screening methods if symptoms occur or in an individual who is at a higher risk for the disease.

2.5 Colon cancer Chemoprevention

The incidence of colon cancer in Western societies such as North America and Europe is much higher compared to areas such as China and Japan. It has been suggested through epidemiological studies that a “Western-style diet” which consists of high-fat, low fiber, and low vitamin D and calcium are some of the major dietary factors involved in the formation of various forms of cancer, specifically cancer of the colon. A recent study involving mice fed a “Western-style diet” demonstrated the activation of oxidative stress in the colon of mice which further

promoted severe inflammation in the colon mucosa, (Erdelyi *et al.,* 1999). Conversely, diets rich in fresh fruit and vegetables along with the absence of highly refined sugars have demonstrated a decrease in the risk for colon cancer, (Singh and Fraser, 1998).

A diet high in animal fat and protein has also been suggested to increase an individual's risk for developing colon cancer. Red meat has been one of the most popular food items that have shown a positive correlation to colon cancer in many populations within the United States, (Singh and Fraser, 1998). This correlation is thought to be due to the cooking of red meat which produces heterocyclic aromatic amines (HAAs), dietary chemicals found to be carcinogenic in animal studies, and the hyper-proliferative effect of dietary heme, (Singh and Fraser, 1998) and (Sesink *et al.,* 2009). White meat or fish have not been found to dramatically increase the risk for colon cancer

Treatment options after the diagnosis of cancer include a combination of chemotherapy, radiation, surgery, and drug options that are specifically targeted at colon cancer. New screening technologies have been able to detect malignancies much earlier, thus making the above treatment options more effective. Genetic screens are also becoming a popular option for those individuals at a higher risk for colon cancer. Inhibition of factors that promote the onset of disease is a more effective method for eradicating cancer before it develops. Therapy regimens and drugs that specifically target early stages of carcinogenesis (pre-malignant diseases) are becoming a beneficial option for colon cancer prevention and treatment.

A review on chemoprevention agents for colon cancer has suggested that a daily intake of aspirin or other anti-inflammatory drugs result in a reduced risk of developing colon polyps and cancer, (Gwyn *et al.,* 2002). Non-steroidal anti-inflammatory drugs are some of the most popular studied chemo-preventive agents against colon cancer. A clinical trial in humans with sulindac,

which is a non-steroidal anti-inflammatory drug, has demonstrated the regression of colon-adenomas in patients with FAP, (Gwyn *et al.,* 2002). A more popular drug marketed under the name of Celebrex has also demonstrated inhibitory effects in FAP patients by methods of targeting COX-2 and has been approved for general care in these patients by the Food and Drug Administration (Gwyn *et al,* 2002). Difluoromethylornithine and hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors are other synthetic agents found to demonstrate chemo-preventive activity in colon cancer *in vivo.*

Non-pharmacological agents such as calcium, folate, vitamins and antioxidants have all demonstrated chemo-preventive effects against colon cancer and are becoming the more popular choice of agents to use due to their natural availability. High calcium and vitamin D intake have demonstrated a moderate chemo-preventive effect in human and animal studies. It is thought that the calcium gradient in crypts of the colon mucosa is disturbed in colon carcinogenesis and that by increasing the level of intracellular calcium, tumor growth is repressed and differentiation of transformed intestine and colon cells is promoted, (Lamprecht and Lipkin, 2003). Vitamin D is also involved in restraining cell proliferation and has been reported to be significant in colon cancer prevention specifically as an apoptosis stimulating factor, (Lamprecht and Lipkin, 2003).

Folate known to be required by cancer cells has also been demonstrated to have an inverse relationship with colon carcinogenesis. (Song *et al,* 2000) reported a decreased incidence of small intestinal and colon adenomas in Apc-mutant min mice that were fed a diet with high folate content. Epidemiological and cohort studies have also demonstrated a correlation between diets low in folate and colon polyp formation. However, chemo-prevention studies with folate *in vivo* have proven to be contradicting as a recent review has also reported that diets high in folate induced dysplastic and hyperplastic abnormalities in the colon, (Lamprecht and Lipkin, 2003).

Antioxidants such as vitamins E, β-carotene and polyphenols to name a few, are capable of protecting our bodies from harmful free radicals. It has been suggested that vitamin E and β-carotene decrease tissue damage by preventing lipid peroxidation through trapping and deactivating free radicals that can damage DNA, thus inhibiting carcinogenesis, (Lamprecht and Lipkin, 2003). Polyphenols are present in fruits, tea, chocolate, peanuts, olive oils and many more products. Numerous *in vitro* and *in vivo* studies have demonstrated a chemo-preventive effect for various forms of polyphenols in colon carcinogenesis. Green tea polyphenols have recently become promising agents in the prevention of colon cancer, and are thoroughly studied in our laboratory. For example, green tea polyphenols showed a reduced appearance of aberrant crypt foci (ACF) in the colon of azoxymethane (AOM)-induced F344 rats fed a standardized green tea preparation compared to mice not administered the dietary tea preparation, (Xiao *et al.,* 2008). Epigallocatechin gallate (EGCG), which is a potent chemo-preventive agent of green tea, has been recognized as an inducer of apoptosis and has demonstrated suppression of the formation and growth of human colon cancers.

2.6.1.*Balanites aegyptiaca*

Balanites aegyptiaca is a species of tree, classified either as a member of the Zygophyllaceae or the *Balanitaceae.* This tree is native to much of Africa and parts of the Middle East. (Aluka specie profile 2009).

There are many common names for this plant. In English the fruit has been called desert date, soap berry tree or bush, Thron tree, Egyptian myrobalan, Egyptian balsam or Zachum oil tree; in Arabicit is known as *lalob*, *hidjihi*, *inteishit*, and *heglig* (*hijlij*). In Hausa it is called *aduwa*, in Swahili *mchunju* and in Amharic*bedena*.(Yves Guinand and Dechassa Lemessa 2009)

Balanite aegyptiaca is a multibranched, spiny shrub or tree which grows up to 10m in height. The leaves are alternate, two foliate, petioles are 3-6 mm long, leaflets are elliptic and have broadly pointed petioles up to 5 mm long. Fruit is an ovoid drupe, 2-5.6 cm long, found on a short thick stalk, and is faintly five grooved. The ripe fruit is brown or pale brown with a brittle coat enclosing a brown or brown-green sticky pulp and a hard stone seed. Seeds are found exalbuminous and embryo is with thick plano-convex, or two-lobed cotyledons and a superior radical. (Iwu, *Maurice M. 1993)*

Traditionally, various parts of *B. aegyptiaca* have been reported to possess medicinal properties in different ethno botanical surveys. (Iwu, *Maurice M. 1993).*



Figure 2.2 *Balanites aegyptiaca*



Figure 2.3 *Balanites aegyptiaca*

The single-seededfruit is edible, but bitter. Many parts of the plant are used as famine foods in Africa; the leaves are eaten raw or cooked, the oily seed is boiled to make it less bitter and eaten mixed with sorghum, and the flowers can be eaten. The tree is considered valuable in arid regions because it produces fruit even in dry times. The fruit can be fermented for alcoholic beverages(meda *et al*., 2013).

Balanites aegyptiacaseed oil has been used in Nigeria as ingredient and substitute to groundnut oil in the preparation of local foods. A four week repeated dose toxicity study of crudeB.aegyptiaca seed oil was performed on male Wister strain rats. The rats were divided into four groups consisting of five animals each and fed diet containing 0, 0.5, 1 and 5% crudeB.aegyptiaca seed oil. Result showed no significant ($p > 0.05$) changes in AST and ALT, except in the 5% group where ALT activity was elevated. No significant ($p>0.05$) changes in

serum total protein, albumin, A/G ratio, serum urea, creatinine, mean final body weight,food consumptionand relative liver and kidney weightwere observed. The results showed that dietary exposure of crudeB.aegyptiacaseed oil in rats did not result in marked changes in the toxicological parameters been assayed. Thus, consumption of the crude oil at the present level of exposure may be of no serious safetyconcern, especially on liver and kidney injury (Journal ofAmerican Science 2009).

2.6.2. ***Ziziphus spina-Christy***

Ziziphus spina-Christy known as Christ thorn is a deciduous tree and nature of warm temperate and sub-tropical region including Africa south Europe, Mediterranean, Australia and tropical America South-East Asia and middle East,Zohary and Flora Palaestina (1972).The genus *Ziziphus* consist of about 100 species of deciduous ever green tree and shrubs throughout the world. Z spina-christy has been the key plant for Iranian traditional medicine. (Sunset Western Garden 1995).

Ziziphus spina-Christy is a shrub sometimes tall tree reaching the height of 20m and a diameter of 60cm; it has very nutritious fruits that are usually eaten fresh or dry. (Sunset Western Garden 1995). *Ziziphus spina-christi* has significance to Jews, Christians and Muslims, and is by some traditions the tree from which Jesus' Crown of Thorns was made.



Figure 2.4 *Ziziphus spina-christ* Plant

The fruit is edible and occasionally sweet, but the flavor and texture is inferior to other ziziphus spp., which have been domesticated in Africa and especially in Northern India. Average size is about 50g with single large seed. The fruit contain 14.16gsugar and about 1.6% vitamin C.

The fruits are applied on cuts and ulcers, they are also used to treat pulmonary ailments and promote healing of fresh wounds (Hector *et al*., 2013).

The toxicological potentials of the pulp, seed and stem bark (aqueous) extracts of Zizyphus spina-christy was investigated in rats. Acute toxicity was conducted following intraperitoneal administration of grade doses of the plant extract. LD50 of the pulp, seed and stem bark (aqueous) extracts were 6500mg/kg, 6250mg/kg and 6000mg/kg body weight respectively. Mortality which was dose dependent occurred at high doses of 6000mg and 8000mg/kg for the pulp, seed and stem bark extracts. Phytochemical analysis of the aqueous extracts (pulp, seed, stem bark) indicated the presence of flavonoids, alkaloids, carbohydrates, tannins and saponins. The results show that all parts of the plant can be useful for medicinal purposes.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.1 Plant Sample

Thefresh *fruits* of *Balanites aegyptica* and *Ziziphus spina-christy* were obtained from the local *kurmi* market *(kasuwar kurmi)* in Kano State and identified at the Herbarium Biological Science Department Ahmadu Bello University Zaria. The *Balanites aegyptica* and *Ziziphus spina-christy* were assigned voucher number 1178 and 1183 respectively.

3.1.2 Experimental Animals

Fifty (50) wistar rats between 7-8 weeks' old were obtained from Department of Human Physiology Ahmadu Bello University, Zaria and transferred to the animal-house in the Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, where the rats were retained and the experiment conducted throughout the period of the study. The animals were fed and given water*ad libitum* and as well allowed to acclimatize for a minimum period of two weeks, while undergoing quarantine. They were maintained under 12- hour light/dark cycle.

3.1.3 Basal Feed

Growers marsh feed produced by Vita Feed Company located at Bukuru Jos Plateau State was used throughout this work.

3.1.4. Chemicals and Reagents

The chemicals used were of analytical grades. N-Methyl-N-Nitrosourea (MNU) was obtained from Sigma-Aldrich chemical company, USA. Other chemicals used include sodium dihydrogen phosphate (monobasic), disodium hydrogen phosphate (dibasic), sodium hydrogen

carbonate, sodium carbonate, hydrogen peroxide, glacial acetic acid, epinephrine, pyridine, n-butanol, sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), potassium dichromate, sodium hydroxide and n-Hexane. Rat Carcinoembryonic antigens' ELISA kit was obtained from Cloud-Clone Corp., Germany.

3.1.5Equipment

The required equipment used for the study include IKEA digital weighing balance, UV spectrophotometer (Jenway 6305 model, 50/60Hz, U.K.), pH meter (Jenway 3150 model, UK.), Weighing Balance (Æ GF-2000, A&D instrument Ltd, Japan), water bath (Grant JB series, Grant instruments Ltd,England), centrifuge (Labofuge 300, Heraeus, Kendro Laboratory products, Germany), Microplate reader, Soxhlet Extractor and Hematocrit Auto-Analyser.

3.2 METHODS

3.2.1 Sample Preparation

The shade dried fruits of *Ziziphus spina-christy* were gently crushed using mortar and pistol to separate pericap from the seed, and the separated pericap was used for the feed formulation.

Also, 1Kg of shed dried fruits of *Balanites aegyptica* was soaked in 1.5 liters' water for 24hr hours after removing the epicap and the mesocap (pulp) was washed completely from the seed which form a thick paste like syrup and the paste was dried in the oven at $40^{0}C$ or under the fan at high speed at room temperature.

