CHEMOPREVENTIVE EFFECT OF *GREWIA MOLLIS* AND *VITEX DONIANA* LEAF SUPPLEMENTATION ON N-METHYL N-NITROSOUREA INDUCED COLON CANCER IN RATS

BY

BAWALLA, IYABO BOLAJI

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY
ZARIA

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BY

BAWALLA, IYABO BOLAJI (B. Sc. Biochemistry (ABU) 2010)
MSc/SCIEN/1009/2011-2012

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE
REQUIREMENT FOR THE AWARD OF MASTERS DEGREE IN BIOCHEMISTRY

MARCH, 2015

DECLARATION

I thereby declare that the work in this thesis "**Chemopreventive effect of *Grewia mollis* and *Vitex doniana* Leaf Supplementation on N-methyl-N-nitrosourea Induced Colon Cancer in Rats**" was performed by me in the Department of Biochemistry, under the supervision of Prof. S.E Atawodi and Prof. I. A. Umar. The information derived from literature has been duly acknowledged in the text a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

Bawalla, Iyabo Bolaji

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MSc/SCIEN/1009/2011-2012	Signature	Date

CERTIFICATION

This project titled **"Chemopreventive effect of *Grewia mollis* and *Vitex doniana* Leaf Supplementation on N-methyl-N-nitrosourea Induced Colon Cancer in wistar Rats**" meets the regulation governing the award of the degree of Masters in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. S.E Atawodi, FAS ---------- ----------

Chairman, Supervisory Committee (Signature) (Date)

Pof I.A Umar ---------- ----------

Member, Supervisory Committee (Signature) (Date)

Pof I.A Umar ---------- ----------

Head of Department (Signature) (Date)

Prof. A.Z. Hassan ---------- ----------

Dean, School of Postgraduate Studies (Signature) (Date)

DEDICATION

This research work is dedicated to Almighty Allah and to the memory of my beloved father late Mr. Abdulganiyu Babatunde Bawalla; your legacy lives on.

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I will forever be grateful to my distinguished supervisor, Prof. S.E Atawodi. Sir may the Almighty God bless you beyond what you think, I can't thank you enough. My gratitude also to Mr. O.A Owolabi for his advice, support and word of wisdom throughout the course of this work, may happiness never cease in your home.

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Mutiat, Idris, Rafiat, Aminat, Jibril, Yakuq, Iliyas, and Hamidat, thank you all for your support. God bless you.

ABSTRACT

Chemopreventive effect of *G. mollis* and *V. doniana* leaf supplementation on N-methyl-N-nitrosourea induced colon cancer in rats was investigated following supplementation in the diet at 0, 2.5, 5 and 10% levels. N-mehtyl-N-nitrosourea was administered intrarectally at a dosage of 1% three times a week for 10 weeks. The carcinoembyronic antigen assay (CEA) showed that the MNU induced colon cancer control fed basal diet had a significant ($P < 0.05$) increased CEA levels compared to MNU induced colon cancer treated groups of the leaves of both *G. mollis* and *V. doniana* supplemented diets except for 2.5% *V. doniana* leaf supplemented diet. However, there was no significant ($P > 0.05$) difference in the CEA levels of the different proportions of *G. mollis* leaf supplemented diets compared to the normal control. The histopathology of the colon of the MNU induced colon cancer treated group showed increase glandular excretion (more globlet cells) of the mucosa and improved epithelial architecture with increasing levels of supplementation of both leaf of *G. mollis* and *V.doniana* compared to the MNU induced colon cancer control with less glandular excretion (more globlet cells) and a distorted epithelial architecture. The histology of the liver and kidney of the MNU induced colon cancer group showed intense congestion of the intertubular spaces and sinusoidal capillaries with intense necrosis compared to the leaf treated groups with moderate necrosis and presence of eosinophilic materials in the lumen of the kidney tubules. There is a significant ($P \square 0.05$) decrease in feed intake of the MNU induced colon cancer control group compared to animals fed the normal control feed (control feed). All the MNU induced colon cancer rats given *G. mollis* and *V. doniana* leaf supplemented diets had no significant change ($P \square 0.05$) in the feed intake compared to the MNU induced colon cancer control. Evaluation of haematological parameter in rats with MNU induced colon cancer showed that there was no significant ($P > 0.05$) change in the white blood cell count, red blood cell count, platelet and the mean corpuscular volume of the MNU induced colon cancer basal control rats compared to normal control of both dietary supplementation of the leaf of *G. mollis* and *V. doniana.* Thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) significantly ($P < 0.05$) increased in the liver and kidney of the untreated MNU induced control group while there was a significant ($P < 0.05$) reduction in the catalase (CAT) compared to the normal control. These results suggest that the leaf of *G. mollis* and *V. doniana* possess high cancer chemopreventive and antioxidant properties justifying their use in folklore medicine.

TABLE OF CONTENT

Title page	ii
Declaration	iii
Certification	iv
Dedication	v
Acknowledgement	vi
Abstract	viii
Table of content	ix
List of Tables	xiii
List of Figures	xiv
List of Plates	xv
List if Appendices	xvi
Abbreviation, Symbols and Glossaries	xviii
Chapter 1	
1.0 Introduction	1
1.1 Statement of Research Problem	4
1.2 Justification	5
1.3 Null hypothesis	6
1.4 General Aim	6
1.5 Specific Objectives	6
Chapter 2	
2.0 Literature Review	8
2.1 Colon cancer	8
2.1.1 Definition	8
2.1.2 Epidemiology	10
2.1.3 Genetics	11
2.2 Colon Cancer and Constituent of food	13
2.2.1 Colon cancer and calcium content	13

2.2.2 Colon cancer and Fiber content 14

2.3 *Grewia mollis* 15

2.3.1 Habitat of *Grewia mollis* 15

2.3.2 Classification of *Grewia mollis* 16

2.3.3 General Description of *Grewia mollis* 16

2.3.4 Nutritional and Medical Uses of *Grewia mollis* 18

2.3.5 Chemical Constituent of *Grewia mollis* 19

2.4 *Vitex doniana* 21

2.4.1 Habitat of *Vitex doniana* 21

2.4.2 Classification of *Vitex doniana* 21

2.4.3 General Description of *Vitex doniana* 22

2.4.4 Nutritional and Medicinal Usess of *Vitex doniana* 23

2.4.5 Chemical constituent of *Vitex doniana* 25

Chapter 3

3.0 Materials and Methods 27

3.1 Materials 27

3.1.1 Chemicals and Reagents 27

3.1.2 Equipment 27

3.1.3 Plant Materials 27

3.1.4 Experimental Diet Formulation 28

3.1.5 Animals 28

3.2 Methods 28

3.2.1 Induction of Colon Cancer 28

3.2.2 Experimental Design and Animal Groupings 28

3.2.3 Body weight and food intake measurement 29

3.2.4 Collection of Organs/Tissue 30

3.2.4.1 Collection and Preparation of Sera sample 30

3.2.4.2 Collection of Organs- Colon, Kidney and Liver 30

3.2.5 Estimation of Endogenous Antioxidant 31

3.2.5.1 Determination of Superoxide Dismutase activity 31

3.2.5.2 Catalase assay 32

3.2.5.3 Lipid peroxidation by measuring the malondialdehyde (MDA) level 33

3.2.6 Determination of Heamatological Parameters 35

3.2.7 Histopathological Analysis of the Tissues 35

3.2.8 Carcinoembryonic antigen assay 36

3.2.8 Statistical Analysis 38

Chapter 4

4.0 Results 39

4.1 Carcinoembryonic antigen assay 39

4.1.1 Levels of Carcinoembryonic antigen on MNU induced colon cancer rats supplemented with the leaf of *G. mollis* and *V. doniana* 39

4.2 Histopathological Studies 41

4.2.1 Effect of *G. mollis* and *V. doniana* leaf supplementation on the Colon of MNU induced Colon Cancer rats 41

4.2.2 Effect of *G. mollis* and *V. doniana* leaf supplementation on the Liver and Kidney of MNU induced Colon Cancer rats 44

4.3 Feed intake and body weight 49

4.3.1 Effect of *G. mollis* and *V. doniana* supplemented diet on Feed Intake of MNU Induced colon cancer rats**.** 49

4.3.2 Effect of *G. mollis* and *V. doniana* Supplementation on average weight gain and percentage change in weight 51

4.4 Heamatological Parameters 53

4.4.1 Effect of dietary supplementation with *G. mollis* and *V. doniana* leaf supplemented diets on Heamatological Parameter 53

4.5 *In vivo* Antioxidant Studies 56

4.5.1 Effect of dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid peroxidation and some Endogenous antioxidant enzymes on the Liver of MNU induced colon cancer in wistar rats 56

4.5.2 Effect of dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid peroxidation and some Endogenous antioxidant enzymes on the Kidney of MNU induced colon cancer in wistar rats 59

Chapter 5

5.0 Discussion 62

Chapter 6

6.0 Summary, Conclusion and Recommendation 66

6.1 Summary 66

6.2 Conclusion 67

6.3 Recommendations 67

References 68

Appendices 78

LIST OF TABLES

Table 4.1: Effect of *G. mollis* and *V. doniana* Supplementation on Average Weekly Weight Gain and Percentage change in weight 52

Table 4.2: Effect of Dietary Supplementation with *G. mollis* Leaf Supplementation on Haematological Parameters of MNU induced colon cancer rats 54

Table 4.3: Effect of Dietary Supplementation with *V. doniana* Leaf Supplementation on Haematological Parameters of MNU induced colon cancer rats 55

Table 4.4: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on some Endogenous Antioxidant Enzymes (SOD and CAT) on the Liver of MNU induced colon cancer wistar rats 58

Table 4.5: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on some Endogenous Antioxidant Enzymes (SOD and CAT) on the Kidney of MNU induced colon cancer wistar rats 61

LIST OF FIGURES

Figure 1.1: Diagram of the digestive system 9

Figure 4.1: Levels of carcinoembryoic antigen (CEA) in rats pre-treated with N-methyl-N-nitrosourea (MNU) given *G. mollis* and *V. doniana* leaf supplemented diets 40

Figure 4.2: Effect of diet supplementation with *G. mollis* and *V. doniana* on feed intake on MNU induced colon cancer rats 50

Figure 4.3: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid perioxidation on the liver of MNU induced colon cancer rats 57

Figure 4.4: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid perioxidation on the Kidney of MNU induced colon cancer rats 60

LIST OF PLATES

Plate 2.1: *Grewia mollis* plant 18

Plate 2.2: *Vitex doniana* plant 23

Plate 4.1a: Effect of *G. mollis* leaf supplementation on colon of MNU induced colon cancer in wister rats 42

Plate 4.1b: Effect of *V. donina* leaf supplementation on colon of MNU induced colon cancer in wister rats 43

Plate 4.2a: Effect of *G. mollis* leaf supplementation on liver of MNU induced colon cancer in wistar rats 45

Plate 4.2b: Effect of *V. doniana* leaf supplementation on liver of MNU induced colon cancer in wistar rats 46

Plate 4.3a: Effect of *G. mollis* leaf supplementation on kidney of MNU induced colon cancer in wistar rats 47

Plate 4.3b: Effect of *Vitex doniana* leaf supplementation on kidney of MNU induced colon cancer in wistar rats 48

LIST OF APPENDICES

Appendix 1.0: Total Protein Standard Curve 78

Appendix 2.0: Table of Carcinoembryoic antigen (CEA) in Rats Pre-Treated with N-methyl-N-nitrosourea (MNU) given *G. mollis* and *V. doniana* leaf supplemented diets 79

Appendix 3.0: Effect of Diet Supplementation with Leaves of *G. mollis* and *V. doniana* on feed intake on MNU induced colon cancer rats. 80

Appendix 4.0: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation on the liver of MNU induced colon cancer rats**.** 81

Appendix 5.0: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation on the kidney of MNU induced colon cancer rats. 82

Appendix 6.0: Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Pre-treatment of MNU induced colon cancer in wistar rats. 83

Appendix 7.0: Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Post-treatment of MNU induced colon cancer in wistar rats. 84

Appendix 8.0: Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Pre-treatment of MNU induced colon cancer in wistar rats. 85

Appendix 9.0: Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Pre-treatment of MNU induced colon cancer in wistar rats. 86

Appendix 10.0: Preparation of the reagents for the determination of Superoxide Dismutase (SOD) activity 87

Appendix 11.0: Preparation of the reagents for catalase assay 88

Appendix 12.0: Preparation of the Reagents for determination of lipid peroxiadation by measuring the malondialdehyde (MDA) level 89

ABBREVIATIONS AND GLOSSARIES

ACF: Aberrant Crypt foci

AOM: Azoxymethane

CAT: Catalase

CCl_4: Carbon tetrachloride

CEA: Carcinoembyronic antigen

CRC: Colorectal Cancer

DMH: 1, 2-dimethylhydrazine

DNA: Deoxyribonucleic acid

FAP: Familial Adenomatous polypsis

GAE: Gallic acid Equivalent

Hb: Haemoglobin

HCT: Heamatocrit

HNPCC: Hereditary nonpolysis Colon cancer

IARC: International Agency for Research on Cancer

MCH: Mean Corpuscular Haemoglobin

MCHC: Mean Corpuscular Haemoglobin Concentration

MCV: Mean Corpuscular Volume

MDA: Malondialdehyde

MNU: N-methyl-N-nitrosourea

NOD-SCID: Non-Obese Diabetic/Severe Combined Immunodeficiency

PLT: Platelet

RBC: Red Blood Cell

SOD: Superoxide dismutase

TBARS: Thiobarbituric Acid Reactive Substance

WBC: White Blood Cell

WHO: World Health Organisation

CHAPTER 1

INTRODUCTION

Cancer is classified as the second leading cause of death after cardiovascular diseases (Heron *et al.* 2009). Worldwide more than 20 million people are living with cancer, a disease estimated to kill 9 million people by 2015 (Darwiche *et al*., 2007). The most frequent cancers are lung, colorectal (CRC), stomach, liver, and breast. CRC is the most common gastrointestinal cancer and a leading cause of death in the world (Jemal *et al.*, 2011). Although surgical excision is the best option for treatment, many patients who undergo therapeutic resection will develop tumor recurrences.

Colorectal cancer (CRC) is one of the most common cancers worldwide, with the highest incidence rates in western countries (Jemal *et al*., 2011). It is estimated that most of the cases of CRC occur sporadically (70–80%), while approximately 15% of CRC cases develop as a result of inherited factors, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal carcinoma (HNPCC) (Souglakos, 2007). Changes in worldwide variations in the incidence rates, together with the results of migrant studies, show that sporadic human CRC may be attributable to various environmental and lifestyle factors, such as dietary habits, obesity, and physical inactivity (Johnson and Lund, 2007).

Colon cancer most often begins as clumps of precancerous cells (polyps) on the inside lining of the colon. Polyps can appear as mushroom-shaped and the growths can also be flat or recessed into the wall of the colon (nonpolypoid lesions). Removing polyps and nonpolypoid lesions before they become cancerous can prevent colon cancer.

The incidence of colon cancer varies widely from country to country throughout the world; colon cancer is a common disease in the United States. Adenocarcinoma of the colon and rectum accounted for approximately 35,000 cases and 55,000 deaths in 1999 (Landis *et al*., 1999). While the mean age of diagnosis is approximately 67 years of age, the incidence rises steadily from age 50 to age 80. Thus fewer than 10% of cancers are diagnosed before age 40.

Current data suggest that epidemiological risk factors for cancer, other than genetic risk factors, include dietary components, such as the amount of fat and fibre in the diet (Cummings and Southgate, 1999) and perhaps the intake of calcium (Bresalier, 1999; Faivre and Bonithon-Kopp, 1999; Mobarhan, 1999), vitamins of the antioxidant class (Slattery *et al*., 1999), and nonsteroidal anti-inflammatory agents such as aspirin and more specific inhibitors of cyclooxygenase (Lipsky, 1999). The risk of colon cancer increases with age, the history of previous polyps or cancer, the family history of cancer, and the history of long-standing inflammatory bowel disease, including ulcerative colitis and even Crohn disease.

There is cause for some optimism, however, as the advent of colonic endoscopy, our improved understanding of the adenoma-carcinoma continuum, and elegant cellular and molecular biologic techniques have made colon cancer now one of the best models for translating research into useful clinical practice. Improvements in fiberoptic technology and sound screening protocols have made elimination of colon cancer as a major cause of death a theoretic possibility. Efforts continue toward the development of more effective strategies aimed at early diagnosis and prevention.

Medicinal plants have been used by diverse cultures around the world and recognized as preventive with regard to the development of cancer (Lee *et al.,* 2006). In addition compelling evidence from epidemiological and experimental studies emphasizes the importance of compounds derived from plants to reduce the risk of cancer and inhibit the development and spread of tumors in experimental animals. Variety of studies have shown that colon cancer stems from nutritional traditions (Moon, *et al*., 1992) and many foods, beverages and their components affect the risk of developing colon cancer (Yamane *et al*., 1991; Steele *et al*., 1994; Wargovich *et al*., 1996; Weisberger, 1998).

