

PREVENTIVE EFFECTS OF DIETARY INCLUSION WITH *CRASSOCEPHALUM
RUBENS* (*JUSS EX JACQ.*) LEAF ON N-METHYL-N-NITROSOUREA-INDUCED
COLORECTAL CARCINOGENESIS IN ALBINO WISTAR RATS

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JULY, 2017

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NIGERIA

JULY, 2017

DECLARATION

I, declare that the work in this Thesis entitled “**Preventive Effects of Dietary Inclusion with *Crassocephalum Rubens (Juss Ex Jacq.)* Leaf on N-Methyl-N-Nitrosourea-Induced Colorectal Carcinogenesis in Albino Wistar Rats**” has been carried out by me in the Department of Biochemistry, Ahmadu Bello University. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project thesis was previously presented for another degree or diploma at this or any other Institution.

Alhassan, Solomon Oguche

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Signature

Date

CERTIFICATION

This thesis report entitled “PREVENTIVE EFFECTS OF DIETARY INCLUSION WITH *CRASSOCEPHALUM RUBENS* (*JUSS EX JACQ.*) LEAF ON N-METHYL-N-NITROSOUREA-INDUCED COLORECTAL CARCINOGENESIS IN ALBINO WISTAR RATS” by Solomon Oguche ALHASSAN meets the regulations governing the award of the Degree of Master of Science Degree in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The ability to prevent the progression of carcinogenesis has been demonstrated in many plants and natural products. This study set out to evaluate the preventive potential of dietary inclusion with *Crassocephalum rubens* on colon carcinogenesis initiated via N-methyl-N-nitrosourea induction. Forty-two wistar rats were divided randomly into 6 groups (n=7) with treatment groups receiving 0%, 2.5%, 5% and 10% dietary inclusion + MNU for 12 Weeks. The study included three groups of controls namely, MNU control, Normal control and 10% dietary inclusion control. At the end of 12 weeks, parameters such as malondialdehyde, endogenous antioxidants enzymes, carcinoembryonic antigen, haematological, histological and immunohistochemistry of MutL Homologue-1 were evaluated. A statistically significant increase ($p \leq 0.05$) in the activities of both superoxide dismutase (SOD) and Catalase (CAT) in the colon, liver and kidney of treated groups when compared with MNU control especially at 5% and 10% dietary inclusion. The reverse was observed for the extent of lipid peroxidation measured by assaying for malondialdehyde in the liver and colon of treated groups when compared with MNU control (0.27 ± 0.01 and 0.38 ± 0.04 nmol/ml/mg protein in the liver and colon respectively). An increase in PCV, RBC, haemoglobin count, WBC, total platelet counts and neutrophils were observed, while a decrease in lymphocyte count was observed in treated groups when compared with MNU controls. A statistically significant ($p \leq 0.05$) decrease in the level of CEA was observed in treated groups compared with MNU control (125 ± 12.42 pg/ml). Histologic and immunohistochemical evaluation of tissue sections also showed a reduction in tissue damage and mild to no expression of the *MLH-1* gene in diet included groups. These results point to the ability of the plant to reduce the effects of chemically induced carcinogenesis and serve as basis for recommending its frequent consumption as part of the diet.

CHAPTER ONE

1.0 INTRODUCTION

Cancer is burgeoning public health crisis with incidence rates steadily rising globally and increasingly representing the largest chronic cause of mortality with over 6 million deaths recorded yearly (Ferlay *et al.*, 2013). In addition, this condition is driving a convergence of both communicable and chronic diseases; low income countries fast becoming confluences with a myriad of biological and social factors fueling this trend. Overall survival rates have improved marginally despite great advances in surgery, chemo- and radiotherapy and other treatment methodologies. Treatments regimes remains prohibitively high and associated with undesirable effects providing some compelling reasons to focus on prevention rather than finding cures (Torre *et al.*, 2015)

Risk factors associated with the leading causes of cancer deaths include tobacco use (lung, colorectal, stomach, and liver cancer), being overweight/obese and physical inactivity (breast and colorectal cancer), and infection (liver, stomach, and cervical cancer). For example, the convergence of smoking patterns between men and women is reflected in the increase of female lung cancer incidence and, sequentially, female lung cancer mortality rates. A substantial portion of cancer cases and deaths could be prevented by broadly applying effective prevention measures, such as tobacco control, vaccination, and predictive screening (Dunn, 2012; Torre *et al.*, 2015).

An extremely promising strategy for cancer prevention is chemoprevention; defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease (Sporn, 1976).

Importantly, plants, vegetables, herbs and spices used in folk and traditional medicine are remain the most potent and promising vehicles of chemopreventive effects with these now the main sources of lead compounds for discovery and development of chemo-preventive therapies. This has become an area of intense scientific enquiry with interest markedly improved by our increasing understanding of the process of carcinogenesis coupled with identification of potential molecular targets to perturb this process, successes registered in chemo prevention of breast, prostate and colon cancer and regulatory approval for at least 10 agents for the treatment of precancerous lesions or cancer risk reduction (Wu *et al.*, 2011).

Several plants and vegetables highly consumed in Africa have been reported by several studies to possess potent chemopreventive properties. Plants such as *Allium cepa* (Linn) and *Allium sativum* (Linn) contain potent phytochemicals that have been used to suppress gastric and skin carcinomas. *Elaeis guineensis* (Jacq), *Kigelia Africana* (Lam.), *Annona muricata* (Linn.), *Vernonia amygdalina* (Del.), *Bryophyllum pinnata* (Lam.) for example also have been tested in various cell culture and animal based models in the prevention of cancers of the colorectum, renal, skin and lungs. Several more have been observed to possess potent antioxidant potential able to prevent or limit the effects of oxidative stress which is an important precursor of cancers (Atawodi, 2011; Alawode, 2013).

The focus of this study is on colorectal cancer which results from the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colon and rectal epithelium to colon polyp which in turn can become cancerous. Individuals who develop polyps are at the highest risk of colon cancer. Most colorectal cancers develop slowly over several years with a growth of tissue or tumour usually beginning as a non-cancerous polypon the inner lining of the colon or rectum (American Cancer Society, 2014).

1.1 Statement of Research Problem

Colorectal cancer is the fourth most common cancer in men and the third most common in women worldwide. Colon and rectal cancers alongside other cancers predominantly linked to western lifestyles most frequently reported in high-income countries are considered one of the clearest markers of the cancer transition rapidly replacing those linked to infectious agents such as cervical, ovarian and liver cancers in countries undergoing rapid socioeconomic transition (Ferlay *et al.*, 2013). Although more prevalent in developed countries developing countries are fast catching up as significant incidence is now be recorded in developing countries where such risk factors as obesity, physical inactivity and harmful use of tobacco are increasingly becoming prevalent (Jemal *et al.*, 2010).

The treatment of colorectal cancer also constitutes a great treatment challenge in resource-constrained countries especially in Sub-Saharan Africa and resistance to anticancer drugs is increasingly being reported. Therefore, more research is required to explore preventive strategies that can be translated from the bench to the population (Burki, 2012).

1.2 Research Justification

According to the World Health Organization, over 80% of the world's populations rely upon traditional, plant-based systems of medicine to provide their first-line primary healthcare. This ubiquitous use has found invaluable importance in the prevention and treatment of cancers since the anticancer properties of a wide array of plants have been recognized for centuries. Compounds such as lignans and podophyllotoxin were isolated from such plants as the mayapple and used to prevent or treat small cell cancers such as cancer of the skin (Wasundara and Vasantha, 2013).

Phytochemicals have the ability to control the DNA damaging factors in cancer cells, regulate DNA transcription and inhibit tumour initiation and progression. They possess numerous other therapeutic benefits such as anti-obesity effects, cardiovascular effects, anti-diabetic effects, immune enhancement, natural antioxidant activity, and anti-inflammatory effects countering factors that predispose to the occurrence of colorectal cancer (Russo *et al.*, 2005; Ugbogu *et al.*, 2013; Wang *et al.*, 2012).

Dietary intake of chemopreventive phytochemicals directly from plants has been associated with decreased risk of cancer and significant survivability of cancer patients (Willett, 2010). These provide safe, affordable and efficacious alternatives to synthetic drugs to individuals in resource constrained settings. Even more importantly, they provide sources for identification of lead compounds that can be optimized for development of conventional therapies. This is supported with the fact that over 60% of anticancer drugs available in the market are of natural origin and plants, vegetables, herbs, and spices used in folk and traditional medicine have now been accepted as one of the main sources of cancer chemo preventive drug and can protect against a wide range of cancers including heart disease and other chronic diseases (Su *et al.*, 2013).

The wide distribution of *Crassocephalum rubens* in Nigeria (Adewale *et al.*, 2016) will make it an excellent dietary supplement for the populace which would be culturally acceptable and thus easily adopted.

1.3 General aim

This study seeks to evaluate the preventive effect of dietary supplementation with the plant *Crassocephalum rubens* on N-methyl-N-nitrosourea- induced colon cancer in Wistar rats.

1.4 Specific Objectives

- i. To assess the possible influence of a *C. rubens* leaf inclusion on oxidative stress status and endogenous antioxidant system in rats chronically exposed to MNU
- ii. To evaluate the hematologic effects of dietary *C. rubens* inclusion in MNU-treated rats
- iii. To determine the effects of dietary inclusion of *C. rubens* on outcomes of colorectal carcinogenesis initiated with MNU in rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Colorectal Cancer

Colorectal cancer (CRC) is the second and third most common cancer in women and men respectively, a combined 19% of all cancers in both sexes. CRC is considered one of the clearest markers of the cancer transition, replacing infection-related cancers in countries undergoing rapid societal and economic changes together with other cancers predominantly linked to western lifestyles, which are already frequently found in high-income countries (Ferlay *et al.*, 2013).

Globally, the distribution of colorectal cancer burden varies widely, with more than two-thirds of all cases and about 60% of all deaths occurring in countries with a high or very high human development index (HDI). Also, a rapid and consistent increase in both incidence and mortality from colorectal cancer is being observed in many medium to high income countries particularly those in Eastern Europe, Asia and South America. However, Colon cancer incidence and mortality rates have been stabilizing or declining in a number of the highest indexed HDI countries like the United States of America, Australia, New Zealand and several Western European countries. The reasons for the recent declining trends in incidence in these countries are ill-defined and likely numerous, but may partially reflect increased early detection and prevention through polypectomy (at least in the USA). This, together with the factors that have brought about declines in incidence, improvements in perioperative care, as well as chemotherapy and radiotherapy, have contributed to the uniformly decreasing trends in colon cancer mortality in many high income settings.

However, because these factors are hardly ever present in Sub-Saharan Africa, the incidence of colon cancer is on the rise (Butler *et al.*, 2013; Ferlay *et al.*, 2013; Kamangar *et al.*, 2006).

Diverse global colon cancer patterns and trends point towards widening disparities and an increasing burden in countries at different phases of transition. Generally, Colon and rectal cancer incidence and mortality rates correlate with the adoption of a western lifestyle but while they are still rising rapidly in many low-income and middle-income countries linked to ongoing societal and economic development, the reverse is observed in highly developed countries where rates are stabilizing or decreasing (Labianca *et al.*, 2010).

In Sub-Saharan Africa, studies report a crude incidence of 4.04 cases per 100000 of the population, an incidence rate much lower than observed in most regions of the world especially in high Income countries. Incidence however is observed to vary across gender and age lines, with incidence higher in males (59%) than in females (39%). The reported most common anatomical site of colon cancer in Africa is the rectum (in 46% of cases), followed by the caecum (17%). These trends are fairly similar to those found in the Western world. A study looking at anatomical sub-site of CRC in Europe found the most common sites to be rectum (31%), sigmoid colon (21%) and caecum (10%) (Graham *et al.*, 2012).

There is evidence to suggest an increased risk of developing colorectal cancers amongst HIV/AIDS infected populations, however the biological mechanism behind this is still poorly understood. Cancers especially Kaposi's sarcoma is strongly associated with HIV/AIDS infection and there are documented cases of its metastasis to the colon or the virus triggering carcinogenesis in other organs, thus HIV/AIDS may significantly contribute to reported incidence and mortality from colon cancer in Africa (Ford *et al.*, 2008).

One of the greatest concerns related to these overall conclusions may be that the incidence of CRC in Africa is systematically under-reported and data on it is generally of poorer quality than in high income countries. Ideally, cancer registries should be population-based but in developing countries, this is often not possible. Problems such as the limited health system infrastructure and cultural and religious obstacles in the reporting of diseases like cancer have contributed to the lack of routine data collection (Valsecchi and Steliarova-Foucher, 2008; Burki, 2012).

A huge transition in colorectal cancer rates has been observed in Nigeria over the last 40 years, with colorectal cancer making a transition from the tenth most common to the fourth most prevalent form of cancer over the last decade. Incidence rates in Nigeria are put at 3.4 cases per 100,000 with a recent study from Ibadan, Nigeria showing the average annual incidence of colorectal cancer at 27 patients per year. In many urban centres, there has been an increase in health risk behaviours associated with colon cancer as with all cancers, such as smoking, decreased physical activity, alcohol consumption and poor diet. There is also evidence of the increase in the health consequences of these behaviours, in terms of increased rates of obesity, diabetes and hypertension (Irabor and Irabor, 2011).

2.2 Risk to Colorectal Cancers

Colorectal cancer most commonly occurs sporadically, and only 25% of the patients have a family history of the disease, suggesting a contribution for shared genes and environment. However, only 5%-6% of colon cancers are due to inherited mutations in major colorectal genes whilst the remaining familial forms likely result from gene-environment interactions. The rates of this cancer increases with industrialization and urbanization, further indicating that environmental factors can likely represent risk factors. Overall, the lifetime risk of developing colorectal cancer is about 1 in 21 (4.7%) for men and 1 in 23 (4.4%) for women (Migliore *et al.*, 2011).

The syndromes of Colorectal cancers (CRCs) are defined on the basis of clinical, pathological, and more recently, genetic findings. Conditions that express adenomatous polyps include Lynch syndrome (also called hereditary non-polyposis colorectal cancer [HNPCC]), familial adenomatous polyposis (FAP), attenuated FAP, and MUTYH-associated polyposis (MAP). Adenomatous polyps are the primary lesions in Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). Finally, hyperplastic polyposis (HPP) is an unusual condition that has a substantial cancer risk and must be distinguished from the other conditions. All of these conditions are inherited, autosomal dominant disorders, except MAP, which is autosomal recessive, and HPP, which is rarely inherited (Jasperson *et al.*, 2010).

The underlying causes of colorectal cancers are complex and heterogeneous. Both environmental factors and genetic events contribute to CRC risk. Among the environmental risk factors for CRC are diets rich in unsaturated fats and red meat, total energy intake, excessive alcohol consumption, and reduced physical activity. As with most cancers, modifiable factors associated with higher CRC risk include excessive alcohol intake, overweight/obesity, tobacco use, and consumption of processed and red meat. Others include family history of CRC, inflammatory bowel disease, hormone therapy in postmenopausal women and aspirin/nonsteroidal anti-inflammatory drug use (Johnson *et al.*, 2014).

Behaviours associated with a decreased risk of colorectal cancer include increased physical activity, postmenopausal hormone therapy, use of non-steroidal anti-inflammatory drugs, and vegetable and fruit intake (Huxley *et al.*, 2009). However, studies showing the association of fruit and vegetable intake with decreased risk of colorectal cancer have been inconsistent. Screening strategies beginning at age 50 have been shown to reduce CRC incidence and mortality through

the removal of precancerous polyps but screening itself may increase observed cancer rates due to early detection of cancers (Hagggar and Boushey, 2009).

Chronic ulcerative colitis and Crohn's disease has been demonstrated to increase the risk of onset of colorectal cancer. Colorectal cancer appearing on the ground of inflammatory bowel disease is the result of a 4-stage process believed to begin from no dysplasia progressing to indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and finally to invasive adenocarcinoma, although colorectal cancer can arise without proceeding through each of these steps (Triantafillidis *et al.*, 2009).

Substantial increases in survival rates for colorectal cancer at all stages has been observed over time however the 5-year prognosis for patients with newly diagnosed metastatic colon cancer continues to be less than 20 %. The relative improvement in 5-year survival over this period and survival has been better in countries with high life-expectancy and good access to modern specialized health care. However, enormous disparities in colorectal cancer survival exist globally and even within regions. This variation is not easily explained but such marked regional disparity in survival is likely due to differences in access to diagnostic and treatment services (Jemal *et al.*, 2011; Siegel *et al.*, 2014; Howlader *et al.*, 2015).

2.2.1 Diets, lifestyle and colorectal cancer risk

An abundance of evidence exist in the literature on the role of nutrition on colorectal carcinogenesis. Often the evidence may be inconclusive due to the lack of randomized trials and because many studies have been overwhelmed by confounding factors such as smoking status, physical activity, obesity and diabetes. However, it has been estimated that nutrition could account for more than one third of cancer deaths and that dietary factors are responsible for 70% to 90% of all cases and increasing evidence points to the relationship between dietary habits and increased

colorectal cancer risk. These dietary associations with colon cancer are characterized as typical of the Western diet, and, indeed, countries adopting a more Western diet are noting an increase in colon cancer risk. Over the past decade, a large number of case-control and cohort studies have added a substantial body of evidence to our understanding of the causes of colon cancer. Although the data are not entirely consistent, several important risk factors have emerged. The epidemiological evidence that physical inactivity or excess energy intake relative to requirements increases risk of this malignancy is quite strong. Intake of red meat appears to increase risk, but protein-rich sources other than red meat probably do not elevate risk and may even reduce the occurrence of colon cancer (Ahmed, 2004; Jemal *et al.*, 2011).

Red meat might be directly linked to the incidence of colorectal cancer or indirectly because diets high in meat may be deficient of other dietary components such as fibre and polyphenols from fruit and vegetables. Also cooking meat at high temperatures in processes such as frying, grilling and broiling may lead to the formation of mutagenic and carcinogenic heterocyclic amines through the interaction of muscle creatinine with amino acids as well as the formation of N-Nitroso compounds. Haem in meat can act as a nitrosating agent promoting the formation of N-Nitroso compounds. Darker meats are more abundant in haem than white meats and therefore, high consumption of red meat (beef, pork, or lamb) could increase the risk of colorectal cancer (Pericleous *et al.*, 2013).