3.2.2 Formulation of Diets

Growers mash produced by Vital Feed Company located at Bukuru Jos Plateau State was bought in Samaru Market Zaria and used throughout the course of the research. The diets were

mixed with the extract of *Balanites aegyptica fruits and Ziziphus spina-christy*according to the percentage of supplementation (2.5, 5 and 10%).

3.2.3 Experimental Animals/Groupings

The Wistar rats were weighed and divided randomly into 10 groups of five (5) animals each. The groupings were as follows:

Group 1: (Control) Animals to be administered normal saline and placed on basal diet.

Group 2: (Control) Animals to be administered N-Methyl-N-nitrosourea (MNU) and given basal diet

Group 3: Animals to be administered N-Methyl-N-nitrosourea (MNU) and given diet supplemented with 2.5% *Balanites aegyptica* extract

Group 4: Animals to be administered N-Methyl-N-nitrosourea (MNU) and given diet supplemented with 5% *Balanites aegyptica* extract

Group 5: Animals to be administered N-Methyl-N-nitrosourea (MNU) and given diet supplemented with 10% *Balanites aegyptica* extract

Group 6: Animals to be administered N-Methyl-N-nitrosourea (MNU) and given diet supplemented with 2.5% *Ziziphus spina-christy* powdered extract

Group 7: Animals to be administered N-Methyl-N-nitrosourea and given diet supplemented with 5% *ziziphus spina-christy* powdered extract

Group 8: Animals to be administered N-Methyl-N-nitrosourea (MNU) and given diet supplemented with 10% *Ziziphus spina-christy* powdered extract

Group 9: (Diet control *Balanites aegyptica*) Animals to be administered Normal saline and given diet supplemented with 10% *balanites aegyptica* extract

Group 10: (Diet control *Ziziphus spina-christy*) Animals to be administered normal saline and given diet supplemented with 10% *Ziziphus spina-christy* powdered extract

3.2.4 Induction of Colon Cancer

Wistar rats in all the groups were fed as specified by the treatment regimens for the first six weeks after quarantine and acclimatization. On the 7^{th} week, each animal in groups 2-8 began to receive 2.0 mg of N-Methyl-N-Nitrosourea (MNU) three time per week at interval of two days while animals in groups 1, 9 and 10 received normal saline and that continued for the following 10 weeks the administration of N-Methyl-N-Nitrosourea and the normal saline were done intra-rectally. The treatment regimens persisted throughout the period of induction.

3.2.5 Body Weight Measurement

The wistar rats were weighed at the commencement of the experiment and weekly during the course of the study experiment.

3.2.6 Animal Sacrifice

The experimental wistar rats were sacrificed by decapitation under anesthesia induced by Chloroform. The sacrifice was carried out one week after the last administration of N-methyl-N-Nitrosourea (MNU). Organs namely; the kidney, liver and colon were excised, divided and stored

appropriately in phosphate buffer (pH 7.4) and refrigerated for biochemical analysis, while organs for histopathological analysis were stored in 10% formal saline. Blood was collected in two separate tubes. Blood needed for hematological analyses was stored in EDTA anticoagulant-containing blood collection tubes while that required for the production of serum was collected in centrifuge tubes from where the serum was transferred after centrifuging at 3500 rpm/10mins into eppendorf tubes and free blood collection tubes. Also, before the sacrifice, blood was collected via an ocular puncture from the living animals for carcino-embryonic antigen level determination.

3.2.7 Tissue Homogenization

Exactly 1 g of liver was homogenized in 9 ml of ice-cold phosphate buffer (pH 7.4). The kidneys were also homogenized in an amount of phosphate buffer (pH 7.4) proportionate to their sizes, in exactly 1:10 of the homogenate volume (9 times the organ size). Homogenates were kept in appropriately labeled tubes and centrifuged at 3500 rpm for 10 minutes. The supernatants were collected with the aid of pasteur pipettes and frozen immediately at $-18^{0}C$ (Vallor *et al*., 2008).

3.2.8.1 Determination of Carcinoembryonic Antigen (CEA)

The level of the carcino-embryonic antigen was determined via enzyme-linked immuno sorbent assay (ELISA) using a kit which was carried out using serum obtained from the blood of both the sacrificed and the living animals.

Assay Principle: The microtitre plate provided in the kit has been precoated with antibody specific to CEA, standards or samples are being added to the appropriate microtitre plates wells with a biotin conjugate antibody specific for CEA. Next avidin conjugated to horse reddish peroxidase (HRP) is added to each micro plate well and incubated. After TMB substrate solutions

have been added, only those wells that contain CEA, biotin conjugate antibody and enzyme conjugate avidin will exhibit change in color.

The enzyme substrate reaction is terminated by the addition of sulfuric acid solution and the colored changed is measured spectrophotometrically at a wavelength of 450nm +_ 10nm. The concentration of CEA in the sample is then determined by comparing the O.D of the sample to the standard curve.

Assay Procedure:

Wells for diluted standard, blank and samples were determined. Seven wells for standard, 1 well for blank were prepared. 100micro liter each of dilution of standard, blank and sample were added into the appropriate wells, and were covered with plate sealer. were Incubated for 2hours at 37degrees Celsius,the liquid of each well and were removed, without washing

One hundred micro liter of detection reagent A working solution was added to each well and Incubate for 1 hour at 37degrees Celsius after covering with the plate sealer. The solutionwas aspirated and washed with 350 micro liter of 1 * wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto washer, and let it seat for 1 to 2 min, the remaining liquid from each well were removed by snapping the plates onto absorbent paper. Totally washed 3 times after the last wash. Remnant of wash buffer were removed by aspirating or decanting, invert the plate and blot it against absorbent paper.

One hundred microliter of detection B working solution was added to each well. And were Incubate for 30 min at 37degrees Celsius after covering it with plate sealer.The aspiration and wash process were repeated for total 5 times as conducted in step 4, then 90microliter of

substrate solution was added to each well, covered with a new plate sealer,and were Incubated for 15 to 25 min at 37degrees Celsius (don't exceed 30min) which was protected from light. The liquid then turn blue when TMB substrate solution was added.

Fifty microliter of stop solution was added to each well,the liquid then turned yellow by addition of stop solution. The liquid was mixed by tapping the side of the plate. (If color change did not appear uniformly the plate was gently tap to ensure thorough mixing). Drops of water and finger print on the bottom of the plate were removed and it wasconfirmed there was no bubble on the surface of the liquid. Then, the microplate reader was run and conduct measurement at 450nm immediately.

Calculation: The concentrations of CEA in the various wells were extrapolated from the standard double reciprocal plots of absorbance versus concentrations of the standards. The line-of-best-fit and the regression equation were generated electronically by the aid of the Microsoft Excel. In the final analyses, the concentrations were determined with calculating the reciprocal of the values extrapolated from the regression equation and multiplied by the dilution factor.

Rec (absorbance) = Rec (concentration) x b + a

Where;**a** and **b** are regression constants.

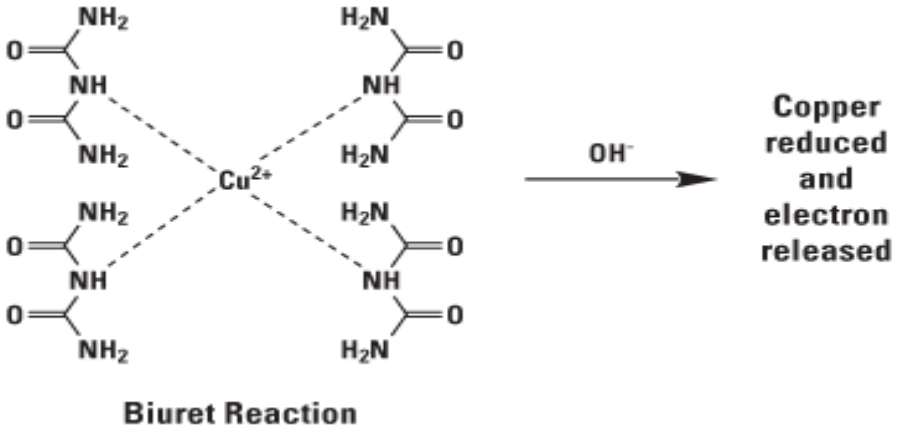
$$\text{Rec (concentration)} = \frac{\text{Rec (absorbance)} - a}{b}$$

$$[\text{C EA}] = \frac{1}{\text{Rec (concentration)}} \mathbf{X} \text{ Dilution Factor}$$

3.2.8.2 Total Protein Assay

The method of (Lowry *et al*, 1951) was adopted in the quantification of total proteins in the targeted tissues.

Assay principle: It involves two major steps. In the first step, the protein molecules react with copper (II) ions (Cu^{2+}) in an alkaline medium in the presence of sodium potassium tartrate in a manner similar to the biuret reaction to form a tetradentate copper complex. In the second step, Folin-Ciocalteu reagent (phosphopolymolybdotungstates mixed acids) is assumed to be reduced by the electrons released in the first step to form heteropolymolybdenum blue believed to absorb maximally at 750 nm.



Biuret Reaction

Data Analyses: The tissue protein concentrations were extrapolated from the standard curve of absorbance versus concentration of the standard as described above.

$$\text{Absorbance} = \text{Concentration} \times b + a$$

Where **a** and **b** are regression constants.

$$[\text{Protein}] = \frac{\text{Absorbance} - a}{b} \times \text{Dilution Factor}$$

It was ensured that the standard was run concurrently with the samples. Mixing of reaction mixture was done as soon as the reagents were added and Lowry reagent C remaining after 24 hours was discarded.

3.2.8.3.1 Assay for concentration of Malondialdehyde

The method of (Ohkawa *et al*, 1979) was adopted in the assay for the concentration of malondialdehyde (MDA) as an index of lipid peroxidation. Alteration in the oxidant- antioxidant profile as a result of oxidative stress due to damage brought about by free radicals is known to occur in cancer, (Saroja *et al*, 1999) and (Bhuvarahamurthy *et al*, 1996) which thereby causes lipid peroxidation to occur.

Assay Principle: Malondialdehyde is one of the products formed during the degradation of lipid peroxides ($LOO^{\cdot}$) formed by the reaction between lipid radicals ($L^{\cdot}$) and molecular oxygen (O_2) or by the abstraction of a hydrogen atom from lipid hydroxyperoxides (LOOH). The assay is dependent on the tendency of the aldehydic products (malondialdehyde [MDA], 4-Hydroxynonena [4-HNE],) to react with thiobarbituric acid to form Thiobarbituric Acid Reacting Substances (TBARS) .

$$LH + Fe^{2+}/NADP \longrightarrow L^{\cdot} + Fe^{3+}/NADPH$$

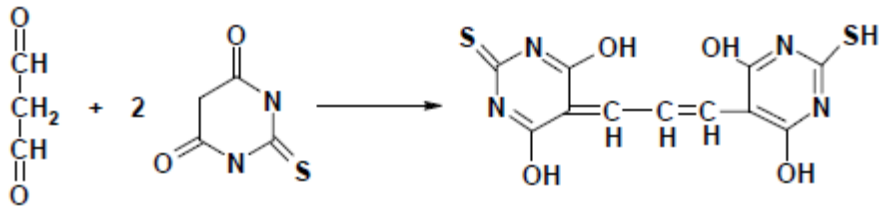
$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$

$$LOO^{\cdot} \longrightarrow MDA + \ldots$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

$$LOOH + Fe^{2+}/NADP \longrightarrow LOO^{\cdot} + Fe^{3+}/NADPH$$

$$LOO^{\cdot} \longrightarrow MDA + \ldots$$



MDA TBA MDA-TBA ADDUCT

Assay Procedure: The reaction mixture containing 40 µl of 8.1 % sodium dodecyl sulphate (SDS), 300 µl of acetate buffer (pH 3.5: 20 % glacial acetic acid, 3M NaOH), 300 µl of TBA (0.8%), 40 µl of homogenate and 120µl of distilled water was incubated in a boiling water bath at 95^0C for 60 minutes and cooled thereafter in ice-cold water. The mixture was further treated with the addition of 200 µl of distilled water and 1.0 ml of n-butanol/pyridine mixture (15:1 v/v), mixed and centrifuged at 3500 rpm for 10 mins. The red (pink) organic layer (upper layer) formed was collected and its absorbance was read spectrophotometrically at 532 nm. The blank contained all components except the sample (homogenate).

3.2.8.4. Analyses of Endogenous Antioxidant Activities

All experimental procedures were performed in duplicate.

