AMELIORATIVE EFFECTS OF APIGENIN ON DEXTRAN SULPHATE SODIUM-INDUCED ULCERATIVE COLITIS AND HEPATOTOXICITY IN MALE AND FEMALE *BALB/c* MICE

BY

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CERTIFICATION

This is to attest that this project work was carried out by OGUNGBE, Bimpe Folasade in the Department of Biochemistry, Faculty of Basic Medical Sciences, University of Ibadan under my supervision.

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DEDICATION

To God, who is the source of my strength and inspiration, is this research report dedicated.

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ABSTRACT

Ulcerative colitis (UC), a multifactorial inflammatory bowel disorder, is on the increase globally and a major risk factor for colorectal cancer. Existing drugs for treatment of UC have adverse effects, hence current research is focused on compounds with anti-inflammatory and antioxidant properties as alternatives. Apigenin (4',5,7-trihydroxyflavone), a natural flavone, possesses anti-inflammatory properties, however, there is dearth of information on its effects on UC. This study was designed to assess the effects of apigenin on dextran sulphate sodium (DSS)-induced UC and hepatotoxicity in both male and female *Balb/c* mice.

Acclimatised male (n=32; 22-26 g) and female (n=32; 19-23 g) mice were divided into four groups (n=8). Group I [Control 0.5mL/kg 1.5% v/v DMSO)], Group II (10 mg/kg Apigenin), Group III (5% w/v DSS) and Group IV (10 mg/kg Apigenin + 5% w/v DSS). The body weights of mice were measured daily. Apigenin was orally administered for 10 days whereas DSS was given for seven days. They were sacrificed after overnight fasting. Colon and liver were excised, weighed and used for biochemical analysis. Colon lengths were measured. Antioxidant indices; Lipid Peroxidation (LPO), hydrogen peroxide concentration (H_2O_2), Superoxide dismutase (SOD), Catalase, reduced Glutathione (GSH) and inflammatory indices; Nitric Oxide (NO) and myeloperoxidase were determined spectrophotometrically. Cytochrome P450 isoform (CYP3A4), P-glycoprotein, inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase-2 expressions were determined by immunohistochemistry. Histological examinations of colon and liver were carried out. Data were analysed using Two-way ANOVA at $\alpha_{0.05}$.

Administration of DSS significantly reduced body weight (-2.83±0.39 vs 0.90±0.43 g) and colon length (7.33±0.33 vs 10.08±0.39 cm) in male mice compared to control. The DSS-treated male group had significantly higher colonic NO (0.66±0.08 vs 0.41±0.06 µmol/mg), myeloperoxidase (3.65±0.36 vs 1.14±0.13 U/mg), LPO (11.09±1.08 vs 6.92±1.32 nmol/mg) and H_2O_2 (0.13± 0.01 vs 0.085±0.01 mmol/mg) but lower catalase (0.93± 0.20 vs 1.57±0.18 U/µg) compared to the female. Hepatic LPO and H_2O_2 significantly increased in the female by 68.6 and 85.6%, respectively compared to male. In the colon, apigenin pretreatment increased SOD in the female by 51.3% and GSH in male (738.0%) and female (336.9%). Apigenin reduced LPO in male by 51.4%, and H_2O_2 in male and female by 52.5 and 48.1%, respectively. Catalase reduced in the

male (0.46± 0.07 vs 1.07± 0.15 U/µg) while NO (0.57± 0.06 vs 0.37±0.03 µmol/mg) and myeloperoxidase (2.76±0.20 vs 1.15± 0.11 U/µg) increased compared to the female. In the liver, apigenin increased GSH in male by 206.0% and decreased LPO in female by 48.0%. Histology of the colon showed that apigenin reduced mucosal inflammation, neutrophilic infiltration and epithelial erosion. The CYP3A4, iNOS and cyclooxygenase-2 expressions were significantly repressed in both sexes and organs while P-glycoprotein was moderately expressed compared to the DSS-treated group.

Dextran sulphate sodium increased inflammation and oxidative stress in male and female mice compared to control. Apigenin attenuated dextran sulphate-induced ulcerative colitis and hepatotoxicity in the male mice better than in the female.

Keywords: DSS-inducedcolitis, Apigenin, Antioxidant indices, Epithelial erosion.

Word count: 484

LIST OF ABBREVIATIONS

API	Apigenin
API+DSS	apigenin and DSS administration
BSA	Bovine serum albumin
CDNB	1-chloro-2, 4-dinitrobenzene
COX-2	Cyclo-oxygenase-2 (Prostaglandin H-synthase)
CYP3A4	Cytochrome P450 (isoform 3A4)
CYP450	Cytochrome P450
DSS	Dextran sulphate sodium
DTNB	5, 5'-Dithiobis (2-nitrobenzoic acid)
EC	Enzyme Commission Number
GIT	Gastro-intestinal tract
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-s-transferase
H & E	hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HER	Human estrogen receptor
HLA	Human lymphocyte antigen
IBDs	Inflammatory bowel disorders
IL-	Interleukins
INOS	Inducible nitric oxide synthase
LPO	Lipid peroxidation
MDA	Malondialdehyde
MDR1	Multidrug resistance 1- protein
MPO	Myeloperoxidase
NAD+	Nicotinamide adenine dinucleotide
NED	N-(1-naphthylethylenediaminedihydrochloride)
NO	Nitric oxide
$NO^{\cdot}$	Nitric oxide radical
P-GP	P-glycoprotein

PGX	Prostaglandin
SOD	Superoxide dismutase
TBXA	Thromboxane A
TBXB	Thromboxane B
UC	Ulcerative colitis

CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Ulcerative colitis (UC), one of the kinds of inflammatory bowel disorders (IBDs) is economically burdensome in endemic areas because of the high cost incurred in finding lasting solutions to the disease. Earlier reports claimed its incidence and occurrence in developed countries but adoption of Western lifestyle in developing nations, migration, inter-racial marriages and other factors are contributory to its increasing incidence in developing nations. Case reports of a middle aged woman, a young girl and some others in some States in Nigeria are confirmations that UC is no longer limited to the Western world but becoming increasingly incident in Nigeria (Ekwunife*et al.,* 2015; Nwankwo and Maduforo, 2010; Senbanjo*et al.,* 2012). This may also mean that lack of technological and medical facilities for proper disease diagnosis in the past as well as low hospital patronage might have led to the rare occurrence reported in the past (Ekwunife*et al.,* 2015).

Among many animal models of ulcerative colitis that are available currently, chemical induction of UC in rodents which involves giving of dextran sulphate sodium (DSS) is a generally used and globally accepted animal model in studies on ulcerative colitis because of its simplicity, ease of colitis induction, close resemblance to human ulcerative colitis as well as reproducibility of results (Boussenna*et al.,* 2015). The mechanism of colitis induction by DSS has been linked to its ability to erode the epithelial lining of the gastro- intestinal tract (GIT), consequentially exposing symbiotic gut microbial flora (e.g. *Shigella* or *Shigella*-like toxin*, Salmonella, Yersinia* and *Clostridium dificile* toxin) which are implicated in GIT inflammation and other gut disorders (Massimo and Paolo, 2001). Upon breakdown of DSS into its carbohydrate and sulphate components, some microflora catabolize the carbohydrate units to generate energy by fermentation while certain microbes such as *Desulfovibrio spp.,* further reduce the sulphate to more toxic compounds like sulfites, hydrogen sulphide and some reactive oxygen species (Akao*et al.,* 2015; Hanning and Diaz-Sanchez, 2015).

Scientific research has revealed that modification of expressions and actions of drug metabolizing enzymes are resultant effects of inflammatory processes. Individuals with active disease often produce high amount of oxidative stress markers and inflammatory cytokines while they overly express the inflammatory proteins including tumor necrosis factors, interleukins,

inducible nitric oxide synthase, cyclo-oxygenase-2) and intercellular adhesion molecules within their colonic mucosa all of which cause tissue damage (Montrose*et al.,*2010). As a result, limiting their production is needful so as to diminish the developmental risks of UC- associated conditions.

Several therapeutic measures are being employed in the treatment of this disease and they include use of immuno-modulatory drugs, amino-salicylates, corticosteroids, etc. and surgery (e.g. colectomy) but none of them has been able to offer a lasting solution to the disease. Patients reportedly experience continuous cycles of remission and relapsing with other unpleasant side effects (Hanaeur, 2004). Hence, experimental studies are ongoing in search of natural agents that can provide cure with negligible side effects accompanying their usage (Awaad*et al.,*2013).

Plants possess various phytonutrients which are of immense medicinal benefits which has enhanced their usage in inhibition, control and management or cure of disorders such as diabetes, arthritis, cancers as well as inflammatory, oxidative and estrogenic conditions. This has widened plant-based research studies (Mostafa*et al.,* 2014). Examples of medicinally important plant phytonutrients include polyphenols, flavonoids, alkaloids, saponins, tannins, among others.

Apigenin (4',5,7-trihydroxyflavone) is naturally found in onions, chamomile tea, Gingko biloba, grapefruits and oranges and likewise in some seasoning agents like parsley, celery, spearmint, and basil, among others (Patel*et al.,* 2007). Over time, studies involving apigenin has increased in an attempt to explore its medicinal uses which can be applied in clinical trials. *In vivo* studies have proven that apigenin possesses minimal cellular toxicity and also selectively acts on cancerous and normal cells, being able to distinguish between both, unlike other flavonoids with similar structures. Notably, this natural flavone has anti-cancer, anti-inflammatory and antioxidant properties which have been scientifically confirmed (Birt*et al.,* 1986). For instance, Liang and co-researchers reported that apigenin and two other related flavonoids, genistein and kaempferol markedly repressed cyclooxygenase-2 and inducible nitric oxide synthase expressions in lipopolysaccharide-activated RAW 264.7 macrophages, thus confirming its anti-carcinogenic and anti-inflammatory potential. (Liang*et al.,* 1999). However, researchers are still studying other mechanisms of its action to ascertain its effectiveness in various disorders.

1.2 Justification for the Study

Despite early diagnosis, IBDs have rising global prevalence with huge cost of management which may cause an exponential effect on health-care systems. In addition, patients experience

other detrimental effects such as impartation of social stigma, impeding of career ambitions and also impairment of their quality of life. Persistent UC is closely connected with pathogenesis of colorectal cancer (Seril*et al.,* 2003), by contributing to induced expression of pro-inflammatory genes, their gene products (inflammatory cytokines), oxidative stress together with abnormal immune response to intestinal bacterial flora in UC patients (Montrose*et al.,* 2010).

Although synthetic drugs have been earlier employed in treatment of colitis, prolonged usage of synthetic compounds such as NSAIDS can bring about anemia, dyspepsia, erosions, gastric ulcers and renal dysfunction (Blackler*et al.,* 2012). There is therefore increasing search for natural anti-ulcerative and anti-inflammatory agents that are generally well tolerated with little or no side effect attached to their usage (Awaad*et al.,* 2013).