Several case-control studies have demonstrated an increase in the risk of colorectal cancer with increased total energy intake and dietary lipids provide a rich source of energy and diets high in lipids. This may lead to the assumption that animal fat may increase the risk of colorectal cancer. However, several large cohort studies do not support exposure-effect relationship between dietary fat on colon cancer risk with analysis showing no energy-independent association between dietary

fat intake and risk of colorectal cancer. It also suggests that simple substitution of fat by other sources of calories is unlikely to reduce meaningfully the risk of colorectal cancer. Reports on consumption of poly unsaturated fatty acids such as omega-3 fatty acids and colorectal cancer risk and also of its potentially protective effects remain contradictory. The advice to reduce intake of saturated fat in order to reduce the risk of colorectal cancer remains only suggestive due to the lack of consistency from clinical studies (Alexander *et al.*, 2009; Pericleous *et al.*, 2013).

The hypothesis that high fibre consumption may be reducing the risk of colorectal cancer has been postulated following the observation of the low incidence of colorectal cancer in African populations that consume a high-fibre diet (Pericleous *et al.* 2013). Fibre is defined as heterogeneous plant material composed of cellulose, hemicellulose and pectin. It has been proposed to work by reducing faecal transit times, diluting and binding carcinogens, altering the proliferation of gastrointestinal epithelium, maintaining colorectal epithelial cell integrity, adsorbing heterocyclic amines affecting bile acid metabolism, and stimulating bacterial anaerobic fermentation to increase the production of short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFAs have been shown to decrease colonic pH and inhibit carcinogenesis (Rieger *et al.*, 1999; Manson *et al.*, 2007).

Consistent and adequate consumption of fruits and vegetables have been demonstrated to strongly protect against colorectal cancer in combination with other beneficial practices. This is attributed to both their fibre content and the presence of chemopreventive phytochemicals of which polyphenols are the most prominent. Evidence from case-control studies, cell culture and animal studies have shown a protective role against colorectal malignancy (Araújo *et al.*, 2011). Divided into five classes; flavonoids, carotenoids, phenolic acids, lignans, stillbenes; these compounds are able to inhibit the initiation and promotion of the carcinogenic process by inhibition of cellular

proliferation, induction of cell cycle arrest, inducing apoptotic pathways and anti-angiogenic and anti-metastatic mechanisms (Szakacs and Pandey, 2006).

Overall, diets high in polyphenols and other phytochemicals such as carotenoids, isothiocyanates and natural phenols have been shown to be protective against colorectal cancer. Foods rich in these compounds includes spices such as mustard seeds and tumeric, fruits including strawberries, cherries, apples, citrus fruit, grapes, watermelons, papaya, apricot and vegetables such as onions, broccoli, carrots, red palm oil, pumpkin, leafy green vegetables and tomatoes (Razis and Noor, 2013).

Alcohol intake may enhance risk of cancers of the distal colon, although the evidence is not entirely consistent. The influence of alcohol may be particularly strong when combined with a diet low in methionine and folate, suggesting that the effect of alcohol may be through antagonism of methyl-group metabolism. The exact mechanism carcinogenesis induced/promoted by alcohol consumption is unclear but proposed pathways include its ability to reduce folate, promote abnormal DNA methylation, delay DNA repair, alter the composition of bile salts or induce Cytochrome p450 to activate carcinogens (Michels *et al.*, 2006).

The combined effect of these dietary factors, as well as modifiable non-dietary factors such as cigarette smoking, suggest that the majority of cases of colon cancer are preventable. In several international studies, risk is associated with an increased intake of dietary fat and a decreased intake of cereal grains and dietary fibre. With analytical studies, risk is associated with a deficiency of vegetables and fruits and a sedentary lifestyle and, perhaps less consistently, with increased dietary energy, meat, cooked meat, sugar, and obesity (Giovannucci and Willett, 1994; Huxley *et al.*, 2009).

2.2.2 Specific gene mutations and colorectal cancer risks

Sporadic colon cancer is considered to be a multifactorial disease characterized by complex interactions between endogenous factors and individual genetic background effectively modulating the risk of onset. Several genes suspected as candidate colon cancer risk factors have been investigated though results obtained are either conflicting or inconclusive. Glutathione S-transferases (GSTs) genes are particularly attractive candidates for CRC susceptibility because they code enzymes involved in the metabolism of environmental carcinogens. Recent meta-analyses of the literature suggest that both GSTM1 and GSTT1 null genotypes are associated with an increased risk of CRC, especially in the Caucasian populations (Ateş *et al.*, 2005; Economopoulos and Sergentanis, 2010).

2.3 Biochemical and Genetic Aspects of Colorectal Cancer

A precise understanding of the genetics of colon and rectal cancers is important for identifying at-risk individuals, improving cancer surveillance and prevention strategies, and developing better diagnostic and therapeutic approaches. Colorectal cancer (CRC) has one of the largest proportions of familial cases of all cancer types. Kindred and twin studies estimated that approximately 30% of all CRC cases are an inherited form of the disease. Approximately 5% of cases are associated with highly penetrant inherited mutations and clinical presentations that have been well characterized. Individuals who have a first-degree relative with CRC diagnosed over age 50 years have a 2–3-fold increased risk for this malignancy. Population-based studies have demonstrated that approximately 20% of all CRC cases occur in a higher risk setting and CRC under age 50 or a first-degree relative pair with CRC. Furthermore, having one first-degree relative with CRC under age 45 years, or having two first-degree relatives affected with CRC confers a 3–6-fold CRC

risk compared to the general population. Sibling pair and parent/child pair studies have identified chromosomal regions that could contain genes that confer this level of risk (Kerber *et al.*, 2005).

The aetiologies of the remaining 20%–30% of inherited CRCs are not completely understood. They are likely to be caused by alterations in single genes that are less penetrant but more common than those associated with the well-characterized syndromes (Lichtenstein *et al.*, 2000; Lynch and de la Chapelle, 2003). Examples include common polymorphisms in genes that regulate metabolism or genes that are regulated by environmental or other genetic factors. Inherited CRCs are also likely to be caused by alterations in multiple susceptibility loci that have additive effects (Jasperson *et al.*, 2010).

An evaluation of family history and several population-based studies have identified several categories of less-penetrant, but potentially more common, causes of susceptibility to colorectal cancer onset. A recent model constructed from a Scottish, genome-wide association study estimated that as many as 170 common but separate genetic variations could confer susceptibility to CRC (Tenesa, 2009). Also population studies have also identified genetic factors associated with increased risk for CRC that include low-penetrant susceptibility loci (identified in genome-wide association studies) and specific polymorphisms. However, no surveillance guidelines are presently given for these CRC risk factors. Interestingly, the low-penetrance susceptibility loci identified by genome-wide association studies have additive effects on CRC risk, whereas CRC risk associated with the common polymorphisms is affected by gene–gene and gene–environmental interactions (Jasperson *et al.*, 2010).

One of the major challenges to genome-wide association studies, however, is that the variants identified at susceptibility loci are not ‘mutations’ that would be predicted to change function or expression of the gene product. They might affect gene expression through non-coding changes or

lead to linkage disequilibrium with other genes that affect CRC risk. Another challenge is that genome-wide association studies do not often detect moderate- or high-penetrant alleles that are uncommon in the population (Dermitzakis and Clark, 2009; Tenesa, 2009). As no specific genetic markers are presently available for common familial CRCs, screening and surveillance are based on family history. In view of the family risks given above, screening recommendations specify that 1) patients with a single first-degree relative over the age of 60 years with colon cancer should receive standard, average-risk colon cancer screening, but starting at age 40 years; 2) patients who have 1 relative with CRC under 60 years or 2 first-degree relatives with CRC should be screened every 5 years by colonoscopy, starting at age 40 years, or at an age 10 years younger than the earliest case in the family; and 3) patients with only second- or third-degree relatives with CRC should receive average-risk screening (Winawer *et al.*, 2011).

2.4 Carcinogenetic Process in Colorectal Cancer

Colorectal cancer (CRC) arises as a result of the accumulation of acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinomas. This is presented in either one of three patterns: inherited, familial, and sporadic. Inherited and familial CRC derive, at least in part, from germline mutations. Inherited CRC accounts for 10 % of cases and presents as well-characterized cancer predisposition syndromes including Lynch syndrome and familial adenomatous polyposis (FAP). Familial CRC accounts for 25 % of CRCs and presents without precisely defined Mendelian inheritance patterns or genetic (Kinzler and Vogelstein, 1996; Roper and Hung, 2013).

Genomic and epigenomic instability distinguishes neoplastic from normal colonic epithelium and is a hallmark feature of colorectal carcinogenesis. At least four kinds of genomic or epigenetic instability have been described in colorectal cancers: (1) chromosomal instability (CIN), (2)

microsatellite instability (MSI), (3) CpG island methylator phenotype (CIMP), and (4) global DNA hypomethylation. Overlap between these categories and imprecise use of these terms has led to confusion and confounds interpretation of the literature (Roper and Hung, 2013; Grady and Pritchard, 2014).

In 1990, Fearon and Vogelstein proposed a multistep genetic model of colorectal carcinogenesis in which inactivation of the adenomatous polyposis coli (*APC*) tumour suppressor gene occurs first in normal colonic mucosa, followed by activating mutations in the *KRAS* gene and subsequent additional mutations (e.g., *PIK3CA*, *TP53*, and *TGF* - β pathway genes). This model assumes the classical CIN pathway associated with 65 - 70% of sporadic colorectal cancers begins with the acquisition of mutations in the adenomatous polyposis coli (*APC*) gene, followed by the mutational activation of the *KRAS* oncogene and the inactivation of the tumour suppressor gene, *TP53*. This pathway comprises aneuploidy, which is an imbalance in the chromosome number, and loss of heterozygosity; LOH. Defects in chromosomal segregation, DNA damage repair, and telomere function along with specific mutations in certain oncogenes and tumour suppressor genes may be responsible for such instability. Aneuploidy and loss of heterozygosity (LOH) are the major players in CIN tumours, which not only constitute most of the sporadic tumours (85%) but also involve familial adenomatous polyposis cases associated with germline mutations in the *APC* gene (Fearon, 1990; Pino and Chung, 2010; Smith *et al.*, 2002).

The CpG island methylator phenotype (CIMP) pathway is characterized by promoter hypermethylation of various tumour suppressor genes, most importantly *MGMT* and *MLH-1* (East, Saunders and Jass, 2008). The CpG Island (CGI) hypermethylation in the promoter region results in the transcriptional inactivation of genes that have tumour suppressive roles or are involved in the cell cycle. Mutations in the *BRAF* gene appear to be an early event in the CIMP tumours. A

total of 759 hypermethylated regions are found. Accordingly, 96% of these regions occur in BRAF mutant tumours. Out of these, 229 regions are localized in the promoter regions enhancing five different pathways, namely the *Wnt* signalling, hedgehog signalling, *bZip* transcription regulation, PI3 kinase, and IGF-protein kinase B signalling pathways. The aberrations in chromatin-remodelling genes, such as ATP-dependent chromatin remodelers, chromodomain helicase 7 (CHD7) and CHD8, may also be associated with CIMP tumours. These mutations may lead to chromatin structure modification and deregulation, which contributes to CIMP (Toyota *et al.*, 1999; van Roon *et al.*, 2013; Tahara *et al.*, 2014).

The MSI pathway involves the inactivation of genetic alterations in short repeated sequences. This activation occurs in CRCs in DNA mismatch repair (MMR) genes, and is a hallmark condition in familial Lynch syndrome (LS), which also appears in ~15% of the sporadic CRC cases. Microsatellite instability occurs because of inactivating mutations in the DNA mismatch repair genes that are responsible for correcting DNA replication errors. The important components of the DNA mismatch repair system are ATPases, *hMSH2*, *hMSH6*, *hMSH3*, *hMLH1*, *hPMS2*, *hPMS1*, and *hMLH375* (Kunkel and Erie, 2005).

The germline mutations that may render these proteins dysfunctional can predispose to cancer as in the case of LS and CRC. MSI is found in 15% of colorectal cancers, with only 3% associated with LS. The rest are sporadic cases caused by the hyper-methylation of the MLH1 gene promoter. In addition, the hyper-methylation of the MMR genes may lead to MSI. This mechanism is often associated with the CIMP pathway. MSI tumours are often associated with proximal colon and poor differentiation but better prognosis. Moreover, the three mechanisms often overlap in CRC (Peltomaki *et al.*, 1993; Triantafillidis *et al.*, 2009; Bak *et al.*, 2014). More than 1, 500 germline variants have been found in the *MMR* genes along with promoter methylation, somatic deletions,

or point mutations with large variation in the *MLH1* and *MSH2* genes although conventional screening is not sufficient to detect them (Suter *et al.*, 2004; de la Chapelle and Hampel, 2010).

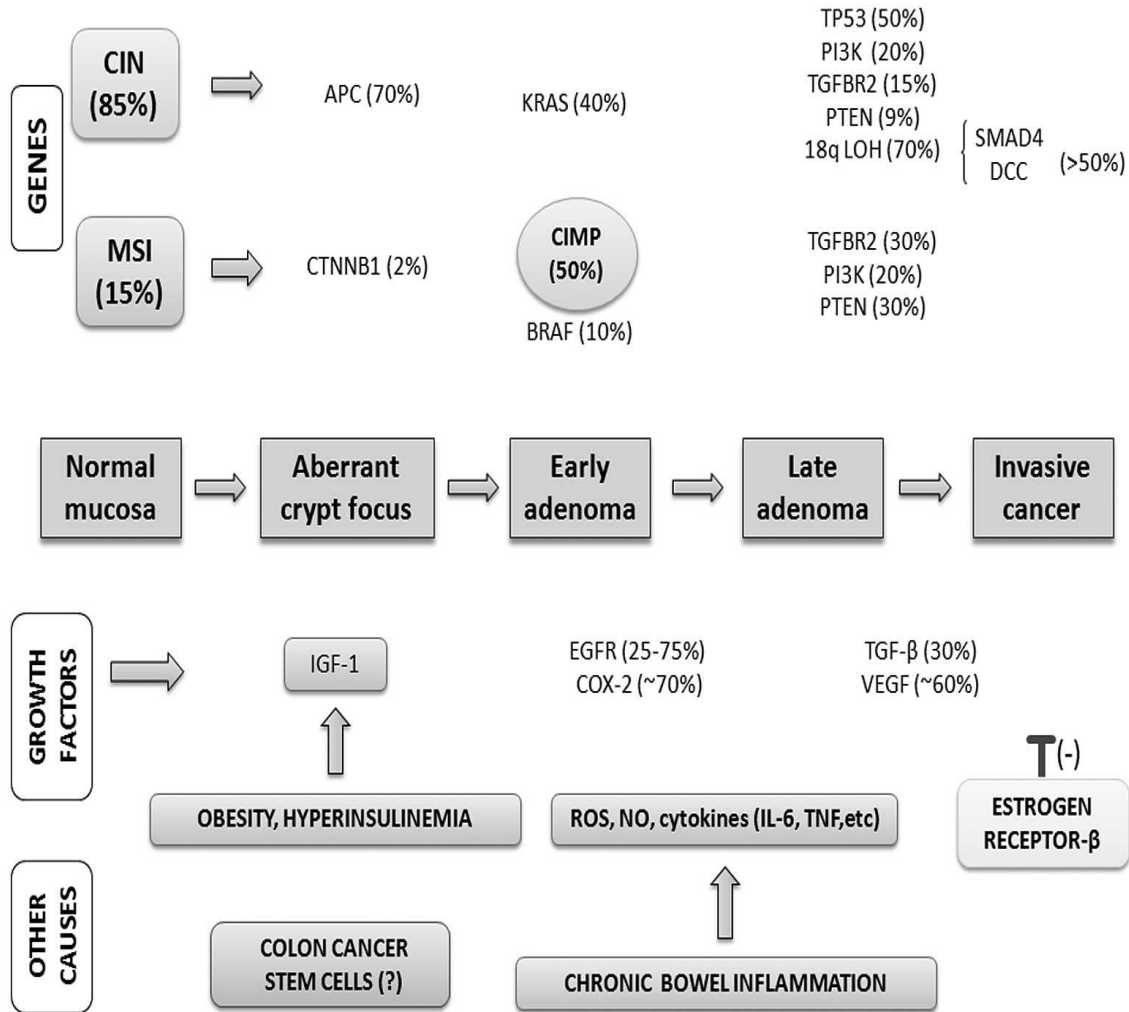


Figure 1: Molecular Players in Colorectal Carcinogenesis (Adopted from Conteduca, 2013)

2.5 Molecular Markers of Colorectal Carcinogenesis

Our growing understanding of the molecular tumorigenesis of colon cancer, progression and metastasis and resistance to chemotherapy has made it easier to identify and select a wide range of tumour markers with excellent diagnostic and prognostic value. Tumor markers are substances (most typically proteins, glycolipids) representing biological structures, which can be attributed to the development of normal cells or carcinogenesis at different cell development stages *e.g.*, tumour-associated antigens (TAAs) which are the largest group of clinically significant markers. As a result, the concentration of TAAs typically correlates with the number (or mass) of specific neoplastic cells. Biomarkers related to DNA, RNA, micro- RNA, epigenetic changes, protein and even antibody expression have been discovered with advances in genomics and proteomics. Although many biomarkers have been described, only a select few have provided prognostic data. This includes markers such as epidermal growth factor receptor (EGFR), BRAF, tumor MSI-H expression (defects in DNA mismatch repair, *MLH-1*, *MSH-2* genes), 18q AI expression, p53 expression and KRAS mutation (Langan *et al.*, 2013).

2.5.1 Some biomarkers applied in the study of colon carcinogenesis

A specific and measurable change in the level or expression pattern of DNA, RNA, protein any metabolite that can be linked to or observed in malignant states can be referred to as a cancer biomarker. These are used as indicators of the biological state of tissues and therefore must have characteristics that enable them to be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. One of the key requirements of biomarkers for detecting colon cancer is that it must allow detection of the disease at initiation or early progression stages and must be sensitive enough

to detect a very significant proportion of cases and specific enough to distinguish cases from non-cases (Tanaka *et al.*, 2010).

Colon cancers markers can be classified differently and identified by different techniques. But the most common method applied in determining the progression or extent of carcinogenesis is via assays consisting of the detection of specific antigen-antibody reactions and the quantification of cancer specific antigens or antibodies in the serum of models. Several specific antigens and molecules that serve as excellent markers are available in this method. One of these is the carcinoembryonic antigen (CEA).

2.5.2 Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen is a member of the immunoglobulin superfamily encoded by 29 genes in humans. It also belongs to a GPI-anchored Carcinoembryonic antigen family predominantly expressed in the gastrointestinal tract, but over expressed in as many as 70% of all human cancers (Beauchemin and Arabzadeh, 2013).

