ATTENUATING EFFECTS OF KOLAVIRON ON EXPERIMENTAL COLITIS IN WISTAR RATS DURING CHRONIC CADMIUM EXPOSURE

BY

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ABSTRACT

Prevalence of Ulcerative Colitis (UC) has been attributed to environmental pollutants from industrialisation due to heavy metals accumulation. Cadmium has been implicated in the aetiology of diseases as well as interference with gut mucosal integrity. Use of synthetic drugs like sulfasalazine in the management of UC often produces unwanted side effects. However, kolaviron has been reported to attenuate UC without producing adverse effects. There is paucity of information on the effect of cadmium exposure on UC attenuation by kolaviron. Hence, its effect on colitis during chronic exposures to cadmium was investigated.

Eighty-five male Wistar rats (110 – 120g) were randomly assigned into 5 groups as follows: Negative control (*negCont*) n=5, and n=20 in each of Positive control (*posCont*), Cadmium (Cd), Cadmium+Kolaviron (Cd+KV) and Kolaviron (KV). Kolaviron was extracted from *Garcinia kola* seeds using Soxhlet apparatus. After four weeks of cadmium (100ppm) and kolaviron (200mg/kg) administration, colitis was induced in all groups except *negCont* by intrarectal administration of 2 mL of 4% acetic acid (AA) and assessed based on symptoms of colitis such as weight loss and stool consistency using a diarrhoea score scale. Five rats were sacrificed before and at 3, 7, 14 days post-colitis induction in each of the treated groups. Colon samples were thereafter collected, homogenised and analysed for concentrations of Malondialdehyde, Nitric oxide (NO), Glutathione (GSH), Sulfhydryl and activities of Superoxide dismutase (SOD), Na^{+}-K^{+} ATPase, Myeloperoxidase by spectrophotometry. Colonic tissue damage was assessed histopathologically (using hematoxylin and eosin Stain) using microscopy. Relative gene expressions of Tumour Necrosis Factor-alpha (TNF-α), Interleukin 10 (IL-10) and Occludin in colonic tissues were quantified by RT-PCR. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The weight loss (%) in Cd group by week 3 relative to *negCont* was (31.05±0.04), while weight gain (%) in *posCont* and Cd+KV relative to Cd group were 36.19±0.73 and 36.17±0.51 respectively. Intrarectal administration of AA resulted in increased stool score in Cd (0.80±0.00) till day 14 post colitis induction compared with *posCont* (0.00±0.00) and Cd+KV (0.00±0.00). Macroscopic score was significantly increased in Cd (2.80±0.25cm) till day 14 compared with *posCont* and Cd+KV (0.60±0.33cm; 0.60±0.25cm). Malondialdehyde were significantly

increased in Cd group by 24.6% and 13.3% on days 7 and 14, respectively. Histology showed healing in all groups post colitis induction unlike in Cd group. The notable increases in Myeloperoxidase and NO during AA-induced colitis and Cd exposure were significantly reduced by kolaviron. Significant decreases in SOD, Na^{+}-K^{+} ATPase activities, sulfhydryl and GSH concentrations upon AA administration were ameliorated in other groups except Cd group. Relative gene expression of TNF-α was up-regulated while IL-10 and Occludin were down-regulated upon induction of colitis; kolaviron caused down-regulation of TNF-α while IL-10 and Occludin were up-regulated on days 7 and 14 of treatment in KV and Cd+KV groups.

Kolaviron ameliorated delayed healing of acetic acid-induced colitis during cadmium exposures in Wistar rats.

Keywords: Ulcerative colitis, Cadmium chloride, Kolaviron

Word count: 470

DEDICATION

To:

God, through Jesus Christ my Lord, in whom lie hidden all the treasures of wisdom and knowledge, and;

The memory of my father; Rev'd AO^2 Adegoke,who eagerly waited to see the completion ofthis but passed on few months ago;your life and the uncompromising principles that guided it will forever be in my memory.

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CERTIFICATION

I certify that this work was carried out by ADEGOKE, Adeoti Gbemisolain the Department of Physiology, College of Medicine, University of Ibadan.

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LIST OF ABBREVIATIONS

AA – Acetic Acid

ANOVA – Analysis of Variance

ANS – Autonomic Nervous System

ARE – Antioxidant Response Element

ATP – Adenosine Triphosphate

B-actin – Beta Actin

BSA – Stock Bovine Serum Albumin

CAT – Catalase

Cd – Cadmium

CD – Crohn's Disease

cDNA – Complementary Deoxyribonucleic Acid

CO_2 – Carbondioxide

DCT-1 – Divalent metal transporter-1

DNA – Deoxyribonucleic acid

DSS – Dextran sodium sulphate

ECM – Extracellular Matrix

EDTA – Ethylene Diamine Tetra Acetic acid

EGF – Epithelial Growth Factor

EGF-R – Epithelial Growth Factor Receptor

GALT – Gut-associated lymphoid tissue

gDNA – Genomic Deoxyribonucleic Acid

GIT- Gastrointestinal tract

GSH – Glutathione

GSH – Reduced glutathione

H&E – Haematoxylin and eosin,

H_2O_2 – Hydrogen Peroxide

H_2O_2– Hydrogen peroxide

H_3PO_4 – Phosphoric Acid

HNO3 – Nitric Acid

HTAB – Hexadecyltrimethlammonium bromide

IBD – Inflammatory Bowel Disease

ICAM-1 – Intercellular adhesion molecule 1

IEC – Intestinal epithelial cells

IL – Interleukin

KCl – Potassium Chloride

KV – Kolaviron

LPO – Lipid peroxidation

LPS – Lipopolysaccharide

MDA – Malondialdehyde

MPO – Myeloperoxidase

mRNA – Messenger RNA

$NaNO_3$ – Sodium Nitrite

NaOH – Sodium Hydroxide

NED – Naphthylethylenediamine

negCont– Negative Control

NLR – Neutrophil-Lymphocyte Ratio

NO – Nitric Oxide

NSAID – Non-Steroidal Anti-inflammatory Drugs

O_2 – Oxygen

$O_2^{-\cdot}$ – Superoxide radicals

PCR – Polymerase Chain Reaction

PECAM-1 – Platelet endothelial cell adhesion molecule 1

posCont – Positive Control

PPM – Part Per Million

RNA – Ribonucleic acid

ROS – Reactive oxygen species

ROS – Reactive Oxygen Species

RT – Reverse Transcriptase

SOD – Superoxide Dismutase

SOD – Superoxide dismutase

TAE – Tris-Acetic Acid-EDTA

TBA – Thiobarbituric Acid

TCA – Trichloric Acid

TNF-α – Tumor necrotic factor α

UC – Ulcerative Colitis

VCAM-1 – Vascular cell adhesion molecule 1

CHAPTER ONE

INTRODUCTION

Inflammatory Bowel Disease (IBD), which includes Crohn's disease and ulcerative colitis,is an idiopathic disorder of the intestine. It is usually characterized by uncontrolled intestinal inflammation resulting from a deranged immune response to the gut microbiota in a genetically susceptible host. It may also be caused byenvironmental factors which influence the normal gut flora and trigger an inappropriate mucosal immune system response (antibody-antigen reaction against the normal mucosal resistance) (Loftus, 2004; Halfvarson *et al*., 2006; Cadwell *et al*., 2008; Petnicki-Ocwieja*et al*., 2009; Kaser *et al*., 2010; Abraham and Medzhitov, 2011).

Genetic IBD research has been helpful in identifying a number of genes that are susceptibleto this disease (Jess *et al*., 2005; Gaya *et al*., 2006); oxidative stress response gene when activated by cytokines like TNF-α, IL-1β, IL-6 and IL-8 released from macrophages has been shown to generate reactive oxygen species (ROS) which plays vital role in the development and maintenance of IBD (Larrick and Wright, 1990; Seo *et al*., 1995; Nagore *et al*., 2010). Also, genetic variants in caspase recruitment domain (CARD) (for example in CARD9 and CARD11) are associated with increased risk of ulcerative colitis (Zhernakova *et al*., 2008; Jostins *et al*., 2012), a two-marker C-type lectin domain family 7 member A (CLEC7A) haplotype,associated with medically refractory ulcerative colitis (Iliev *et al*., 2012) and IL-1 to IL-6, IL-10, IL-12 to IL-13, IL-15, IL-17 to IL-24, IL-26 and IL-27 ligands and/or receptors; several tumour necrosis factor (TNF) superfamily ligands and receptors; interferon and interferon-regulatory factors (Van Limbergen *et al*., 2014), mucosal barrier integrity genes such as adherens junction protein E-cadherin (CDH1), G protein $G\alpha_{12}$(GNA12), protein tyrosine phosphatase family member (PTPN2) (Khor *et al*., 2010)and many others.However, none of these genes alone have been identified as sufficient enough to initiate the disease (Kaser *et al*., 2010; Kabi *et al*., 2012).

Environmental risk factors including smoking, appendectomy and exposure to antibiotics (Cosnes *et al*., 2002; Cosnes, 2004; Pinsk *et al*., 2007; De Vroey *et al*., 2010; Hviid *et al*., 2011; Molodecky *et al*., 2012)oral contraceptives, diet, appendectomy, breast feeding, vaccination, infections, and childhood hygiene (Loftus, 2004; Halfvarson *et al*., 2006; Molodecky and

Kaplan, 2010) may be involved in IBD pathogenesis because of their many indirect link to the disease. However, these factors too do not completely explain the rising incidence of the disease.Moreover, reported observations have not been consistent, making more studies necessary in understanding the etiology and pathophysiology of IBD.

Inflammatory Bowel Disease has become a common finding in the diagnosis of gastrointestinal tract diseases in developed and developing countries(Kaplan *et al*., 2010; Ananthakrishnan *et al*., 2011) with temporal trends indicative of increasing incidence and prevalence across various regions of the world (Molodecky *et al*., 2012). Increasing prevalence rates is evident among immigrants from low-incidence regions moving to developed countries and a correlation of prevalence rates with industrialization in Hong Kong and mainland China (Leong *et al*., 2004; Zheng *et al*., 2005). In Nigeria also, there are increases in reported cases; from Benin (Ohanaka and Njoku, 2006; Obaseki and Forae, 2014), Lagos (Senbanjo *et al*., 2012), Zaria (Ukwenya *et al*., 2011), Ife (Alatise *et al*., 2012), and Owerri (Ekwunife *et al*., 2015). This increase has largely been attributed to environmental influences as a result of urbanization and industrialization, better diagnostic tools and increased awareness by physicians (Ekwunife *et al*., 2015).These highlightsthe potential role of industrialization and its resultant environmental pollutants in the pathophysiology of IBD.

Industrialization has led to an increased accumulation of environmental pollutants, particularly heavy metals, in our surrounding ecosystems. One of such heavy metal is cadmium, which is found in both natural and industrial environments (Waalkes *et al*., 1992) due to their wide natural distribution and extensive use in the industry (Wu *et al*., 2008). Cadmium is an element of the group IIb in the periodic table, air, water, food and cigarette represent significant sources of cadmium exposure (Friberg *et al*., 1986). It presents a high rate of soil to plant transference (Satarug *et al*., 2002) making it to be found easily in plant and food products.Acute intoxication with cadmium is however rare; more frequently it is chronic such that even at low doses, the metal is still toxic due to its ability to accumulate. Its biological half-life in humans is 10–30 years (Wu *et al*., 2008). This cumulative effect on the organs or tissues results in metabolic disorders and, subsequently, pathological changes in some organs (Hughes *et al*., 2000; Vasquez *et al*., 2015).

Cadmium is toxic to many organs, including liver, kidney, lung, testis and brain (Oliveira *et al*., 2012) moreover, it can enter the brain parenchyma and neurons causing neurological alterations in humans and animal models, leading to lower attention, olfactory dysfunction and memory deficits (Lukawski *et al*., 2005; Nishimura *et al*., 2006).

On the gastrointestinal tract, oral cadmium intake can damage the mucosa lining of the stomach (Singh *et al*., 2012), and also, in the colon because only about 5% of the total orally ingested cadmium is absorbed in the intestines, the remaining 90-95% is excreted, this way, colonic epithelial cells are exposed to cadmium in fecal matter as well as to cadmium present in the circulation (Singh *et al*., 2011).

Medicinal plants' components such as flavonoids, phenols and their metabolites may be good prospectus for metal chelation *invivo*. Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups (Michalak *et al*., 2006). Specialized phenol storing cells which occur in several plant species (Martyn *et al*., 1993) and flavonoids possess ability to chelate (bind) metal ions and quench singlet oxygen (Hall and Cuppett, 1997). This appears to contribute to their antioxidant activity *invitro*(Cheng and Breen, 2000; Mira *et al*., 2002). Metal-chelating activities of flavonoids may be beneficial in pathological conditions of heavy metal excess, as such flavonoids or their metabolites together with phenols may be able to function as effective metal chelators *invivo*(Frei and Higdon, 2003).

Plants and spices used in folk and traditional medicine have gained wide acceptance for the treatment of various acute and chronic diseases (Aruoma *et al*., 2006).Polyphenols and flavonoids are products from plants, and they possess potential benefits for human health due to their biological activities (Terashima *et al*., 2002), they have potentials for reducing chronic inflammation in experimental model of IBD (Havsteen, 1983). Flavonoids have free radical scavenging ability and inhibitory potential for pro-inflammatory cytokines (Mora *et al*., 1990). They exhibit their antioxidant properties through chelating with transition metals, which participate in reactions generating free radicals (Malesev and Kuntic, 2007).

For example, the biflavonoids of *Garciniakola* have been shown to be pharmacologically active with several pharmacokinetic advantages over simple monomeric flavonoids as they survived

first-pass metabolism (Iwu, 1986). Kolaviron is a fraction of the defatted ethanol extract of *Garcinia kola*, containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone as its major components (Iwu, 1985).

1.1 Statement of Problem and Justification

Previous studies have shown that cadmium depletes antioxidant substances such as glutathione and protein bound sulfhydryl groups and thereby induces oxidative stress (Mojzis and Nistiar, 2000;Waisberg *et al*., 2003; Cinar *et al*., 2010; Goncalves *et al*., 2010)which results from the production of reactive oxygen species (ROS), interferes with endogenous antioxidant systems and expression of certain proteins (Gupta *et al*., 1991) which play significant roles in inflammatory disorders.

In contrast to cadmium is kolaviron (a *Garcinia kola* extract), a biflavonoid complex that hasbeen found to have anti-inflammatory (Iwu, 1986; Olaleye *et al*., 2000), antiulcer (Ibironke *et al*., 1997) and antioxidant properties. *Garcinia* bi-flavonoids prevent lipid peroxidation invivo (Farombi *et al*., 2000; Adaramoye *et al*., 2005) by inhibiting hydroxyl and superoxide anion radicals which are known to play important role in the process of lipid peroxidation (Farombi and Nwaokeafor, 2005), and lipid peroxidation has been implicated in many pathological conditions including ulcerative colitis and cadmium toxicities (Mora *et al*., 1990).

In spite of the known anti-inflammatory and antioxidant properties of kolaviron, it is not known if it has potential to reverse the actions of pro-inflammatory agents such as cadmium during colitis and also, what dosage of kolaviron may be appropriate in this condition.

1.2 Aim of Study

The study is designed to investigate the effect(s) of kolaviron on colitis and its healing in cadmium exposed rats and to explore the possible underlying mechanisms by assessing various macroscopic, microscopic, biochemical and molecular parameters.

1.3 Objectives

The objectives of the study were to:

i. Study the effect(s) of cadmium on some blood and biochemical parameters.
ii. Study the effects of cadmium on colon histopathology during colitis.
iii. Study the effect(s) of cadmium exposure on colitis healing.
iv. Study the effect of kolaviron and its possible effective dosage during colitis in cadmium exposed rats.
v. Study the effect of kolaviron on full blood count in acetic acid induced colitis during cadmium exposures in rats.
vi. Study the effect of kolaviron on body weight, stool consistency, colon macroscopic scores in colitis during cadmium exposures in rats.
vii. Assess the effect of kolaviron on histomorphology of the rat's colon in colitis during cadmium exposures.
viii. Assess the effect of kolaviron on levels of protein, MDA,NO, GSH, sulfhydryland the activities of SOD, catalase, and MPO in colitis during cadmium exposures in rats.
ix. Assess the effect of kolaviron on the gene expression of TNFα,IL-10 andoccludinin colitis during cadmium exposures in rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Gastrointestinal Tract

The gastrointestinal tract (Figure 2.1) consists of mouth, esophagus, stomach, small intestine, colon and anus. It gives a link between the external and internal environment and is one of the largest surfaces in the body, lengthening up to 9metres (Kong and Singh, 2008).Approximately 60 tons of food passes through the gastrointestinal tract in a normal lifetime (Bengmark, 1998; Johnson, 2001). Food is obviously important for living, but its passage through the gastrointestinal tract can constitute threat to health especially when it has contaminants or allergens in it. Thus, the gastrointestinal tract functions in digesting, absorbing nutrients from food and water, and removing dietary antigens, viable micro-organisms and bacterial products (endotoxins, hydrogen sulphide, phenols, ammonia, and indoles) which can be detrimental on both the intestinal mucosa and host health (Barrie and Lee, 1992; Macfarlane and Macfarlane, 1995).

2.1.1 The Colon

The colon is a 5-6-foot-long muscular tube composed of lymphatic tissues, blood vessels, connective tissues, and specialized muscles for carrying out the functions of water absorption and removal. The colon has no villa (multiple, minute projections of the intestinal mucous layer which serves to absorb food and nutrients) and produces no digestive enzymes. It is like a tube of circular muscle lined with a layer of moist mucous cell that secrete mucus which acts as a lubricant for the transport of intestinal constituents (Colon Function and Health Information, 2010).

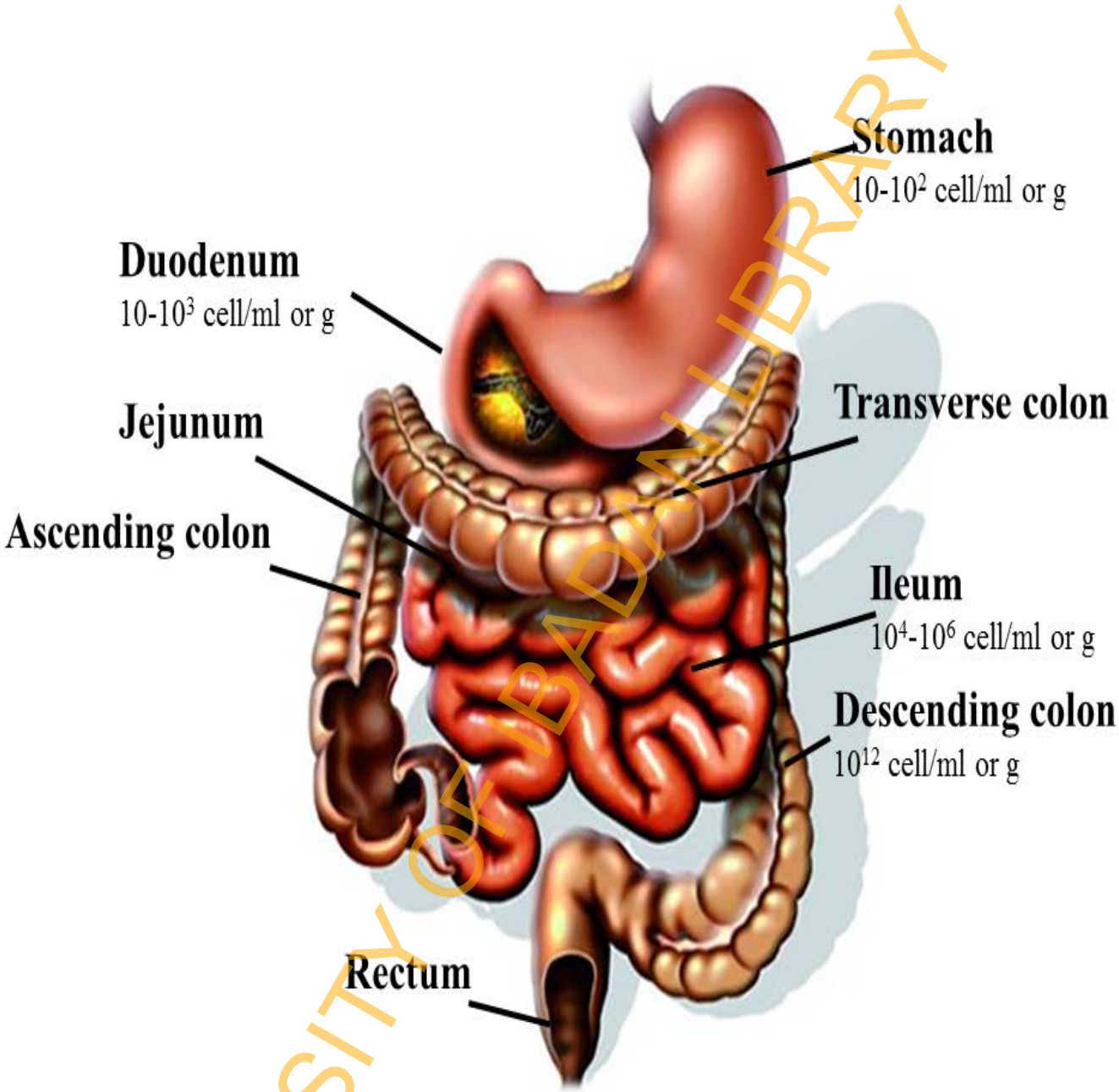


Figure 2.1: Overview of the human gastrointestinal tract (Gibson, 2004; Thomassen*etal*.,2011).

The smooth folds of the colon are spotted with glands that resemble skin pores. It has small pouches called haustra and these are caused by sacculations which give the colon its segmented appearance. Taenia coli run the length of the colon and because it is shorter, the colon becomes sacculated between the taenia, forming the haustra. As the stool moves through the colon, fluids are removed and absorbed into the body. The consistency of the stool is dependent upon many things; including how long the stool stays in the colon, how much water has been absorbed from the waste, and the amount of fibre and fluids in the diet. Stool consistency can be hard lumps, soft formed, very loose or watery (Colon Function and Health Information, 2010). The colon is anatomically sectioned into four parts (Figure 2.2);

Ascending Colon

The ascending colon is one of four sections of the large intestine; it is connected to the small intestine by the caecum. The ascending colon runs through the abdominal cavity, upwards toward the transverse colon for approximately eight inches (20cm).One of the main functions of the colon is to reabsorb water and other important minerals and vitamins from waste material into the body. As the waste material leaves the small intestine it moves into the caecum and then the ascending colon where this process of extracting starts. The waste material is moved upwards toward the transverse section of the colon by peristalsis (Lichtenstein *et al*., 1998).

Transverse Colon

The transverse colon is the part of the colon from the hepatic flexure to the turn of the colon by the spleen (splenic flexure). The transverse colon hangs off the colon attached to it by a wide band of tissue called the greater omentum. On the posterior side, the transverse colon is connected to the posterior abdominal wall by a mesentery called transverse mesocolon. The transverse colon is ensuited in the peritoneum and is therefore mobile (unlike the parts of the colon immediately before and after it). Cancers form more frequently further along the large intestine as the contents become more solid (water is removed) in order to form faeces. The proximal two-thirds of the transverse colon is perfused by a branch of superior mesenteric artery (SMA) called middle colic artery, while the latter third is supplied by branches of the inferior mesenteric artery (IMA).

The area between these two blood supplies, which represents the embryologic division between the mid and hind gut is an area sensitive to ischemia(Lichtenstein *et al*., 1998).

Descending Colon

The descending colon is the part of the colon from the splenic flexure to the beginning of the sigmoid. The function of the descending colon in the digestive system is to store food that will be emptied into the rectum. It is retroperitoneal in about two-thirds of humans. In the other third, it has a (usually short) mesentery and the arterial supply comes through the left colic artery(Lichtenstein *et al*., 1998).

Sigmoid Colon

The sigmoid colon is the part of the large intestine after the descending colon and before the rectum. The name sigmoid means S-shaped. Its walls are muscular, and it contracts to increase the pressure inside the colon, causing stool to move into the rectum. The sigmoid colon is supplied with blood from several branches (usually between 2 and 6) of the sigmoid arteries, a branch of the inferior mesenteric artery (IMA)(Lichtenstein *et al*., 1998).

2.1.2 Blood Supply and Lymphatics

Arterial supply to the colon comes from branches of the superior mesenteric artery (SMA) and inferior mesenteric artery (IMA). Flow between these two systems communicates through a marginal artery that runs parallel to the colon for its entire length.Historically, it has been believed that the meandering mesenteric artery (of Moskowitz), is a variable vessel connecting the proximal superior mesenteric artery to the proximal inferior mesenteric artery which can be very useful if either vessel is occluded(Lichtenstein *et al*., 1998).

Venous drainage usually depicts colonic arterial supply, with the inferior mesenteric vein draining into the splenic vein and the superior mesenteric vein joining the splenic vein to form the hepatic portal vein that then enters the liver. Lymphatic drainage from the entire colon and proximal two-thirds of the rectum is to the Para-aortic lymph nodes that then drain into the cisterna chili. The lymph from the remaining rectum and anus can either follow the same route, or drain to the internal iliac and superficial inguinal nodes(Lichtenstein *et al*., 1998).

2.1.3 Transport of Electrolyte in the Normal Colon

The epithelial layer covering the inner surface of the mammalian colon is responsible for the transport of electrolytes. The colon in addition to its motor function also has non-motor functions of secretion and absorption, moving salt and water from its mucosa to the blood or vice versa (Barbry and Hofman, 1997). The mammalian colon absorbs up to 1.8 L of electrolyte-rich fluid per day, this accounts for about 90% of the salt and water entering the proximal colon from the ingested and digested food and water (Halm and Halm, 2000).

The rate of Na^+ absorption is most important in colonic water absorption; this can be electrogenic through the sodium channel (ENaC) on the surface epithelium and upper crypts of the distal colonic mucosa or electroneutral through parallel Na^+/H^+ and $Cl^-/HCO3^-$ exchanger in both crypts and surface epithelium of the proximal and distal colonic mucosa (Dawson, 1991; Kockerling and Fromm, 1993;Sandle, 1998).

Colonic mucosal secretion occurs in the surface epithelium and crypts where itenables clearing of the crypts from mucus, secreted from goblet cells and columnar epithelial cells (Strabel and Diener, 1995; Mall *et al.*, 1998). Release of K^+ to the luminal side is potentially driven and largely maintained by the extracellular NaCl (ENaCl). During absorption of NaCl, polarized distribution of transport proteins is required for secretory salt transport. Secretory epithelial cells contain Cl^- and K^+ channels in their luminal membranes, allowing for secretion of KCl. In addition, after secretory stimulation and upon inhibition of absorption, paracellular transport of Na^+ facilitates secretion of NaCl (Schultheiss and Diener,1997; Grotjohann *et al.*,1998; Kerstan *et al.*, 1998; Mall *et al.*, 2000).

2.1.4 Histological Features of the Colon

The histology of the colon is closely related to that of other parts of the gastrointestinal tract; it comprises the mucosa, the submucosa, the muscularis mucosa and the serosa (Figure 2.3).

The Mucosa: It is the innermost layer which has simple columnar epithelium shaped into straight tubular crypts. The mucosa surface of the large intestine is relatively smooth; devoid of plicae circulars or intestinal villi. Crypts of Lieberkum are present and usually longer and straighter than those of the small intestine. Several mucous glands secrete mucous into the

hollow lumen of the large intestine to lubricate its surface and protect it from rough food particles. It has a higher portion of goblet cells interspersed among absorptive cells which is a significant difference in cellular composition from the small intestine. Although absorptive cells remain more numerous throughout the tract, goblet cells too appear to be much (bulging against the adjacent absorptive cells) such that its epithelium appears to consist mostly goblet cells. The crypt epithelium harbours the stem cells which replenish the epithelium every few days, enteroendocrine cells, and (in the caecum and proximal colon) paneth cells. The crypts are differentiated by lamina propria, which is closely related to that of small intestine with loose connective tissue infiltrated by inflammatory cells, capillaries and tiny strands of smooth muscle (Atuma *et al*., 2001; Johansson *et al*., 2011).

The mucosa has two purposes; to protect against luminal content and to serve as a selective barrier for nutrients, water, ions and others. The mucosa is highly folded in the small intestine resulting in the formation of crypts and villi, but in the colon, there are only crypts (Warberg, 2005; van der Flier*et al*., 2009). The mucosa consists of a layer of intestinal epithelial cells (IEC), which is covered by a mucus layer. Under the epithelial cells in the Lamina propria the host immune cells reside in the gut-associated lymphoid tissue (GALT).

The mucus layer

The IEC are covered by a mucus layer that protects it from luminal content, including irritants, microbial attachment, and invasion (Maynard*et al*., 2012; Kim and Khan, 2013). This layer consists ofmucin, IgA, trefoil factor, antimicrobial peptides, water and ions, and has a gel-like structure (Johansson, 2011; Johansson *et al*., 2011; Maynard*et al*., 2012; Kim and Khan, 2013). Mucin, more precisely mucin-2 (MUC2), is the main component of the mucus layer in the intestine, and is secreted by goblet cells in the epithelial layer. When MUC2 is released from the goblet cells its volume increase, and it immediately becomes part of the mucus layer (Johansson *et al*., 2011).

In the small intestine the mucus layer is not well defined, whereas it is well-defined and dense in the colon (Atuma *et al*., 2001; Johansson *et al*., 2011). In the colon, the mucus layer can be divide into two layers. A firm layer close to the IEC, where no bacteria reside, and a loose layer above, where bacteria can reside. This loose layer above is considered to arise by proteolytic

cleavage of MUC2 (Johansson *et al*., 2008). Despite the presence of bacteria in the loose part of the mucus layer, the IEC are still protected from the majority of the luminal content. As the mucus protects the IEC, the maintenance of the mucus layer is important for the intestinal integrity.

The Submucosa: It surrounds the mucosa with a layer of blood vessels, nerves and connective tissue, which supports the outer layers of the large intestine. The submucosa of the lower gastrointestinal tract is relatively unspecialized (Myers, 2007).

The Muscularis Mucosa: The muscularis mucosa of the colon forms a thin layer beneath the deep ends of the crypts. The appearance of the muscularis externa is different from that of the small intestine. The inner circular layer of muscle forms the usual sheath around the large intestine, but the outer longitudinal muscle layer forms three flattened strands, the taenia coli. Only a thin layer of longitudinal muscle surrounds the inner circular muscle layer between the taenia coli. The adventitia forms small pouches (appendices epiploicae) filled with fatty tissue along the large intestine (Myers, 2007).

The serosa: The serosa forms the outermost layer and is a thin layer of simple squamous epithelial tissue that secretes watery serous fluid to lubricate the surface of the large intestine, protecting it from friction between abdominal organs and the surrounding muscles and bones. Serosa is attached to the mesentery (Myers, 2007).

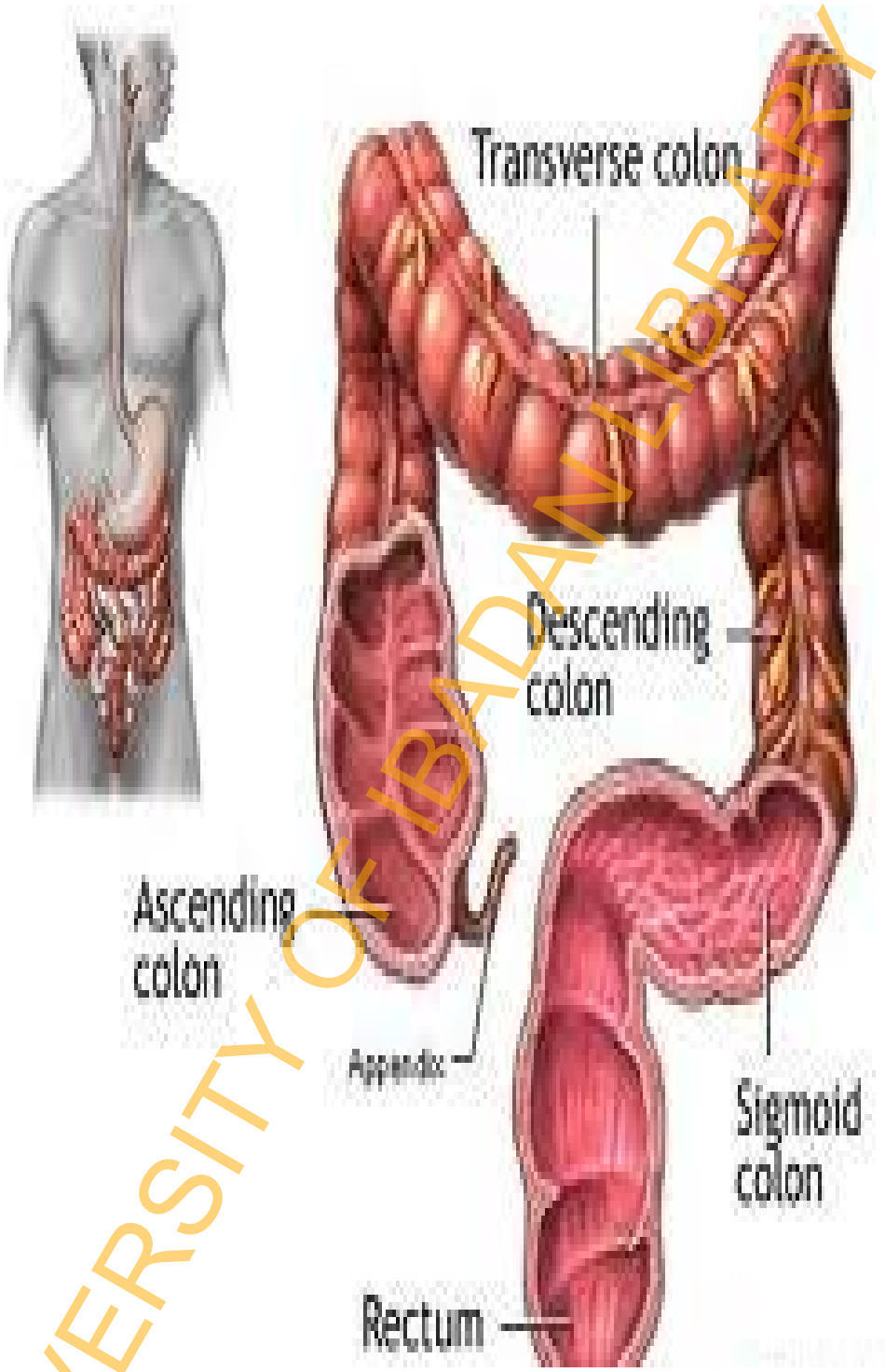


Figure 2.2: The Anatomical sections of the Colon (Lichtenstein *et al.*, 1998).

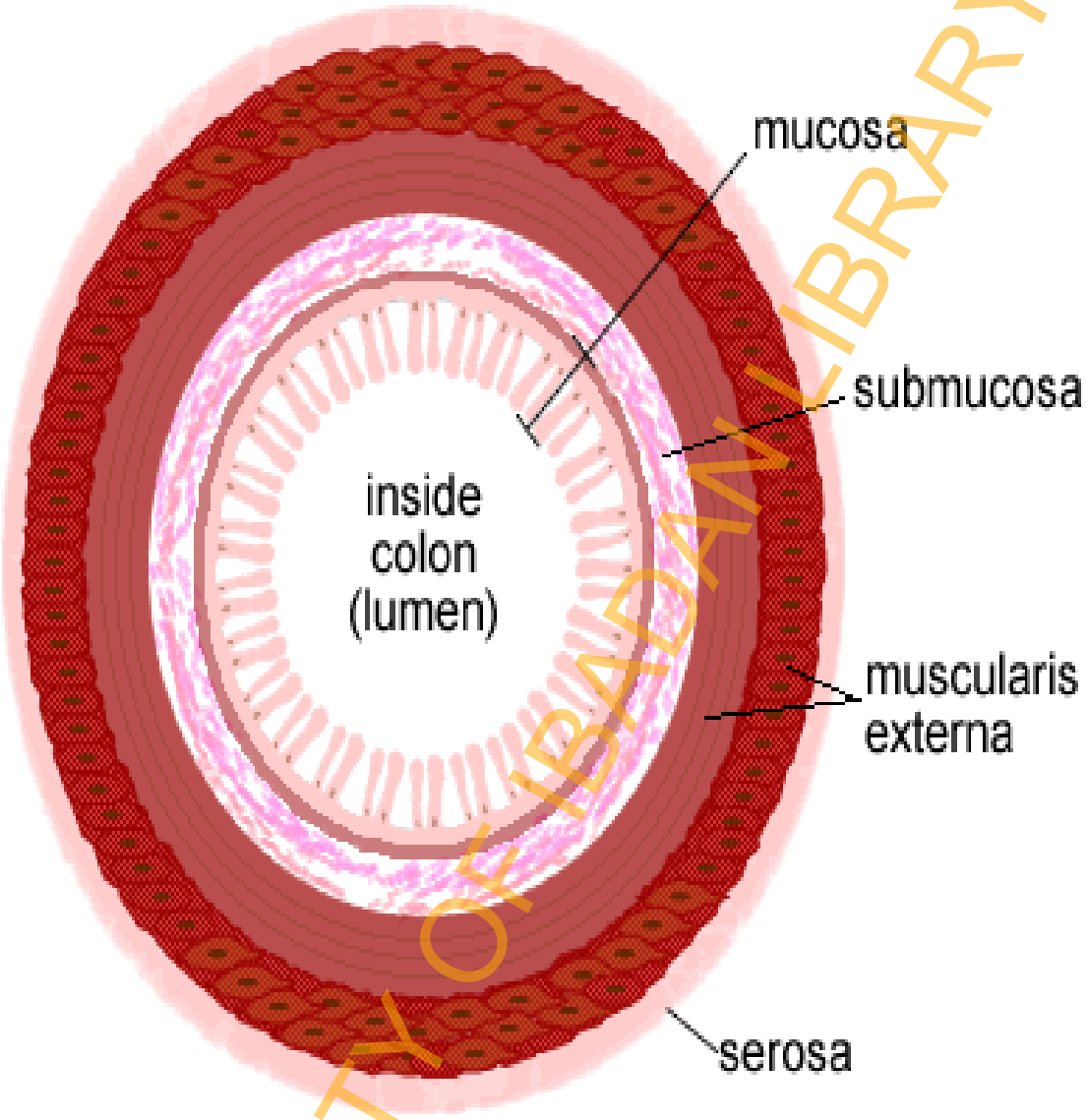


Figure 2.3: Cross section of the colon(Myers, 2007).

2.1.5 The Epithelial Layer

The epithelial layer (Figure 2.4)is about 20µm thick, it is the outer cell layer of the intestinal wall, making it the final cell barrier between the luminal content and the host (Ismail and Hooper, 2005). Generally, IEC are polarised cells with an apical surface towards the intestinal lumen with microvilli, while the basolateral surface, towards the underlying tissue, does not have villi. The selective barrier is constructed by the IEC and contact between adjacent cells. IEC have to initiate an immune response upon bacterial invasion, but also maintain homeostasis upon contact with the gut microbiota (Ismail and Hooper, 2005), as its derangement may lead to a constant inflammatory state. In order to have this complex barrier function andat the same time, absorb nutrients from the lumen, the epithelial layer consists of several cell types each with their own specific task. These include M cells, goblet cells, Paneth cells, and absorptive cells termed enterocytes in the small intestine and colonocytes in colon(van der Flier*et al*., 2009; Maynard*et al*., 2012). Also enteroendocrine cells, that secrete hormones are part of the IEC layer (van der Flier*et al*., 2009). The IEC emerges from stem cells, that differentiate to the different cell types during the migration from the crypt to the villi (van der Flier*et al*., 2009; Sancho*et al*., 2003).

The Epithelial Intestinal Cells:

Enterocytes

Enterocytes (and colonocytes) are highly polarised and are the main cell type in the epithelial barrier constituting up to 80 percent of IEC in the epithelial layer (van der Flier*et al*., 2009). As the enterocytes are the predominate cells in the epithelial barrier, they and their interactions are mainly responsible for maintaining the barrier integrity. Besides this important task, the enterocytes absorb and transport nutrients from the intestinal lumen and into the blood stream (van der Flier*et al*., 2009), and secretes antimicrobial peptides as β-defensins and cathelicidin (Ismail and Hooper, 2005).

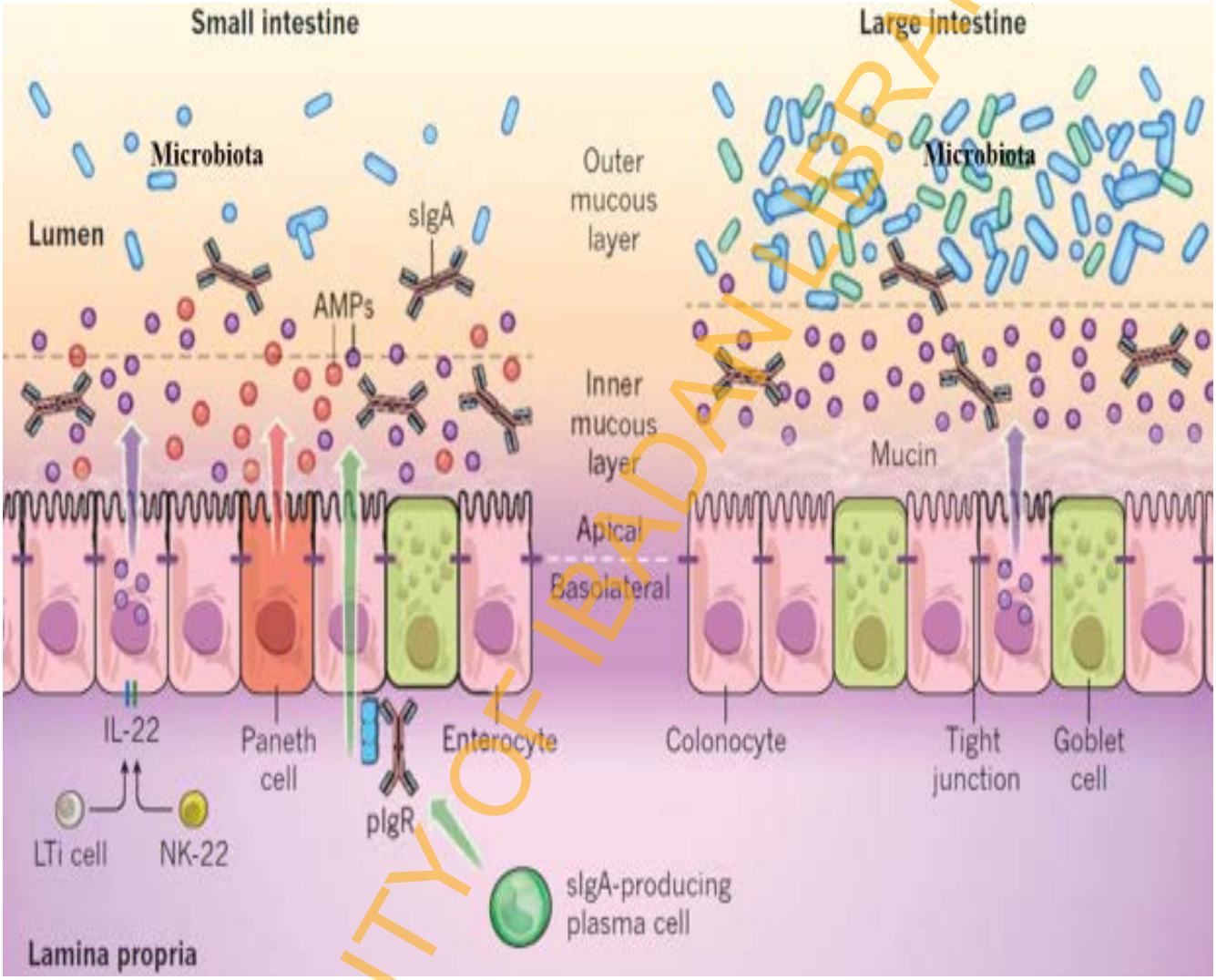


Figure 2.4: The epithelial layer in the small and large intestine(Maynard *et al.*, 2012).