Sex and genetic makeup are important risk factors in ulcerative colitis development. Ulcerative colitis affects both male and female individuals. While many studies claim higher susceptibility of males to UC than females, controversies still exist as regards this. Maternal inherited gene expression and functions have been reported to make significant impact on the incidence of the disorder in children (Balzola*et al.,*2010). In addition, birth defects (such as limb loss or deformation in children from IBD-affected mothers) and higher risk of disease development seen in children have been associated with maternal-IBD inherited defects (Kalkan and Dağli, 2012).

Sex influences the pharmacokinetics and metabolism of apigenin. Apigenin is metabolized by both sulphate and glucuronide conjugation mechanisms. Studies carried out using rodents revealed a higher proportion of sulphate conjugates being recovered from mature male rats while a higher proportion of glucuronide conjugate was recovered from juvenile male and female rats as well as adult female rats (Gradolatto*et al.,* 2005). Metabolism of dextran sulphate sodium (DSS) on the other hand follows a deconjugation pathway being aided by the large intestinal microflora and glucuronidases with the production of dextran (or dextrin – the carbohydrate moiety of DSS) and sulphate ions (which are degraded into toxic sulphite ions by gut microorganisms) (Akao *et al.,* 2015; Hanning and Diaz-Sanchez, 2015).

Apigenin like other polyphenols undergoes biotransformation after consumption which may affect its activity as the metabolites produced may have an increased or a decreased activity relative to the parent compound (Holst and Williamson, 2008). Apigenin is also known to be poorly absorbed and slowly eliminated, it is hoped that the unabsorbed part in the intestinal tract will effectively delay the onset of UC and also attenuate its severity.

1.3 Aim

The general focus of this project work is to check potential of apigenin (10mg/kg/day orally administered for ten days) in improving health status of male and female *Balb/c* mice which orally received 5% DSS (administered through their drinking water) for seven days.

1.4 Objectives

1.4.1 Macroscopic Assessment

To evaluate sex influence on susceptibility to DSS-induced ulcerative colitis through measurement of disease activity indices which include measurement of change in body mass, colon length, ratio of the weight of colon to its length, presence of bloody diarrhea and occult blood in the faeces of the animals used.

1.4.2 Biochemical Assessment

To assess the effect of apigenin on the following biochemical markers in colon and liver post-mitochondrial fractions

- oxidative stress markers e.g. hydrogen peroxide (H_2O_2) concentration and lipid peroxidation (using malondialdehyde - MDA concentration as marker)
- inflammatory markers of ulcerative colitis through measurement of myeloperoxidase (MPO) activity and nitrite ion concentration
- antioxidant status through measurement of catalase activity, reduced glutathione concentration, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase activities.

1.4.3 Histological Assessment of the Colon and Liver

To assess extent of erosion/ulceration of colonic epithelium, presence of inflammatory infiltrates, vascular congestion, loss of goblet cells and crypt abscess.

1.4.4 Immunological-Histological Chemistry (IHC)

To determine the expressions of

- Inducible form of nitric oxide synthase (iNOS and cyclooxygenase-2

- An isoform of cytochrome P450, phase I drug metabolizing/detoxifying enzyme -– CYP3A4
- Multidrug resistance protein-1, a drug efflux transporter (p-glycoprotein)

1.5 Hypothesis

- Sex determines susceptibility to colitis induction and severity of DSS-induced ulcerative colitis
- Sex affects apigenin metabolism
- Apigenin metabolism alters activities of drug metabolizing enzymes and expression of drug efflux transporter (P-glycoprotein) which may reduce DSS-induced toxicity

➢ The data obtained from this study will reinforce knowledge of similarities and differences in the mechanisms behind sex-related response to apigenin treatment. This can be useful clinically in understanding the efficacy of apigenin treatment in male and female IBD-patients.

CHAPTER TWO
LITERATURE REVIEW

2.1 Inflammatory Bowel Disease

In humans, clinical presentation of inflammatory bowel disease (IBD), include ulcerative colitis (UC) and Crohn's disease (CD). In both conditions, there are alterations in gastro-intestinal motility resulting from inflammatory reactions (Collins, 1996). Patients with UC usually experience repeated sessions of relapse and remittance with symptoms like diarrhoea, rectal bleeding and anaemia, (Low*et al.,* 2013)which result from intestinal inflammation, oedema and ulceration (Hale and Greer, 2012). The UC is chronic in nature and the colon is primarily affected continually, beginning from the rectum to the distal (sigmoidal and descending colon) and proximal (transverse, ascending and caecum) section of the colons. When the whole colon is affected, the term "pancolitis" is used. The inflammation is usually limited to the mucosa while ulceration and crypt abscess are commonly observed by histopathological examination (Hale and Greer, 2012).

2.1.1 Incidence and Prevalence

Globally, UC is steadily rising. About 24.3, 19.2 and 6.3 patients per 100,000 population were recorded in Europe, North America and Asia (including Middle East). Bimodal age range has also been observed in the incidence and prevalence of UC. Prevalence is common between 15 to 30 years and 50 to 70 years(Low*et al.,* 2013). A cohort study among USA veterans showed a pronounced bimodal age prevalence between 25-34 years and 55-64 years (Hou*et al.,* 2013). However, according to Low and co-workers (2013), age and sex differences did not have any significant effect on UC risk among their studied patients.

The rise in IBD prevalence is associated with huge financial and health consequences. Medical cost for UC treatment in the United States was once proposed to be approximately $15,020 per patient (Rubin*et al.,* 2014). There also arises the need for dose changes, drug switching, and augmentation for patients but all these have adverse event risks with reduced expected health benefits that make patients to eventually discontinue therapy while searching for better and more affordable treatments that can eventually cure the disorder (Low*et al.,* 2013).