Growing attention is being focused on food components with potential cancer inhibiting effects, in the hope of identifying chemopreventive diets or dietary supplements for human use. Among potentially chemopreventive food components are polyphenols and flavonoids, characterized by hydroxylated aromatic rings that are ubiquitously present in foods of plant origin, have long been recognized to possess anticarcinogenic properties (Mutoh, 2000). Polyphenols present in food have been demonstrated to decrease various types of experimental carcinogenesis. In recent years, identification of effective chemopreventive polyphenols in diets or dietary supplements for human use is of much interest. Treatments with such polyphenols result in cell cycle arrest (Lepley *et al*., 1996), thereby reducing the growth and proliferation of cancerous cells through apoptosis or programmed cell death (Hall, 1994).

Recent research by Shagal *et al.* (2012) showed that the leaf of *Grewia mollis* contains several phytochemicals such as tannin, phenols, flavonoid, glycoside, saponin etc. Thus it can be use as a food supplement for the management/prevention of colon cancer. Various parts of the plant are used in food and medicine. In Nigeria, the stem bark powder or mucilage is used as a thickener in local cakes made from beans or corn flour

commonly called “Kosai” and “Punkasau” in Hausa (Nigeria), respectively. The dried stem bark is ground and the powder mixed with beans or corn flour thereby enhancing the texture of the food product. Some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations (Martins *et al.,* 2008; Muazu *et al.,* 2009). In addition, the mucilaginous property of the bark is used traditionally by the Yoruba people of Nigeria at child birth.

Vitex doniana is widely distributed in Nigeria and many parts of the world. Chemical constituents of the plant include glycosides, flavonoids, alkaloids, essential fatty acid (Arokiyaraij *et al*., 2009). In ethnomedicine, *V. doniana* is employed in the treatment of a variety of diseases. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea and dysentery (Irvine, 1961; Etta, 1984). The roots and leaves are used for nausea, colic and in epilepsy ((Bouquet *et al*., 1971; Iwu, 1993). In eastern parts of Nigeria, the young leaves are used as vegetable for sauces and porridge for meals indicating that the leaves can be used as supplements in food for the management/prevention of colon cancer.

However, this work attempts to compare the chemopreventive effect of *G. mollis* and *V. doniana* supplement diet on colon cancer since the fresh leaves are commonly consumed by human.

1.1 STATEMENT OF RESEARCH PROBLEM

Colorectal cancers are the third most frequent cancers in the world and second leading cause of death in United States, affecting both sexes (Kinzler *et al*., 1996) with over 1.2 million new cases and 608,700 deaths estimated to have occurred in 2008 (Jemal *et al*., 2011) and it accounts for 10-50% of all gastro-intestinal tract malignancies in Nigeria.

Although chemotherapy and radiotherapy have been applied as the surgical adjuvant treatments of colon cancer, they vary in success rates for local recurrence, disease-free survival, and overall survival (Martenson *et al.*, 2004). In addition, these treatments may present some side effects such as an increased risk for infections, hair loss, fatigue, lip sores, nausea, vomiting, diarrhea, and bloody stools. Postulated mechanisms include reduction in the activity of several cancer causing bacteria, desmutagenic and anticarcinogenic properties (Collins and Gibson, 1999; Chau and Cunningham, 2006).The major problems of colon cancer treatment are the unpredictable post operative survival rate and the reoccurrence. These are besides the complications that accompany resection and the damages caused by radiotherapy in addition to the toxicity challenge of the adjuvant chemotherapy (Boni *et al*., 2007).

Besides the risks involved in the orthodox treatment of colon cancer, the cost is quite exorbitant (Ladabaum *et al*., 2003) and unaffordable to most poor patients, a reason many patients in developing countries prefer to die in silence or allow their cases to be handled by quacks who give them analgesics for cancer treatment or associate the disease as spiritual. Additionally, most of the screening facilities for early diagnosis of the disease are not available in these countries.

1.2 JUSTIFICATION

Presently the use of orthodox medicine in the management of colon cancer is expensive and out of reach for most Nigerians in addition to the low post-operative survival rate. Hence there is need to search for a cheaper alternative intervention which when supplemented with diet will prevent or reduce the risk of development of colon cancer.

Recent urbanization/civilization has resulted in upsurge of confectionary food outlets in major cities resulting in many Nigerians changing their dietary habit from a fibre rich

diet, which was common practice to a highly refined carbohydrate and fat diet which increases the risk of colon cancer. Diet interventions and natural bioactive supplements have now been extensively studied to reduce the risks of colon cancer, as a means of prevention instead of cure. The addition of *G. mollis* and *V. doniana* to these refined diets will go a long way in preventing/managing the risk of developing colon cancer.

V. doniana and *G. mollis* are widely distributed in most part of the Nigeria giving individuals assess to them when necessary, thus fortification/ supplementation against colon cancer can become a routine as individuals can include these vegetables in their meals.

1.3 NULL HYPOTHESIS

Dietary supplementation with the leaf *G. mollis* and *V. doniana* does not prevent colon cancer.

1.4 GENERAL AIM

The aim of this work is to compare the effects of dietary supplementation with *G. mollis* and *V. doniana* leaf on N-methyl-N-nitrosourea (MNU) induced colon cancer in Wistar rats.

1.5 SPECIFIC OBJECTIVES

i. To compare the histopathologically potential of *Grewia mollis* and *Vitex doniana* leaf supplemented diet to chemoprevent colon cancer.

ii. To determine the effect of *Grewia mollis* and *Vitex doniana* leaf supplemented diets on feed intake, body weight and haematological parameters of rats with N-methyl –N- nitrosourea induced colon cancer.

iii. To establish the possible relationship between the antioxidant effect of *Grewia mollis* and *Vitex doniana* leaf supplemented diet in colon cancer chemopreventive capacity.

CHAPTER 2

LITERATURE REVIEW

2.1 COLON CANCER

2.1.1 Definition

Colon cancer is a disease of the large intestine which begins at a structure called the caecum, located in the right lower quadrant of the abdomen, and continues through all portions of the abdomen to its junction with the rectum, located in the deep pelvis (Yeatman, 2001). The colon is an organ shaped like a question mark. It is approximately 1.5 metres long and is divided into four segments based on the vascular supply to each segment. The right colon, otherwise known as the ascending colon, receives its blood supply from the right colic and the ileocolic artery. The most proximal portion of the right colon is termed the caecum and is identified by the appendix, which a small appendage attached to three longitudinal bands of muscle is called the taeniae coli. The transverse colon connects the right colon to the left colon. Its blood supply is derived from the middle colic artery and, like the right colic artery; it is derived from the superior mesenteric artery, a branch of the aorta. The juncture at which the right and transverse colon join it's termed the hepatic flexure and the juncture at which the transverse colon and left colon join is termed the splenic flexure. Flexures are points at which the large bowel is tethered and supported in order to prevent excessive twisting that could lead to a condition called volvulus. Volvulus is a serious problem that can result in diminished blood supply to the bowel, with subsequent necrosis and perforation. The transverse colon also serves as a tethering point for the greater omentum, which is a large fatty apron of tissue that essentially covers the entire abdomen, separating the small and large bowel from the anterior abdominal wall (Yeatman, 2001). The purpose of the omentum is to 'police' the abdomen, identifying

holes or perforations of the bowel wall and ceiling them. In addition, it seems to be an adhesive target for neoplastic cells, which otherwise float free in the abdomen. The left colon connects the transverse colon to the sigmoid colon, which is named for its S-shaped appearance in the abdomen. The left colon and sigmoid colon are supplied by the left colic and sigmoidal arteries, branches of the inferior mesenteric artery. While the wall of the right colon generally has the largest diameter, the sigmoid colon frequently has a narrow diameter and a thicker wall. It is often the site of formation of diverticula, which are small herniations through the bowel wall and can be sites for faecal impaction, abscess formation and perforation. The colon proper ends at the junction of the sigmoid colon with the rectum, which is marked by its disappearance into the deep pelvis and is covered by visceral peritoneum and perirectal (mesorectal) fat (American Cancer Society: Colorectal cancer, 2011).

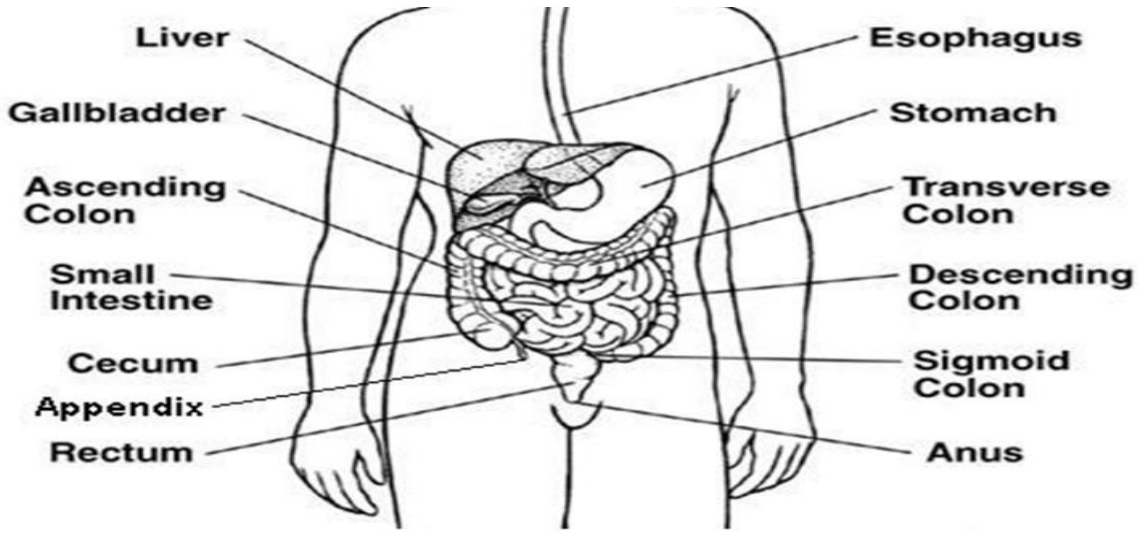


Figure 2.1: Diagram showing the digestive system (American Cancer Society: Colorectal cancer, 2011)

Colon cancer is a mucosal disease. In other words, all colon cancers are derived from the mucosal lining of the bowel wall. From the inside out, the bowel wall is composed of multiple layers, which include the mucosa, the submucosa, the muscularis propria (containing circular and smooth muscle layers) and the serosa. The innermost layer of the bowel wall, the mucosa, is a single layer of columnar epithelial cells, some of which produce large amounts of mucus and are thus termed goblet cells. This is the site of the earliest genetic changes that lead to the development of cancer cells. It is a region where normally cells are continuously dividing to replenish those that are shed from the bowel wall into the lumen (Yeatman, 2001).

Underneath this mucosal layer lies the submucosa, the strength layer of the bowel. This layer contains blood vessels, lymphatics and terminal nerve fibres. It is an important layer with regards to the genesis of cancer because once a tumour has invaded into this region of the bowel wall it can gain entrance to the blood supply and lymphatic system, permitting distant spread throughout the body. Thus, the earliest stage of colon cancer, termed Dukes' stage A, represents a stage at which cancer is limited to the submucosa and has not spread to lymph nodes or distant organ sites. The likelihood of survival following resection of these early stage cancers is very high. Dukes' B tumours have invaded into and through the muscular layer of the bowel wall and thus carry an increased risk of distant spread; Dukes' C tumours have already spread to regional lymph nodes (Yeatman, 2001).

2.1.2 Epidemiology

While the incidence of colon cancer varies widely from country to country throughout the world, colon cancer is a common disease in the United States (Yeatman, 2001).

The majority of colon cancers are not inherited but rather are considered sporadic, having developed from an accumulation of mutations throughout the course of a lifetime. Approximately 10% of colon cancers are considered inherited: a genetic mutation in genomic deoxyribonucleic acid (DNA) (involving all cells in the body) has been passed on from one generation to another. In addition to inherited cancers, familial cancer predispositions may exist that are independent of the hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis (FAP) diseases. To date, these predisposing factors are not well defined.

Current data suggest that epidemiological risk factors for cancer, other than genetic risk factors, include dietary components, such as the amount of fat and fibre in the diet (Cummings and Southgate, 1999) and perhaps the intake of calcium (Bresalier, 1999; Faivre and Bonithon-Kopp, 1999; Mobarhan, 1999), vitamins of the antioxidant class (Slattery *et al*., 1999), and nonsteroidal anti-inflammatory agents such as aspirin and more specific inhibitors of cyclooxygenase (Lipsky, 1999).

2.1.3 Genetics

Whereas cancer is a genetic disease, only 10% of colon cancers are actually inherited from family members. In fact, the majority of colon cancers are considered sporadic in nature and are derived from accumulated genetic changes. These changes occur within the epithelial cells of the mucosal surface of the bowel wall throughout the lifespan of the person. Once a critical number of genetic changes has occurred (usually 5–6), a cancer may develop. A genetic model for the development of colon cancer has recently evolved (Vogelstein and Kinzler, 1993); this is largely due to extensive gains in the understanding of human genetics, as a direct result of the human genome project.

Current understanding suggests that colon cancer is a disease of disrupted growth control. Early genetic alterations occur in the colonic epithelial cells lining the bowel wall. The genetic changes involve mutations or alterations in the genetic code responsible for producing specific proteins. These mutational alterations or deletions result in the production of malformed proteins, which are absent or can no longer function normally. As a result, cells may be 'turned on' to grow without limits and may resist normal mechanisms that result in cell death, a process of cellular suicide called apoptosis.

There basically two types of important growth genes that are affected in cancer, oncogenes and tumour suppressor genes. Oncogenes represent the mutated forms of pre-existing normal genes, called protooncogenes, which have normal growth-stimulatory functions in the cell. Only when these genes are mutated in specific regions that they can take on constitutively active (always turned on) growth functions. Tumour suppressor genes are the second class of genes that are affected in the genesis of colon cancer. While the specific function and identity of many of these genes in this class are not yet identified, it is clear that specific genes are deleted in the development of cancers and that their absence results directly in tumour formation. In the normal cell, protooncogenes provide growth stimulus and tumour suppressor genes provide growth control. In the tumour cell, mutated protooncogenes and mutated or deleted tumour suppressor genes permit continuous unchecked growth stimuli (Yeatman, 2001).

2.2 COLON CANCER AND CONSTITUENTS OF FOOD

2.2.1 Colon Cancer and Calcium Content

Calcium is a mineral that has been intensively investigated in terms of its role in the prevention of colorectal cancer. There are a fair amount of plausible biological mechanisms (indirect and direct) explaining the physiological role of calcium. The indirect pathway is expected be the effect of the formation of insoluble bile acid complexes (Govers *et al*., 1994), thus decreasing the proliferative role of secondary bile acids (Welberg *et al*., 1993). The direct pathway is associated with the role of calcium played at the cellular level, and this pathway is also related to the action of vitamin D, as the effect of the intracellular concentration of calcium, activation of calmodulin, phosphorylation of some cellular enzymes and activation of signaling pathways leading to cell differentiation or apoptosis (Lamprecht and Lipkin, 2003).

Despite the presence of a relatively good body of evidence from basic research (meaning an experimental or theoretical work undertaken primarily to acquire new knowledge of the underlying foundations of phenomena and observable facts, without any particular application or use in view), human studies have not shown consistent results. Majority studies performed before the year 2000 failed to confirm the presence of a clear beneficial effect of calcium (Bergsma-Kadjik *et al*., 1996; Washington, 1997; Martinez and Willett, 1998; Kampman *et al*., 2000). There have also been studies on the use of calcium supplements, showing an up to 34% reduction in a rate of colorectal adenoma recurrence among patients with a history of adenomas (Hofstad *et al*., 1998; Baron *et al*., 1999; Bonithon-Kopp *et al*., 2000). The results of most recent studies are also inconsistent (Wactawski-Wende *et al*., 2006; Huncharek *et al*., 2009). However, there is increasing evidence suggesting that calcium plays a prophylactic role

(Washington, 2007; Ruder *et al*., 2011; Lofano *et al*., 2013). There are some possible explanations for the lack of an observed benefit from calcium on the development of colorectal cancer. One is the effect of dose, as some of the investigations evaluated the risk across relatively low dosages (Lin *et al*., 2005); some others were not designed for that purpose (Wactawski-Wende *et al*., 2006); some had relatively small sample sizes (Bonithon-Kopp *et al.,* 2000); and finally, calcium - especially dietary calcium is one of several dietary components, and analyses performed probably did not enable researchers to isolate the effects of calcium from the role of some other dietary nutrients.