Goblet cells

Goblet cells are the main mucin producers in the epithelial layer, after differentiation they contain granules with mucin, which they release during migration from the crypt. Once reaching the villi the goblet cells have secreted the mucin granules, but also organelles trap between the granules, hence the goblet cells are released into the intestinal lumen, leading to a constant replacement of the goblet cells (Kim and Khan, 2013). Goblet cells are found in the entire intestine, but the number of goblet cells increases downwards through the gastrointestinal tract (Kim and Khan, 2013). Subsequently the level of mucin and thickness of the mucus layer also increases leading to the thick mucus layer in the colon. In addition to secreting mucingoblet cells also release intestinal trefoil factor (TFF) and resistin-Like Molecule-β (RELM-β) that is part of the innate immune system (Sharma *et al*., 2010).

Paneth Cells

Paneth cells are a pyramid shape cell type that does not migrate from the crypt towards the villi but reside at the crypt in the small intestine (Porter *et al*., 2002; Sancho*et al*., 2003; van der Flier*et al*., 2009). In the crypt the Paneth cells take part in the innate immune system as they have granules containing lysozymes, α-defensins, and antimicrobial peptides that are released upon close contact with bacteria but also by cytokine and hormones stimuli (van der Flier*et al*., 2009; Ismail and Hooper, 2005; Porter *et al*., 2002). These anti-microbial peptides are considered to be important for maintaining intestinal homeostasis, since they have been shown to be important for defense against inflammation (Natividad and Verdu, 2013). In addition to the antimicrobial peptides, Paneth cells have been suggested to produce cytokines (Porter *et al*., 2002), making them important for communication between the epithelial layer and the underlying immune system.

M cells

In addition to the already stated cells, a cell type with a different structure termed M cells is found. In humans they do not have microvilli, but micro-folds on the apical side of the membrane. These cells are mainly placed above the Peyer's patches in the small intestine, which

are part of the GALT. But M cells are also found in the colon. M cells are filled with lymphocytes, as the basolateral side of the M cells has pockets that the lymphocytes can reside in (Miller *et al*., 2007). Antigens are transported from the intestinal lumen across the M cells to the immune cells, resulting in antigen presentation and activation of immune cells. For example, B cells that secrete IgA (Kindt*et al*., 2007; Miller *et al*., 2007). M cells are therefore rather important for the communication between the luminal environment and the immune system.

In summary the main barrier between the luminal content and the underlying host tissue is a layer of IEC covered by a mucus layer. Maintenance of this barrier is therefore important for intestinal integrity. Impairment of the barrier may occur by changes in the thickness of the mucus layer, loss of IEC, or decreased interaction between the epithelial cells.

2.1.6 Tight Junction Proteins

Interaction between IEC is important for maintaining intestinal barrier integrity. IEC interacts through tight junctions (TJ), desmosomes, adhesion junctions, and gap junctions (Sharma *et al*., 2010; Suzuki, 2013). Tight junctions(Figure 2.5) are mainly responsible for controlling the paracellular fluxbetween the epithelial cells, hence the permeability of the epithelial barrier, while desmosomes and adhesion junctions are important for communication and binding between the cells (Suzuki, 2013). Tight junctions are complexes of several proteins that reside in the apical section of the epithelial cell (Suzuki, 2013), these proteins include both membrane bound and intracellular proteins that interact between adjacent cells, but also with the cytoskeleton in the cell (Sharma *et al*., 2010; Suzuki, 2013). This helps in stabilising the TJ, but also makes it possible for the cytoskeleton to regulate interactions between IEC (Suzuki, 2013)

Occludin

Occludin (Ocln) is a transmembrane protein with four transmembrane regions, that are linked with two extracellular loops, and one intracellular loop. The extracellular loops of occludin interact between adjacent IEC, forming a barrier between the cells. On the cytoplasmic side occludin has a short N-terminal and a longer C-terminal that interacts with the intracellular proteins in the tight junction (Suzuki, 2013). The importance of occludin in the formation and

maintenance of tight junction has been examined in occludin-deficient mice models and Caco-2 cells (Saitou*et al*., 2000; Schulzke*et al*., 2005; Al-Sadi*et al*., 2011). Occludin deficiency did not affect electrical resistance across intestinal sections, or mannitol permeability in mice (Schulzke*et al*., 2005; Saitou*et al*., 2000). But the mice suffered from chronic inflammation in the gastric epithelium (Saitou*et al*., 2000)and had decreased secretion of gastric acids (Schulzke*et al*., 2005). Based on these results the authors considered occludin to have a regulatory function in the tight junction or regulation of IEC differentiation, but occludin was not considered to be essential for formation of tight junction(Saitou*et al*., 2000; Schulzke*et al*., 2005).

Contradictory to this, Al-Sadi and co-workers found that depletion of occludin in mice and Caco-2 cells increased the flux of macromolecules, indicating that occludin is important for the paracellular flux of macromolecules (Al-Sadi*et al*., 2011). However, this was not connected to a decrease in electrical resistance across the cell layer (Al-Sadi*et al*., 2011). Since electrical resistance, must indicate the permeability of ions, which are able to pass through the same areas as macromolecules. Al-Sadi and co-works did not observe changes in this parameter.

Based on these studies it is difficult to clarify if occludin is essential for the formation of the tight junction, however, as depletion of occludin affected the expression of another tight junction protein claudin-2 at mRNA and protein level (Al-Sadi*et al*., 2011), and occludin-deficient mice had inflammation in the gastric epithelium (Saitou*et al*., 2000), occludin may have importance for intestinal integrity. Regulation of occludin's interactions and localisation have been linked to phosphorylation (Suzuki, 2013). For example, some studies have shown that phosphorylation and de-phosphorylationof occludin by protein kinase C isoforms and the phosphatases PP2A and PPI regulates the formation and break down of the tight junction complex, respectively (Rao, 2009).

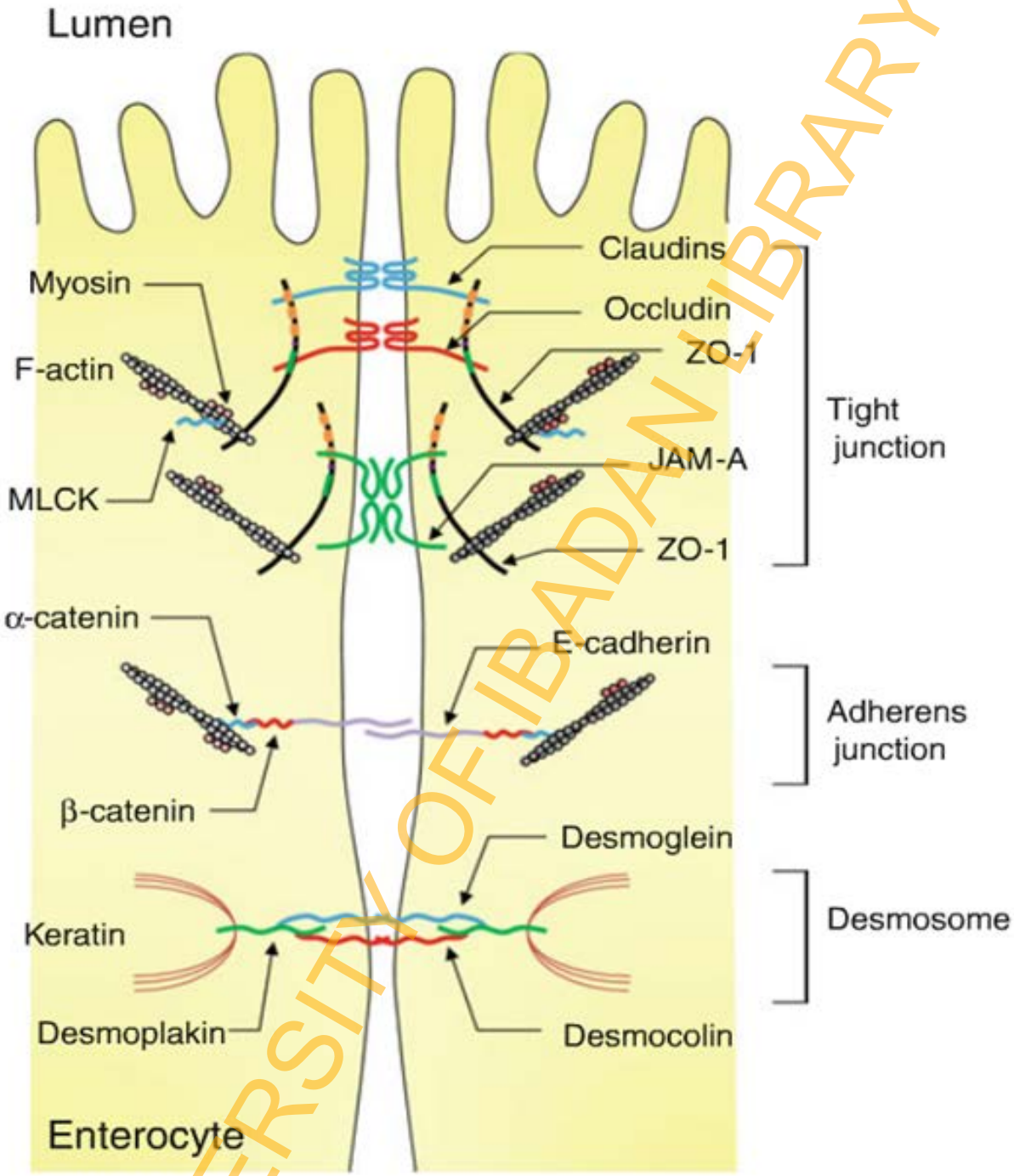


Figure 2.5: Interaction between epithelial cells through tight junction (TJ), adherens junction, and desmosome (Suzuki, 2013).

2.2 Pathophysiology of the Colon

The following are the most common diseases or disorders of the colon: angiodysplasia of the colon, appendicitis, chronic functional abdominal pain, colitis, colorectal cancer, constipation, Inflammatory Bowel disease (Crohn's disease and ulcerative colitis), diarrhoea, diverticulitis, diverticulosis, aganglionosis, irritable bowel syndrome, pseudo membranous colitis, and toxic mega colon (Johnson,2001).

2.3 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is an idiopathic inflammatory disorder of the gastrointestinaltract characterized by inflammation and injury to the intestinal mucosa. Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of IBD (Figure 2.6). Ulcerative colitis primarily affects themucosal lining of the colon and rectum, whereas Crohn's diseaseaffects all layers of the intestine and sometimes, this may extend to any part of the gastrointestinal tract. Inflammatory mediators like tumor necrosis factor-α (TNF-α), interferon-ϒ (IFN-ϒ), and interleukin (IL)-12 produced by infiltrating $CD4^+$ T cells and macrophages play key roles in these disease exacerbation. The etiology of bothCrohn's disease and ulcerative colitis remains unclear, it could be as a result of an aberrant immune response to an environmentaltrigger in a genetically susceptible individual (Sartor, 1997; Maharshak *etal*., 2010).

2.4 Ulcerative Colitis

Ulcerative colitis (UC) is an idiopathic, chronic inflammatory bowel disease (IBD) primarily affecting the colon and the rectum (Podolsky, 1991) and exhibits a clinical course with remission and exacerbations, characterized by abdominal pain, rectal bleeding and diarrhea. The present view is that these conditions arise as a result of the convergence of a genetically determined susceptibility to inflammation and environmental factors (Podolsky, 1991; Maharshak*etal*., 2010). Environmental factors, immunological factors (Figure 2.7), and reactive oxygen species (ROS) have however, been implicated in its pathogenesis (Keshavarzian *et al*., 1990; Millar *et al*., 1996; Fiocchi, 1998 and Sartor, 2006). Its incidence is on the increase in Nigeria, probably because of an increasing industrialization and western dietary lifestyles.

Ulcerative colitis is characterized by increased mucosal infiltration by neutrophils and monocytes which, having become activated, release pro-inflammatory cytokines-tumor necrosis factor α (TNF- α), interferon-γ (IFN- γ), interleukin-1β (IL-1β) and free oxygen radicals which induce increased expression of iNOS, COX-2, nuclear transcription factor NF-kB, protein p66, and enhancement of the lipid-peroxidation processes. These changes cause destruction of the mucous membrane of the large intestine, for example impairment of mucous barrier, swelling, ulcers, erosions, and hemorrhages (Hosoi *et al*., 2001; Martin *et al*., 2005). The destructive changes in the intestinal mucosa due to ulcerative colitis are associated with increased numbers of active forms of oxygen, enhanced synthesis of nitric oxide, expression of inducible nitric oxide synthase (iNOS) by epithelial cells, macrophages, and neutrophil infiltration into the damaged mucous membrane.

2.4.1 Colitis

Colitis is a term used to describe the inflammation (a protective attempt by organisms to remove injurious stimuli and to initiate healing process) of the colon. Colitis causes inflammation and sores called ulcers in the top layers of the large intestine. The inflammation makes the stomach empty frequently, causing diarrhea. Ulcers form in places where the inflammation has destroyed colon lining cells and is called ulcerative colitis (Head and Jurenka, 2003).

2.4.2 Types of Colitis

There are different forms of colitis, this includes; chemical colitis, ischemic colitis, diversion colitis and ulcerative colitis.

A. Chemical Colitis

Chemical colitis is a form of colitis caused by the introduction of harsh chemicals to the colon by an enema or other procedure. Chemical colitis can resemble ulcerative colitis, infectious colitis, and pseudo membranous colitis endoscopically. Prior to 1950, hydrogen peroxide enemas were commonly used for certain conditions, this practice often result in chemical colitis (Harish *et al.*, 2006). Harsh chemicals such as compounds used to clean colonoscopies, are sometimes accidentally introduced into the colon during colonoscopy or other procedures and can also lead to chemical colitis.

B. Ischemic Colitis

Ischemic colitis is a medical condition in which inflammation and injury of the colon result from inadequate blood supply. Although uncommon in the general population, ischemic colitis occurs with greater frequency in the elderly, and is the most form of bowel ischemia (Brandt and Boley, 2000; Higgins *et al*., 2005).

C. Diversion Colitis

Diversion colitis is an inflammation of the colon which can occur as a complication of ileostomy or colostomy, often occurring within the year following the surgery. It can also occur in a neovagina created by colovaginoplasty, sometimes several years after the original procedure. In many milder cases of ileostomy and colostomy, diversion colitis is left untreated and disappears naturally.

D. Ulcerative Colitis

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) of unknown cause that exhibits a clinical course with remission and exacerbations, characterized by abdominal pain, rectal bleeding and diarrhea primarily affecting the colon and rectum (Head and Jurenka, 2003).

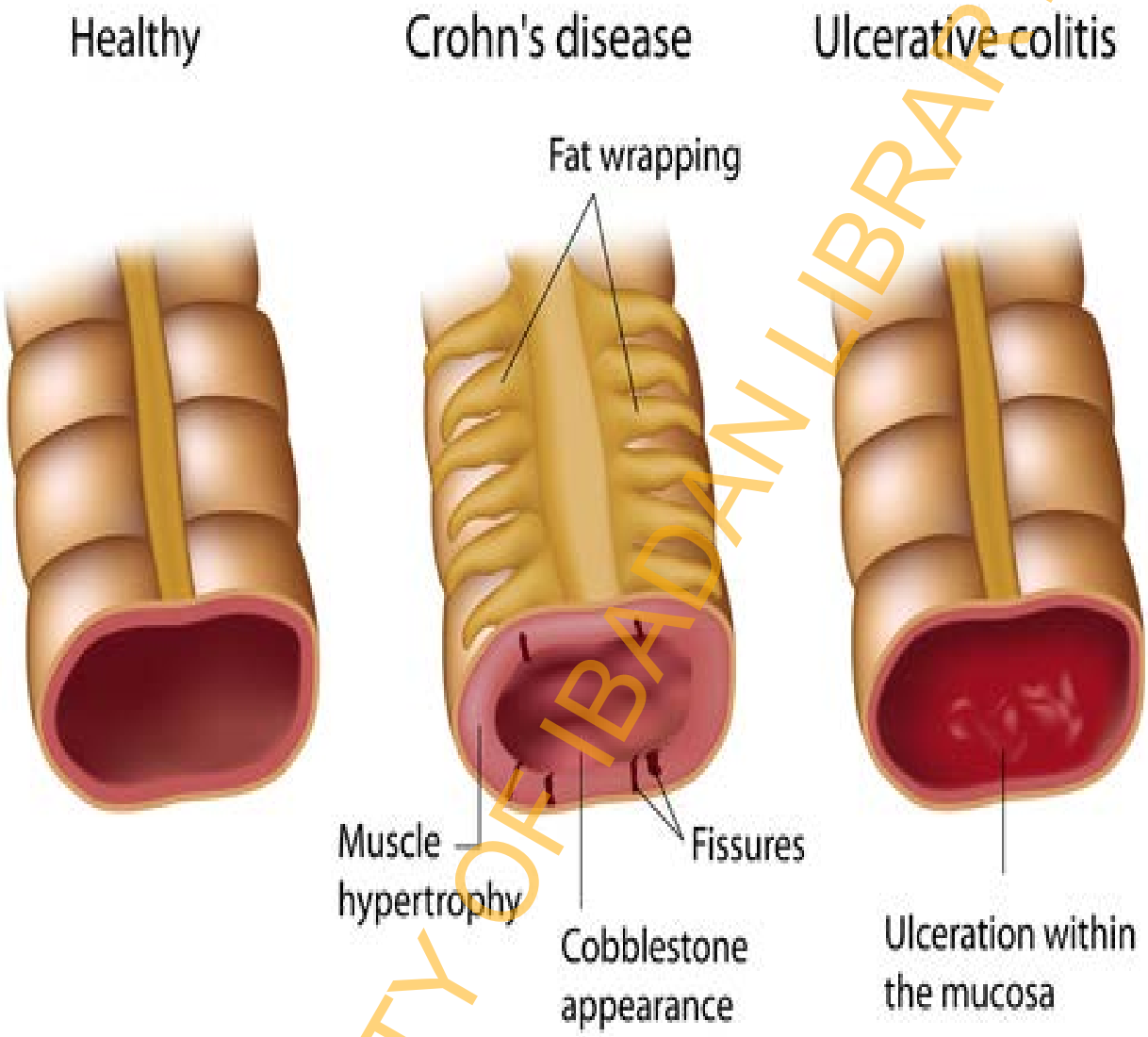


Figure 2.6: Macroscopic appearance of IBD in humans(Anatomic.us, 2013).

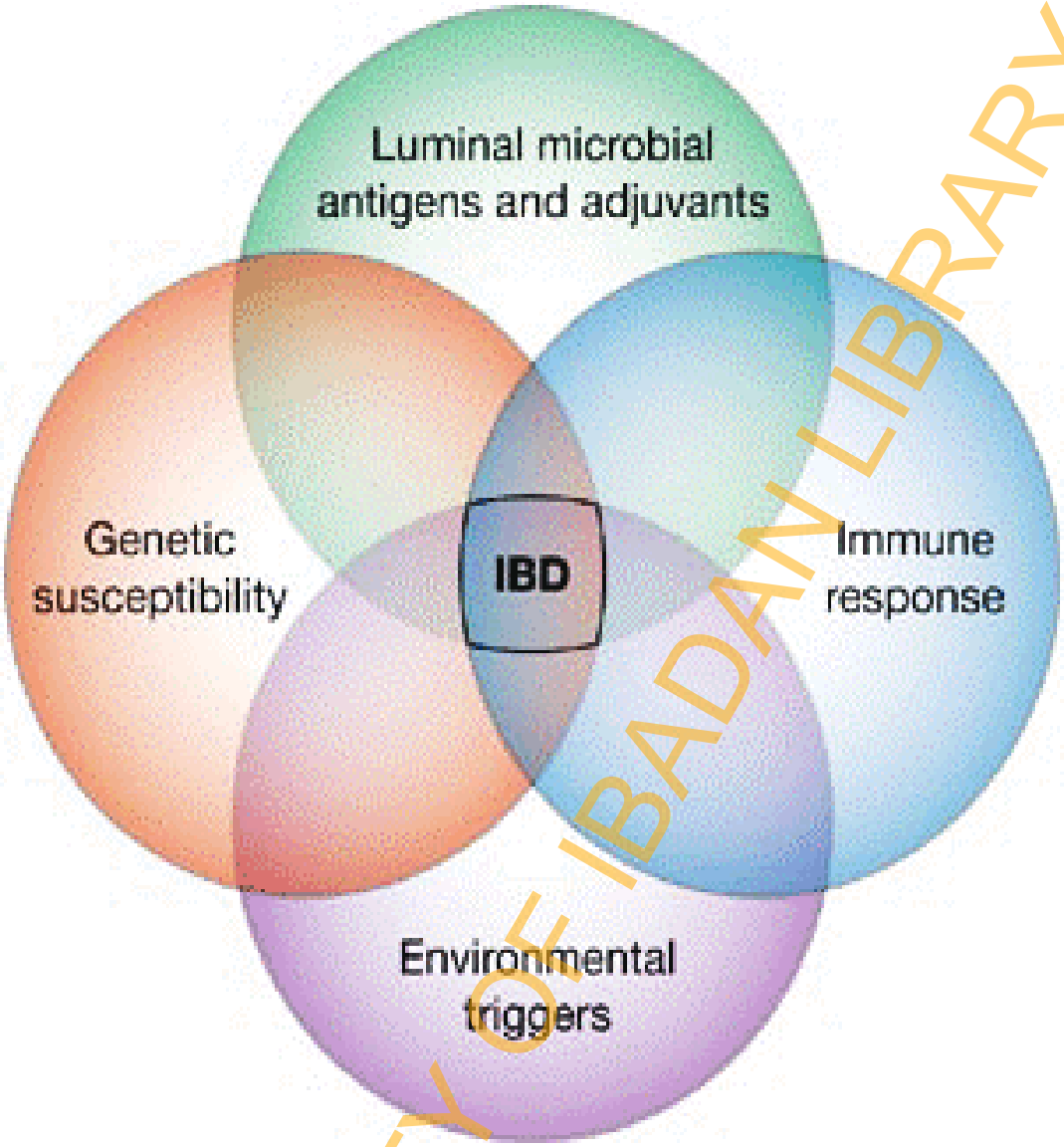


Figure 2.7: Factors implicated in the pathogenesis of Inflammatory Bowel Disease(Sartor, 2006).

2.4.3 Description and Symptom

The predominant symptom of ulcerative colitis is diarrhea which is usually associated with blood in the stool. It is characterized by superficial infiltration of the bowel wall by inflammatory cells, resulting in multiple mucosal ulcerations and crypt abscesses. The lesions are contiguous, typically extending retrograde from the rectum, involving the descending, transverse, or the entire colon. Bowel movements are frequent but small in volume as a result of rectal inflammation, other symptoms include pain in the lower quadrant or rectum. Systemic features, including fever, malaise, and weight loss are more common if a greater portion of the colon is affected. Decreased appetite and interference with the body's ability to absorb nutrients and fluid from the food eaten due to frequent abdominal pain and diarrhea may be the cause of weight loss. Elderly patients often complain of constipation rather than diarrhea because rectal spasm prevents passage of stool (Head and Jurenka, 2003).

The initial attack of ulcerative colitis may be fulminant with bloody diarrhea, but the disease more commonly begins indolently, with non-bloody diarrhea progressing to bloody diarrhea. Ulcerative colitis can present initially with any extent of anatomic involvement ranging from disease confined to the rectum to pancolitis. Most commonly, ulcerative colitis follows a chronic intermittent course with long periods of quiescence interspersed with acute attacks lasting weeks to months. However, a significant percentage of patients suffer a chronic continuous course (Stenson, 2002).

Ulcerative colitis affects the colon and rectum and typically involves only the innermost lining or mucosa, manifesting as continuous areas of inflammation and ulceration, with no segments of normal tissue. The Crohn's and Colitis Foundation of America define several varieties of ulcerative colitis; disease involving only the most distal part of the colon and the rectum is termed ulcerative proctitis; disease from the descending colon down is referred to as limited or distal colitis; and disease involving the entire colon is called pancolitis (Head and Jurenka, 2003).

The natural history of intestinal bowel disease is characterized by relapse and remission, and several factors are known to trigger relapses, including infection, ingestion of non-steroidal anti-

inflammatory drugs and changes in smoking habits (Head and Jurenka, 2003). Animal models of intestinal bowel disease have been useful in confirming these clinical observations, and have demonstrated, for example, the deleterious effect of non-steroidal anti-inflammatory drugs on colitis (Reuter *et al*., 1996). Ulcerative colitis may be insidious, with gradual onset of symptoms, or the first attack may be acute and fulminant. More mild symptoms include a progressive loosening of the stool, abdominal cramping, and diarrhea. As the disease progresses from mild to more severe, the patient may also experience weight loss, fatigue, loss of appetite that may result in nutrient deficiencies, mucus in the stool, severe rectal bleeding, fever, and anemia (Head and Jurenka, 2003).

2.4.4 Epidemiology

It is estimated that 1-2 million Americans suffer from inflammatory bowel disease; approximately half of these have ulcerative colitis. The disease accounts for 250 million annual physician visits; 30,000 hospitalizations; and a loss of over one million workdays per year. The onset of ulcerative colitis is most common between 15 and 40 years of age(Kim *et al*., 2004; Markowitz *et al*., 2009), with a second peak between 50 and 80years of age. The genetic susceptibility is thought to be mainly in the regulation of mucosal immune responses, and this is reflected experimentally by the spontaneous development of colitis in transgenic animals deficient in essential immune-regulatory molecules in a non-germ free environment (Elson *et al*., 1995).

Ulcerative colitis can occur anytime in life, but is usually diagnosed prior to age 30. The disease appears to affect men and women equally. Approximately 20 percent of people with ulcerative colitis have a close relative with inflammatory bowel disease. The geographic distribution of ulcerative colitis and Crohn's disease is similar worldwide (Podolsky, 2002), with highest incidences in the United States, Canada, the United Kingdom, and Scandinavia. Higher incidences are seen in northern locations compared to southern locations in Europe (Shivananda *et al*., 1996) and the United States (Sonnenberg *et al*., 1991) and Jewish people of European descent have 3-6 times more likelylihood to develop the disease. Regions with a low incidence of ulcerative colitis include Asia, Japan, Africa, and South America.

In Western Europe and in the USA, ulcerative colitis has an incidence of approximately 6 to 8 cases per 100.000 populations and an estimated prevalence of approximately 70 to 150 per 100.000 populations (Ardizzone, 2003). In China, ulcerative colitis has been thought uncommon; however, an analysis (Jiang and Lui, 2002) indicated that a sharp rise of the ulcerative colitis has been observed over the last decade. In United Kingdom, ulcerative colitis affects one in every 500 people. In North America, the prevalence varies from 37.5 to 238 per 100000 people (Jacobsem *et al.*, 2006). Breast feeding, appendectomy (Mitchell *et al.*, 2002), and smoking are associated with reduced risk of ulcerative colitis. Consumption of "Western diet," left-handedness, (Morris *et al.*, 2001) and depression (Kurina*et al.*, 2001), may increase risk for ulcerative colitis.

2.4.5 Diagnosis of Ulcerative Colitis

Since the early symptoms of ulcerative colitis are similar to inflammatory bowel disease (IBD), Crohn's disease, diverticulitis, and colorectal cancer, a complete patient history is essential. In addition, it is initially necessary to rule out infectious causes of diarrhea and cramping with stool cultures and parasite analysis. Other tests that may be performed early in the diagnostic process are fecal occult blood and a complete blood count (CBC) to check for intestinal blood loss and anemia. If ulcerative is not ruled out, confirmation is usually via either flexible sigmoidoscopy or colonoscopy (Head and Jurenka, 2003).

2.4.6 Factors in the Etiopathogenesis of Ulcerative Colitis

Although the exact cause of ulcerative colitis remains unknow, the condition appears to be related to a combination of genetic and environmental factors. Whole genome scans have found susceptibility genes for ulcerative colitis on chromosomes1 and 4, although these loci have not been uniformly confirmed(Rutgeerts and Geboes, 2001).Among the pathological findings associated with ulcerative colitis are an increase in certain inflammatory mediators, signs of oxidative stress, a deranged colonic milieu, abnormal glycosaminoglycan (GAG) content of the mucosa, decreased oxidation of short chain fatty acids (SCFAs), increased intestinal permeability, increased sulphide production, and decreased methylation. While no one factor has been identified as the initial trigger for ulcerative colitis, pieces of the puzzle have been

elucidated; fitting them together to create a complete picture remains to be accomplished (Head and Jurenka, 2003). Factors leading to itspathogenesisinclude:

i. Genetic factors and hereditary susceptibility
ii. Environmental factors
iii. Alteration in gut flora (deranged colonic mileau)
iv. Increase in inflammatory mediators (host immune response)
v. Oxidative stress
vi. Autoimmunity
vii. Increased intestinal permeability
viii. Nitric oxide involvement

2.4.7 Electrolyte disorders in Ulcerative Colitis

Studies have suggested that electrolyte deficiencies in UC patients may be life-threatening, with the main transport abnormality being decrease in net sodium and chloride absorption, resulting in impaired water absorption and secretion (Duthie *et al*., 1964; Head *et al*., 1969). Active UC has been associated with a very low transmucosal electrical potential difference (PD) and loss of the characteristic ability of the mucosa to absorb sodium against considerable electrochemical gradients (Edmonds and Pilcher, 1973). In addition, the increased plasma-to-lumen sodium flux rate suggesting increased leakiness of the mucosa and the loss of the active sodium absorption mechanism comprise active UC features. However, during healing of UC, PD increases and the absorptive disturbances in sodium transport are limited, while at full recovery epithelial function is normal. On the other hand, potassium secretion rate difference at various stages of the disease is less prominent. In a normal colon, the PD with the lumen negatively charged would tend to facilitate the flux of potassium into the lumen and lead to the establishment of an intraluminal steady-state concentration substantially greater than that of blood, the nearly normalsecretion of potassium in UC with a PD almost zero suggests that potassium loss to the lumen was relatively excessive (Edmonds and Pilcher, 1973).

2.4.8 Oxidative Stress in Ulcerative Colitis

Signs of increased oxidative stress in ulcerative colitis may be secondary to inflammation; free radicals can produce damage to mucosal proteins in ulcerative colitis, this may involve TNF-α production of reactive oxygen species (ROS); ROS in turn activate nuclear factor- kappa B (NF-kB), which then enhances further TNF- α production, propagating a vicious Cycle. Ulcerative colitis patients have been found to have significantly lower plasma levels of vitamins A and E and several carotenoids (D'Odorico*et al*., 2001). Mucosal biopsies of ulcerative colitis patients have been shown to increase reactive oxygen intermediates, DNA oxidation products (8-OHdG), and iron in inflamed tissue.

2.4.9 Neutrophils in Ulcerative Colitis

One of the most prominent histological features observed in UC is infiltration of neutrophils into the inflamed mucosa (Yuji *et al*., 2007). Disease activity in UC is linked to an influx of neutrophils into the mucosa and subsequently into the intestinal lumen, resulting in the formation of so-called crypt abscesses. In addition, circulating activated neutrophils – major source of inflammatory cytokines, are elevated with increased survival time in active ulcerative colitis.

The sequence of events in the extravasation of neutrophils from the vascular lumen to the extravascular space is divided into margination and rolling, adhesion and transmigration, and migration in interstitial mucosal tissues towards chemotactic stimulants, which are regulated by the interaction of adhesion molecules located on the surface of neutrophils and endothelial cells (Yoshida *et al*., 2001).

With stimuli, such as various cytokines and inflammatory mediators, neutrophils roll slowly on endothelial cells through interactions between L-selectin and carbohydrate antigen on neutrophils, and P- and E-selectin on endothelial cells. Eventually, the neutrophils adhere strongly to endothelial cells via CD11/CD18 glycoproteins and endothelial adhesion molecules of immunoglobulin superfamily, including the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1). Their expression is stimulated by molecule such as inflammatory cytokines and lipopolysaccharide (LPS). After firmly binding to the endothelial surface, the neutrophils transmigrate between cells along the intercellular junction. Platelet endothelial cell adhesion molecule 1 (PECAM-1) – a cell-cell adhesion molecule, is a

likely candidate for mediating this process. After passing the endothelial junctions, leukocytes are able to cross the basement membrane by focally degrading it with secreted collagenases. After extravasation, neutrophils migrate towards the site of intestinal mucosal injury along a chemical gradient of chemotaxis.

2.4.10 Antioxidants in ulcerative colitis

Antioxidant defense mechanisms are decreased in IBD. The intermediate products of oxygen metabolism (superoxide, hydroxyl radicals and H_2O_2) in dextran sodium sulphate (DSS) and acetic acid (AA)-induced colitis is controlled by various cellular enzymes including superoxide dismutase (SOD), catalase (CAT) and non-enzymes as reduced glutathione (GSH) (Buffinton and Doe, 1995; Furrie *et al*., 2004).

2.5 Cytokines in Inflammation

Studies have suggested that TNF-α is essential for neutrophils to migrate; local influx of neutrophils plays an important role in the cascade of events leading to tissue, but not systemic, TNF-α production (Souza *et al*., 2000a, b, 2004a). Systemic concentrations of TNF-α appear to be the best correlate of lethality in our system (Souza *et al*., 2001, 2002c). An initial tissue release of TNF-α, possibly mast cell-derived, may be essential and sufficient for an early wave of neutrophil influx to occur. An amplification circuit is then installed in which neutrophil influx facilitates TNF-α production and TNF-α production facilitates neutrophil influx (Souza *et al*., 2001, 2002c).

Several studies have demonstrated that IL-10 modulates pro-inflammatory cytokine production and tissue injury (Frangogiannis *et al*., 2000; Zingarelli *et al*., 2001; Souza et al. 2003a). Treatment with anti-IL-10 was associated with increased TNF-α concentration, tissue injury and lethality, demonstrating a role for endogenous production of IL-10 in modulating exacerbated tissue pathology and lethality (Souza *et al*., 2003a). Additionally, IL-10 appears to be involved with preconditioning; Oreopoulos *et al*., (2004) showed that hypertonic saline pretreatment prevented liver enzyme release concomitant with a reduction in liver neutrophil sequestration.

This effect appeared to be secondary to inhibition of TNF-α generation and enhancement of IL-10 expression.

2.6 Reactive Oxygen Species and Oxidative Stress in the Gut Mucosa

Reactive oxygen species (ROS) are highly reactive; when they are generated close to cell membranes, possibly by intestinal epithelial cells, they induce oxidative stress and oxidized membrane phospholipids (lipid peroxidation), which may continue in a form of a chain reaction. The main sources of ROS in the inflamed mucosa are activated leukocytes and neutrophils which are capable of producing superoxide and cascade of various reactive species leading to the formation of reactive hydroxyl radicals and peroxides. These products cause impairment in cell membrane function via lipid peroxidation in inflammatory bowel disease. In activated neutrophils, NADPH oxidase in cell membranes becomes activated, and an electron transfer takes place from NADPH in cells to oxygen inside and outside cells, and the oxygen that received electrons becomes superoxide radicals (O_2^-),which is rapidly converted to hydrogen peroxides (H_2O_2) by spontaneous dismutation or enzymatic superoxide dismutase(SOD), and hydroxyl radicals ($^{\cdot}OH$), which are formed non-enzymatically in the presence of Fe2+ as a secondary reaction (Yoshikawa and Naito, 2002;Naito *et al.*, 2005).There is substantial evidence for the involvement of oxidative stress and profound alterations in the biosynthesis of the labile free radical nitric oxide (**.**NO)from L-arginine in the pathogenesis of colitis (Myers *et al.*, 1997; Aguilar-Nascimento *et al.*, 1999; Barbosa *et al.*,2003; La *et al.*, 2003).

Oxidative stress may be a direct and proximal cause of mucosal ulceration in flare-ups of ulcerative colitis. At the opposite end of the gastrointestinal tract there is evidence for the role of oxygen radicals in the formation of gastric ulcers, (Smith *et al.*, 1987) which can be suppressed in some experimental models by intravenous administration of SOD. Perhaps similar oxidant dependent mechanisms might be operative in ulceration of the colon as well. Certainly the biological oxidants involved in Fenton chemistry are capable of altering structural components of tissue, including proteins, carbohydrates, lipids, and nucleic acids (Slater, 1972; Gutteridge and Toeg, 1982).

One possible model for such direct implication of free radical reactions in extension of crypt abscesses involves hydrogen peroxide generated from either spontaneous or SOD catalyzed dismutation of O_2- derived from activated neutrophils within the cul de sac of the crypt abscess. Assuming that extracellular catalase levels are relatively insufficient, a stream of hydrogen-peroxide-rich mucus and exudate, created within the abscess, would emerge from the crypt orifice to encounter ferrous iron previously reduced in the relatively hypoxic environment of the colonic lumen. This efflux of hydrogen peroxide from the crypt orifice would occur whenever H_2O_2 generation exceeds the catalase activity of mucus and bacteria within the crypt. As the two steams of material mix near the crypt opening, Fenton's reaction is likely to take place, leading to the creation of toxic oxidants that induce first denaturation of the local mucus barrier, followed by lipid peroxidation within the nearby surface mucosa. This oxidative stress could lead to cell death and epithelial sloughing. The resulting epithelial cell loss may produce a crater that enlarges and deepens as the mixing zone is displaced more toward the bottom of the crypt. Lateral extension could then occur by the same process, producing ulcer tracts separating pseudo polyps. The linear patterns of ulcerations might even correspond to the distribution of underlying blood vessels where oxygen tension is highest.

Another possible explanation for the linear patterns of the ulcers is that the flow of colon contents toward the anus carries a streak of activated neutrophils and hydrogen peroxide “downstream” of the crypt opening, producing a streak like pattern of oxidative injury. In this way ulceration might be initiated by Fenton chemistry as H_2O_2 escapes from crypt openings to react with fecal ferrous iron at the mucosal surface (Babbs, 1992).

The study of the mechanisms involved in cell damage mediated by oxidative compounds as well as the evaluation of biomarkers of the cellular defense system in such conditions could greatly help to prevent appearance and development of oxidative stress related diseases (Mario *et al*., 2005). Most cells can produce superoxide (O_2•-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) on demand. Other sources of free radicals include redox cycling of xenobiotics, exposure to physicochemical agents like ionizing radiations such as X-rays and γ-rays, visible light or UV in the presence of oxygen and an endogenous compound or a drug that act as photosensitizer (Devasagayam *et al*., 2004), cigarette smoke, (Devasagayam and Kamat, 2002) and antibodies

(Wentworth *et al*., 2003). They are also generated through environmental pollutants, automobile exhaust fumes, radiation, air pollutants, and pesticides, (Halliwell *et al*., 1992).

The important beneficial role of free radicals, include the following:

1. Generation of ATP (universal energy currency) from ADP in the mitochondria via oxidative phosphorylation.

2. Detoxification of xenobiotics by cytochrome P450 (oxidizing enzymes).

3. Apoptosis of defective cells (function as anti-tumourigenic species).

4. Defence against infectious agents.

5. Generation of prostaglandins and leukotrienes, which have many regulatory functions

6. Function as second messengers in intracellular signaling cascades.

7. Induction of a mitogenic response.

2.7 Industrialization and Heavy Metal Deposits

Industrialization has led to an increased accumulation of environmental pollutants particularly, heavy metals, in our surrounding ecosystems. One of such heavy metal is cadmium, an environmental toxicant that occurs naturally in ores with zinc, lead and copper. Cadmium can be emitted into the air during volcanic emission. It became commercial in the 20th century due to agricultural and industrial applications (WHO, 2000; Jarup, 2003). Occupational exposure to cadmium, such as working with cadmium containing pigments, plastic, glass, metal alloys and electrode material in nickel - cadmium batteries, and non-occupational exposure, such as food, water and cigarette smoke induces uptake of Cadmium from the environment into the body through pulmonary and enteral pathways (Waisberg *et al*., 2003). The intracellular release of cadmium is responsible for the generation of reactive oxygen species, glutathione depletion, lipid peroxidation, protein cross-liking, DNA damage, culminating ultimately in oxidant induced cell death (Brennan, 1996; Shaikh *et al*., 1999; Jurezuk *et al*., 2004 and Babu *et al*., 2006).

Cadmium is transported in the blood by red blood cells and high-molecular-weight proteins such as albumin (Goyer, 1991). Normal blood cadmium levels in adults are <1µg/dL. Although cadmium is widely distributed throughout the body, most (50 to 70% of the body burden) accumulates in the kidneys, liver (Goyer, 1991) and gastrointestinal tract (ATSDR, 1989; 2008).

Cadmium burden, especially in the kidneys, tends to increase in a linear fashion with age up to about 50 or 60 years of age after which the kidney levels remain somewhat constant or slightly decline (Goyer, 1991). There is evidence that the placenta is a partial barrier to cadmium, and that the fetus is exposed to only small amounts of maternal cadmium (ATSDR, 1989; 2008).

As with most metallic elements, there are little or no direct metabolic conversions of cadmium, but rather binding to various biological components, such as protein and non-protein sulfhydryl groups and anionic groups of various macromolecules (ATSDR, 1989; 2008). Of special importance, is the binding protein, metallothionein which is very effective in binding cadmium and some other metals, this is useful in determining the concentration of cadmium in the body (e.g. concentration of cadmium in the kidneys).

2.7.1 Sources of Human Cadmium Exposure

Most of human cadmium exposure (Figure 2.8) comes from ingestion of food, and most of that arises from the uptake of cadmium by plants from fertilizers, sewage sludge, manure and atmospheric deposition (Van Assche, 1998).

2.7.2 Applications of Cadmium

Cadmium may also be used (Figure 2.9) as plating material in food-processing plants, solder used to seal cans, old galvanized pipes and new plastic (PVC) pipes passed for drinking water, dental amalgams and appliances (Metal allergy, 2011;ARL, 2012).

2.7.3 Primary Targets of Oral Exposure

Kidney: Renal tubular proteinuria is the primary toxic effect of long-term cadmium exposure. Gastrointestinal tract: Exposure to high levels of cadmium and cadmium compounds, especially acute may cause irritation, vomiting, nausea, and diarrhea (Young, 1991).