There is strong evidence that CEA is involved in multiple biological aspects of neoplasia such as cell adhesion, metastasis, suppression of cellular immune mechanisms, and inhibition of apoptosis (Soeth *et al.*, 2001; Duxbury, Ito *et al.*, 2004).

In monitoring the carcinogenic process or screening colon and rectal carcinomas, the Carcinoembryonic antigen (CEA) still remains the “gold standard” and remains the marker of choice when studying dissemination of the disease during therapy. The sensitivity of CEA in colorectal cancer increases with advancing tumour stage. Serum concentrations of CEA are elevated in 50% of patients with tumour extension to the lymph nodes and in 75% of patients with distant metastasis (Bast *et al.*, 2001).

Carcinoembryonic antigens have epitopes that are specific to the neoplasm and epitopes that connect antibodies against nonspecific cross-reacting antigens (NCAs). Monoclonal or polyclonal antibodies may identify several antigens belonging to glycoproteins of CEA family. Inflamed or necrotic tissues react with CEA antibodies, making up a specific pattern (Duffy *et al.*, 2007; Oremek *et al.*, 2007). The specificity of CEA is usually associated with the site of metastases. In metastatic colorectal cancer of lungs, the CEA elevation is not sufficiently enough and also could be significantly affected by chemotherapy; totally, cures ascribable to CEA monitoring are commonly scarce. Despite the aforesaid limitations, CEA is currently used as a serological diagnostic factor (Roayaei *et al.*, 2014).

2.5.3 Immunohistochemical markers in the study of colorectal cancer

Immunohistochemistry is becoming an important method for the monitoring of the process of colorectal carcinogenesis and has become widely adopted as a screening method for this cancer. with the development of antibodies suited for immunohistochemistry against the proteins encoded by these MMR genes, immunohistochemistry has evolved into a widely used screening method for the identification of colon cancers and its forms such as HNPCC (Shia *et al.*, 2009).

The underlying principle utilized in this method as applied in colorectal carcinogenesis is the detection of microsatellite Instability, one of four very crucial pathways in the initiation of carcinogenesis by assaying for protein products of mutated mismatch repair genes. Microsatellite instability is primarily characterized by mutations in microsatellite chromosomes, *i.e.* repeated DNA sequences. These sequences are particularly exposed to errors in the mutation repair system that consist in the loss or multiplication of nucleotide sequence repetitions, which results in

shortening or extension of microsatellite regions in neoplastic cells (de la Chapelle and Hampel, 2010).

Mutations arising out of these processes are eliminated by mismatch repair genes (*MMR*) such as *MSH2*, *MSH6*, *PMS2* and *MLH1*, which makes some researchers believe that MSI can be caused by mutations in these genes. DNA mismatch repair (MMR) is a multi-protein intracellular process for recognizing and repairing non-native DNA structures. In the absence of normal MMR, the cellular mutation rate increases and this deficiency is evidenced by high levels of microsatellite instability (MSI), i.e. insertions and deletions in repetitive DNA sequences, and an accumulation of single nucleotide alterations. In agreement with the ‘mutator hypothesis’ (Loeb *et al.*, 2008), which predicts an association between the increased mutation rates and tumorigenesis, mice that carry MMR gene deficiencies display elevated levels of MSI and have an increased susceptibility to cancer. Microsatellite instability can be classified into microsatellite instability-high (MSI-H), and microsatellite instability-low (MSI-L), depending on the percentage of loci that correlate to MSI characteristics. Tumour cells that lack MSI features are designated as MSS (Poulogiannis *et al.*, 2010).

In humans, heterozygosity for mutations in MMR genes results in predisposition to hereditary non-polyposis colorectal cancer (HNPCC), an autosomal-dominant syndrome characterized by early-onset colorectal cancer and other cancers. The basic mechanisms and protein components of MMR are conserved in a broad range of species, including *Escherichia coli*, the yeast *Saccharomyces cerevisiae* and humans (Jiricny, 2013).

2.6 Animal Models in the Study of Colon Cancer

The complex interplay of genetic and epigenetic events that characterize colorectal carcinogenesis and the apparent disadvantage of relying solely on extrapolation from cell culture models necessitate the use of animal models relevant to the molecular characteristics of colorectal cancer (CRC). Studies in the earlier part of the 20th century demonstrated that colorectal tumorigenesis could be induced and studied in experimental models. The use of animal models classified as spontaneous and artificially transplanted systems has significantly expedited the delineation of molecular pathogenesis of colorectal carcinogenesis and also contributed to the development of newer preventive and therapeutic strategies. These models offer several advantages: 1. they are highly reproducible and can be readily tested on animals with different genetic backgrounds 2. the pathogenesis especially in the early stages to a significant extent mirrors the occurrence of human CRC and 3. As with mice, the availability of extensive genetic information on individual animal lines and the ever-increasing number of transgenic, knockout and knock-in genetic models makes them veritable tools (Johnson and Fleet, 2013).

Three important criteria must be met for results from animal models to be beneficial in human cancer treatment or chemoprevention. First, the cancer that develops in the animal model should be limited to the large intestine so that researchers can study the development of the disease without the confounding effects of disease in other tissues. Second, the histologic and molecular features of colorectal lesions should be similar to those observed in human tissue. Finally, the models should capture the complex cellular interactions that are relevant to human colon cancer (Johnson *et al.*, 2013).

For the study of colorectal cancer, specific requirements are that 1. Animal CRC models must possess the molecular pathways similarly involved in human CRC, 2. These pathways must

correlate with factors that affect the frequency of the disease in human populations, 3. The chemopreventive/therapeutic agent-induced signal is sufficient to carry out the project with an affordable and statistically significant number of mice (Vanessa *et al.*, 2016). The high frequency of tumours generated within the distal colon of mice and the subsequent development of adenocarcinomas from multiple adenomas, also validate the importance of this species for studying the pathogenesis of colon cancer. However, the most notable disadvantage of these models is the general lack of invasive and metastatic phenotype (Rosenberg *et al.*, 2009).

As in human populations, genetic background of laboratory animals is a significant factor in carcinogenesis. Different species show differing susceptibility to colorectal cancer risk such as chronic exposure to carcinogens. Genetically defined inbred mouse strains that differ in their sensitivity to colon carcinogens offer distinct advantages for studying organ-specific carcinogenesis. Some of these differences however can be attributed to the environmental conditions under which the animals are maintained and with the route of exposure to carcinogen also playing a vital role in tumour incidence. A number of potential molecular mechanisms that may contribute to differential cancer sensitivity have been considered. These include rates of carcinogen activation/detoxification, the extent of acute DNA alkylation and the efficiency of repair mechanisms (Bissahoyo *et al.*, 2005; Megaraj *et al.*, 2014).

The dog has also become an attractive model for comparative oncology research with several key similarities between canine and human colorectal cancer. Tumour location and histological characteristics are quite similar between both species and immunohistochemical evaluation of canine colorectal adenomas revealed cytoplasmic and nuclear accumulation of β -catenin, suggesting that dysregulation of the WNT signaling pathway is also an important driver of colorectal carcinogenesis in the dog as in humans. However, the utility of dogs in the study of

colorectal cancer is however limited by the low prevalence of the disease in dog population (McEntee and Brenneman 1999).

2.6.1 Limitations of animal models in the study of colon cancers

Currently a number of animal models are available to dissect various facets of the pathogenesis of Colon cancer and to quantify risk. Mutant mouse models provide a unique opportunity in studying numerous adenomas under defined experimental conditions and uniform genetic background. However, use of animal models in studying human disease has its own limitations. For example, in carcinogen-induced models of CRC, the tumor incidence and latency period could be modulated by amount of carcinogen used – higher amount of carcinogen leading to higher incidence of tumors. However, high ethanol consumption reduced carcinogen (DMH)-induced tumorigenesis suggesting that DMH model is not useful in determining the role of alcohol in Colon cancer. Careful consideration is essential for the selection of animal model to study a particular agent and requires validation in two or more models for the unequivocal demonstration. Future advances in animal model development will require a combination of dietary and genetic manipulation of rodents or other inexpensive animals to more accurately mimic the various factors that contribute to colorectal neoplasia in humans. It is imperative to expect that any one model will not answer all the questions about the CRC chemoprevention or therapeutic intervention strategies under investigation (Suman *et al.*, 2013).

2.7 Use of N-methyl-N-nitrosourea-induced Colon Cancer Models

A large number of chemicals with mutagenic or carcinogenic potential have been discovered and are currently deployed in laboratory controlled induction of cancers. Classified as either direct or indirect agents (requiring enzymatic processing into active carcinogenic metabolites for action) one of the most common class is the alkyl-Nitroso compounds; N-methyl-N-nitro-N-

nitrosoguanidine (MNNG) and N-methyl-N-Nitrosourea (MNU); direct alkylating agents that do not require metabolic activation and thus are potent topical carcinogens. Intra-rectal instillation with these compounds have been reported to induce colorectal tumours in rodent models. In rats and mice, most of the induced colonic tumours are sessile polypoid lesions. Because biochemical activation is not required, these carcinogens are ideal for inducing colon tumours in animals and studying the modifying effects of xenobiotics without involving the metabolism of the initiating carcinogen (Rosenberg *et al.*, 2009). MNU-induced colon cancer has been used to test a host of other potential preventive interventions against colorectal cancer e.g. 1alpha-hydroxy-24-ethylcholecalciferol (a vitamin D analogue) dietary restriction, dietary fatty acids, ursodeoxycholic acid (Johnson *et al.*, 2014).

Because MNNG and MNU given intra-rectally selectively induce tumours in the distal colon and rectum, these models have been widely used. The major weakness of MNNG and MNU models are that proper intrarectal injection poses a significant technical challenge for most workers and quantification of carcinogens dose instilled intra-rectally is difficult. Another challenge is the possibility of off-site effects with thymic lymphomas and pulmonary cancers reported in a study (Suman *et al.*, 2012).

2.8 Role of Oxidative Stress in Carcinogenesis

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. In the last decade, growing number of reports investigating association between ROS and carcinogenesis have been published. Reports have proposed various consequences of oxidative stress that may be linked to carcinogenesis (Valko *et al.*, 2007; Mena *et al.*, 2009).

ROS can promote many aspects of tumor development and progression, which can be classified into the following biological processes: (a) cellular proliferation (e.g., extracellular-regulated kinase 1/2 (ERK1/2) activation), (b) evasion of apoptosis or anoikis (e.g., Src, NF- κ B and phosphatidylinositol-3 kinase (PI3K)/Akt activation), (c) tissue invasion and metastasis (e.g., metalloproteinase (MMP) secretion into the extracellular matrix (ECM) and Met overexpression), and (d) angiogenesis (e.g., the release of vascular endothelial growth factor (VEGF) and angiopoietin) (Sosa *et al.*, 2013).

Genetic factors play key roles in the transforming events that lead to cancer development. The high metabolism of cancer cells is generally associated with an increase in ROS; however, such levels are less deleterious in cancer cells than they would be in normal cells. For example, although the ROS level increases by a modest degree, tumorigenic cells can induce a new redox balance, resulting in cellular adaptation and proliferation. This is a striking feature of cancer cells that allows for the acquisition of resistance to oxidative stress inducers relative to normal cells. The resistance to oxidative stress is one of the major adaptive advantages that permit cancer cells to increase their metabolic rate and proliferation and to escape free radical damage. However, this adaptive response to high doses of ROS is not enough to explain the high metabolic rate of cancer cells (Visconti and Grieco, 2009).

The damage that these ROS can cause to the cell not only depends on their intracellular concentration but also on the equilibrium between the ROS and the endogenous antioxidant species. When the pro-oxidant/anti-oxidant equilibrium is lost, oxidative stress is generated, altering and damaging many intracellular molecules, including DNA, RNA, lipids and proteins. The oxidative effects of ROS are controlled by exogenous antioxidants such as vitamins E and C, and by endogenous antioxidants such as scavenger enzymes (i.e. superoxide dismutase, catalase

and glutathione peroxidase), bilirubin, and uric acid. Natural antioxidants are the cell's defense mechanisms that scavenge reactive species, and they can be classified into different groups according to their properties: endogenous antioxidants, natural antioxidants and synthetic antioxidants. Endogenous antioxidants include glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, L-carnitine, melatonin, enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRXs). PRXs are a ubiquitous family of antioxidant enzymes (PRX I-VI) that also control cytokine-induced peroxide levels and mediate signal transduction in mammalian cells. For example, PRX III scavenges up to 90% of H₂O₂, and PRX V behaves more effectively as a scavenger of peroxynitrite (Schieber and Chandel, 2014).

Natural antioxidants coexist in a delicate balance with oxidative inputs. Other antioxidants can be obtained from the diet, such as ascorbic acid (Vitamin C), tocopherol (Vitamin E), beta-carotene (Vitamin A), lipoic acid, uric acid, and glutathione and polyphenol metabolites. Examples of synthetic antioxidants include N-acetyl cysteine (NAC), pyruvate, and selenium, butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate (Skrzydłowska *et al.*, 2005; Papaioannou *et al.*, 2011; Sosa *et al.*, 2013).

2.9 Colon Cancer Chemoprevention

Consistent increase in global cancer rates combined with the prohibitive costs of treatment and deleterious side effects drives increasing interest in strategies for prevention. An obvious strategy of primary prevention of cancer and other mutation-related diseases is to avoid or minimize exposures to recognized risk factors through risk assessment, risk management, and risk communication. A complementary approach is to render the host organism more resistant to the attack of mutagens and carcinogens, and to inhibit progression of the disease. Various appealing

approaches to these are being considered by researchers and health experts but one enormous potential is chemoprevention, which is defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease making use of suitable pharmacological agents or of dietary agents, under the form either of macronutrients, micronutrients, or non-nutritive phytochemicals (Sporn, 1976; De Flora and Ferguson, 2005).

With deeper insight and understanding into the precise details of cancer initiation and progression and identification potential molecular targets for interfering with this process, intense research is being devoted to this approach. Interest has been further stimulated by successes in the chemoprevention of breast, prostate and colon cancer, and the fact that there are now 10 FDA-approved agents for the treatment of precancerous lesions or cancer risk reduction (Wu *et al.*, 2011).

The introduction of drugs that suppress cholesterol synthesis, modify platelet aggregation or lower blood pressure has led to a steady fall in heart disease over the past 3 decades. This highlights the potential impact that chemoprevention could have on cancer morbidity and mortality if successfully demonstrated on a population scale (Hansson, 2005).

Approaches to chemoprevention are broadly categorized as primary, secondary and tertiary. Primary chemoprevention involves the administration of agents to the general 'healthy' population or to those without overt disease but with particular risk factors. Examples may include the administration of agents such as oltipraz, which induce phase I or II enzymes to modify carcinogen metabolism in an exposed population. Secondary chemoprevention involves the identification of individuals with premalignant lesions and administration of agents to prevent progression to invasive cancer. This would encompass the use of non-steroidal anti-inflammatory drugs

(NSAIDs) such as the widely trialled Aspirin in patients with colorectal adenomas. Definitions of primary and secondary chemoprevention vary and some groups now combine the two scenarios under the term primary chemoprevention. Tertiary chemoprevention is defined as the administration of agents to prevent recurrence or second primary cancers in individuals who have undergone successful treatment of early disease (Kelloff *et al.*, 1996; Xiao and Yang, 2008).

Most strategies generally deploy long-term, continuous treatment or supplementation with chemopreventive agents but while this is likely to produce major preventive effects; unexpected serious adverse events are not unlikely. This raises the question of whether intermittent dosing schedules might reduce toxicity while retaining benefit, a concept referred to as short-term intermittent therapy to eliminate pre-malignancy (SITEP). Recent preclinical studies have validated the effectiveness of a novel SITEP approach whereby short-term intermittent therapy eliminates premalignant cells via apoptosis that is induced by synthetic lethal interactions. Synthetic lethality allows personalized, selective elimination of premalignant clones without harming normal cells (Wu and Lippman, 2011).

2.9.1 Mechanisms of chemoprevention

Chemoprevention may involve perturbation of a variety of steps in tumour initiation, promotion, and progression. Numerous potential mechanisms have been described and attempts have been made to broadly classify agents according to the effects they have on different stages of carcinogenesis (De Flora and Ferguson, 2005). The different stages of carcinogenesis are aptly summarized as initiation, promotion, and progression stages. Initiation involves direct DNA binding and damage by carcinogens and it is rapid and irreversible. Promotion, which involves epigenetic mechanisms, leads to pre-malignancy and is generally irreversible. Progression, which

is due to genetic mechanisms, is the period between pre-malignancy and the cancer and is also generally irreversible.

Various classification schemes of chemopreventive agents based on their hypothesized mechanism of action and stage during the carcinogenetic process where they act have been made. Compounds that inhibit cancer initiation are traditionally termed 'blocking agents'. They generally act at the Initiation phase by preventing contact and reaction between carcinogens with critical target sites or molecules. They may act by preventing the interaction between chemical carcinogens or endogenous free radicals and DNA, thereby reducing the level of damage and resulting mutations which contribute not only to cancer initiation but also progressive genomic instability and overall neoplastic transformation (Steward and Brown, 2013).

Several specific mechanisms of protection or inhibition of cancer initiation utilized by blocking agents have been highlighted and studied. They include decreased cellular uptake and metabolic activation of pro-carcinogens and/or enhanced conjugation, removal of reactive electrophiles, free radical scavenging, induction of repair pathways, down-regulation of chronic inflammatory responses and the production of reactive oxygen and nitrogen species to mention a few (Valko *et al.*, 2007; Yu and Kong, 2007).

Suppressing agents are those that act once initiation has occurred interfering the promotion and progression of initiated cells. These compounds would kill initiated/transformed cancer cells *in vitro* and *in vivo* xenografts via diverse anti-cancer mechanisms. The major reported mechanisms contributing to this activity involve the inhibition of signal transduction pathways (for example, by targeting nuclear factor (NF)- κ B), the activation of signalling kinases, caspases and the mitochondria damage/cytochrome c pathways to perturb the effects of tumour promoters (Michels *et al.*, 2006), which would otherwise lead to cell proliferation. Recent reports suggest interference

with cancer cell metabolism and energy homeostasis via effects on pathways such as Adenosine monophosphate Kinase (AMPK) and mammalian target of rapamycin(mTOR) signaling may be an attractive goal for chemopreventive agents. Other mechanisms of chemoprevention include the induction of apoptosis and inhibition of angiogenesis (Shu *et al.*, 2013).

2.9.2 Dietary phytochemicals and chemoprevention

A large body of epidemiologic evidence links consistent consumption of fruit and vegetables with lowered risk of colorectal cancer and has been suggested as a non-expensive and practicable strategy in reducing incidence and thus mortality from colorectal cancer as with other chronic diseases (Slavin and Lloyd, 2012).