Recent research by Galas *et al.* (2013) show that an increase of dietary calcium intake by 100 mg/day was associated with 5% decrease of colorectal cancer risk while higher dietary calcium intake (100mg/day) was associated with 30% decrease in colorectal cancer risk. Calcium intake increases the formation of insoluble calcium-phosphate-bile acid complexes in feces, decreases the concentration of soluble fatty acids and decreases lytic activity of fecal water (Van der Meer *et al*., 1991). Calcium is also suspected to promote differentiation and restraining growth of colonic cells through intracellular release of calcium, calmodulin activation and phosphorylation of intracellular enzymes (Lamprecht and Lipkin, 2001). Also it was found that calcium may act through the activation of calcium-sensing receptors present on the luminal surface of intestinal epithelial cells (Saidak *et al*., 2009). Their activation can influence cell differentiation (Whitfield, 2009).

2.2.2 COLON CANCER AND FIBER CONTENT

Currently, there is no accepted international definition of dietary fibre. The basic accepted definition proposed by Trowell and associates in 1976 (Trowell *et al*., 1976)

stated that dietary fibre is composed of the remnants of plant cells resistant to hydrolysis by human alimentary enzymes and that it includes all indigestible polysaccharides (celluloses, hemicelluloses, oligosaccharides, pectins, gums, waxes) and lignin. Dietary fibre is classified as soluble (ie. pectin, agar) or insoluble (cellulose, heteroxylans and lignified cell walls (wheat bran)). Evidence suggests that soluble fibres are less likely to protect against cancer than insoluble dietary fibres (Ferguson and Harris, 1996). The fraction of starch that escapes digestion in the small intestines is called resistant starch. It is not included in the classic definition of dietary fibre, yet has been proposed to function as dietary fibre (Prosky, 2000). Direct and indirect mechanisms have been proposed on how dietary fibres are protective against CRC (Ferguson and Harris, 1996; Lupton and Turner, 1999; Sowa and Sakai, 2000). Direct mechanisms include the reduced exposure of the colonic mucosal cells to carcinogens or tumours promoters due to their absorption, dilution or shortened transit time that results from increased fibre. Indirect mechanisms include the products of degradation of the fibre by bacterial enzymes in the colon. These products such as butyrate may reduce the activity of tumour promoters. There are some conflicting results, but overall animal and human experimental studies support the potential protective effects of dietary fibre (Alberts *et al*., 1996; Kritchevsky 1999; Reddy 1999). The source of fibre also plays an important role. Wheat bran consistently had the most protective effects in the experimental animal and human studies, compared with pectin, oat or corn bran.

2.3 *Grewia mollis*

2.3.1 Habitat of *Grewia mollis*

Grewia mollis occurs widely in tropical Africa, from Senegal and Gambia eastward to Somali and southward to Angola, Zambia and Zimbabwe. In Nigeria, the shrub or small

plant is widely distributed in northern part of the country. It grows in area with annual average rainfall of 600- 1400 mm, from sea level in West Africa up to 2200 m altitude in East Africa. It grows on a range of soil types and is highly resistant to fire. *Grewia mollis* is often gregarious, with branch-suckering leading to the formation of thickets.

2.3.2 Classification of *Grewia mollis*

The taxonomic system of Agiosperm Phylogeny Group classification (APG III) was used

Kingdom (Regnum): Plantae

Phylum (Cladus): Angiosperms

Sub-phylum (Cladus): Eudicots

Class (Cladus): Core eudicots

Sub-class (Cladus): Rosids

Sub order (Cladus): Eurosids II

Order (Ordo): Malvales

Family (Familia): Malvaceae

Sub family (Sub familia): Grewioideae

Genus: *Grewia*

Species: *mollis* Juss

Other names: Dargaza (Hausa), Ora-igbo (Yoruba)

2.3.3 General Description of *Grewia mollis*

Grewia mollis a shrub or small tree up to 10.5m tall often multi- stemmed (the stem diameter is up to 30cm). The young branches are densely stellate-pubescent, turning

dark gray to purple with age. The outer bark is black, thick, rough, flaking and deeply fissured and the inner bark is yellowish to brown and fibrous.

Leaves alternate and are simple; stipules lanceolate and are 5-10 mm long, slightly hairy and caduceus (Plate 2.1). Petiole are 4-13 mm long, grey to reddish brown pubescent; blade are elliptical to elliptical-oblong, base cunate or broadly rounded or obliquely truncate, apex are acute to slightly acuminate, margin are toothed with 3-veined from the base, glabrous to sparsely minutely stellate- pubescent above and densely greyish to browish white pubescent beneath (Louppe *et al*., 2008).

The flowers are yellow and bisexual; sepals 6-10 mm long; petals obviate to oblong, 4-6 mm long, ± 2 mm wide, and sometimes notched at apex. The ovary is 1.5 -2 mm long and densely hair (Louppe *et al*., 2008)

Fruits are globe drupe 4-8 mm by 5-8 mm with fine whitish hair, yellow turning black, the endocarp are hard, woody and rugose. The fruits are green when younger and turning yellow when older (Whitehouse *et al*., 2001; Louppe *et al*., 2008)



Plate 2.1: *Grewia mollis* plant (Adapted from http://www.prota4u.org)

2.3.4 Nutritional and Medicinal Uses of *Grewia mollis*

The different parts of *Greewia mollis* have been used for different purposes across the regions and places where the plant grows/cultivated. In the Democratic Republic of Congo, the bark is kneaded with water into a viscous substance that is added to sauce while in Gabon the inner bark is sometimes eaten as food. In Nigeria, the inner stem bark and leaves of *G. mollis* are used as thickener(mucilaginous) and commonly used in soup; dried and ground then mixed with bean-meal or corn flour to make cakes called "Kosai" and "Punkasau" in Hausa (northern Nigeria) respectively (Gill, 1992; Obidah *et al*., 2010).

The flower, buds and young shoots are added to soup and sauces as garnishing while in Sudan, the young leaves are cooked and eaten as vegetables. The fruits are eaten raw or boiled (Louppe *et al*., 2008). The ash from the wood, leaves, stem and roots are used as salt substitute (Louppe *et al*., 2008).

Herbs humans ascribe varied medicinal functions to different parts of the plant. Mucilages from the stem bark and leaves are applied to ulcers, cuts, sores, and snake bites and also drink orally for constipation (Louppe *et al*., 2008; Obidah *et al*., 2010). A decoction of the root and stem bark are administered orally for the treatment of cough, diarrhoea, anal prolapsed, rheumatism and palpitation (Louppe *et al*., 2008; Obidah *et al*., 2010; Idika and Niemogha, 2008) while extract from the stem bark and leaves are used for the treatment of fever and rickets. Women drink the macerated decoction of the leaves and stem bark during child birth to reduce the pains of the process (Louppe *et al*., 2008; Obidah *et al*., 2010). The sap squeezed out of the root shavings are applied under the eyelid for the treatment of sores in the eyes (Louppe *et al*., 2008).

2.3.5 Chemical Constituent of *Grewia mollis*

According to Onwuliri *et al*. (2006), the ethanolic extract of the stem bark of *Grewia mollis* contains tannins, saponins, flavonoids, glycosides, balsam, phenols, terpenes, and steroids but alkaloids were absent. The extract exhibited toxic properties at the lethal dose (LD_{50}) of 1500 mg/kg body weight. The convoluted tubules of the kidney but no structural effects on the liver and heart were observed suggesting that the extract may be safe in humans but should be used with caution on patients with renal failure.

Studies by Obidah *et al*. (2010) on the toxic effects of the stem bark of *Grewia mollis* showed that the addition of the pulverized stem bark to the normal diet of male Wister rats at concentrations of 0, 1, 5, and 10% given to them as feed for 4 weeks, resulted in no deaths, and remarkable changes in appearance were not observed in the treated animals. However, rats fed with 10% dietary level showed significant increases in serum transaminases activities which were accompanied by decreased food intake. There was no observed effect on serum alkaline phosphatase activity, urea, creatinine, triglycerides, cholesterol, glucose concentrations, and body and organ weights in their study. These workers concluded that dietary exposure of rats to *Grewia mollis* stem bark powder at high concentrations (10%) may cause some adverse effects, especially liver injury.

Asuku *et al.* (2012) evaluated the methanolic extract of *Grewia mollis* leaves for its antioxidant and hepatoprotective properties by an *in vivo* procedure. They reported significant hepatoprotective potential evidenced by the lowering of serum levels of bilirubin, aspartate aminotransferase, and alanine aminotransferase and decreasing of malondialdehyde levels in rats pretreated or posttreated with carbon tetrachloride (CCl_4). They concluded that *Grewia mollis* leaves contain potent antioxidant compounds that could offer protection against hepatotoxicity and ameliorate preexisting liver damage and oxidative stress conditions.

Saleem *et al*. (2012) assessed the nutritive value of the leaves and fruits of three grewia species under semiarid environment, and the results of the study indicated that the three species could be introduced as a source of fodder in animal production farms and silvopastoral systems.

2.4 *Vitex doniana*

2.4.1 Habitat of *Vitex doniana*

Vitex doniana occurs in a variety of habitat from forest to savanna, often in wet localises and along rivers, and on termite mounds, up to 2000 m altitude. It occurs in regions with a mean annual rainfall of 750-2000 mm. It is the most commonly found on alluvial soil. In Central Africa it is often the first species to establish when gallery forests evolve in low-lying areas in the savanna. In Nigeria, it is found in the middle belt areas particularly Kogi, Benue, and parts of savanna regions of Kaduna, Sokoto and Kano states (Etta, 1984)

2.4.2 Classification of *Vitex doniana*

The taxonomic system of Agiosperm Phylogeny Group classification (APG III) was used

Kingdom (Regnum): Plantae

Phylum (Cladus): Angiosperms

Sub-phylum (Cladus): Eudicots

Class (Cladus): Core eudicots

Sub-class (Cladus): Rosids

Sub order (Cladus): Eurosids

Order (Ordo): Lamiales

Family (Familia): Verbenaceae

Genus: *Vitex*

Species: *doniana*

Authority: Sweet

Name: *Vitex doniana* Sweet

Other names: Dinya (Hausa), Ori-nla (Yoruba), Ucha-koro (Igbo)

2.4.3 General Description of *Vitex doniana*

Vitex doniana is a deciduous small to medium- sized tree that grows up to 25m tall with a heavy rounded crown. The bark is rough, pale brown or light grey with numerous vertical fissures. Branchlets are not hairy (Plate 2.2). Leaves are opposite, glabrous, 14-34 cm long, usually with 5-leaflets on stalks 6-14 cm long. Leaflets distinctly stalked ovate, obovate to elliptical, 8-22 cm long, 2-9 cm wide. Leaf tips are rounded or emarginated, leaf bases cuneate, dark green above, pale greyish- green below, thickly leathery, with a few scattered stellates hairs on the upper surface, otherwise without hairs.

Flowers are petals white except on large lobe, which are purple, in dense opposite and axillary cymes. Flowers are small, blue or violet, 3-12 cm in diameter, only a few being open at a time. Fruits are obovoid to oblong-ellipsoid drupe 2-3 cm long, green when young, turning purplish-black on ripening and with starchy black pulp. Each fruit contains 1 hard, conical seed that is 1.5-2 cm long and 1-1.2 cm wide (Burkill, 2000).



Plate 2.2: *Vitex doniana* plant

2.4.4 Nutritional and Medicinal Uses of *Vitex doniana*

Young leaves of *Vitex doniana* are cooked and eaten as vegetable or in sauces. The blackish pulp of the fruits is edible and sweet, and eaten raw (taste like prunes). It is nutritious, as it has high lipid content (Glew *et al*., 1997), and the pulp contains vitamin C, vitamin A and protein are also present in the fruits (Iwu, 1986). Beverage can be made from the fruit juice, and boiled fruits are the basis for alcoholic liquor and wine. It has been reported that syrup similar to honey was produced from the fruit and that

physicochemical and sensory results showed that it can be substitute for other syrup as a nutritive sweetener (Egbekun *et al*., 1996).

Vitex doniana has numerous applications in traditional medicine. The leaf sap is used as an eye drop to treat conjunctivitis and other eye complaints. Leaf decoction is applied externally as a galactogogue and against headache, stiffness, measles, rash, fever, chickenpox and hemiplagia, and internally as a tonic, anodyne and febrifuge, and to treat respiratory diseases. Pastes of pounded leaves and bark are applied to wounds and burns. Leaf infusions are added to alcoholic drinks to make them stronger. A root decoction is administered orally to treat ankylostomasis, rachitis, gastro-intestinal disorder and jaundice. The powdered bark added to water is taken to treat colic, and the bark extract are used to treat stomach complaints and kidney troubles (Adejumo *et al*., 2013).

Dried and fresh fruits are eaten against diarrhoea and as remedy against lack of vitamin A and B. The twigs are used as chewing sticks for teeth cleaning. The stem bark is given to cattle to treat diarrhoea, dysentery and liver problems. Report has showed the use of the stem bark of *Vitex doniana* to control postpartum bleeding after child birth (Ladeji *et al*., 2005) due to its high potassium and phosphorus content. Other reported uses of the tree include its stem bark extract for the control of hypertension and its anti-hepatotoxic effect and treatment of stomach ache, pains, disorders and indigestion (Ladeji and Okoye, 1996; Ladeji *et al*., 1996).

The anti- hypertensive (Olusola *et al*., 1997) and antidiabetic (Owolabi *et al*., 2011) effects of the stem bark have also been reported. Extract of stem bark of *Vitex doniana*

have also demonstrated some level of *in vitro* trypanocidal activity against *Trypanosome brucei brucei* (Atawodi, 2005).

2.4.5 Chemical Constituent of *Vitex doniana*

The value for the proximate composition of *Vitex doniana* leaves were: moisture content 77.03%, ash content 1.65%, fat 2.9%, fiber 2.75%, protein 8.10% and carbohydrate 7.57% (Adejumo *et al*., 2013). *Vitex doniana* contains vitamins, macro and micro nutrients in different proportion. Among the anti nutritive factors found in the young leaves of *Vitex doniana* include tannis, saponin, alkaloids and trace of cardiac glycoside (Adejumo *et al*., 2013) which can be controlled by boiling. It can therefore be concluded that the young leaf is highly rich in nutrients and contains the nutrients level that fall within other popular edible vegetables.

Mustapha *et al.* (2012) evaluated the effect of ethanolic extract of *Vitex doniana* stem bark on peripheral and central nervous system of laboratory animals. The result showed that the extract has significant local anesthetic effect when compared to xylocaine. Antinociceptive activity of the ethanolic extract was evaluated using acetic acid induced pain and heat. The extract demonstrated significant antinociceptive activities dose dependently when compared to control, the extract also increased the sleeping time together with the pentobarbitone from 72.3 ± 3.07 at a doses (100 mg/kg of extract and 35 mg/kg of the pentobarbitone) to 181 ± 0.35 at a dose of 400mg/kg and 35 mg/kg), respectively. Thus this plant could be a good source of psychotherapic agent.

Kadejo *et al.* (2013) evaluated the in *vivo* antioxidant effect of aqueous root bark, stem bark and leaves of *Vitex doniana* in carbon tetrachloride induced liver damage and non

induced liver damaged albino rats. The study showed that there was a significant ($P > 0.05$) increase in TBARS and a significant ($P > 0.05$) decrease in the SOD and CAT of the liver of the CCl_4 induced not treated groups. However, there was no significant ($P > 0.05$) difference between the TBARS, SOD, and CAT in the liver of the induced treated groups and the normal control. In the kidney, the study showed that there was no significant ($P > 0.05$) difference in TBARS level between the normal and the induced groups. They therefore concluded that application of *Vitex doniana* plant would play an important role in increasing the antioxidant effect and reducing the oxidative damage in that formed both in the liver and kidney.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Reagents

All chemicals were of analytical grades. Required chemicals include N-methyl-N-nitrosourea (MNU) procured from Sigma Chemical Coy and Rat Carcinoembryoic antigen assay kit purchased from Wkea Med Supplies Corp. China.

Others are formal-saline, graded alcohol, xylene, paraffin, eosin and hematoxylin etc.

3.1.2 Equipment

The major equipment used for the study were dissecting set, mortal and pistol, syringes, cannula, microscope, hand glove, cages, feeders, electrical weighing balance, automated haematological analyser (Sysmex XS series), microplate reader (BioRad model 680), centrifuge(Labofuge 300 centrifuge -Heracus), spectrophotometer (Jenway 6305 spectrophotometer).

3.1.3 Plant Materials

Fresh leaves of *Vitex doniana* were collected from their habitat in the month of March to June around Ahmadu Bello University Zaria and fresh leaves of *Grewia mollis* were obtained in Zaria city, Zaria around the same month as *Vitex doniana*. The leaves were taken to the Herbarium in the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University Zaria for identification and vouchers 1162 and 925 were deposited. The leaves were destalked, washed separately and dried under the sun for 3 hours then at room temperature. The dried samples were pulverized using pestle and mortar, to obtain the powder form. This was then stored at room temperature before using for diet formulation.

3.1.4 Experimental Diet Formulation

Standard diet of Vital Feed (Vital Feeds Company-Grower mash) bought from Samaru, Zaria was used in the entire study. The diet was mixed with 2.5, 5 and 10% *Grewia mollis* leaf powder and 2.5, 5 and 10% *Vitex doniana* leaf powder.