3.2.8.4.1 Assay for Catalase Activity

The method of (Sinha, 1972) was employed in the assay for catalase activities.

Assay Principle: Potassium dichromate reacts with hydrogen peroxide (H_2O_2) in the presence of acetic acid to form an unstable blue precipitate of perchromate which upon heating decomposes to produce a clear green solution of chromic acetate which absorbs maximally at a wavelength of 570nm. The sample catalase decomposes H_2O_2 to form water and molecular oxygen. This enzyme-substrate reaction is terminated by the acetic acid while potassium dichromate reacts with the excess H_2O_2, upon the introduction of the dichromate/acetic acid mixture.

$$H_2O_{2\,(l)} \xrightarrow{\text{Catalase}} H_2O_{(l)} + O_{2\,(g)}$$

Assay Procedure: A total volume of 1.32 ml solution of the reaction mixture was prepared.Exactly 660 µl of 50 mM phosphate buffer (pH 7.0) was added to 530 µl of hydrogen peroxide (0.2M) in a test-tube. The reaction was initiated by the addition of 130 µl of the sample (diluted). The mixture was swirled gently and an aliquot of 330 µl of the reaction mixture was transferred into 660 µl of potassium dichromate/acetic acid mixture (1:3 v/v) in 4 separate test-tubes at 1 minute interval to stop the reaction. The blue precipitate formed was heated at about 95^0C for 10 minutes to obtain a green solution and the absorbance was read spectrophotometrically at 570 nm.

A calibration curve was prepared using 1.0-200 mM of hydrogen peroxide to determine the concentration of hydrogen peroxide reacted with potassium dichromate in the reaction mixtures. The specific activity of catalase was expressed as [H_2O_2] consumed/min/mg protein. The concentration of H_2O_2 consumed per minute was determined electronically with the aid of the Microsoft Excel from the standard curve of absorbance versus concentration. Average change in absorbance/minute/sample was determined by plotting absorbance against time. The slope of the graph gave the average change in H_2O_2 absorbance per minute with which average concentration of H_2O_2 consumed per minute was extrapolated from the standard regression equation.

Absorbance (H_2O_2) = x b + a

Where **a** and **b** are regression constants.

[H_2O_2] Absorbance (H_2O_2) - a **(**Standard)

$$= \frac{\qquad\qquad\qquad\qquad}{b}$$

Absorbance = Time (mins) x $\underline{b}$ + a (Sample)

Where $\underline{b}$ is the ΔAbsorbance/minute and the independent coefficient of the sample regression equation. The concentration of hydrogen peroxide consumed per minute was obtained thus:

$$[H_2O_2]/\text{minute} = \frac{b\text{- a}}{b} \text{ X Dilution Factor}$$

Where $\underline{b}$ is the sample independent coefficient while **b** and **a** arethe standard regression coefficients.

The specific activity of catalase was calculated thus:

$$\text{Specific Activity} = {}^{1}\!/\!_{[\text{Protein}]} \left(\frac{\text{b- a}}{b}\right) \text{Dilution Factor}$$

3.2.8.4.2 Assay for Superoxide Dismutase Activity

The method of (Fridovich and Mistra, 1972) was employed in the assay for the activity of superoxide dismutase (SOD).

Assay Principle: It is dependent on the ability of superoxide dismutase to inhibit autoxidation of epinephrine by converting the superoxide anion (O_2^-), which is responsible for the propagation of epinephrine oxidation to its unstable autoxidation product, adrenochrome which absorbs maximally at 480 nm, to hydrogen peroxide and oxygen.

The auto-oxidation reaction is initiated intrinsically, may be owing to the presence of metal impurities, by abstracting a hydrogen atom from the epinephrine (RH_3^-) to convert it to the activated form, $RH_3^{\cdot}$, which reacts with molecular oxygen to form adrenochrome (R) and a superoxide radical. The rate of formation of the adrenochrome is a function of the concentration

of the superoxide anion present, and the activity of the superoxide dismutase if present at a pH above 8.5. (McCord, and Fridovich, 1996)

One unit of SOD is the amount of the enzyme required to inhibit the autoxidation of epinephrine by 50% under the specified conditions, such as pH 10.2 and temperature of 30^0C.

$$RH_3^- + Me^n \longrightarrow RH_3^{\cdot} + Me^{n-1}$$

$$RH_3^{\cdot} + O_2 \longrightarrow RH_2 + O_2^- + H^+$$

$$RH_2 + O2^- + H^+ \longrightarrow RH^{\cdot} + H_2O_2$$

$$RH^{\cdot} + O_2 \longrightarrow R + O_2^- + H^+$$

Assay Procedure: A total volume of 3.0 ml reaction mixture was prepared. Exactly 2.5 ml of 50 mM carbonate buffer (pH 10.2) was added to 200 µl of the diluted sample (homogenate) in a test-tube. The reaction mixture was transferred into a 4 ml-cuvette used for equilibrating the spectrophotometer at 480 nm. An aliquot of 300 µl of 0.3 mM solution of epinephrine (prepared in 0.005 M glacial acetic acid maintained at pH 1.3 to 2.0 with HCL to form a clear solution) was added and absorbance readings were recorded for 5 minutes at 1-minute interval. The reference readings were recorded at the same wavelength prior to sample readings for the same period to serve as a guide for dilution of samples and as the base of the final calculations. The reference cuvette contained 2.5 ml of the buffer, 200 µl of distilled water and 300 µl of epinephrine solution.

Calculation: The specific activity (unit/mg/ml protein) of SOD was calculated electronically with the aid of the Microsoft Excel. The average rate of formation of adrenochrome (concentration of adrenochrome formed per minute) in the presence of the samples (r_s) or in the reference cuvette (r_b), was obtained by determining the average change of absorbance of adrenochrome per minute from the slopes of graphs of absorbance versus time (min).

Absorbance = Time (mins) x r_s + *a* (Sample)

Absorbance = Time (mins) x r_b + a (Reference)

The rate ratio was calculated thus:

$$\text{Rate Ratio} = \frac{r_s}{r_b}$$

The percentage inhibition was determined thus:

$$\% \text{ Inhibition} = \frac{r_b - r_s}{r_b} 100$$

The unit of SOD was calculated thus:

$$[\text{SOD}] \text{ (U/ml)} = 0.02 \left(\frac{r_b - r_s}{r_b} 100 \right) \frac{\text{Dilution Factor}}{\text{Sample Volume}}$$

Finally, the specific activity of SOD was calculated thus:

$$\text{Specific Activity} = 0.02 \Big/ [\text{Protein}] \left(\frac{r_b - r_s}{r_b} 100 \right) \frac{\text{Dilution Factor}}{\text{Sample Volume}}$$

3.3. Histopathological Analysis of Harvested Organs

The dissected colon tissues were removed and fixed in 10 % formol saline which was prepared by mixing formaldehyde (200 ml) in distilled water containing sodium chloride (17g). The tissues are then placed in separate plastic cups containing the formol saline according to their various groups for 48 hours. The tissues were processed using the Shandon Southern Automatic tissue processor and sections of 5 microns were made using Leitz base sledge microtome. The sections were stained using haematoxylin and eosin technique.

3.4 Hematological Analysis

Blood collected from the various animals were analyzed in Biochemistry department using Sysmex KX2IN Heamatology autoanalyser of Drucker diagnostic innovative solutions for healthier world U.S.A. Blood parameters assayed were red blood cells, white blood cells, platelets and hemoglobin.

3.5 Statistical Analysis

The data generated from the study were analyzed using Excel and SPSS 2.0 applications. All results are expressed as ± standard deviation using one-way ANOVA

CHAPTER FOUR

4.0RESULTS

4.1 Body Weight of the Experimental Wistar Rats

The results obtained from the determination of the experimental wistar rats a day before sacrifice are presented in Table 4.1.

There was no significant (p 0.05) difference in body weight of the control normal saline group compared to the control MNU group. However, in the treated groups there was significant (p 0.05) increase in the body weights of wistar rats fed with 10% *Balanites aegyptica* and *Ziziphus spina-christy* fruits extract supplemented diet compared to the animals fed with 2.5% and 5% *Balanites aegyptica* and *Ziziphus spina-christy* fruits extract supplemented diets respectively. However, the body weights of the wistar rats in the diet control groups fed with 10% *Balanites aegyptica* and *Ziziphus spina-christy* fruits extract (Normal saline) supplemented was a significantly (p 0.05) higher than the wistar rats fed with 10% *Balanites aegyptica* and *Ziziphus spina-christy* fruits extract (MNU) supplemented diet.

Table 4.1 Body Weights of the Experimental Wistar Rats Treated with MNU and Diets Supplemented with Varying Levels of *Balanites aegyptica* and *Ziziphus spina-christy*

Treatment	Body Weight Change (g)
Control Normal Saline	9.30 ± 1.06^{c}
Control MNU	6.33 ± 0.51^{ac}
MNU + 2.5 % *balanites aegyptica*	5.01 ± 0.56^{ab}
MNU + 5 % *balanites aegyptica*	5.25 ± 0.61^{ab}
MNU + 10 % *balanites aegyptica*	7.70 ± 0.60^{b}
MNU + 2.5 % *ziziphus spina-christy*	6.72 ± 0.56^{bc}
MNU + 5 % *C. ziziphus spina-christy*	7.14 ± 0.61^{c}
MNU + 10 % *ziziphus spina-christy*	7.14 ± 0.60^{c}
10 % *balanites aegyptica* diet control	8.27 ± 0.00^{a}
10 % *C. ziziphus spina-christy* diet control	8.59 ± 0.00^{d}

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different (P>0.05). N-Methyl-N-Nitrosourea (MNU)

4.2 Feeding Rates of the Experimental Wistar Rats

The feeding efficiency rates of the various experimental animals measured are presented in table 4.2

Feeding efficiency rate observed in the control normal saline group were significantly (p□0.05) increased compared to the control MNU group. Conversely in the treated groups there was no significant (p□0.05) difference in feeding efficiency rate of animals fed with *Balanites aegyptica* supplemented diets. However, in the other treated groups, wistar rats fed with *Ziziphus spina-christy* supplemented diet did not differ significantly (p□0.05) in their feeding efficiency rate from each other. The results also showed that the wistar rats in the diet control group fed with 10% *Ziziphus spina-christy* supplemented diet showed a significant (p□0.05) increase in feeding efficiency rate compared to wistar rats fed with 10% *Balanites aegyptica* supplemented diet.

Table 4.2 Feeding Rates of the Experimental Wistar Rats Treated with MNU and Diets Supplemented with Varying Levels of *Balanites aegyptica* and *Ziziphus spina-christy*

Treatment	g/100gkg body weight
Control Normal	73.87 ± 3.74 [d]
Control MNU	67.10 ± 2.87 [bcd]
MNU + 2.5 % *balanites aegyptica*	52.41 ± 3.92 [abc]
MNU + 5 % *balanites aegyptica*	55.46 ± 4.93 [abc]
MNU + 10 % *balanites aegyptica*	56.82 ± 4.49 [abc]
MNU + 2.5 % *ziziphus spina-christy*	67.56 ± 2.74 [cd]
MNU + 5 % *ziziphus spina-christy*	68.66 ± 3.16 [cd]
MNU + 10 % *ziziphus spina-christy*	71.50 ± 3.55 [cd]
10 % *balanites aegyptica* diet control	53.80 ± 2.13 [ab]
10 % *ziziphus spina-christy* diet control	62.45 ± 3.32 [abcd]

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different (P>0.05). N-Methyl-N-Nitrosourea (MNU)

4.3 Concentrations of Carcino-embryonic Antigen in the Serum of the Wistar Rats Treated with MNU and Diets Supplemented with Varying Levels of *Balanites aegyptica* and *Ziziphus spina-christy*

Serum concentrations of carcinoembryonic antigen of experimental wistar rats treated with MNU and diets supplemented with different levels of *Balanites aegyptica* and *Ziziphus spina-christy* are presented in Table 4.1 Carcinoembryonic antigen level was observed to increase significantly (p□0.05) in the control MNU induced group compared to the control normal saline group. Results also showed that concentrations of carcino-embryonic antigen in rats group fed with 2.5% *Balanites aegyptica* and *Ziziphus spina-christy* supplemented diets increased significantly (p□0.05) compared to the other treated groups. However, the level of carcino-embryonic antigen in wistar rats in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet was not significantly (p□0.05) different from those fed with 10% *Ziziphus spina-christy* supplemented diet.