Epidemiological and pre-clinical data suggest that various natural phytochemicals and dietary compounds possess chemopreventive properties, and in-vitro and animal studies support that these compounds may modulate signalling pathways involved in cell proliferation and apoptosis in transformed cells, enhance the host immune system and sensitize malignant cells to cytotoxic agents (Kotecha *et al.*, 2016).

With varying phytochemical profile, the consumption of a diverse range of food offers increased protection as extracts exhibit strong antioxidant and anti-proliferative effects especially in combination as several studies have demonstrated additive and synergistic effects of phytochemicals in fruits, vegetables and whole foods are responsible for observed effects. Combinatorial effects have also been observed where any one of the single agents is inactive. Apart from interactions among dietary micronutrients, drug-phytochemical interactions have also been observed, indicating possibilities for improved cancer therapeutic strategies. Our understanding of

the molecular mechanisms underlying such synergistic effects is still limited, but it appears that different combinations of complementary modes of actions are involved (de Kok *et al.*, 2004).

The evidence suggests that antioxidants or bioactive compounds are best acquired through whole-food consumption, not from expensive dietary supplements. We believe that a recommendation that consumers eat 5 to 10 servings of a wide variety of fruits and vegetables daily is an appropriate strategy for significantly reducing the risk of chronic diseases and to meet their nutrient requirements for optimum health (Liu, 2013; Kasote *et al.*, 2015).

Studies on a wide spectrum of plant secondary metabolites extractable as natural products from fruits, vegetables, teas, spices, and traditional medicinal herbs have identified various bioactive plant phytochemicals that regulate multiple cancer-inflammation pathways and epigenetic cofactors, are cost effective, exhibit low toxicity, and are readily available. Advances in genomics and metabolomics have enabled biologists to better investigate the potential use of immunomodulatory natural products for treatment or control of cancerous diseases. More recently, evidence has emerged that specific combinations of phytochemicals may be far more effective in protecting against cancer than isolated compounds (Shu *et al.*, 2010; Wang *et al.*, 2012).

Evidence that phenolic compounds may have a potential inhibitory effect on cancer invasion and metastasis is increasingly being reported in the scientific literature. Curcumin, resveratrol, and their related derivatives are the most studied compounds in this topic so far, Gallic acid, chlorogenic acid, caffeic acid, carnosol, capsaicin, 6-shogaol, 6-gingerol, and their corresponding derivatives are also suggested to be the active members of the phenolic family on anti-invasion and anti-metastasis (Yang *et al.*, 2001; Johnson and Mukhtar, 2007; Pandey and Rizvi, 2009).

2.10 The Evaluated Plant: *Crassocephalum rubens* (Juss ex Jacq)

2.10.1 Habitat and distribution of *C. rubens*

Crassocephalum rubens (Juss ex Jacq) is an erect, annual herb growing up to 80 cm tall with leaves arranged spirally and sessile and inflorescent heads, arranged in a terminal corymb. It is included in the Asteraceae family of plants and in tropical Africa *Crassocephalum rubens* comprises about 24 species, many of which have medicinal uses (Burkill, 1985).

2.10.2 Culinary uses of *C. rubens*

The leaves of *Crassocephalum rubens* are commonly eaten in south-western Nigeria, less so in other humid zones of West and Central Africa. They are mucilaginous and used for soups and sauces (Adjatin *et al.*, 2013).

2.10.3 Ethno-medicinal uses of *C. rubens*

In countries where it is consumed, *Crassocephalum rubens* is used medicinally as a stomachic and to treat liver complaints and colds, and externally to treat burns, sore eyes (filaria), earache, and leprosy and breast cancer. In East Africa it is used as an antidote against any form of poisoning. The plant is locally known as “*Ichokolo*” in Igala and “*Bologi*” in Yoruba and is traditionally applied as a nutraceutical and believed to have antibiotic, anti-helminthic, anti-inflammatory, anti-diabetic, anti-malaria and blood regulation properties and also treats indigestion, liver complaints, colds, intestinal worms, and hepatic insufficiency in addition to its nutritional value (Adjatin *et al.*, 2012).

2.10.4 Nutritional composition of *C. rubens*

Nutritional evaluation of the plant by Adjatin *et al* (2013) reveal the raw protein and crude lipid contents of the leaves expressed in percentage of dry matter are 26.43% and 2.75% respectively. The content of vitamin C for 100 g of fresh leaf of *C. rubens* is 3.60 mg. The contents of sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), Manganese (Mn), and copper (Cu) are 2129.04 mg, 4469.91 mg, 434.13 mg, 3845.88 mg, 1.6 mg, 8.22 mg, and 2.6 mg, respectively.

2.10.5 Phytochemical constituents of *C. rubens*

Phytochemical screening of the plant revealed the presence of a wide variety of pharmacologically important compounds such as tannins, coumarins, combined anthracene derivatives C-heterosides, flavonoids, mucilage, reducing compounds and steroids. The chemical composition of the essential oil extracted from the fresh leaves of *Crassocephalum rubens*, has revealed the presence of limonene (48.8%), myrcene (30.7%), E-(β)-ocimene (7.4%) and α -thujene (4.6%) as the main components (Adjatin *et al.*, 2013).

2.10.6 Studies on pharmacological activity

Studies on the minimal Inhibitory Concentration (MIC) determined on some bacteria Gram+ (*Staphylococcus aureus*, *Streptococcus faecalis*), bacteria Gram- (*Escherichia coli*, *Salmonella typhi*) and against a pathogenic yeast (*Candida albicans*) vary from 0.54 mg/mL to 4.38 mg/mL. On toxicity evaluation of the plant, the LC₅₀ values of 0.374 mg/ml for leafy extract of the plant has been reported (Adjatin *et al.*, 2013).

Studies of its hepatoprotective capacity of the plant against CCl₄-induced liver damage was evaluated in animal models was carried out by Adewale *et al.*, (2016). All animals were sacrificed 24 h after the administration of CCl₄. Administration of the plant led to a significantly decreased levels of enzyme markers of liver damage ALT, AST, GGT and ALP when compared to controls.

The plant was also demonstrated to prevent lipid peroxidation and necrotic liver damage in test animals. The hepatoprotective effect of the ethanol extract of *C. rubens* may be traced to the presence of phytochemicals inherent in the plant.



Plate I: *Crassocephalum rubens* (Juss ex Jacq.)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals and Reagents

The carcinogen, N-methyl-N-nitrosourea and carcinoembryonic antigen assay (CEA) kit were procured from Sigma Chemical Co, England and Cloud-clone Corp, Houston, Texas respectively and were maintained under recommended conditions till time of use. Others such as buffer salts, formal-saline, graded alcohol, xylene, paraffin, eosin and hematoxylin were obtained from reputable chemical vendors. All other reagents and chemicals were of the highest analytical grade.

3.2 Equipment and Apparatus

Equipment utilized in this study include blender, dissecting set, centrifuge, Spectrophotometer, homogenizer, syringe, cannula, microscope, electrical weigh balance, water trough, cages, feeders, microtome machine.

3.3 Sample Collection and Preparation

Crassocephalum rubens leaves were collected from Anyigba, Kogi State and its environs. Identification and verification of the plant will be done at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria (Voucher number: 533). Leaves and twigs of the plants were air dried and at room temperature and finely pulverized with a mortar and pestle and then weighed.

3.4 Experimental Feed

Growers mash from Vital Feed (Vital Feed Ltd, Nigeria) was used for the entire study. The diet was thoroughly mixed with the *Crassocephalum rubens* according to the percentage of inclusion forming 0%, 2.5%, 5% and 10% as part of the animal daily diet.

3.5 Experimental Animals

Apparently healthy male Albino rats of the wistar strain weighing 80-100g were purchased from the animal unit of the National Veterinary Research Institute (NVRI) Vom, Plateau state, Nigeria and housed under appropriate conditions in standard cages with wooden shavings as beddings at the Animal house of the department of Biochemistry, Ahmadu Bello University Zaria and allowed free access to water and fed standard diet *ad libitum* for two weeks to acclimatize.

At the end of the acclimatization period, all animals were weighed and randomly allocated to six (6) groups of seven animals each (n=7) based on percentage dietary inclusion. The experimental groups were fed according to each group's percentage of inclusion or standard diet for six (6) weeks and subsequently induced with MNU or normal saline. Animal groups were named accordingly thus

- i. Normal control
- ii. MNU Control
- iii. MNU+2.5% Inclusion
- iv. MNU+5% Inclusion
- v. MNU+10% Inclusion
- vi. 10% dietary Control

3.6 Procedure for Induction of Colon Cancer

After four (4) weeks of feeding, the rats in the experimental groups received an intra-rectal instillation of freshly prepared MNU solution for 12 weeks using a feeding tube.

3.7 Collection of Organs and Tissue Samples and Homogenization

The animals were sacrificed by decapitation under anaesthesia (Chloroform) at the end of the 16th week of the start of the experiment. The rats were dissected and the Colon, Liver and Kidney removed and fixed quickly in 10% formal-saline for subsequent analyses.

The three organs; the colon, liver and the kidney were quickly obtained, washed with ice-cold saline to remove blood and weighed (absolute weight) using a digital weighing scale and refrigerated. The colon was grossly examined for presence of tumours.

The colon, liver and kidney (100 mg tissue/mL buffer) were homogenized separately using mortar and pestle in 50 mM phosphate buffer (pH 7.4). The homogenate was then centrifuged at 10,000 rpm for 10 minutes and the supernatant collected with pipettes for analysis.

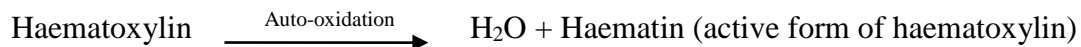
3.8 Carcinoembryonic Antigen Assay

The concentration of carcinoembryonic antigen (CEA) was determined by an enzyme-linked immunosorbent assay (ELISA) test method. The assay system utilizes a monoclonal antibody directed against a distinct antigenic determinant on the intact CEA molecule immobilized in the microtiter wells. A mouse anti-CEA antibody conjugated to horseradish peroxidase (HRP) is present in the antibody-enzyme conjugate solution. The test sample was then allowed to react simultaneously with the two antibodies, resulting in the CEA molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 1 hour incubation at room temperature, the wells were washed with water to remove unbound labelled antibodies. A solution of TMB Reagent was added and incubated for 20 minutes, resulting in the development of a blue colour. The reaction is stopped with the addition of Stop Solution changing the colour to yellow. The concentration of CEA is directly proportional to the colour intensity of the test sample. Absorbance was measured spectrophotometrically at 450 nm.

3.9 Estimation of Endogenous Antioxidants

3.9.1 Assay for Activity of the Enzyme Superoxide Dismutase

The activity of the enzyme superoxide dismutase (EC. 1.15.1.1) which decomposes superoxide anion into hydrogen peroxide and oxygen was assessed. The method utilized was based on monitoring the auto-oxidation rate of haematoxylin as originally described by Martin *et al* (1987). In the presence of SOD enzyme, the rate of auto oxidation is inhibited and the percentage of inhibition is linearly proportional to the activity of SOD present within a specific range. Sample SOD activity is determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample.



Forty microliters (40 μ L) of homogenate supernatant was added to 920 μ L of phosphate buffer (0.05M, pH7.8). A reagent blank was also prepared by replacing the sample with 40 μ L of sample dilution buffer (0.85% NaCl). The mixture was incubated for 2 minutes at 25⁰C before the addition of 40 μ L of haematoxylin. Following the addition of 40 μ L of haematoxylin, absorbance of the sample test and reagent test was read at 560nm at every minute for 4 minutes against the sample blank which was distilled water.

3.9.2 Assay for the activity of the enzyme Catalase

Catalase (EC. 1.11.1.6) activity was determined according to the method described by (Aebi *et al.*, 1968), by monitoring the decomposition of H₂O₂. In 1ml of reaction mixture containing potassium phosphate buffer (pH 7.0), 250 ml of tissue homogenate and 60 mM H₂O₂ was added to initiate the reaction. The reaction was spectrophotometrically measured at 240 nm for every minute for 4 minutes and H₂O₂ consumption was calculated using extinction coefficient, 1.56 x 10⁻⁵ mM⁻¹cm⁻¹.

1.

3.10 Evaluation of lipid peroxidation by measuring Malondialdehyde (MDA) levels

The concentration of thiobarbituric acid reactive substances (TBARS) in the tissue homogenate of major organs was estimated by the method of Fraga *et al.*, (1988). The formation of Malondialdehyde is the basis for the well-known TBA method used for evaluating the extent of lipid peroxidation. One (1) ml of the tissue homogenate was treated with 2.0 ml of thiobarbituric acid–trichloro acetic acid–hydrochloric acid reagent and mixed thoroughly. The mixture was incubated in a boiling water bath for 15 min, cooled and centrifuged at 3000 rpm for 10 min and the supernatant was taken and the absorbance was read at 535 nm against the blank reagent.

3.11 Haematological Analysis

The blood of sacrificed animals was collected into EDTA bottles for analysis of haematological parameters such as haemoglobin concentration (Hb), white blood cell count (WBC), red blood cell count (RBC), packed cell volume (PCV), total protein, Lymphocyte and Neutrophil count.

3.12 Histological Analysis

Tissue samples from the colon, liver and kidney were rapidly fixed in 10 % formal saline and dehydrated in ascending grades of alcohol (70%, 80%, 90%, 95%, and absolute 100 %) for 2 hours each. The tissue was cleared in xylene and subsequently transferred through a pure paraffin in an oven for 1 hour and then into a second pot of melted paraffin for additional 2 hours (infiltration). The tissues were then immersed in a mould containing molten paraffin wax and then allowed to solidify (embed) for sectioning. Sections of 3 µm in thickness were prepared according to standard micro techniques onto glass slide and stained with haematoxylin and eosin. Sections were viewed at ×10 and ×40 magnification.

3.13 Immunohistochemical Analysis

The assay procedure was based on the avidin-biotin immunoperoxidase method. Antigen sites were retrieved using citric acid on 2-3 microns of formalin fixed paraffin embedded colon tissue. Peroxidase, biotin and protein blocks were done using hydrogen peroxide. The specimens were first incubated with Peroxidase Block for 5 minutes to quench endogenous peroxidase activity. The specimens are then incubated for 5 minutes with a protein block to suppress nonspecific binding of subsequent reagents, followed by a 15-minute incubation with an appropriately characterized and diluted mouse primary antibody or negative control reagent. This is followed by sequential 15-minute incubations with anti-mouse immunoglobulin-HRP, fluoresceyl-tyramide hydrogen peroxide (amplification reagent) and anti-fluorescein-HRP. Staining is completed by a five-minute incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which resulted in a brown precipitate at the antigen site (Hsu *et al.*, 1981).

3.14 Statistical Analysis

Data from this study was analysed using SPSS version 20.0 (Armonk, NY: IBM Corp) and Microsoft Excel 2010. Results were reported as Mean \pm Standard deviation and the statistical significance between the controls and experimental groups were determined by one-way ANOVA with Tukey's HSD Post-hoc analysis for multiple comparisons. Statistical test was performed at $p \leq 0.05$ level of significance.

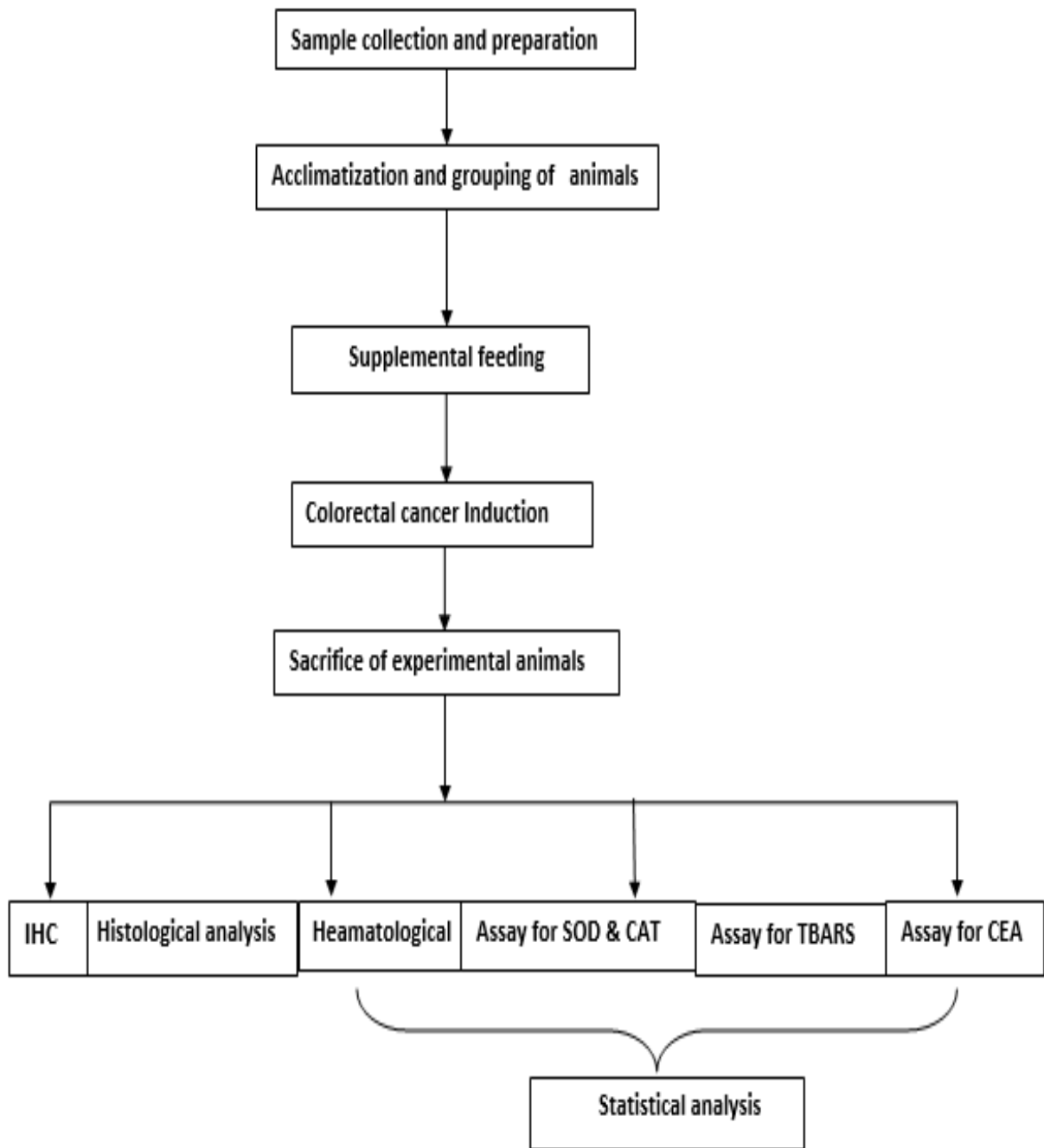


Figure 3.1: A diagram of the experimental protocol for this research

CHAPTER FOUR

4.0 RESULTS

4.1 Analysis of Endogenous Antioxidant Enzymes

The activity of the enzyme responsible for the dismutation of the superoxide radicals (O_2^-) to Hydrogen Peroxide (H_2O_2), Superoxide dismutase in colon, liver and kidney of the various experimental groups in this study was evaluated. From Table 4.1, a statistically significant increase ($p \leq 0.05$) in SOD activity was observed in the colon of all groups that received dietary supplementation (2.5%, 5% and 10%) with *C. rubens* when compared to MNU control (20.8 ± 2.7 U/ml). Increase in SOD activity was observed to increase with percentage supplementation as 2.5% (23.33 ± 4.2 U/ml), 5% (30.33 ± 6.5 U/ml), 10% (34.6 ± 3 U/ml) dietary inclusion + MNU and 10% dietary (37.5 ± 5.1 U/ml) control SOD activity was observed.