3.1.5 Animals

A total of 80 male Wistar rats weighing 70-100 g were procured and housed in Department of Pharmacology, Faculty of Pharmaceutical Science, Ahmadu Bello University Zaria and allowed free access to water and feed standard (Vital feed Grower's mash) *ad libitum* for two weeks to acclimatise before commencement of the experiment.

3.2 METHODS

3.2.1 Induction of Colon Cancer

After six (6) weeks of feeding with the leaf supplemented diets of *V. doniana* and *G. mollis*, freshly prepared N-Methyl-N-nitrosourea (MNU) were administered to each animal in the groups 2 (a and b), 4 (a and b), 5 (a and b) and 6 (a and b) for ten (10) weeks of continual *V. doniana* and *G. mollis* leaf supplement diet feeding.

3.2.2 Experimental Design and Animal Groupings

The rats were weighed and randomly divided into two sets of six (6) groups for each plant of five animals each (n= 5). The groups are as follows:

Group 1: Normal control group (The animals in this control group were fed unsupplemented normal feed without the carcinogen induction)

Group 2: MNU induced colon cancer rats fed normal unsupplemented diet

Group 3A: Normal saline induced normal rats+ 10% *Grewia mollis* leaf supplemented diet

Group 3B: Normal saline + 10% *Vitex doniana* leaf supplementation control

Group 4A: MNU induced+ 2.5% *Grewia mollis* leaf supplementation

Group 4B: MNU induced+ 2.5% *Vitex doniana* leaf supplementation

Group 5A: MNU induced+ 5% *Grewia mollis* leaf supplementation

Group 5B: MNU induced+ 5% *Vitex doniana* leaf supplementation

Group 6A: MNU induced + 10% *Grewia mollis* leaf supplementation

Group 6B: MNU induced + 10% *Grewia mollis* leaf supplementation

After sixteen (16) weeks of dietary supplementation with the leaf of *G.mollis* and *V.doniana*, which is ten (10) weeks stoppage of induction all the animals were scarified. Colon cancer was confirmed by determining the level of carcinoembryoic antigen using the carcinoembryoic antigen assay kit.

3.2.3 Body Weight and Food Intake Measurement

Bodyweights and food intake of all groups was measured throughout the study. The animal weights were measured immediately prior to the commencement of the supplement feeding at weekly intervals to the end of the study (Obidah *et al*., 2010). Final body weight gain and percentage change in body weight were calculated as follows:

Percentage change in body weight = $\frac{\text{Final weight}_{(g)} - \text{Initial weight}_{(g)}}{\text{Final weight}_{(g)}} \times 100$

The animal diet were weighed weekly and given to animals daily. Food intake was calculated by subtracting the mass of the feed remaining from the known mass provided to the rats and the food intake measured (Obidah *et al*., 2010).

3.2.4 Collection of Organs/Tissue

3.2.4.1 Collection and Preparation of Sera Sample

The animals were sacrificed by anaesthetizing them using chloroform at the end of the 16th week. Blood samples were collected from the head wound in a plain bottles (for Carcinoembryonic antigen assay) and in Ethylene diamine tetraacetic acid (EDTA) coated bottles (for haematological parameters). The blood samples collected in the plain bottles were allowed to clot and the serum separated by centrifugation using Labofuge 300 centrifuge (Heracus) at 3500 rpm for 5 minutes and the supernatant (serum) collected was kept in the refrigerator for Carcinoembryoic antigen assay.

3.2.4.2 Collection of organs- colon, kidney and liver

Immediately after the blood was collected, the kidneys and liver were quickly excised, trimmed of connective tissues, rinsed with cold normal saline to eliminate blood contamination and blotted on filter paper.

The distal colon and rectum were removed and cleansed of fecal material by cutting the large bowel longitudinally, rinsed with normal saline and blotted on filter paper, then stored in ice bag. Then 10% homogenates of the liver and kidney were prepared by homogenizing the organs using mortal and pistol with 50 mM phosphate buffer pH 7.4.

It was then centrifuged at 4000rpm for 15 minutes. The supernatant was collected using Pasteur pipette and kept in the refrigerator for further use.

3.2.5 Estimation of Endogenous Antioxidants

3.2.5.1 Determination of Superoxide Dismutase (SOD) activity

Superoxide dismutase activity was measured using the method described by Mistra and Fridovich (1972).

Principle: The ability of superoxide dismutase to inhibit autoxidation of epinephrine by converting the superoxide anion ($\mathbf{O_2^-}$) which is responsible for propagation of epinephrine oxidation to its unstable intermediate, adrenochrome (absorbs maximally at 480 nm), to water and oxygen. The auto-oxidation reaction is initiated intrinsically, may be owing to the presence of metal impurities, by abstracting a hydrogen atom from the epinephrine ($\mathbf{RH_3^-}$) to convert it to the activated form, $\mathbf{RH_3}$ which reacts with molecular oxygen to form adrenochrome (**R**) and a superoxide radical. The rate of formation of the adrenochrome is a function of the concentration of the superoxide anion present, and the activity of the superoxide dismutase if present at a pH above 8.5.

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

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

$$RH_2 + O_2^- + H^+ \longrightarrow RH^{\cdot} + H_2O_2$$

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

Procedure: Exactly 0.2 ml of tissue homogenate (diluted 10- 20 times) was added to 2.5 ml of 50 mM carbonate buffer. The reaction was started by addition of 0.3 ml epinephrine (prepared in 0.005 M glacial acetic acid adjusted to pH 2.0 with HCl to form a clear solution). The reference mixture contained 2.5 ml of 50 mM carbonate

buffer, 0.2 ml distilled water and 0.3 ml of 0.03 mM epinephrine. Absorbance readings were taken at 480 nm for 5 minutes at an interval of 1 minute each.

SOD concentration in the sample was calculated thus:

Absorbance $_{\text{Reagent test}}$ (A_R) = Absorbance $_{\text{Reagent tes5}}$ - Absorbance $_{\text{Reagent test1}}$

Absorbance $_{\text{Sample test}}$ (A_s) = Absorbance $_{\text{Sample test5}}$- Absorbance $_{\text{Sample test1}}$

% inhibition = $[1 - A_s / A_R] \times 100 \times$ Dilution factor

Specific activity (µmol/mg of protein) = 0.02 × % inhibition / mg of protein

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

3.2.5.2 Catalase assay

Catalase activity was determined by the method described by Sinha (1972).

Principle: The assay depends on the measure of the rate of disappearance of hydrogen peroxide (H_2O_2) in the presence of catalase. It further involves an intermittent suspension/stoppage of the enzymatic reaction followed by a reaction between the excess hydrogen peroxide and potassium dichromate to form chromic acetate when heated. Chromic acetate absorbs at 570nm and its concentration (colour intensity) is inversely related to the catalase activity.

Catalase scavenges hydrogen peroxide converting it to water and molecular oxygen.

$$2H_20_2 \xrightarrow{\text{Catalase}} 2H_20 + O_2$$

Procedure: Exactly 1.32 ml solution of the reaction mixture was prepared. 660 µl of 0.005 M phosphate buffer was added to 530 µl of 0.2 M hydrogen peroxide. The reaction was initiated by addition of 130 µl of tissue homogenate. The mixture was swirled gently and 330 µl of reaction mixture was transferred into 660 µl of dichromate/acetic acid mixture at 1minute interval to stop the reaction. The blue precipitate obtained was heated at about 95^0C for 10 minutes to obtain a green solution which was read spectrophotometrically at 570 nm. A calibration curve was prepared by using 0-160 µmol of hydrogen peroxide in the absence of tissue homogenate to determine the amount of hydrogen peroxide present in the homogenates.

Calculations:

The concentration of H_2O_2 was calculated using =

$$\frac{\text{Increase in absorbance of substrate} \times \text{dilution factor}}{\text{Increase in absorbance of standard} \times \text{protein concentration}}$$

Catalase specific activity was expressed as µmol/mg of protein

3.2.5.3 Determination of Lipid peroxidation by measuring the malondialdehyde (MDA) level

The method of Ohkawa *et al.* (1979) was adopted for the assay of malondialdehyde as an index of lipid peroxidation.

Principle: Malondialdehyde is one of the products formed during the degradation of lipid peroxides (**LOO**$^{\cdot}$) formed by the reaction between lipid radicals (**L**$^{\cdot}$) and molecular oxygen (O_2) or by the abstraction of a hydrogen atom from lipid hydroxyperoxides

(**LOOH)**. The assay is dependent on the tendency of the aldehydic products (malondialdehyde [MDA], 4-Hydroxynonena [4-HNE],) to react with thiobarbituric acid to form thiobarbituric acid reacting substances/species (TBARS) which is a pink complex that absorbs maximally at 523 nm.

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

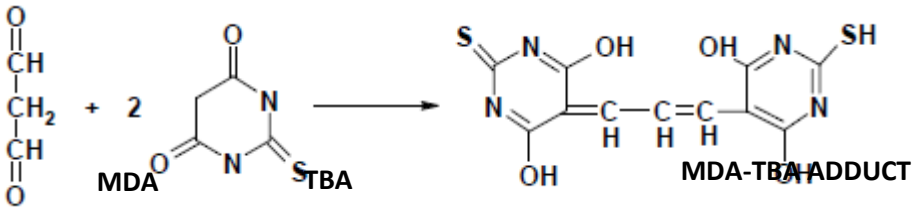
$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$

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

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

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

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



Procedure: Precisely 40 µl of SDS was pipette into test tubes labelled as; sample test and blank and 0.3 ml of acetate buffer (pH 3.5) was added to each tube. Then 40 µl of tissue homogenate was added to sample test only while 40µl of distilled water was added to the blank only. This was then followed by the addition of 120 µl of distilled water to both test tubes. Exactly 0.3 ml of 0.8% TBA was then added to each tube and the mixture heated in a water bath at 95^0C for 60 minutes. The mixture was then allowed to stand in cold water. Precisely 0.2 ml of distilled water and 1 ml n-butanol/pyridine mixture were then added to the reaction mixture. The mixture was then mixed (vortexed) and centrifuged at 3500 rpm for 10 minutes. The pink organic (upper

layer) formed was then removed and absorbance read spectrophotometerically at 535 nm wavelength.

Calculation

The concentration of TBARS is expressed in terms of malondialdehyde (MDA) in µmol/mg of protein.

Molar extinction of MDA = $1.56 \times 10^5 cm^{-1}m^{-1}$

MDA concentration = Absorbance/ $1.56 \times 10^5 cm^{-1}m^{-1}$

Specific activity of MDA (µmol/mg of protein) = MDA concentration/ mg of protein

3.2.6 Determination of Haematological Parameters

The blood samples collected into EDTA bottles were used to the determine the haematological parameters and they include: haemoglobin concentration (Hb), hematocrit count (HCT), white blood cell count (WBC), red blood cell count (RBC), platelet (PLT) and haematological indices (mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV)) with the aid of an automated haematology analyzer.

3.2.7 Histological Analysis of The Tissues

Following 18-24 hours fixation in 10% formal-saline, the colons, kidneys, livers were dehydrated through ascending grades of alcohol (70%, 90%, 95% and absolute (98%)) for 2 hours each. This was followed by clearing in xylene (to remove alcohol) and the tissues were subsequently passed through a molten paraffin wax (impregnation) for 2 hours. The tissues were then immersed in a mould containing molten paraffin wax, which was allowed to solidify (embedding) while the tissue was inside. The tissues

trapped in the wax were then trimmed for sectioning in a microtome machine at 3 µm. The sections were then finally stained with the staining agents, haematoxylin and eosin, for microscopy (Fereshteh *et al*., 2009).

3.2.8 Carcinoembryonic Antigen Assay

The level of carcinoembryonic antigen (CEA) was determined by enzyme-linked immunosorbent assay (ELISA) using rat carcinoembryonic antigen (CEA/CD66) kit.

Principle: The assay system utilizes purified rat CEA/CD66 to coat microtiter plate well to make the solid- phase antibody, then the added CEA/CD66 to the wells. The combine CEA/CD66 antibody with the enzyme labelled become antibody-antigen–enzyme-antibody complex. A monoclonal anti-CEA conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution (Zamcheck and Martins 1981), the color change is measured spectrophotometrically at 450 nm wavelength.

Reagents

Content	**Specification**
Standard	13.5 ng/ml
Standard diluent	1.5 ml
Enzyme conjugate	6 ml
Sample diluent	6 ml
Substrate A	6 ml
Substrate B	6 ml
Stop solution (sulphuric acid)	6 ml
Wash solution	20 ml

Method

Accurately 10 wells on the microtiter plate were set for standard; 100 µl of standard was added to the first and second wells. 50 µl of the standard diluent was added to the first and second wells, it was mixed and 100 µl was taken from each well and added to the third and fourth well. 50 µl of standard dilutent was also added to the third and fourth wells and mixed. 50 µl was taken from the third and fourth wells and discarded, and then another 50 µl was taken from the third and fourth wells into the fifth and sixth wells and mixed. To the fifth and sixth well 50 µl of standard diluent was added and mixed. This was done for up to the tenth well to get a concentration of 9 ng/ml, 6 ng/ml, 3 ng/ml, 1.5 ng/ml, and 0.75 ng/mg for plotting the standard curve.

To the remaining wells 40 µl of sample diluent was added, then 10µl of sample diluted 5folds was also add to all the wells except for the blank wells, and then mixed gently. The plate was then closed with the plate membrane and incubated for 30 minutes at 37^0C. The incubated mixture was then removed by aspirating content of the plate into the sink and the wells completely washed with the wash solution (diluted 30 folds with distilled water) for four or five times then blotted by hitting the plate onto an absorbent paper/paper towel until no moisture appears. Then 50 µl of enzyme conjugate was added to each well except the blank wells. The plate was incubated for 30 minutes at 37^0C and washed as done previously. Exactly 50 µl of substrate A and substrate B were added to each well and incubated for 15 minutes at 37^0C, after which 50 µl of stop solution was added to each well and mixed properly. The absorbance was then determined spectrophotometrically in a microplate reader at 450 nm wavelength.

Calculation

The mean absorbance values (A450) for each set of reference standards, controls and treated samples were calculated. A standard curve was plotted using the mean

absorbance obtained from each reference standard against its concentration in ng/ml on Microsoft excel, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis. The mean absorbance value of each sample was used to determine the corresponding concentration of CEA in ng/ ml from the standard curve.

3.2.9 Statistical Analyses

The statistical significance between the control and other groups of experimental animal were expressed as mean ± SD. The SPSS program (version 20) was used for the Analysis of Variance (AVOVA) while the Duncan's multiple range test (DMRT) was used for comparison of means. Statistical test was performed at $P< 0.05$ level of significance.

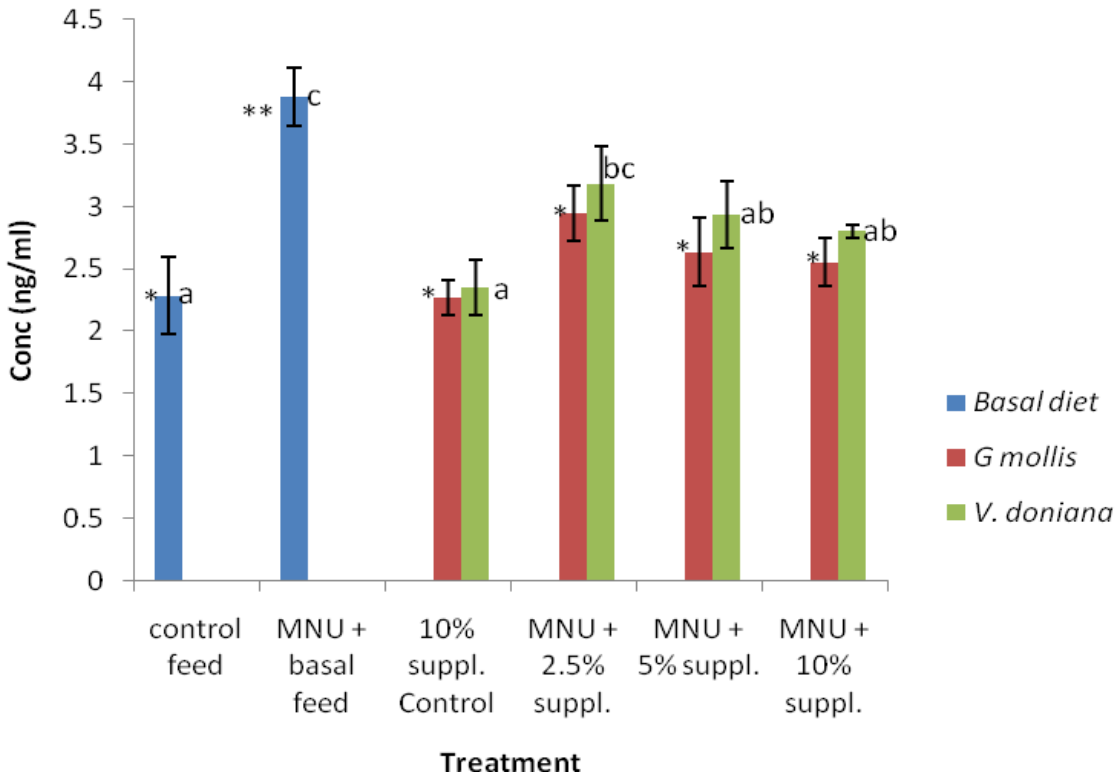
CHAPTER 4

RESULTS

4.1 CARCINOEMBRYONIC ANTIGEN ASSAY

4.1.1 Levels of carcinoembryonic antigen on MNU induced colon cancer rats supplemented with the leaf of *G. mollis* and *V. doniana* diets.