Table 4.3 Serum Concentrations of Carcino-embryonic Antigens of the Wistar Rats Treated with MNU and Diets Supplemented with Varying Levels of *Balanites aegyptica* and *Ziziphus spina-christy*

Treatment	Concentration (ng/ml)
Control Normal Saline	1.47 ± 0.17^{bc}
Control MNU	1.91 ± 0.10^{cd}
MNU + 2.5 % *balanites aegyptica*	2.06 ± 0.11^{cd}
MNU + 5 % *balanites aegyptica*	1.60 ± 0.11^{bc}
MNU + 10 % *balanites aegyptica*	1.34 ± 0.05^{c}
MNU + 2.5 % *ziziphus spina-christy*	2.13 ± 0.07^{d}
MNU + 5 % *ziziphus spina-christy*	1.29 ± 0.08^{ab}
MNU + 10 % *ziziphus spina-christy*	1.59 ± 0.17^{bc}
10 % *balanites aegyptica* diet control	1.14 ± 0.28^{a}
10 % *ziziphus spina-christy* diet control	1.27 ± 0.15^{a}

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different ($P>0.05$).N-Methyl-N-Nitrosourea (MNU)

4.4 Effect of Dietary Supplementation with *Balanites aegyptica* and *Ziziphus spina-christy* on Lipid Peroxidation, antioxidant enzymes and Protein Content in the Liver of MNU Induced Colon carcinogenesis in Wistar Rats

4.4.1 Malondialdehyde (MDA) Concentration.

In table 4.2 the concentration of malondialdehyde (MDA) in the control MNU group was significantly ($p \square 0.05$) different compared to the MDA concentration in the control normal saline group. However, the concentration of MDA of the wistar rats fed with 10 % *Balanites aegyptica* supplemented diet was significantly ($p \square 0.05$) lower than those wistar rats fed with 2.5 % and 5 % *Balanites aegyptica* supplemented diets. Similarly, wistar rats fed with 2.5% *Ziziphus spina-christy* supplemented diet had significantly ($p \square 0.05$) higher concentration of MDA compared to those fed with the 5% and 10% *Ziziphus spina-christy* supplemented diets. However, in the diet control groups there was no significantly ($p \square 0.05$). difference in MDA concentration.

4.4.2 Endogenous Antioxidants

4.4.2.1 Catalase Activity

In table 4.2Catalase activity in the control MNU group was significantly ($p \square 0.05$) different from the control normal saline group. Similarly, in the treated groups the catalase activity of the wistar rats fed with *Balanites aegyptica* supplemented diet were significantly ($p \square 0.05$) different from each other. However,in the treated group fed with *Ziziphus spina-christy*the groups were not significantly different. The wistar rats in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet showed a significantly ($p \square 0.05$) increase in catalase activity compared to wistar rats receiving 10% *Ziziphus spina-christy* supplemented diet.

4.4.2.2 Superoxide Dismutase (SOD) Activity

In Table 4.2there was significant (p□0.05) difference in SOD activity in the control normal saline group compared to the control MNU group. Among the treated groups the SOD activity of the wistar rats fed with 10 % *Balanites aegyptica* supplemented diet was significantly (p□0.05) elevated compared to the SOD activity obtained in the wistar rats fed with 2.5% and 5% *Balanites aegyptica* supplemented diets respectively. Conversely, the wistar rats fed with *Ziziphus spina-christy* supplemented diets showed no significant (p□0.05) SOD activity in 5% and 10% treated groups respectively. The results also showed that the SOD activity in the wistar rats in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet did not differ significantly (p□0.05) to those wistar rats receiving 10% *Ziziphus spina-christy* supplemented diet.

4.4.3Protein Content

In table 4.2the results obtained from the determination of protein content showed that there was significant (p□ 0.05) difference in the protein concentrations in the control MNU group compared to that obtained in the control normal saline group. However, in the treated groups the protein concentration of the wistar rats fed with 10% *Balanites aegyptica* supplemented diet showed a significant (p□ 0.05) increase in protein concentration compared to the wistar rats fed with 2.5% and 5% *Balanites aegyptica* supplemented diets, respectively. Similarly, wistar rats fed with 2.5% *Ziziphus spina-christy* supplemented diet had significantly (p□ 0.05) amount of protein compared to those of wistar rats fed with the 5% and 10% *Ziziphus spina-christy* supplemented diets. However, the animals in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet had a (p□ 0.05) increase protein concentration than the animals receiving 10% *Ziziphus spina-christy* supplemented diet.

Table 4.4 Effect of Dietary Supplementation with *Balanites aegyptica* and *Ziziphus spina-christy* on Lipid Peroxidation, antioxidant enzymes and Protein Content on the Liver of MNU Induced Colon carcinogenesis in Wistar Rats

Treatment	[Protein] (mg/ml)	Catalase Activity (Unit/mg protein)	Superoxide dismutase Activity (Unit/mg protein)	Malondialdehyde (µM/mg/ml protein)
Normal Control	10.40 ± 0.23^{bcd}	111.9 ± 6.6^{bc}	79.2 ± 2.2^{abc}	5.07 ± 0.13^{c}
Control MNU	21.14 ± 1.02^{abcde}	80.45 ± 9.2^{b}	65.6 ± 4.7^{a}	16.40 ± 0.00^{ac}
MNU+ 2.5% *Balanites aegyptica*	14.98 ± 1.01^{e}	107.1 ± 7.0^{bc}	71.3 ± 2.1^{cd}	11.22 ± 0.01^{ab}
MNU+5% *Balanites aegyptica*	14.07 ± 1.03^{e}	105.1 ± 1.4^{a}	$83,7 \pm 4.1^{abc}$	6.22 ± 0.20^{b}
MNU+10% *Balanites aegyptica*	$12.36 \pm 1,49^{de}$	117.8 ± 5.8^{ab}	100.2 ± 1.5^{d}	5.16 ± 0.16^{c}
MNU+2.5% *Ziziphus spina-christy*	18.94 ± 1.11^{bcde}	111.0 ± 4.8^{cd}	73.6 ± 5.4^{abc}	12.23 ± 0.01^{bc}
MNU+5% *Ziziphus spina-christy*	14.12 ± 1.28^{abc}	115.3 ± 6.4^{cd}	88.9 ± 2.1^{ac}	11.16 ± 0.10^{a}
MNU+10% *ziziphus spina-christy*	13.39 ± 1.20^{abc}	120.5 ± 9.4^{cd}	90.9 ± 6.0^{ac}	10.13 ± 1.11^{a}
10% *Balanites aegyptica*	11.05 ± 1.47^{a}	134.3 ± 8.0^{bc}	84.4 ± 5.1^{bc}	6.06 ± 0.40^{b}
10% *Ziziphus spina-christy*	10.81 ± 1.16^{cde}	119.6 ± 0.00^{e}	76.66 ± 6.34^{bc}	6.40 ± 0.043^{b}

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different (P>0.05).N-Methyl-N-Nitrosourea (MNU)

4.5 Effect of Dietary Supplementation with *balanites aegyptica* and *ziziphus spina-christy* on Lipid Peroxidation, antioxidant enzymes and Protein Content on the Kidney of MNU Induced Colon carcinogenesis in Wistar Rats

4.5.1 Malondialdehyde (MDA) Concentration.

In table 4.5 the results showed that there was significant (p□0.05) difference in the malondialdehyde (MDA) concentrations of the control MNU group compared to the control normal saline group. Similarly, the concentration of MDA of the wistar rats fed with the *Balanites aegyptica* supplemented diets did not differ significantly (p□0.05) from each other. However, wistar rats treated with *Ziziphus spina-christy* supplemented diets showed a significant (p□0.05) increase in MDA concentration compared to the 5% and 10% *Ziziphus spina-christy* supplemented diet groups respectively. The results also showed that the diet control groups wistar rats fed with 10% *balanites aegyptica* supplemented diet and 10% *Ziziphus spina-christy* supplemented diets showed no significant (p□0.05). difference in MDA concentration.

4.5.2 Endogenous Antioxidants

4.5.2.1 Catalase Activity

In table 4.5 the results obtained from the analyses of the endogenous antioxidants showed that there was significant (p□0.05) difference in catalase activity in the control MNU group compared to the control normal saline group. The catalase activity of the wistar rats fed with 10% *Balanites aegyptica* supplemented diet was significantly (p□0.05) higher in catalase activity compared to the 2.5% and 5% *Balanites aegyptica* supplemented diet groups. However, the concentration of catalase of the wistar rats group fed *Ziziphus spina-christy* supplemented diet differ significantly (p□0.05) from each other. The catalase activity of the wistar rats in the diet control groups fed with 10% *Balanites aegyptica*supplemented diet was significantly higher(p□0.05) as comparedwith 10% *Ziziphus spina-christy* supplemented diet, (Table 4.3).

4.5.2.2 Superoxide Dismutase (SOD) Activity

In table 4.5 the activity of superoxide dismutase (SOD) in the control MNU group of wistar rats differ significantly (p□0.05) from the control normal saline group. The SOD activity in the animals fed with 10% *Balanites aegyptica* supplemented diet increased significantly (p□0.05) compared to the wistar rats fed 2.5 % and 5 % *Balanites aegyptica* supplemented diets. However, there was no significant (p□0.05) difference in SOD activity in the wistar rats fed with *Ziziphus spina-christy* supplemented diets. Similarly, the wistar rats in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet had no significant (p□0.05) difference in SOD activity compared to the wistar rats fed with 10% *Ziziphus spina-christy* supplemented diet.

4.5.3Protein Content

In table 4.5 the protein concentrations in the control normal saline group was significantly (p□0.05) lower compared to that obtained in the control MNU group. The result also showed that the protein concentration in the wistar rats fed with 2.5% *Balanites aegyptica* supplemented diet increased significantly (p□0.05) compared to the wistar rats fed with 5% and 10% *Balanites aegyptica* supplemented diets groups. Likewise, the wistar rats fed with 2.5% *Ziziphus spina-christy* supplemented diet had significantly (p□0.05) higher protein content compared to the wistar rats fed with the 5% and 10% *Ziziphus spina-christy* supplemented diets. The protein content of the wistar rats in the diet control groups fed with 10% *Balanites aegyptica* supplemented diet did not differ significantly (p□0.05) from those wistar rats fed with 10% *Ziziphus spina-christy* supplemented diets.

Table 4.5 Effect of Dietary Supplementation with *Balanites aegyptica* and *Ziziphus spina-christy* on Lipid Peroxidation, antioxidant enzymes and Protein Content on the Kidney of MNU Induced Colon carcinogenesis in Wistar Rats

Treatment	Protein (mg/ml)	Catalase Activity(Unit/mg protein)	Superoxide dismutase Activity (Unit/mg protein)	Malondialdehyde (µM/mg/ml protein)
Normal Control	9.45 ± 0.34^{de}	622 ± 75^{ab}	79.4 ± 4.6^{ab}	7.12 ± 5^{ab}
Control MNU	17.92 ± 0.52^{ad}	238 ± 23^{a}	65.0 ± 4.9^{b}	15.4 ± 3.34^{a}
MNU+ 2.5% *Balanites aegyptica*	12.11 ± 0.06^{cd}	304 ± 22^{b}	73.8 ± 1.0^{a}	10.19 ± 2.10^{ab}
MNU+5% *Balanites aegyptica*	11.17 ± 0.15^{bc}	372 ± 19^{ab}	80.2 ± 2.3^{bc}	7.18 ± 3.02^{ab}
MNU+10% *Balanites aegyptica*	10.44 ± 0.53^{a}	489 ± 22^{c}	89.4 ± 2.3^{c}	7.87 ± 2.23^{ab}
MNU+2.5% *Ziziphus spina-christ*	11.59 ± 1.18^{bcd}	407 ± 19^{ab}	88.9 ± 3.6^{ab}	13.86 ± 1.11^{ac}
MNU+5% *Ziziphus spina-christ*	11.13 ± 1.05^{bd}	392 ± 17^{ac}	66.6 ± 20^{ab}	9.12 ± 2.08^{a}
MNU+10% *Ziziphus spina-christ*	9.47 ± 0.48^{b}	$579 \pm 40.^{bc}$	97.2 ± 26^{ab}	8.10 ± 1.91^{a}
10% *Balanites aegyptica*	10.59 ± 1.08^{de}	704 ± 32^{ad}	87.8 ± 9.3^{ac}	12 ± 2.94^{ab}
10% *Ziziphus spina-christ*	9.42 ± 1.16^{de}	604 ± 23^{bd}	82.2 ± 2.4^{ac}	12 ± 2.11^{ab}

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different (P>0.05).N-Methyl-N-Nitrosourea (MNU)

4.6 Effect of Dietary Supplementation with *Balanites aegyptica* and *Ziziphus spina-christy* on the Hematological Parameters of MNU Induced Colon carcinogenesis in Wistar Rats

In table 4.6the hematological parameters observed in the red blood cells, white blood cells and hemoglobin were not significantly (p 0.05) different across the groups Although platelets counts were significantly (p 0.05) reduced in the control MNU group compared to the control normal saline group, also the red blood cell count is significantly lower in the MNU treated groups as compared the normal control group and other treated groups.