2.1.2 Etiology and Pathogenesis

Etiological factors of ulcerative colitis and in general inflammatory bowel disorders (IBD) include among others genetic, environmental, microbial and immunological responses as well as continual intake of trans unsaturated fatty acids (Ananthakrishnan, 2015; Ananthakrishnan*et al.,* 2014; Low*et al.,* 2013).

2.1.2.1 Genetic causes:

Development of IBDs is affected by a person's genetic composition as well as evidence of family history, especially those genes that are maternally inherited. Such inheritance factors from mothers have been shown to encode gene products implicated in UC development. This has been used to explain why disease risk is normally high among individuals with family history and descendants of IBD-affected mothers compared with those without previous family history of disease occurrence. Recently, Genome-Wide Association Studies (GWAS) further recognized no less than 47 UC risk alleles. Some of the candidate genes identified that give potentially significant information on the pathogenesis of UC include interleukin 1 receptor 2 (IL-1R2), interleukin 8 receptors alpha and beta (IL8RA-IL8RB), interleukin 7 receptor (IL7R), interleukin 12-beta, death associated protein-1, positive regulatory domain-1, Janus kinase-2, interferon regulatory factor 5, guanine nucleotide binding protein alpha 12 (GNA12) and Lymphocyte-specific protein-1. These genes code for proteins, enzymes and cytokines which are directly or otherwise involved in various cellular processes. Their roles include among others

i. Activation of immune responses through enhanced immune cells proliferation as well as their survival and differentiation
ii. Apoptosis, autophagy, assembly of tight junction proteins in epithelial cells, by associating with zonular occluden-1 (ZO-1) and Src (a non-receptor tyrosine kinase protein also known as proto-oncogen protein tyrosine kinase); neutrophil transmigration and regulation of other cytokines.

Besides, direct variants in DNA sequences, alterations in epigenetics, as well as histone modifications, noncoding RNA and DNA methylation form genetic risk factors of IBD (Anderson*et al.,* 2011; Legaki and Gazouli, 2016; Low*et al.,* 2013).

2.1.2.2 Environmental causes:

Based on recent studies, incidence of IBD positively correlates with environmental agents (seasonal changes), smoking, breastfeeding, oral contraceptives, antibiotics, infanthood hygiene,

appendectomy, infection and diet (Legaki and Gazouli, 2016). This however contradicts previous studies in which inconsistent results were found on IBD incidence and some of these factors. It can however be concluded that genetic susceptibility has a major role to play in this regard (M'koma, 2013).

2.1.2.3 Microbial agents and immunological responses:

Certain GIT microflora are known to exhibit significant impact in the pathogenesis of UC(Abraham and Cho, 2009; Uronis*et al.,* 2009). Although some of these microorganisms live as commensals with their hosts, many of them are still capable of causing the disorder most especially after administration of chemical agents that can disrupt the epithelial layer and homeostasis of the GIT. Examples are the gram negative *Salmonella strains* (*S. typhi* and *S. Dublin)* and the adherent invasive *Escherichia coli* (E. *coli)* (Low*et al.,* 2013).

Alteration in immunological response energized by microbial agents in the intestinal tract plays significant roles in the initial and chronic stages of UC. There is accumulating evidence that persistent intestinal infections, defects in mucosal barrier and the dysfunction of the mucosal immunological system have significant effects on the pathogenesis of IBD. For instance, mucosal $CD4^+$ T cells supposedly induce chronic inflammatory response and prolong it. This is brought about by an elevation in the formation of IL-21 and T-helper 2-related cytokines such as interleukins- 4 and 13. Pro-inflammatory molecules potentially stimulate intestinal mucosal functions *in vitro*, and cause proliferation of T-immune cells and macrophages, expression levels of chemokines and adhesion molecules, coupled with synthesis of other pro-inflammatory cytokines (Xu*et al.,* 2014).

Numerous immunological cells, including innate and humoral immune cells, are normally found in the lamina propria of the GIT at physiological conditions. Innate immunity prevents the entry and spread of pathogens within the body and offers protection through mechanisms involving the mucus layer, epithelium, and lamina propria. The mucus, with its various isotypes of constitutive proteins (called mucins or MUC), is the first defense mechanism in the various sections of the intestine. UC patients normally express decreased and deregulated MUC2 which might be due to the cytokine imbalance. The intestinal epithelium which is the second line of defense contains a single layer of cells with differing functions, including enterocytes, entero-endocrine cells and goblet cells which rapidly replicate themselves. Epithelial cells are joined to one another by tight junctions which also makes a clear distinction among the components of epithelial cells from

adjacent ones. (Xu*et al.,* 2014). Lamina propria contains innate as well as immune cells and functions as the third level of intestinal protective mechanism. Strong identification of microbial metabolites (antigens) which could likely provoke an abnormal immunological reaction and intolerance to normal gut microflora in human patients with IBDs, is enhanced by dendritic cells. During inflammatory conditions, the innate and immune cells become activated and penetrate into the intestinal mucosa. Once activated, they associate few stromal cells to start expressing increased concentrations of cells involved in processes like inflammation, cell-surface adherence, membrane signaling and response. List of such cells include complement of differentiations (CD)- 54 and 62L, chemokine receptors (CCR)- 5, 6 and 9, with integrins, especially integrin α4β7 (Xu*et al.,* 2014).