2.7.4 Other Targets

The liver, bones, testes, and cardiovascular system have been shown to be affected to various degrees by cadmium (Young, 1991).

2.7.5 Cadmium Uptake and the Gastrointestinal Tract

The uptake through the human gastrointestinal tract is approximately 5% of an ingested amount of cadmium, depending on the exact dose and nutritional composition (Taylor, 1988). An average smoker has an additional intake of 30µg per day (Foulkes, 1985). Several factors can increase this amount, such as low intakes of vitamin D, calcium, and trace elements like zinc and copper.

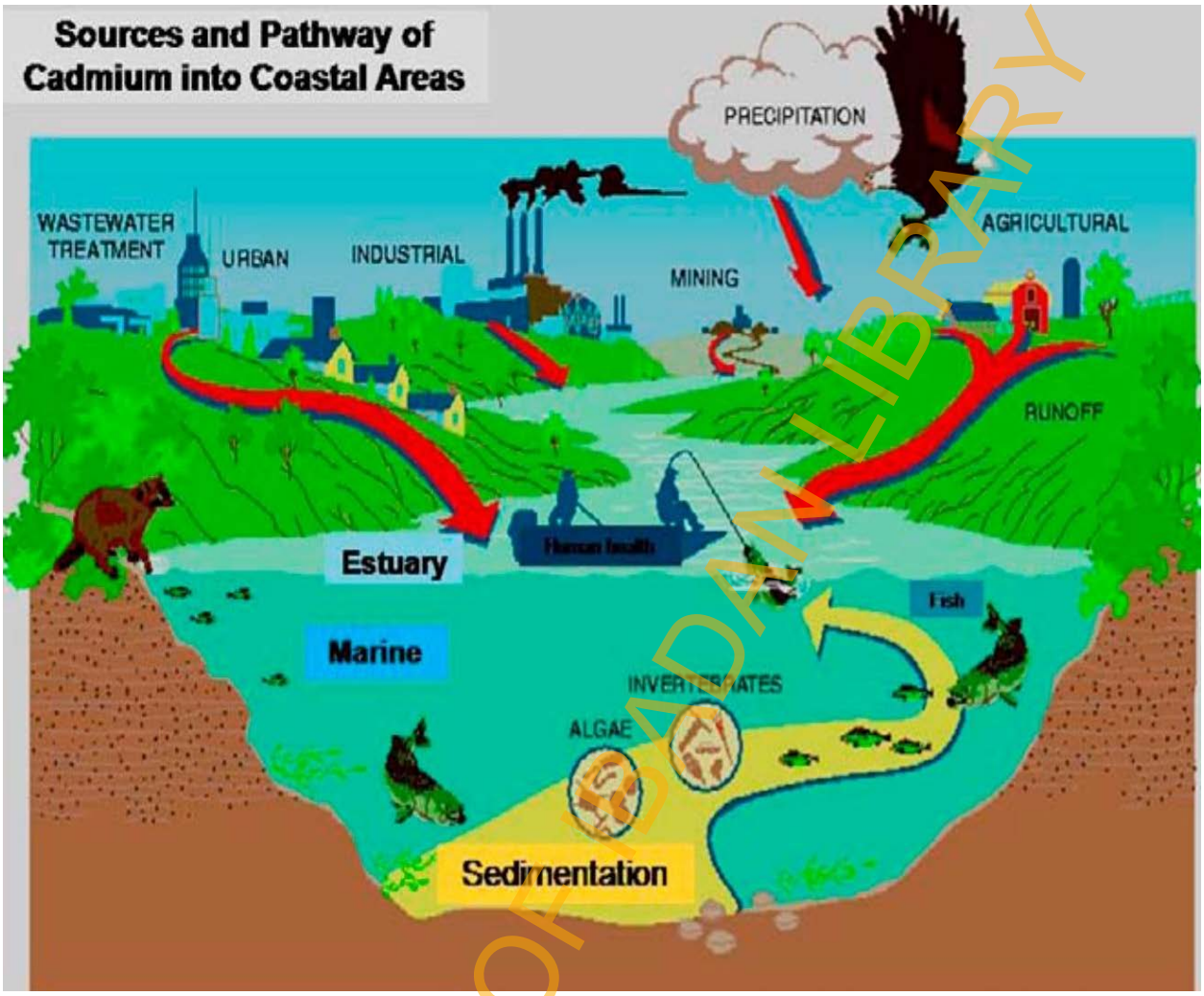


Figure 2.8: Sources and pathway of cadmium into the aquatic environment (what when.how.com, 2013).

Figure 2.9: Applications of cadmium (Metal allergy, 2011).

It is assumed that zinc and calcium's molecular homology could be a reason for a compensatory higher cadmium resorption (Jarup *et al*., 1998). Foulkes was able to show such a competitive resorption of Cadmium in an animal model: In rat jejunum, the cadmium uptake was depressed by relatively high concentrations of other polyvalent cations, including Pb, Ni, Cr^{3+}, Sr, and Mg (Flanagan *et al*., 1978).

Furthermore, a high fiber diet increases cadmium intake (Gunshin *et al*., 1997) but the most important metabolic parameter for cadmium uptake is a person's possible lack of iron. People with low iron supplies showed a 6% higher uptake of cadmium than those with a balanced iron stock (Elinder *et al*., 1976). This is the main reason for the higher cadmium resorption in people with anaemia and habitual iron deficit, such as children or menstruating women. Low iron blood levels stimulate the expression of DCT-1, a metal ion transporter in the gastrointestinal tract, serving as a gate for cadmium resorption (Barbee and Prince, 1999).

2.7.6 Mechanism of action of Cadmium

Once taken up by the blood, the majority of cadmium is transported bound to proteins, such as albumin and metallothionein. The first organ reached after uptake into the gatrointestinal-blood is the liver; here cadmium induces the production of metallothionein. After consecutive hepatocyte necrosis and apoptosis, Cd-metallothionein complexes are washed into sinusoidal blood. From here, parts of the absorbed cadmium enter the entero-hepatical cycle via secretion into the biliary tract in form of Cadmium-Glutathione conjugates. Enzymatically degraded to cadmium-cysteine complexes in the biliary tree, cadmium re-enters the small intestines (Lansdown and Sampson, 1996).

The main organ for long-term cadmium accumulation is the kidney (Nordberg, 2004), its half - life period here is about 10 years. A life-long intake can therefore lead to a cadmium accumulation in the kidney, consequently resulting in tubulus cell necrosis.

The blood concentration of cadmium serves as a reliable indicator for a recent exposition, while the urinary concentration reflects past exposure, body burden and renal accumulation (Taylor, 1988); excretion is fromfeces and urine.

2.8 Oxidative damage to biological membranes

In living organisms, various ROS can form by different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells (Halliwell and Gutteridge, 1989). ROS induce some oxidative damage to biomolecules which may lead to ageing, cancer, and other diseases (Kehrer, 1993; Aruoma, 1994). As a result of this, ROS have been implicated in more than 100 diseases, including malaria, AIDS, heart disease, stroke, arteriosclerosis, diabetes, ulcerative colitis and cancer (Tanizawa *et al*., 1992; Hertog *et al*., 1993).

2.9 Antioxidants and protection against ROS-induced damage

The term antioxidant refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells.Antioxidants,when present at low concentrations compared with those of an oxidizable substrate, such as fats, proteins carbohydrates or DNA, significantly delays or prevents the oxidation of the substrate (Halliwel, 1990). Humans have evolved highly complex antioxidant systems (enzymic and nonenzymic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. The antioxidants can be endogenous or obtained exogenously, for example as a part of a diet or as dietary supplements.

Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity and may also be classified as antioxidants. An ideal antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary (exogenous) antioxidants may be required to maintain optimal cellular functions. The most efficient enzymatic antioxidants involve in scavenging free radicals include glutathione peroxidase, catalase and superoxide dismutase (Mates *et al*., 1999).

Nonenzymatic antioxidants include vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds (McCall and

Frei, 1999). Some antioxidants can interact with other antioxidants regenerating their original properties; this mechanism is often referred to as the antioxidant network (Sies *et al*., 2005).

2.9.1 Classification of antioxidants

Antioxidants can generally be grouped into 3 classes, including:

1. Enzymic antioxidants: e.g. Superoxide dismutase, Catalase, Glutathioneperoxidase;

2. Low molecular weight substances: e.g. alpha-tocopherol, beta-carotene,urate,ascorbate;

3. Metal-ion chelators: e.g. lactoferrin, transferring, cerruloplasmin.

Superoxide dismutases (SODs) remove the superoxide radical by accelerating its conversion to H_2O_2.Human cells have a SOD enzyme which contains manganese at its active site (MnSOD) in the mitochondria. Another isoenzymeof SOD with copper and zinc at the active site (Cu, ZnSOD) is also presentbut largely in the cytosol (Michelson *et al*.,1977; Fridovich, 1983). Catalases in the peroxisomes convert H_2O_2 into water and O_2 and help to dispose of H_2O_2generated by the action of oxidase enzymes located in these organelles.

Glutathione peroxidase (GSHPx) is more important as an H_2O_2-removing enzyme in human cells (Flohe, 1982). It requires selenium, as selenocysteine at the active site, for its action. Glutathione peroxidase enzymes remove $H_2O_2$by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a FAD containing enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power.

2.9.2 Mechanisms of action of antioxidants

Antioxidants, capable of neutralizing free radicals or their actions react directly with the free radicals formed during oxidation reactions and convert them to less reactive compounds by donating a hydrogen atom rather than blocking the initial free radical generation reaction. These antioxidants act at different levels and this forms the basis for their classification. The different levels include prevention, interception and repair.

Free-radical scavengers, which react with peroxyl radicals before the polyunsaturated fatty acids react with peroxyl radicals, can prevent lipid peroxidation. Chain-breaking antioxidants donate

hydrogen atoms to peroxyl radicals and convert them to more stable and non-radicalproducts (Decker, 1998). Antioxidantradicals formed from hydrogen-donating antioxidants can react with alkyl, alkoxyl, andperoxyl radicals and generate non-radical stable compounds.

Metal chelators form complex ions or coordination compounds with metals by occupying all metal coordination sites and preventing metal redox cycling. Metal chelators can convert metal ions into insoluble metal complexes or generate steric hindrance, which can prevent the interactions between metals and lipid intermediates. Metal chelators includephosphoric acid, citric acid, ascorbic acid, polyphenols such as quercetin, carnosine, some amino acids, peptides, and proteins such as transferrin and ovotransferrin (Decker 1995; Halliwell *et al*., 1995). Flavonoids can form complexes with metals and inhibit metal initiating lipid peroxidation.

Singlet oxygen quenchers work by 2 types of singlet oxygen-quenching mechanisms: physical and chemical quenchings. Physical quenching converts singlet oxygen into triplet oxygen by either energy transfer or charge transfer without generating any other intermediates. Chemical quenching is involved with the generation of intermediates, such as oxidized products.

Primary Antioxidants

These prevent and attempt to stop the formation of new ROS either by converting existing free radicals into less harmful molecules before they have a chance to react or by preventing formation of free radicals from other molecules. These include superioxide dismutase (SOD) that catalyses the dismutation of superoxide to $H_2O_2$and catalase that breaks it down to water (Sies, 1996; Cadenas and Packer, 1996). Others are glutathione peroxidase and the metal binding proteins ferritin and ceruloplasmin.

Secondary Antioxidants

These act mainly by the interception of free radicals through the process of radical scavenging. Scavenging of peroxyl radicals are also affected. The effectors includevarious antioxidants like vitamins C and E, glutathione and other thiol compounds, carotenoids, flavonoids, uric acid, bilirubin and albumin.

Tertiary Antioxidants

These repair free radical damaged biomolecules. They include methionine sulphoxide reductase and DNA repair enzymes such as the ligases, polymerases and nucleotidases (Halliwell and Aruoma, 1993; Cadenas and Packer, 1996;Sies,1996).

2.9.3 Antioxidant enzymes

Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. Glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase, superoxide dismutase, heme oxygenase and biliverdin reductase, are the most important antioxidant enzymes. The enzyme superoxide dismutase converts two superoxide radicals into one hydrogen peroxide and one oxygen molecule. To eliminate hydrogen peroxide before the Fenton reaction can create a hydroxyl radical, organisms use catalase and/or glutathione peroxidase. The brain, which is very vulnerable to free radical damage (due to high metabolic rate, high unsaturated fat in neurons, and the fact that neurons are post-mitotic) has seven times more glutathione peroxidase activity than catalase activity (Marklund, 1982). Moreover, glutathione peroxidase is found throughout the cell, whereas catalase is often restricted to peroxisomes. Lifespan of transgenic mice has been extended about 20% by overexpression of human catalase targeted to mitochondria (Schriner, 2005).

The regulatory gene sequence for producing antioxidant enzymes, both constitutively and by induction, is called the antioxidant response element (ARE). The most important transcription factors for regulating and upregulating the ARE are Nuclear Factor Erythroid 2 p-45 related factors 1 and 2 (especially NF-E2-related factor 2, Nrf2). Nrf1 and Nrf2 arephase 2 detoxification enzyme inducers, which mean they also induce enzymes which conjugate toxins to increase water solubility and excretion.

Superoxide dismutase

The Superoxide dismutase (SOD) molecule in the cytoplasm contains copper and zinc atoms (Cu/Zn−SOD), whereas the SOD in mitochondria contains manganese (Mn−SOD). Unlike exogenous antioxidants, which are generally depleted by antioxidant action, antioxidant enzymes are not depleted because they act catalytically. An exogenous orally effective SOD mixture has

been shown to protect against hyperbaric oxidation damage to DNA that cannot be prevented with Vitamin E or N-Acetyl Cysteine (Muth, 2004). Superoxide dismutase without glutathione peroxidase or catalase (CAT) to remove hydrogen peroxide is of little value. Insects lack glutathione peroxidase, but experiments have been performed on fruit flies made transgenic by having extra genes for SOD, CAT or both. The flies that were given extra genes for SOD or CAT (but not both) had no more than a 10% increase in mean lifespan, with no increase in maximum lifespan. But fliesthat had extra genes for both SOD and CAT showed maximum lifespan increase by as much as a third, while showing less protein oxidative damage and better physical performance (Orr and Sohal, 1994). A similar experiment using SOD/CAT mimetics in nematode worms increased mean lifespan 44% (Melov *et al*.,2000). And selective inbreeding of bread-mold fungus resulted in strains with lifespans more than 6 times longer than wild-type - a change that was shown to be due to increased expression of antioxidant enzymes (Graf and Eaton,1990). Females express both more Mn−SOD and more glutathione peroxidase than males, and this has been suggested to be the reason females live longer than males in mammalian species (Vina, 2005).

Catalase

Catalase is usually located in a cellular organelle called the peroxisome (Alberts *et al*., 2002). It is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). Catalase has one of the highest turnover numbers of all enzymes; one catalase enzyme can convert 40 million molecules of hydrogen peroxideto water and oxygen each second (Goodsell, 2004). Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon *et al*., 2007). It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7 (Maehly *et al*., 1954), and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5) (Aebi, 1984). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species (Toner *et al*., 2007).

The reaction of catalase in the decomposition of hydrogen peroxide is:

$2H_2O_2 \rightarrow 2H_2O + O_2$

This can be tested by taking a solution of the enzyme and dipping a piece of paper into it, then seeing how long it takes to rise in a tube of hydrogen peroxide.

Glutathione

Glutathione (GSH) is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side-chain. It is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella *et al*., 2003). Thiol groups are reducing agents, existing at a concentration of approximately 5mM in animal cells. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form glutathione disulfide (GSSG). Glutathione is found almost exclusively in its reduced form, since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Pastore *et al*., 2003).

Functions of Glutathione

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is possible due to the relatively high concentration of glutathione in cells (up to 5mM in the liver). GSH can be regenerated from GSSG by the enzyme glutathione reductase.

Glutathione has multiple functions:

1. It is the major endogenous antioxidant produced by the cells, participating directlyin the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Scholz *et al*., 1964).
2. It is essential for the immune system to exert its full potential, e.g., (1) modulating antigen presentation to lymphocytes, thereby influencing cytokine production and type of response (cellular or humoral) that develops, (2) enhancing proliferation of lymphocytes, thereby increasing magnitude of response, (3) enhancing killing activity of cytotoxic T cells and natural killer (NK) cells, and (4) regulating apoptosis, thereby maintaining control of the immune response.
3. It plays a fundamental role in numerous metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system, especially the immune system, the nervous system, the gastrointestinal system and the lungs.

2.9.4 Nutrient Antioxidants

These antioxidants belong to endogenous compounds which cannot be synthesized by the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace elements (selenium, manganese, zinc), flavonoids, omega-3 and omega- 6 fatty acids. These antioxidants from our diet play an important role in helping endogenous antioxidants for the neutralization of oxidative stress. The nutrient antioxidant deficiency is one of the causes of numerous chronic and degenerative pathologies. Each nutrient is unique in terms of its structure and antioxidant function (Willcox *et al*., 2004; Donaldson, 2004).

Vitamin E

Vitamin E is a fat-soluble vitamin with high antioxidant potency, it is a chiral compound with eight stereoisomers: α, β, γ, δ tocopherol and α, β, γ, δ tocotrienol. Only α-tocopherol is the most bioactive form in humans. Studies in both animals and humans indicate that natural dextrorotary d-α-tocopherol is nearly twice as effective as synthetic racemic dl-α-tocopherol (Nguyen *et al*.,

2006). Because it is fat-soluble, α-tocopherol safeguards cell membranes from damage by free radicals. Its antioxidant function mainly resides in the protection against lipid peroxidation. Vitamin E has been proposed for the prevention against colon, prostate and breast cancers, some cardiovascular diseases, ischemia, cataract, arthritis and certain neurological disorders (Mayo Clinic Medical Information, 2005). The dietary sources of vitamin E are vegetable oils, wheat germ oil, whole grains, nuts, cereals, fruits, eggs, poultry, meat (Willcox *et al.*,2004; Mayo Clinic Medical Information, 2005). Cooking and storage may destroy natural d-α-tocopherol in foods (Mayo Clinic Medical Information 2005).

Vitamin C

Vitamin C also known as ascorbic acid is a water-soluble vitamin. It is essential for collagen, carnitine and neurotransmitters biosynthesis (Li and Schellhorn, 2007). Health benefits of vitamin C are antioxidant, anti-atherogenic, anti-carcinogenic, immunomodulator. The positive effect of vitamin C resides in reducing the incidence of stomach cancer, and in preventing lung and colorectal cancer. Vitamin C workssynergistically with vitamin E to quench free radicals and also regenerates the reduced form of vitamin E. However, the intake of high doses of vitamin C (2000mg or more/ day) has been the subject of debate for its eventual prooxidant or carcinogen property (Naidu, 2003; Li and Schellhorn, 2007). Natural sources of vitamin C are acid fruits, green vegetables, and tomatoes. Ascorbic acid is a labile molecule; therefore, it may be lost during cooking (Naidu, 2003).

Beta-carotene

Beta-carotene is a fat soluble member of the carotenoids which are considered provitaminsbecause they can be converted to active vitamin A. Betacarotene is converted to retinol, which is essential for vision. It is a strong antioxidant and is the best quencher of singlet oxygen. However, beta-carotene supplement in doses of 20mg daily for 5-8 years has been associated with an increased risk of lung and prostate cancer and increased total mortality in cigarette smokers (Mayo Clinic Medical Information, 2005). Betacarotene 20-30mg daily in smokers may also increase cardiovascular mortality by 12 to 26% (Mayo Clinic Medical Information, 2005). These adverse effects do not appear to occur in people who eat foods high in

beta-carotene content. Beta-carotene is present in many fruits, grains, oil and vegetables (carrots, green, plants, squash, spinach) (Willcox *et al*., 2004).

Lycopene

Lycopene, a carotenoid, possesses antioxidant and antiproliferative properties in animal and *in vitro* studies on breast, prostate and lung cell lines, although anticancer activity in humans remains controversial (Willcox *et al*.,2004; Dahan *et al*., 2008; Seren *et al*., 2008). Lycopene has been found to be very protective, particularly for prostate cancer (Dahan *et al*., 2008). Several prospective cohort studies have found associations between high intake of lycopene and reduced incidence of prostate cancer, though not all studies have produced consistent results (Seren *et al*., 2008). The major dietary source of lycopene is tomatoes, with the lycopene in cooked tomatoes, tomato juice and tomato sauce included, being more bioavailable than that in raw tomatoes (Donaldson, 2004).

Selenium (Se)

Selenium is a trace mineral found in soil, water, vegetables (garlic, onion, grains, nuts, soybean), sea food, meat, liver, yeast (Willcox *et al*., 2004). It forms the active site ofseveral antioxidant enzymes including glutathione peroxidase. At low dose, health benefits of Se are antioxidant, anti-carcinogenic and immunomodulator (Pham-Huy *et al*., 2001). Selenium is also necessary for the thyroid function (Higdon *et al*., 2007). Exceeding the tolerable upper intake level of 400 µg Se/day can lead to selenosis which is a selenium poisoning characterized by gastrointestinal disorders, hair and nail loss, cirrhosis, pulmonary edema and death (Higdon *et al*.,2007). Selenium deficiency can occur in patients on total parenteral nutrition and in patients with gastrointestinal disorders. In certain China areas with Se poor soil, people have developed a fatal cardiomyopathy called Keshan disease which was cured with Se supplement (Higdon *et al*.,2007). The role of Se in cancer prevention has been the subject of recent study and debate. Results from clinical and cohort studies about cancer prevention, especially lung, colorectal, and prostate cancers are mixed (Young and Woodside, 2001; Higdon *et al*., 2007).

2.10 Phytochemicals

Phytochemicals, by the strictest definition, are chemicals that are produced by plants. Currently, the term is being used only for those plant chemicals that may have health related effects but are not considered essential nutrients (proteins, carbohydrates, fats, minerals, and vitamins). Although, these compounds play no known role in nutrition (non-nutrients), many of them have properties including antioxidant, anti-mutagenic, antioestrogenic, anti-carcinogenic and anti-inflammatory effects that might potentially bebeneficial in preventing disease and protecting the stability of the genome. During the 1980s and 1990s, numerous laboratories began studying phytochemicals to mine plants for bioactive substances that might be used as medicines (nutriceuticals) or for other chemical applications. Many compounds are showing great promise as disease fighters in the body, boosting production or activities of enzymes, which then act by blocking carcinogens, suppressing malignant cells, or interfering with the processes that can cause heart disease and stroke.

2.10.1 Phytochemicals as therapeutics

There is evidence from laboratory studies that phytochemicals in fruits and vegetables may reduce the risk of cancer, possibly due to dietary fibers, polyphenol antioxidants and anti-inflammatory effects. Specific phytochemicals, such as fermentable dietary fibers, meet significant scientific agreement to be allowed by the US Food and Drug Administration (FDA). Phytochemicals have been used as drugs for millennia. For example, Hippocrates may have prescribed willow tree leaves to abate fever. Salicin, having anti-inflammatory and pain-relieving properties, was originally extracted from the white willow tree and later synthetically produced to become the stapleover-the-counter drug called Aspirin. An important cancer drug, Taxol (paclitaxel), is a phytochemical initially extracted and purified from the Pacific yew tree.

Some phytochemicals with physiological properties may be elements rather than complex organic molecules. Abundant in many fruits and vegetables, selenium, for example, is involved with major metabolic pathways, including thyroid hormone metabolism and immune function (Brown *et al*., 2001). Particularly, it is an essential nutrient and co-factor for the enzymatic synthesis of glutathione, an endogenous antioxidant (Papp *et al*., 2007). Many phytochemicals have anti-inflammatory properties in vitro, including turmeric and chia. Inflammation is a factor

in many diseases of aging including Alzheimer's and arthritis. Turmeric is also reported to be active against skin cancer (melanoma).

2.11 Flavonoids

Polyphenolic compounds are the most ubiquitous class of plant chemicals, and consist of compounds with a hydroxyl group (-OH) attached to an aromatic hydrocarbon. These compounds are antioxidants usually from natural origins such as fruits, vegetables, nuts, seeds, flowers, and tree barks. They are of diverse chemical structure and characteristics and are majorly classified into flavoniods, tannins, coumarins, lignans and many more. These polyphenolic compounds have been found to be involved in the alleviation of some ROS degenerative diseases such as aging, arthritis, cancer and diabetes. Flavonoids are a special class of polyphenolic plant secondary metabolites. The term flavonoids encompass a wide range of compounds which are all based on the C6-C3-C6 (phenylbenzopyran) carbon skeleton. Flavonoids are located inside the cells or on the surface of various plant organs and have various functions in plants (Marais *et al*., 2006).

2.11.1 Classification of Flavonoids

The underlying basic structure of all flavonoids consists of 2 aromatic rings, the ‘A’ and ‘B’ rings linked by a 3-carbon bridge. For most flavonoids, the 3-carbon bridge combines with an oxygen molecule and the ‘A’ ring to form a third central ring structure, the ‘C’ ring (Beecher, 2003). The central ring structure is known as a chromane ring and is a shared structure of flavonoids and tocopherols. Chalcones, a sub-class of flavonoids, lack the central ‘C’ ring. Flavonoids are categorized into several groups based on specific structural features: the major flavonoids, isoflavonoids, and neoflavonoids (Marais *et al*., 2006). The major flavonoids, isoflavonoids and neoflavonoids are isomers that differ according to the position of the aromatic B-ring moiety. Other variations of the flavonoid structures include the chalcone and aurone flavonoid families that are referred to as minor flavonoids. The group of major flavonoids is further subcategorized based on differences in structureof the central chromane ring.Different

degrees of saturation of the chromane ring occur, with flavanones and flavanols having no carbon-carbon double bonds in the middle ring; flavones having one C-C double bond; and anthocyanidins possessing two C-C double bonds. Flavanones and flavanols are differentiated based on the presence of a carbonyl (C=O) group at position 4 on the chromane ring of flavanones which is absent in flavanols. This C=O functional group is also seen in flavones. Flavonols are very similar to flavones differing only by the addition of a hydroxyl at the 3-position on the central ring. Flavanols usually have a hydroxyl at the 3-position and are therefore termed flavan3-ols, helping to avoid confusion with the similar sounding flavonols. Anthocyanidins are unique in that the oxygen atom of the chromane ring has a positive charge.

2.11.2 Antioxidant Activity of Flavonoids

Flavonoids appear to be particularly potent antioxidants *in vitro* and act as effective scavengers of free radicals. The flavonoids, however, are not all equally effective, with definite structural requirements necessary for the greatest antioxidant effect. The presence of a 2, 3 double bond in the C-ring, a catechol structure in the B-ring, and hydroxylation at position 3 and 5 of the A ring appear to impart increased redox potential (Bors *et al*., 1990). Direct scavenging of free radicals is one of the major mechanisms of antioxidant activity by flavonoids. Several other mechanisms of antioxidant activity of flavonoids have been proposed including scavenging of transition metal ions (Mira *et al*., 2002), and inhibition of enzymes responsible for antioxidant production. In terms of the latter property, flavonoids have been shown to inhibit several pro-oxidant enzymes including xanthine oxidase (Van Hoorn *et al*., 2002), glutathione S-transferase, nitric oxide synthase (Raso *et al*., 2001), and NADH oxidase (Morre *et al*., 2000) amongst others. The antioxidant effects of flavonoids *in vitro* are well established, and have been confirmed using several methodologies (Rice-Evans and Miller, 1996; Jovanovic *et al*.,1994). However, studies examining the effects of flavonoids in humans have demonstrated conflicting results. However, despite all inconsistencies, studies examining total antioxidant capacity of human plasma after consumption of flavonoid rich diets have almost all noted significant increase (Lotito and Frei, 2006). Discrepancies between the strong antioxidant capacity of flavonoids *in vitro* and their antioxidant efficacy *in vivo* may be due to the poor absorption and extensive metabolization of flavonoids leading to poor bioavailability. The serum levels of other dietary antioxidants such as

vitamins C and E, uric acid, and glutathione are typically over 100 times greater than flavonoids even after the consumption of flavonoid rich foods (Lotito and Frei, 2006).

2.11.3 Adverse Effects of Flavonoids

No adverse effects have been associated with high dietary intakes of flavonoids from plant-based foods. This lack of adverse effects may be explained by the relatively low bioavailability and rapid metabolism and elimination of most flavonoids.

2.11.4 Pro-oxidant Effects of Flavonoids

While flavonoids are best known for their antioxidant properties, it has been shown that under certain conditions, flavonoids may be pro-oxidant. This property has been proposed to account for several biological effects of flavonoids, such as apoptosis, that are induced in the setting of oxidative stress. These pro-oxidant effects have also been observed for other phenolic antioxidants including tocopherols, ascorbate, urate, curcumin and N-acetylcysteine. The balance between antioxidant and pro-oxidant effects of these compounds is dependent on several factors in the cellular environment, particularly on the presence of transition metal ions. Various protective features of human physiology make it unlikely that the pro-oxidant properties of flavonoids will have such dramatic effects in the human body. For example, flavonoid-quinone toxicity is rapidly prevented in vivo by dithiols (Boots *et al.*, 2005).Flavonoids also undergo considerably less auto-oxidation in vivo as a result of the sequestration of free transition metal ions in carrier proteins such as transferrin. Thus, the pro-oxidant effects of flavonoids are expected to be considerably less pronounced in vivo.

2.12 Metal Chelation

Metal ions, such as iron and copper, can catalyze the production of free radicals. The ability of flavonoids to chelate (bind) metal ions appears to contribute to their antioxidant activity in

vitro(Mira *et al*., 2002; Cheng and Breen, 2000). In living organisms, most iron and copper are bound to proteins, limiting their participation in reactions that produce free radicals. Although the metal-chelating activities of flavonoids may be beneficial inpathological conditions of iron or copper excess, it is not known whether flavonoids or their metabolites function as effective metal chelators in vivo(Frei and Higdon, 2003).

2.13 *Garcinia kola*

Garcinia kola (Figure 2.10), Heckel (a species of flowering plant in the Clusiaceae or Guttiferae family), commonly known as bitter kola, male kola or false kola, are eaten in West and Central Africa. It is commonly used in traditional hospitality, cultural and social ceremonies. It is a medium sized and largely cultivated forest tree indigenous to sub-Saharan Africa and has been referred to as a 'wonder plant' because almost every part of it has been found to be of medicinal importance (Hutchinson and Dalziel, 1956). The plant extraction has been traditionally used for ailments such as laryngitis, liver diseases and cough (Ayensu, 1978). The seeds are used to prevent or relieve colic, cure head or chest colds and relieve cough (Iwu, 1993). The seed also has anti-inflammatory, antimicrobial, antidiabetic and antiviral (Iwu, 1986) as well as antiulcer properties (Ibironke *et al*., 1997).

Scientific classification

Kingdom: Plantae - Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division: Magnoliophyta - Flowering plants

Class: Magnoliopsida

Order: Theales

Family: Clusiaceae

Genus: *Garcinia*

Species: *Garcinia kola* (Heckel)

Garciniakola seeds are eaten in Nigeria as they are known to have a high content of biflavonoid. The toxicity is very low, the oral 50% lethal dose being above 5000 mg/Kg (Nwankwo *et al*., 2000). The edible nut which is highly valued is commonly known as bitter kola or 'false kola'. It is a masticatory and is a major kola substitute offered to guests at home and shared at social ceremonies. It is commonly called Agbilu in Igbo land, Namijin goro in Hausa and orogbo in Yoruba land of Nigeria (Aluka, 1985). It produces a characteristic orange-like pod with seeds covered with a skin or husk.

The seeds are used in folk medicine and in many herbal preparations for the treatment of ailments such as laryngitis, liver disorders, and bronchitis (Iwu, 1982). The seed also has anti-inflammatory, antimicrobial, antidiabetic and antiviral (Iwu, 1986) as well as antiulcer properties (Ibironke *et al*., 1997). The seed is employed as a general tonic and it is believed to have aphrodisiac properties. *Garcinia kola* seeds have also been indicated in the traditional African medicine in the treatment of inflammatory disorders. The biflavonoid mixture isolated from the seeds of *Garcinia kola* is known as kolaviron. Various extracts of *Garcinia kola* have been found to elicit a number of biochemical properties including hepatoprotection, antidiabetic properties and antigenotoxic potentials.

The roots and stems are cut into short chew-sticks used for cleaning teeth. *Garcinia kola* stick among ten common Nigerian chewing sticks examined for antibacterial properties, displayed good activity (Taiwo *et al*., 1999). *Garcinia kola* seeds have also been indicated in the traditional African medicine in the treatment of inflammatory disorders.

2.13.1 Biological Properties of *Garcinia kola*

Garcinia kola and its extracts have been shown to elicit a number of biological activities in various experimental models. Rats chronically fed diets containing powdered seeds of *Garcinia kola* at the level of 10% w/w for 6 weeks displayed marked inhibition of gastrointestinal motility, protection against castor oil induced diarrhea and prolonged pentobarbital sleeping time (Braide, 1991). *Garcinia* biflavonoid complex, also known as kolaviron have been shown to elicit

hypoglycaemic effect on both fasted normoglycemic and alloxan-diabetic rabbits (Iwu *et al*., 1990). Also the complex inhibited rat lens aldose reductase activity, an enzyme found in the lens and other tissues which have been implicated in many diabetic complications such as neuropathy and retinopathy (Iwu, 1990; Crabbe *et al*., 1998; Kinoshita *et al*., 1979).

Figure 2.10: *Garciniakola* seeds (Researcher design)

The biflavanones of *Garcinia kola* have been shown to be pharmacologically active with several pharmacokinetic advantages over simple monomeric flavonoids as they survived first-pass metabolism which inactivates most flavonoids (Iwu, 1986). In addition to the above notable biological effects of *Garcinia* biflavonoid complex, studies have also shown the ability of this complex to protect against hepatotoxicity induced by phalloidin, amanita, 2-acetylaminofluorene, carbon tetrachloirde, paracetamol, aflatoxin, dimethyl nitrosamine in rodents (Farombi *et al*., 2000; Farombi, 2000; Farombi *et al*., 2005; Farombi *et al*., 2009).

Chemical Composition of *Garcinia kola*

Studies involving bioassay-guided fractionation of *Garcinia kola* seed have yielded complex mixtures of phenolic compounds, triterpenes and benzophenones. Aplin *et al*., (1967) isolated cycloartenol and its 24-methylene derivatives from the petroleum spirit extract of the seeds. The ethyl acetate-soluble fraction of the acetone extract contains C-3/8-link biflavanone GB1, GB2, GB1a andkolaflavanone (Cotterhill *et al*., 1978). These biflavanones and their glycosides were also isolated from the stem back (Iwu, 1982). Theether soluble fraction of the alcoholic extract yielded apigenin-5, 7, 4-trimethyl ether, apigenin-4-methylether, fisetin, amento-flavone, kolaflavanone and GB1 (Iwu, 1982). Benzophenone and kolanone with potent antimicrobial properties were isolated from the light petroleum extract of *Garcinia kola* seeds (Hussain *et al*., 1982). Subsequently, *Garcinia*biflavonoid complex containing GB1, GB2 and kolaflavanone, a defatted fraction of alcoholic extract of *Garcinia kola* seeds was isolated (Iwu, 1985). This fraction is also popularly referred to as kolaviron. Recently, GB1 one of the major components of *Garcinia*biflavonoid was isolated from the roots of Garcinia kola. It showed inhibitory effects against methicilin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistantenteroccoci (VRE). Terahima *et al*., (2002), also reported two new chromanols, garcinoic acid and garcinal, together with δ-tocotrienol isolated from *Garcinia kola* seeds.

2.14 Kolaviron

Kolaviron is an extract of *Garciniakola*, a biflavonoid complex which contains Garcinia biflavanone (Gb1, Gb2) and kolaflavanone in an approximate ratio of 2:2:1 (Cotterhill *et al*., 1978and Iwu *et al*., 1990). Garcinia biflavonoids have been found to inhibit lipid peroxidation invivo (Farombi *et al*., 2000 and Adaramoye *et al*., 2005). Lipid peroxidation has been implicated in many pathological conditions including ulcerative colitis and cadmium toxicities (Mora *et al*., 1990).

Kolaviron is a fraction of the deffated ethanol extract of *Garcinia kola*, containing *Garcinia*biflavonoids GB1, GB2 and kolaflavanone as its major components in an approximate ratio of 2:2:1 (Cotterhill *et al*., 1978; Iwu, 1985; Kubanga, 1987 and Iwu *et al*., 1990). Garcinia biflavonoids have been found to inhibit lipid peroxidation invivo (Farombi *et al*., 2000 and Adaramoye *et al*., 2005). Lipid peroxidation has been implicated in many pathological conditions including ulcerative colitis and cadmium toxicities (Mora *et al*., 1990).

Some studies have also, revealed the protective effects of kolaviron againsthepatotoxicity and oxidative stress induced by 2-acetylaminofluorene (Farombi *et al*.,2000), carbontetrachloride (Farombi, 2000), aflatoxin B1 (Farombi *et al*.,2001) andpotassium bromate-induced nephrotoxicity (Farombi *et al*.,2002a) via scavenging ofreactive metabolites and induction of increases in carcinogen detoxifying enzymes(Farombi *et al*.,2002b; Farombi, 2003). In addition, the ability of kolaviron to inhibithydroxyl and superoxide anion radicals, which are known to play an important rolein theprocess of lipid peroxidation, has also been demonstrated (Farombi, 2003).

The structure of kolaviron possesses many hydroxyl groups on the dual B-rings, as well as dual 4-keto groups in the C-rings. These structural features confer on kolaviron theantioxidantactivity of radical scavenging utilizing its many hydroxyl groups on the dual B rings and metal ion chelation utilizing an abundant availability of 3- or 5-hydroxyl and 4- ketosubstituents or hydroxyl groups in the ortho-position in the B-ring.

2.14.1 Antioxidant and Radical Scavenging Effects of kolaviron (*Garcinia*Biflavonoid)

A plethora of mechanisms have been proposed and implicated in chemoprevention of various degenerative diseases including cancer and antioxidant actions have been suggested as one of the prominent mechanism. Chemoprevention by natural products against oxidative damage and chemical carcinogens may therefore be related to their intrinsic antioxidant properties. The antioxidant and scavenging activity of *Garcinia* biflavonoid complex has beeninvestigated in a range of established *in vitro* assays involving reactive oxygen species. The study showed that kolaviron elicited significant reducing power and a dose-dependent inhibition of oxidation of linoleic acid (Farombi *et al*., 2002b). Kolaviron inhibited H_2O_2, and was more effective than the standard antioxidants BHA and δ-carotene and equivalent in power to δ-tocopherol. Kolaviron also significantly scavenged superoxide generated by phenazine methosulfate NADH.

Furthermore, kolaviron scavenged hydroxyl radicals as revealed by significant inhibition of the oxidation of deoxyribose. Hydroxyl radical is highly reactive oxygen centered radical, which attacks all proteins, DNA, polyunsaturated fatty acids in membranes and almost any biological molecule it touches (Hussain *et al*., 1982; Aruoma, 1999). The inhibitory activity of kolaviron in deoxyribose assay may relate directly to prevention of the propagation of the process of lipid peroxidation and modulation of other biomarkers of oxidative stress in animal model (Farombi *et al*., 2000; Farombi, 2000). Farombi *et al*., (2004) related the chemoprotective effect of kolaviron to its*in vivo* antioxidant effects.Thus, kolaviron reduced background levels of protein oxidation marker (2-aminoadipic semi-aldehyde)in plasma and liver and γ-glutamylsemi-aldehyde (GGS) as well as malondialdehyde in liver (Farombi *et al*., 2004). Inaddition, kolaviron reduced damage to proteins and lipids induced by Fe3+/EDTA/ascorbate mixtures*ex vivo*(Farombi *et al*., 2004). Furthermore, kolaviron dose dependently inhibited the intracellular ROS productioninduced by H_2O_2in HepG2 cells detected as 2, 7-dichlorofluorescein diacetate (DCF) fluorescence (Eddy *et al*., 1987; Nwankwo *et al*., 2000).

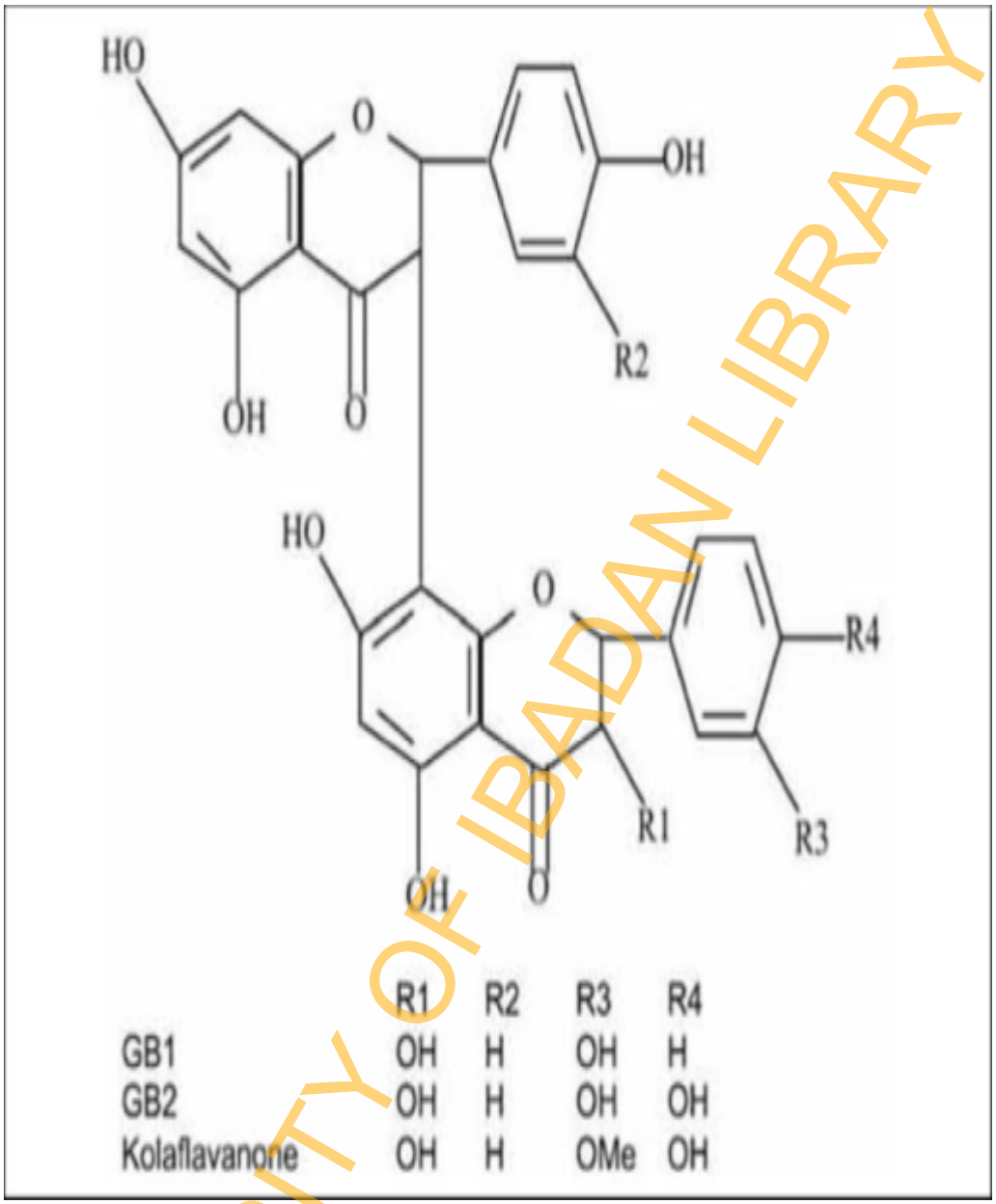


Figure 2.11: Structure of Kolaviron (Farombi *et al.*, 2012)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals, Solvents and Reagents

Cadmium chloride (Burgoyne Burbidges & Co Mumbai-India), sodium chloride, 5% ketamine (Rottexmedica, Germany), KI, $CuSO_4$. $5H_2O$, NaOH, KCl, NaH_2PO_4, Na_2HPO_4 (BDH Chemical Limited, England), o-Dianisidine dihydrochloride, Hexadecyltrimethylammonium Bromide and Sodium nitrite were all procured from Tokyo Chemical Industry, Co., Limited, Japan,Formalin, BSA (sigma Chemical Co., USA), TCA, TBA, Tris-KCl buffer, $K_2Cr_2O_7$ (Hopkins & Williams, England), methylated spirit, acetic acid, Griess reagent, Ellman's reagent, Biuret reagent, Oligo-dT primers, Random Hexamers, Total RNA kit, cDNA Synthesis kit (PCR-511S), Jena Bioscience (ABI, Foster City, CA).