Compared with MNU control (23.9 ± 6.1 U/ml), a statistically significant increase ($p \leq 0.05$) in the activity of SOD in the liver was observed in groups that received 2.5% (30.2 ± 5.4 U/ml), 5% (31.25 ± 4.6) and 10% (33.4 ± 3.4 U/ml) supplementation + MNU. Normal control (42.7 ± 3.45) and 10% dietary control (41.67 ± 4.2) also showed a statistically significant increase when compared with MNU control as presented in Table 4.1

As presented in Table 4.1, no statistically significant increase in the activity of superoxide dismutase was observed in the kidney of groups that received 2.5% (24 ± 6.0 U/ml) and 5% (25.5 ± 6.0 U/ml) supplementation + MNU when compared with MNU control (23.34 ± 4.0 U/ml). However, a significant increase in activity was observed in 10% dietary inclusion + MNU group (30.2 ± 6.2) and 10% dietary controls. All treatment groups were predictably observed to have SOD activity lower than in Normal control (35.8 ± 4.2 U/ml)

Table 4.1: Effects of Dietary Inclusion with *Crassocephalum rubens* for 12 weeks on Superoxide dismutase Activity (u/ml) in Key Organs

Groups	Colon	Liver	Kidney
Normal	40.67±3.4 ^a	42.70±3.5 ^a	35.80±4.2 ^a
MNU Control	20.80±2.7 ^b	23.90±6.1 ^b	23.34±4.0 ^b
2.5% Suppl. + MNU	23.33±4.2 ^b	30.20±5.4 ^c	24.00±4.5 ^b
5% Suppl.+ MNU	30.33±6.5 ^c	31.25±4.6 ^c	25.50±6.0 ^b
10% Suppl. + MNU	34.60±3.0 ^c	33.40±3.4 ^c	30.20±6.2 ^a
10% dietary control	37.50±5.1 ^{ac}	41.67±4.2 ^a	32.30±4.5 ^a

Results within a column (organ) with different alphabet superscripts are statistically significant at $p \leq 0.05$ when compared using ANOVA and Tukey's HSD post-hoc analysis.

The enzyme catalase is responsible for the conversion of reactive hydrogen peroxide to water and molecular oxygen in biological systems. Catalase activity was assayed for in colon, liver and kidney of the various experimental groups in this study and results are presented in Table 4.2.

In the colon of experimental groups, a statistically significant increase ($p \leq 0.05$) in the activity of this enzyme was observed in groups that received 2.5% (40.25 ± 9.10 U/mg/ml), 5% (46.60 ± 6.00 U/mg/ml) and 10% (46.38 ± 5.30 U/mg/ml) supplementation + MNU when compared with the MNU control group (29.50 ± 4.60 U/mg/ml). Comparing these groups with both normal control (55.70 ± 8.70) and 10% dietary control (53.62 ± 8.30 U/mg/ml) revealed a decrease in Catalase activity that was however not statistically significant at $p \leq 0.05$.

In the liver, a statistically significant increase in the activity of catalase was observed when dietary supplemented groups were compared with MNU control. The catalase activity of 45.50 ± 6.50 , 47.40 ± 7.50 , 50.90 ± 10.70 , 57.43 ± 6.70 U/mg/ml reported in 2.5% + MNU, 5% + MNU, 10% +MNU and 10% dietary control groups respectively.

In the kidney, no statistically significant increase ($p \leq 0.05$) was observed in the 2.5% + MNU (34.25 ± 3.90 U/mg/ml) and 5% + MNU (34.74 ± 3.40 U/mg/ml) dietary supplemented groups when compared to MNU control (31.00 ± 2.00 U/mg/ml). A statistically significant increase in catalase activity was however observed in groups that received 10% dietary inclusion + MNU (37.00 ± 1.60 U/mg/ml) and 10% dietary control (57.33 ± 6.50 U/mg/ml) group when compared with control.

Table 4.2: Effects of Dietary Inclusion with *Crassocephalum rubens* for 12 weeks on Catalase activity (U/ml/ mg protein) in Key Organs of Experimental Groups

Groups	Colon	Liver	Kidney
Normal	55.70±5.70 ^a	58.10±3.00 ^a	60.80±3.70 ^a
MNU control	29.50±4.60 ^b	39.87±2.70 ^b	31.00±2.00 ^b
MNU + 2.5%	40.25±5.08 ^a	45.50±3.50 ^a	34.25±3.90 ^b
MNU + 5%	46.80±6.00 ^a	47.40±3.50 ^a	34.74±3.40 ^b
MNU + 10%	46.38±5.30 ^a	50.90±4.20 ^a	37.00±1.60 ^b
10% dietary control	53.62±3.30 ^a	57.43±5.70 ^a	57.33±6.50 ^a

Results within a column (organ) with different alphabet superscripts are statistically significant at $p \leq 0.05$ when compared using ANOVA followed by Tukey's HSD post-hoc analysis.

4.2 Analysis of Assay for Index of Lipid Peroxidation

Reactive oxidative species induce lipid peroxidation releasing products that play important roles in pathological processes. Malondialdehyde has been pointed out as the main product to evaluate lipid peroxidation. In this study, malondialdehyde was determined by its reaction with thiobarbituric acid (TBARS), the reaction product quantified by spectroscopy and results are presented in Table 4.3.

In the colon of experimental groups, a statistically significant decrease ($p \leq 0.05$) in the concentration of MDA-TBARS (nmol/ml/mg tissue) was observed in groups that received 2.5% (0.17 ± 0.02), 5% (0.11 ± 0.04) and 10% (0.11 ± 0.02) supplementation + MNU when compared with the MNU control group (0.27 ± 0.01). Compared with Normal group (0.025 ± 0.08) these groups demonstrated a statistically significant increase in MDA-TBARS concentration.

In the liver, a statistically significant decrease in the concentration of MDA-TBRAS (nmol/mol/mg tissue) was observed in dietary supplemented groups when compared with MNU control (0.38 ± 0.03). Treatment groups of 2.5%, 5%, 10% dietary inclusion + MNU had a MDA-TBARS concentration of 0.29 ± 0.03 , 0.21 ± 0.01 , 0.2 ± 0.01 respectively.

In the kidney, no statistically significant difference ($P \leq 0.05$) in concentration was observed in all dietary inclusion groups when compared to MNU control (0.23 ± 0.01). Normal control (0.1 ± 0.005) and 10% dietary control (0.11 ± 0.03) groups however showed a statistically significant decrease in malondialdehyde concentration.

Table 4.3: Effects of Dietary Inclusion with *C. rubens* on Thiobarbituric Acid Reactive substances (TBARS) Concentration (nmol/ml/mg protein tissue)

Groups	Colon	Liver	Kidney
Normal	0.025±0.01 ^a	0.20±0.02 ^a	0.10±0.005 ^a
MNU control	0.27±0.01 ^b	0.38±0.04 ^b	0.23±0.01 ^b
MNU + 2.5%	0.17±0.02 ^d	0.29±0.03 ^c	0.23±0.04 ^b
MNU + 5%	0.11±0.04 ^c	0.21±0.01 ^a	0.19±0.01 ^b
MNU + 10%	0.11±0.02 ^c	0.20±0.01 ^a	0.17±0.016 ^b
10% dietary control	0.10±0.01 ^c	0.18±0.03 ^a	0.11±0.03 ^a

Results within a column (organ) with different alphabet superscripts are statistically significant at $p < 0.05$ when compared using ANOVA followed by Tukey's HSD post-hoc analysis.

4.3 Analysis of Haematological Parameters/Blood Cells

Haematological parameters can serve as an indirect indicator of the carcinogenetic process as malignancies have been associated with deviations from normal blood composition in many cases. Several haematologic parameters with links to carcinogenesis were assayed for in this study and the results are presented in Table 4.4.

In this study, no statistically significant difference ($p \leq 0.05$) in packed cell volume (%) was observed in all groups when compared with MNU Controls. However, the Normal control group had a significantly elevated PCV when compared to other groups. Also, compared with MNU control, no statistically significant difference in haemoglobin concentration was observed in all groups except the groups that received 5% dietary inclusion + MNU.

Assay for total platelet volume (g/dL) also revealed no statistically significant difference between all groups but will all dietary supplemented groups showing elevation when compared with MNU control. However, a statistically significant elevation in White Blood Count ($10^3/L$) was observed in dietary supplementation groups and normal control when compared with MNU control. Differences in Total Red Blood Count ($10^3/L$) was generally not statistically significant when dietary inclusion + MNU groups were compared with MNU control except in the 5% dietary inclusion + MNU group.

Determination of percentage neutrophil count also showed a statistically significant increase ($P \leq 0.05$) in neutrophil count when all groups were compared with MNU control. Dietary supplementation also resulted in a statistically significant elevation in neutrophil count when compared with Normal control. However, a statistically significant decrease in lymphocyte count (%) was observed in dietary groups when compared with MNU control. Lymphocyte count was however statistically similar ($P \leq 0.05$) when Normal and MNU control groups were compared.

Table 4.4 Effects of Dietary Supplementation with *C. rubens* or 12 weeks at Different Percentages on Key Hematological Parameters in Treated Groups

	Normal control	MNU control	2.5% Suppl. + MNU	5% Suppl. + MNU	10% Suppl. + MNU	10% Suppl. + Saline
<i>Packed Cell Volume (%)</i>	59.75±3.50 ^a	41±5.90 ^b	44.50±7.06 ^b	43.75 ±1.71 ^b	42.20± 8.70 ^b	48.75±5.40 ^b
<i>Haemoglobin (g/dl)</i>	14.56±0.56 ^a	13.62±1.90 ^a	19.88±1.7 ^b	14.80±2.35 ^a	14.04±2.90 ^a	16.55±0.56 ^a
<i>Total Platelet (g/dl)</i>	7.40±0.28 ^a	6.90±0.54 ^b	7.68±0.72 ^a	7.83±0.46 ^a	7.40±0.75 ^a	8.04 ±0.79 ^a
<i>White Blood count (10³/l)</i>	7.38±1.40 ^{ab}	5.56±0.90 ^a	12.08±3.74 ^b	11.76±2.27 ^b	8.83±3.18 ^{ab}	10.08±5.26 ^b
<i>Red Blood Count (%)</i>	7.40±0.41 ^a	6.90±0.94 ^a	10.03±0.54 ^b	7.16±1.96 ^a	7.40±1.14 ^a	7.16±1.96 ^a
<i>Neutrophil count (%)</i>	27.00±3.16 ^a	19.00±6.98 ^b	26.75±4.19 ^c	32.33±9.61 ^c	38.40±6.97 ^c	40.00±8.37 ^c
<i>Lymphocyte count (%)</i>	70.50±3.80 ^a	77.00±8.42 ^a	66.83±9.32 ^{ab}	67.17±9.4 ^{ab}	69.25±4.35 ^b	58.80±3.30 ^b

Results within a column (organ) with different alphabet superscripts are statistically significant at $p \leq 0.05$ when compared by ANOVA followed with Tukey's HSD post-hoc analysis.

4.5 Analysis of Level of Colon Cancer Marker

Carcinoembryonic antigen (CEA) is an excellent biomarker of colon cancer progression and outcome. Elevated levels strongly correlate with cancer presence and possible metastasis. This antigen was assayed for in experimental groups in this study. A statistically significant decrease ($p \leq 0.05$) in CEA concentration (pg/ml) was observed in all groups when compared with MNU control (125.56 ± 4.2). Groups that received 2.5%, 5% and 10% dietary inclusion + MNU instillation had CEA concentration of 96.60 ± 4.97 , 68.80 ± 3.44 and 50.57 ± 6.80 respectively. Ten percent dietary control had a lower CEA level (35.8 ± 7.16) when compared with other groups including the Normal control group (46.24 ± 3.43).

Table 4. 4: Effects of Dietary Inclusion with *C. rubens* at varying Percentages on the Level Carcinoembryonic Antigen as a Biomarker of Colon Cancer in Experimental Groups

Experimental Groups	CEA (pg./ml)
Normal control	46.24±3.43 ^a
MNU control	125.56 ±12.42 ^b
MNU + 2.5% dietary inclusion	96.60±4.97 ^b
MNU + 5% dietary inclusion	68.80±3.44 ^a
MNU + 10% dietary inclusion	50.57± 6.80 ^a
10% dietary control	35.8 8± 7.16 ^a

Results within a column (organ) with different alphabet superscripts are statistically significant at $p \leq 0.05$ when compared by ANOVA followed with Tukey's HSD post-hoc analysis

4.6 Histological and Immunochemical Evaluation of Tissue Sections

Histologic evaluation of Formalin-fixed, paraffin-embedded sections of the colon, liver and tissue of representative animals from the various groups were carried out in this study to ascertain the ability of the plant to protect against tissue damage even with long-term exposure to the chemical carcinogen MNU.

An essentially normal colon epithelium was observed in normal control, however examination of the colon of MNU controls groups revealed full thickness ulceration of the mucosa with highly necrotic and desquamated villi. The presence of intestinal polyps was however not recorded. The group that receive 2.5% dietary inclusion with MNU also showed deep mucosal ulceration and glandular necrosis with inflammation of the epithelium. With 5% dietary inclusion with MNU, only a moderated mucosal ulceration with some glandular necrosis while 10% dietary inclusion + MNU instillation showed mild mucosal ulceration and some inflammation. Colon tissue from 10% dietary control showed only moderate mucosal surface ulceration and some sclerosis.

On examination, no damage was observed in the liver of normal control. However, marked destruction of hepatocytes with severe necrosis and clearing was observed in MNU control group. Animals from the group that received 2.5% and 5% dietary inclusion with MNU instillation also showed marked hepatic damage, however the 10% supplemented group with MNU instillation and 10% dietary controls showed little hepatic damage on microscopic examination.

Tissues section of the kidney were also examined for histologic damage. Normal control showed essentially no damage to the kidney however that from the MNU control group was characterized by marked necrosis of the renal tubule with cellular infiltration with inflammatory cells and an almost totally absent glomeruli. Groups that received 2.5% and 5% dietary inclusion with MNU

instillation were observed to show marked dilation of the renal tubules, cellular infiltration and necrosis of the glomeruli while those that received 10% dietary inclusion showed mild fibrosis and hypoperfused glandular damage.

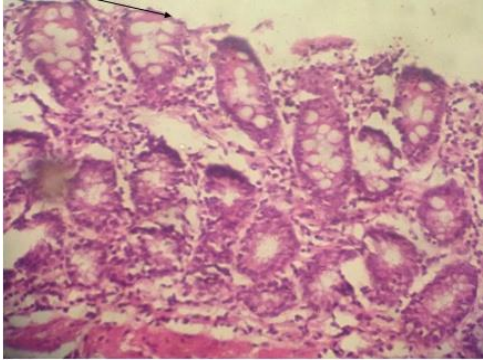


Plate I A: Normal controls: Essentially normal colon (x10)

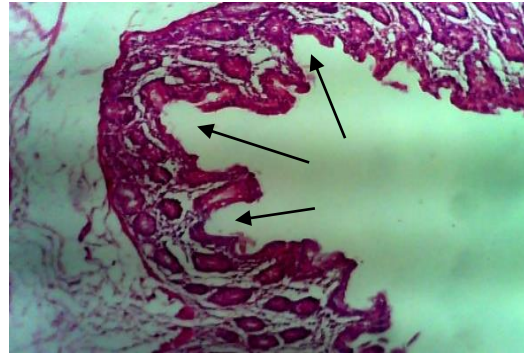


Plate I B: MNU controls: Deep mucosal ulceration, highly necrotic and desquamated villi (x10)

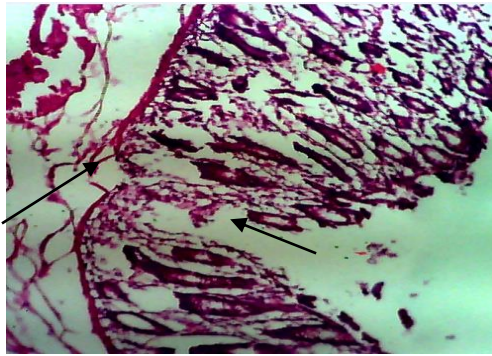


Plate I C: 2.5% Suppl. + MNU: Deep mucosal ulceration and glandular sclerosis, Necrotic and desquamation of the villi and inflammation (x10)

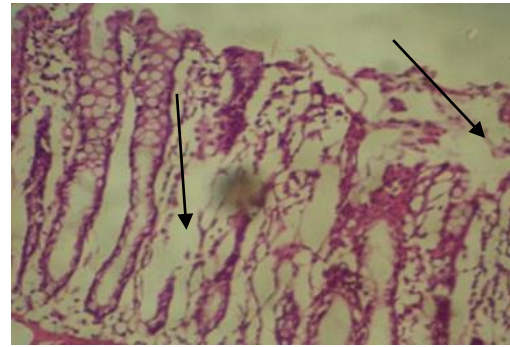


Plate I D: 5% Suppl. + MNU: Moderate surface mucosal ulceration, glandular necrosis and desquamation of the villi and crypts (x10)