2.2 Animal Models of Ulcerative Colitis

Research studies on animal models have a lot of significance. Through animal models researchers can readily assess the factors responsible for the development of human IBD because differences exist in human anatomy and physiology. It becomes possible to investigate the pathogenic factors and secondary effects of ulceration leading to disorders in hepatic functions and protein metabolism in the intra- and extra-cellular electrolytes as well as other systemic complications (Gennari and Weise, 2008). Also, better and more efficient management protocols (and possibly cure) may be developed as considerable progress is being made in studies on the disease while the influence of various management protocols on the course of UC can also be studied using animal models. In addition, since an association between diet and IBD pathogenesis has been affirmed, a more efficient diet structure may be generated to ensure the total remission of the disorder.

It should be noted however, that no single model of UC and IBDs as a whole is able to solely mimic all the symptoms manifested in human patients. Hence, it may become necessary to employ different models to study the effect of a single therapeutic protocol on IBD to ascertain its efficacy and shortcomings (Barnett and Fraser, 2011).

Series of animal models have been developed for over fifty years, using varieties of species of animals with different inducing agents, in the course of research studies on UC**.** These models range from colitis induction using chemicals, to adoptive (immune cell) transfer in addition to genetic modification (gene knockout and gene transfer).

In the initial model of UC, it was discovered that different species of laboratory animals fed with certain seaweed extracts (carrageenan) presented symptoms that were similar to human IBD. Thereafter, these models became modified and resulted in newer ones which involve induction using chemical agents and bacterial infection, all of which can be performed on animals including mice, rats, porcine, rabbits, hamster, monkey and cotton top tamarin as well as lower animals such as zebra fish and drosophila. In addition, gene variants relevant to IBD (such as transgenic and gene knock-out) are also being broadly used in mice and rats (Barnett and Fraser, 2011; Cummings*et al.,* 2003).

Animals models used in ulcerative colitis studies display some clinical characteristics which closely mimic the ones observed in patients with UC. Common symptoms include significant loss of body mass, anaemia, mucoid and blood-stained stool, diarrhea and mild to severe rectal bleeding. The use of these models has greatly facilitated and provided useful insight into the mechanisms of UC development and efficacy of different treatment protocols. Histopathological results do reveal useful comparison between animal models and human UC. Pathological damages such as mucosal ulceration, loss of colonic haustral segment and disappearance of normal vascularized mucosa being replaced with friable and granular mucosa together with formation pseudo-polyps and polyploids. Apart from ulcerations seen at the different stages of UC progression and mucosal healing, there are other histological features seen in animals, including mucosal inflammatory changes (acute, subacute, and chronic), occasional neutrophilic exudates found in glandular lumen of crypts of Lieberkuhn, lymphocytic infiltrate seen in the lamina propria and cystic dilatation or distortion and glandular epithelial hyperplasia (DeRoche*et al.,* 2014).

Major advantages of animal models of IBD include possibility of investigating the pathogenic factors and secondary effects of ulceration leading to hepatic malfunction, protein metabolism disorders, electrolyte imbalance and other systemic complications (Gennari and Weise, 2008). Furthermore, studies involving influence of various management protocols on the course of UC are also made possible using animal models.

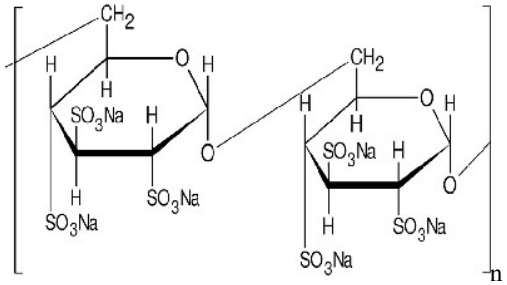
2.2.1 Chemically Induced Model of Ulcerative Colitis

Assortments of chemical compounds are used to induce UC in laboratory animals. Examples of these agents include carragenin, dextran sulfate sodium (DSS), oxazolone, 2,4,6-

TriNitroBenzeneSulphonic Acid (TNBS), acetic/ethanoic acid, azoxymethane, etc. Both TNBS and DSS are able to destroy the intestinal barrier within a short period of administration. However, features seen in TNBS models resemble CD more closely while the damages caused by DSS simulates UC (Barnett and Fraser, 2011).

2.2.1.1 Dextran sulphate sodium-induced ulcerative colitis

2.2.1.1.1 Structure of Dextran Sulfate Sodium (DSS) $(C_6H_7Na_3O_{14}S_3)n$,



Dextran sulfate sodium is sulfated polysaccharide with diverse molecular weights between 5 kDa and 1400 kDa. Dextran, a polyanionic derivative of dextrin, is a polymer of anhydrous glucose with about 95% alpha-D-(166) linkages and the rest (i.e. 5% containing 163 linkages) involved in the branching of dextran.

Dextran sulphate sodium is applicable in different research fields and has been used for selective isolation of lipoproteins by precipitation. Previous studies had shown the ability of sulphated polysaccharides, DSS inclusive, to selectively precipitate low-density lipopolysaccharide (LDL) from serum. An interaction was proposed to exist between the precipitated LDL and the sulphated polysaccharide through which they are implicated in cardiovascular disorders. As a follow-up to earlier studies, Nishida and Cohan (1970) explored the exact groups involved in the DSS-LDL interaction using DSS-precipitated serum LDL (modified by acylation and succinylation). They concluded that the charged protein moiety of LDL and not the acyl groups were majorly involved in the interaction with sodium salt of dextran sulfate (Nishida and Cogan, 1970). In addition, DSS found usage in enhancing formation of a hybrid between a probe and DNA immobilized to the membrane (Wahl*et al.,* 1979). Also, Kent and colleagues in 1987 demonstrated the ability of DSS to cause DNA to be released from histone protein-DNA complexes (Kent*et al.,* 1958). Ishioka and co researchers in 1987 reported its use in animal

models of colorectal cancer (Barnett and Fraser, 2011) and intestinal colitis (Okayasu*et al.,* 1990).