3.1.1 Cadmium Salts

Cadmiumchloride salts of analytical grade was purchased from Burgoyne Burbidges & Co Mumbai-India and was appropriately administered to the groups of animals in their drinking water.

3.2 Plant Materials and Extract Preparation

Garcinia kola seeds was obtained in a local market in Ikirun, Osun State, Nigeria and was identified by a botanist; Esimekhuai, D.P.O. in the department of Botany, University of Ibadan, Ibadan, Nigeria. A total of 5kg of peeled seeds was sliced, pulverized with electric blender and then air dried. Kolaviron was isolated according to the method of Iwu *et al*., 1990; briefly, the powdered seeds were extracted with n-hexane (cold extraction) for 24 hr. The defatted dried product was re-packed and extracted with acetone. The extract was concentrated and extracted with ethyl acetate (6×300 ml). The concentrated ethyl acetate yielded kolaviron;

Kolaviron yield (%) = $\frac{\text{Weight of extract (1940g)}}{\text{Weight of plant sample (5000g)}} X\ 100$

= 38.8%.

3.3 Experimental Design, Animals and Groupings

This study was split into three;

Phase 1 was done to know the effect of graded doses of cadmium on colitis healing.

Phase 2 (kolaviron graded dose study) was carried out to ascertain the efficacy and appropriate dosage of kolaviron that may be useful during colitis in cadmium (100ppm) exposed rats.

Phase 3 investigated the mechanisms involved in the actions of kolaviron during colitis healing in cadmium exposed rats.

Although IBD affects all age groups, studies have shown that two peaks in the agegroup have been noted; the primary peak in the age group occurs in young people of 15 to 40 years and the secondary peak is seen more commonly at 50 to 60 years (Nikolaus and Schreiber, 2007). This informed the choice for the weight of animals used in this study (110 – 120g) which falls in the primary peak; which occurs in young people.

All animals in phases 1, 2 and 3 of this study were acclimatized for two weeks, kept under standard conditions with food (vital feed; crude protein 14%, fat 7%, crude fibre 10%, calcium 1.0%, phosphorous 0.35%) and water made accessible to them freely.

Cadmium chloride ($CdCl_2$) was dissolved in drinking water in 25, 50 and 100ppm in phase 1 and $CdCl_2$was dissolved in drinking water at 100ppmphase 2 and 3 of this study; all $CdCl_2$preparations were made freely accessible to the animals appropriately, according to their groupings and study. Administration by oral route was chosen because it is the main route of exposure to cadmium in human and animals. The concentration of $CdCl_2$ and the period of treatment were selected on the basis of previous studies (Chowdhury *et al.*, 1987; Waalkes and Rehm, 1999b; Asagba and Eriyamremu, 2007).

Colitis was induced after a four-week cadmium exposure in each of phases 1, 2 and 3; a modified method of colitis induction previously described by MacPherson and Pfeiffer, 1978; Millar *et al.*, 1996 was followed.

Kolaviron was administered once daily with an oral cannula at a dose of 50mg/kg, 100mg/kg, 200mg/kg in phase 2 and only 200mg/kg in phase 3(Iwu, 1985; Farombi *et al*., 2000).

Body weight was recorded weekly and rats were sacrificed at predetermined intervals of **Day 0, Day 3, Day 7 and Day 14**in all studies. At the time of the sacrifice, blood was collected by intra-cardiac puncture; this was then used for blood analysis, including blood cadmium level assay. Colon tissues were removed immediately and weighed; parts were processed immediately for gene expression, biochemical assays and light microscopic studies.

Where Day 0 represents before induction of colitis,

Day 3 represents three days' post colitis induction,

Day 7 represents seven days' post colitis induction and

Day 14 represents fourteen days' post colitis induction.

Phase 1: Effects of graded doses of cadmium (25, 50 and 100ppm) on the normal colon and on colitis healing;

Eighty male Wistar rats (110-120g) were randomly assigned into four groups of twenty rats each; Control group was given drinking water, three other groups received cadmium chloride ($CdCl_2$) at 25ppm, 50ppm and 100ppm respectively in drinking water (Table 3.1).Administration of cadmium in the appropriate groups was sustained throughout the experiment and colitis was induced in all the groups after four weeks of cadmium exposure. The Animals were divided into four groups as follows:

A. Colitis control

B. Cadmium (25ppm) exposed animals + Colitis

C. Cadmium (50ppm) exposed animals + Colitis

D. Cadmium (100ppm) exposed animals + Colitis

Table 3.1: Grouping, exposures, treatments and experimental procedurein Phase 1

Groups	Number of Animals	Exposure	Treatment	Experimental Procedure
posCont	20	None	None	Induction of colitis
Cad25	20	25ppm $CdCl_2$	None	Induction of colitis
Cad50	20	100ppm $CdCl_2$	None	Induction of colitis
Cad100	20	100ppm $CdCl_2$	None	Induction of colitis

Where *posCont* = Positive Control;

Cad25 = Cadmium at 25ppm;

Cad50 = Cadmium at 50ppm and

Cad100 = Cadmium at 100ppm.

Phase 2: Effects of graded doses of of kolaviron (50, 100, 200mg/kg) on colitis healing during cadmium exposures;

Eighty-five male Wistar rats weighing 110-120g were used; these were randomly assigned into five groups.Negative control (*negCont*) group was given tap drinking water, positive control (*posCont*) received tap drinking water and vehicle (Dimethylsulphoxide (DMSO)) and the three other groups received cadmium chloride ($CdCl_2$) in drinking water at 100ppm, respectively (Table 3.2). These treatments continued throughout the experiment. Rats were grouped with five rats in negative control, and twenty rats each of the others as follows;

A. Negative control (*negCont* group
B. Positive control (*posCont* group)
C. Cd 100ppm + 50mg/kg KV + colitis (50mg/kg KV group)
D. Cd 100ppm + 100mg/kg KV+ colitis (100mg/kg KV group)
E. Cd 100ppm + 200mg/kg KV + colitis (200mg/kg KV group)

Phase 3: Mechanisms of action of kolaviron during cadmium exposures and colitis healing;

Eighty-five male Wistar rats (110 – 120g) were randomly assigned into five groups (Table 3.3). Appropriate treatments were given to the different groups throughout the period of the experiment. Cadmium chloride was dissolved in drinking water of appropriate groups and kolaviron was administered to the appropriate rat groups once daily throughout the experiment. Rats were grouped with five rats in negative control, and twenty rats each of the other groups as follows;

A. Negative control (*negCont* group)

B. Positive control (*posCont* group)

C. Cadmium (100ppm) treated animals + Colitis (Cd group)

D. Cadmium (100ppm) + Kolaviron (200mg/kg) treated animals + Colitis (Cd+KV group)

E. Kolaviron (200mg/kg) treated animals + Colitis (KV group)

Table 3.2: Grouping, exposures, treatments and experimental procedure in Phase 2

Groups	Number of Animals	Exposure	Treatment	Experimental Procedure
negCont	5	None	None	None
posCont	20	None	Vehicle (DMSO)	Acetic acid (4%) intra-rectal induction of colitis
Cd 100ppm + 50mg/kg KV	20	100ppm $CdCl_2$	Kolaviron (50mg/kg)	Acetic acid (4%) intra-rectal induction of colitis
Cd 100ppm + 100mg/kg KV	20	100ppm $CdCl_2$	Kolaviron (100mg/kg)	Acetic acid (4%) intra-rectal induction of colitis
Cd 100ppm + 200mg/kg KV	20	100ppm $CdCl_2$	Kolaviron (200mg/kg)	Acetic acid (4%) intra-rectal induction of colitis

Where *negCont* = Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron.

Table 3.3: Grouping, exposures, treatments and experimental procedure in Phase 3

Groups	**Number of Animals**	**Exposure**	**Treatment**	**Experimental Procedure**
negCont	5	None	None	None
posCont	20	None	Vehicle (DMSO)	Acetic acid (4%) intrarectal induction of colitis
Cd	20	100ppm $CdCl_2$	None	Acetic acid (4%) intrarectal induction of colitis
Cd+KV	20	100ppm $CdCl_2$	Kolaviron (200mg/kg)	Acetic acid (4%) intrarectal induction of colitis
KV	20	None	Kolaviron (200mg/kg)	Acetic acid (4%) intrarectal induction of colitis

Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron.

3.3.1 Determination of Blood Cadmium levels

One (1) ml of blood was collected (ocular) from rats after four weeks of cadmium treatment, all blood samples were digested with HNO_3(2ml in each) and left overnight. The samples were heated in a water bath for 30 minutes at 100°C. They were removed, allowed to cool, diluted with 12 ml of distilled water and then filtered (Asagba and Eriyaremu, 2007; Gonçalves et al., 2012). Cadmium concentrations were assessed from the filtrates using an atomic absorption spectrophotometer. The concentration of cadmium was measured in parts per million (ppm). Blood cadmium concentration was determined at the Central Research Laboratory, University of Ibadan, Ibadan.

3.3.2 Induction of colitis

Colitis was induced after cadmium had been appropriately administered for four weeks; a modified method of colitis induction previously described by MacPherson and Pfeiffer, 1978; Millar *et al*., 1996was followed – rats were fasted for 24 hours but were given access to water *adlibitum* and were thereafter, anaesthetized with 35mg/kg intraperitoneal ketamine, then an improvised Teflon cannula was inserted intrarectally into the colon 8cm proximal to the anus of the animals to induce colitis by administering 2mL of 4% acetic acid, this was retained for 55 seconds, after which it was withdrawn (Figure 3.1). The rats were thereafter monitored and scored for the presence of diarrhea (Masonobu*et al.*, 2002), at predetermined intervals.

3.3.3 Stool consistency scoring

Stool consistency was scored daily post colitis induction using the method described by Masonobu*et al.*, 2002, but with slight modifications as follows:

0 – (normal),

1 – (soft but still formed),

2 – (very soft),

3 – (diarrhea).

3.3.4 Differential Counting of White Blood Cell Count

Test method

i. A drop of fresh blood was gently dropped to one end of a clean free slide, allowing the drop to spread.
ii. Holding the slide and 'spreader' at a suitable angle, the spreader was pushed along the slide, drawing the blood behind it until the whole blood had been smeared. The smeared slide was allowed to dry off.
iii. The slide was placed in a staining rack for staining.
iv. Drops of stain were applied on the film until the whole film was covered with stain. The drops added were counted. The undiluted stain was allowed to act for one and half minutes.
v. After the one and half minutes, twice the number of drops of distilled water was applied to dilute the stain. It was mixed by rocking and by blowing through an ordinary pipette. The film was allowed to stain for 10 minutes; the excess stain was then washed with distilled water. The excess water was drained, and the back side of the slide was wiped with a clean, dry filter paper and kept in a vertical position to drain and dry.
vi. After drying the well stained film, a drop of immersed oil on the lower third of the blood film was placed and covered with a clean cover glass.
vii. The film was observed microscopically; the cells were focused using 10X objective lens with the condenser iris closed sufficiently to see the cells clearly. The staining and distribution of cells were also checked.

The blood film was examined and the absolute number of each different white blood cell type were counted as observed in each field and calculated. Neutrophil/lymphocyte ratios were estimatedfrom the observed counts of cells.

3.3.5 Gross Assessment of Colon Morphology

On appropriate days of the experiment, animals were randomly picked from each group and sacrificed, colons (8cm) was resected and colon was flushed out with cold buffered saline solution and opened by an out-in turning.

3.3.6 Macroscopic Scoring

The macroscopic morphologywas scored using a scoring scale as follows:

0= no damage;

1= hyperemia, no ulcers;

2= linear ulcer with no significant inflammation;

3= linear ulcer with inflammation at one site;

4= two or more sites of ulceration/inflammation;

5= two or more major sites or of ulceration and inflammation or one site of ulceration/inflammation extending > 1 cm along the length of the colon;

6 – 10= If damage covers >2cm along the length of the colon, the score is increased by one from each additional centimeter of involvement (Peran *et al*., 2005).

3.3.7 Assessment of integrity of Colon by histopathology:

On specific days of the experiment, animals were randomly picked from each group and sacrificed for histological study. Histological studies were performed by slight modification of the method previously described by Ogihara and Okabe, (1993).

Hematoxylin and Eosin Staining

Principle:

In order to microscopically view a tissue specimen that has been processed, sectioned (thinly sliced), and mounted on a slide, the specimen must be stained in order to make the cells visible.

The basic nature of histology slide staining is to stain the slide with two or more contrasting dyes that will highlight specific areas or entities with one color, and leave a counterstaining background colour. The standard or 'routine stain' is the hematoxylin and eosin stain, better known as the 'H&E'stain. The H & E stain uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. Hematoxylin is a dark purplish dye that will stain the chromatin (nuclear material) within the nucleus, leaving it a deep purplish-blue color. Eosin is an orangish-pink to red dye that stains the cytoplasmic material including connective tissue and collagen, and leaves an orange-pink counterstain. This counterstain acts as a sharp contrast to the purplish-blue nuclear stain of the nucleus, and helps identify other entities in the tissues such as cell membrane (border), red blood cells, and fluid.

Procedure:

Step 1 (Fixation): Colonic samples taken from the distal part of each colon were fixed in 10% buffered formalin for histological examinationwere. This was immediately immersed in neutral buffered 10% formal- saline for 48 hours.

Step 2 (Dehydration): Water was removed from the tissue by putting it in ascending grades of alcohol (70%, 80%, 90%, 2 changes of 100%) one hour each. Ascending concentrations of alcohol was used to prevent sudden rush out of water from the tissues, so that the cell will not be distorted or damaged.

Step 3 (Clearing): Alcohol was then removed from the tissues because it is not miscible with paraffin. The tissues were infiltrated with xylene, which replaced the alcohol and was also miscible with paraffin. Xylene also made the opaque tissue transparent, therefore the name clearing stage. The tissue was passed twice through xylene, and it spent about 2 hours each time.

Step 4 (Embedding): Then each small pieces of tissue, including ulcers, were embedded in paraffin; infiltrated in 2 changes molten paraffin wax one hour each. It was allowed to cool on a frozen surface then removed from mold.

Step 5 (Microtomy): The tissue was trimmed to expose tissue surface with mocrotome, and was cooled on ice. 5µ of tissue was sectioned.

Step 6 (Mounting of Paraffin Sections): Float using 2% alcohol into a warm water of about 2^0C below melting point of wax. Use clean, grease free slide to pick the floating section. The other side of the slide was cleaned and placed on hot plate after proper labeling for about 3 hours for the section to be completely fixed and the slide to dry.

Step 7 Staining: The section was deparaffinized in 2 changes of xylene for 4 minute each so that the stains can permeate. The slide was then immersed in a descending concentration of alcohol (ie. 100%, 90%, 80%, and 70%) for about 1 minute in each alcohol solution so as to dehydrate it. The slides were rinsed in water and placed in Erhlich haematoxylin for about 15 minutes. The slides were dipped in 1% acid-alcohol (2 dips) and rinsed in running water for about 3 minutes till the colour of the section to become blue. The slides were counterstained in eosin for about 2 minutes and briefly rinsed in water. The slides were immersed in ascending grades of alcohol (70%, 80%, 90%, and 100%) for about 30 seconds so as to dehydrate the preparation. The preparation was cleared of alcohol by dipping it in xylene for 1minute. After these, the slide was blotted and mounted under a cover slip using dibutylphthalate xylene (DPX), and air bubbles were prevented from getting in. The slide was then read under the microscope using x100 and x400 magnifications, and lesions were noted. A photomicrograph of the slide preparation was then taken.

Figure 3.1: Intra-rectal method of Inducing Colitis (Researcher design)

3.3.8 Preparation of Tissues for Biochemical Assays

Excised tissues were quickly rinsed in ice-cold 1.15% KCl after which they were blotted and weighed. Tissues were then minced with scissors in 4 volumes of ice-cold 0.1M phosphate buffer, pH 7.4, and homogenized using a Tefflon homogenizer. The resulting homogenates were centrifuged at 10,000g, 4°C for 10minutes. The supernatant was collected and processed for biochemical estimations.

Reagents:

1. Homogenizing buffer (0.1M Phosphate buffer, pH 7.4)

Di-Sodium hydrogen phosphate Na_2HPO_4(35.822g) (BDH Chemical Limited, England) and 15.603g of NaH_2PO_4 were dissolved in 900ml of distilled water. The pH was adjusted to 7.4 and then made up to 1 liter with distilled water.

2. Potassium Chloride (1.15%)

Potassium chloride (1.15g) (BDH Chemical Limited, England) was dissolved in distilled water and made up to 1000ml and stored at 4°c.

Determination of Protein Concentration

The protein concentrations of the various samples were determined by means of the Biuret method as described by Gornal *et al*., (1949) a slight modification: potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions as cuprous oxide.

Principle

Proteins form a coloured complex with cupric ions in an alkaline solution as exemplified bythe Biuret reagent containing CuSO4, KI and sodium potassium tartarate. The protein and Biuret reagent form complexe with maximum absorbance at 540nm. The procedure is usually calibrated with a standard BSA curve.

Reagents

Sodium hydroxide (0.2M)

Eight grams (8g) of NaOH (BDH, England) was dissolved in distilled water and made up to 1 litre.

Stock Bovine Serum Albumin (standard)

Twenty milligram (20mg) of BSA (sigma Chemical Co., USA) was dissolved in 2ml distilled water to give a stock solution of 10mg protein/ml.

Burriets Reagent

Three grams (3g) of $CuSO_4$. $5H_2O$ (BDH Chemicals, England) were dissolved in 500ml of 0.2M NaOH 5g of potassium iodide, KI (BDH Chemicals, England) was added and the solution made up to a litre with 0.2M NaOH.

Estimation of Protein in Test Samples

Procedures identical to those described above for the standard curve was used except that suitable dilutions of the test samples (colon supernatant) were made with distilled water. The colon supernatants were diluted 100 times with distilled water. This was done to reduce the level of protein in the samples to the sensitivity range of Biuret method. 1ml of the diluted sample was taken and added to 3ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples was usually extrapolated from the standard curve and multiplied by 100 to get the actual amount in the fraction.

Induction of Oxidative Stress (LPO Assessment)

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out by the method of Varshney and Kale (1990).

Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of free MDA produced.

Reagents

1. **Trichloroacetic acid (TCA) (30%)**

   Nine grams (9g) of TCA (CCl_3COOH) was dissolved in distilled water and made up to 30ml with same.

2. **Thiobarbituric acid (TBA) (0.75%)**

   This was prepared by dissolving 0.225g of TBA in 0.1M HCl and made up to 30ml with same.

3. **Tris-KCl buffer (0.15M) (pH 7.4)**

   Potassium chloride (KCl) (1.12g) and 2.36 g of tris base were dissolved separately in distilled water and made up to 100ml with same. The pH was then adjusted to 7.4.

Procedure

An aliquot of 0.4ml of the sample was mixed with 1.6ml of Tris-KCl buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000g for 15 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in unit/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}Cm^{-1}$.

$$\text{MDA (unit/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample} \times \text{mg protein}}$$

Determination of Catalase Activity

Catalase activity was determined according to the method of Sinha, 1971.

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents

1. **Dichromate Solution (5% $K_2Cr_2O_7$)**

   Potassium dichromate($K_2Cr_2O_7$) 5g (Hopkins & Williams, England) was dissolved in 80ml of distilled water and made up to 100ml with same.

2. **Hydrogen peroxide (0.2M H_2O_2)**

   Hydrogen peroxide (H_2O_2) 11.50ml of 30% (w/w) was diluted with distilled water in a volumetric flask and the solution made up to 500ml.

3. **Dichromate/acetic acid**

   This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

4. **Phosphate buffer (0.01M, pH 7.0)**

   Dissolved 3.5814g of Na_2HPO_4 $12H_2O$ and 1.19g $NaH_2PO_4.2H_2O$ in 900ml of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

Colorimetric Determination of H_2O_2

Different amounts of H_2O_2, ranging from 10 to 100 µmoles were taken in small test tubes and 2ml of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml and the optical density measured with a spectrophotometer at 570nm. The concentrations of the standard were plotted against the absorbance.

Determination of catalase activity of samples

One (1) ml of supernatant fraction of the testicular homogenate was mixed with 19ml distilled water to give a 1: 20 dilutions. The assay mixture contained 4ml of H_2O_2 solution (800µmoles) and 5ml of phosphate buffer, pH 7.0 in a 10ml flat bottom flask. 1ml of properly diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. 1ml portion of the reaction mixture was withdrawn and blown into 2ml dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

Calculation

Catalase activity was calculated by plotting the standard curve and the concentration of the remaining H_2O_2 was extrapolated from the curve.

H_2O_2 consumed = 800µmoles – H_2O_2 remaining

$$\text{Catalase activity} = \frac{H_2O_2 \text{ consumed}}{\text{mg protein}}$$

Determination of Superoxide Dismutase (SOD) Activity

The level of SOD activity was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\bullet-}$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2^{\bullet-}$ introduced increased with increasing pH (Valerino & Mc Cormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet-}$) radical and hence inhibitable by superoxide dismutase.

Reagents

1. Carbonate buffer (0.05M) (pH 10.2)

Distilled water (200ml) was used to dissolved3.58g of $Na_2CO_3.10H_2O$ and 1.05g of $NaHCO_3$. The pH was adjusted to 10.2 and then made up to 250ml with distilled water.

2. Adrenaline0.3mM

Adrenaline (0.01g) (epinephrine) was dissolved in 200ml-distilled water. Always prepared fresh when needed.

Protocol

One (1) ml of sample was diluted in 9ml of distilled water to make a 1 in 10 dilutions. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation

Increase in absorbance per minute = $\frac{A_3 - A_0}{2.5}$

Determination of Total Nitrite

Nitrite determination was done using the method described by Ignarro *et al*., (1987). The assay relies on a diazotization reaction that was originally described by Griess in (1879). The procedure is based on the chemical reaction which uses sulfanilamide and naphthylethylenediamine dihydrochlorate (NED) under acidic condition. Sulfanilamide and NED compete for nitrite in the Griess reaction.

Procedure

Griess reagent was prepared by mixing equal volume of 0.1% NED and 1% sulfanilic acid in 5% phosphoric acid. 50µl of sample was added to a 96 plate well in duplicate and 100µl of griess reagent was added. The mixture was incubated at room temperature protected from light for 10minutes to allow for colour development. The absorbance was measured within 30 minutes in a microplate reader at 550nm.

Procedure for the Estimation of Reduced Glutathione (GSH) Level

The method of Beutler *et al*., (1963) was followed in estimating the level of reduced glutathione (GSH) in liver supernatants.

Procedure

Sample (0.2ml) was added to 1.8ml of distilled water and 3ml of 4% Sulphursalicyclic acid was mixed with the sample. This was centrifuged at 3,000g for 4 minutes. Thereafter, 0.5ml of the supernatant was added to 4.5ml of Ellman reagent. A blank was prepared with 0.5ml of the diluted precipitating agent and 4ml of phosphate buffer and 0.5ml of Ellman's reagent. The absorbance of the reaction mixture was read within 30 minutes of colour development at 412nm against a reagent blank.

Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity

A. Material Preparation

• Reaction Buffer: 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA

• Ellman's Reagent Solution: Dissolve 4 mg Ellman's Reagent in 1 ml of Reaction Buffer.

B. Measure Absorbance

1. For each unknown sample to be tested, prepare a tube containing 50 µl of Ellman's Reagent Solution and 2.5 ml of reaction Buffer.

2. Add 250 µl of each unknown to the separate test tubes prepared in Step 1. As a blank, add 250 µl of Reaction Buffer to a separate test tube prepared in Step 1.

Note: For the unknown(s), make dilutions so that the 250 µl sample applied to the assay reaction has a sulfhydryl concentration less than 1.0 mM. Concentrations exceeding 1 mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.

3. Mix and incubate at room temperature for 15 minutes.

4. With a spectrophotometer set to 412 nm, zero the instrument on the blank and then measure absorbance of each sample.

5. Calculate the amount and concentration of sulfhydryls in the sample from the molar extinction coefficient of TNB (14,150 M-1cm-1).

Determination of Na+/K+ ATPase Activities

This assay was carried out by the method of Ronner *et al.*, (1977) as modified by Bewaji *et al.*, (1985). Protocol is shown in the table below.A reaction mixture containing an aliquot of 200mM NaCl/ 40mM KCl/ 60mM tris buffer (pH.7.4), 80mM $MgCl_2.6H_2O$, 20mM EGTA and enzyme source was incubated for at 37^0C. Thereafter, 8mM ATP was added and then incubated for 30min at same temperature. 5% SDS was then added to stop the reaction and centrifuge at 3000rpm for 5min at 4^0C. Reagent mixture (H_2SO_4-Ammonium molybdate-Ascobate) was added and allowed to stand at room temperature for 20 min for colour development, after which the absorbances were read at 725nm. The absorbances obtained were then extrapolated from the standard phosphate curve to obtain concentration of inorganic phosphate. Na^+/K^+-ATPase was expressed as µmole P_i/mg Prot./hr.

Table 3.4:Protocol for the determination of Na^+/K^+ATPase activity

Reagents	Volume
40 mM KCl/60 mM Tris HCl, pH 7.4	400µl
80 mM $MgCl_2.6H_2O$	100 µl
Enzyme Source	100 µl
Incubated at 37^0C for 5 minutes	
8 mM ATP	100 µl
Incubated at 37^0C for 30 minutes	
10% TCA (ice cold)	300 µl
Incubate at 4^0C for 20 minutes	
9% Ammonium molybdate	20 µl
2% Ascorbic acid	40 µl

3.3.9 Procedure for Gene Extraction

RNA Extraction

RNA was extracted from inflamed sections of the colon using Jena Bioscience RNA kit (ABI, Foster City, CA) according to the manufacturer's instructions as described in appendix I.

Principle of the Technique

Total RNA kits use the reversible binding properties of column activation buffer which is a silica-based material. This is combined with the speed of mini-column spin technology. A specifically formulated high salt buffer system allows RNA molecules greater than 200 bases to bind to the matrix. Tissues are first homogenized and then lysed under denaturing conditions using lysis buffer that practically inactivates RNases. Samples were then applied to the spin columns to which total RNA binds, while cellular debris and other contaminants are effectively washed away. Excess contaminants and debris were removed in a two-step washing process with the primary and secondary wash buffers. Excess ethanol was removed by spinning column for 2 minutes after the secondary washing step. This is necessary to prevent the inhibition of PCR reagents during cDNA and PCR procedure. High quality RNA was finally eluted in elution buffer.

cDNA Synthesis

The RNA extract obtained from the sample was then used as template for the synthesis of cDNA.cDNA was synthesized using Jena Bioscience SCRIPT cDNA Synthesis kit (PCR-511S). All procedures were carried out according to the manufacturer's manual as described below:

- The reaction mix was prepared by mixing up the components in a nuclease-free microtube. The procedure was performed on ice and the components were mixed properly by pipetting gently up and down. Water, RNA and primers were mixed together before the remaining components were added. The components were added in the following compositions for a 20µl reaction (Table 3.5).

First-strand cDNA synthesis

- The reaction mix was transferred into the thermal cycler where it was incubated at 42°C for 10 minutes followed immediately by incubation at 50°C for 60 minutes.

Heat Inactivation

- The mix was further heated to 70°C for 10 minutes to inactivate the Reverse Transcriptase.

Principles of the Assay

The assay uses SCRIPT Reverse Transcriptase which is a genetically engineered version of M-MLV Reverse Transcriptase (M-MLV RT) that does not have RNase H activity and increased thermal stability. The enzyme is a RNA-dependent DNA polymerase that can synthesize a complementary DNA strand using single-stranded RNA or DNA as template. The reaction is initiated by a primer which could be of the three types; Oligo-dT primers, Random Hexamers, or sequence specific primers. Oligo-dT primers will initiate synthesis of cDNA using only RNA sequence with poly A sequence, random hexamer primers will initiate synthesis of cDNA using the total RNA sequence present in the extract while the specific primers initiates cDNA synthesis of specific RNA sequence of interest. In this assay, the Random Hexamer primers were used. SCRIPT RT buffer complete contains all the necessary salts such as $MgCl_2$ that are required to catalyse the RT activity.

The dNTP Mix is composed of the four DNA nucleotides; that is deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanidine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), which serves the building block for the synthesis of new DNA strands. DDT is a reducing agent which helps to stabilize RNA strand by breaking hydrogen bond thereby relieving RNA templates of their secondary structures. The RNAse inhibitors also help prevent the activity of RNAse which can degrade RNA template during cDNA synthesis. Prevention of RNA degradation is also enhanced by the use of RNAase free reagents and materials such as powder free gloves.

Determining Gene Expression

The cDNA synthesized from the extracts obtained from the homogenate of individual rat colon sample were used for quantification of gene expression by quantitative real-time PCR using Sybr®Green PCR Master Mix (green master) in the Jena Bioscience Kit that contained Sybr Green as the fluorescence dye in the assay. The PCR amplification was performed using Applied Biosystem 7500 fast Real-Time PCR System machine.

Principle of the Assay

The fluorescence monitoring chemistry of this assay is based on the use of DNA-intercalating dye, SYBR®Green I which serve as the reporter fluorophore. It binds double-stranded DNA produced during the polymerase chain reaction. During the reaction cycle, the instrument detects and records the increase in fluorescence produced as a result of intercalation of the dye over time. The SYBR®Green assay requires a validated primer pair in addition to the regular PCR master mix components.

The assay requires experimental controls, one of which is the internal control. It is required to normalize the signal value of each sample in order to ensure that the differences between samples are the results of biological difference and not a mere product of loading inconsistency.

Real-Time (qPCR) Primers

The primers used in the assay to amplify each gene of interest were previously described primers from different publications. Each primer was test run on Amplifx application software using specific gene sequence obtained from the genebank data base. The pairs of primers from were purchased from Jena Bioscience GmbH, Lobstedter Str. 71|07749 Jena, Germany.

The oligonucleotides stock samples were re-suspended to 100picoM in nuclease free H_2O, and an aliquot was further diluted at a ratio 1:9 (10X) in sterile nuclease free water. The final dilution was used for the quantitative PCR mix preparation in subsequent assays. The primers used with their cycling conditions are listed in table 3.7.

qPCR Master Mix Set-up

The reaction mix was prepared using the cDNA synthesis from each sample as template. All procedures were performed on ice. The concentration of each component of the PCR mix was prepared (Table 3.6).

qPCR Cycling Conditions and Instrument Set-up

a) A set-up using nuclease-free water in place of the template "NTC" (No Template Control) was used as a negative control ineach 96 plate well reaction. The PCR plates were centrifuge to ensure that there were no bubbles formed in the wells. The Applied Biosystem 7500 Real-Time PCR software was operated and the conditions for amplification were set. Each well was also labeled before the start of PCR reaction.
b) Default PCR conditions for primers used for amplifying each gene is as stated on table 3.6.
c) The set-up was saved before running; the program to generate all fluorescence data during PCR automatically.

Table 3.5: cDNA synthesis mix preparation components and volume

Reagents	Volume (for 20µl reaction)
RNAse-free water	7 µl
SCRIPT RT Buffer complete (1X)	4 µl
DTT stock solution (5mM)	1 µl
dNTP Mix (500uM each)	1 µl
RNAse Inhibitor (40 units)	1 µl
Random Hexamers (50pmol)	0.5 µl
SCRIPT Reverse Transcrptase (100units)	0.5 µl
RNA template	5µl

Table 3.6: qPCR mix preparation components and volume

Components	Volume (for 20µl reaction)
Nuclease-free water	4.8µl
Reverse primer (5µM)	0.6µl
Forward primer (5µM)	0.6µl
2X SYBR Green mix10µl	
Template cDNA 4µl	
TOTAL 20µl	

Table 3.7: Oligonucleotide primers and cycling conditions used for real time PCR

Gene	Primer sequence	Cycling conditions
β-actin	sense 5′ TTTGAGACCTTCAACACCCC 3′ antisense 5′ ATAGCTCTTCTCCAGGGAGG 3′	95°C 5min; 40 cycles of 95°C 1min, 60°C 1min and 72°C 1.5min; 72°C 5min
TNF-α	sense 5′ CGA GTG ACA AGC CCG TAG CC 3′ antisense 5′GGA TGA ACA CGC CAG TCG CC 3′	95°C 5min; 35 cycles of 95°C 30sec, 60°C 1min and 72°C 30sec; 72°C 10min
IL-10	sense 5′ TGC CTTCAG TCA AGT GAA GAC T 3′ antisense 5′ AAA CTC ATT CAT GGC CTT GTA 3′	95°C 5min; 40 cycles of 95°C 1min, 60°C 1min and 72°C 1.5min; 72°C 5min
Occludin	sense 5′ GCCTTTTGCTTCATCGCTTCC 3′ antisense 5′ AACAATGATTAAAGCAAAAGCCAC 3′	95°C 5min; 40 cycles of 95°C 30sec, 58°C 1min and 72°C 1min; 72°C 10min

3.4 Statistical Analysis

Analysis of data was performed using a statistical software – Graph pad prism (version 5.0). Results were expressed as mean ± standard error of mean(S.E.M), statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Differences were considered significant at *p< 0.05.

CHAPTER FOUR

RESULTS

Phase 1

4.1 Effect of Cadmium on body weight after four weeks of exposure

There were significant decreases in weekly percentage weight gain by weeks 3 and 4 (128.07±3.40% and 123.57±3.34%) in *Cd100* group when compared with *posCont* (141.75±4.75% and 139.65±4.51%) (Figure 4.1).

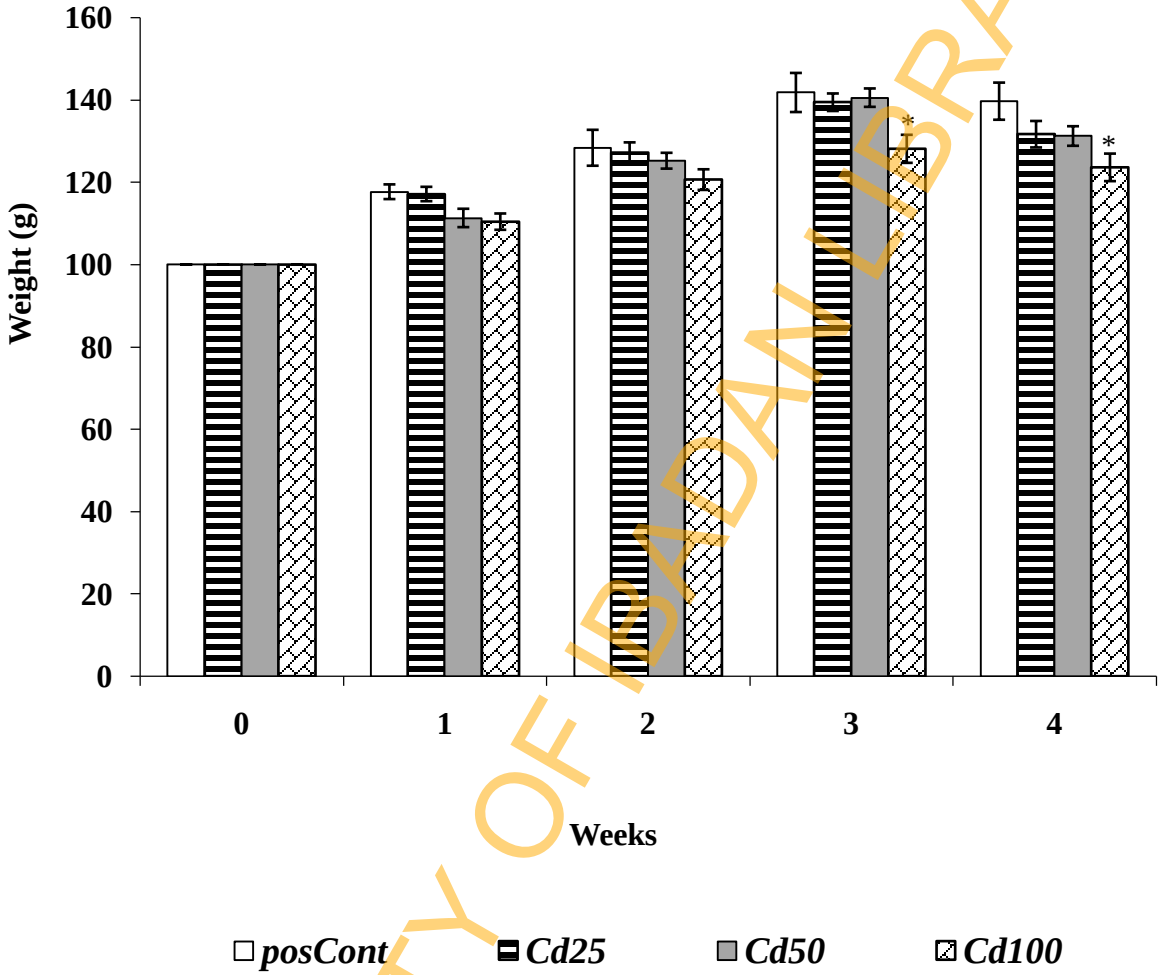


Figure 4.1: Weekly percentage body weight gain

Each bar is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. * = $p<0.05$ values differ significantly from *posCont* group.

4.2. Effect of Cadmium on kidney, liver and spleen weights

There were significant increases in the kidney weight in *Cd100*group(0.52±0.01g) when compared with *posCont* group (0.40±0.01g) by day 7 (Table 4.1), other increases were not statistically significant.

Table 4.1: Kidney, Liver and Spleen weights (g)

Kidney	***posCont***	***Cd25***	***Cd50***	***Cd100***
Day0	0.40±0.03	0.39±0.01	0.43±0.02	0.40±0.01
Day3	0.40±0.02	0.42±0.04	0.42±0.02	0.45±0.01
Day7	0.40±0.01	0.40±0.02	0.44±0.02	0.52±0.01*
Day14	0.40±0.03	0.41±0.01	0.43±0.02	0.50±0.03
Liver	***posCont***	***Cd25***	***Cd50***	***Cd100***
Day0	3.80±0.22	3.70±0.19	4.25±0.32	4.13±0.29
Day3	4.40±0.19	4.44±0.43	4.47±0.56	4.59±0.39
Day7	4.65±0.32	4.77±0.33	4.78±0.31	4.98±0.39
Day14	4.54±0.29	4.70±0.32	4.74±0.28	5.68±0.27
Spleen	***posCont***	***Cd25***	***Cd50***	***Cd100***
Day0	0.40±0.04	0.41±0.01	0.51±0.03	0.52±0.05
Day3	0.36±0.03	0.42±0.06	0.44±0.04	0.42±0.04
Day7	0.36±0.01	0.36±0.02	0.49±0.08	0.52±0.01
Day14	0.36±0.01	0.36±0.03	0.47±0.07	0.52±0.01

Each value is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. * = $p<0.05$ values differ significantly from *posCont* group.

4.3 Blood Cadmium Concentration(Phase 1)

Blood Cadmium concentration(Figure 4.2) was significantly high in *Cd50* (0.04±0.00ppm) and *Cd100* group (0.04±0.00ppm) when compared with *posCont*group (0.03±0.00ppm) by the end of four-week cadmium exposure.

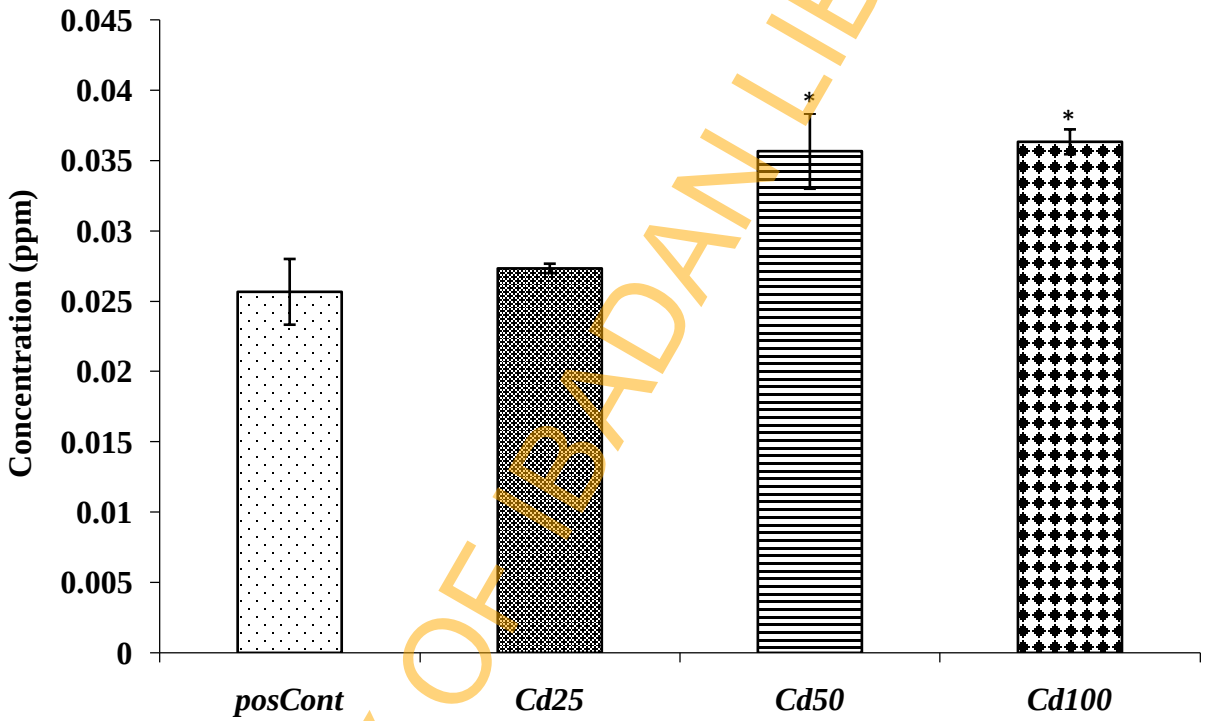


Figure 4.2: Blood cadmium concentration after four weeks of oral exposures (Phase 1)

Each bar is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. *=$p<0.05$ values differ significantly from *posCont* group.