Table 4.6 Effect of Dietary Supplementation with *Balanites aegyptica* and *Ziziphus spina-christy* on Hematological Parameter of MNU Induced Colon carcinogenesis in Wistar Rats

Animal Groupings	White Blood Cell (µL)	Hemoglobin (dL)	Red Blood Cells (µL)	Platelets (µL)
Normal Control	9.66±2.24 [ab]	152.00 ± 0.68[a]	9.34± 0.64 [a]	950.67± 62.17 [a]
Control MNU	9.85± 1.81 [ab]	151.14 ± 0.92[a]	6.99 ± 0.54 [a]	779.33 ± 10.50[b]
MNU+ 2.5% *Balanites aegyptica*	10.09±2.90 [ab]	145.86 ± 5.24[a]	7.64± 0.34 [a]	814.33 ± 6.03 [a]
MNU+5% *Balanites aegyptica*	7.76 ± 1.15 [a]	153.14± 9.30[a]	5.00± 0.46 [a]	945.67± 51.97[b]
MNU+10% *Balanites aegyptica*	9.76 ± 2.07 [ab]	143.75± 7.92[a]	8.78 ± 0.46 [a]	818 ± 60.23 [a]
MNU+2.5% *Ziziphus spina-christ*	11.39± 2.40 [b]	156.00 ±10.50[a]	8.67± 0.84 [b]	847.33 ± 21.73[a]
MNU+5% *Ziziphus spina-christ*	11.28± 2.31 [b]	158.13± 17.96 [a]	8.51± 0.80 [a]	781.33± 9.82[ab]
MNU+10% *Ziziphus spina-christ*	9.1± 2.43 [ab]	148.38 ±11.28	8.19 ± 0.50 [a]	780.67± 23.09[ab]
10% *Balanites aegyptica*	8.60 ± 1.23 [ab]	144.00 ± 8.76[a]	7.36 ±0.44 [a]	820±29.70 [a]
10% *Ziziphus spina-christy*	7.83 ± 1.45 [ab]	144.50 ± 10.79 [a]	9.02 ± 0.62 [a]	824 ± 23.46[a]

Values are presented as mean ± Standard Deviation. Values bearing the same superscripts are not significantly different (P>0.05).

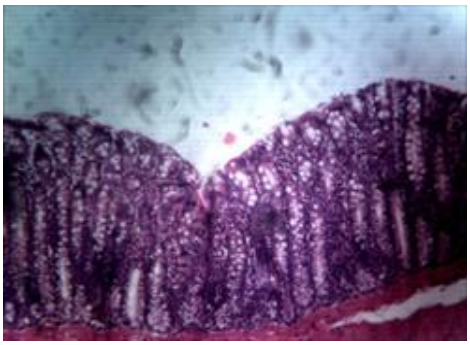


PLATE 1a. Normal control (x60)
Normal control colon shows normal colon mucosa

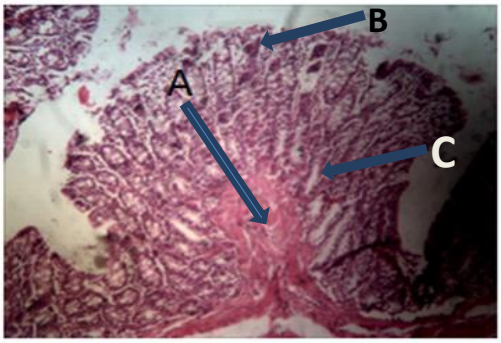


PLATE 1b. Control MNU (x60)
MNU control colon architecture shows **A;** hyperchromiticity, B, Atrophy of gland, C: necrosis

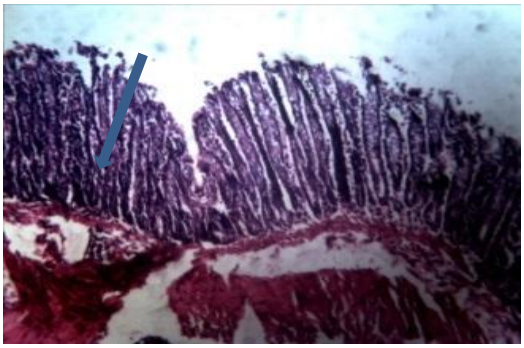
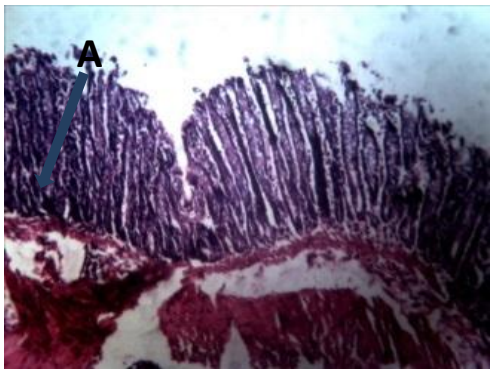


PLATE 1c. MNU + 2.5% *Balanite aegyptica* (x60)
MNU + 2.5% *Balanite aegyptica* suppl. colon shows A; Hyperchromicity



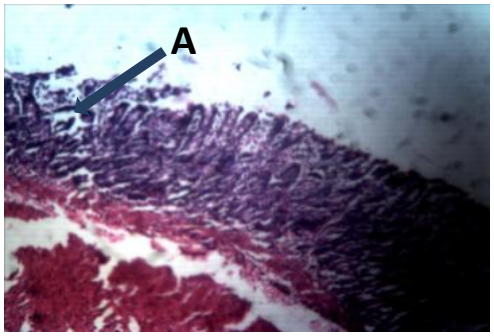


PLATE 1d. MNU + 5% *Balanite aegyptica* (x60)
MNU + 5 % *Balanite aegyptica* suppl. colon shows A; atrophy

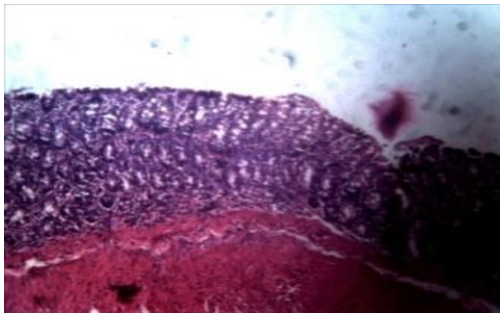


PLATE 1e. MNU + 10% *Balanite aegyptica* (x60)
MNU + 10 % *Balanite aegyptica* suppl. colon shows normal features.

4.7.1 Effect of dietary supplementation with *Balanites aegyptica* on the histology of the colon of MNU induced colon carcinogenesis

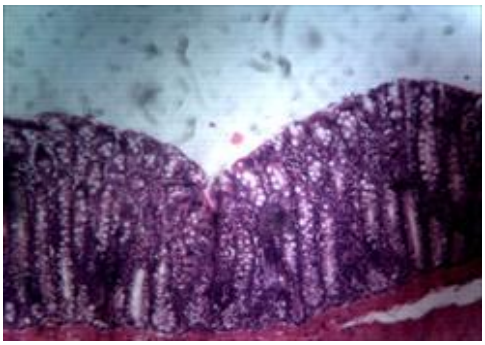


PLATE 2a. Normal control (x60) Normal control colon shows normal colon mucosa

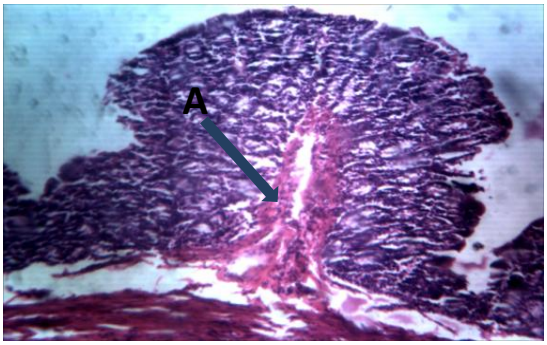


PLATE 2d. MNU + 5% (x60) MNU+ 5% *Ziziphus spina-christy* suppl. Colonshows A; pedunculated hyperplastic polyp

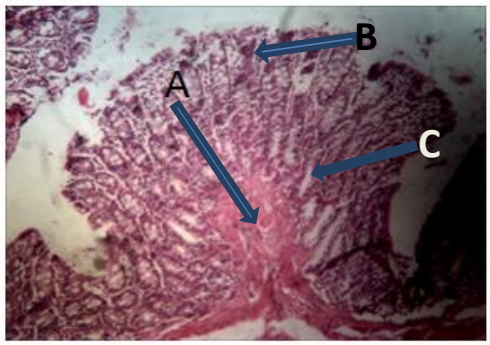


PLATE 2b. Control MNU (x60)
MNU control colon architecture shows **A;** hyperchromiticity, B, Atrophy of gland, C: necrosis

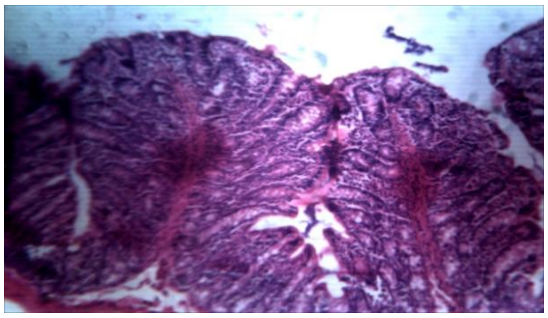


PLATE 2e. MNU + 10% (x60)
MNU + 10% *Ziziphus spina-christy* suppl. Colon shows normal feat

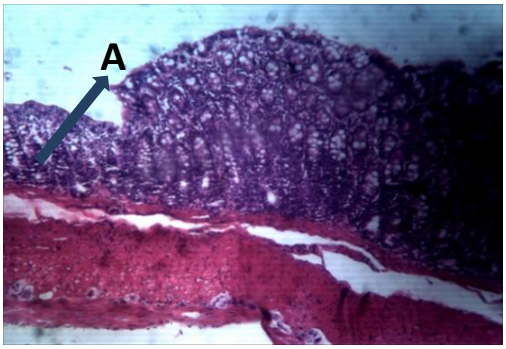


PLATE 2c. MNU + 2.5% (x60)
MNU + 2.5% *Ziziphus spina-christy* suppl. Colon shows A; Hyperchromicity

4.7.2 Effect of dietary supplementation with Ziziphus *spina-christy* on the histology of the colon of MNU induced colon carcinogenesis.

CHAPTER FIVE

DISCUSSION

The observed increase in body weight in the 10 % *Balanites aegyptica* supplemented diet group may be as a result of increased feed consumption and a reduction in oxidative stress which would have otherwise led to carcinogenesis as a result of increased antioxidants. The insignificant consumption rate recorded across the groups may be due to the fact that the rate of carcinogenesis had not progressed to the point of inhibiting feed intake in the treated groups.

Free radical scavenging effects of aqueous extracts of leaves of Balanites aegyptica at different concentrations were measured with ascorbic acid as standard compound by using DPPHmethod. From theresults it can be shown that Free radical scavenging capacity increased with increasing extracts concentration (Thamaraiselvi, 2014).

The carcinoembryonic antigen level was observed to be elevated in the control MNU and 2.5% *Balanites aegyptica* and *Ziziphus spina-christy* supplemented diet groups indicating that the carcinogen had elicited an immunological response on the wistar rats. However,the low levels of carcinoembryonic antigen in the other treated group may be attributed to the endogenous antioxidants activity which served as free radical scavengers and the presence of polyphenols and flavonoids in the supplemented diets. This observation is in agreement with Atawodi (2014), who reported that the compound (polyphenol) possessed anticancer properties and it is present in*Balanites aegyptica* and *Ziziphus spina-christy*.

The presence of calcium in the basal supplemented diets may also have an anticancer role in colon cancer. This finding is supported by Terry *et al.*, (1999), in a study that included more

than 61,000 Swedish women, colorectal cancer risk was approximately 28 percent lower among individuals who had the highest calcium intakes (approximately 800–1000 mg per day) compared with those with the lowest calcium intakes (approximately 400–500 mg per day). Data from this study also suggested that the benefit associated with calcium was limited to the distal colon. In a study that involved more than 34,000 postmenopausal Iowa women, high intakes of calcium (approximately 1280 mg per day or more) compared with low calcium intakes (approximately 800 mg per day or less) from both the diet and supplements were associated with a 41 percent reduction in risk of rectal cancer. According to Zheng *et al.* (1998) reduced risks of rectal cancer were also observed for dietary calcium alone.