2.2.1.1.2 Induction of colitis by DSS administration

Administration of DSS to animals is most commonly done by dissolving it in their drinking water (usually 1%-5%) to induce colitis. Once taken, DSS initiates inflammatory bowel disorder, which is lethal and resembles human ulcerative colitis with clinical symptoms such as diarrhoea and bloody stool. In addition, there are other damaging effects, like being toxic to epithelial cells, increasing permeability of intestinal barrier as well as activating macrophages (Ajayi*et al.,* 2015).

Intestinal microflora have an impact in the susceptibility to the disease. Reports have shown that mechanism of colitis induction by DSS has been linked to its ability to exert injurious effect on the epithelial layer of the intestinal lumen thereby exposing lamina propria (LP) and submucosal sections to antigens and enteric bacteria localized in the lumen (e.g. *Shigella* or *Shigella*-like toxin*, Salmonella, Yersinia* and *Clostridium dificile* toxin), resulting eventually in inflammation. These symbiotic micro-organisms become parasitic and trigger the specific inflammation and other GIT disorders (Low*et al.,* 2013; Massimo and Paolo, 2001).Upon breakdown of DSS into its carbohydrate and sulphate components, some microflora catabolise the carbohydrate units to generate energy by fermentation while certain microbes such as *Desulfovibrio spp*. further reduce the sulphate to more toxic compounds like sulfites, hydrogen sulphide and some reactive oxygen species (Akao*et al.,* 2015; Hanning and Diaz-Sanchez, 2015). Laboratory animals treated with DSS also develop UC as DSS binds to average-chain fatty acids in the colon, causing colonic epithelial lesion and induction of acute inflammation. The earliest pathological changes in DSS-induced model include progressive disruption of colonic crypts with influx of inflammatory agents such as neutrophils and cluster of differentiation-4 (CD4+) T cells, in addition to macrophages, to the damaged segments of the colon. (Low*et al.,* 2013). Chronic colitis usually occurs upon withdrawal of DSS with presence of activated immune T-cells at locations of inflammation but the damaged (eroded) parts of the epithelium become regenerated after several days to weeks when DSS has been withdrawn (Albert*et al.,* 2010).

2.2.1.1.3 Severity of DSS-induced colitis:

Colitis onset and severity may vary based on several factors such as concentration or dosage of DSS administered, its molecular weight, date of manufacture, the company/manufacturer, the

batch, duration of DSS exposure (sub-acute, acute or chronic), as well as genetic factors (i.e. strain and sub-strain of animals). It has been shown that strains of mice specifically *Balb/c* and C3H/HeJ are at higher risk of developing UC than wild type mice, while rats are more susceptible than mice.

Age, gender, intestinal flora (e.g. germ-free or sterile as against specific pathogen-free) and stress are also important factors that affect efficacy of DSS-induced colitis. Male mice are reported to be more susceptible than their female counterparts(Cheah*et al.,* 2013). Though no correlation has been reported between disease susceptibility and the volume of DSS-supplemented water consumed, it is important to measure this volume in cases where other agents (which may alter water consumption) are administered with DSS (Cheng*et al.,* 2009), (Chung*et al.,* 2008).

2.2.1.1.4 Impact of molecular mass of DSS on susceptibility to UC

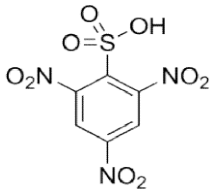
The molecular mass of DSS is a key factor which determines susceptibility of animals to colitis (De Smet*et al.,* 2009) or colitis-induced dysplastic lesions (Dharmani*et al.,* 2011). It also determines the adversity of colitis (Elvington*et al.,* 2015) as well as carcinogenicity (Hakansson*et al.,* 2009). Treatment of *Balb/c* mice with 40kDa DSS causes the most severe colitis while colitis resulting from 5kDa treatment was milder. On the other hand, mice that were administered 500 kDa DSS did not develop any injury in their large bowel (Hao*et al.,* 2012). Similarly, there are reports with evidence that the 54kDa DSS can initiate cancer of the colon but the 9.5 and 520 kDa DSS are non-carcinogenic as at the time of the studies (Hasegawa*et al.,* 2013). Hudovic and co-workers observed (through histochemical studies) the inability of high molecular weight DSS to induce UC. This was based on the mechanism that the molecules could not penetrate the mucosal membrane due to their large molecular mass, which also restricted their distribution and concentration in various tissues (Hudcovic*et al.,* 2012). In addition, molecular mass of DSS equally affects the localization of colitis. For instance, administration of 40kDa DSS to mice leads to diffuse colitis mainly found in mid-section and one-third part of the large intestine starting from distal end, whereas treatment with DSS (5 kDa) causes fairly sparse lesions which are majorly seen in the cecum and upper colon (Islam*et al.,* 2008).

2.2.1.1.5 Advantages of DSS model:

The DSS model of colitis induction is reportedly advantageous over other models of colitis. To begin with, the forms of colitis such as chronic, acute or relapsing, resulting from DSS

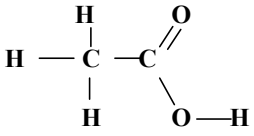
administration can be readily replicated by varying DSS dosage as well as duration in experimental animals. Moreover, the dysplasia resulting from DSS administration resembles the chronic sign seen in humans with UC. In addition, based on differences in sensitivity expressed by different strains of mice, 'susceptibility genes' can be identified. Furthermore, recent review has shown that data from animal studies, particularly UC-related carcinogenesis can be translated to human disease (Boussenna*et al.,* 2015).