4.4 Effect of cadmium on stool consistency from days 1 through 10 post colitis induction

There were significant increases in stool consistency (Table 4.2) at day5 in *Cd100* groups (2.50±0.22) when compared with *posCont*group (1.50±0.22).

Table 4.2: Stool Consistency Scores During Colitis

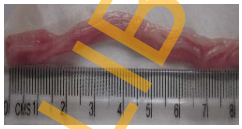
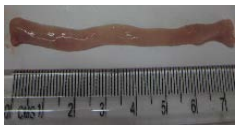
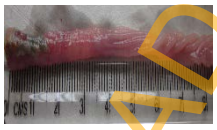
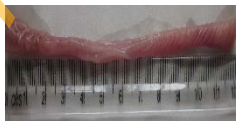
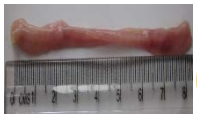
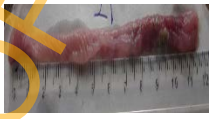
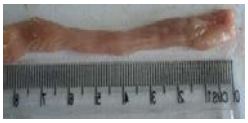
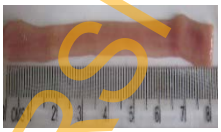
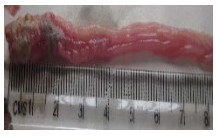
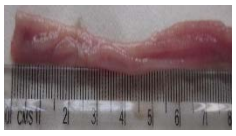
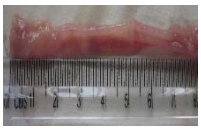
	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9	Day10
posCont	1.83 ±0.31	2.17 ±0.17	2.33 ±0.21	2.00 ±0.00	1.50 ±0.22	1.67 ±0.33	1.83 ±0.31	1.00 ±0.00	0.00 ±0.00	0.00 ±0.00
Cd25	1.67 ±0.21	2.33 ±0.21	2.33 ±0.21	2.00 ±0.00	1.83 ±0.17	1.83 ±0.31	2.17 ±0.17	1.00 ±0.58	0.00 ±0.00	0.00 ±0.00
Cd50	1.67 ±0.21	1.67 ±0.21	2.00 ±0.26	2.33 ±0.21	2.17 ±0.17	2.33 ±0.33	2.33 ±0.21	1.33 ±0.33	0.33 ±0.33	0.00 ±0.00
Cd100	2.00 ±0.26	2.00 ±0.37	1.83 ±0.31	2.00 ±0.26	2.50 ±0.22*	2.33 ±0.21	2.33 ±0.21	1.67 ±0.33	0.67 ±0.33	0.67 ±0.33

Each value is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. * = $p<0.05$ values differ significantly from *posCont* group.

4.5 Effect of cadmium on colon macroscopic scores (Phase 1)

Macroscopic scores from the *Cd50* and *Cd100* by days 0, 3, 7 and 14 were increased (Table 4.3), these increases where however, not statistically significant.

Table 4.3: Macroscopic scores and presentations of colonic tissues (Phase 1)

Groups		Day0	Day3	Day7	Day14
posCont	**Scores**	0.0±0.0	4.67±0.33	2.00±0.0	0.0±0.0
	Gross				
Cd25	**Score**	0.0±0.0	5.90±1.07	2.67±0.67	0.33±0.33
	Gross				
Cd50	**Score**	0.0±0.0	5.83±0.60	2.67±0.67	0.67±0.33
	Gross				
Cd100	**Score**	0.0±0.0	6.23±0.79	3.17±0.60	1.00±0.0
	Gross				

Each value is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm.

4.6 Effect of cadmium on Neutrophil/Lymphocyte Ratio (Phase 1)

Neutrophil/Lymphocyte ratio(Figure 4.3), was significantly increased in *Cd50* (0.43±0.04; 0.80±0.06) and *Cd100*(0.49±0.03; 0.80±0.03) when compared with *posCont*(0.36±0.03; 0.63±0.04) on day0 and7, respectively.

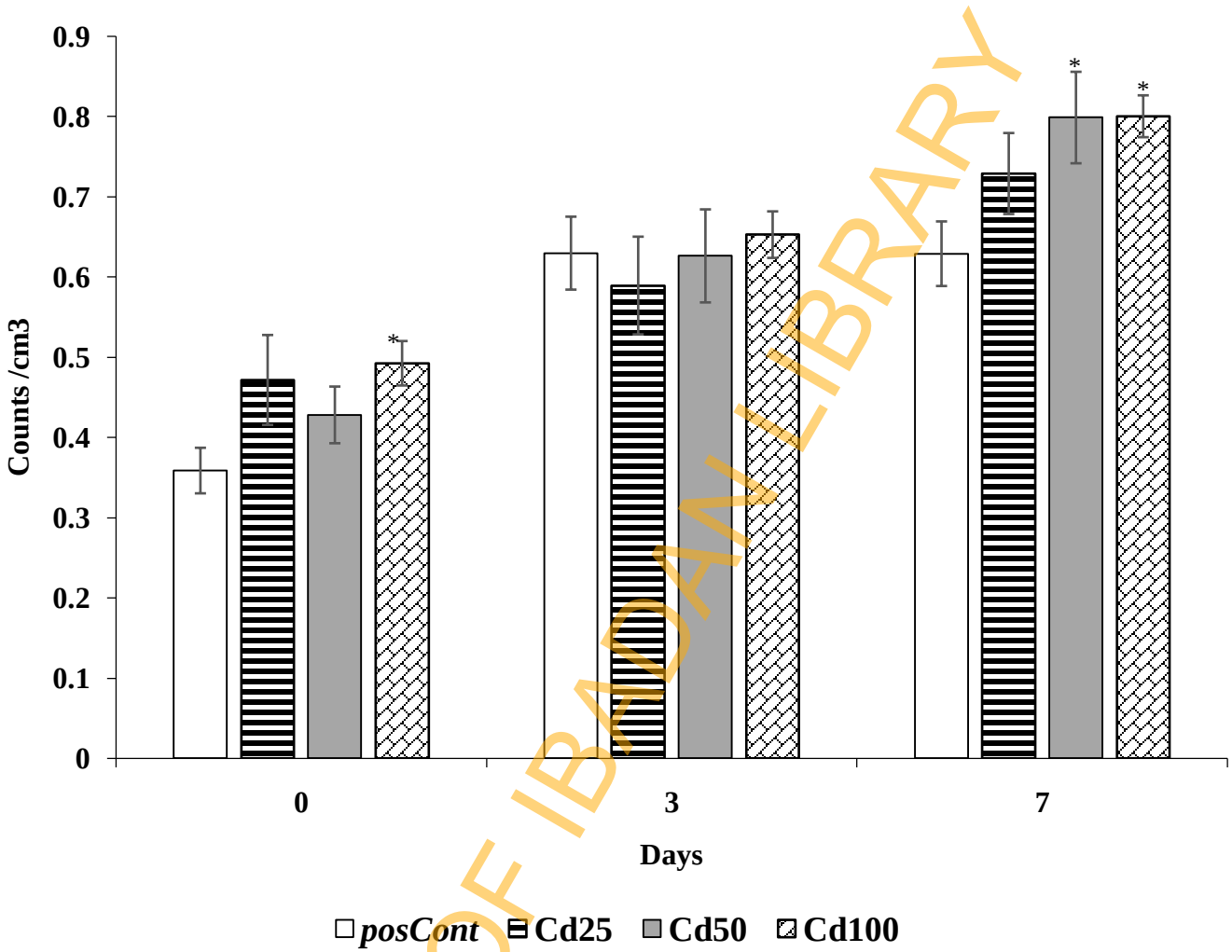


Figure 4.3: Neutrophil/Lymphocyte Ratio (Phase 1)

Each bar is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. * = $p<0.05$ values differ significantly from *posCont* group.

4.7 Effect of cadmium on Malondialdehyde (MDA) Concentration

Malondialdehyde concentrations were significantly increasedin*Cd100* groupwhen compared with *posCont* group at day 0, also, in *Cd25*, *Cd50* and *Cd100* groups when compared with *posCont at* days 7 and 14 (Figure 4.4).

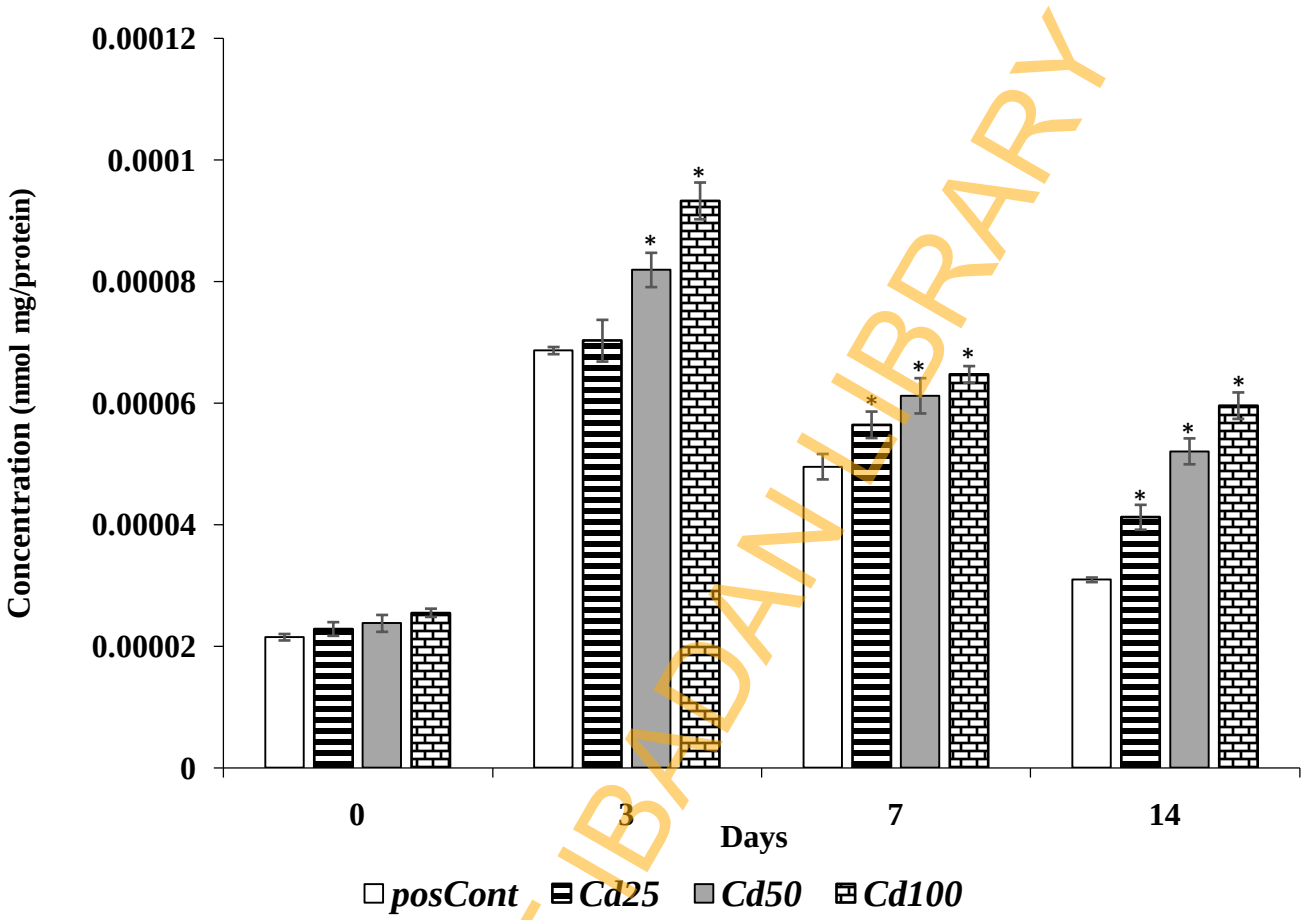


Figure 4.4: Malondialdehyde Concentration (Phase 1)

Each bar is expressed as mean ± S.E.M of 5 rats. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. * = $p<0.05$ values differ significantly from *posCont* group.

4.8 Effect of Cadmium on Colonic Histology (Phase 1)

Lymphocyte infiltration of different levels in the mucosa, edema, erosion, ulcer and regenerating crypts are seen in *Cd25*, *Cd50* and *Cd100* groups when compared with *posCont* (Plate 1).

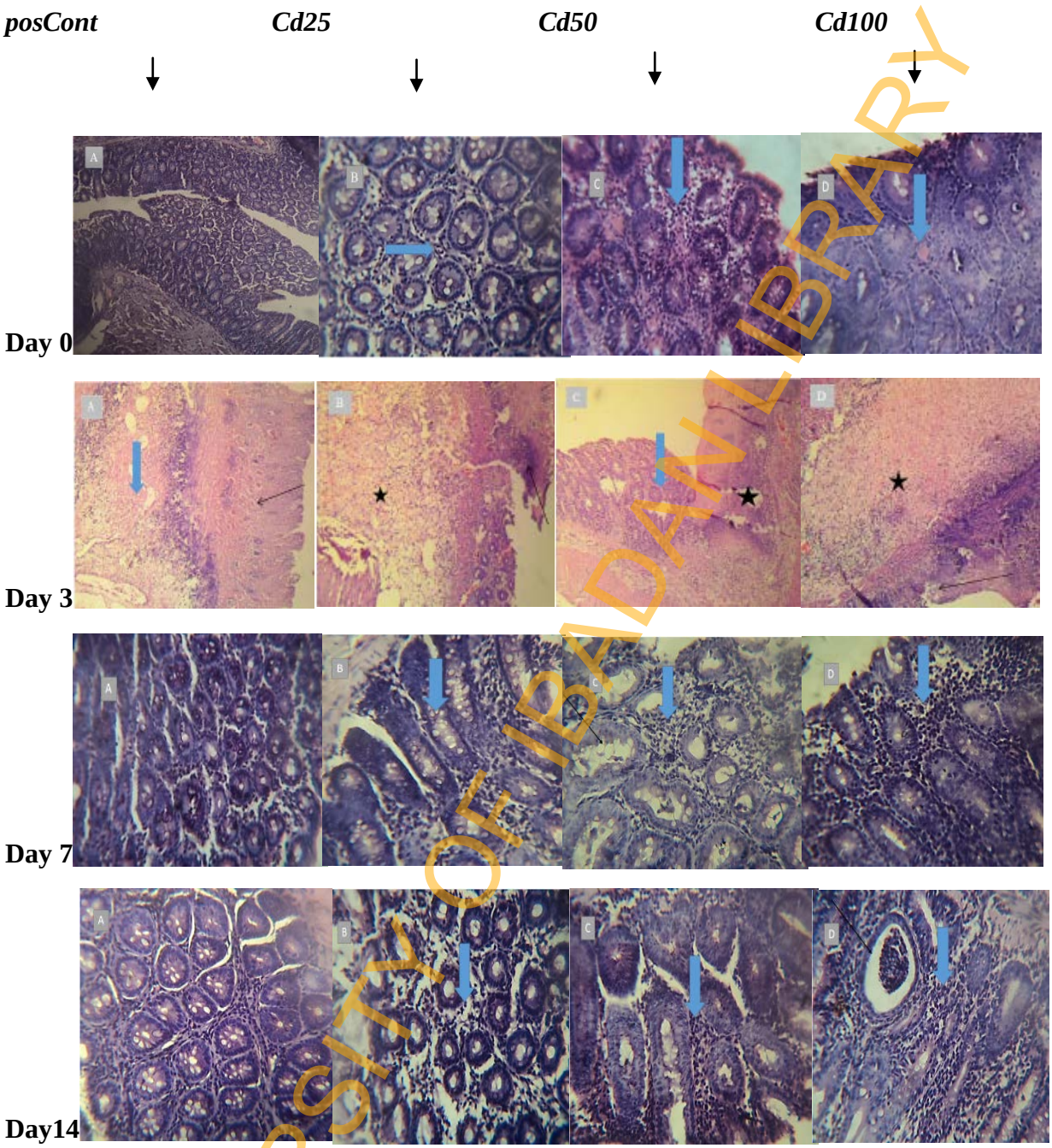


Plate 1: Photomicrographs from colon sections of positive control rats and those treated with cadmium chloride; (A) *posCont*), (B) *Cd25*, (C) *Cd50* and (D) *Cd100*; before induction of colitis by acetic acid (Day 0); and days 3, 7 and 14 post-colitis induction. Where *posCont* = Positive Control; *Cd25* = Cadmium at 25ppm; *Cd50* = Cadmium at 50ppm and *Cd100* = Cadmium at 100ppm. Arrows show different levels of lymphocyte infiltration in the mucosa, edema, erosion, ulcer and regenerating crypts. H&E X400

Phase 2

4.9 Effect of cadmium and graded doses of kolaviron on percentage body weight before and during colitis healing.

Percentage body weight (Figure 4.5)was significantly decreased in 50mg/kg group (30.63±0.99%) at week 3 when compared with *negCont* and *posCont*groups (34.84±0.49%; 34.13±0.87%), respectively.

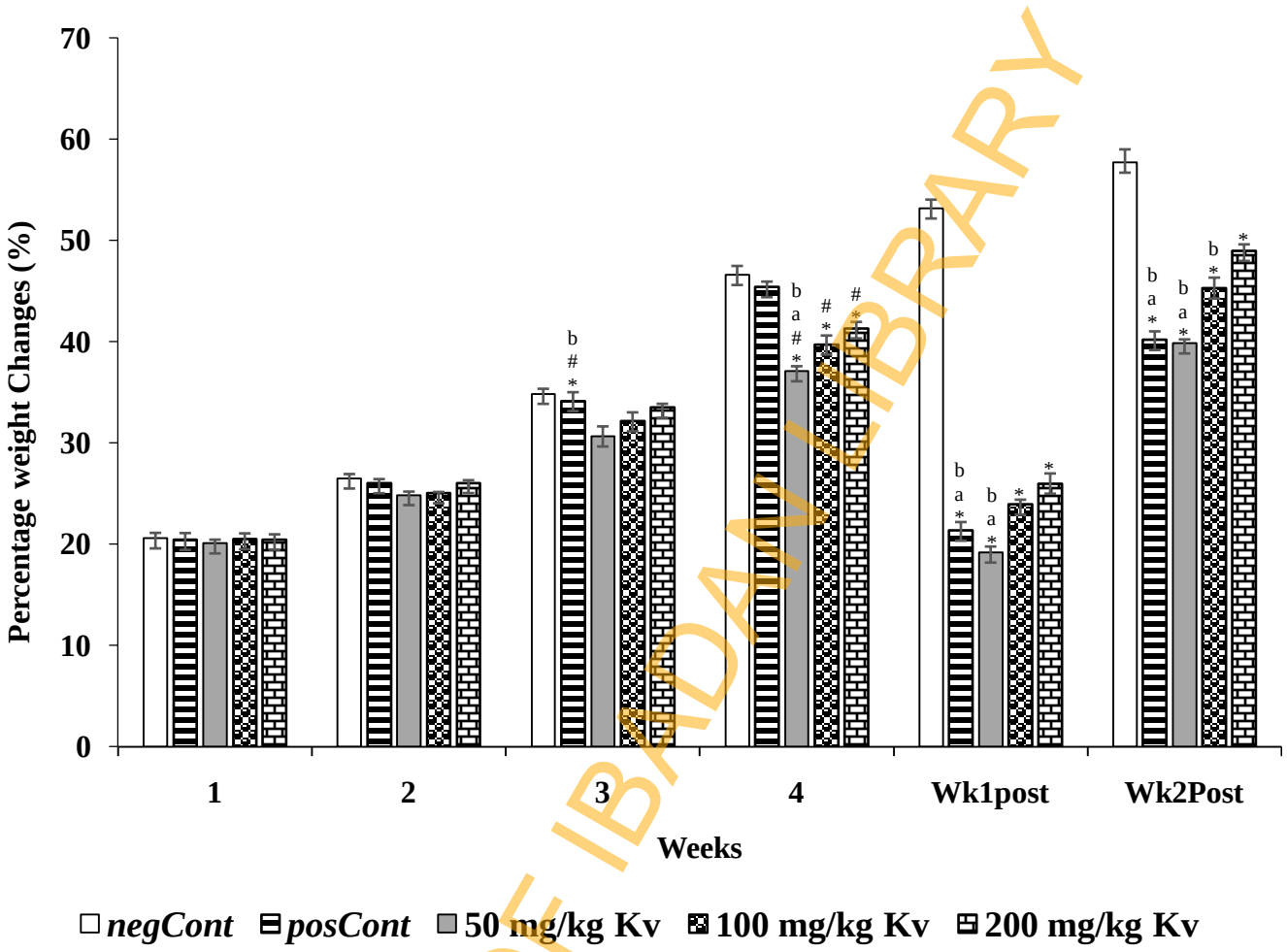


Figure 4.5: Graded doses of kolaviron on mean percentage body weight before and during Colitis Healing in cadmium exposed rats.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron, 200mg/kg = Cadmium+50mg/kg kolaviron, Wk1post = Week 1 post-colitis and Wk2post = Week 2 post-colitis. $^{*, \#, a \text{ and} b}$ = $p<0.05$ values differ significantly from *negCont*, *posCont* or 50mg/kg, 100mg/kg and 200mg/kg groups, respectively.

4.10 Effect of graded doses of kolaviron on colon weight

Colon weight was significantly increased in each of *posCont*, 50mg/kg and 100mg/kg groups (1.62±0.03g; 1.58±0.04g; 1.51±0.04g) at days 7 post colitis induction (Figure 4.6).

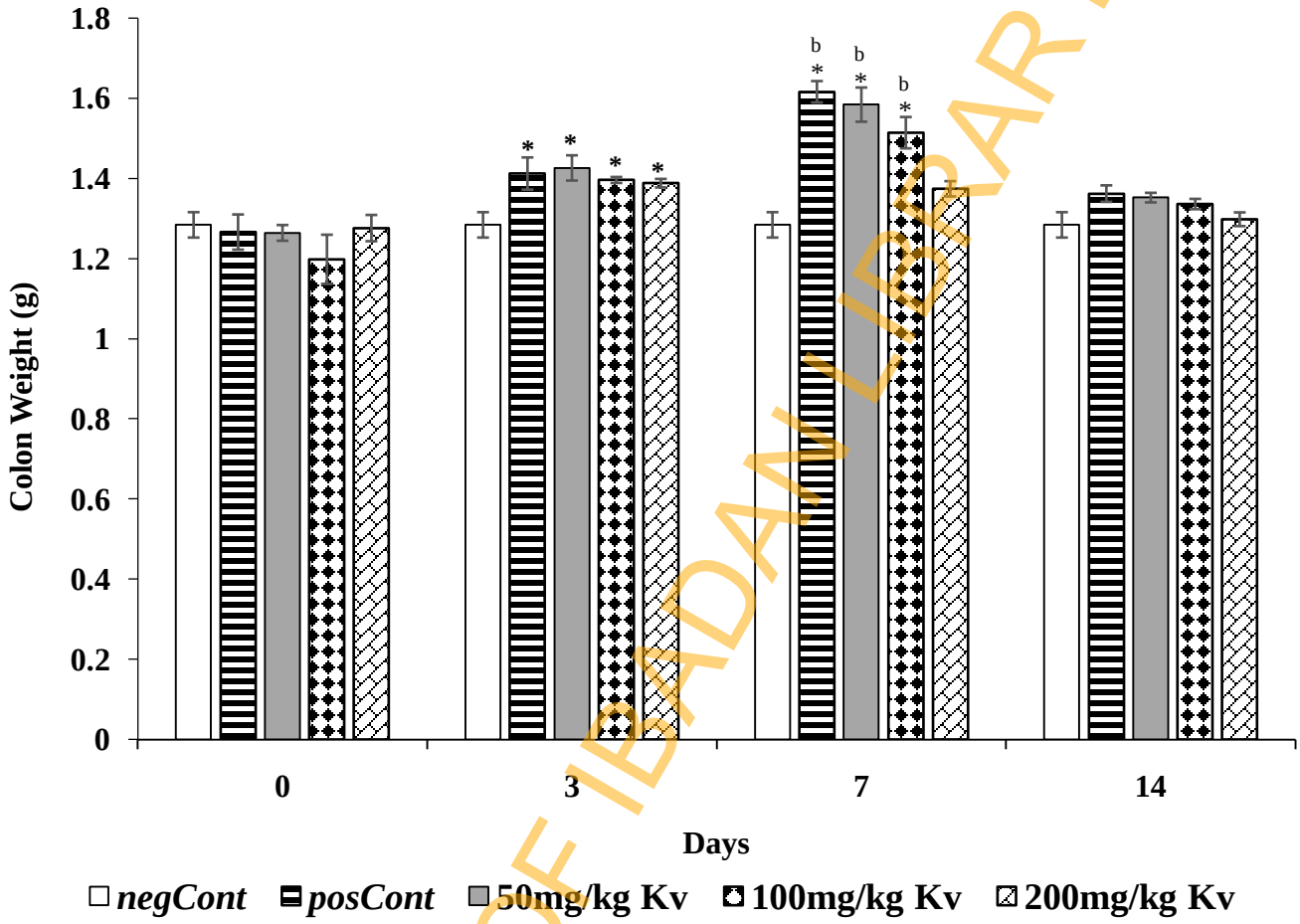


Figure 4.6: Graded doses of kolaviron on mean colon weight before and during Colitis Healing in cadmium exposed rats.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron.*and b = $p<0.05$ values differ significantly from *negCont*and 200mg/kg groups, respectively.

4.11 Effect of graded doses of kolaviron on stool consistency score

Stool consistency scores (Figure 4.7), were significantly increased in each of the groups that was induced with colitis at day 3, this increase was sustained in *posCont*, 50mg/kg and 100mg/kg groups (2.00±0.00; 1.80±0.20;1.40±0.25) till day 7.

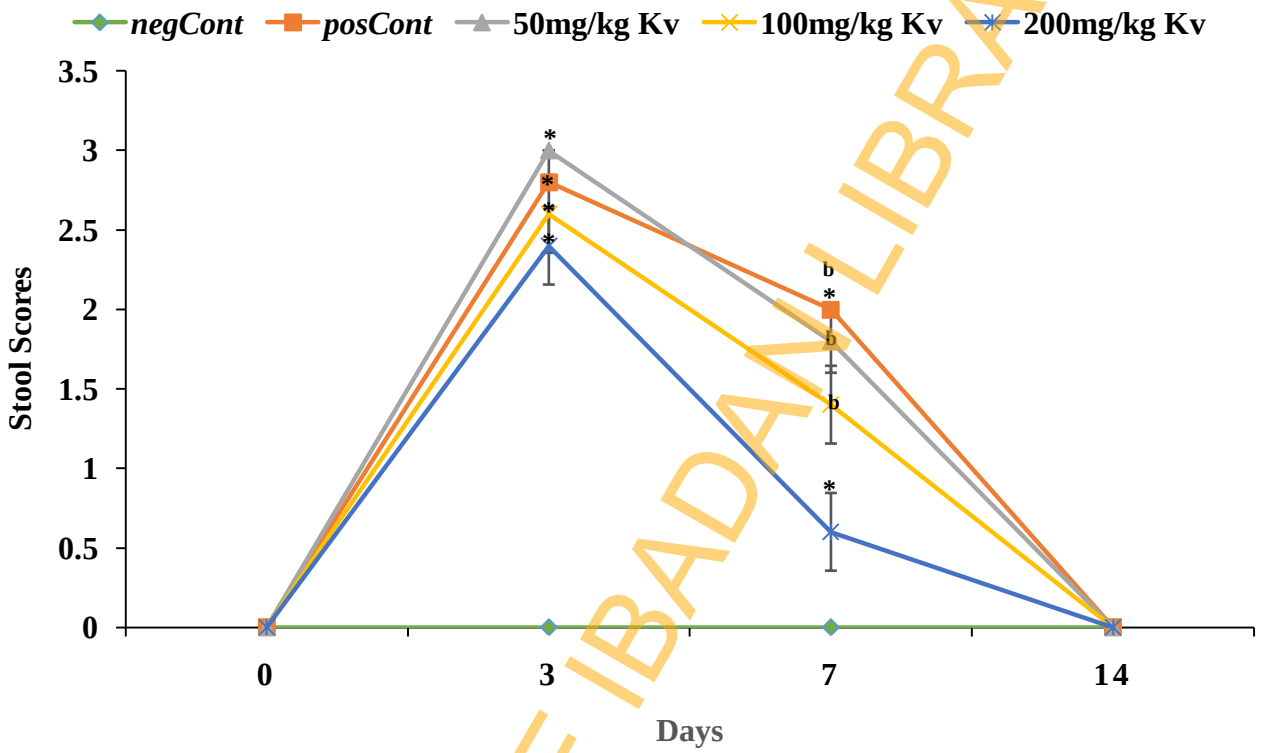


Figure 4.7: Graded doses of kolaviron on mean stool scores before and during Colitis Healing in cadmium exposed rats.

Each line represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron. * and b = $p<0.05$ values differ significantly from *negCont*and 200mg/kg groups, respectively.

4.11 Effect of graded doses of kolaviron on Neutrophil/Lymphocyte ratio (NLR) during cadmium exposures.

Neutrophil/Lymphocyte ratio was significantly increased in each of *posCont*, 50mg/kg and 100mg/kg groups (0.54±0.01; 0.60±0.01; 0.50±0.01) day 3 (Figure 4.8).

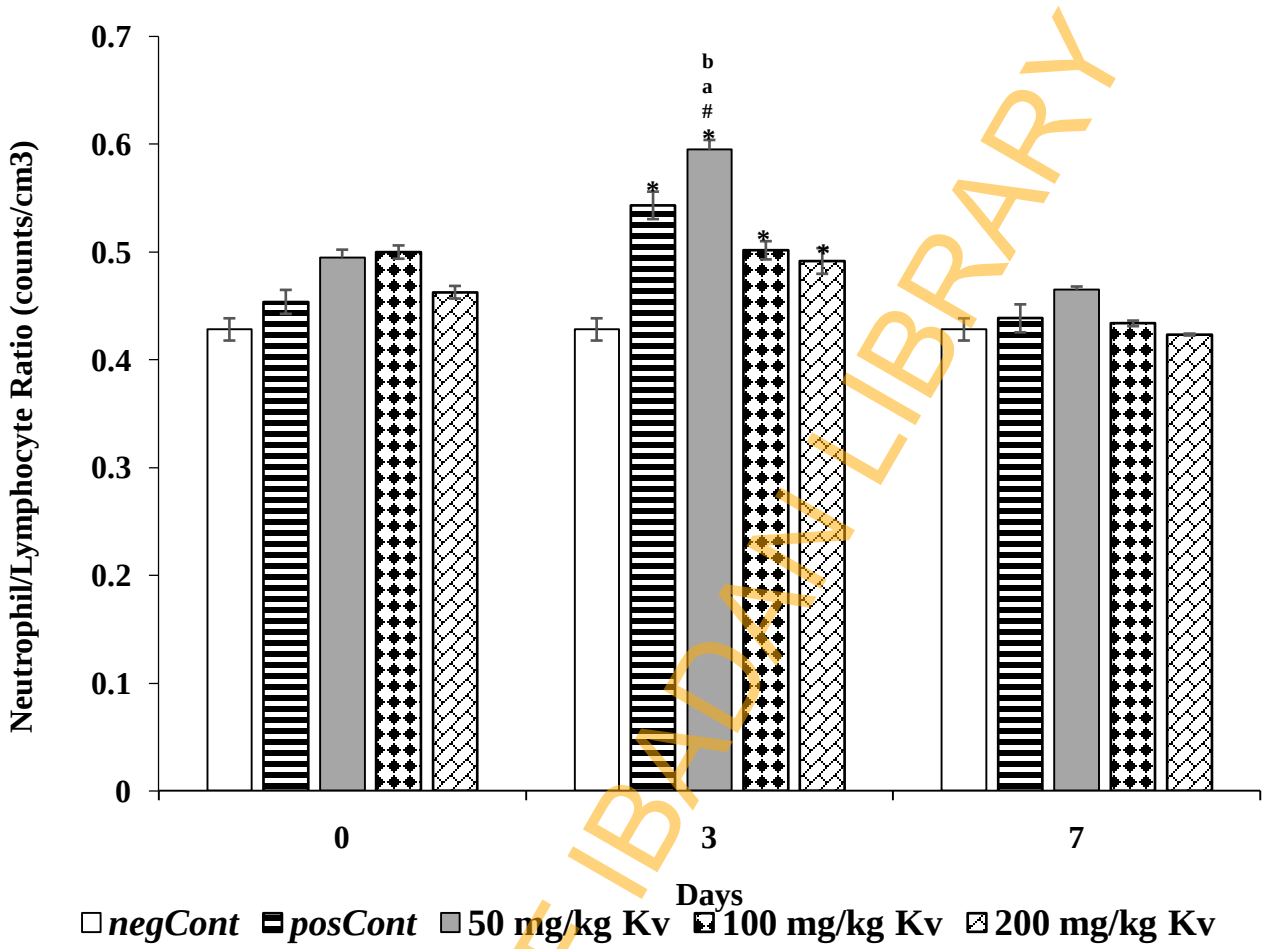


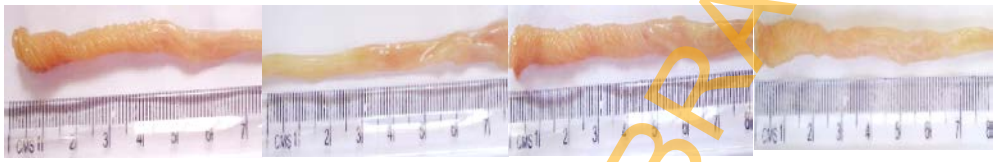
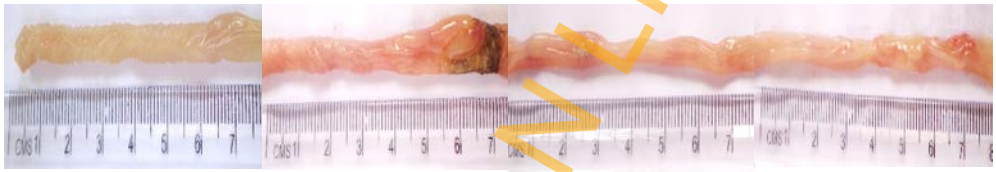
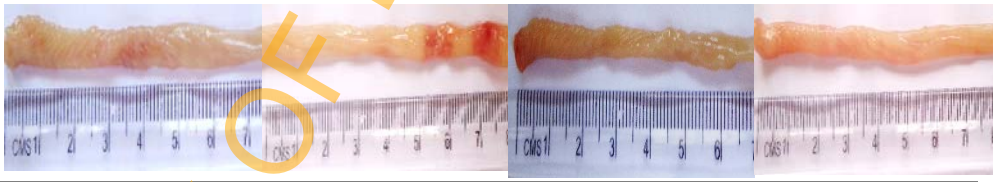
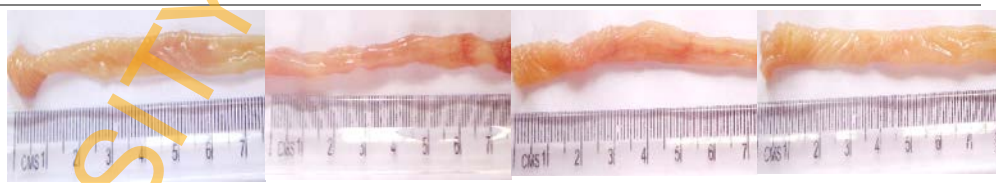
Figure 4.8: Neutrophil/Lymphocyte Ratio

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, 100mg/kg and 200mg/kg groups, respectively.

4.12 Effect of graded doses of kolaviron on colon macroscopic score

Macroscopic scores were significantly increased in each of *posCont*, 50mg/kg, 100mg/kg and 200mg/kg groups (3.80±0.38; 4.80±0.50; 3.60±0.25; 1.6±0.25) on day 3, and *posCont* and 50mg/kg (0.80±0.20; 1.20±0.25) on day 7 (Table 4.4).

Table 4.4: Colonic Gross Appearances and Macroscopic scores for Phase 2

Groups		Day0	Day3	Day7	Day14
negCont	**Scores**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Gross				
posCont	**Score**	0.0±0.0	3.80±0.37*[b]	0.80±0.37*[ab]	0.20±0.20
	Gross				
50mg/kg KV	**Score**	0.0±0.0	4.80±0.49*[b]	1.20±0.20*[ab]	0.40±0.24
	Gross				
100mg/kg KV	**Score**	0.0±0.0	3.60±0.25*[b]	0.00±0.00	0.00±0.00
	Gross				
200mg/kg KV	**Score**	0.0±0.0	1.60±0.20*	0.00±0.00	0.00±0.00
	Gross				

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron. [*,a and b] = $p<0.05$ values differ significantly from *negCont*, 100mg/kg and 200mg/kg groups, respectively.

4.13 Effect of graded doses of kolaviron on MDA of colonic homogenate

MDA levels of colon homogenate on day 3 was significantly increased in *posCont* (6.72±0.10) 50mg/kg (7.00±0.1) groups respectively and on day 7, *posCont* (6.36±0.11) 50mg/kg (6.36±0.05) (Figure 4.9).

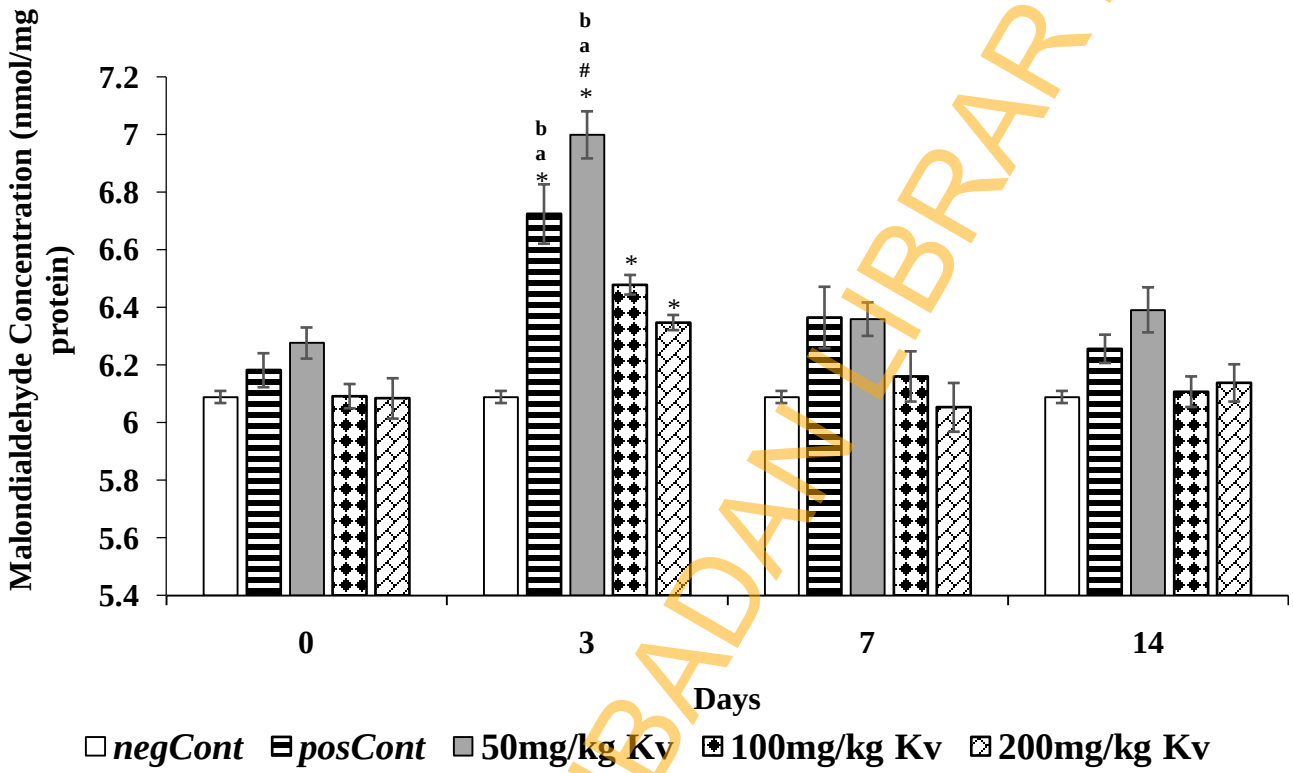


Figure 4.9: Mean malondialdehyde (MDA) concentration for Phase 2

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, 100mg/kg and 200mg/kg groups, respectively.

4.14 Effect of Kolaviron on Colonic Reduced Glutathione Concentration, SOD and Catalase activities during Colitis and Cadmium Exposures.

Reduced glutathione concentration, SOD and catalase activities were significantly decreased at day 3 post- colitis induction in each of *posCont*, 50mg/kg and 100mg/kg groups (Table 4.5).