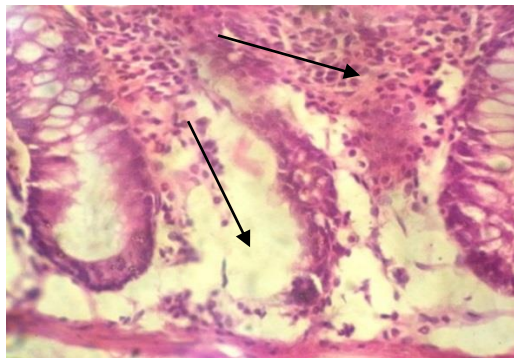


Plate I E: 10% Suppl. + MNU: Mild mucosal ulceration and glandular necrosis. Villi are sclerotic and show inflammation (x10)

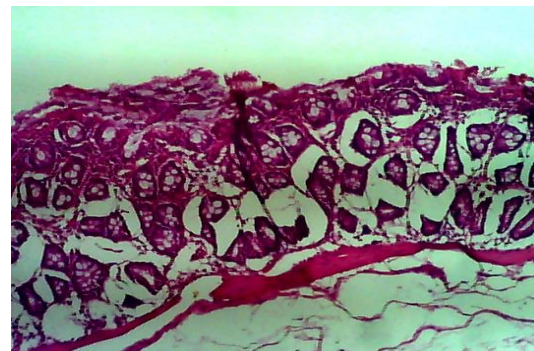


Plate I F: 10% dietary control: Moderate surface mucosal ulceration, some glandular sclerosis (x10)

PLATE I (A-F): Photomicrograph images from histopathological evaluation of formalin fixed paraffin-embedded colon tissue sections from treated and control groups (x10 Mag)

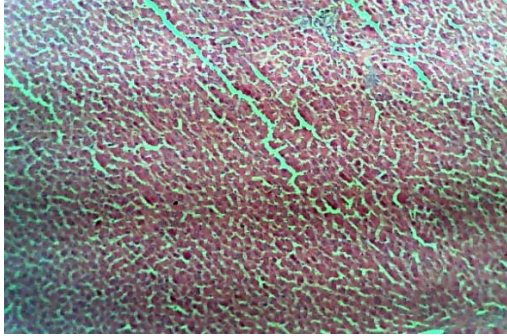


Plate II A: Normal control: Essentially normal liver (x40)

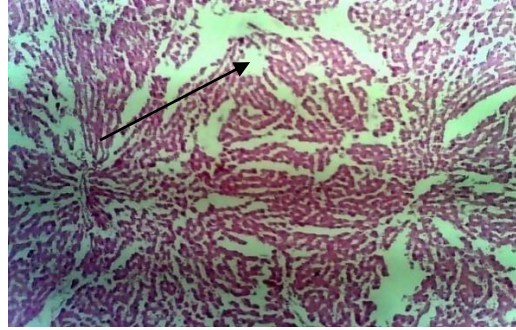


Plate II B: MNU control: Marked hepatic destruction and severe necrosis and cytoplasmic clearing (x40)

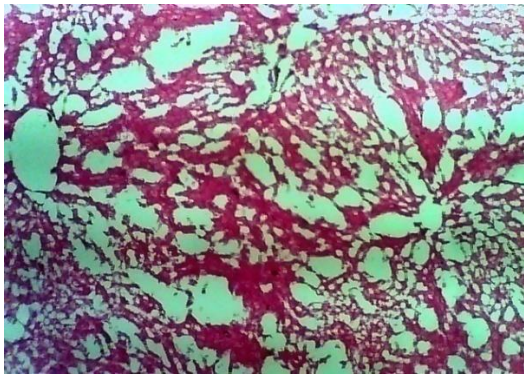


Plate II C: 2.5% suppl. + MNU: Marked hepatic destruction and cytoplasmic clearing of hepatocytes and vacuolation (x40)

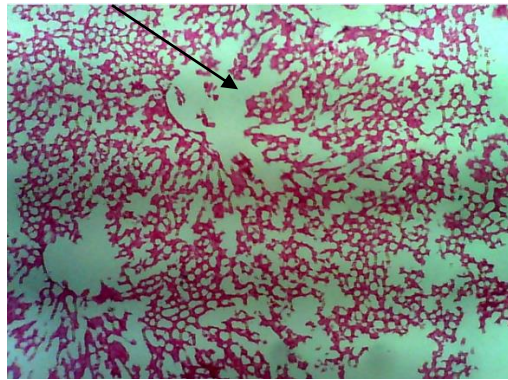


Plate II D: 5% Suppl. + MNU: Marked hepatic destruction, cytoplasmic clearing and vacuolation (x40)

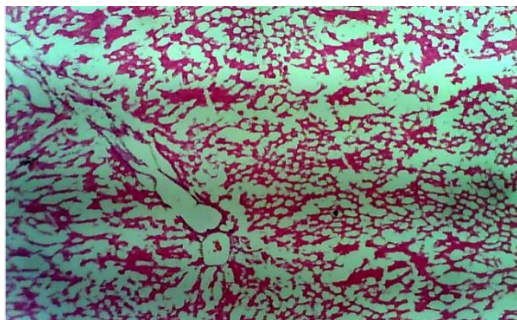


Plate II E: 10% Suppl. + MNU: Normal liver with some hepatic clearing (x40)

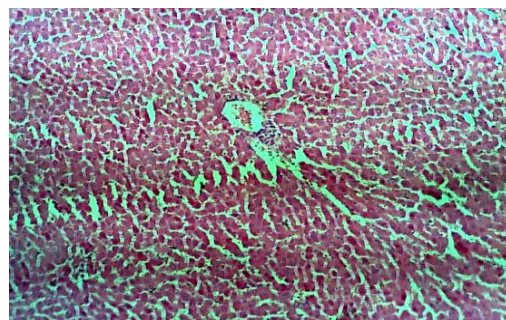


Plate II F: 10% Suppl. + Saline: Normal liver (x40)

PLATE II (A-F): Photomicrograph images from histopathological evaluation of formalin fixed paraffin embedded Liver tissue sections from experimental groups (x40 mag)

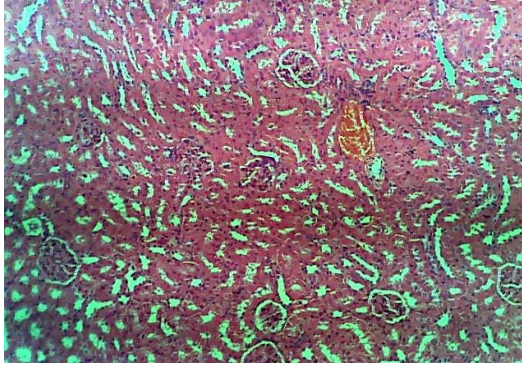


Plate III A: Normal control: Essentially normal kidney (x 10)

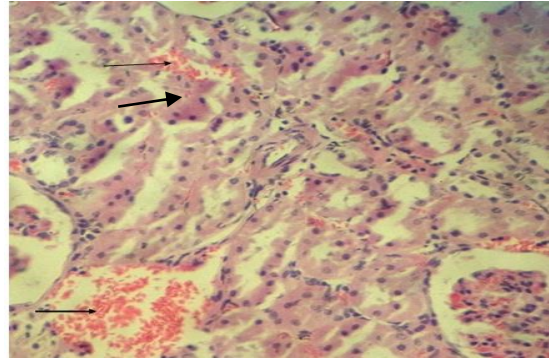


Plate III B: MNU controls: Necrosis of renal tubule, interstitial fibrosis, cellular infiltration with neutrophils, glomeruli almost gone. (x10)

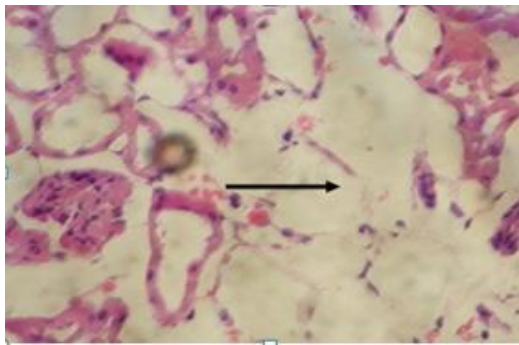


Plate III C: 2.5% Suppl. + MNU: Marked dilation of tubules, severe necrosis/hypoperfused glomeruli (x10)

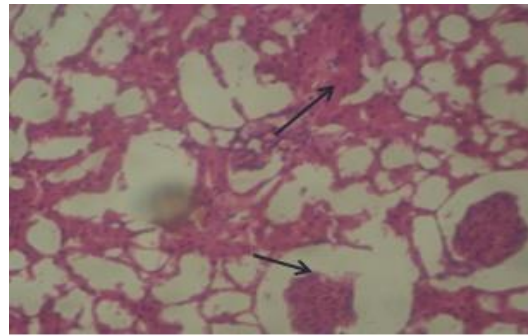


Plate III D: 5% Suppl. + MNU: Dilation of tubule and glomeruli, cellular infiltration, some necrosis of the glomeruli is also observed (x40)

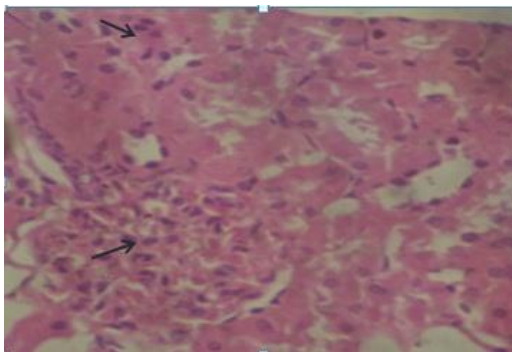


Plate III E: 10% Suppl. + MNU: Marked dilation of tubules, Fibrosis is seen, mild hypoperfused glandular damage and inflammation (x10)

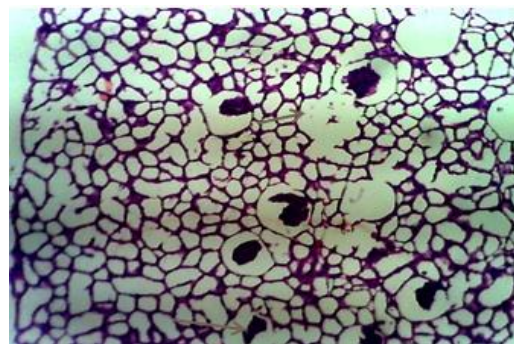


Plate III F: 10% dietary control: Tubules are mildly dilated. Mild necrosis and hypoperfused glomeruli (x10)

PLATE III (A-F): Photomicrograph images from histopathological evaluation of formalin fixed, paraffin embedded Kidney tissue sections from experimental and control groups (x10 Mag)

4.6 Immunohistochemical Analysis of the Colon of groups that received Dietary Inclusion with various percentages of *C. rubens* for expression of *mutl* homologue -1 protein.

Immunohistochemistry is an excellent method in studying the expression of key proteins whose roles have been elucidated in the pathogenesis of cancers. One of such proteins in colorectal carcinogenesis is the mismatch repair protein, Mutl-1.

In this study, no expression of the protein was observed in the MNU control, 2.5%, 5% and 10% dietary inclusion + MNU groups. However, a mild and pronounce expression was observed in Normal control and 10% dietary control groups respective.

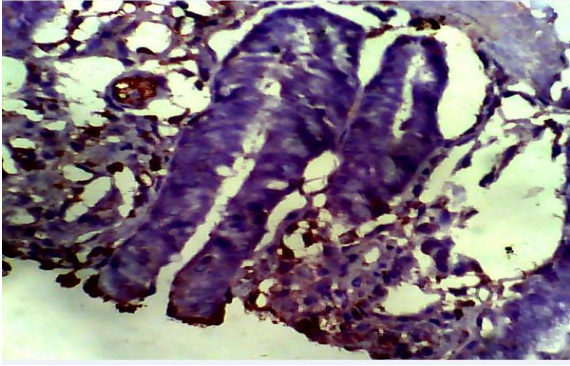


Plate IV A: Normal controls: Expression of MLH1 protein



Plate IV B: MNU control: No expression of MLH-1 protein

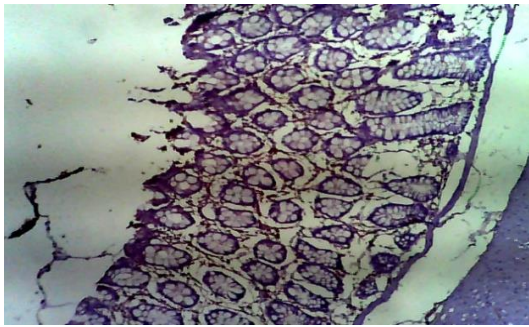


Plate IV C: 2.5% suppl. + MNU: No expression of MutL protein

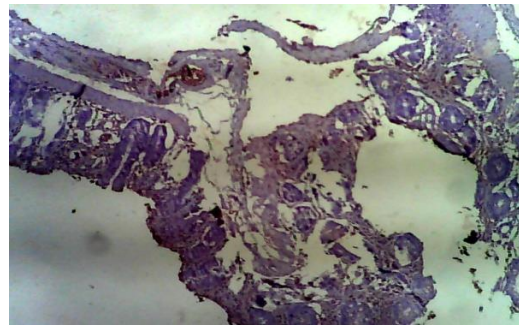


Plate IV D: 5 % suppl. + MNU: No expression of MutL protein

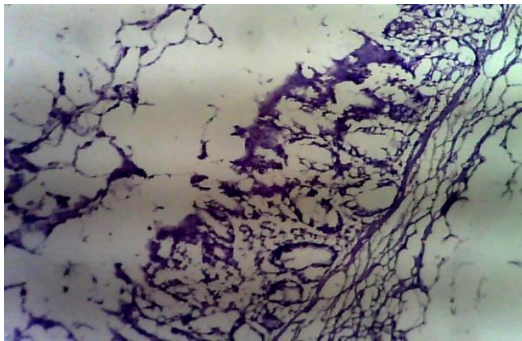


Plate IV E: 10% + MNU: NO Expression of MutL-1

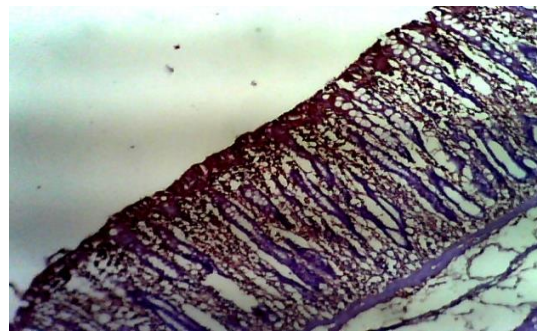


Plate IV F: 10 % dietary control: Expression of MutL-1 protein

PLATE IV (A-F): Photomicrograph images from immunohistochemical analysis for expression of MutL homologue 1 protein in colon tissue sections. (x10 Mag)

CHAPTER FIVE

5.0 DISCUSSION

N-Methyl-nitrosourea (MNU) induced carcinogenesis is an established model in the study of colorectal pathogenesis in animal models. Being an alkylating requiring no metabolic activation, its mode of action in triggering carcinogenesis is via direct methylation of DNA bases. It can also act to trigger oxidative stress via the production of reactive oxygen species and other inflammatory responses that are precursors to full carcinogenesis (Johnson and Fleet, 2013).

This study was aimed at evaluating the effectiveness of the plant (*C. rubens*) in interfering with the onset of carcinogenesis by several strategies: preventing the effects of oxidative stress such as lipid peroxidation and adduct formation by effectively scavenging reactive oxygen species, preventing necrotic damage to tissues, preventing MNU- induced alteration to key cellular processes such as DNA mismatch repair and limiting cancer progression indicated by up regulation of such molecular markers as carcinoembryonic antigen.

Several evidence highlights the role of reactive oxygen species as important precursors of carcinogenesis (Valko *et al.*, 2007). Under a sustained environmental stress, ROS are constantly produced and may induce significant damage to cellular structures chiefly the plasma membrane, protein and nucleic acids via mechanisms such as peroxidation, oxidation and adduct formation. This in turn can induce or contribute to somatic mutations and neoplastic transformation. Cancer initiation and progression has also been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation (Visconti and Grieco, 2009; Reuter *et al.*, 2010).

Determining the amount of malondialdehyde (a key product from the peroxidation of plasma membrane lipids and modulator of such cellular processes that feature in carcinogenesis such as cell proliferation and differentiation (Cejas *et al.*, 2004), and two of this enzyme systems; Superoxide Dismutase and Catalase, responsible for the dismutation of superoxide radicals and conversion of dangerous hydrogen peroxide to water in supplemented and control groups provided a basis for assessing the potential of the plant in dealing directly with oxidative stress.

Results show that dietary supplementation with *C. rubens* led to a lowering of the extent of lipid peroxidation across all treatment groups when compared to MNU controls for all organs studied. Results were statistically significant at $p \leq 0.05$ except of the kidney with better outcomes in this regard observed for groups what received 10% and 5% supplementation. The levels of MDA were also statistically comparable between the Normal group and Positive control (10% Supplement without MNU instillation). This agrees with findings by (Adewale *et al.*, 2016), which demonstrated the ability of the *C. rubens* to lower MDA levels in carbon- tetrachloride induced liver damage in rats at doses of 150, 300 and 450 mg/kg body weight. The ability of this plant to lower the extent of lipid peroxidation is likely due to its content of antioxidant phytochemicals. Phytochemicals; products of secondary metabolism in plants serve a variety of functions ranging from protection from ultraviolet radiation to preventing invasion by pathogens. Phytochemical analysis of the *C. rubens* has shown a considerable concentration of polyphenols and flavonoids such as quercetin, catechins, cathetic tannins, gallic tannins, coumarins, combined anthracene derivatives C-heterosides (Adjatin *et al.*, 2013), all of which have shown excellent chemopreventive properties in animal models with positive results observed. Various mechanisms directly linked to chemoprevention are deployed by these and include the induction of

detoxification enzymes and effective scavenging of free radicals implicated in cancer, ageing and neurodegenerative diseases amongst others (Pandey and Rizvi, 2009).

Dietary supplementation of *C. rubens* also seemed to elevate the activity of endogenous antioxidant enzyme systems responsible for the conversion of harmful reactive free oxygen species to innocuous products as demonstrated by an increase in the activity of Superoxide dismutase and Catalase in groups that received *C. rubens* supplementation at various percentages. A statistically significant increase in Superoxide dismutase and catalase activity was observed in the liver and colon of groups that received dietary supplementation with the plant. Similar results were obtained when the hepatoprotective ability of ethanol extracts of the plant was assessed in CCL₄-induced oxidative stress (Adewale *et al.*, 2016).