The levels of carcinoembryonic antigen (CEA) on MNU induced colon cancer rats supplemented with the leaves of *G. mollis* and *V. doniana* for a period of 16 weeks is shown in Figure 4.1. The result showed a significant (P□ 0.05) increasein CEA levels in the MNU induced colon cancer control group compared to the normal control feed group for both *G. mollis* and *V. donina* supplement diets. There was a significant (P< 0.05) decrease in the CEA levels of all the MNU induced colon cancer group given *G. mollis* leaf supplemented diet compared to the MNU induced colon cancer control group. However for the *V. doniana* supplement diet, there was no significant (P> 0.05) difference in the CEA level of the MNU induced colon cancer control compared to the *V. doniana* leaf supplemented diet control.



Values with different superscript are significantly different (asterisk(s)* for *G. mollis* and alphabet(s) for *V. doninana*)

Figure 4.1: Levels of carcinoembryonic antigen on MNU induced colon cancer rats supplemented with the leaves of *G. mollis* and *V. doniana* diets.

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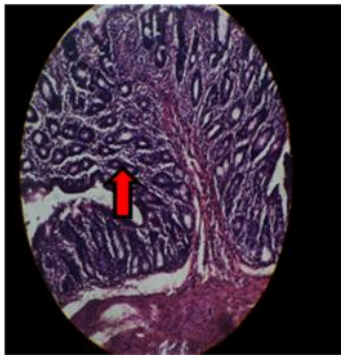
4.2 HISTOPATHOLOGICAL STUDIES

4.2.1 Effect of *G. mollis* and *V. doniana* leaves Supplementation on the Colon of MNU induced Colon Cancer rats.

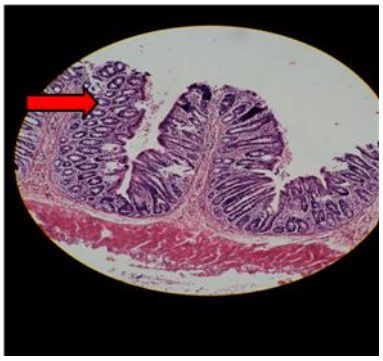
The histopathological section of the colon of MNU induced colon cancer rats given 2.5, 5 and 10% leaves supplemented diets of *G. mollis* and *V. doniana* respectively for 16 weeks is shown in plate 4.1 (a and b). The histopathological examination of the colon section of the control feed (4.1a I) showed normal architecture of the epithelium and more glandular excretion of the globlet cells. The colon of MNU induced colon cancer given basal feed (disease control group 4.1a II) showed distorted architecture of the epithelium with less glandular excretion of globlet cell of the mucosa. The MNU induced colon cancer treated groups showed normalization of the globlet cells with increasing levels of supplementation for both leaves of *G. mollis* (4.1a IV, V and VI) and *V. doniana* (4.1b IV, V and VI) supplemented diets. The result for the leaves supplemented control which is 10% *G. mollis* (4.1a III) and *V. doniana* (4.1b III) supplemented diet control respectively, were similar to the normal control with moderate/ more globlet cells.



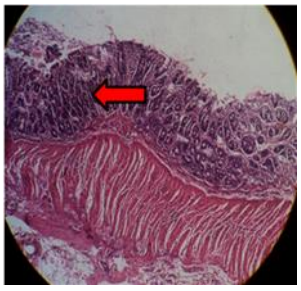
4.1a I



4.1a II



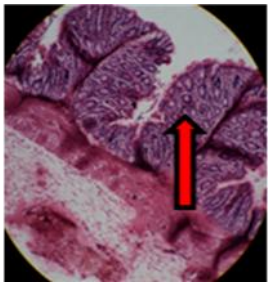
4.1a III



4.1a IV



4.1a V



4.1a VI

Plate 4.1a: Effect of *G. mollis* Leaves Supplementation on Colon of MNU Induced Colon Cancer in Wister Rats. (H & E STAIN × 200)

4.1a I**:** Control feed **(**Normal mucosa lining**)**

4.1a II: MNU induced + basal feed (Less glandular excretion with distorted mucosa)

4.1a III: 10% *G. mollis* supplement control **(**Normal mucosa lining**)**

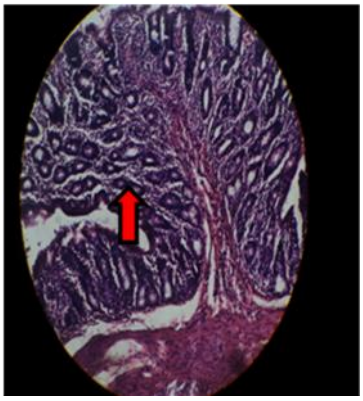
4.1a IV: MNU+2.5% *G. mollis* supplementation (Less glandular excretion**)**

4.1a V: MNU+ 5% *G. mollis* supplementation **(**Few glandular excretions**)**

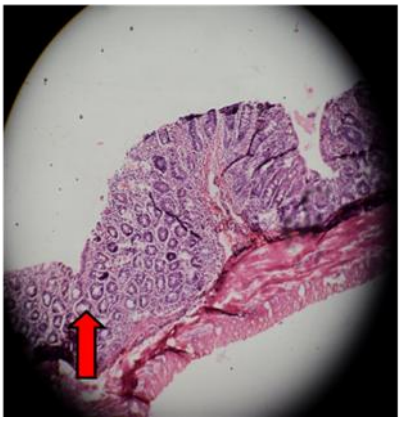
4.1a VI: MNU+ 10% *G.mollis* supplementation (More glandular excretions)



4.1b I



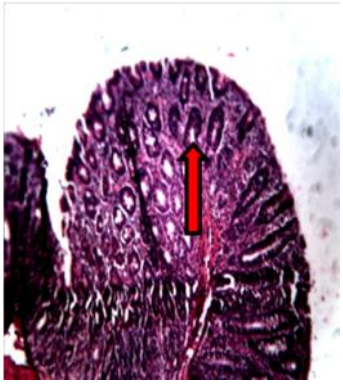
4.1b II



4.1b III



4.1b IV



4.1b V



4.1b VI

Plate 4.1b: Effect of *V. doniana* Leaves Supplementation on Colon of MNU Induced Colon Cancer in Wister Rats. (H & E STAIN × 200)

4.1b I: Control feed **(**Normal mucosa lining**)**

4.1b II: MNU + basal feed (Less glandular excretion)

4.1b III: 10% *V. doniana* supplementation (Normal mucosa lining)

4.1b IV: MNU+2.5%*V.doniana* supplementation (Less glandular excretion)

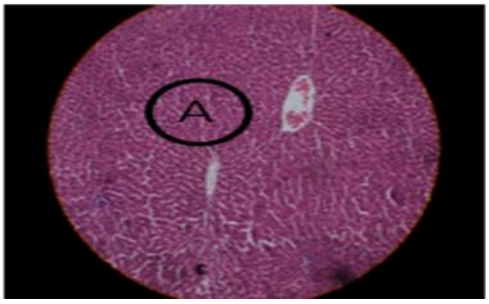
4.1b V: MNU+5% *V.doniana* supplementation (Few glandular excretions)

4.1b VI: MNU + 10% *V.doniana* supplementation (More glandular excretion)

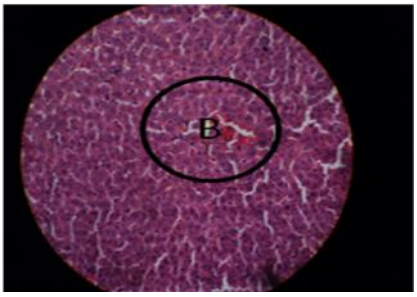
4.2.2 Effect of *G. mollis* and *V. doniana* Leaf Supplementation on the Liver and Kidney of MNU induced colon cancer rats.

The histopathology of the liver of MNU induced colon cancer in rats treated with dietary supplementation of the leaves of *G. mollis* and *V. doniana* for a period of 16 weeks in showed in plate 4.2 a and b, respectively. There were normal observable hispathological findings in the liver of the control feed animals (the normal control 4.2a and b I). The liver of MNU induced colon cancer control rats (4.2a and b II) had congested sinusoidal capillaries and central vein with few scattered areas of necrosis of the hepatic cells. The MNU induced *G. mollis* (4.2a IV, V and VI) and *V. doniana* (4.2b IV, V and VI) leaves supplemented diet treated groups also had congested sinusoidal capillaries and central vein with few focal area of necrosis of the hepatocytes with almost normalisation as the level of treatment increases. The control groups of both plants (4.2a and b III) had normal hepatocytes as seen in the normal control.

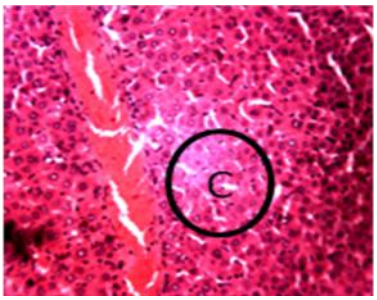
However, the induction of colon cancer using MNU resulted in scattered areas of tubular epithelium necrosis with congested intertubular spaces as showed on plate 4. 3a (I) and 4.3b (I) in the kidney of both *G. mollis* and *V. doniana* leaves dietary supplementation, respectively. There were also eosinophic materials within the lumen of the seminiferous tubules. On daily feeding of the animals with different levels of the supplemental diet of leaves of *G. mollis* (4.3a IV, V and VI) and *V. doniana* (4.3b IV, V and VI) there was no notable change in the features of the kidney.



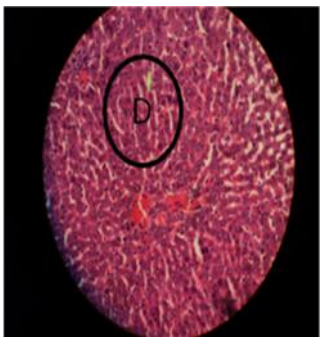
4.2a I



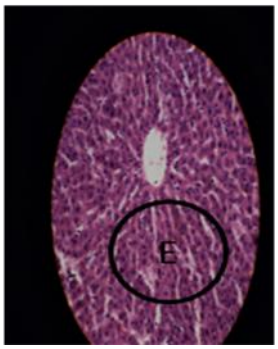
4.2a II



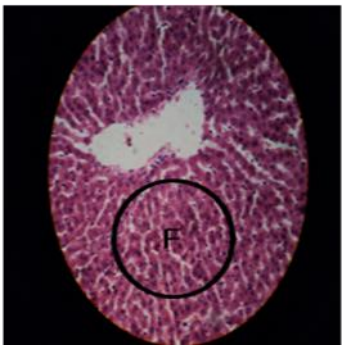
4.2a III



4.2a VI



4.2a V



4.2a VI

Plate 4.2a: Effect of *G. mollis* Leaf Supplementation on Liver of MNU Induced Colon Cancer in Wistar Rats. (H & E STAIN × 200)

4.2a I: Control feed (A: Normal hepatocytes)

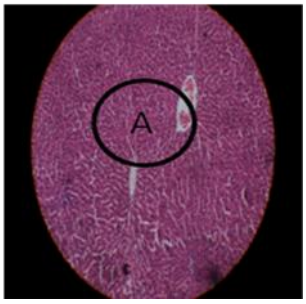
4.2a II: MNU + basal feed (B: Congested sinusoidal capillaries and vein with hepatic necrosis)

4.2a III: 10% *G. mollis* supplement control (C: Normal hepatocytes)

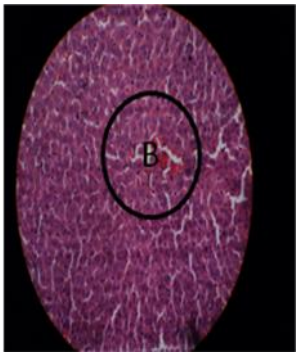
4.2a IV: MNU + 2.5% *G. mollis* supplementation (D: Congested vein and sinusoidal capillaries with necrosis of hepatocytes)

4.2a V: MNU+5% *G. mollis* supplementation (E: Vascular congestion with hepatic necrosis)

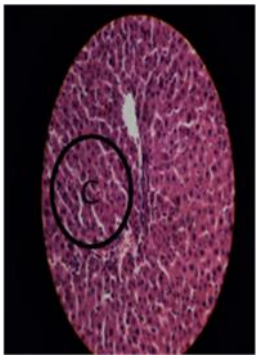
4.2a VI: MNU + 10% *G. mollis* supplementation (F: Slight vascular congestion)



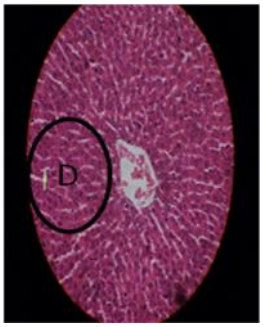
4.2b I



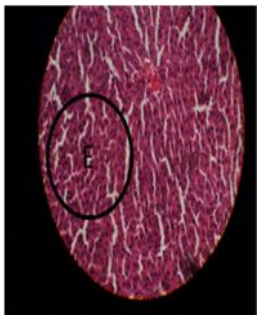
4.2b II



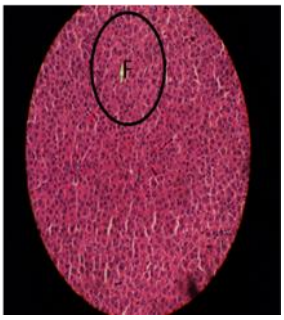
4.2b III



4.2b IV



4.2b V



4.2b VI

Plate 4.2b: Effect of *V. donina* Leaves Supplementation on Liver of MNU Induced Colon Cancer in Wistar Rats. (H & E STAIN × 200)

4.2b I: Control feed (A: Normal hepatocytes)

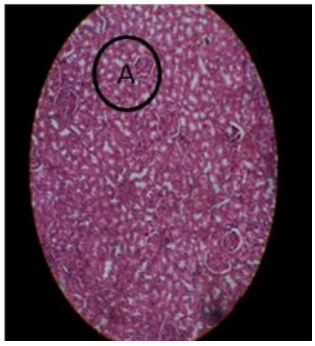
4.2b II: MNU + basal diet (B: Congested sinusoidal capillaries and vein with hepatic necrosis)

4.2b III: 10% *V. doniana* supplement control (C: Normal hepatocytes)

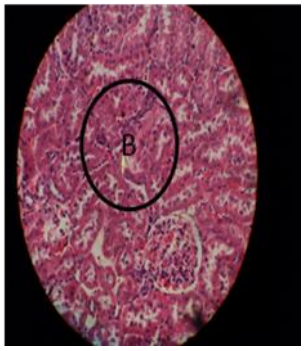
4.2b IV: MNU+2.5% *V. doniana* supplementation (D: Necrosis of the hepatocytes)

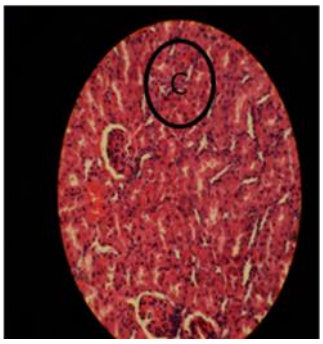
4.2b V: MNU+5%*V. doniana* supplementation (E: Vascular and sinusoidal capillaries congestion)

4.2b VI: MNU+10% *V. doniana* supplementation (F: Congested sinusoidal capillaries)



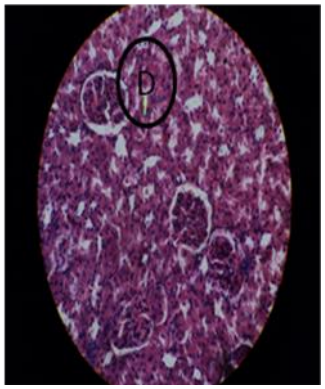
4.3a I

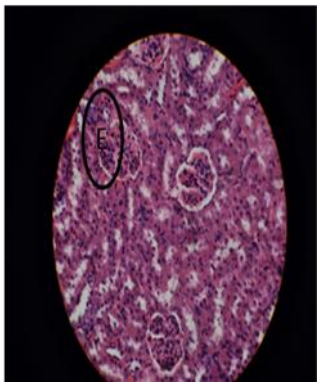


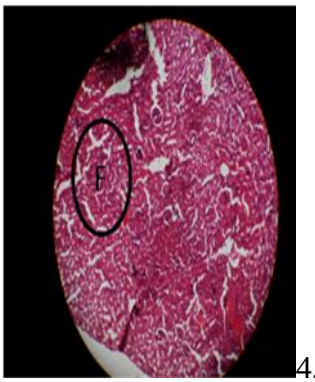


4.3a II

4.3a III







4.3a IV

4.3a V

4.3a VI

Plate 4.3a: Effect of *G. mollis* Leaves Supplementation on Kidney of MNU Induced Colon Cancer in Wister Rats. (H & E STAIN × 200)