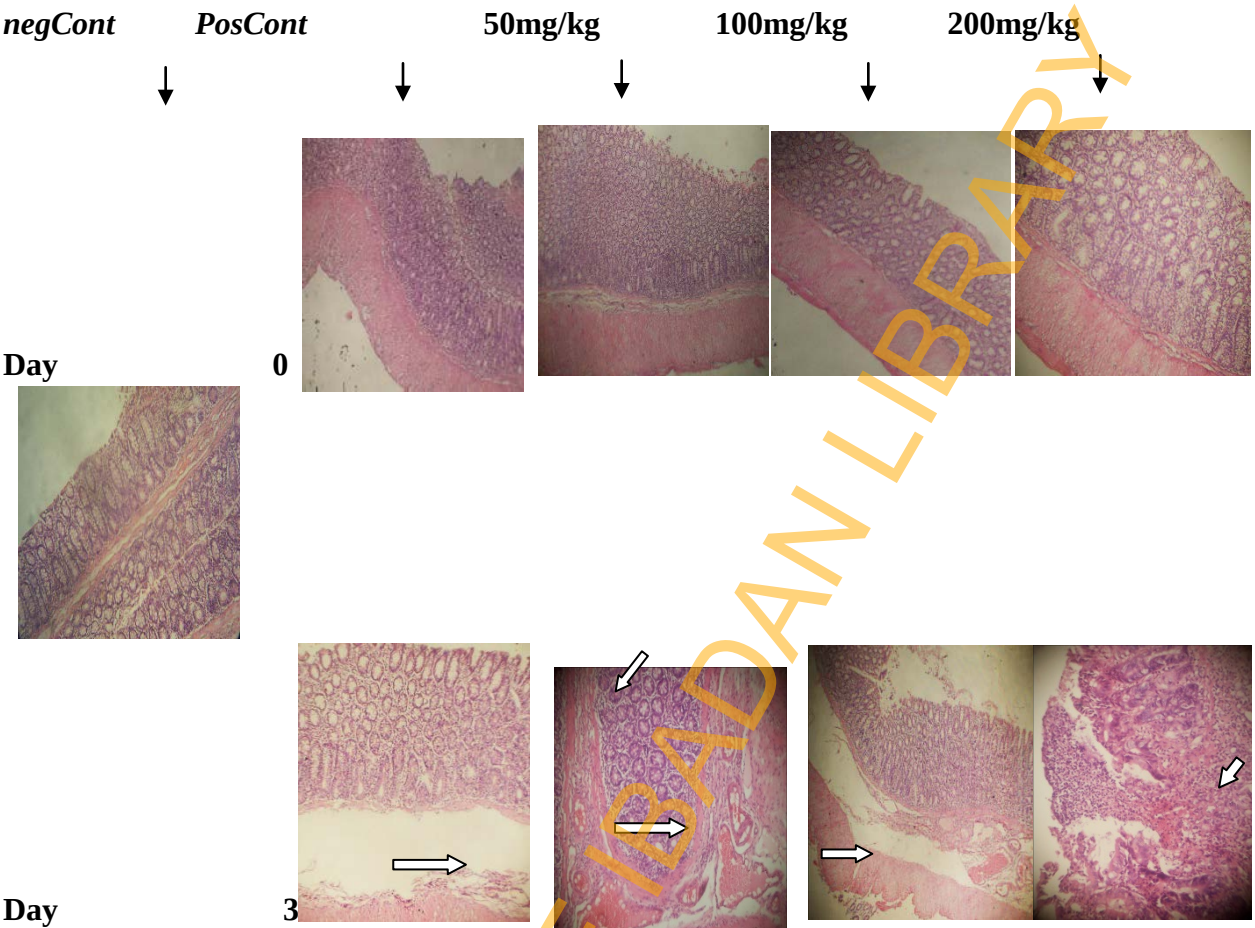
Table 4.5: Reduced Glutathione Concentration (nmol mg/protein), SOD (U/mg protein) and Catalase (U/mg protein) activities

Reduced glutathione	***negCont***	***posCont***	**50mg/kg**	**100mg/kg**	**200mg/kg**
Day0	67.89 ±3.51	66.29 ±1.24	65.97 ±1.94	67.34 ±2.42	68.30 ±2.27
Day3	67.89 ±3.51	56.54 ±2.72*****	57.91 ±3.57*****	58.78 ±3.76*****	63.02 ±3.78
Day7	67.89 ±3.51	63.65 ±3.07	67.91 ±9.13	67.01 ±0.83	71.16 ±3.93
Day14	67.89 ±3.51	69.86 ±1.43	70.83 ±6.38	73.13 ±4.71	77.51 ±5.86
Superoxide Dismutase	***negCont***	***posCont***	**50mg/kg**	**100mg/kg**	**200mg/kg**
Day0	376.63 ±33.31	365.91 ±41.34	344.31 ±36.02	370.54 ±29.39	370.54 ±28.11
Day3	376.63 ±33.31	239.95 ±13.22*****	265.05 ±11.93*****	315.32 ±27.82	315.32 ±37.64
Day7	376.63 ±33.31	287.80 ±24.55	279.58 ±24.03	369.55 ±49.28	369.55 ±25.53
Day14	376.63 ±33.31	338.16 ±34.75	340.54 ±27.00	378.01 ±32.17	378.01 ±28.64
Catalase	***negCont***	***posCont***	**50mg/kg**	**100mg/kg**	**200mg/kg**
Day0	718.85 ±12.36	716.77 ±13.56	719.59 ±6.42	724.78 ±7.24	726.97 ±7.14
Day3	718.85 ±12.36	668.58 ±7.98*****	661.66 ±7.98*****	661.20 ±12.54*****	670.55 ±11.67*****
Day7	718.85 ±12.36	686.01 ±11.47	691.58 ±6.56	701.81 ±3.90	716.95 ±7.42
Day14	718.85 ±12.36	718.96 ±13.85	718.73 ±9.30	724.04 ±6.32	729.37 ±4.77

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; 50mg/kg = Cadmium+50mg/kg kolaviron; 50mg/kg = Cadmium+100mg/kg kolaviron and 200mg/kg = Cadmium+50mg/kg kolaviron. *= $p<0.05$ values differ significantly from *negCont*.

4.15 Effect of Cadmium on Colonic Histology (Phase 2)

Arrows point to different levels of lymphocyte infiltration in the mucosa, edema, erosion, ulcer and regenerating crypts are seen in *posCont*, 50mg/kg, 100mg/kg and 200mg/kg groups during colitis groups when compared with *negCont* group (Plate 2).



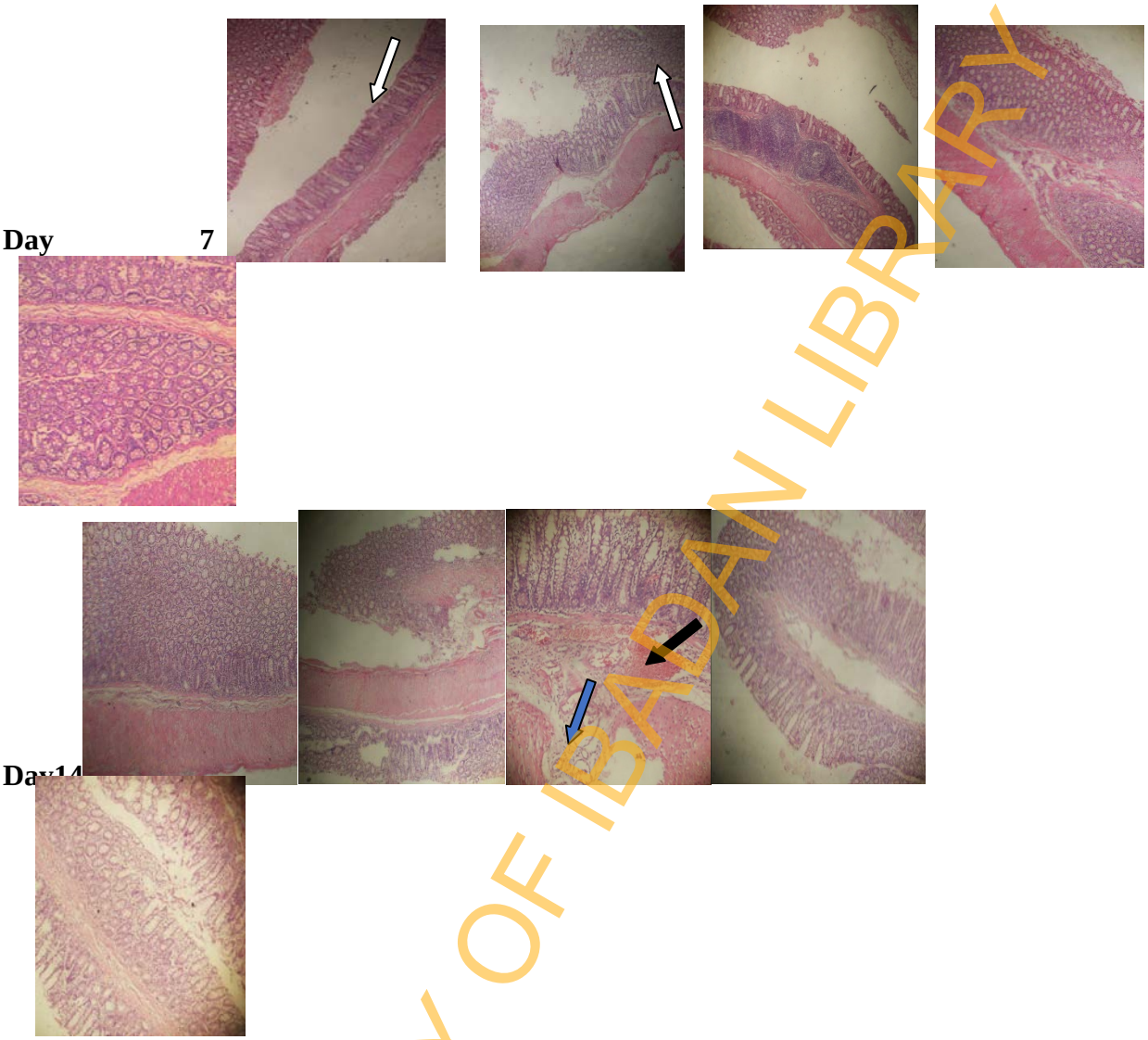


Plate 2: Photomicrographs from colon sections of control rats and those exposed to cadmium and treated with graded doses of kolaviron; 50mg/kg, 100mg/kg and 200mg/kg during colitis; before induction of colitis by acetic acid (Day 0); and days 3, 7 and 14 post-colitis induction. Arrows show different levels of lymphocyte infiltration in the mucosa, hyperemia, erosion, ulcer lymphoid tissue and granulation tissue. H&E X400

Phase 3 Results

4.16 Water Cadmium Concentration:

The results from this study (Figure 4.10) showed significant increases in the different concentrations of cadmium; Tapwater+0.0025g$CdCl_2$(0.11±0.00ppm), Tapwater+0.005g$CdCl_2$ (0.19±0.00ppm) and Tapwater+0.01g$CdCl_2$ (0.66±0.00ppm) when compared with Tapwater (0.01±0.00ppm).

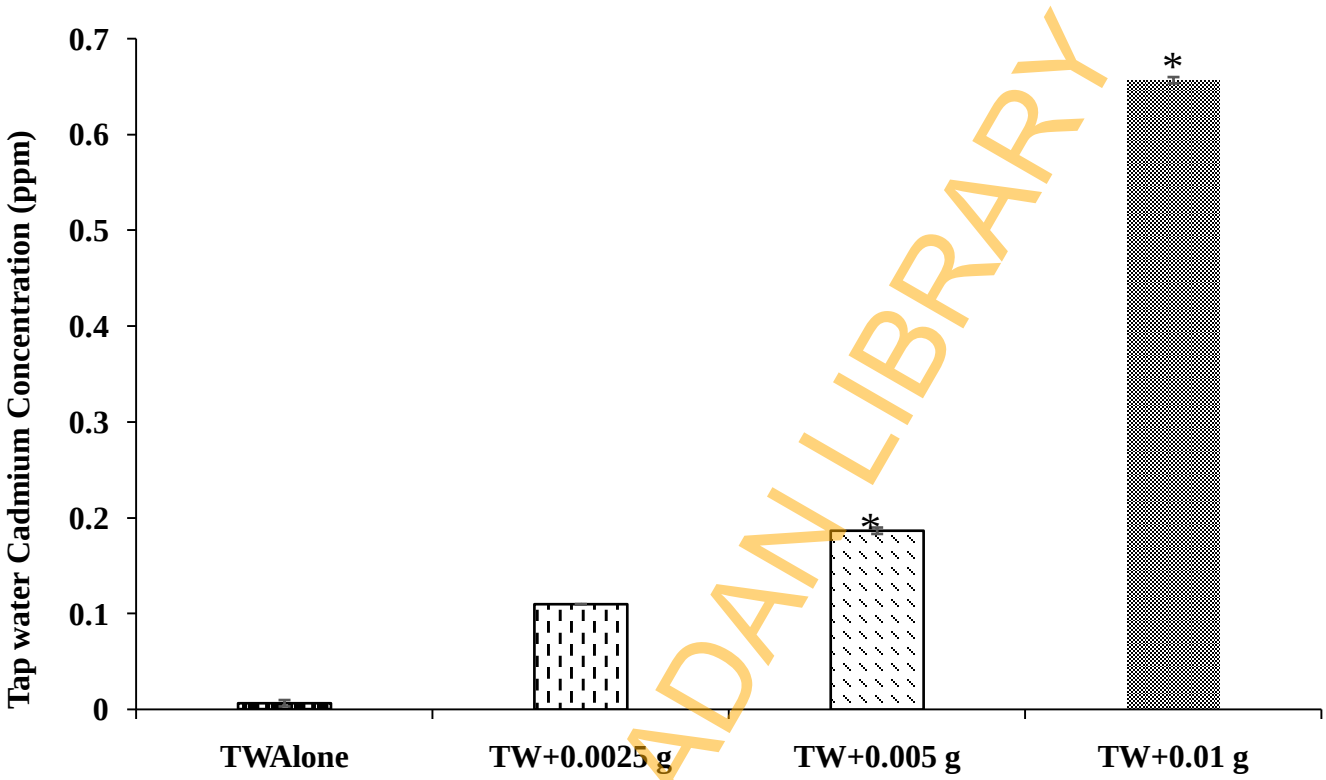


Figure 4.10: Watercadmium concentration.

Where TW Alone = TapWater Alone; TW+0.0025g = TapWater+0.0025g of $CdCl_2$; TW+0.005g= TapWater+0.005g of $CdCl_2$; TW+0.01g= TapWater+0.01g of $CdCl_2$. *= $p<0.05$ values differ significantly from TapWater alone.

4.17 Blood Cadmium Concentration (Phase 3):

The results from this study (Figure 4.11) showed significant decreases blood cadmium concentration; *negCont*(0.01±0.00ppm), *posCont* (0.01±0.00ppm), Cd+KV(0.01±0.00ppm)and KV (0.01±0.00ppm) when compared with Cd (0.02±0.00ppm).

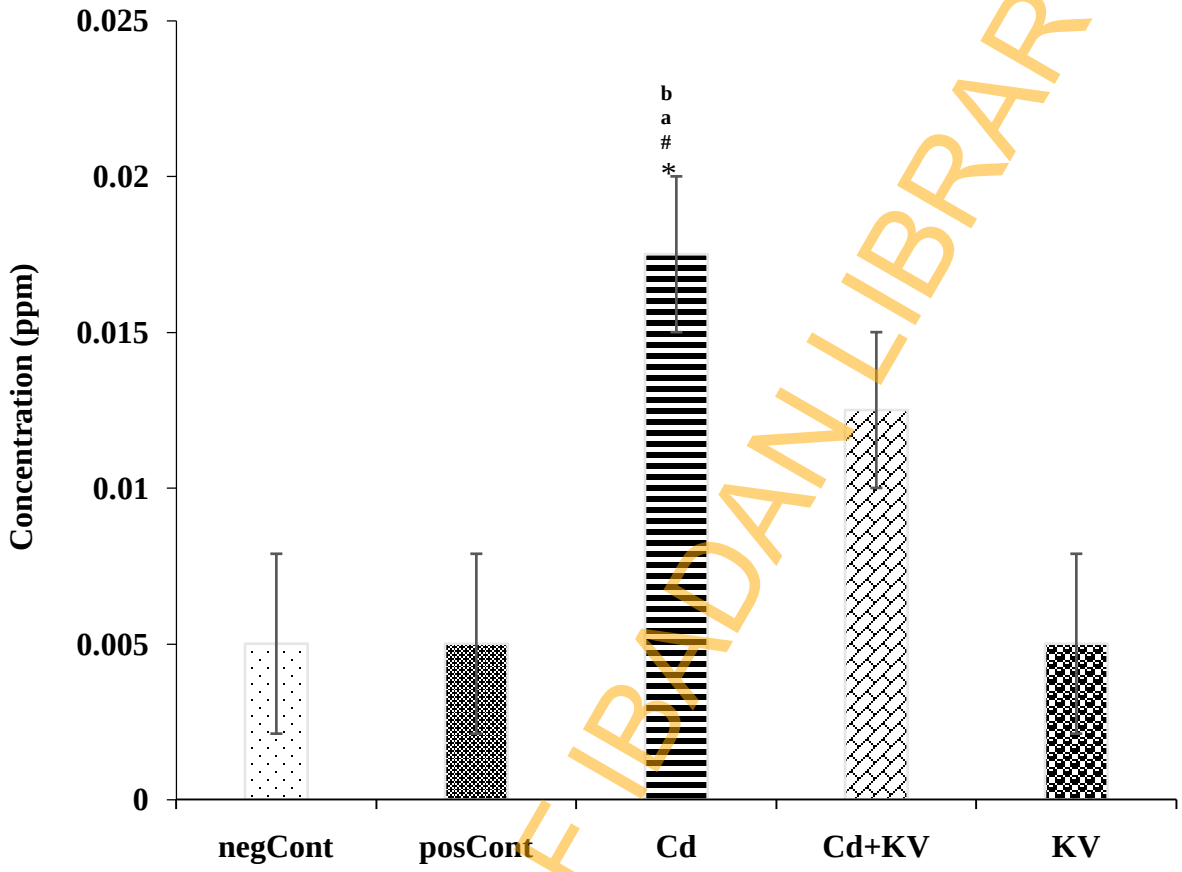


Figure 4.11: Blood cadmium concentration after four weeks of exposure and treatment (Phase 3).

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.18 Effect of Cadmium and Kolaviron on Neutriphil/Lymphocyte ratio (NLR):

The results from this study (Figure 4.12) showed significant decreases in NLR; *negCont* (0.43±0.01), *posCont* (0.46±0.06), Cd+KV(0.45±0.02) and KV (0.41±0.00) when compared with Cd (0.51±0.02) on day 3.

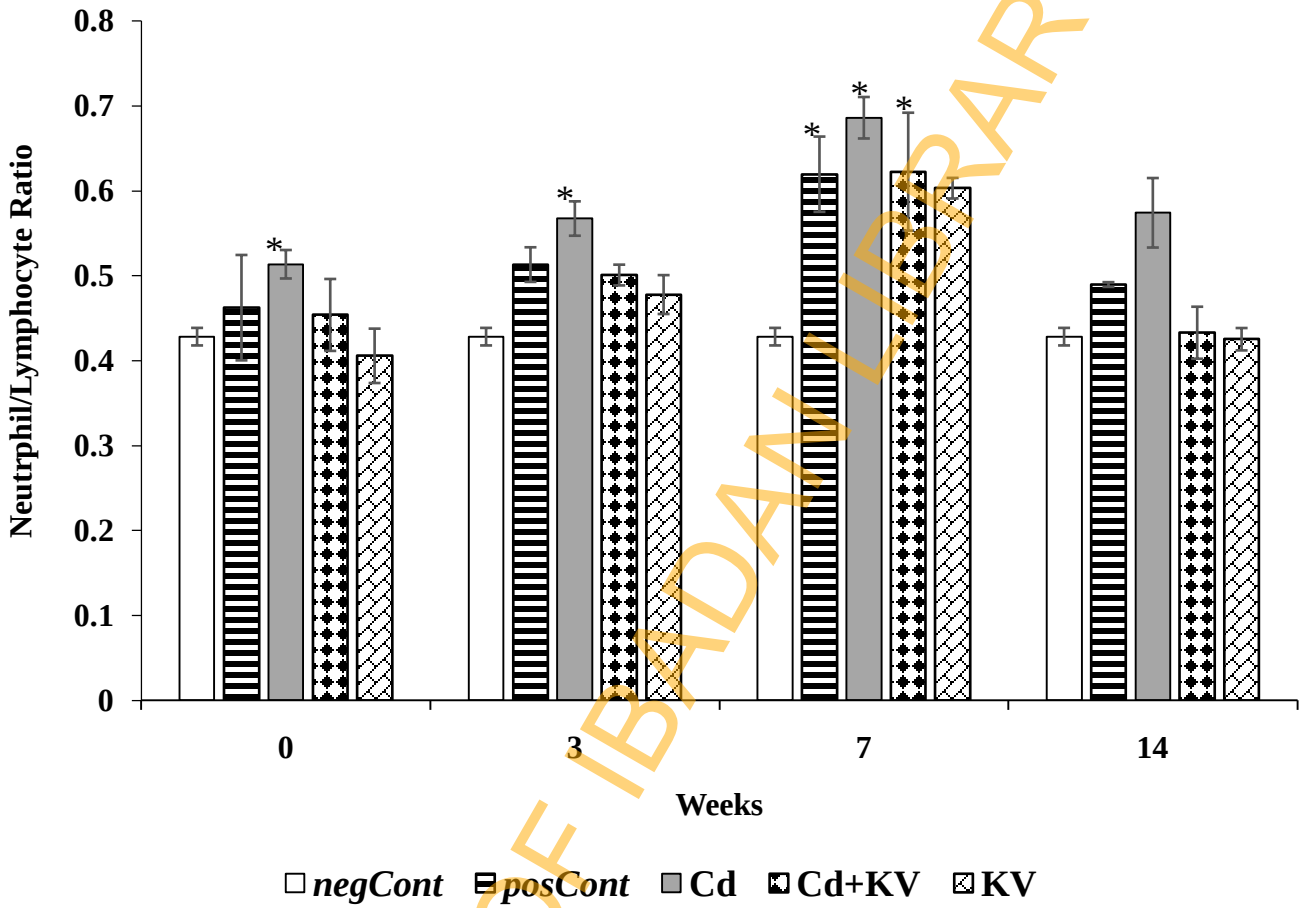


Figure 4.12: Effect of Cadmium and Kolaviron on Neutriphil/Lymphocyte ratio on predetermined days of the experiment.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. * = $p<0.05$ values differ significantly from *negCont* group.

4.19 Effect of Cadmium and Kolaviron on Weekly Percentage Body Weight:

The results from this study (Figure 4.13) showed significant increases in percentagebody weight; *negCont* (36.35±0.45%), *posCont* (36.19±0.73%), Cd+KV(36.17±0.51%) and KV (36.54±0.00%) when compared with Cd (31.00 ±0.04%) on week 3.

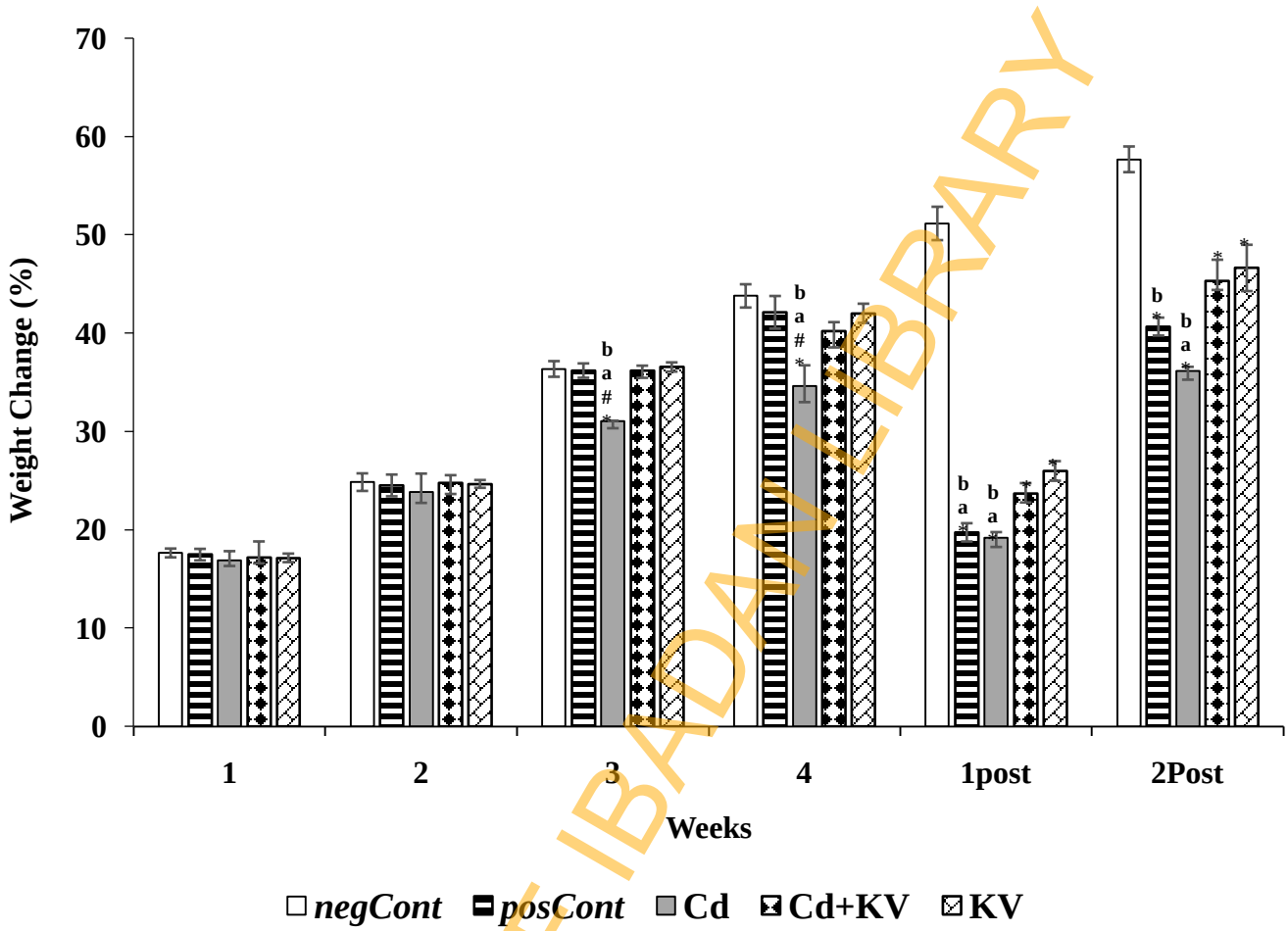


Figure 4.13: Weekly Percentage body weight changes in rats before and post-colitis induction during cadmium and kolaviron administration.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.20 Effect of Cadmium and Kolaviron on Colon/Weight Ratio:

The results from this study (Table 4.6) showed significant increases in colon weight/ratio.Oral administration of kolaviron at 200mg/kg decreased colon weight/length ratio on day 14 post colitis induction such that weight/length ratio was not significant when compared with *negCont* group.

Table 4.6: Colon Weight/Length Ratio

Weight/Length Ratio (g/cm)	*negCONT*	*posCONT*	Cd	Cd+KV	KV
Day0	0.08±0.00	0.10±0.01	0.09±0.00	0.08±0.01	0.09±0.00
Day3	0.08±0.00	0.13±0.01*	0.13±0.00*	0.12±0.01*	0.12±0.01*
Day7	0.08±0.00	0.12±0.01*	0.13±0.01*	0.13±0.00*	0.11±0.01*
Day14	0.08±0.00	0.10±0.01*	0.10±0.00*	0.09±0.00	0.09±0.00

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. * = $p<0.05$ values differ significantly from *negCont* group.

4.21 Effect of Cadmium and Kolaviron on Kidney, Liver and Spleen Weights:

The results from this study (Table4.7) showed significant increases in liver and spleen weight. This results also showedincrease in kidney weight on day 14 when compared with *negCont* group. Liver weight was significantly increased in Cd group on days 0, 3, 7 and 14 when compared with *negCont*, *posCont*, Cd+KV and KV groups.

Table 4.7: Kidney, Liver and Spleen Weights (g)

Kidney Weight(g)	***negCont***	***posCont***	**Cd**	**Cd+KV**	**KV**
Day0	0.40±0.03	0.41±0.01	0.46±0.04	0.42±0.02	0.41±0.02
Day3	0.40±0.03	0.40±0.03	0.45±0.01	0.45±0.02	0.41±0.04
Day7	0.40±0.03	0.42±0.01	0.45±0.02	0.44±0.01	0.43±0.01
Day14	0.40±0.03	0.44±0.02	0.50±0.02*	0.44±0.02	0.44±0.02
Liver Weight(g)	***negCont***	***posCont***	**Cd**	**Cd+KV**	**KV**
Day0	3.79±0.16	3.79±0.15	4.88±0.43*$^{\#ab}$	4.03±0.10	3.75±0.13
Day3	3.79±0.16	3.82±0.31	4.80±0.24*$^{\#b}$	4.32±0.26	3.81±0.19
Day7	3.79±0.16	4.07±0.18	4.88±0.14*$^{\#ab}$	4.34±0.24	4.05±0.10
Day14	3.79±0.16	4.52±0.06	5.68±0.17*$^{\#ab}$	5.00±0.06	4.62±0.07
Spleen Weight(g)	***negCont***	***posCont***	**Cd**	**Cd+KV**	**KV**
Day0	0.33±0.02	0.34±0.01	0.38±0.01	0.35±0.01	0.32±0.03
Day3	0.33±0.02	0.34±0.04	0.45±0.03*$^{\#b}$	0.41±0.01	0.34±0.01
Day7	0.33±0.02	0.33±0.02	0.45±0.03*$^{\#ab}$	0.38±0.01	0.33±0.03
Day14	0.33±0.02	0.35±0.02	0.47±0.01*$^{\#ab}$	0.40±0.02*	0.38±0.01

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. $^{*, \#, a \text{ and } b}$ = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.22 Effect of Cadmium and Kolaviron on Stool Consistency Scores:

The results from this study (Figure 4.14) showed significant increases in stool scores at day 3 *posCont*, Cd, Cd+KV and KVgroups (2.6±0.25; 2.6±0.25; 2.4±0.25 and 2.4±0.25), respectively compared with *negCont* (0.00±0.00). Increases in stool scores lasted in Cd group (0.8±0.2) till day 14.

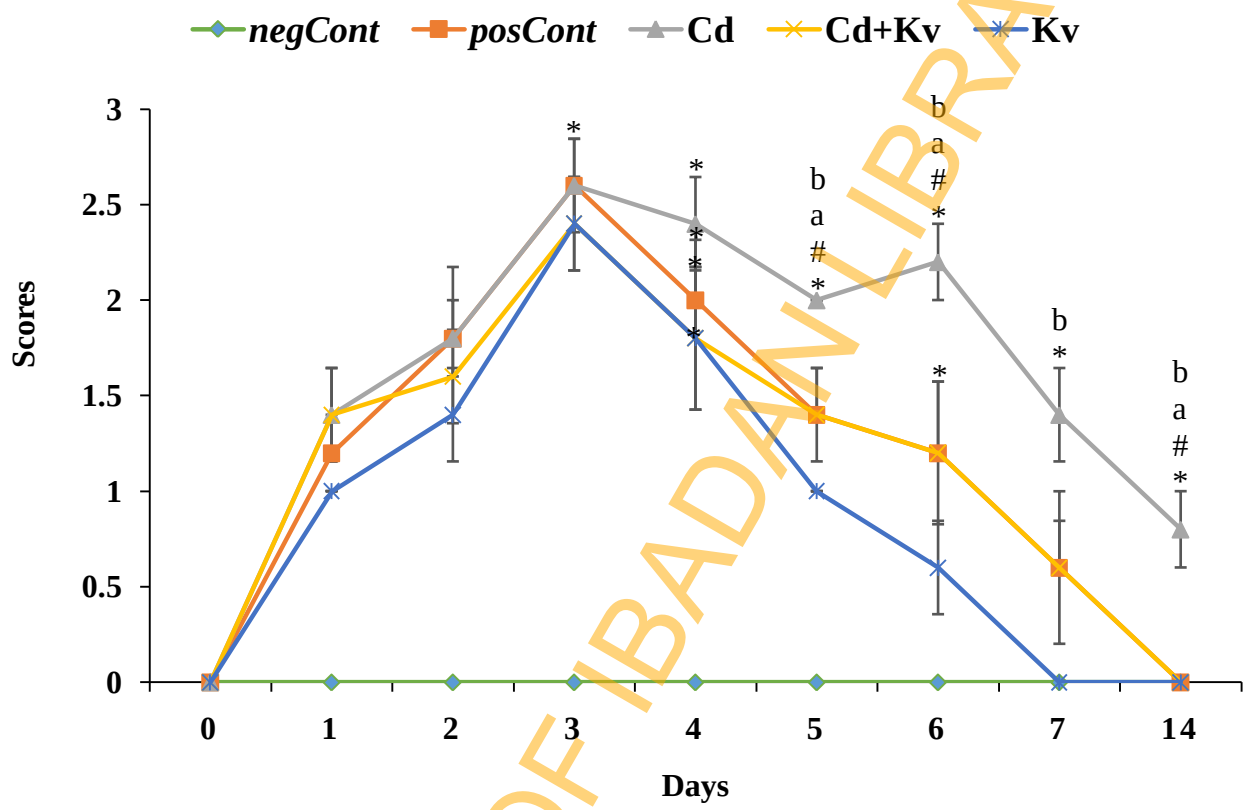


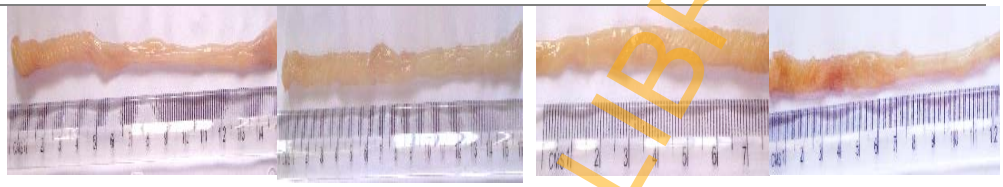
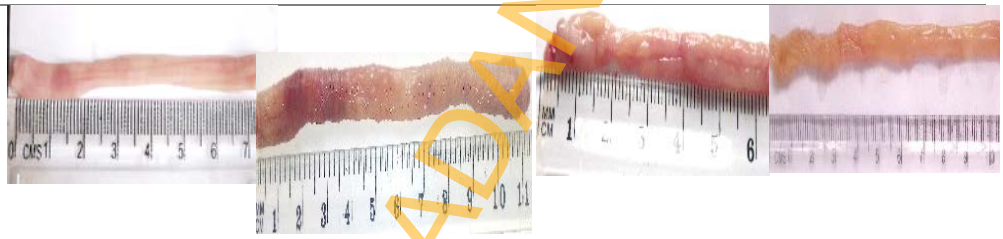
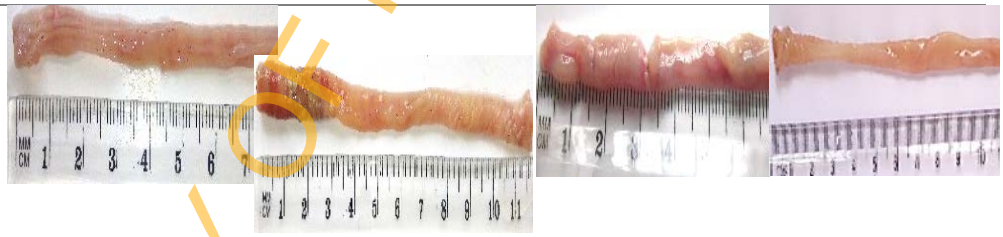
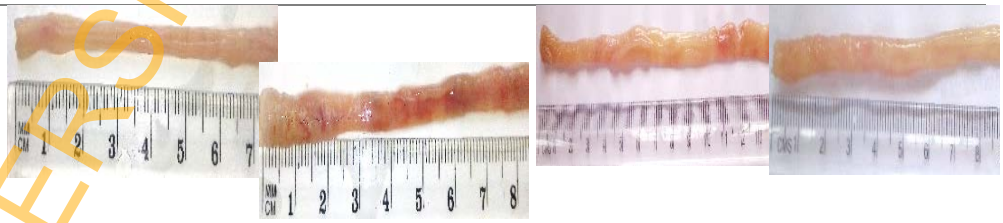
Figure 4.14: Effect of Cadmium and Kolaviron on Stool Consistency Score during colitis healing.

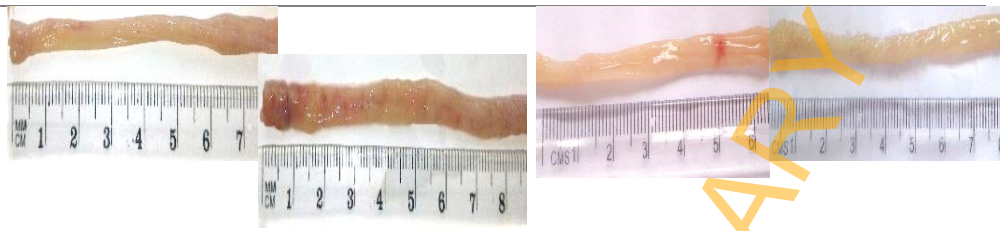
Each line represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.23 Effect of Cadmium and Kolaviron on Macroscopic Scores (Phase 3):

The results from this study (Table 4.8) showed significant increases in macroscopic scoresat day 3 in *posCont*, Cd, Cd+KV and KV groups (4.20±0.60; 6.60±0.49; 6.20±0.49; 4.20±0.20), respectively when compared with *negCont* (0.00±0.00). This increase was sustained till day 14 in Cd group (2.80±0.25) when compared with other groups.

Table 4.8: Colonic gross appearance and macroscopic scores for Phase 3

Groups		Day0	Day3	Day7	Day14
negCont	**Scores**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Gross				
posCont	**Score**	0.0±0.0	4.20±0.60*	3.00±0.67*	0.60±0.33
	Gross				
Cd100	**Score**	0.0±0.0	6.60±0.49*$^{\#b}$	4.20±0.00*$^{\#ab}$	2.80±0.25*$^{\#ab}$
	Gross				
Cd+KV	**Score**	0.0±0.0	6.20±0.49*$^{\#b}$	3.20±0.37*	0.60±0.25
	Gross				
KV	**Score**	0.0±0.0	4.20±0.20*	2.20±0.20*	0.00±0.0

Gross	

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.24 Effect of Cadmium and Kolaviron on Histomophormetry (Crypt depth, Submucosa width and goblet cell density):

The results from this study (Table 4.9) showed that crypt depth was significantly reduced in *posCont*, Cd, Cd+KV and KV groups (37.61±1.69; 37.24±4.18; 39.90±2.97; 41.88±4.08µm), respectively when compared with *negCont* group (53.32±3.80µm) on day 3. Significant decreases in crypt depth was sustained in crypt depth till day 7 in *posCont* and Cd groups (42.28±2.84; 40.46±1.01µm), respectively. Significant increases in submucosa width were seen in all colitis induced groups from day 3 till day 7. Goblet cell density as seen on figure 4.15, were significantly decreased in Cd group on days 3, 7 and 14 ($2.2*10^{-2}$±0.00; $2.5*10^{-2}$±0.00; $2.6*10^{-2}$±0.00), respectively when compared with *negCont* group ($2.6*10^{-2}$±0.00).

Table 4.9: Effect of Cadmium and Kolaviron on Histomorphometry

Crypt depth (µm)	***negCont***	***posCont***	**Cd**	**Cd+KV**	**KV**
Day0	53.32 ±3.80	52.88 ±2.41	54.20 ±2.34	53.34 ±3.38	53.39 ±3.48
Day3	53.32 ±3.80	37.61 ±1.69*	37.24 ±4.18*	39.90 ±2.97*	41.88 ±4.08*
Day7	53.32 ±3.80	42.28 ±2.84*	40.46 ±1.01*	45.86 ±3.37	46.05 ±1.24
Day14	53.32 ±3.80	51.57 ±1.53	49.82 ±3.57	53.54 ±4.10	54.39 ±2.67
Submucosa width (µm)	***negCont***	***posCont***	**Cd**	**Cd+KV**	**KV**
Day0	35.59 ±2.28	35.51 ±3.71	38.08 ±2.99	37.27 ±2.09	36.09 ±3.38
Day3	38.33 ±2.16	74.75 ±1.74*	79.87 ±1.30*	74.65 ±3.51*	69.40 ±4.62*

Day7	39.06 ±2.51	63.43 ±5.33*	68.36 ±3.02*	61.56 ±5.77*	53.90 ±2.55*
Day14	41.74 ±1.49	59.82 ±4.51	63.01 ±8.98	53.48 ±5.63	51.66 ±3.76

Each value represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. * = p<0.05 values differ significantly from *negCont* group.

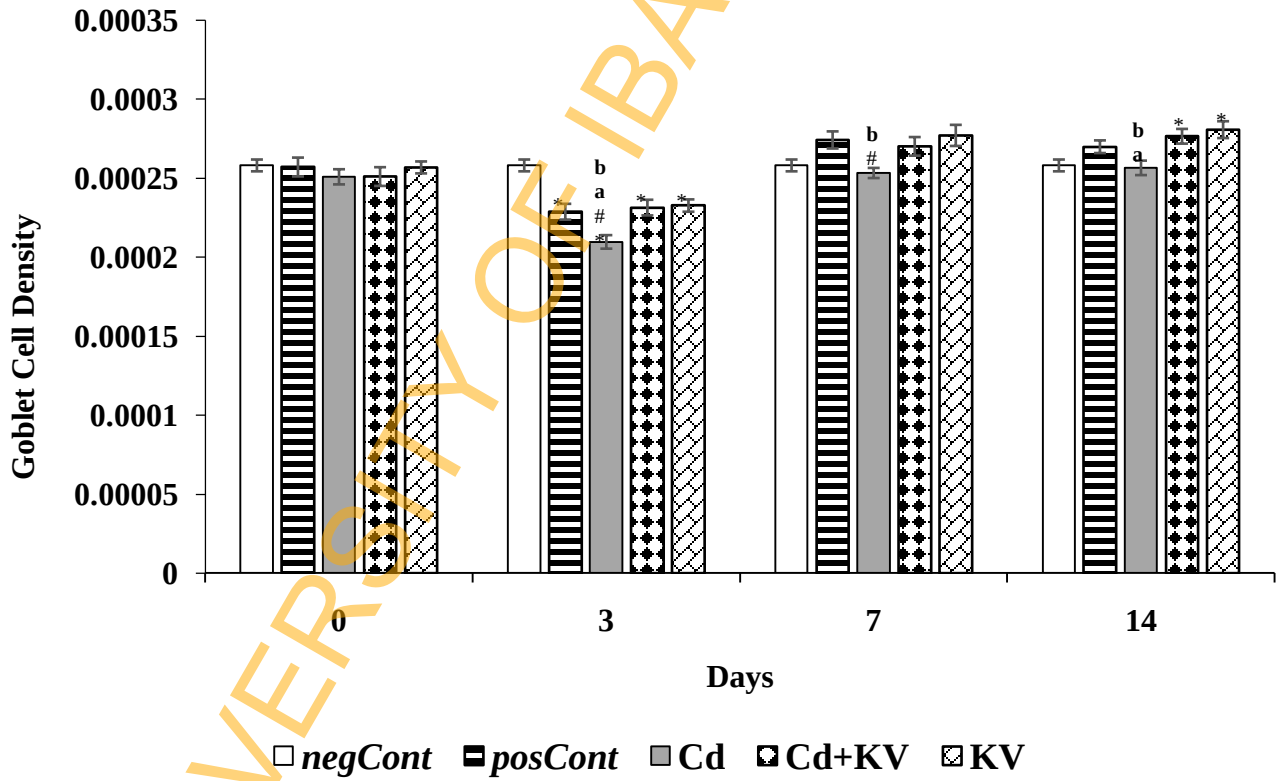
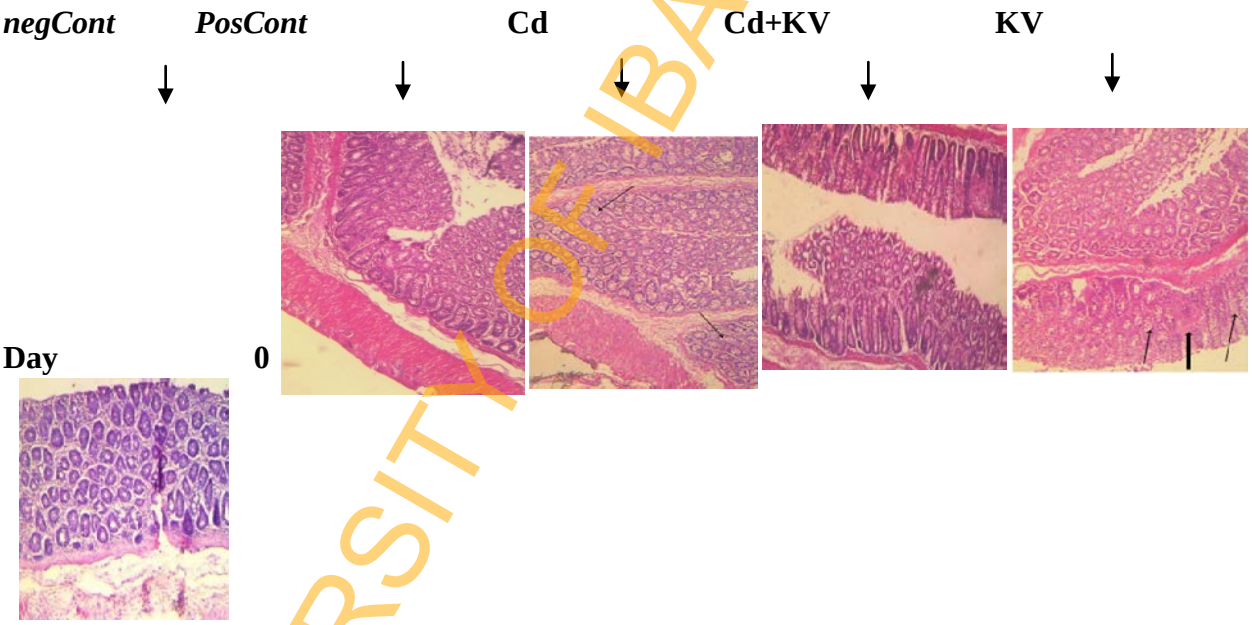


Figure 4.15: Effect of Cadmium and Kolaviron on goblet cell density.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.25 Effect of Cadmium on Colonic Histology for Phase 3 Study

Arrows point to different levels of lymphocyte infiltration in the mucosa, edema, erosion, ulcer and regenerating crypts are seen in *negCont*, *posCont*, Cd, Cd+KV and KVgroups during colitis groups when compared with *negCont* group (Plate 3).