The significant decrease in malondialdehyde (MDA) content in the wistar rats treated with 10 % *balanites aegyptica* and *ziziphus spina-christyziziphus* supplemented diet may be attributed to the protection derived from the antioxidants which caused a reduction in oxidative stress that may have led to lipid peroxidation. Jaswal *et al*., (2014) reported positive effect of combined dietary antioxidants supplementation (vitamin E, A and C with no mention of the dosage) on the reducing of lipid peroxidation levels compared to control group after 3 months supplementation in RA (Rheumatoid arthritis) patients. According to Nourmohammadi *et al*., (2016) studied the antioxidant supplementation with vitamin C 300 mg, zinc 5mg and vitamin A 25000 International Unit (IU) as every other day for 12 weeks that reduced the level of lipid peroxidation. We used serum levels of malondialdehyde as a marker of lipid peroxidation and previous studies indicated the increased lipid peroxidation in patients with RA.

According to Torabian *et al.*, (2009)consumption of nuts increased plasma polyphenol concentrations, total antioxidant capacity and reduced plasma lipid peroxidation. Schinella *et al.* (2010) evaluated the ability of cocoa powder a rich source of flavonoids and polyphenols to

bring about reduction in lipid peroxidation in brain homogenates and human plasma using nonenzymatic generation systems. Cocoa powder inhibited lipid peroxidation in both the homogenates and the plasma. Also malondialdehyde (MDA) has been decreased in the erythrocytes of patients with colorectal cancer. Gerber *et al*. (1996) and Saintot *et al*., (1996) have reported similar findings in patients with breast cancer. They have also observed a decrease in plasma MDA with tumor size and progression. A similar decrease in tissue lipid peroxide was observed in oral squamous cell carcinoma (Subapriya *et al.,* 2002). Saroja *et al*., (1999) have also reported diminished lipid peroxidation in oral tumor tissue and a decrease in phospholipid content and an increase in cholesterol: phospholipid ratio Saroja *et al.,* (1999). MDA is reported to be an unstable intermediate in the peroxidation sequence of unsaturated fatty acids. It may be metabolised further or be transported, (Roy *et al.,* 2004).

The endogenous antioxidants CAT and SOD were generally increased in the 10% *balanites aegyptica* and *ziziphus spina-christy* supplementation diet groups probably owing to the increased quantities of polyphenols and flavonoids present in the groups and this could be the reason for the colon exhibited normal histological features in same groups. The observation corroborates with the report of Atawodi (2011), who observed that reduced lipid peroxidation, increased endogenous antioxidants.

Epigallocatechin gallate (EGCG) is the most abundant polyphenolic flavonoid in green tea. Catechin and its derivatives, including EGCG, are widely believed to function as antioxidants. where we demonstrated that both EGCG and green tea extract (GTE) cause oxidative stress-related responses in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* under weak alkaline conditions in terms of the activation of oxidative-stress-responsive transcription factors. GTE as well as EGCG induced the nuclear

localization of Yap1 in *S. cerevisiae*, which was repressed by the addition of catalase and addition of superoxide dismutase (Iso *et al.,* 2015).

In table 4.6 the Hematological parameters for white blood cells, red blood cells, and hemoglobin were no significant different between the treated and control probably owing to the fact that the cancer had not reached its metastatic state however, platelet counts for the control MNU were found to be reduced compared to the control normal saline.In a study where the cancer has reached its metastatic state, the study compares MNU-induced mice to the control group. MNU-treated samples showed an abnormal elevated level of leucocytes (4-fold), monocytes (10-fold) and neutrophils (43%). It has been reported that abnormally high neutrophil, monocyte or leucocyte counts are associated with poor survival in patients with metastatic carcinoma (Donskov *et al*., 2014). Monocytes and neutrophils as 'bud guys' for outcomes of interleukin-2 with and without histamine in metastatic renal cell carcinoma – Results from a randomized phase II trial. Brit J Cancer 94:218–26; Schmidt *et al*. (2013).

In a study by Verna S., Pretreatment levels of peripheral neutrophils and leukocytes as independent predictors of overall survival in patients on Cancer Stage IV Melanoma: American Joint Committee Results of the EORTC 18951 biochemotherapy trial. Verma S,(2014). Neutrophil–lymphocyte ratio as a prognostic factor in colorectal cancer. The decrease in lymphocyte count in MNU-treated sample compared to other HEAM-treated groups and its reported association with a more aggressive behavior of the tumor. Characterization of peripheral blood and ovarian cancer infiltrating lymphocytes: Effect of HIV infection on phenotypic expression and proliferative response. HEAM (Afterbirth) also normalized the leucocyte, neutrophil, monocyte and eosinophil counts and increased the lymphocyte count after 28 weeks

of treatment in MNU-induced hepatocarcinogenesis mice, thus providing additional evidence of its beneficial effect and in this particular case highlighting

The high protein content in the MNU control group as compared to other treated groups suggests that chemical carcinogens such as MNU can initiate protein synthesis. This observation supports Takaki and Shigeru (2006) findings who establish that various chemical carcinogens such as Methyl methanesulfonate (MMS) N-Methyl-N-nitro-N-nitrosoguanidine, (MNNG), Sodium deoxycholate, (DOC) can enhance the synthesis of several proteins including p32.

Hradec (2014), demostrate that the effect of series of carcinogenic N-nitroso compounds; alkylnitrosoureas and M-methyl N-nitrosourea (MNU) on enzymatic reactions involved in protein synthesis was studied in cell-free systems from rat liver,the addition of most of this N-nitroso compounds stimulate the formation of aminoacyl-tRNA complex in test systems from rat liver whereas analogous preparations from Escherichia coli did not show this effect. The polymerization of phenylalanine and the binding of aminoacyl-tRNA to ribosome were only slightly and apparently non-specifically inhibited in the presence of the test compounds, on the other hand, the binding of mRNA to ribosome was markedly stimulated after the addition of most carcinogens were tested. It appears that the carcinogens intervene specifically with one of the early steps in peptide initiation, Since the binding of mRNA to ribosome is known to be an important rate-limiting step in protein synthesis, the N-nitroso carcinogens and triazenes may thus control the expression of genetic message at the translation level.

Histology result obtained showed that the control normal saline group had normal colon features/architecture, compared to the control MNU which showed Hyperchromicity, atrophy of gland and necrosis in the colon.The 10 % *Balanites aegyptica* and *Ziziphus spina*-

*christy*supplemented diet groups had normal colon features which may be as a result of high level of antioxidants phytochemicals constituents in both plants with respect to supplementation.

In the bove study, the observed anti-carcinogenic properties of the PapPE, PomPE and SE, may be attributed to their antioxidant phytochemicals constituents, these extracts acted as a colon protective agent against the damage caused by AOM-induced insult, and this observation was in consistent with previous studied reported by Al-Numair*et al.*,(2014). The antioxidant potential properties of the extracts prevented the generation of ROS in the colinic cells as presented by its ability to quench and scavenge free radicals' formation the study postulated that the presence of extracts with AOM by pre and post treatment normalized the levels of the colonic intracellular antioxidant capacity to nearly normal. The observed chemopreventive effect of the assayed extracts was exerted by its capacity as a potent antioxidant agent, hence boosting the colonic cellular antioxidant capacity of rats treated with PapPE/AOM, PomPE/AOM and SE/AOM versus rats treated with AOM (Waly et al.,2016).

CHAPTER SIX

6.0 **CONCLUSION AND RECOMMENDATIONS**

6.1 **Recommendation**

This research work was restricted to the colon. For further study, this research can be carried out on other organs to ascertain the diverse potential of *Balanites aegyptica* and *Ziziphus spina-christy* and their cancer chemopreventive properties.

Further work on the curative aspect of *Balanites aegyptica* and *Ziziphus spina-christy* on carcinogenesis of the colon and other organs is recommended.

6.2 **Conclusion**

This study suggests that dietary supplementation with 10% *Balanites aegyptica* and *Ziziphus spina-christy* has chemopreventive effect on N-Methyl-N-Nitrosourea induced colon carcinogenesis in wistar rats.

Both *Balanites aegyptica* and *Ziziphus spina-christy* are potent in the chemoprevention of carcinogenesis but *Balanites aegyptica* ismore potent than *Ziziphus spina-christy*

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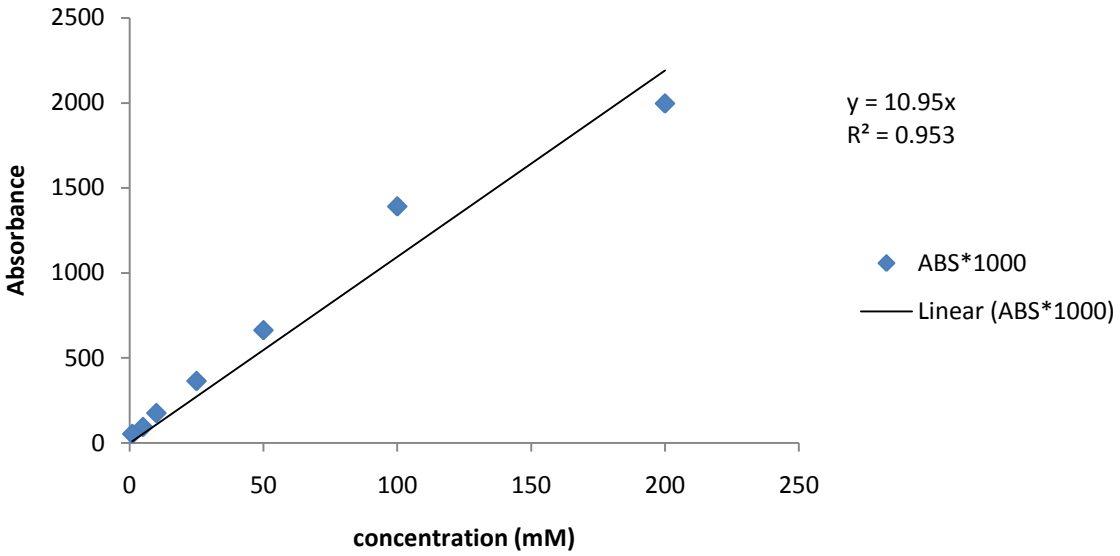
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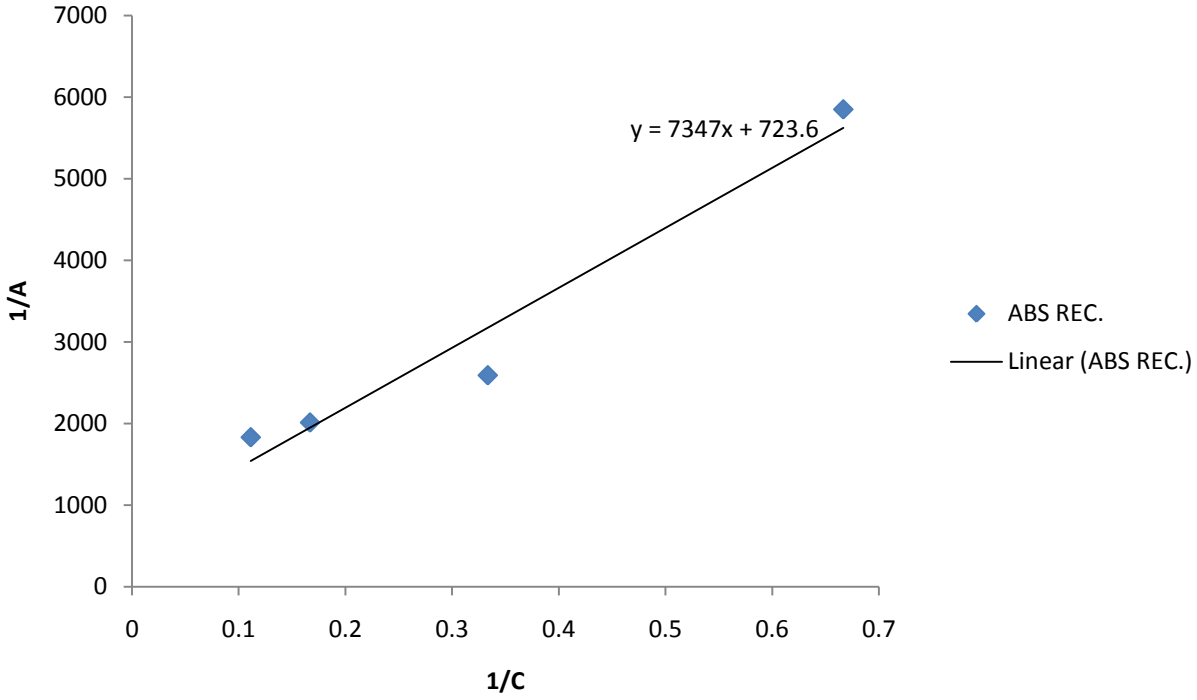
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APPENDICES