2.2.1.2 2,4,6, Trinitrobenzenesulfonic acid-induced ulcerative colitis



Trinitrobenzenesulfonic acid (TNBS) is a highly reactive nitroaryl acid with very strong oxidizing properties. This chemical agent is normally administered intrarectally and simultaneously with ethanol to induce diffuse inflammation of the colon. Characteristic features of this model include increased leukocyte infiltration, edema as well as ulceration. The colon becomes necrotic, in addition to being severely damaged. Observation of necrotic portions of the colon shows that they are usually enclosed by areas of acute inflammation. This model of colitis possesses features which are similar to features of Crohn's disease. However, it also resembles DSS model by upregulation of two particular genes, matrix metallopeptidase14 (Mmp14) and TIMP metallopeptidase inhibitor 1 (TIMP1). Moreover, the intestinal inflammation resulting from TNBS instillation is pan-ulcerative colitis (pancolitic), that is, the entire colon is affected. Furthermore, it slowly develops, reaches the peak one week after administration and continues in the mice for as long as they remain alive. There is also mucosal permeability leading to neutrophil infiltration and increase in myeloperoxidase activity. In addition, interferon-gamma (IFN- γ) expression becomes increased and the inflammatory process involves T-helper 1 (Th1) response, which is based on interleukin- (IL-)12 expression (Boirivant*et al.,* 1998). The mechanism by which inflammation results from TNBS administration is initiated when macrophages recognize and degrade altered mucosal cells and proteins. The TNBS, when instilled, has been shown to react with active phospholipids located on surface of colonic mucosa (for instance, phosphatidyl choline and phosphatidyl inositol) causing decreased mucosal hydrophobic integrity and increased inflammation (Barnett and Fraser, 2011).

2.2.1.3 Acetic (Ethanoic) acid-induced ulcerative colitis



Acetic acid (CH_3COOH)

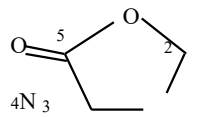
Intra-rectal instillation of diluted ethanoic acid is another means of causing chemical damage to the epithelial layer of colonic mucosa. This model brings about a temporary phenotype that mimicks UC. MacPherson and Pfeiffer first demonstrated this model in rats by instilling 10%–50% acetic for 10 seconds through the rectal opening, following which the lumen was flushed with normal saline three times. Diffuse colitis was detected in those rats in a concentration-dependent form. Histopathological features observed include ulceration of the distal part of the colon (which sometimes extend to the lamina propria) and crypt abnormalities (Low*et al.,* 2013). Regeneration of the ulcerated mucosa begins within few days or weeks in mice and rats as the case may be.

Over the years, researchers began to focus on ways of optimizing this model by adopting many changes such as altering the concentration of acetic acid injected as well as the duration of exposure. This became necessary as rectal injection of high concentrations of acetic acid caused intestinal perforations. Therefore, the protocol was modified to involve low concentration (e.g. 4%) and shorter duration of exposure (between 15 to 30 seconds).

The acetic acid model is advantageous in terms of affordability, accessibility and ease of infusion. In addition, this model was shown to be useful for studies aiming to determine effectiveness of anti-oxidative substances in the management of UC. Vitamin E (α-tocopherol), N-acetyl cysteine (NAC), trimetazidine, and melatonin are few examples among such. In situations where the efficacy of immune-modulatory drugs needs to be assessed, this model is useful but tests should be carried out about twenty-four (24) hours after acetic acid injection (Low*et al.,* 2013).

2.2.1.4 Oxazolone-induced model of colitis:

1



($C_3H_3NO_2$)

4-ethoxymethylene-2-phenyloxazol-5-one 5-(4*H*)-oxazolone isomer

Intra-rectal instillation of oxazolone, dissolved in ethanol (hapten) into murine animals causes an acute form of colitis that is distinct by initiation of a T helper (Th)2-type immunological reaction (Kojima*et al.,* 2004) with notable rise in the levels of interleukins- 4 and 5 production, which is preventable by *in vivo* co-administration of anti–IL-4. In addition, there is normal or low IFN-γ production. Accompanying symptoms include loss of body mass, bloody diarrhea, colonic ulceration and epithelial cell loss (Boirivant*et al.,* 1998).

Inflammation resulting from oxazolone injection is very rapid and reaches its climax within a few days following administration. Macroscopic and microscopic observation of colons of affected animals normally reveal that the inflammation, which is normally superficial with characteristic ulceration, is limited to distal half of their section of the colons. Moreover, cellular exudates are present with a mixture of infiltrates i.e. lymphocytes, granulocytes, and eosinophils. Diseased animals may die or gain full recovery depending on the severity of the colitis resulting from this model.

2.2.2 Bacterial-Induced Ulcerative Colitis

Microbial agents commonly employed in the induction of ulcerative colitis in animals include *Salmonella typhimurium* (*S. typhi*) *and Adherent Invasive Escherichia coli* (AIEC). Both are useful models in acute colitis studies.

2.2.2.1 Salmonella typhimurium infection

In order to induce inflammatory processes with histopathological features that are comparable to those seen in human colitis, it is necessary to first treat the animal with oral antibiotic cocktail, majorly to disrupt commensal microorganisms in the colon and allow formation of new colonies of the desired pathogenic microbe, in this case, S. typhi. High density growth is usually observed for S. typhi within twenty-four hours post infection. Loss of epithelial crypt, erosion, and neutrophilic infiltration, which characterize this model are normally observed from fifth to

seventh day post infection. This model is therefore useful in studies involving acute ulcerative colitis (Low*et al.,* 2013).