4.3a I: Control feed (A: Normal glomerulus and tubules)

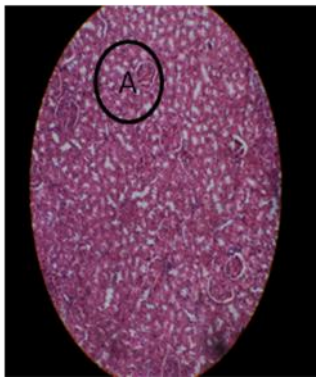
4.3a II: MNU + basal feed (B: Congested intertubular spaces with intense tubular necrosis and eosinophilic materials)

4.3a III: 10% G. *mollis* supplement control (C: Normal glomerulus and tubules)

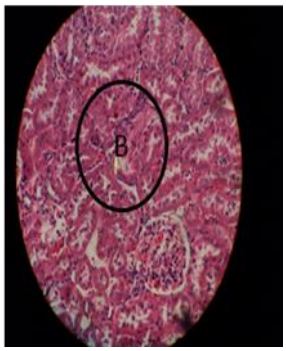
4.3a IV: MNU+ 2.5% *G. mollis* supplementation (D: Areas of tubular necrosis)

4.3a V: MNU+ 5% *G. mollis* supplementation (E: Moderate necrosis of renal tubular epithelium with eosinophilic materials in the lumen)

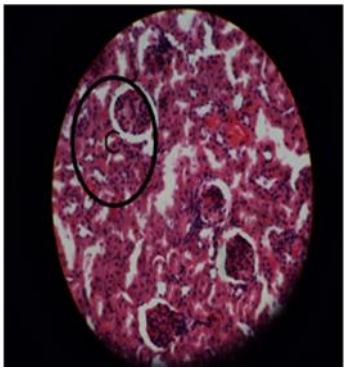
4.3a VI: MNU+ 10% *G. mollis* supplementation (F: Slight necrosis of the tubules)



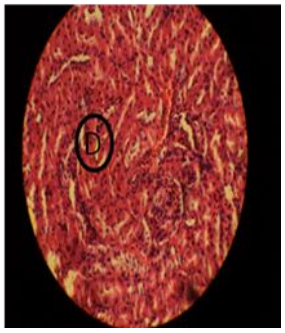
4.3b I



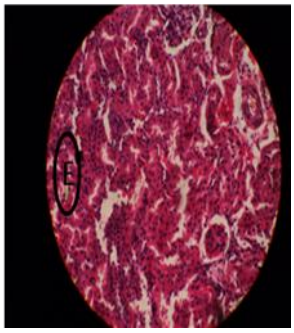
4.3b II



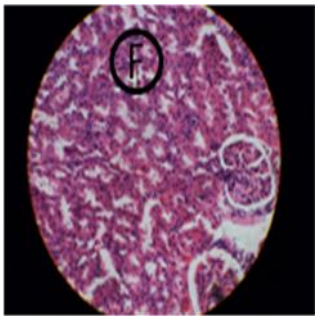
4.3b III



4.3b IV



4.3b V



4.3b VI

Plate 4.3b: **Effect of *V. doniana* Leaves Supplementation on Kidney of MNU Induced Colon Cancer in Rats. (H & E STAIN × 200)**

4.3b I: Control feed (A: Normal glomerulus and tubules)

4.3b II: MNU + basal feed (B: Congested intertubular spaces with intense tubular necrosis and eosinophilic materials)

4.3b III: 10% *V. doniana* supplement control (C: Normal glomerulus and tubules)

4.3b IV: MNU+2.5% *V. doniana* supplementation (D: Intense areas of tubular necrosis)

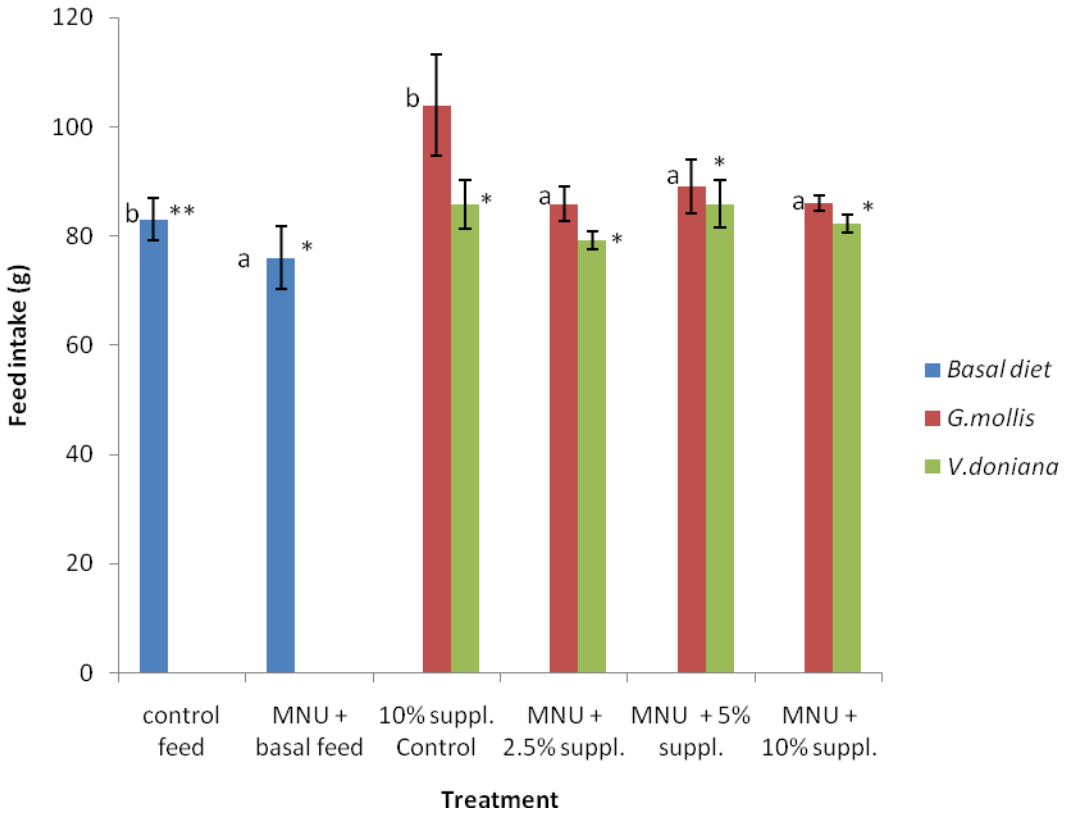
4.3b V: MNU+5% *V. doniana* supplementation (E: Moderate necrosis of renal tubular epithelium with eosinophilic materials in the lumen)

4.3b VI: MNU+10% *V. doniana* supplementation (F: Slight necrosis of the tubules)

4.3 FEED INTAKE AND BODY WEIGHT

4.3.1 Effect of *G. mollis* and *V. doniana* supplemented diet on Feed Intake of MNU Induced colon cancer rats.

The result of effect of *G. mollis* and *V. doniana* supplemented diet on feed intake of MNU induced colon cancer rats is shown on Figure 4.2. There was a significant (P□ 0.05) decrease in feed intake of the MNU induced colon cancer control group compared to animals fed the normal control feed (control feed). However, there was no significant (P□ 0.05) different between all the MNU induced colon cancer fed*G. mollis* and *V. doni*na leaves supplemented diets compared the MNU induced colon cancer control group expect for the 10% *G. mollis* supplemented diet group which was significantly (P< 0.05) increased.



Values with different superscript are significantly different (alphabet(s) for *G. mollis* and asterisk(s)* for *V. doninana*)

Figure 4.2: Effect of diet supplementation with leaves of *G. mollis* and *V. doniana* on feed intake on MNU induced colon cancer rats.

4.3.2 Effect of *G. mollis* and *V.doniana* Supplementation on Average Weight Gain and Percentage Change in Weight

The effect of *G. mollis* and *V. doniana* supplementation on average weight gain and percentage change in weight is shown on Table 4.1. The results shows that there was no significant ($P > 0.05$) difference in weekly weight gain between the MNU colon cancer basal control and the normal control fed animals of both *G. mollis* and *V. doniana*. However, the 10% supplemented diet control of *G. mollis* had a significantly ($P < 0.05$) increased average weight gain compared to the normal control fed while there was no significant ($P > 0.05$) difference in weekly weight gain between all MNU induced treated groups *V. doniana* supplemented diets compared to the normal control. There was no significant ($P > 0.05$) difference in the percentage change in weight between the MNU induced colon cancer control and the normal control.

Table 4.1: Effect of *G. mollis* and *V.doniana* leaves Supplementation on Average Weight Gain and % Change in Weight on MNU induced colon cancer in rats.

TREATMENT	AVERAGE WEIGHT GAIN (g)		CHANGE IN WEIGHT (%)	
	G.mollis	*V.doniana*	*G.mollis*	*V.doniana*
Control feed	5.69 ± 1.10[ab]	5.69 ± 1.10[a]	51.00± 6.46 [a]	51.00±6.46[a]
MNU + basal feed	5.30 ± 3.63[ab]	5.30 ± 3.63 [a]	49.28 ± 8.01[a]	49.28±8.01[a]
10% suppl. control	8.64 ± 4.17[b]	6.39 ± 1.26[a]	51.12 ± 14.65 [a]	45.53±3.78[a]
MNU + 2.5% suppl.	5.99 ± 1.93[ab]	5.30 ± 1.52[a]	50.36 ± 7.13[a]	48.32±6.24[a]
MNU + 5% suppl.	3.08 ± 1.44[a]	5.97 ± 0.85[a]	37.93 ± 4.02[a]	48.32±12.06[a]
MNU +10% suppl.	3.72 ± 314[a]	6.86 ± 2.91[a]	36.60 ± 5.82[a]	30.59 ± 9.20[a]

Values with different superscript down the column are significantly different (P<0.05)

4.4 HEAMATOLOGICAL PARAMETERS

4.4.1 Effect of *G. mollis* and *V. doniana* Leaves Supplemented diets on Haematological Parameters of MNU Induced Colon Cancer rats.

The effect of dietary supplementation with *G. mollis* and *V. doniana* leaves supplemented diet on haematological parameters of MNU induced colon cancer rat over a period of 16 weeks is shown in Tables 4.2 and 4.3. The results showed that there was no significant ($P > 0.05$) difference in the white blood cell count (WBC), red blood cell count (RBC), platelet (PLT) and mean corpuscular volume (MCV) of the MNU induced basal diet control compared to the normal (basal diet) control of both plants. There was a significant ($P < 0.05$) decrease in the haemoglobin (Hb) and hematocrit (HCT) and a significant ($P < 0.05$) increase in the mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) levels of the MNU induced basal control group compared to the normal controls group of *G. mollis* and *V. doniana*. However, there was a significant ($P < 0.05$) reduction in the levels WBC, RBC, Hb, and HCT in *G. mollis* leaves dietary supplement groups compared to the normal control group.

Table 4.2: Effect of *G. mollis* Leaves Supplemented diet on Haematological Parameters of MNU induced Colon Cancer rats

Treatment	WBC($\times10^9$/L)	RBC($\times10^{12}$/L)	Hb (g/L)	PLT(10^9/L)	HCT (%)	MCV(fL)	MCHC(g/L)	MCH(pg)
Control feed	15.33 ± 4.80^{bc}	151.88 ± 4.64^{c}	8.88 ± 0.27^{c}	811.37 ± 102.94^{b}	50.03 ± 1.80^{b}	56.34 ± 1.45^{ab}	303.75 ± 4.33^{a}	17.13 ± 0.50^{a}
MNU+ basal feed	17.05 ± 3.94^{c}	143.63 ± 9.86^{bc}	7.68 ± 0.44^{b}	794.75 ± 115.12^{b}	44.01 ± 2.35^{a}	57.19 ± 1.61^{ab}	327.00 ± 9.18^{b}	18.69 ± 0.79^{c}
10%suppl. control	18.76 ± 6.8^{c}	130.14 ± 11.93^{a}	7.41 ± 0.71^{ab}	655.43 ± 175.42^{a}	42.81 ± 4.74^{a}	51.69 ± 15.04^{a}	304.14 ± 9.67^{a}	17.50 ± 0.61^{ab}
MNU+2.5%suppl.	17.05 ± 3.94^{c}	132.75 ± 16.89^{ab}	7.60 ± 0.86^{ab}	703.75 ± 44.54^{ab}	43.41 ± 5.60^{a}	57.14 ± 1.61^{ab}	305.38 ± 10.21^{a}	17.41 ± 0.64^{ab}
MNU + 5% suppl.	9.72 ± 1.74^{a}	136.17 ± 2.49^{ab}	7.68 ± 0.29^{b}	683.83 ± 55.68^{ab}	44.37 ± 1.28^{a}	57.93 ± 0.97^{ab}	306.50 ± 4.85^{a}	17.72 ± 0.46^{ab}
MNU +10%suppl.	10.68 ± 2.28^{a}	126.67 ± 3.93^{a}	7.00 ± 0.17^{a}	720.67 ± 113.56^{ab}	41.83 ± 1.99^{a}	59.78 ± 2.18^{b}	302.67 ± 9.16^{a}	18.03 ± 0.50^{b}

Values with different superscript down the column are significantly different ($P<0.05$)

WBC: white blood cell count, RBC: red blood cell count, Hb: haemoglobin, HCT: heamatocrict, PLT: platelets, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration, MCH: mean corpuscular haemoglobin, Suppl.: supplementation

Table 4.3: Effect of *V. doniana* Leaves Supplementation on Haematological Parameters of MNU induced Colon Cancer rats.

Treatment	WBC($\times10^9$/L)	RBC($\times10^{12}$/L)	Hb (g/L)	PLT(10^9/L)	HCT (%)	MCV(fL)	MCHC(g/L)	MCH(pg)
Control feed	15.33 ± 4.80 [a]	151.88 ± 4.64[d]	8.88 ± 0.27[b]	811.37±102.94[b]	50.03±1.80 [c]	56.34±1.45[a]	303.75±4.33[a]	17.13±0.50[a]
MNU+basal feed	17.05±3.94 [a]	143.63±9.86[cd]	7.68±0.44[a]	794.75±115.12[b]	44.01±2.35[bc]	57.19 ±1.61[a]	327.00±9.18[b]	18.69±0.79[b]
10%suppl. Control	15.86±8.66 [a]	124.38±15.52[a]	6.81 ± 0.95[a]	546.63±156.69[a]	41.28±3.71[ab]	60.96 ±5.10[b]	300.00 ± 13.78[a]	18.19±0.91[b]
MNU+2.5% suppl.	15.01± 3.82 [a]	136.13±7.92[abc]	7.38±0.40[a]	662.63±120.99[a]	41.80±2.34[ab]	56.73 ±1.58[a]	324.00 ± 8.19[b]	18.40±0.69[b]
MNU + 5% suppl.	11.51 ± 1.85 [a]	139.75±6.75[bcd]	7.60 ± 0.31[a]	557.38 ± 95.12[a]	44.96±2.04[bc]	59.14±1.69[ab]	310.50 ± 6.39[a]	18.35±0.42[b]
MNU + 10% suppl.	16.70 ± 4.25 [a]	128.38±24.14[ab]	6.81 ± 0.95[a]	657.00 ± 90.47[a]	36.29±13.56[a]	62.01 ±6.09[b]	302.14 ± 14.55[a]	18.63±0.96[b]

Values with different superscript down the column are significantly different (P<0.05)