The exact mechanism by which this positive induction of activity is accomplished remains to be elucidated. However, two plausible explanations for this phenomenon are proposed: positive induction of the expression of genes coding for the endogenous antioxidant enzyme (Thakur *et al.*, 2014) and the reduction in the concentration of ROS available to overwhelm antioxidant enzyme systems thus decreasing their activity (Kasote *et al.*, 2015). This latter is the most likely. Enzyme activity is dependent on several factors one of which is the concentration of substrates. Decomposition of H₂O₂ by the catalytic activity of catalase follows the fashion of a first-order reaction and its rate is dependent on the concentration of H₂O₂. Enzyme kinetics allude to the fact that when enzymes systems are saturated by reactants, activity peaks and begins to drop. The exogenous antioxidant property of the plant thus ensures that enzymes systems are not overwhelmed by the concentration of radicals leading to a fall in activity but that available enzymes are freed up to function maximally (Berg *et al.*, 2006).

A high frequency of deviations from normal peripheral blood composition and constituents has been observed in subjects with active carcinogenesis. This has especially been linked to defects in the hematopoietic process. The cytopenias, or reductions in erythrocyte, neutrophil, and platelet numbers that are frequently observed may be a result of defective haematopoiesis or increased hematopoietic cell consumption or destruction (Zuckerman, 1998).

The key hematologic consequence of carcinogen exposure in animal models is the disruption of normal hematopoiesis. The mechanisms are related to elimination of pluripotent stem cells, damage to the bone marrow microenvironment required for normal hematopoiesis to occur, inhibition of production of hematopoietic growth factors, and/or production of hematopoietic inhibitory cytokines (Pontel *et al.*, 2015).

In this study, differences in the quantity of several vital blood components were observed when the values from plant supplemented groups were compared with MNU control groups. Elevation in Packed Cell Volume and Neutrophil count were observed. However, for parameters such as total white blood count, Hemoglobin concentration and total platelet count, no significant difference was observed. An interesting observation however was the elevation in Lymphocyte concentration which was elevated in MNU controls than in other groups. These observations are consistent with what is expected during the carcinogenetic process: interference with normal hematopoiesis.

There is an astonishingly tight control over daily hematopoietic cell production in normal circumstances and rapid responses in stress situations such as generated during sustained chemical exposure in this case a carcinogen may alter this. However, a number of disorders that affect any of the components of this production and normal destruction process may result in the development

of anemia, neutropenia, thrombocytopenia, or a combination of these cytopenias (Zuckerman, 1998).

Carcinogen transformed cells are also able to induce pathological changes to normal hematologic parameters via the recruitment of inflammatory cells that express the CXC and CC group of chemokines. In addition to potentially mutagenic infectious agents that sustain chronic inflammation, inflammatory cells create a microenvironment favourable for the transformation of normal proliferating cells. Inflammatory cells, in particular phagocytic cells, generate reactive oxygen and nitrogen species that contribute to the formation of the mutagenic agents such as Malondialdehyde and peroxynitrite (Chantrain *et al.*, 2008).

Extensive research has been performed to identify colon cancer-specific antigens in blood. However, there are only two blood-based biomarkers available to monitor colorectal cancer patients namely carcinoembryonic antigen (CEA) and Carbohydrate Antigen 19- 9 (CA19-9). Carcinoembryonic antigen, a high molecular weight glycoprotein, is found in embryonic tissue and colorectal malignancies. Since its discovery in 1965 (Bast *et al.*, 2001), it has remained the most acceptable tumor marker to monitor CRC recurrence to date. Elevated CEA levels are considered a poor prognostic factor for resectable CRC and correlate with cancer progression thus making it a good choice as prognostic marker after diagnosis and in monitoring disease progression (Roayaei *et al.*, 2014).

This study like several others have demonstrated the potential benefit of dietary consumption of plants and other natural products to not only prevent but also manage the outcomes of cancers. The deployment of these is invaluable in ensuring good prognosis after cancer is detected (Ravasco *et al.*, 2005; Rock *et al.*, 2012). Carcinoembryonic antigen levels in animals that received dietary inclusion with *C. rubens* were considerably lower than in MNU control (Table 4.5). The decrease

in CEA levels was observed to occur in a dose-response manner, indicating that higher concentration of the plant had more beneficial CEA-lowering capacity. The exact mechanisms by which this plant, like others, lowers CEA levels has not been elucidated but a few mechanisms which all may contribute to chemoprevention may offer some explanation. Some of these include, modulation of immune and anti-inflammatory response (basically since CEA is an antigenic protein) related to gene expression, interference with estrogenic/antiestrogenic activity, induction of cell cycle arrest or apoptosis, inhibition of oxidation, and changes in cell signalling pathways (Su *et al.*, 2013)

Immunohistochemistry is increasingly being deployed in the study of colon carcinogenesis arising from high frequency microsatellite instability especially in the early stages, to profile the expression of critical genes implicated in colon cancer onset and progression (Lanza *et al.*, 2002). One of these genes is the *MLH-1* gene whose product MutL homologue 1 plays a vital role in combination with other proteins (MutS homolog 2, MutS homolog 6 and Post-meiotic segregation 2, PMS 2) in DNA mismatch repair. Recent studies have demonstrated the predictive and diagnostic value of this method as applied to these proteins almost equivalent to direct Microsatellite instability testing using sequencing methods (Kheirelseid *et al.*, 2013).

In this study, no expression of the MutL homolog-1 was demonstrated in colon tissue sections from MNU control and 2.5% dietary inclusion + MNU groups, indicating colon carcinogenesis. This immunostaining is observed in almost all of colon adenocarcinomas arising from microsatellite instability and agrees with findings by other investigators who also suggested that MLH-1 hypermethylation is implicated as the most common cause of an absence of MMR protein (Joost *et al.*, 2014). However, in groups that received varying percentages with dietary supplementation of leaves of *C. rubens* together with MNU instillation, some level of expression

of this protein was observed. Quantifying the level of expression also reveals that increasing the in percentage supplementation also resulted in increased expression of this protein. This proved the ability of the plant to suppress carcinogenesis by protecting MMR genes from MNU-induced mutations that can trigger carcinogenesis. While the exact mechanism of how this action is effected remains to be elucidated, the most probable explanation is the interference of dietary phytochemicals present in the plant with the transfer of methyl groups from MNU to the DNA in colon cells. *MLH-1 gene promoter hypermethylation* translates to epigenetic silencing of its expression into the MutL protein (van Roon *et al.*, 2013).

The value of supplementation with *C. rubens* also extends beyond chemoprevention, as it can also be deployed alongside standard drugs in colon cancer therapy. It has been reported by various studies that DNA mismatched repair deficient cells are resistant to the alkylating agents (e.g. melphalan and busulphan), methylating agents (e.g., temozolomide), the platinum-containing agents (e.g., cisplatin and carboplatin), antimetabolites (e.g. fluorouracil and thioguanine) and topoisomerase inhibitors (e.g., doxorubicin) (Helleman *et al.*, 2006; Hewish *et al.*, 2010).

Understanding the nature of early appearing lesions in colorectal carcinogenesis should contribute to both clarification of the mechanisms underlying neoplasia in colon and efforts to find effective preventive agents. Carcinogen induced colon carcinogenesis is a multistep process with morphological and histological features similar to those seen in human sporadic colon carcinogenesis. It is widely accepted today that the adenoma to carcinoma sequence is characterized by recognizable histological changes that start with dysplastic aberrant crypts or intraepithelial neoplasia (Tanaka, 2009; Perše and Cerar, 2011). These lesions then have the potential to progress to advanced adenomas, which have a significant potential to transform into adenocarcinomas. This understanding is necessary in order to accurately identify and interpret

alterations that occur in the colonic mucosa when evaluating natural or pharmacological compounds in rat colon carcinogenesis.

In this study, a range of specific histologic changes in the assessed tissue sections were observed in the different experimental groups. Histologic assessment of the colon, showed deep mucosal ulceration, marked necrosis and desquamation in the villi epithelium of MNU controls and 2.5% supplementation + MNU groups. This significant damage is a precursor to neoplastic transformation of colonic epithelium. However, groups that received higher percentages of dietary inclusion only showed significantly milder tissue damage indicating the potential of the plant in protecting tissues exposed to the carcinogen.

Also in this study, the potential off-site histologic effects of the carcinogen MNU and ability of the plant to offer protection from histologic damage was also assessed by evaluating tissue sections of the liver and kidney for histologic changes. MNU instillation without dietary inclusion with leaves of *C. rubens* to offer some protection in MNU controls led to marked hepatic destruction, necrosis and cytoplasmic damage. Low level of supplementation with MNU administration as seen in groups that received 2.5% and 5% inclusion also offered little protection to the liver with hepatic necrosis and cytoplasmic clearing clearly observed by not as comprehensive as that observed in MNU control. Histologic assessment of the kidney also revealed a highly necrotic renal tubule with and cellular infiltrations with neutrophils in MNU controls. Dietary inclusion however, offered marginal protection as significant damage to the kidney was also observed in groups that received varying percentages of supplementation with MNU administration.

The mechanism of protection afforded by this plant can be related to the prevention of oxidative stress which in turn prevents chronic inflammation that has been reported to result in tissue damage that precludes carcinogenesis. Excessive and uncontrollable production of ROS for a longer period

of time results in persistent injury of cells in the tissue and consequently persistent inflammation creates a sustained inflammatory/oxidative environment that leads to a vicious circle (Reuter *et al.*, 2010), which can damage healthy neighboring epithelial and stromal cells and over a long period of time may lead to carcinogenesis. Besides, cells produce soluble mediators, which act by further recruiting inflammatory cells to the site of injury and produce more reactive species. Chronic inflammation has been linked to various steps involved in carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Reuter, Simone, 2010; Perše and Cerar, 2011).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

The molecular basis and etiology of colorectal cancer has largely been elucidated with advances in laboratory and population studies. It has also been proven that this cancer like others is preventable by interfering with carcinogenetic process either by blocking its onset or preventing its progression to premalignant stages. Unfortunately, the incidence and mortality from colorectal cancer continues to be on the increase largely due to ignorance and reluctance to adoption beneficial practices such as the frequent and adequate consumption of fruits and dietary vegetables and undergoing of appropriate screening for risk prediction or early detection.

Plants secondary metabolites such as isoprenoids, phenolics and alkaloids and other natural products have been demonstrated to offer substantial preventive benefits and also serve as leading sources of novel cancer preventing agents whose potential have been demonstrated by both *in vitro* methods or use of animal models. This study has demonstrated the preventive capacity of the vegetable *C. rubensin* preventing colon carcinogenesis via regular inclusion in the diet. With a statistically significant increase in the activity of beneficial endogenous antioxidant enzymes, lowering of the extent of lipid peroxidation and concentration of carcinoembryonic antigen and ability to sustain the expression of beneficial mismatch repair genes while limiting the extent of tissue damage, it has been demonstrated as offering significant protection from the effects of chemically induced carcinogenesis.

6.2 CONCLUSION

This study was undertaken to evaluate the potential of a widely consumed indigenous vegetable, *Crassocephalum rubens* assumed to offer protection against the onset of colon carcinogenesis by evaluating key molecular players in its pathogenesis. Its results

- i. Have demonstrated the positive potential of the plant in sustaining a beneficial antioxidant status that is able to prevent oxidative stress induced by sustained chemical assault by a well known carcinogen thus lowering the risk of carcinogenesis.
- ii. Show that the plant is able to boost the body's ability to effectively scavenge for free radicals and reactive oxygen species which can precipitate carcinogenesis by attacking key cellular structures and macromolecules by events such as plasma membrane lipid peroxidation, DNA oxidation and methylation, alteration of key cellular signaling cascades and tissue necrosis by enhancing the activity of antioxidant enzyme system and scavenging for free radicals using its beneficial phytochemical constituents
- iii. Reveal that *C. rubens* possess the valuable potential in sustaining normal hematopoiesis by defending against bone marrow suppression characteristic of carcinogenesis from chronic carcinogen exposure. The ability of the dietary inclusion with the plant to limit the extent of necrotic damage to key organs and to also prevent alterations to the expression of key proteins and genes involved active mismatch repair of compromised DNA was also demonstrated in this study.

6.3 RECOMMENDATIONS

In summary, this study demonstrates the potential of the plant in limiting the effects of chemically induced carcinogenesis while adding to the wealth of knowledge and strengthening the evidence that plants especially consumed fruits and vegetables are excellent options in the deployment of chemoprevention as an effective strategy in reducing the incidence and mortality from colon cancer as with all cancers. This strengthens our recommendations that the utilization of drugs or natural products derived from dietary supplementation alone or in combination may effectively control the development of colorectal adenomas and carcinomas. More knowledge of the mechanisms of carcinogenesis will hopefully lead to the development or optimization of agents from locally consumed vegetables and fruits that are specific, effective, nontoxic and scalable to meet the populations preventive needs.

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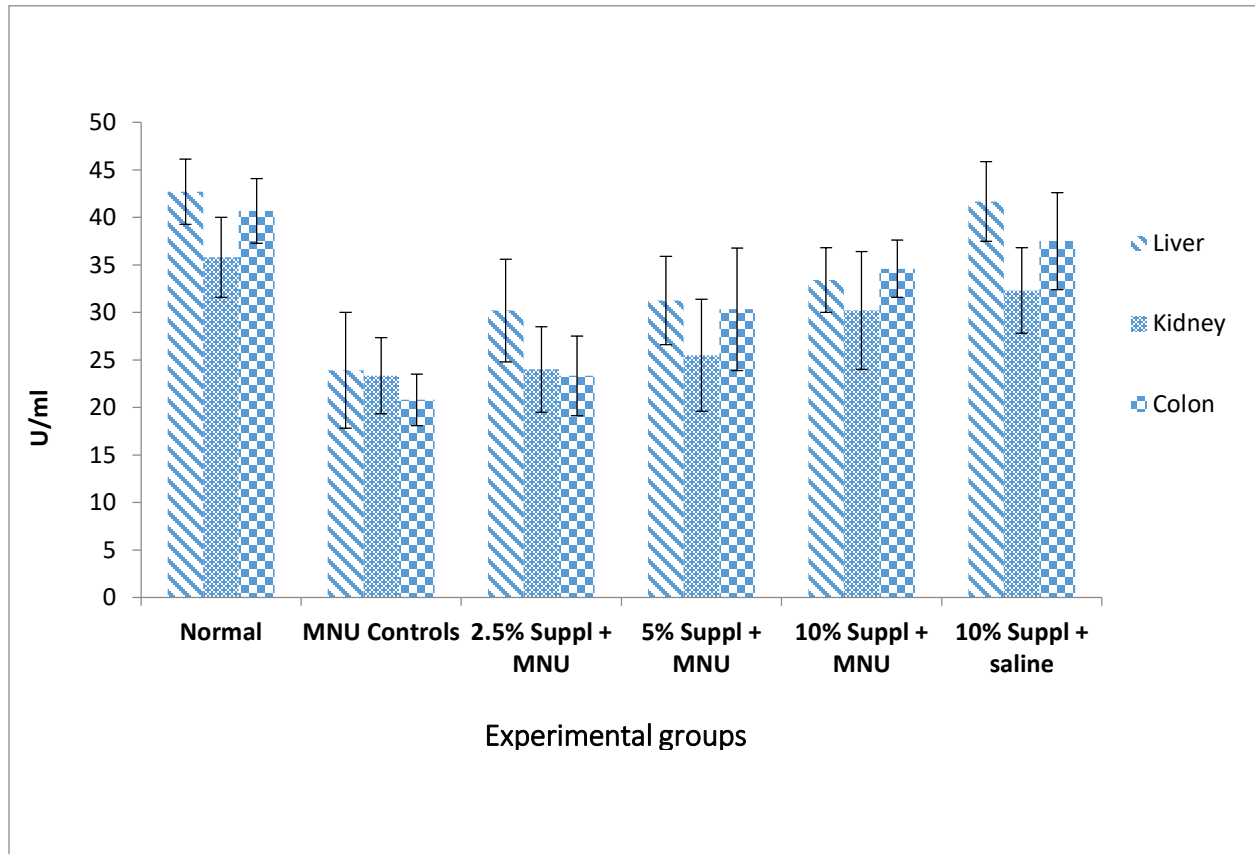
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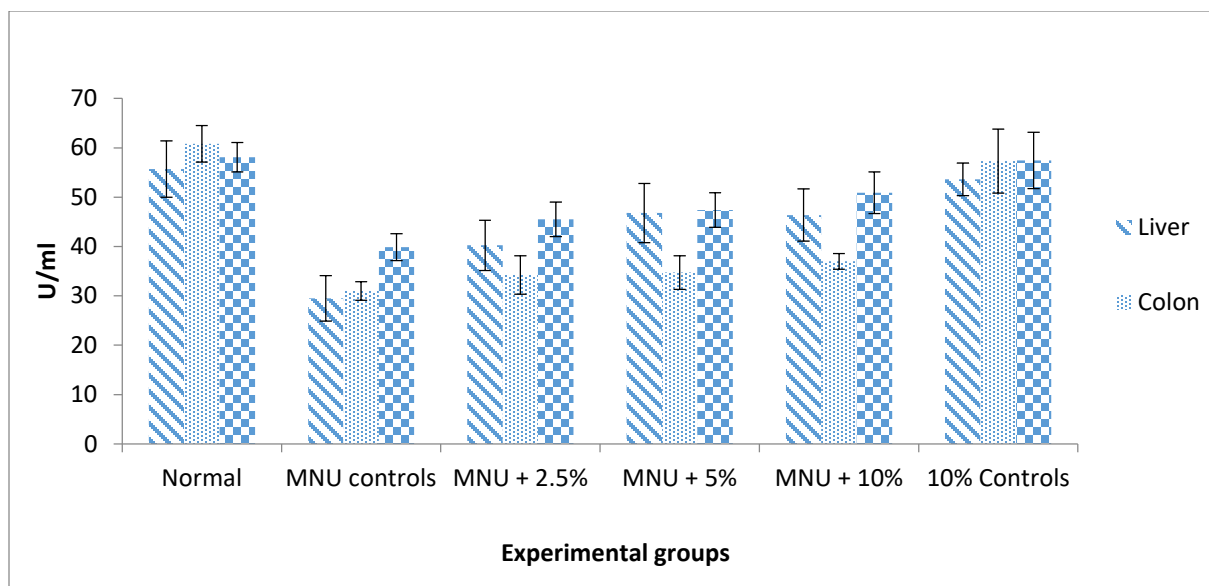
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APPENDICES



Appendix 1: The Figure above presents results of the assay for Super oxide dismutase activity measured in U/ml in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each organ (Liver, Kidney and Colon) evaluated in between experimental groups.