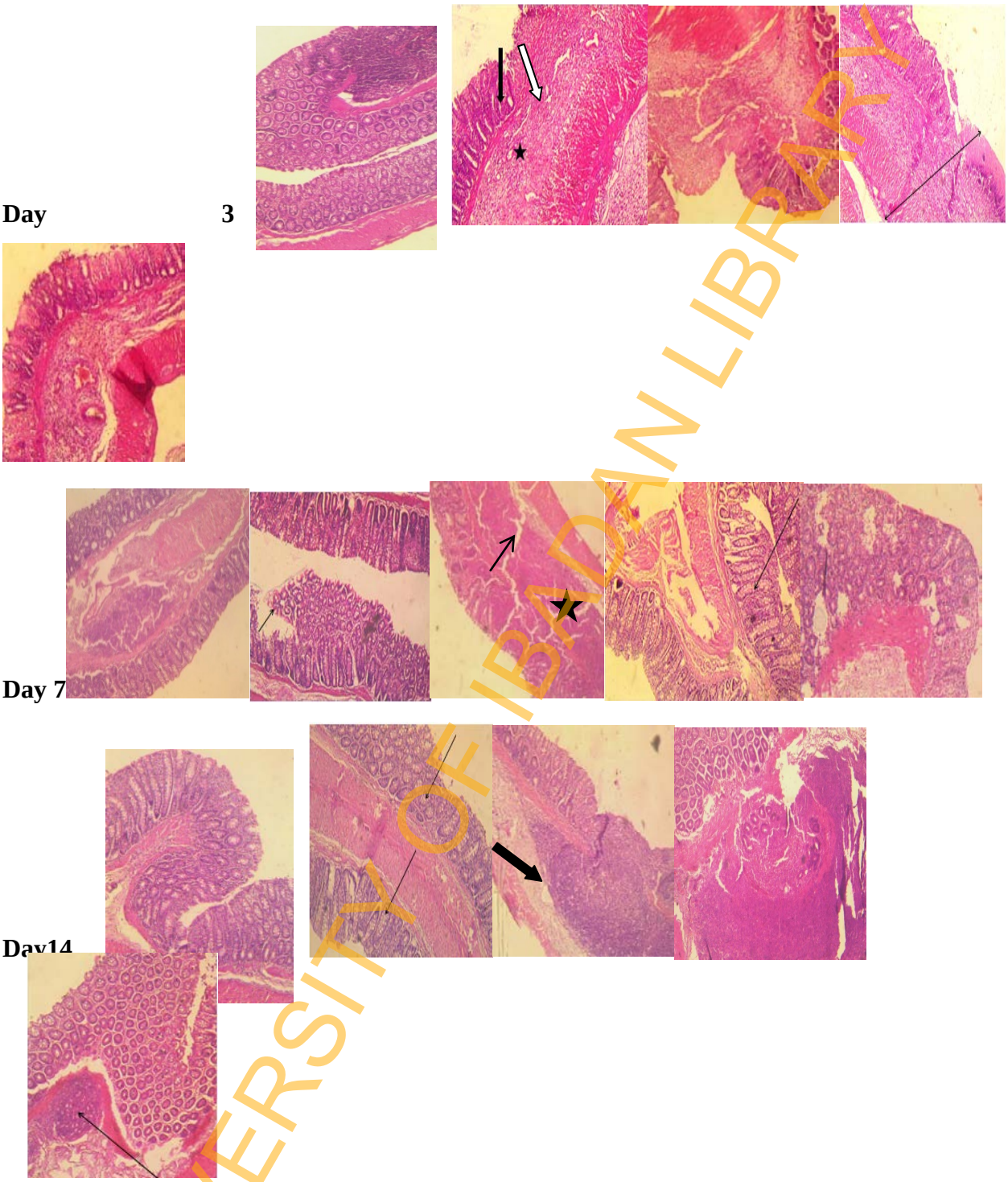


Plate 3: Photomicrographs from colon sections of *negCont*, *posCont*, Cd, Cd+KV and KV groups during colitis healing; Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron.; before induction of colitis by acetic acid (Day 0); and days 3, 7 and 14 post-colitis induction. Arrows show different

levels of lymphocyte infiltration in the mucosa, edema, erosion, ulcer and regenerating crypts. H&E X400

4.26 Effect of Cadmium and Kolaviron on Colonic Malondialdehyde (MDA) concentration:

The results from this study (Figure 4.16) showed significant increases in MDA concentration in Cd group on days 0, 3, 7 and 14 (2.46±0.04; 3.07±0.01; 2.63±0.05; 2.39±0.02 nmol/mg protein), respectively when compared with *negCont* group (2.11±0.05).

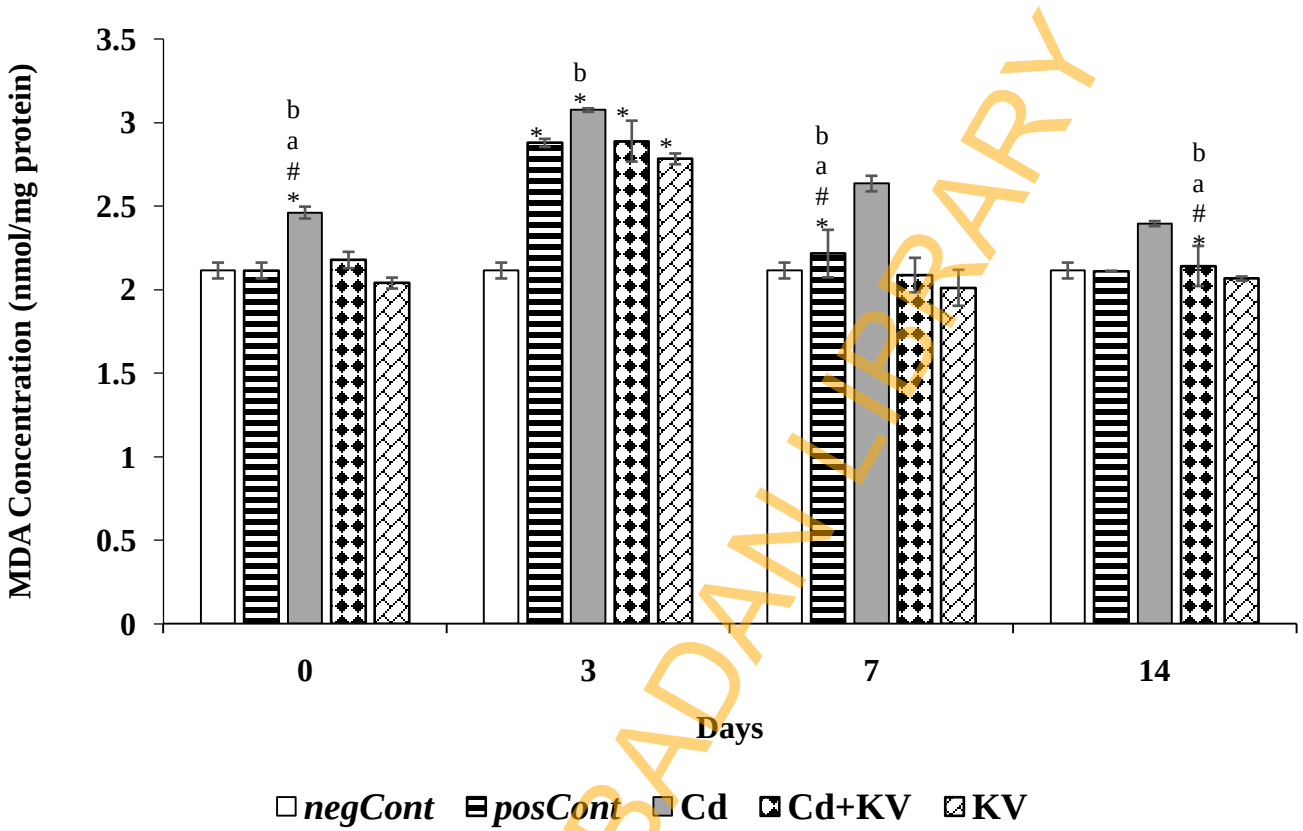


Figure 4.16: Effect of Cadmium and Kolaviron on Colonic Malondialdehyde (MDA) Concentration during Colitis Healing (Phase 3).

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.27 Effect of Cadmium and Kolaviron on Colonic Myeloperoxidase (MPO) Activity during Colitis Healing:

The results from this study (Figure 4.17) showed significant increasesin MPO concentration on days 3 and 7 in Cd group (0.28±0.01; 0.21±0.01 U/mg tissue), respectively when compared with *negCont* group (0.12±0.00 U/mg tissue).

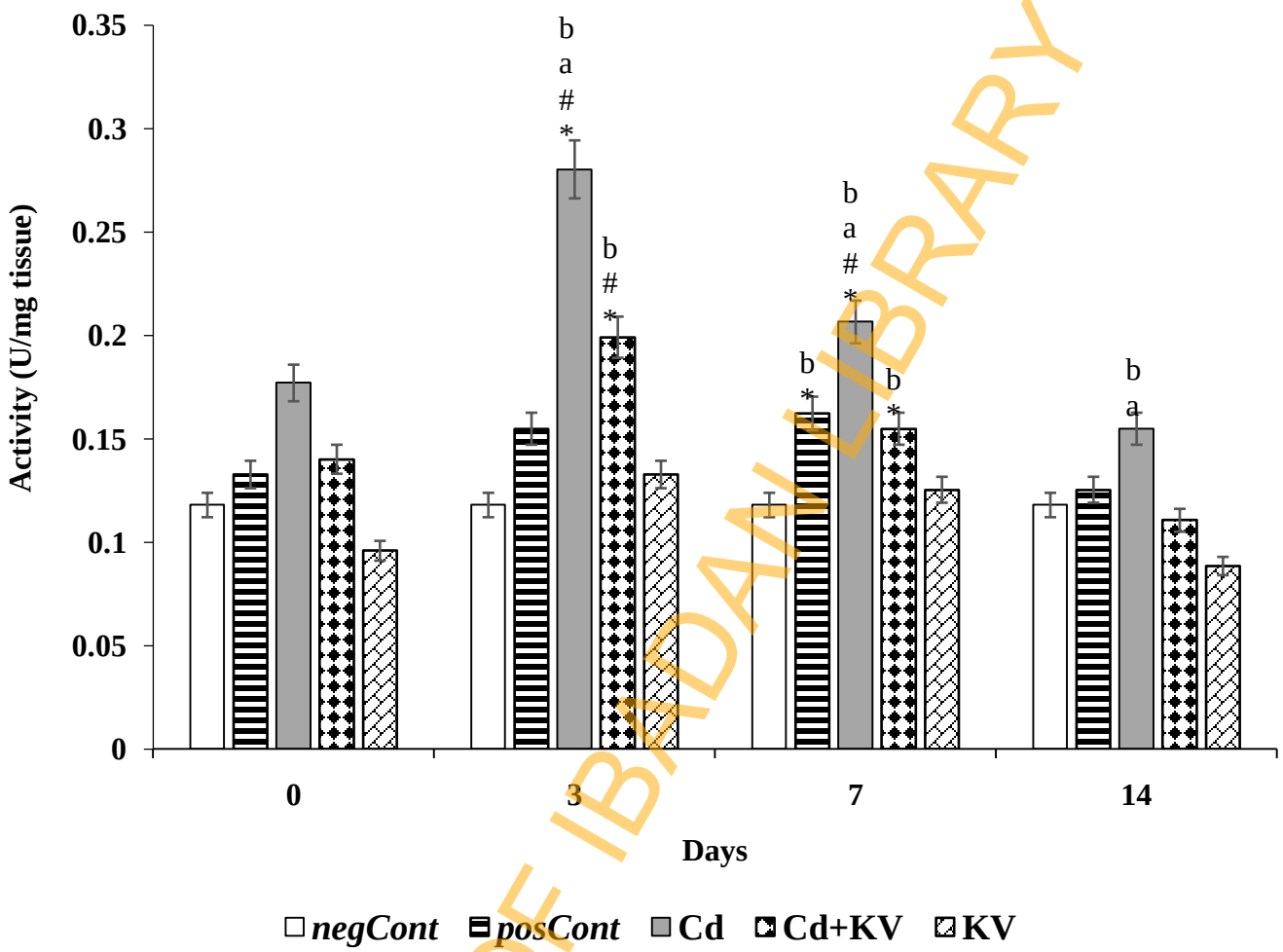


Figure 4.17: Effect of Cadmium and Kolaviron on Colonic Myeloperoxidase (MPO) Activity.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.28 Effect of Cadmium and Kolaviron on Colonic Nitric Oxide (NO) concentration:

The results from this study (Figure 4.18) showed significant increases in NO concentration from days 3 through 14 in Cd group (165.29±5.91; 148.55±6.62; 143.2±1.67µg/tissue) when compared with *negCont* group (126.37±2.39µg/tissue).

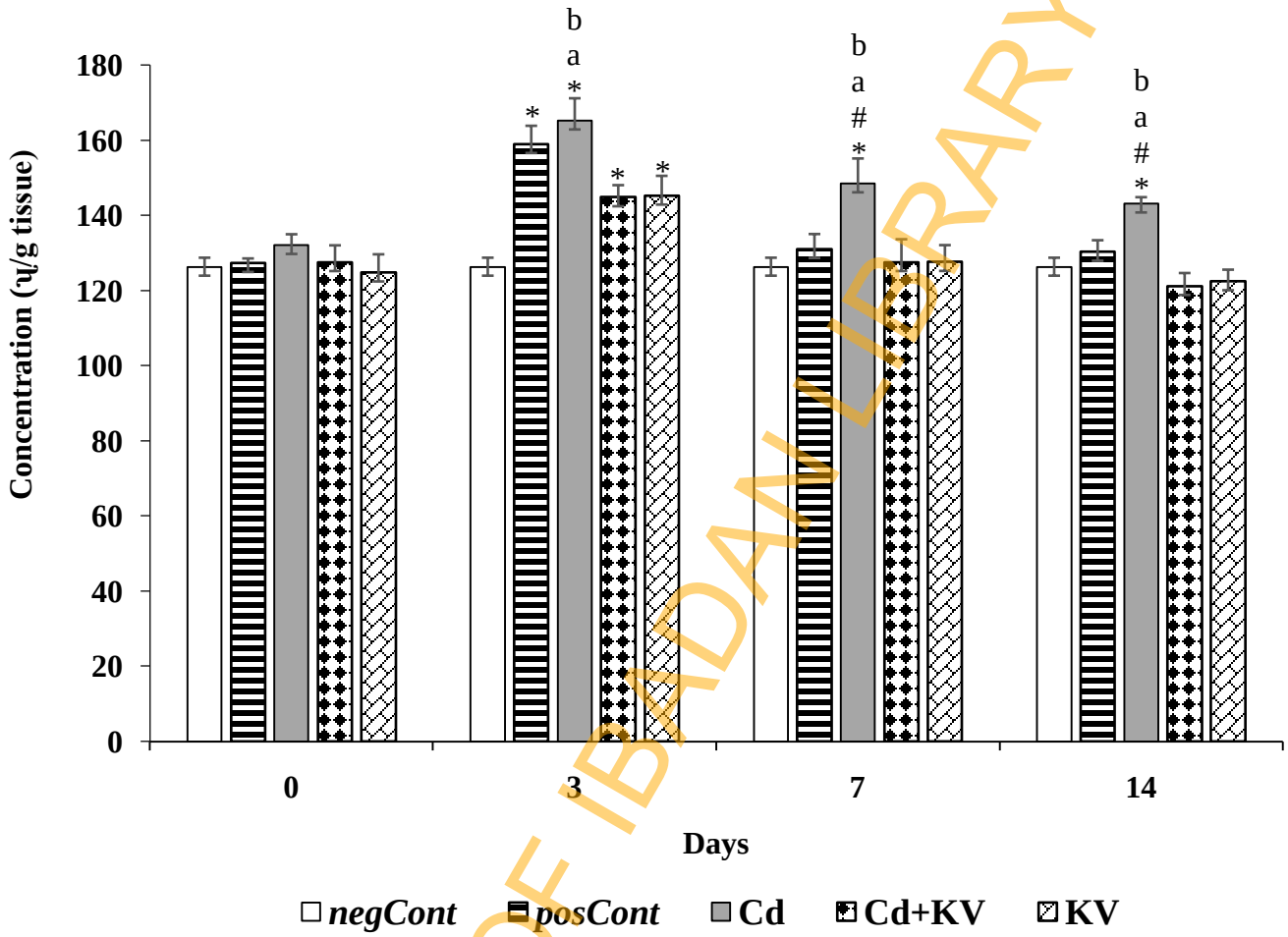


Figure 4.18: Effect of Cadmium and Kolaviron on Colonic Nitric Oxide (NO) Concentration.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.29 Effect of Cadmium and Kolaviron on Colonic Superoxidedismutase (SOD) Activity:

The results from this study (Figure 4.19) showed significant decreases in SOD activity at day 3 in *posCont*, Cd, Cd+KV and KV groups (180.45±3.32; 175.46±2.33; 180.38±3.19; 187.38±4.02 U/mg protein), respectively and at day 7 in Cd group (184.70±4.32 U/mg protein) when compared with *negCont* group (208.09±6.28 U/mg protein).

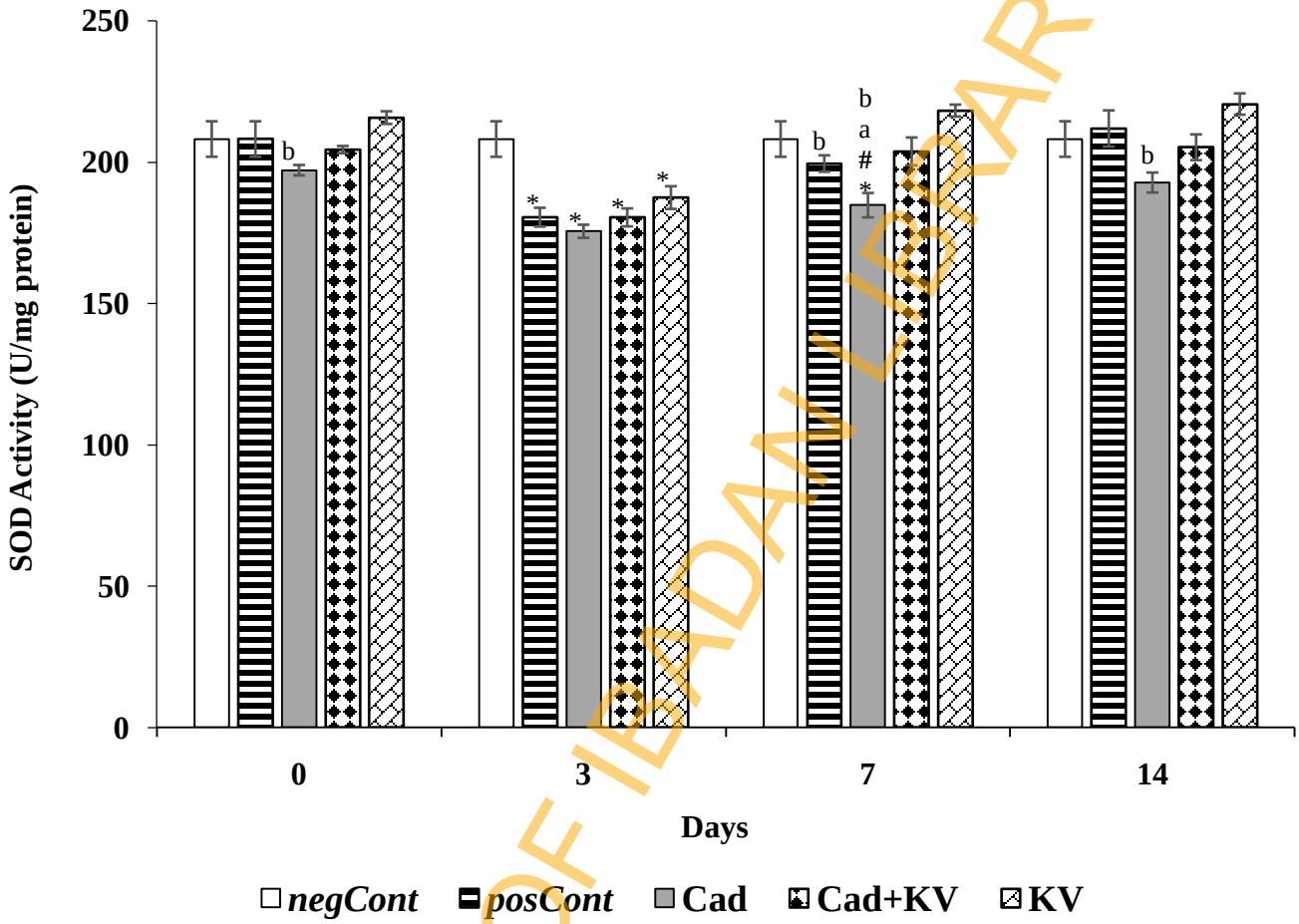


Figure 4.19: Effect of Cadmium and Kolaviron on Colonic Superoxide dismutase (SOD) activity.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.30 Effect of Cadmium and Kolaviron on Colonic Sulfhydryl concentration:

The results from this study (Figure 4.20) showed significant decreases in sulfhydryl concentration at day 7 and 14 in Cd group (2.60±0.02; 3.60±0.39 M), respectively when compared with *negCont* group (4.10±0.32 M).

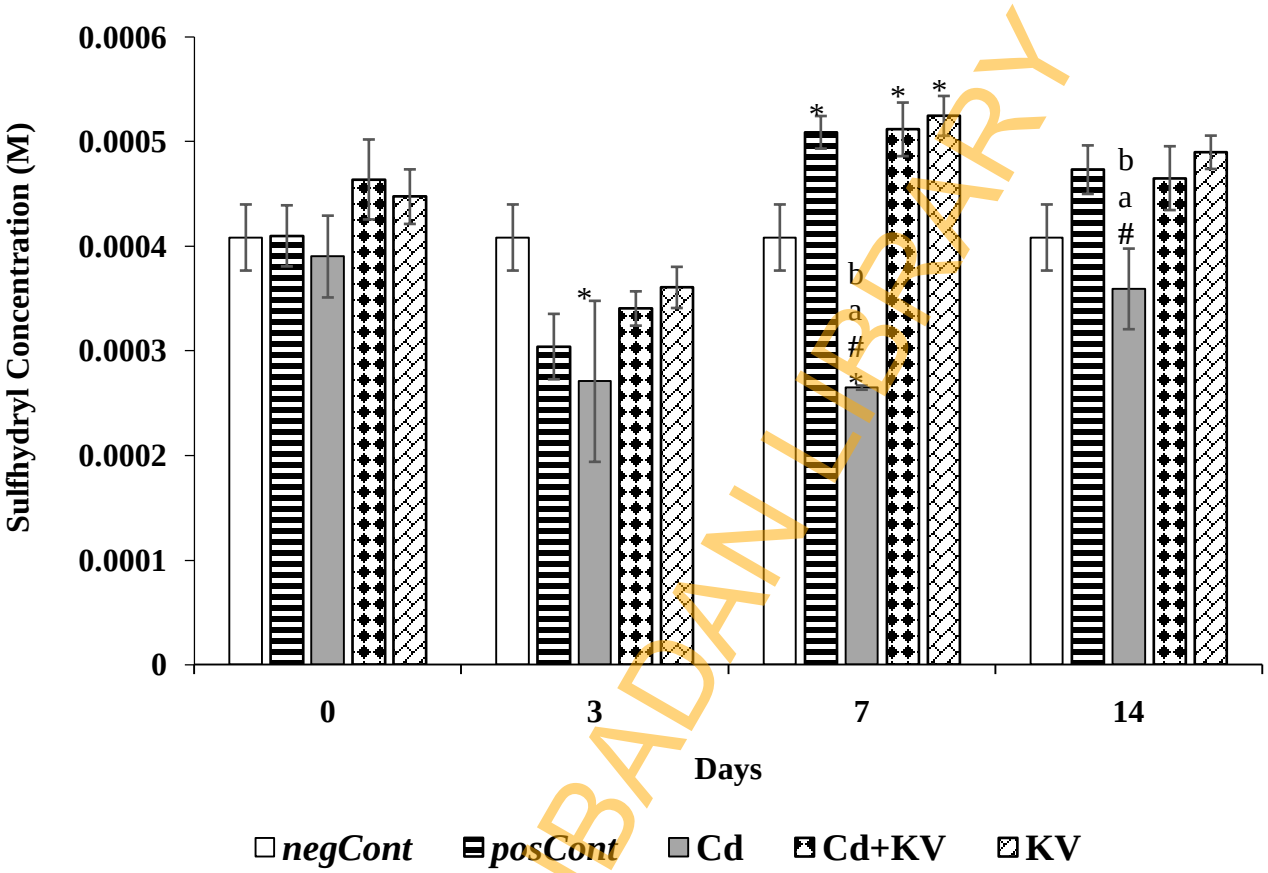


Figure 4.20: Effect of Cadmium and Kolaviron on Colonic Sulfhydryl Group Concentration.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.31 Effect of Cadmium and Kolaviron on Colonic reduced glutathione (GSH) concentration:

The results from this study (Figure 4.21) show significant decreases in Cd group (32.68±0.19; 32.99±0.67 nm mg/protein) at day 3 and 7, and at day 14 (47.43±0.44; 45.38±0.69 nm mg/protein) in *posCont* and Cd groups, respectively when compared with *negCont* group (52.08±0.97 nm mg/protein).

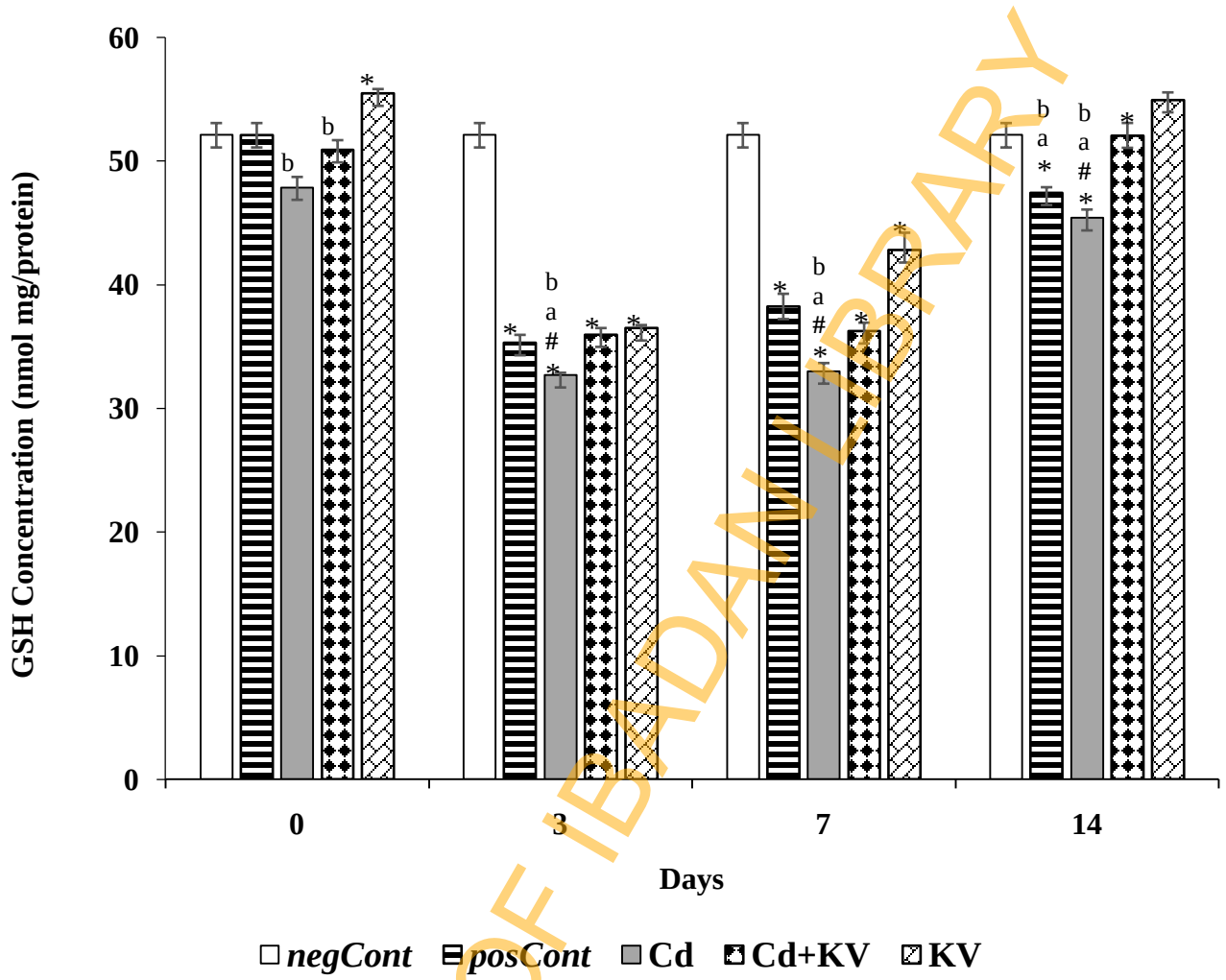


Figure 4.21: Effect of Cadmium and Kolaviron on Colonic Reduced Glutathione Concentration

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.32 Effect of Cadmium and Kolaviron on Colonic Sodium-Potassium (Na^+-K^+)Pump Activity:

Sodium-Potassium(Na^+-K^+) ATPase activities (Figure 4.22) were significantly decreased in Cd group(0.12±0.03; 0.13±0.01; 0.28±0.01nmol/ hr/mg tissue) at days 3, 7 and 14, respectively when compared with *negCont* group (0.60±0.02nmol/ hr/mg tissue).

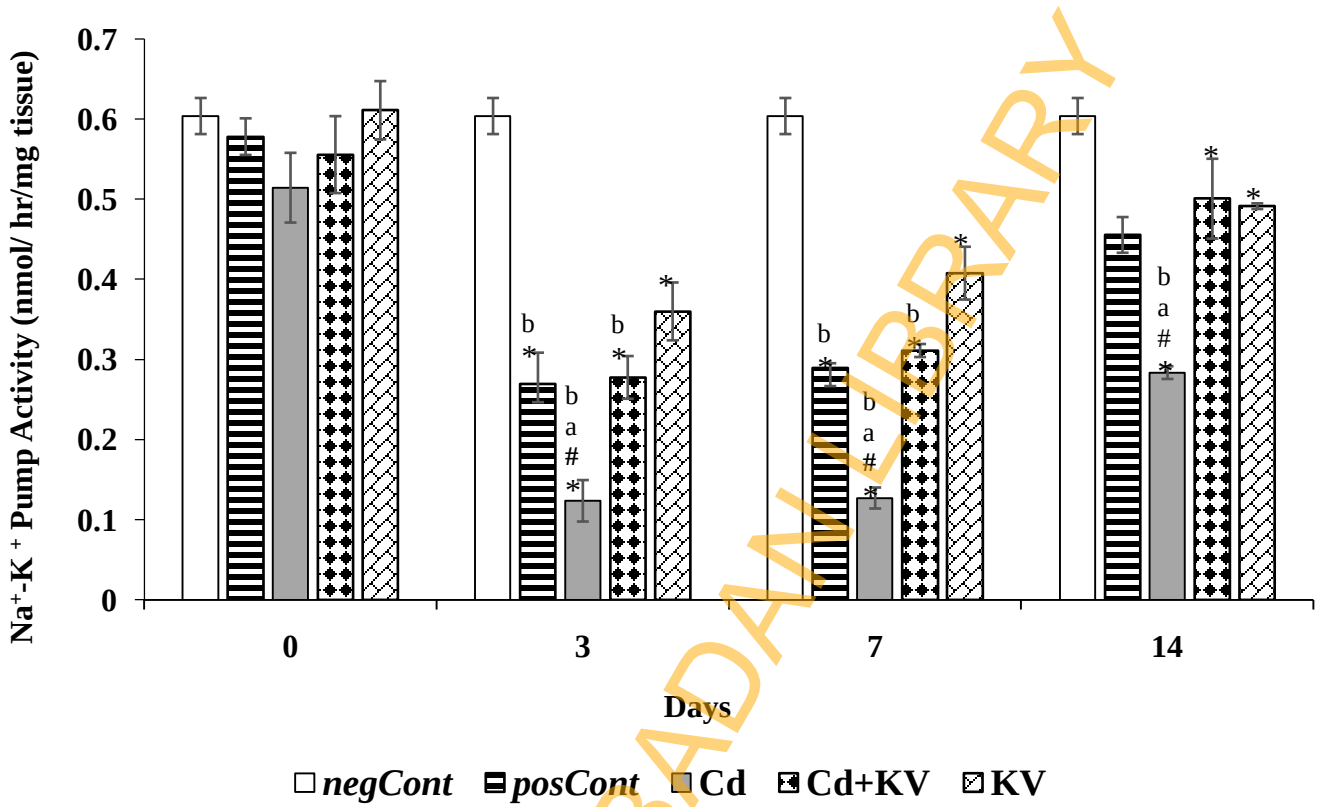


Figure 4.22: Effect of Cadmium and Kolaviron on Colonic Na^{+}-K^{+} ATPase Pump Activity.

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Control; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. *, #, a and b = $p<0.05$ values differ significantly from *negCont*, *posCont*, Cd+KV and KV groups, respectively.

4.33 Effect of Cadmium and Kolaviron on Colonic Gene Expression of Tumour Necrosis Factor Alpha:

There were up-regulations up to 0.8-fold in the relative gene expression of TNF-α (Figure 4.23) at days 3 and 7 in each of *posCont*, Cd, Cd+KV and KV groups. The up-regulation had however, decreased to a 0.2-fold change by day 14 in *posCont*, Cd and Cd+KV groups and in KV group, a down-regulation of up to a 0.1-fold change below *negCont* group.Fold changes werecalculated based on the difference in Ct values for TNF-α gene in colonic tissues, which were normalized againt β-actin and relative to negative control.

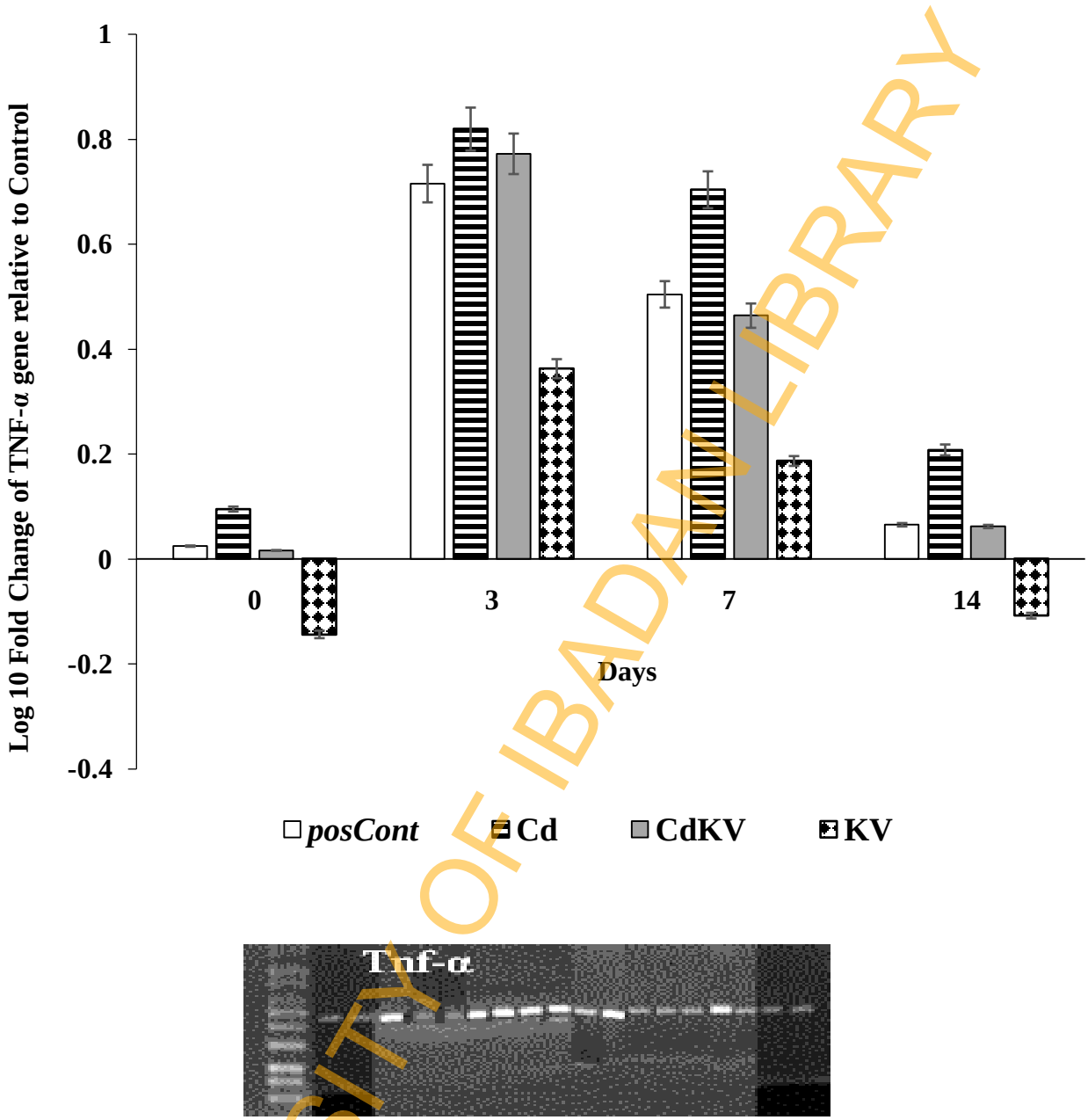


Figure 4.23: Effect of Cadmium and Kolaviron on Colonic gene expression of TNF-α

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control, represented by the horizontal line; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. Inset is the gel electrophoresis photograph of Tnf-α gene expression.

4.34 Effect of Cadmium and Kolaviron on Colonic Gene expression of Occludin:

There were down-regulations in the relative gene expression of Occludin (Figure 4.24) at days 3 and 7 in each of *posCont*, Cd, Cd+KV and KV groups. The down-regulation reached a 1-fold change by day 14 in Cd group, and in Cd+KV and KV groups, an up-regulation up to a 0.5-fold change above *negCont* group.Fold changes werecalculated based on the difference in Ct values for Occludin gene in colonic tissues, which were normalized againt β-actin and relative to negative control.

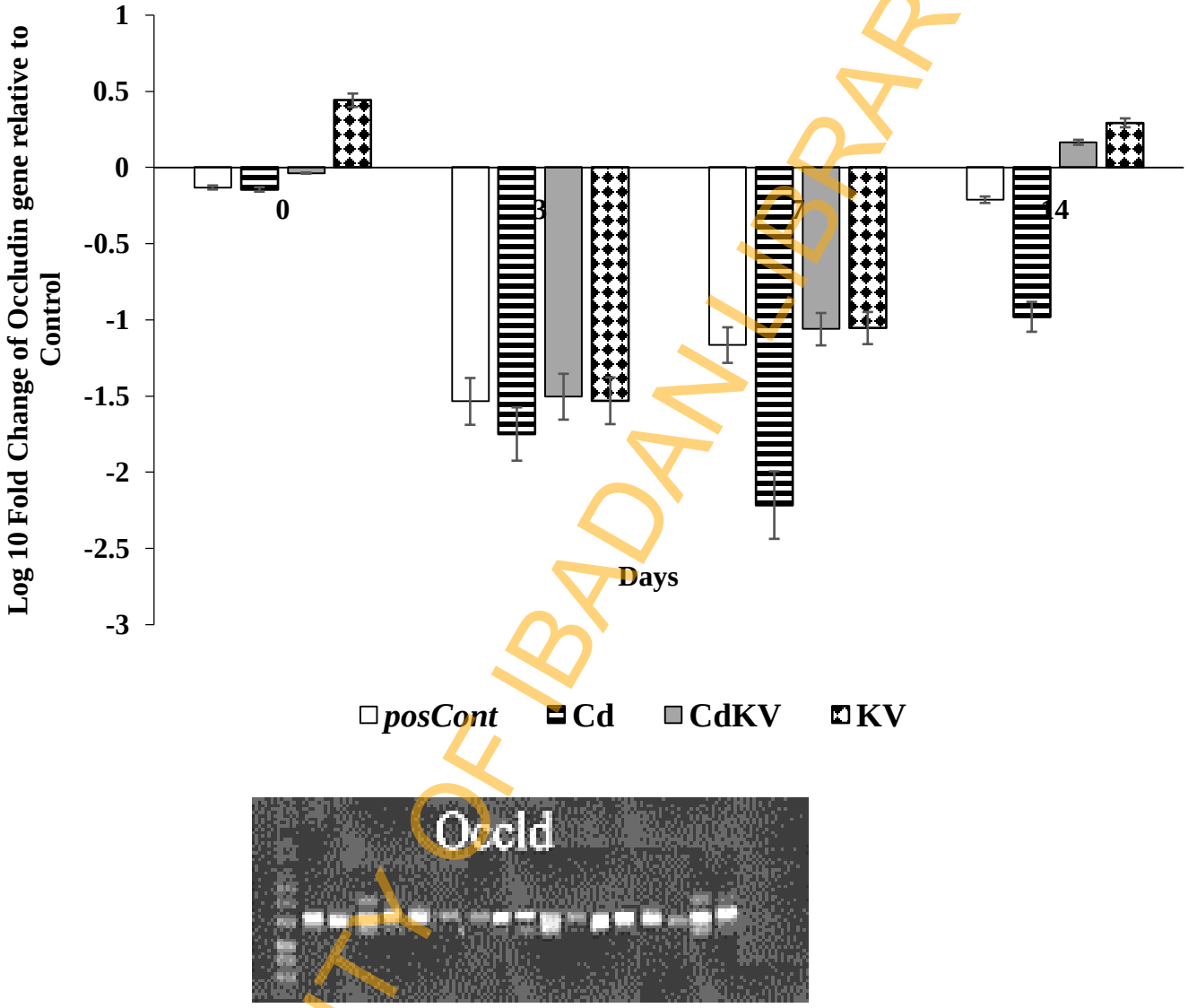


Figure 4.24: Effect of Cadmium and Kolaviron on Colonic gene expression of Occludin

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control, represented by the horizontal line; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. Inset is the gel electrophoresis photograph of Occludin gene expression.