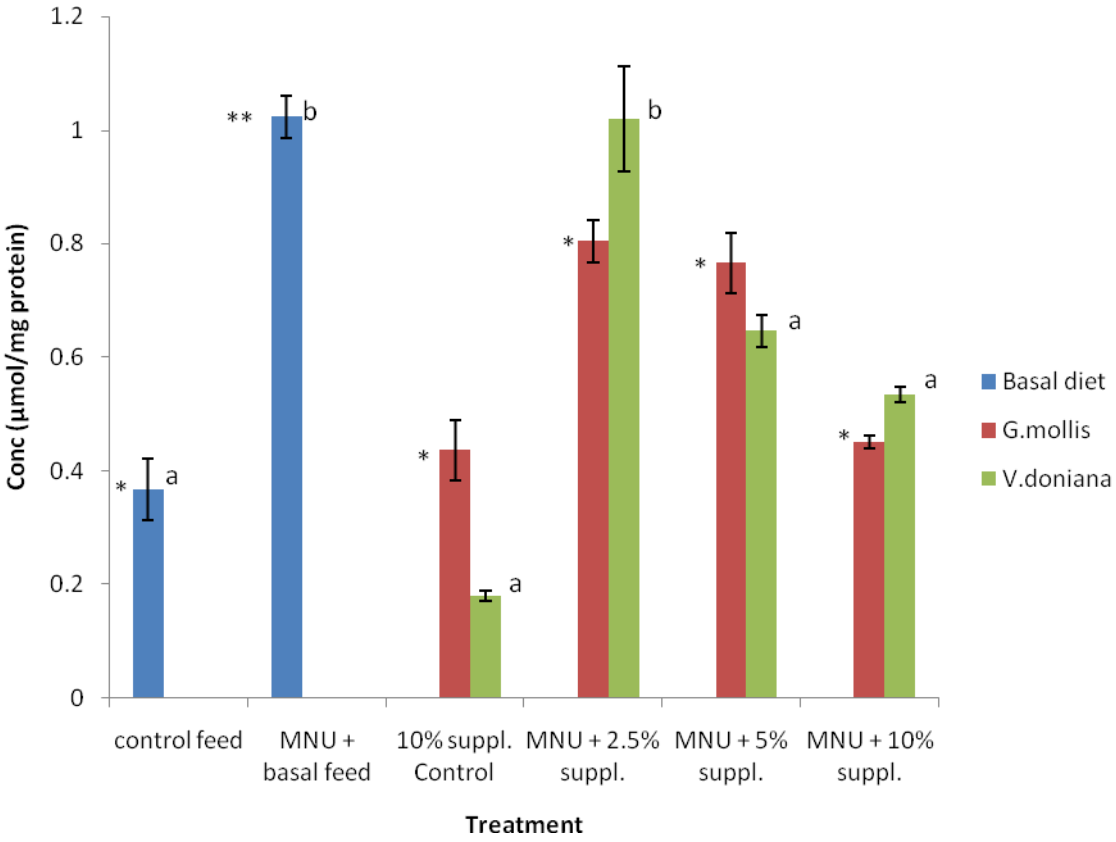
WBC: white blood cell count, RBC: red blood cell count, Hb: haemoglobin, HCT: heamatocrict, PLT: platelets, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration, MCH: mean corpuscular haemoglobin, Suppl.: supplementation

4.5 *IN VIVO* ANTIOXIDANT STUDIES

4.5.1 Effect of Dietary Supplementation with Leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation and Some Endogenous Antioxidant Enzymes on the Liver of MNU induced Colon Cancer rats

The effect of dietary supplementation with the leaves of *G. mollis* and *V. doniana* on lipid peroxidation of MNU induced colon cancer in wistar rats after 16 weeks is shown in Figure 4.3. The result showed that there was 58% increase in the TBARS level in the MNU induced colon cancer group compared to the basal diet group. There was no significant ($P > 0.05$) difference

The effect of dietary supplementation with the leaves of *G. mollis* and *V. doniana* on some endogenous antioxidant enzyme: catalase (CAT) and superoxide dismutase (SOD) of MNU induced colon cancer in rats after 16 weeks is shown in Table 4.5. The result showed that there was no significant ($P> 0.05$) difference between the SOD of the control feed, MNU induced not treated and the treated group of the leaves supplemented diets of both *G. mollis* and *V. doniana*. However, the catalase of the MNU induced basal diet control group was significantly ($P< 0.05$) lowered compared to the control feed group for both *G. mollis* and *V. doniana* leaf supplements. There was no significant ($P< 0.05$) difference in the catalase level between the MNU induced basal control and leaf supplement diet of both plants treated groups expect for the 10% treated and control groups, respectively.



Values with different superscript are significantly different (asterisk(s)* for *G. mollis* and alphabet(s) for *V. doninana*)

Figure 4.3: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation on the liver of MNU induced colon cancer rats.

Table 4.4: Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on some Endogenous Antioxidant Enzymes (SOD and CAT) on the Liver of MNU induced colon cancer wistar rats

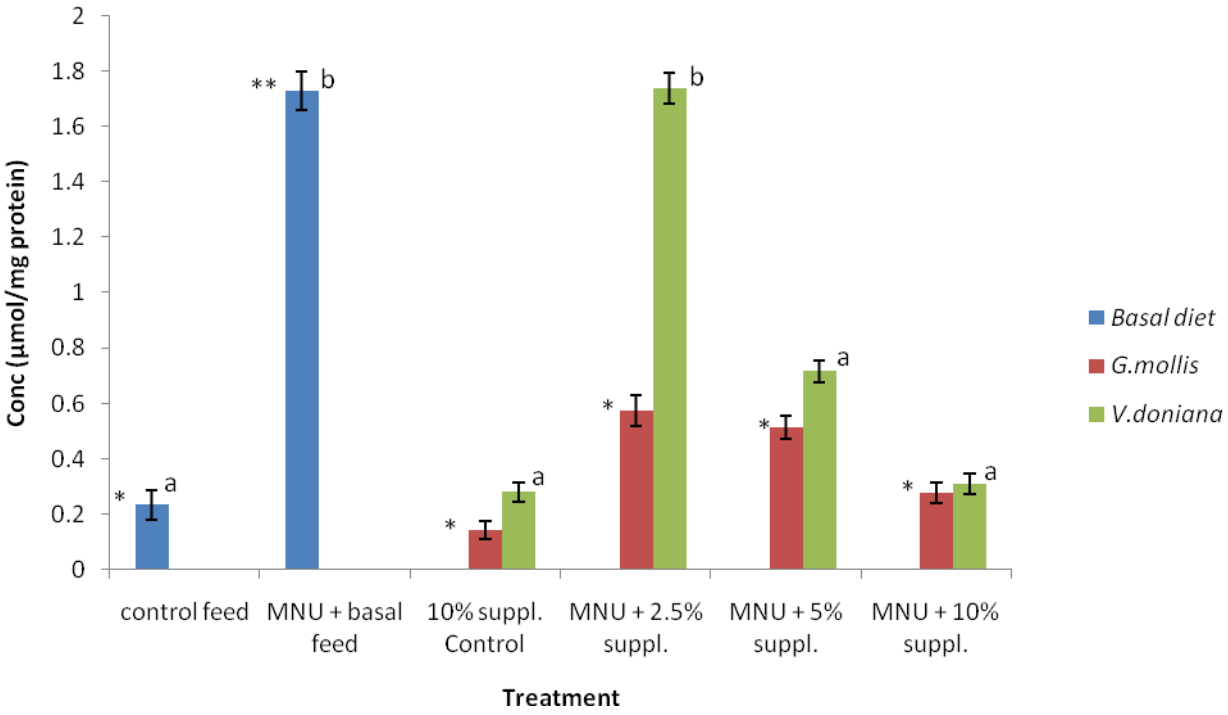
TREATMENT	SOD (µmol/mg protein)		CATALASE (µmol/mg protein)	
	G.mollis	*V.doniana*	*G.mollis*	*V.doniana*
Control feed	1.50±0.40[a]	0.91 ± 0.34 [a]	26.11±0.35[c]	26.11 ± 0.35[b]
MNU + basal feed	0.84±0.13 [a]	2.09 ± 0.40[b]	13.2 ± 0.78[a]	13.22 ± 0.78[a]
10% suppl. control	1.69±0.30 [a]	0.53 ± 0.33 [a]	23.41±2.66[bc]	22.65 ± 1.66[b]
MNU + 2.5% suppl.	0.79±1.08 [a]	2.08 ± 1.29[b]	12.61 ± 4.51[a]	13.16 ± 1.59[a]
MNU + 5% suppl.	1.36±0.09 [a]	0.88 ± 0.46 [a]	16.56± 3.92[ab]	14.83 ± 1.87[a]
MNU +10% suppl.	1.32±0.30 [a]	0.75 ± 0.45 [a]	22.12±4.22[bc]	20.85 ± 5.70[b]

Values with different superscript down the column are significantly different ($P<0.05$)

4.5.2 Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation and some Endogenous Antioxidant Enzymes on the Kidney of MNU induced colon cancer wistar rats

The effect of dietary supplementation with the leaves of *G. mollis* and *V. doniana* on lipid peroxidation on the kidney of MNU induced colon cancer wistar rats for a period of 16 weeks is shown in Figure 4.4. The result showed that there was a significant ($P< 0.05$) increase in thiobarbituric acid-reactive substances (TBARS) of the MNU induced colon cancer control group compared to the basal control group. There was a significant ($P<0.05$) decrease in the TBARS levels of all the MNU induced treated groups of both leaves compared to the MNU induced colon cancer control group except for the 2.5% *V. doniana* leaves supplemented diet group which was significantly ($P< 0.05$) higher.

The effect of dietary supplementation with the leaves of *G. mollis* and *V. doniana* on some endogenous antioxidant enzymes on the kidney of MNU induced colon cancer wistar rats for a period of 16 weeks is shown on Table 4.7. The result showed that there was a significant ($P< 0.05$) decrease in the catalase level of the MNU induced colon cancer control group compared to the control feed group for both *G. mollis* and *V. doniana* leaf supplemented diets. Similarly, there was no significant ($P> 0.05$) different in the SOD levels between the MNU induced colon cancer group and the control feed group for both plants. There was a significant ($P< 0.05$) decrease in the catalase level of MNU induced colon cancer control group compared to the normal control groups of both *G. mollis* and *V. doniana* leaf supplement diet. There was also no significant ($P> 0.05$) difference in the levels of SO D and catalase of all the induced treated groups when compared to the normal control expect for 2.5% *V. doiniana* leaf supplemented group which was significantly ($P< 0.05$) increased.



Values with different superscript are significantly different (asterisk(s)* for *G. mollis* and alphabet(s) for *V. doninana*)

Figure 4.4: Effect of Dietary Supplementation with leaf of *G. mollis* and *V. doniana* on Lipid Perioxidation on the kidney of MNU induced colon cancer wistar rats.

Table 4.5: Effect of Dietary Supplementation with leaf of *G. mollis* and *V. doniana* on some Endogenous Antioxidant Enzymes on the Kidney of MNU induced colon cancer wistar rats.

TREATMENT	SOD (µmol/mg protein)		CATALASE (µmol/mg protein)	
	G. mollis	*V. doniana*	*G. mollis*	*V. doniana*
Control feed	1.42 ± 1.79^{ab}	1.42 ± 1.79^{a}	22.67 ± 4.39^{c}	22.67 ± 4.39^{a}
MNU + basal feed	1.09 ± 0.20^{ab}	1.09 ± 0.20^{a}	14.23 ± 1.31^{ab}	14.23 ± 1.31^{b}
10% suppl. Control	2.33 ± 1.01^{b}	2.35 ± 1.87^{a}	23.41 ± 2.66^{c}	23.46 ± 1.27^{a}
MNU + 2.5% suppl.	0.78 ± 0.59^{ab}	1.72 ± 0.31^{a}	12.61 ± 4.50^{a}	19.80 ± 1.20^{a}
MNU + 5% suppl.	0.35 ± 0.01^{a}	1.22 ± 0.14^{a}	19.34 ± 2.32^{bc}	19.84 ± 8.78^{a}
MNU +10% suppl.	0.60 ± 0.43^{ab}	1.26 ± 0.28^{a}	19.13 ± 2.30^{bc}	20.85 ± 5.70^{a}

Values with different superscript down the column are significantly different ($P < 0.05$)

CHAPTER 5

DISCUSSION

N-methyl-N-nitrosourea (MNU) a direct acting carcinogen is one of the most used colon (large bowel) carcinogen for experimental induction of colon cancer (Tsubura *et al*., 2011). MNU has been administered to different murine species for inducing carcinogenesis in several organs, such as the ovary (Ting *et al*., 2007), skin (Brown *et al*., 1990), thymus (de Silva *et al*., 2003) and colon (Zhou *et al*., 2000). Narisawa *et al.* (2000) reported that capsanthin-rich paprika has effect on MNU induced colon cancinogenesis in F344 rats. It was therefore proposed that foods containing multiple antioxidant phytochemicals, which play role in attenuating the cancer risk, are far more effective for cancer prevention than a single chemical constituent in the food. The carcinogenic action of MNU can be applicable for the establishment of organ-specific models for human cancer.

The carcinoembryonic antigen (CEA) of colorectal cancer is the most useful tumor marker to determine prognosis and to monitor patients with resectable colorectal cancer for early recurrence, and it is now widely tested (Gold and Freedman, 1965; Goldenberg *et al.,* 1981). The increased CEA level in the serum of rats administered MNU was also reported by Ebrahimzadeh *et al.* (2005). This increase may be as a result uncontrollable or continuous division of the inner layer of the bowel (mucosa). Although, CEA is a tumour marker used currently in colorectal cancer, it has low diagnostic sensitive. (Sugarbaker *et al*., 1976; Nilsson *et al*., 1992; Eskelinen *et al*., 1994). The significantly decrease in the levels of CEA in rats given different proportions of the supplement diet with the leaves of *G. mollis* and *V. doniana* may suggest that leaves of *G. mollis* and *V.*

doniana supplemented diets may contain anticarcinogenic properties which show great antioxidant capacity thereby yielding a better overall colon carcinogenesis.

Histopathological examinations give more explanation of the CEA analysis. The histopathological findings of the colon in the MNU induced group showed less glandular excretion (less number of globlet cells) of the mucosa and a loss/ distorted architecture of the epithelium. This finding is in agreement with (Donnelly *et al*., 2004; Narisawa *et al*., 1998). Upon treatment with different proportions of the leaf supplemented diet, the globlet cells increased in number and the epithelium had close to the normal architecture. This effect was more obvious in *G. mollis* leaves supplement diets 10% which may be due to protective effect of the plants. The histopathological findings of the liver and kidney of the MNU induced colon cancer group showed that MNU caused intense congestion of intertubular spaces and sinusoidal capillaries and necrosis indicating its hepatoxicity and nephrotoxicity. Following the appropriate levels of supplementation, hepatocytes showed moderate necrosis while they were eosinophilic materials in the lumen of the kidney tubules. This may be as a result of regeneration and repair of the liver and kidney respectively.

The dietary supplemented control (10% supplement control) of the leaves of both plant had additional nutrient from the leaves of the plant compared to normal and colon cancer control groups. This could be the reason behind the increased feed intake of the rats in those groups. The feed intake of the MNU colon cancer control was low compared to MNU induced treated group with different levels of the supplement diets. This effect could also be as a result of the disease condition of the rats. The weekly weight gain of MNU induced rats receiving the different levels of dietary

supplementation of *G. mollis* and *V. doniana* were similar with that of the colon cancer control, this findings is in accordance with that reported by Narisawa *et al*, (2000)

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 (Valko *et al.*, 2007). In the last decade, growing number of reports investigating association between ROS and carcinogenesis have been published. Researchers have proposed various consequences of oxidative stress that may be linked to carcinogenesis (Cejas *et al.*, 2004; Valko *et al.*, 2006; Mena *et al.*, 2009). There was increased TBARS in the liver and kidney of the MNU induced rats, this observation is in accordance with previous work (Hendrickse *et al.*, 1994*;* Skrzydlewska *et al***.,** 2001b), who reported increase plasma and tissue MDA concentrations in colorectal patients this may be as a result of cellular membrane degeneration and DNA damage due to oxygen radical production (Skrzydlewska *et al.*, 2005). Recent research by Perse (2013) revived that the gut mucosa possesses various protective mechanisms to neutralize effects of increased production of free radicals, that is, thick secreted mucosal layer, which represents important physical and chemical protective defence to luminal content and strong antioxidative protective mechanism. The body has an effective defence mechanism to prevent and neutralise free radicals-induced damage. This is accomplished by a set of endogenous antioxidant enzyme such as superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase (SOD) is an important element of cellular defense against the toxicity of oxygen free radicals. It catalyzes the dismutation of superoxide free radical to hydrogen peroxide and molecular oxygen (Klimczak *et al.*, 2010). The increased SOD activity in colon cancer cells may be an indicator of greater need for protection against the toxic effects of ROS however, MnSOD also protects

cells against cytotoxic effects (Czeczot *et al.,* 2005). This finding is in accordance with that reported by Skrzydlewska et al., 2001a; Strzelczyk *et al*., 2012 and Kocot *et al*., 2013. Treatment with the appropriate levels of supplement diets of both leaves of *G. mollis* and *V. donianan,* significantly reduced the SOD levels. Catalase (CAT) is an enzymatic antioxidant widely distributed in animal tissues, and the highest activity is found in the red blood cells and the liver. CAT protects cells from the accumulation of hydrogen peroxide by dismutating it to form water and oxygen or by using it as an oxidant in which it works as a peroxidise (Lenzi *et al*., 1993).This present study showed there was a decrease CAT level in the MNU colon cancer induced group compared to the basal diet control group both in the liver and kidney. This decrease is in agreement with the report of Skrzydlewska *et al* (2005). Upon treatment with appropriate levels of supplementation the CAT levels increased with increasing levels of supplementation.