Appendix 2: The Figure above presents results of the assay for Catalase activity measured in U/ml in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each organ (Liver, Kidney and Colon) evaluated in between experimental groups

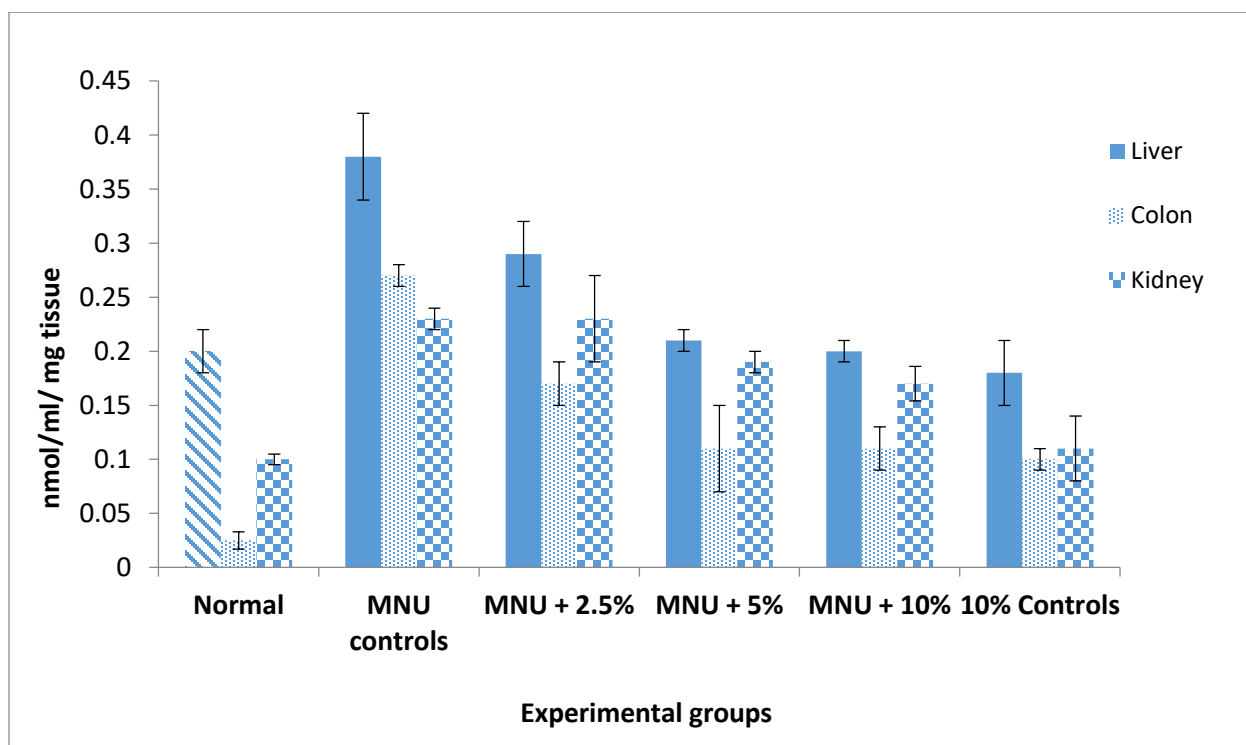


Figure 3: The Figure above presents results of the assay for Thiobarbituric Reactive Acid substances in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each organ (Liver, Kidney and Colon) homogenate assayed.

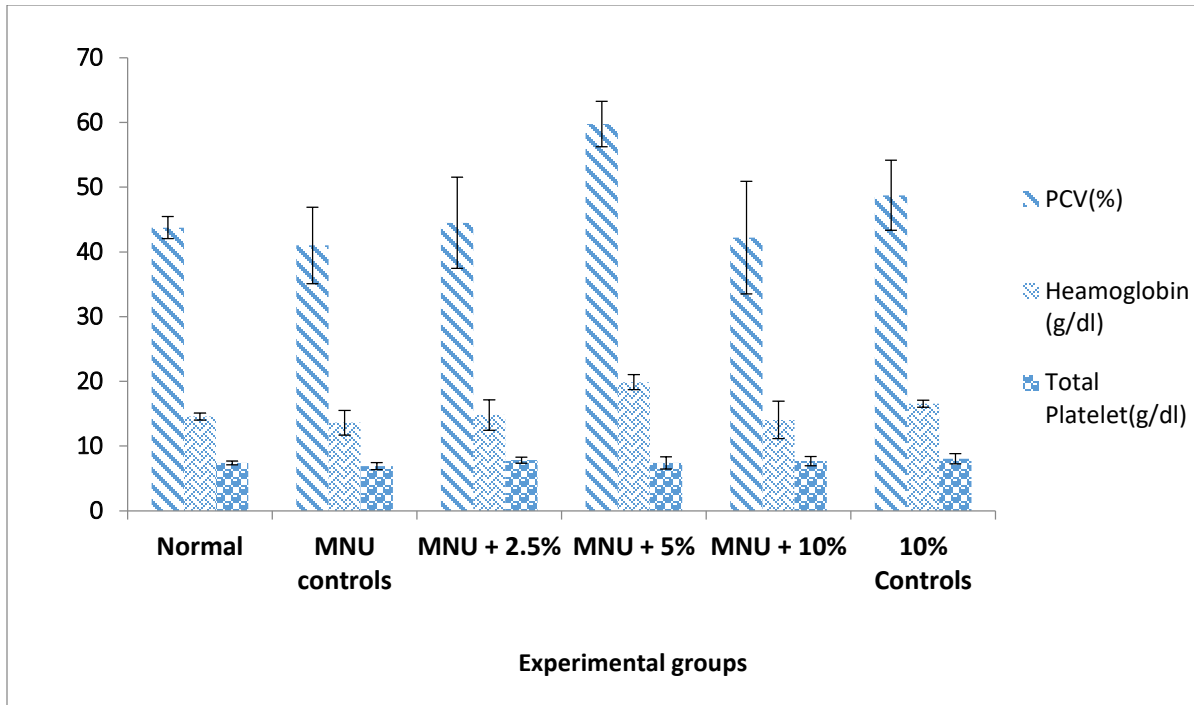


Figure 4: The Figure above presents results from the measurement of Hematological parameters in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each parameter in the different groups

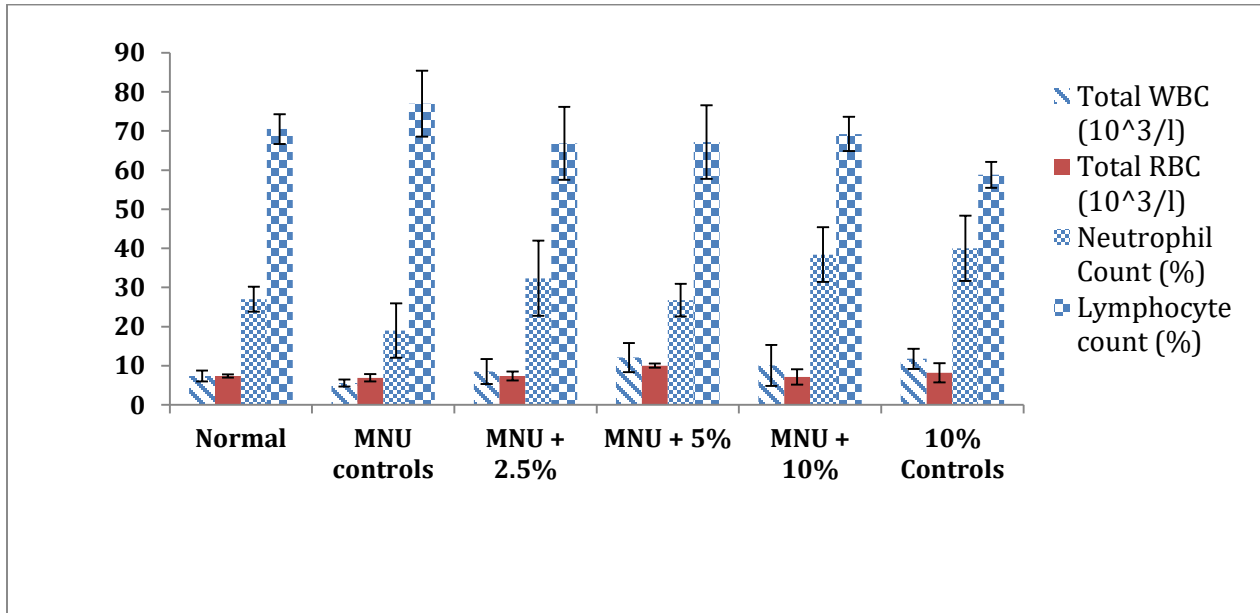


Figure 4b: The Figure above presents results from the measurement of Hematological parameters in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each parameter in the different groups.

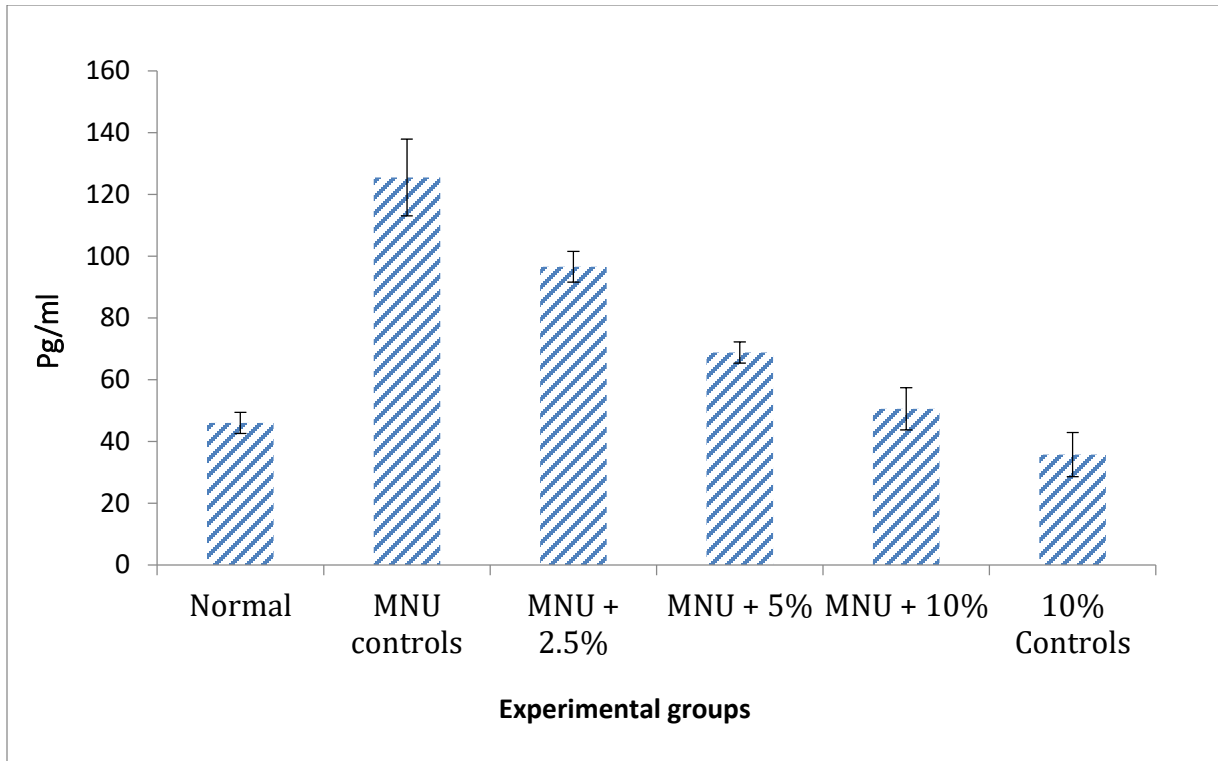


Figure 5: The Figure above presents results from the measurement of Carcinoembryonic antigen levels in Picograms/ ml in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each parameter in the different groups.

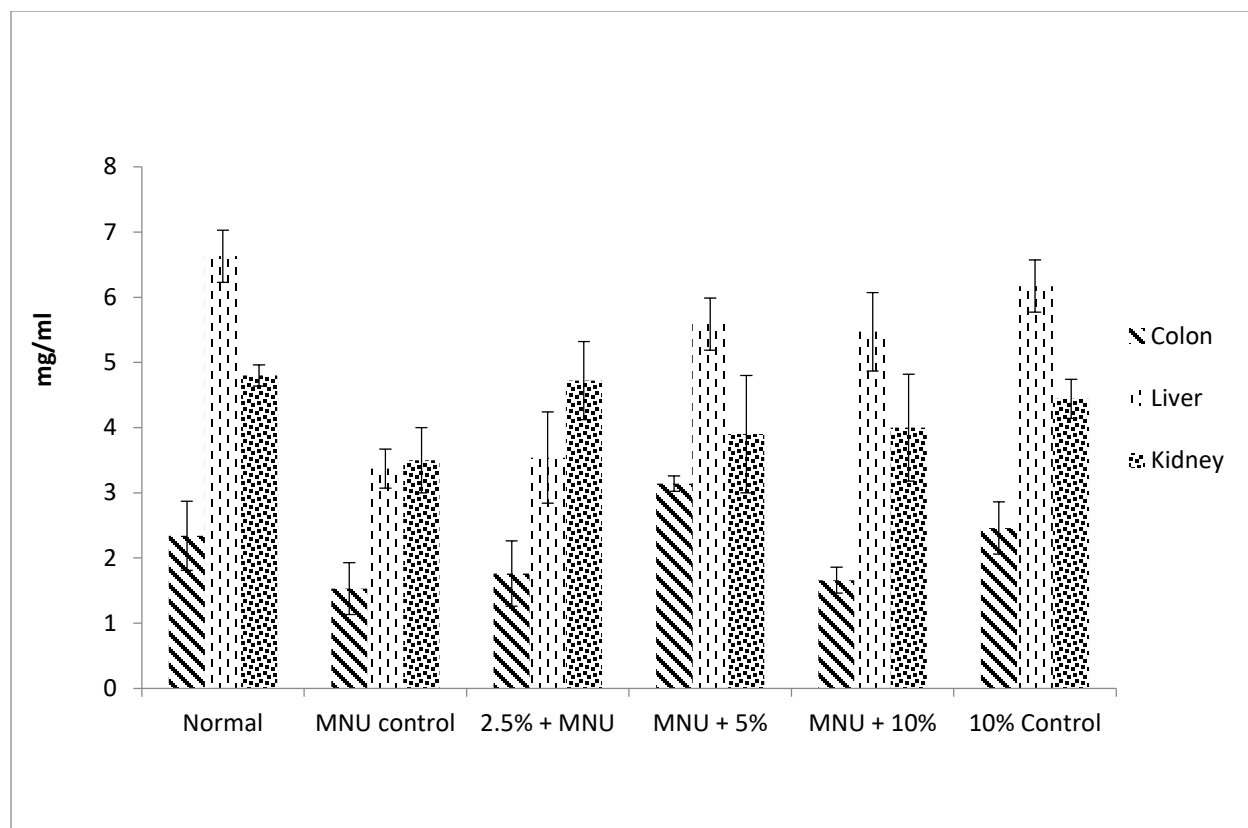


Figure 6: The Figure above presents results from the measurement of Total protein (mg/ml) in tissue in experimental groups in the form of a bar chart. Error bars represent standard deviations from the mean for each parameter in the different groups.

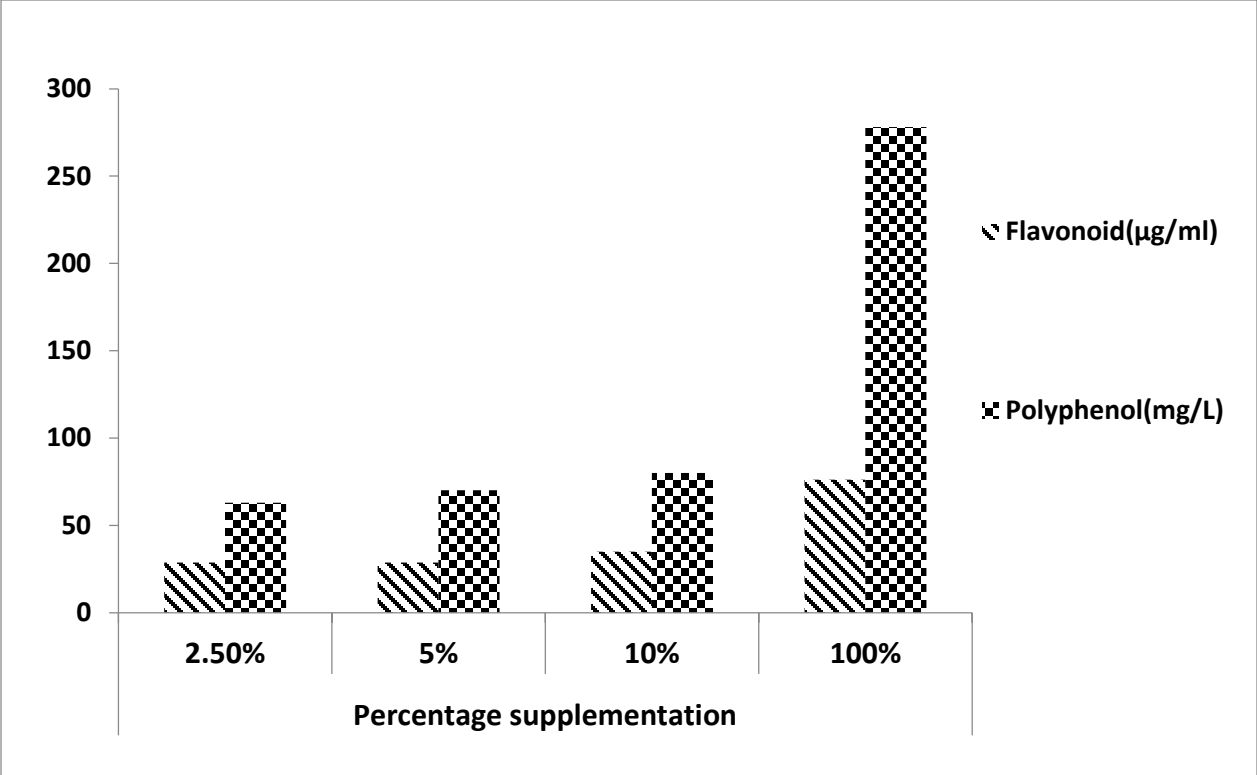


Figure 7: The figure above presents the amount of Flavonoid (Quercetin) and Polyphenol (Gallic acid) per percentage supplementation in each treatment group.