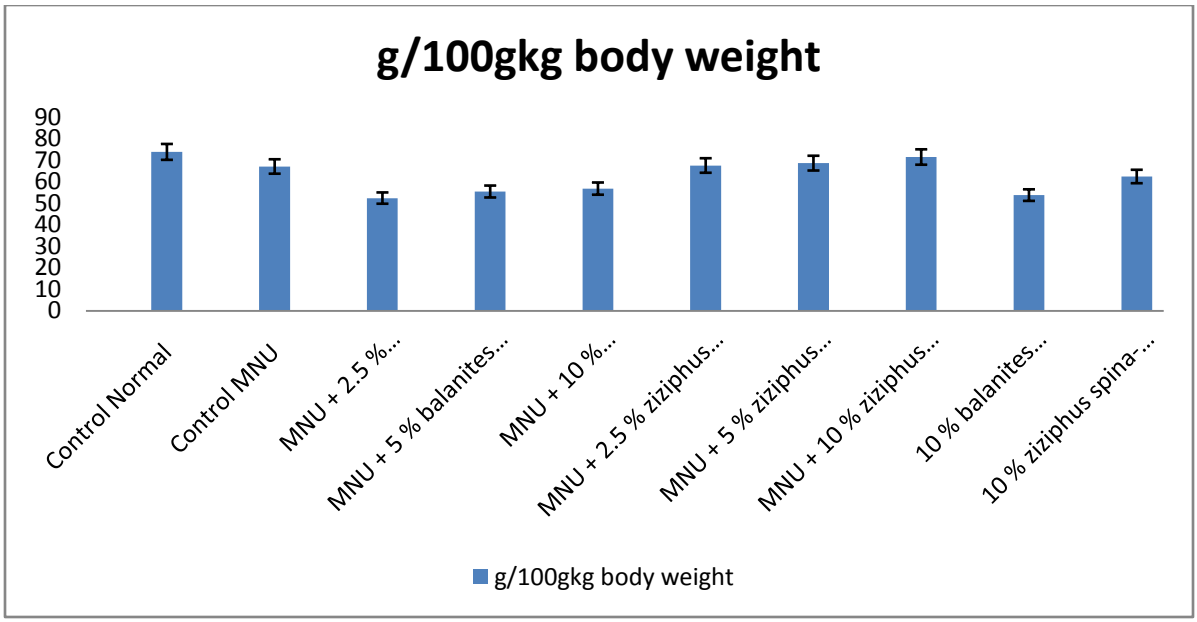
APPENDIX I:CATALASE STANDARD CURVE



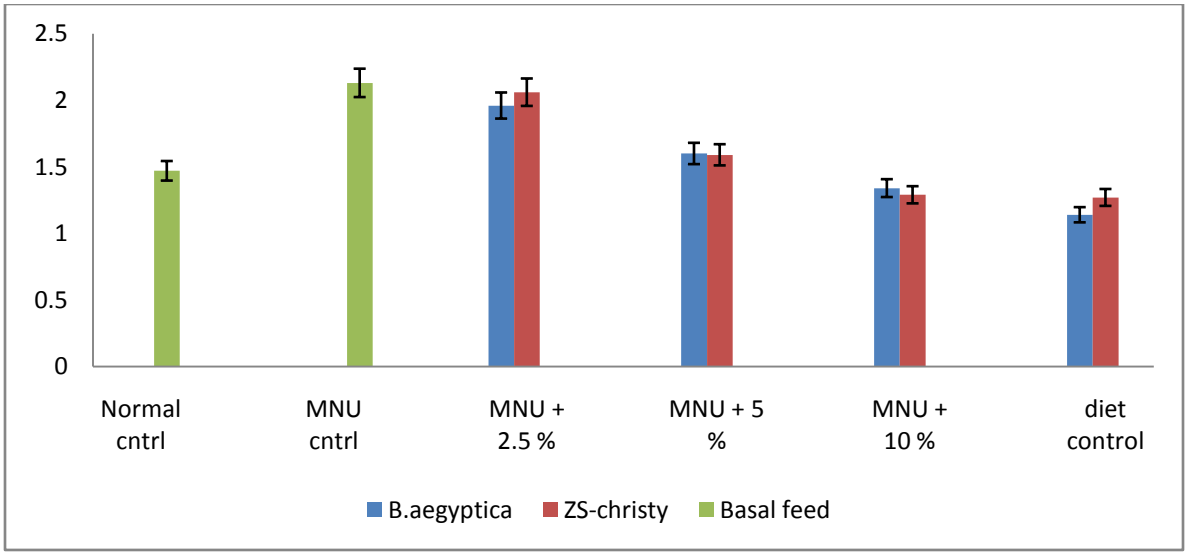
APPENDIX II:CEA STANDARD CURVE

Percentage Composition(%)	Quantity of Sample (g)	Quantity of the Mash (g)	Qty of Total Compounded feed (g)
		BALANITE AEGYPTICA	
2.5	25	975	1000
5	50	950	1000
10	100	900	1000
		ZIZIPHUS SPINA-CHRISTY	
2.5	25	975	1000
5	50	950	1000
10	100	900	1000

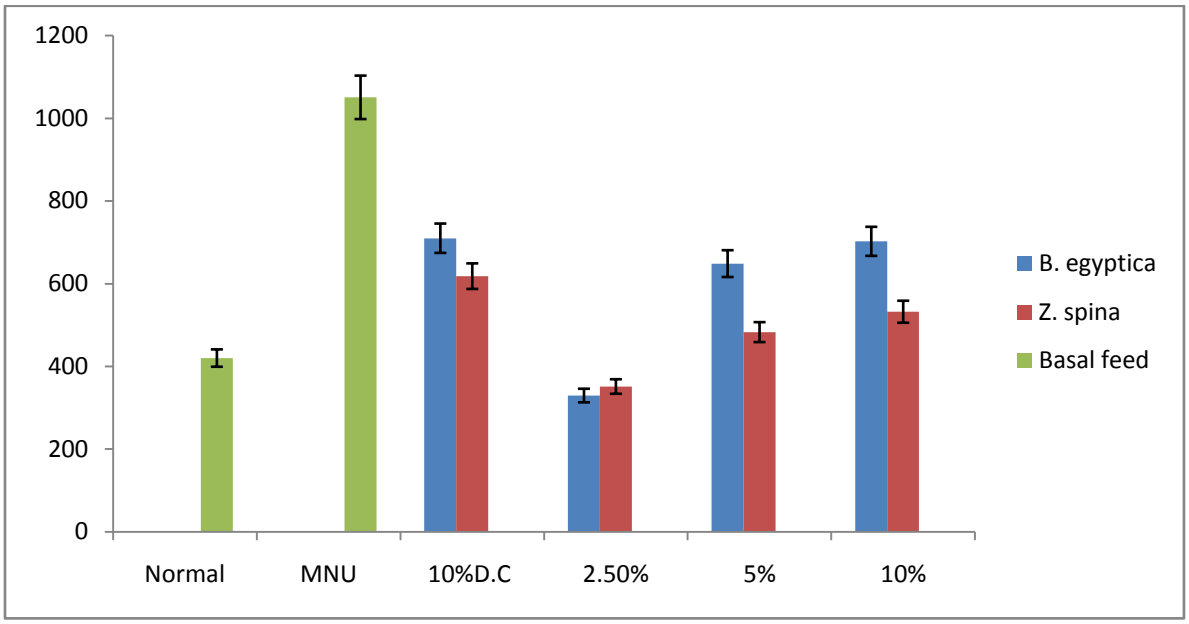
APPENDIX III: LEVEL OF SUPPLEMENTATION



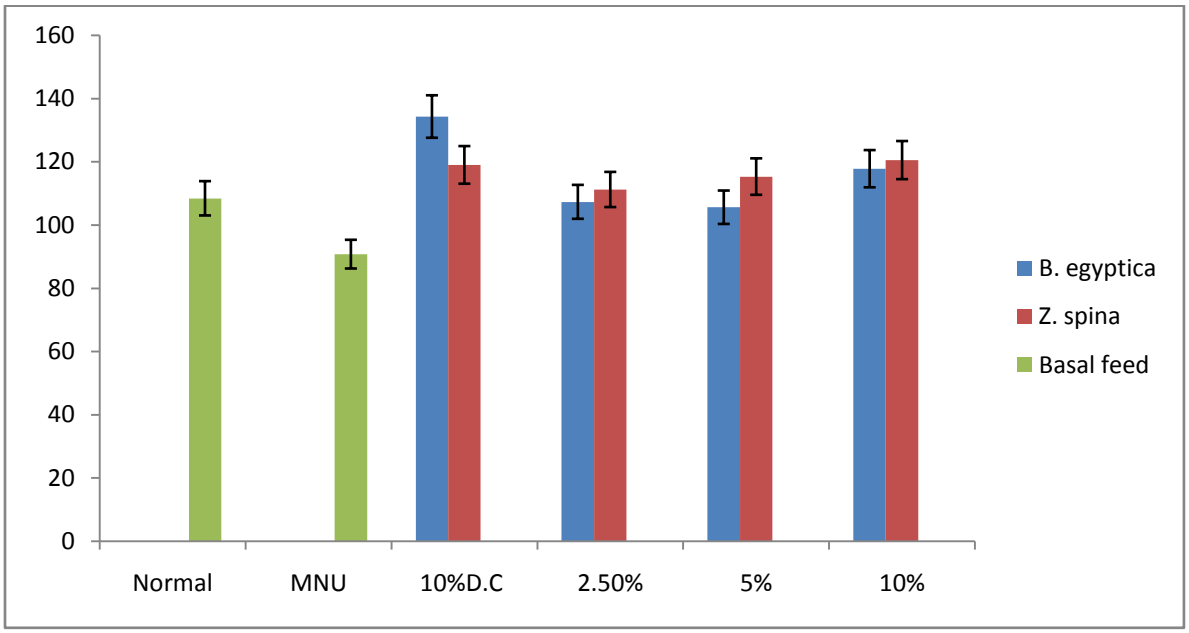
APPENDIX V: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Feed Rate in N-methyl N- Nitrosourea Induced Colon Cancer in Male Wistar Rats



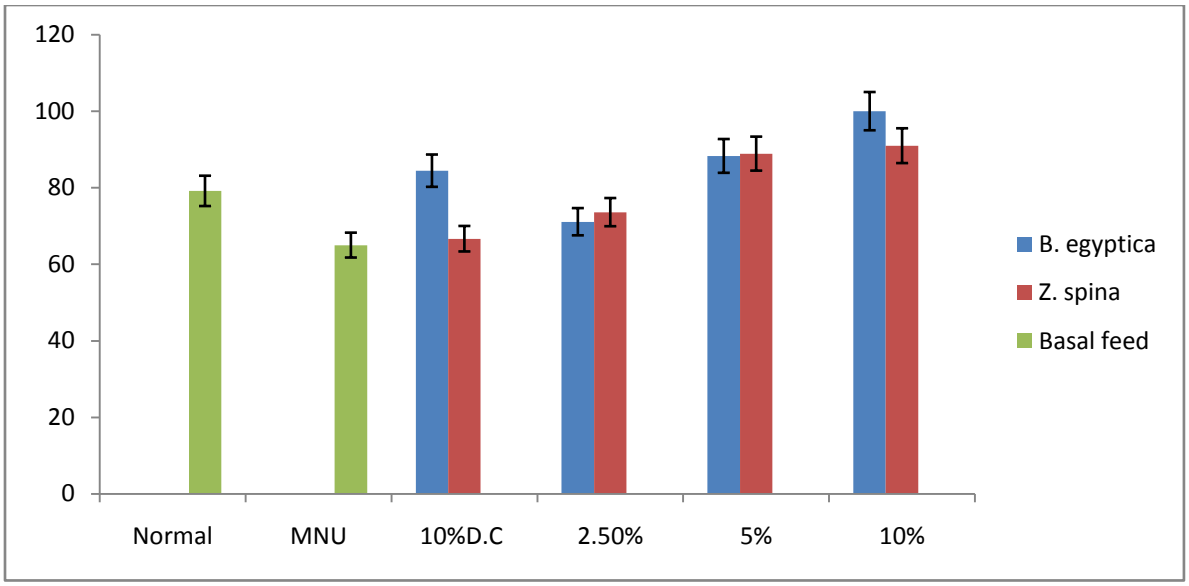
APPENDIX VI: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Serum Carcino Embryonic Antigen (CEA) Levels in N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