Thus, this result of this present study and the findings of previous studies showed that colorectal carcinogenesis is associated with serious oxidative stress and that advancement of oxidative-antioxidative disorders is followed by progression of colorectal cancer.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMEDATION

6.1 SUMMARY

i. The supplemented diets of the leaves *G. mollis* and *V. doniana* possessed significant anticarcinogenic activity by reducing CEA level in the serum of MNU induced colon cancer rats.

ii. The increase glandular excretion of globlet cell of the colonic mucosa also confirm the colon cancer chemopreventive effect of the supplemented diets of the leaves of *G. mollis* and *V. doniana* compared to MNU induced colon cancer group.

iii. *G. mollis* and *V. doniana* supplemented diets significantly lowered the TBARS and SOD activity in the liver and kidney of MNU induced colon cancer rats and significantly increase the Catalase activity indicating its antioxidant activity.

6.2 CONCLUSION

The results of this study showed that dietary supplementation with the leaves of *G. mollis* and *V. doniana* possess possible anticarcinogenic activity with the highest activity in 10% leaves supplementation of *G. mollis* and *V. doniana* this is evident with decrease CEA levels and improvement in the glandular excretion of the globlet cells of the mucosa. Dietary supplementation with the leaves of *G. mollis* and *V. doniana* possesses significant antioxidant activity. This present findings therefore, indicate the existence of an abnormal balance between the oxidative and protective mechanisms in MNU induced colon cancer animals. The observations strongly suggest that treatment with antioxidants in the initial stages of the disease may be useful as secondary therapy to prevent oxidative damage.

6.3 RECOMMENDATIONS

1. The isolation and extraction of the phytochemical constituents of the leaves of both plants responsible for the pharmacological activities before supplementation especially for those of *G.mollis* should be studied**.**
2. The study should be carried out for a longer period of time (20-30weeks), focusing mainly on the colon for both histopathological and biochemical assys.

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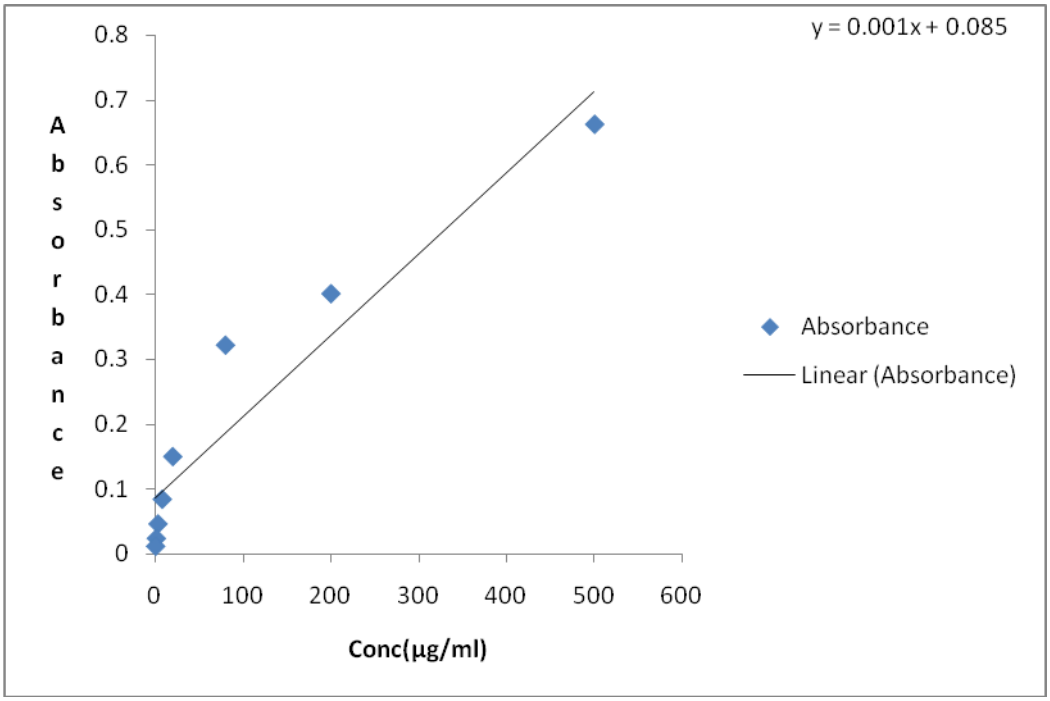
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APPENDICES

APPENDIX 1.0



Total Protein Concentration Standard Curve

APPENDIX 2.0

Levels of carcinoembryonic antigen (CEA) on MNU induced colon cancer rats supplemented with the leaf of *G. mollis* and *V. doniana* diets.

TREATMENT	CEA (ng/ml)	
	G. mollis	*V. doniana*
Control feed	2.28 ± 0.30^{a}	2.28 ± 0.30^{a}
MNU + basal feed	3.88 ± 0.23^{b}	3.88 ± 0.23^{c}
10% supplementation control	2.27 ± 0.14^{a}	2.35 ± 0.23^{a}
MNU + 2.5% supplementation	2.95 ± 0.22^{a}	3.18 ± 0.23^{bc}
MNU + 5% supplementation	2.64 ± 0.28^{a}	2.93 ± 0.27^{ab}
MNU + 10% supplementation	2.55 ± 0.19^{a}	2.80 ± 0.05^{ab}

APPENDIX 3.0

Effect of diet supplementation with leaves of *G. mollis* and *V. doniana* on feed intake on MNU induced colon cancer rats.

TREATMENT	FEED INTAKE (g)	
	G.mollis	*V.doniana*
Control feed	83.07 ± 3.93	83.07 ± 3.93
MNU + basal feed	76.01 ± 5.68	76.01 ± 5.68
10% supplementation control	103.88 ± 9.26	85.76 ± 4.50
MNU + 2.5% supplementation	85.89 ± 3.14	79.25 ± 1.58
MNU + 5% supplementation	89.04 ± 5.01	85.82 ± 4.34
MNU + 10% supplementation	86.05 ± 1.42	82.23 ± 1.70

APPENDIX 4.0

Effect of Dietary Supplementation with leaves of *G. mollis* and *V. doniana* on Lipid Perioxidation on the liver of MNU induced colon cancer rats.

TREATMENT	TBARS (μmol/mg protein)	
	G.mollis	*V.doniana*
Control feed	0.37 ± 0.05	0.37 ± 0.05
MNU + basal feed	1.02 ± 0.04	1.02 ± 0.04
10% supplementation control	0.44 ± 0.05	0.18 ± 0.01
MNU + 2.5% supplementation	0.80 ± 0.04	1.02 ± 0.09
MNU + 5% supplementation	0.77 ± 0.05	0.65 ± 0.03
MNU + 10% supplementation	0.45 ± 0.01	0.53 ± 0.01

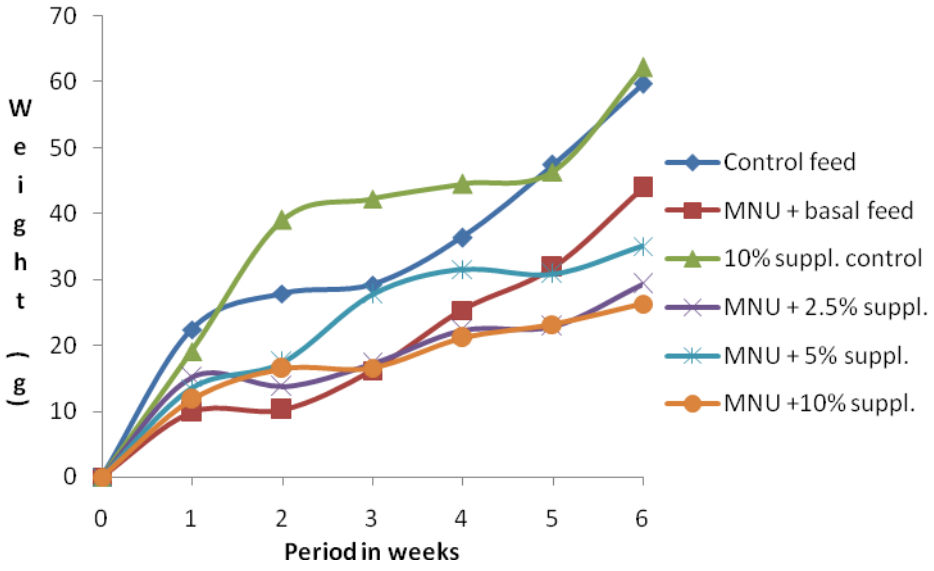
APPENDIX 5.0

Effect of Dietary Supplementation with leaves of *Grewia mollis* and *Vitex doniana* on Lipid Perioxidation on the kidney of MNU induced colon cancer wistar rats.

TREATMENT	TBARS (μmol/mg protein)	
	G.mollis	*V.doniana*
Control feed	0.23 ± 0.01	0.23 ± 0.01
MNU + basal feed	1.73 ± 0.11	1.73 ± 0.11
10% supplementation control	0.14 ± 0.03	0.28 ± 0.01
MNU + 2.5% supplementation	0.57 ± 0.08	1.74 ± 0.07
MNU + 5% supplementation	0.51 ± 0.05	0.72 ± 0.02
MNU + 10% supplementation	0.28 ± 0.01	0.31 ±0.02

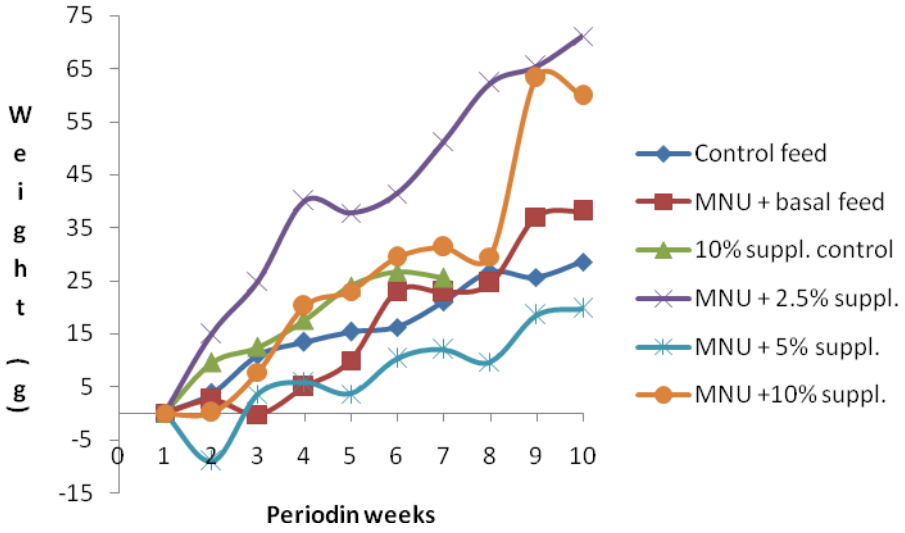
APPENDIX 6.0

Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Pre-treatment of MNU induced colon cancer in wistar rats.



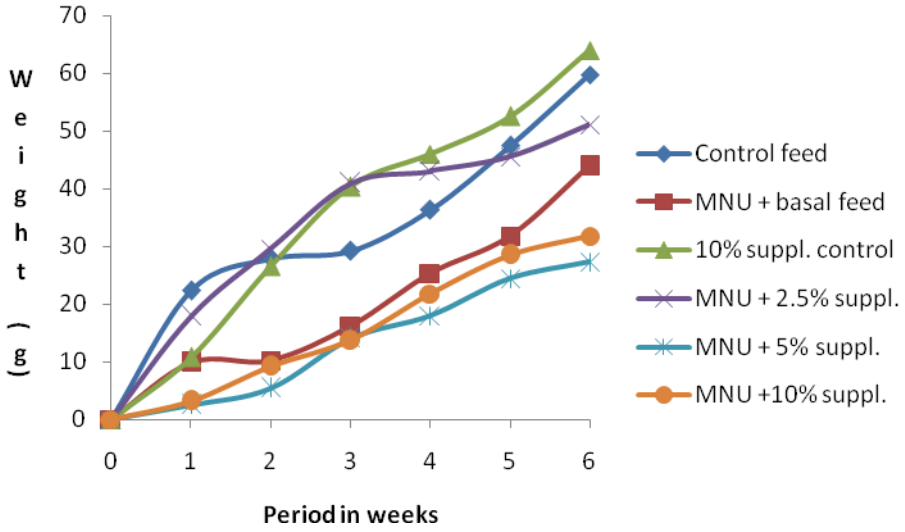
APPENDIX 7.0

Effect of *G. mollis* Supplementation on Percentage Cumulative Change in Weight for Post-induction of MNU induced colon cancer in wistar rats.



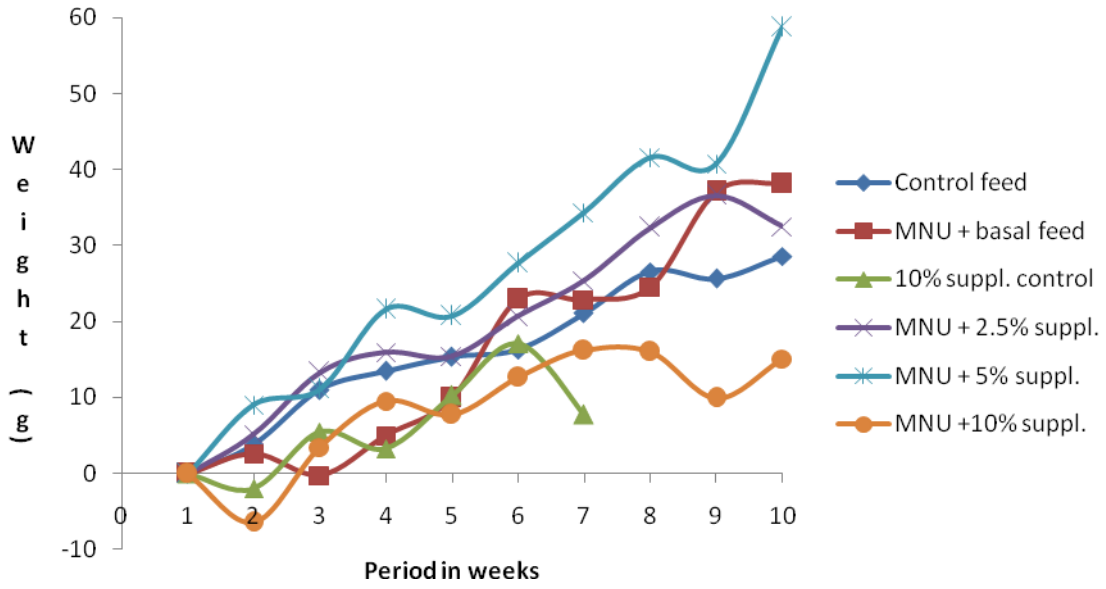
APPEDIX 8.0

Effect of *V. doniana* Supplementation on Percentage Cumulative Change in Weight for Pre-induction of MNU induced colon cancer in wistar rats.



APPENDIX 9.0

Effect of *V. doniana* Supplementation on Percentage Cumulative Change in Weight for Post-induction of MNU induced colon cancer in wistar rats.



APPENDIX 10.0

Preparation of the reagents for the determination of Superoxide Dismutase (SOD) activity

Sodium carbonate buffer (pH 10.2, 50mM): 14.3g of Na_2CO_3 and 4.2g of $NaHCO_3$ were dissolved in distilled water and made up to 100ml in a volumetric flask. The buffer was adjusted to pH 10.2.

Epinephrine (0.03mM): 0.0549g of epinephrine was dissolved in distilled water and made up to 1000ml in a measuring cylinder.

APPENDIX 11.0

Preparation of the reagents for catalase assay

Hydrogen peroxide (0.2M): 0.6ml of H_2O_2 was dissolved in little quantity of distilled water and made to100ml and stored in a dark room.

Phosphate buffer (0.005M, pH 7.0).

Dichromate/ Acetic acid: 5% potassium heptaoxochromate (VI), $K_2Cr_2O_7$, was mixed with glacial acetic acid (100%) in the ratio 1:3, and was stored in amber bottle at room temperature.

APPENDIX 12.0

Preparation of the Reagents for determination of lipid peroxiadation by measuring the malondialdehyde (MDA) level

Thiobarbituric acid TBA (0.8%): Exactly 0.8g of TBA was dissolved in little amount of distilled water and the volume made up to 100 ml in a volumetric flask.

Sodium dodecyl sulphate SDS (8.1%): Accurately 8.1g of SDS was dissolved in distilled water and the volume made to 100ml in a measuring cylinder.

Acetic buffer, pH 3.5 (20% glacial acetic acid): Distilled water was added to 20ml of acetic acid, the volume was then made up to 100 ml in a volumetric flask.

n- Butanol/pyridine (15:1 v/v): 10 ml of pyridine was added to 150 ml of n- butanol. The mixture is then stored in a 250 ml amber bottle for further use.