4.35 Effect of Cadmium and Kolaviron on Colonic Gene expression of Interleukin-10:

There were down-regulations up to 3-fold in the relative gene expression of IL-10 (Figure 4.25) at day 7 in each of *posCont*, Cd and Cd+KV groups. The down-regulation remained a 3-fold change at day 14 in Cd group, and in KV groups, an up-regulation up to a 1.5-fold change above *negCont* group at day 14.Fold changes werecalculated based on the difference in Ct values for IL-10 gene in colonic tissues, which were normalized againt β-actin and relative to negative control.

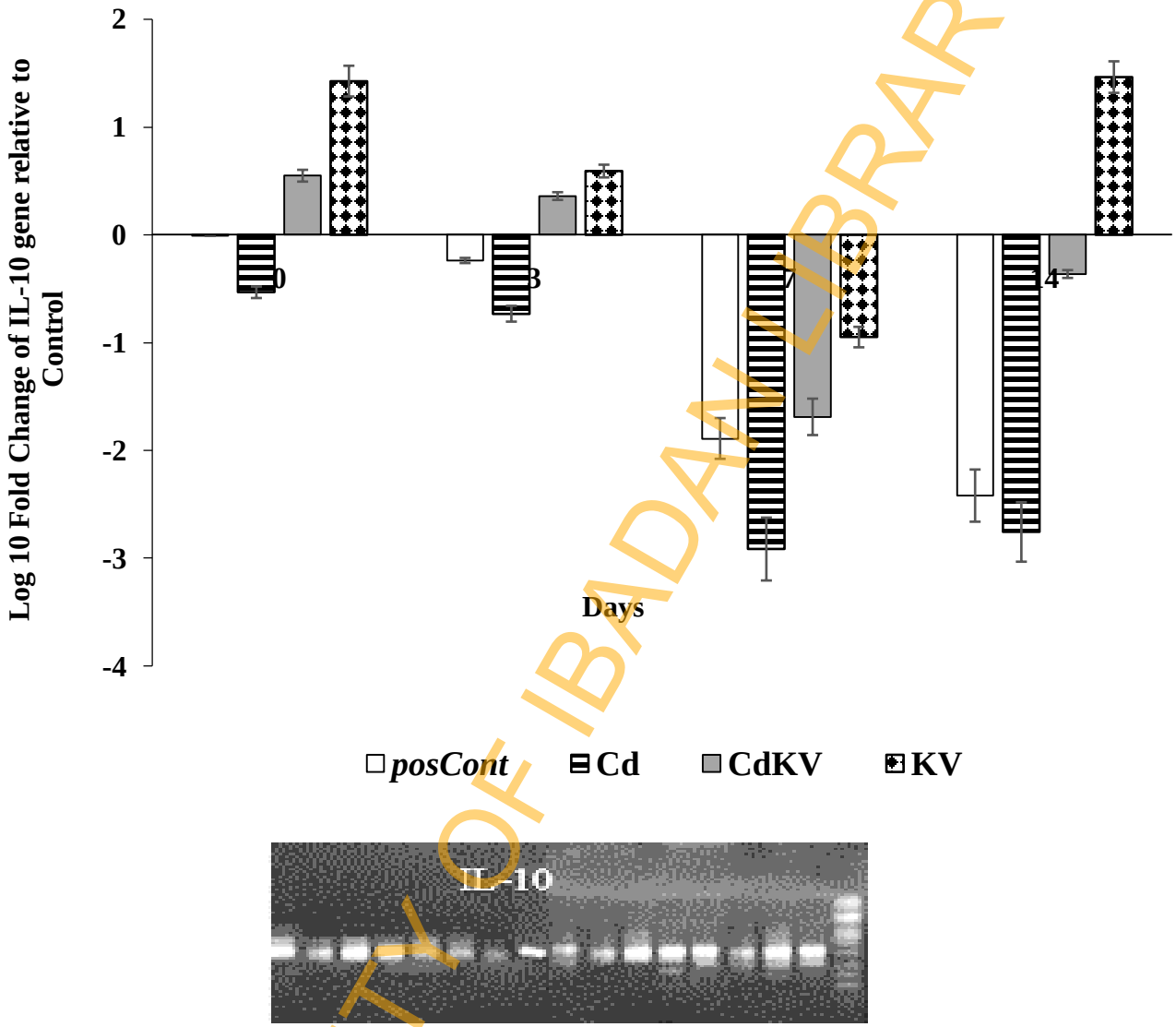


Figure 4.25: Effect of Cadmium and Kolaviron on Colonic gene expression of IL-10

Each bar represents mean ± S.E.M of 5 rats. Where *negCont* = Negative Control, represented by the horizontal line; *posCont* = Positive Control; Cd = Cadmium; Cd+KV = Cadmium+Kolaviron and KV = Kolaviron. Inset is the gel electrophoresis photograph of IL-10 gene expression.

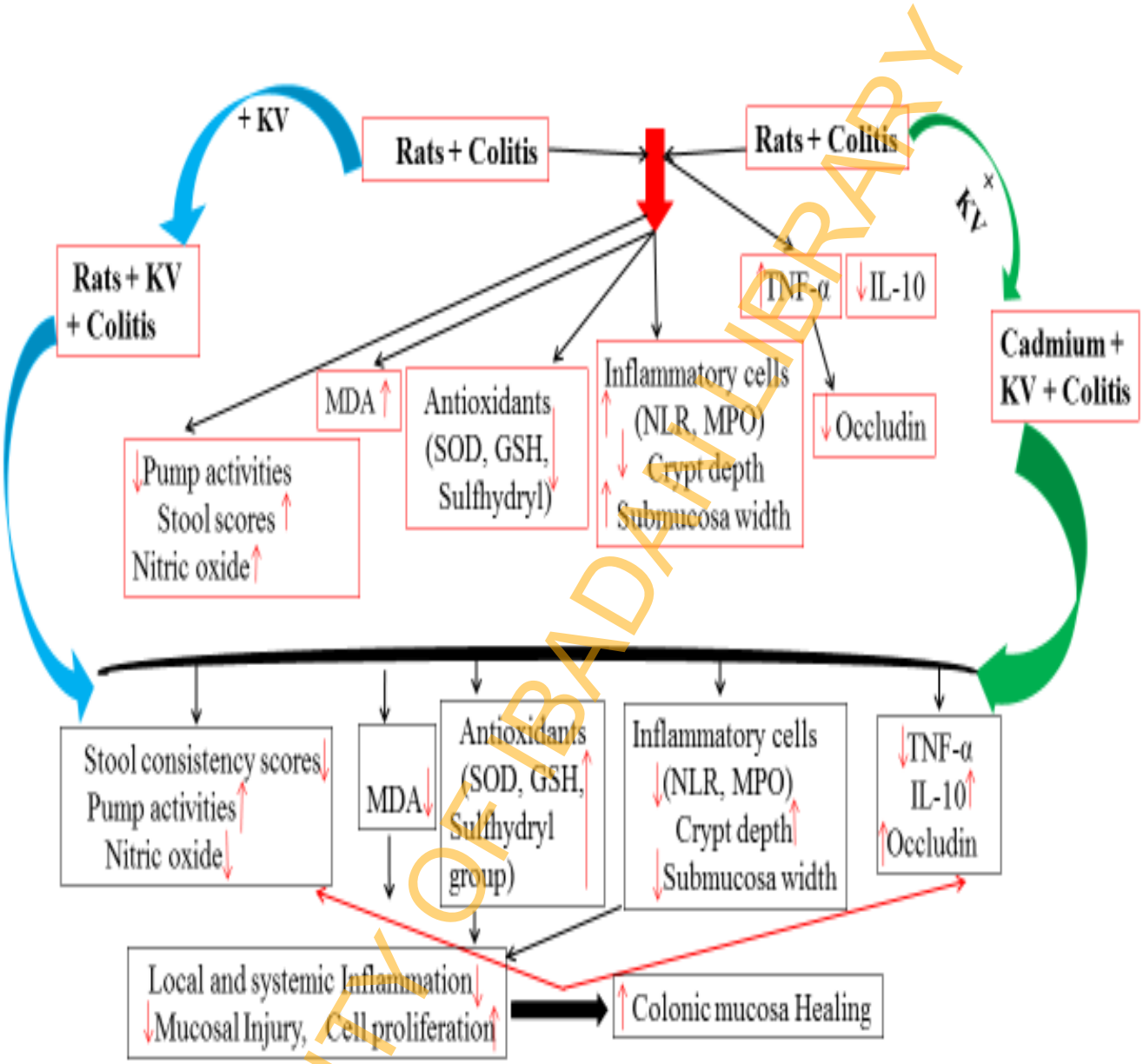


Figure 4.26: Proposed Mechanism of Action of Kolaviron During Colitis Healing in Cadmium Exposed Rats (Researcher design).

CHAPTER FIVE

DISCUSSION

Induction of colitis by acetic acid in experimental animals is one of the reliable methods used in producingexperimental model of inflammatory bowel disease (Niu *et al*., 2013), it mimics the pathological condition of ulcerative colitis as in humans (Otari *et al*., 2012); causing increased oxidative stress, infiltrated and activated neutrophils and consequently lipid peroxidation, this is usually caused by alterations in mucosal antioxidant defense system (Jagtap and Niphadkar, 2011).

Phase 1 of this study showed that intra-rectal administration of acetic acid (AA) caused an increase in stool consistency scores, neutrophil/lymphocyte ratio and lipid peroxidation; these increases were more pronounced in *Cd100* group. This may be due to inflammation reactions within the colonic mucosa caused by AA in addition to the already accumulating effect from cadmium. However, AA has been documented to induce localized inflammation, desquamation and loss of mucosal integrity leading to epithelial injury (Kandhare *et al*., 2012).

Phase 2 of this study showed that daily administration of kolaviron attenuated the effects of cadmium (100ppm) during colitis. The 200mg/kg dosage showed maximum efficacy among others (50, 100 and 200mg/kg) during colitis in cadmium (100ppm) exposed rats. Farombi *et al*., (2013) had earlier reported the efficacy of kolaviron at 200mg/kg in a 5-day treatment of DSS induced colitis in rats. However, this study differed from theirs's in that, rats had an earlier 4-week exposure to cadmium before they were induced with colitis, the exposure continued throughout the experiment.

Phase 3 of this studyinvestigated the mechanisms involved in the amelioration of cadmium (100ppm) effects by kolaviron at 200mg/kg during colitis healing.

Cadmium decreased weekly percentage weight gain, this finding is consistent with previous reports of Kotsonis and Klaassen (1977); Borzelleca *et al*., (1989); Kozlowska *et al*., (1993) and Horiguchi *et al*., (1996) who reported that Sprague-Dawley rats that received cadmium by gavage exhibited a decreased body weight. These decreases in body weight gain may be due to increased degeneration of lipids and proteins (Erdogan *et al*., 2005), decreased growth rate and

decreases in nutrient digestion and absorption (Elsenhans, 1999 and Eriyamremu *etal*., 2005). Decreased body weight and decreased growth rate have however, been common findings in studies where experimental animals are orally exposed to cadmium (Horiguchi *et al*., 1996; Asagba *et al*., 2002 and FEDRIP, 2012). Kolaviron reversed the negative effects of cadmium on on body weight, such that weekly percentage weight in Cd+KV groupwas increased. This may be indicative of kolaviron's potentials to modulate the effects of cadmium on growth and body weight.

Stool consistency scores were increased in all groups post colitis induction; this increases in stool consistency scores may be associated with fluid and electrolyte loss which is indicating an impaired colon during experimental colitis (Eisenhut, 2006; Lakatos, 2006). Studies have shown alterations in colonic reabsorption of water and ions during active colonic inflammation (Massironi *et al*., 2013). The increase in stool consistency scores after induction of colitis that was sustained in the Cd group may be due to alterations in the absorptive functions of the epithelium. Treatment with Kolaviron however, decreased stool consistency scores such that even the Cd+KV group had reduced stool scores earlier. i.e days 7 and 14, stool consistency had returned to normal in KV and Cd+KV groups.

The increase in blood cadmium concentration after four weeks of exposure indicates sufficient exposure (Taylor, 1988; Jin *et al*., 2002). Although the level was generally low and this may be because even exposure to high cadmium concentration orally or intravenously still presents as extremely low in the blood (ARL, 2012); this probably is because cadmium exposures from drinking water or foodsinduces metallothionein(a zinc and cadmium binding protein which serves as a binder and chelator) in the intestinal epithelium(Eriyamremu *etal*., 2005; Asagba, 2009; Klaassen *etal*., 2009; Liu *etal*., 2009 and Xue *etal*., 2009).

However, reports on absorption and resultant bioavalability of cadmium is contradictory; some authors conclude that binding implies a smaller absorption (Min *et al*., 1991), but other studies have shown that metallothioneins do not affect cadmium absorption and blood concentration (Flaig *et al*., 2003).The decrease in blood cadmium concentration in the Cd+KV group may be due to kolaviron's flavonoidal ability to bind metals and free radicals; generally, polyphenols and flavonoids can chelate metals (Farombi and Nwaokeafor, 2005; Ebrahimzadeh *et al*., 2008).

Neutrophil/Lymphocyte ratio (NLR) is an index that can be used to measure disease progression particularly during inflammation (Celikbilek *et al*., 2013). The observed increase in NLR in *Cd50* and *Cd100* on days 0 and 7 may be indicative of continuous activation of inflammatory cells by cadmium. In Phase 3, increase in NLR observed in all the groups at days 3 and 7 post colitis induction may probably be due tothe previously activated inflammatory cells during colitis still being stimulated by cadmium. Increases in NLR were however sustained till day 14 in the Cd group alone.

Macroscopic scores are useful in assessing gross lesions or alterations during tissue injury. Scores were increased significantly in all the groups on day 3 post colitis induction. This may be due to inflammation resulting from intra-rectal administration of AA. Tahan *et al*., (2011) and Somani *et al*., (2014) had earlier reported similar observations. On days 7 and 14, other groups presented with reduced macroscopic scores except Cd group which still had a high macroscopic score till day 14. Reduction in macroscopic scores in the Cd+KV group may be suggestive that Kolaviron was able to modulate the effects of cadmium on macroscopic scores.

Weight/length ratio of inflamed colonic tissue is considered a reliable and sensitive indicator for the severity and extent of inflammation. The increase in weight/length ratio observed after AA colitis may indicate ulcers and edema caused by active inflammation, this finding is consistent with other studies in induction of colitis with acetic acid caused increase in weight/length ratio (Cestari *et al*., 2011; Sánchez-Fidalgo *et al*., 2010).

Kidney is a well perfused organ which metabolises toxic substances found in the circulation and passes them out of the body through urine. Chapatwala *et al*., (1982) reported an insignificant increase in total kidney weight gain when exposed to different doses of cadmium for four weeks. Horiguchi *et al*., (1996); Asagba and Eriyamremu, (2010) also, observed kidney swelling after subcutaneous and oral exposures of cadmium in rats. A significant increase in right kidney weight was observed in Phase 3 only on day 14 post colitis induction in the Cd group, there were however no such increases in the Cd+KV group exposed to same concentration of cadmium but administered with kolaviron.

The liver is a major organ of metabolism, and is responsible for the metabolism of cadmium and other toxicants. Chapatwala *et al*., (1982) observed similar increases in liver weight. Horiguchi *et al*., (1996); Tu *et al*., (2007); Asagba and Eriyamremu, (2010), observed hepatomegaly after subcutaneous and oral exposures of cadmium in rats.Similarly, Dwivedi, (2015) reported that relative liver weight of rats increased when treated with cadmium for 30 days and 60 days.

Increases was observed in liver weight of rats in the Cd group were not seen in the Cd+KV group. This may be because cadmium accumulates in liver inducing ROS resulting in DNA strand breaks and lipid peroxidation (Madejczyk *et al*., 2015), and kolaviron may have prevented increases in liver weight due to its antioxidant capacities thereby preventing accumulation of cadmium in hepatocytes.

The spleen is an immune system related organ which can be useful in indicating immune dysfunction (Bencko *et al*., 1988). In this study, increases in spleen weight were observed in Cd group on days 0 and 3, 7 and 14 post colitis induction. Increase in spleen weight may be suggestive of cadmium's ability to exert direct toxic effect on spleen andthis may be due to significant alteration in oxidative stress-related indices, appreciable cytotoxic changes (Institoris *et al*., 2001 and 2002) and complex immunomodulatory effect in spleen.

Kolaviron was able to attenuate that effect in the Cd+KV group as the increases in spleen weight in this group was negligible. This too may be attributed to the antioxidant the antioxidant effects of kolaviron. Reports from the worksof Horiguchi *et al*., (1996) showed spleenomegally, and similarly, Tu *et al*., (2007), Asagba and Eriyamremu, (2010),andDwivedi, (2015) showed significant increase in relative weights of spleen.Increases in organ (kidney, liver and spleen) weights as observed in this study may be due to the toxic properties of cadmium or adaptive mechanisms in an attempt to combat systemic toxicity (Tandon *et al*., 2012).

Histological evaluation showed that cadmium caused erosion of colonic epithelium, increased severity of colonic injury, and delayed healing in *Cd50*,*Cd100*and Cd groups at day 0; this may be indicative of a continuous stimulation of colonic inflammatory cells due to a sustained exposure to cadmium. Tahan *et al*., (2011); Zalups and Ahmad, (2003) reported a similar finding, where oral administration of cadmium compounds caused desquamation of the colonic

epithelium.Singh*et al*., (2011) also, observed that mice dosed with $CdCl_2$ (50 mg/kg) showed histopathological damage evidenced by emptied goblet cells, lacerated and suppressed mucosa, destruction of surface epithelium, marked decline in mucin production, and hemorrhage. Such histopathological damage as observed in the *Cd50*, *Cd100* and Cd groups werenot seen in the Cd+KV group except on day 3 when histology of colon sections of all groups induced with colitis showed erosion of the epithelium, mucosal ulcerations, crypt distortion and infiltration of cells day 3 post colitis induction while control rats had normal cryptal arrangement.Some of these histopathology signs were still seen in *Cd50*, *Cd100* and Cd groups till day14 whereas, other groups had a fairly intact epithelium by day 7.

In normal intestine the crypts are deep and full of goblet cells on their bases. Crypt depth reflects inflammation and mucosal cell proliferation (Frankel *et al*., 1994) and intestinal mucosal regeneration starts from the base of the crypt and cell proliferation increases crypt depth. In diseased conditions like colitis, there is shortening in cryptal depth (Saha *et al*., 2015) and submucosa width is enlarged during colitis and other pathologic conditions of the colon. In this study, crypt depths were shortened and submucosa widths were increased in all groups on day 3 however, crypth depth in Cd+KV and KV groups returned to normal by day 7.These findings may be suggestive of kolaviron's potentialto stimulate cell renewal and proliferation.

MDA is a marker that reflects the extent of ROS accumulation in the body in response to oxidative stress (Hou *et al*., 2011). Previous reports have shown that MDA is one of the products of lipid peroxides and that it marks for peroxidation in cells (Somani *et al*., 2014). The notable increase in MDA concentration in *Cd50* and *Cd100* group by day 3 may be indicating oxidative stress which is a common finding during cadmium exposures (Shukla *et al*., 2000; Zalups and Ahmad 2003; Sharma *et al*., 2010). *Cd25* group on the same day had MDA values not different from that of control; it may be that cadmium at 25ppm was not sufficient enough to increase oxidative stress or that endogenous antioxidant enzymes and molecules were stimulated by cadmium and they were able to maintain biochemical homeostasis. An adaptive response to cadmium by cells may also, have been induced thereby causing resistance to MDA, Hart and colleagues (2001), found out that repeated exposures to cadmium at low concentrations to alveolar epithelia cells can result in the development of an adaptive survival response. However,

increases in MDA seen in*Cd50*and Cd100 groups on days 7 and 14 may be as a result of stimulated endogenous enzymes that have been exhausted from cadmium-stimulated reactions.

Antioxidants, in general, are compounds and reactions which dispose, scavenge and suppress the formation of ROS (Valko *et al*., 2007). Cd group had a raised MDA level throughout in this experiment, even before the induction of colitis (day 0), this is indicative of lipid peroxidation. The ability of kolaviron to protect against lipid peroxidation process however, was evidenced by decrease in the levels of malondialdehyde (MDA) in colon homogenates of rats in the KV group and even in those in the Cd+Kv group as against those in the Cd group which had increased MDA on days 0, 3, 7 and 14. This may be due to the antioxidative stress activity of Kolaviron.

Myeloperoxidase (MPO) is a heme protein secreted by phagocytes, especially neutrophils, it marks for local leukocyte infiltration and inflammation (Zheng *et al*., 2000; Young *et al*., 2011). Activated neutrophils produce superoxide anion, through NADH oxidase, which reduces molecular oxygen to the superoxide anion radical through the enzyme MPO (Talero *et al*., 2006). Accumulation of leucocytes in the colon could contribute to leucocyte-mediated mucosal tissue damage; breakdown of the mucosal barrier and subsequent inflammatory response (Mantovani *et al*., 2001). MPO activity has been shown to increase in inflamed mucosa and ruptured colonic barrier and this may be reflective of disease's progression (Joo *et al*., 2015).

Increases in colonic MPO activity was observed following induction of colitis, similar increases in the MPO activity both in animals and in human beings with ulcerative colitis have been reported (Yukitake, *et al*., 2011; Wadie *et al*., 2012; Joo *et al*., 2015). Increase in MPO activity was consistently increased in Cd group till day 14. This increase in the colonic MPO activity may indicate increase in the magnitude of immune response in the Cd group.Increases in the expression of TNF-α as observed in this study has been suggested to be a main chemo-attractant for neutrophils (Wang and Mao, 2007; Singh *et al*., 2011), this also, may be the reason for an increased MPO concentration as they catalyse the reaction activity of neutrophils. Some studies show that the effect of cadmium on the intestinal immune system is responsible for the pathophysiologies observed after exposure to this cadmium; Zhao *et al*., (2006) found an increase in expression of the proinflammatory cytokine MIP-2 (macrophage inflammatory protein-2), in myeloperoxidase activity, and infiltration of inflammatory cells in the lamina

propia. This may be the cause of the prolonged increase in MPO activity as observed in the Cd group. Cd+KV group however,did not show similar prolonged increase in MPO activity and TNF-α expression and this may be attributed to the antioxidant and anti-inflammatory properties of kolaviron on the inflammatory cells and their product (Farombi *et al*., 2009; Olaleye *et al*., 2010).

Nitric oxide derived from iNOS in epithelial cells plays an important role in the pathogenesis of inflammatory disease (Talero *et al*., 2006). It is induced by various inflammatory cytokines TNF-α, IFN-γ or LPS (Talero *et al*., 2006). Nitric oxide reacts with superoxide and inhibits key enzymes in the mitochondrial electron transport chain. It leads to production of potent cytotoxic oxidant (peroxynitrite) (Liu and Wang, 2011) which can aggravate of colitis. Studies have reported that NO controls mucosal blood flow and maintains mucosal barrier integrity in both healthy mucosa (Dijkstra *et al*., Kubes, 1992; Magierowski *et al*., 2015).Some others reported that it contributes to disease progression (Magierowski *et al*., 2015).

In this study however, NO concentration was increased in all the groups on day 3 post colitis induction, this increase was however, not prolonged such that by day 7, NO concentration in other groups were not different from*negCont* group except Cd group which maintained increased NO concentration till day 14.This may be as a result of the infiltration of cell that was still enhanced in the Cd group till day 7 as evidencedin the histology slides.Inflammatory cells express iNOS after been attracted to injury site by cytokines, these then together with cytokines like TNF-α enhances NO production, which then reacts with superoxide anion radical to produce a more deleterious peroxinitrite.Kolaviron may have caused a reduction in NO as observed in this study, due to its inhibitory effect on iNOS (Farombi *et al*., 2009; Olaleye *et al*., 2010).

Superoxide dismutase (SOD) scavenges superoxide radical by converting it to hydrogen peroxide (H_2O_2) (Zelko *et al*., 2002; Jurkovič *et al*., 2008). SOD activity was reduced in all the groups on day 3, this may be indicative of oxidative stress, but the decrease was reversed by days 7 and 14 in Cd+KV and KV groups, as against Cd group which still had a reduced activity of SOD.Cadmium group had the most reduced activity of SOD, this may be due to large quantities of reactive oxygen species produced and which may have overwhelmed the system (Sarkar *et al*., 2013).Antioxidant enzymes, mainly superoxide dismutase and catalase are first line defensive

enzymes against free radicals (Visavadiya and Narasimhacharya, 2005), they maintain a variety of defenses against reactive oxygen species toxicity and oxidative stress in cells. Superoxide dismutase (SOD) scavenges superoxide radical by accelerating its conversion to hydrogen peroxide (H_2O_2) (Zelko *et al*., 2002; Jurkovič *et al*., 2008). CAT acts in the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen.

According to Sakthivel *et al*., 2014, SOD levels increase in ulcerative colitis induced by intrarectal acetic acid. The elevation of SOD and other antioxidants can be explained by the fact that in IBD, inflammation and oxidative stress are present and this SOD increase would protect the tissue against oxidative damage. However, in studies on ulcerative colitis induced by acetic acid (Rise *et al*., 2012; Aleisa *et al*., 2014) and TNBS (RabeloSocca *et al*., 2014; Wang *et al*., 2010; Xu *et al*., 2015), SOD activity was reduced, which clearly indicates increased levels of oxidative stress, which may damage cells through lipid peroxidation of membranes and oxidation of cellular proteins (Aleisa *et al*., 2014). Administration of kolaviron in Cd+KV and KV groups increased SOD activities.

Sulfhydryl and GSH plays an important role in coordinating the body's antioxidant defense, they are non-protein thiol that causes detoxification of heavy metals (Ahmad *et al*., 2012; Witaicenis *et al*., 2012). It has been shown that cells exposed to cadmium depletes their antioxidant defense mechanisms, results in reduction of glutathione-SH related proteins and antioxidant enzyme activity (Sarkar *et al*., 2013).This study showed that sulfhydryl and GSH concentration decreased in the Cd group on day 0 before induction of colitis and on days 3, 7 and 14 post colitis induction.Some authors have reported reduction in GSH concentration in whole blood by metals due to its interaction to form the oxidized glutathione (GSSG) or metal complexes (Khan *et al*., 2010). The reduction in sulfhydryl and GSH concentration of Cd group may therefore, suggest conjugation with cadmium to form complexes in an attempt to maintain cellular homeostasis.

Furthermore, decreases in GSH concentration on day 3 post colitis induction may be due to an increased rate of consumption of GSH during colitis.Various studies have shown that GSH concentration may be reduced during experimental colitis (Rise *et al*., 2012; Witaicenis *et al*., 2012; Prabhu and Guruvayoorappan, 2014; RabeloSocca *et al*., 2014).Sulfhydryl and GSH depletion were restored with Kolaviron administration, even in Cd+KVgroup.

The biflavonoid complex of Kolaviron contains a hydroxyl group in the position three (3-OH) of the C-ring, a C-4 carbonyl, C-5 and C-7 hydroxyl groups of its A-ring (Farombi *et al*., 2007; 2013).These hydroxyl and carbonyl group can react with hydroxyl radicals and hydrogenperoxides.It may therefore be one of the mechanisms by which Kolavironinhibited oxidative reactions and displayed its free radical scavenging ability.

During ulcerative colitis, associated colon mucosal damage causes loss of absorptive functions of the Na^+-K^+ ATPase.Na^+-K^+ ATPase is an enzyme responsible for the transmembrane exchange of water and ions to maintain transcellular gradient (Sundaram *et al*., 1997; Sundaram *et al*., 1998). Data from this study indicated that Na^+-K^+ATPase activity was decreased in all groups post colitis induction. This is in agreement with studies of Sundaram and West (1997) and Massironi *et al*., (2013). However, by day 14 Cd+KV and KV groups had regained this pump (Na^+-K^+ATPase) activity such that, the activity of Na^+-K^+ ATPase was not different from *negCont* group.This ameliorative activity of kolaviron may be responsible for the decrease earlier observedin stool constituency score of Cd+KV and KV groups. This response may also be due to the anti-inflammatory activities of Kolaviron.Administration of Kolaviron has been found to suppress inflammatory mediators in earlier studies (Olaleye *etal*., 2010).

TNF-α is a major cytokine involved in initiation of inflammation during inflammatory bowel disease (Jarry *etal*., 2008).Up-regulation of TNF-α gene expression observed before induction of colitis in Cd group and upon induction of colitis in all the other groups is in accordance with previous reports; Wang and Mao, (2007) measured the expression of TNF-α in inflamed mucosa of ulcerative colitis patients. Increases in TNF-αas observed in this study may have contributed to colonic mucosal damage; Kim *et al*., (2014) reported a similar finding and Duizer *et al*., (1999) also, reported epithelial damage in cell line models of the intestinal epithelium.

However, administration of kolaviron even in the cadmium+kolaviron group down-regulated the expression of TNF-α gene and up-regulated IL-10 and Occld gene expressions in rats during AA induced colitis healing.Previous *in vitro* observations has been reported on the ability of kolaviron to down-regulate the expression of inflammatory mediators (Olaleye *etal*., 2010 and Farombi *et al*., 2009, 2013).This anti-inflammatory property of kolaviron may relate to its ability to down-regulate NF-κB expression which has been shown to play a central role in the

production of TNF-α (Faronbi *et al*., 2009; Zhao *et al*., 2012). Some studies have also found that not only TNF-α, but a mixture of cytokines (TNF-α, interferon-γ, interleukin-1β) down-regulates gene expression of occludin (Sappington *et al*., 2003; Cui *et al*., 2010).

This study however, showed that administration of kolaviron even in the cadmium+kolaviron group downregulated the expression TNF-α gene and upregulated IL-10 and Occld gene expressions in rats during AA induced colitis (Quesnell *et al*., 2007; Cui *et al*., 2010).This suggests that TNF-α may directly cause a down regulation of occludin gene expression and consequently, cause abnormal tight junction and epithelial barrier (Quesnell *et al*., 2007; Cui *et al*., 2010).

IL-10 is an immunomodulatory cytokine that is responsible for preventing spontaneous colitis and can be constitutively expressed and secreted by human normal colonic mucosa epithelial cells (Jarry *et al*., 2008). Depletion of IL-10 in the mucosa has been shown to induce both down-regulation of the IL-10 and up-regulation of TNF-α (Jarry *et al*., 2008). This study revealed a down-regulation of rat's colonic IL-10 and occludin gene expression even before induction of colitis in Cd group and after colitis induction in all the other groups relative to *negCont* group which served as a baseline. Kim *et al*., (2014) observed that increase in TNF-α due to sub-chronic exposure to cadmium produces an effect on gut immune homeostasis and innate immunity; this may be the cause of the down-regulation observed in the gene expression of IL-10 in the Cd group.

However, groups that were administered with kolaviron(Cd+KV and KV) had an up-regulation IL-10 and occld gene expressions during exposures to cadmium and AA induced colitis.This may also, be the reason for the decreased NO concentration observed in this study; decreases in the concentration of NO and reactive oxygen species production have been related to the protective actions of IL-10 gene expression (Li *et al*., 2014).

5.1 Summary of findings, Conclusions and Recommendations

Summary of findings:

This study clearly shows that;

1. Cadmium Chloride can cause erosion of the colonic mucosal epithelium during sub-chronic oral exposures to cadmium.
2. Cadmium Chloride can cause an increase in neutrophil/lymphocyte ratio in the systemic circulation during sub-chronic oral exposures and Acetic acid-induced colitis.
3. Cadmium Chloride can induce oxidative stress in the colonic mucosal epithelium during sub-chronic oral exposures by increasing colonic malondialdehyde concentration.
4. Cadmium Chloride can cause decreases in the concentration and activity of endogenous antioxidants during sub-chronic oral exposures and Acetic acid-induced colitis.
5. Cadmium Chloride can increase the colonic NO concentration, MPO activity and the gene expression of TNF-α during sub-chronic oral exposures and Acetic acid-induced colitis.
6. Cadmium Chloride can decrease the colonic gene expressions of IL-10 and Occludin during sub-chronic oral exposures and Acetic acid-induced colitis.
7. Cadmium Chloride can cause delay in mucosal healing during sub-chronic oral exposures and Acetic acid-induced colitis.
8. Kolaviron can chelate cadmium chloride during sub-chronic oral exposures and Acetic acid-induced colitis.
9. Kolaviron can reverse the negative effects of cadmium chloride during sub-chronic oral exposures and Acetic acid-induced colitis and thereby increase mucosal healing.

Conclusion:

In conclusion, chronic cadmium intake delayed the healing if acetic acid induced colitis by increased neutrophil/lymphocyte ratio, lipid peroxidation and colonic inflammation. Kolaviron

reversed the observed side effects of cadmium on colitis and may be a useful dietary intervention in cases of cadmium exposure.

Recommendations:

Cadmium is a metal of toxic health effects and therefore poses public health concern, also, its prevalence in the industry and environment makes it difficult to completely eliminate. The under listed measures may be taken to reduce or counteract exposure risks;

1. There should be proper disposal of batteries especially Ni-Cad batteries to avoid indiscriminate burning and consequent inhalation in the environment.
2. Water sources must be tested before use to know if they contain cadmium and if they do, filters that can remove cadmium and other metals from drinking water can be used.
3. It must be ensured that healthy diet of organically grown plants, fruit and vegetables should be included in meals to counteract exposure risks and decrease absorption of cadmium.
4. Routine checks to determine the level of cadmium in the body should be done.
5. If cadmium levels are too high, chelation therapy with organic plant products or extract such as kolaviron may be used.
6. When using fungicides or fertilizers that contain cadmium on lawn, garden or farm land, safety instructions must be properly read and strictly adhered to. Protective safety gear like dust nose masks should be worn.

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APPENDIX I

TOTAL RNA PURIFICATION (JENA)

1. Sample purification and cell lysis
    - Collect 20-50mg fresh sample in a microcentrifuge tube
    - Add 300µL of buffer (2ME) and homogenize the material using appropriate apparatus (hard operated pellet pestle or motor driven grinder)
    - Add additional 200µL of lysis buffer (2ME added) to the homogenized sample and vortex for 15-30 seconds

      Note: sample should not exceed 10% of the lysis buffer volume.
    - Centrifuge at 10,000g for 10 minutes.

      Optional: in case the debris still remains in the supernatant;

      a) Add 500µL chloroform and vortex for 15-20 seconds
      b) Centrifuge at 10,000g for 10 minutes
      c) Transfer the supernatant (if you added chloroform, the upper aqueous phase should be transferred into a microcentrifuge tube).
2. Column Activation
    - Place a spin column into a 2ml collection tube
    - Add 100µL activation buffer into the spin column
    - Centrifuge at 10,000g for 30 seconds
    - Discard flow through, immediately proceed to next step.
3. Column Loading
    - Add 300µL (0.6 volume of cell lysis) isopropanol to the prepared lysate and vortex.
    - Transfer the mixture directly into the spin column
    - Centrifuge at 10,000g for 30 seconds
    - Discard flow through.
4. Primary Column Washing
    - Apply 100µL of washing buffer (ethanol added) to spin column
    - Centrifuge at 10,000g for 30 seconds

- Discard flow through.

5. Secondary Column Washing
    - Repeat as for primary column washing
    - Centrifuge again at 10,000g for 2 minutes to remove residual ethanol
6. Elute
    - Add 40-50µL elution buffer
    - Incubate at room temperature for 1 minute
    - Centrifuge at 10,000g for 1 minute
    - Store RNA at -20°C or -80°C.

APPENDIX II

CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)

Materials Required

- Jena PCR kit
- Disposable powder free gloves
- Microcentrifuge
- Pipette tips with filter plugs
- Pipettors
- Plypropylene tubes
- Vortexer
- MicroAmp Optical Caps or ABI PRISM™ Optical Adhesive Cover

PCR Mix Set-up

- The reaction mix was prepared using the cDNA synthesized from each samples as template.
- All procedures were performed on ice.
- The concentration of each components pf PCR mix was prepared in PCR tubes as shown on the table below:

PCR masre mix (ready to use)	2.5µl
Template cDNA	2.5µl
5µM Forward primer	0.5µl
5µM Reverse primer	0.5µl
Nuclease free water	6.5µl
TOTAL	12.5µl

APPENDIX III

AGAROSE GEL ELECTROPHORESIS

Materials needed:

- Agarose Base
- TAE Buffer
- 6X Sample Loading Buffer
- DNA ladder standard
- Electrophoresis chamber
- Power supply
- Gel casting tray and combs
- DNA stain (sybr green)
- Gloves
- Pipette and tips

TAE Buffer Recipies:

- 4.84g Tris Base
- 1.14ml Glacial Acetic Acid
- 2ml 0.5M EDTA (pH 8.0)

Agarose Gel Electrophoresis Protocol

I. Preparing the agarose gel:

- 1g of Agarose powder was measured into a 500ml flask
- TAE Buffer (100ml) was added to the flask
- The agarose was melted in a microwave until the solution becomes clear
- The solution was cooled to about 50-55℃ by swirling the flask occasionally to cool evenly
- 8µl of Sybr green dye was added and mixed properly

- The casting tray was set-up with the combs properly inserted with two layers of tape
- The melted agarose solution was poured into the casting tray and allowed to cool until it solidifies,
- The combs were carefully pulled out and the tray edges were removed,
- The gel was placed in the electrophoresis chamber and
- Enough TAE Buffer having the same strength as that used in the gel preparation was poured into the gel tank so that there was about 2-3mm of buffer over the gel.

II. Loading of gel

- The order in which each sample, controls and ladder will be loaded on the gel was recorded.
- 5µl of each sample/sample loading buffer mixture was carefully pipetted into separate wells in the gel.
- 6µl of the DNA ladder standard with loading dye was also pipetted into at least one well of each row on the gel.
- The gel tank was covered and electric current was switch on. The electric instrument was set-up as follows;

  Current……120Amp

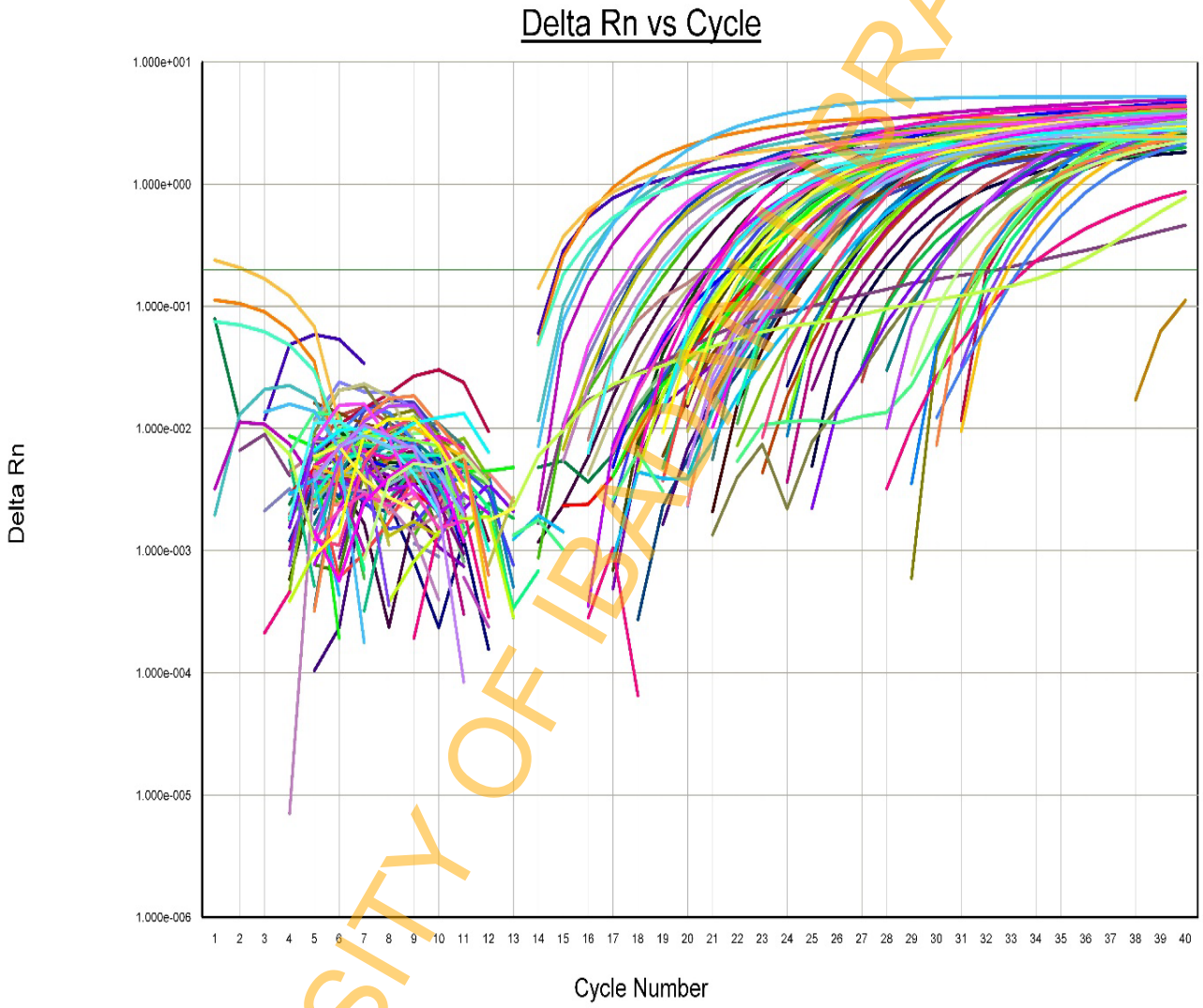
  Voltage……400volt

  Time……38mins

III. Visualization of the gel

- The gel was visualized under ultraviolet light using the BioRad imaging machine and the gel picture was obtained automatically on the computer using the BioRad image lab software application.

APPENDIX IV



qPCR Amplification Plot Representative of Samples