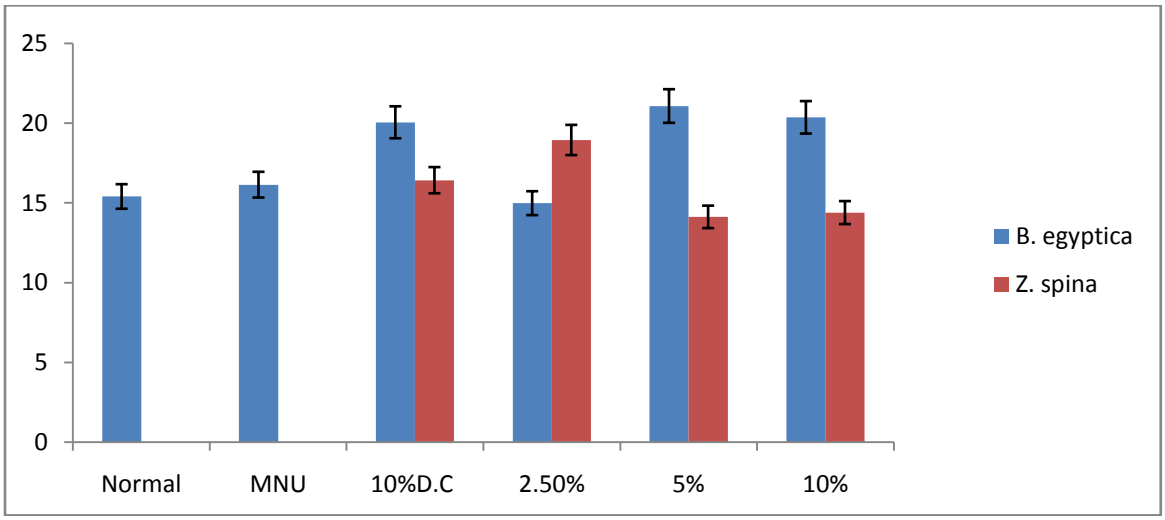
APPENDIX VII: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Liver Malondialdehyde concentration on N- methyl N- Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



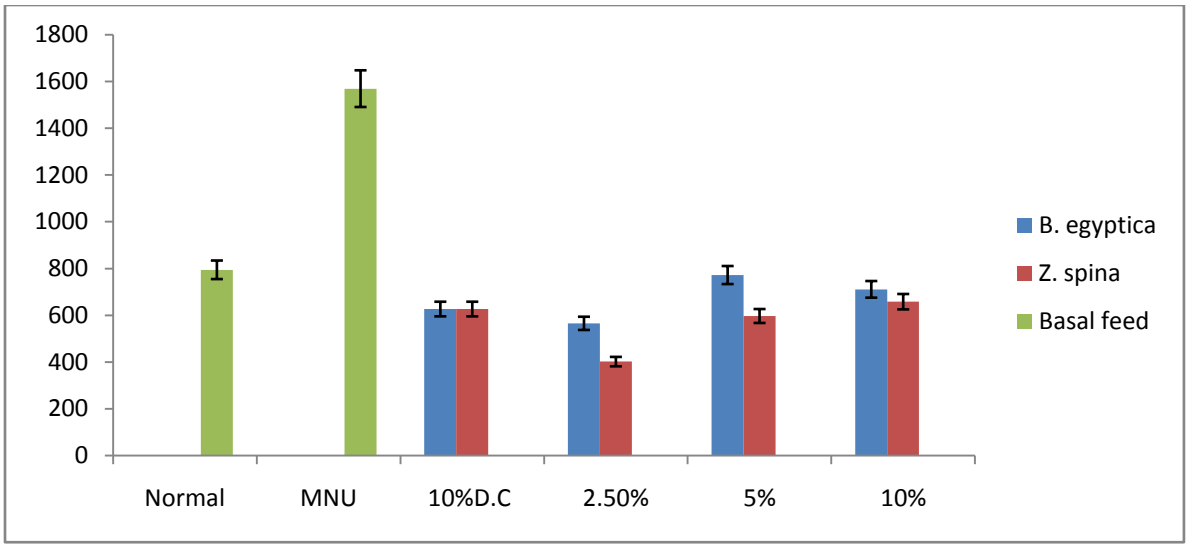
APPENDIX VIII: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Liver Catalase Activity on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



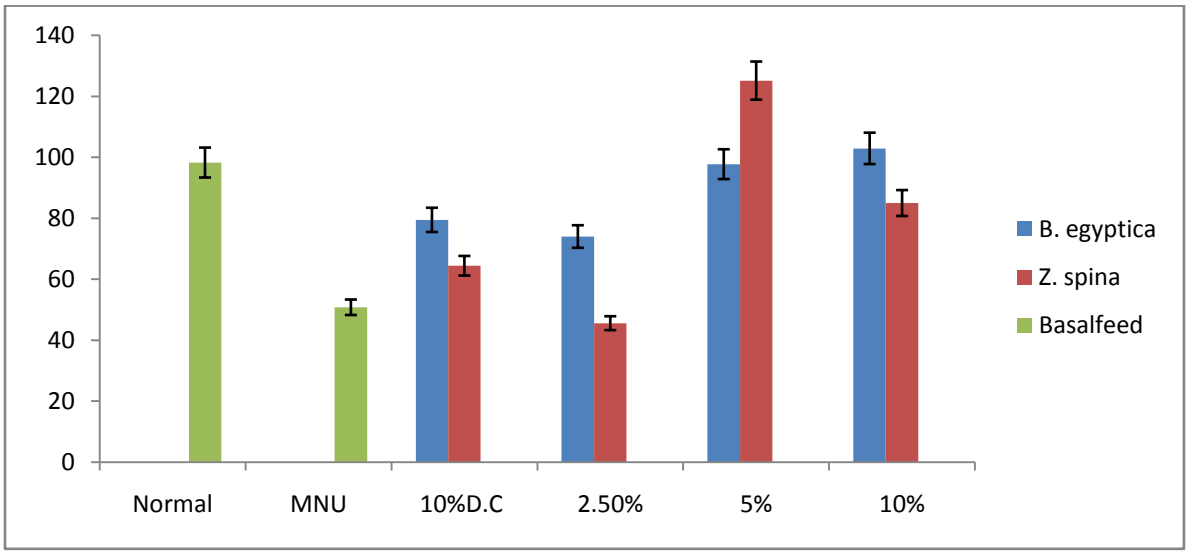
APPENDIX XI: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Liver Superoxide Dismutase Activity on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



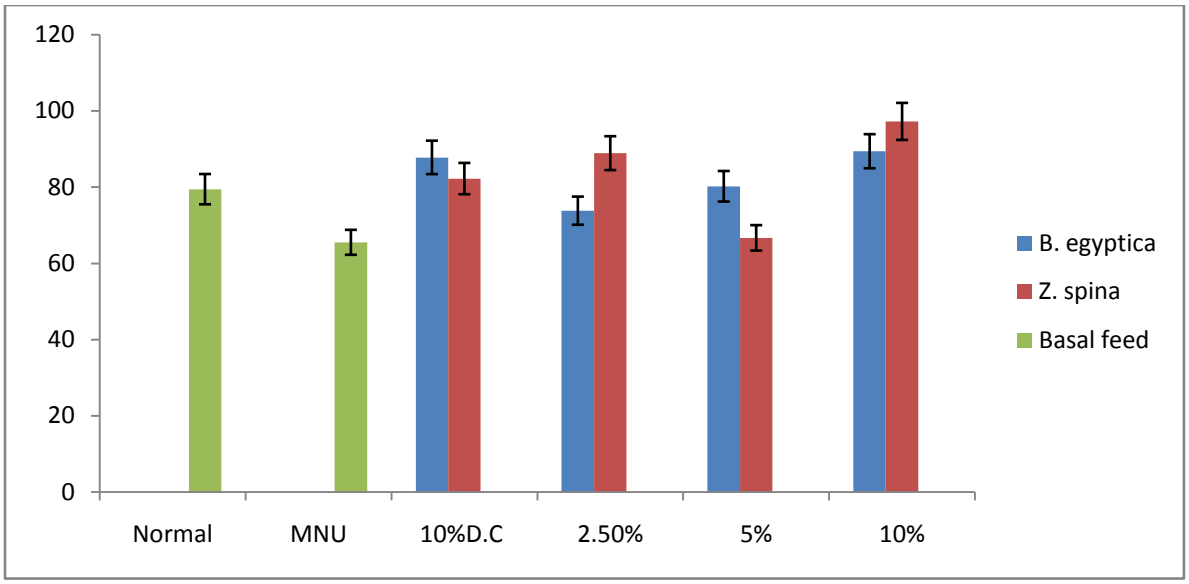
APPENDIX X: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Liver Protein Activity on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



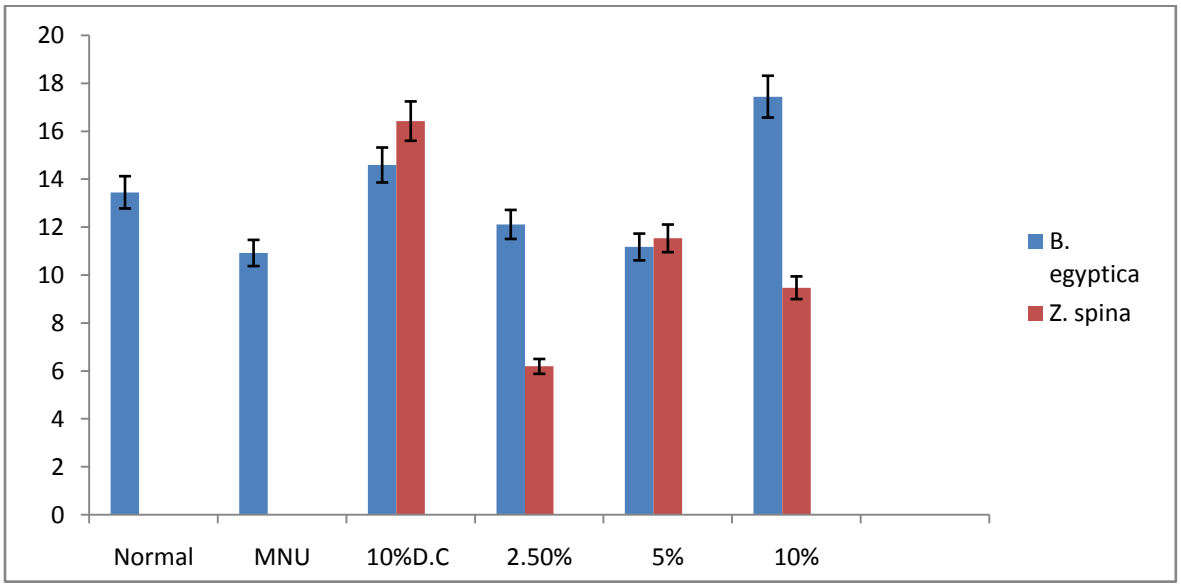
APPENDIX XI: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Kidney Malondialdehyde concentration on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



APPENDIX XII: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Kidney Catalase Activity on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



APPENDIX XIII: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Kidney Superoxide Dismutase Activity on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU



APPENDIX XIV: Effects of *Balanites aegyptica* and *Ziziphus spina-christy* Supplementation on Kidney Protein on N-methyl N-Nitrosourea Induced Colon Cancer in Male Wistar Rats, Control feed: Control Normal Saline, MNU + Basal Diet: Control MNU

Contributions to knowledge

This study establishes that;

- *Balanites aegyptica* and *Ziziphus spina-christy* have been shown to have significance ($p<0.05$) endogenous antioxidants effect on MNU induced colon carcinogenesis.
- *Balanites aegyptica* and *Ziziphus spina-christy* demonstrated protective effect on hematological parameters (white blood cells, red blood cells) in MNU induced colon carcinogenesis.
- *Balanites aegyptica* and *Ziziphus spina-christy* reduce the severity of MNU induced colon damage and colon carcinogenesis