2.2.2.2 Adherent–invasive E. coli (AIEC):

The AIEC has equal affinity for intestinal epithelial/mucosal cells (Xu*et al.,* 2014). To induce colitis through this model, it is necessary to cause mild epithelial damage, using low concentrations of chemical agents like DSS throughout the AIEC infection period. This model has phenotypic features which resemble UC features seen in patients, such as loss of body mass, rectal bleeding and infiltration of colonic mucosa by neutrophils (Low*et al.,* 2013).

2.2.3 Genetic Model of UC

With the aid of Innovative genetic technologies, variations in susceptible genes are associated with increased possibility of developing IBDs. Knowledge of this was enhanced using transfected animals (both gene-knockout and transgenic). For gene knockout Common variant genes include IL-2 receptor

Innovative genetic technologies have helped in the recognition of a numerous genes, in which animals with their variants normally have greater probability of developing self-generated colitis. The severity of colitis however differs as some of these models still need additional means of initiating the inflammatory processes. These genetic models include gene knockout (KO) mice models (that lack specific genes) and transgenic models (which possess mutated forms - gene variants. Examples of knockout models include T cell receptor mutant, deletion at locations for IL-2 receptor-alpha, interleukin-2, interleukin-22 and TNF-3'untranslated region. Transgenic mutation in murine and rat includes IL-7, signal transducer and activating transcription (STAT)-4 and HLA B27 (Barnett and Fraser, 2011; Low*et al.,* 2013; Wirtz and Neurath, 2007; Yang and Pei, 2006).

2.3 Markers of Ulcerative Colitis:

Inflammation is a primary problem in IBD generally. It is the body's immune defence against invading pathogens and affects the entire large bowel in a continuous manner in ulcerative colitis. Patients with ulcerative colitis may likely experience inflammation in other parts of their bodies such as joints (arthritis), vertebrae (spondylitis) and eyes, as well as skin and mouth ulcers, liver and gallbladder disorders.

In humans with active colitis, the concentrations of inflammatory cytokines and reactive oxygen species (ROS) are often higher within patients' their colonic mucosae (Wang*et al.,* 2015). These oxidative and inflammatory species elicit damaging effects on target tissue, conditions known to be severe events in UC development. Oxidative stress is a secondary event in addition to inflammation. Assessment of colitis incidence is initially done through macroscopic calculation of disease activity indices) and further involves various laboratory protocols including histological assessment (microscopic observation of H&E-stained section of the colons), biochemical assays (quantifying the levels of (i) pro-inflammatory cytokines, (ii) inflammatory enzyme activities and expressions e.g. MPO, iNOS and COX-2 and (iii) oxidative stress markers, using spectroscopic, electrophoretic and immuno-histochemical techniques.

2.3.1 Disease Activity Indices (Vasina*et al.*, 2010)

Disease activity indices of ulcerative colitis include reduction in body mass (resulting from loss of appetite), retarded growth, weakness, fever, diarrhoea, occult blood in stool, shortened and thickened colon length, mild and severe bleeding from the rectum and anaemia consequential to intestinal inflammation, edema and ulceration. These are normally scored in patients or experimental animals using predefined grading methods (da Silva*et al.,* 2014).

2.3.2 Macroscopic and Histological Assessment of Colonic Damage

Colonic damage can only be assessed in animal models after they are sacrificed. However in patients, colonoscopy (sigmoidoscopy) is normally used before surgical excision of the damaged section of the colon (colectomy). In animal models, the distal segment of colon is excised and opened longitudinally, then immediately viewed under a stereomicroscope in order to score visible damage (Morris*et al.,* 1989).

For histopathological assessment (microscopy), fixatives such as phosphate buffered formalin are normally used to preserve the colon immediately after removal. It is then embedded in paraffin wax from which a small section (usually about 5µm) is prepared for histological assessment of other damages by a pathologist.

Pathological changes which include ulceration and erosion of the mucosa, inflammatory cell infiltration, mucosal wall thickening, goblet cell depletion, loss of crypt architecture, crypt

abscess, mucin depletion etc. (depending on the severity of the disease), are normally scored (using light microscope with varying magnification) and graded according to a given protocol.

2.3.3 Myeloperoxidase Assay

Myeloperoxidase is encoded by MPO and Mpo genes in humans (*Homosapiens*) and in mice (*Mus musculus*). It is a heme-containing protein located on chromosomes 17:58,269856-58,280,935 bp in humans and 11:87793581-87804413 bp in mice, which means it is about 11,079 and 10,832 bp long in the two species, respectively (Morishita*et al.,* 1987).

The enzyme – myeloperoxidase with enzyme commission number (EC 1.11.2.2), is involved in decomposing lipid peroxides, including hydrogen peroxide. The enzyme is synthesized during myeloid differentiation in bone marrow (Koeffler et. al., 1985). It's level of expression is reportedly high in neutrophil granules and to a lesser extent in monocytes which are sites of completion of its synthesis as well as storage. It is later released upon leukocyte activation and degranulation (Lópes*et al.,* 2009; Zhang*et al.,* 2001).

Myeloperoxidase possesses antimicrobial effects and can produce free radicals and diffusible oxidants during its actions. In addition, it catalyses H_2O_2-dependent oxidation of chloride anions to form hypochlorous acid (Hendersson et al., 2003).

During respiratory burst, MPOs present in neutrophils are activated and become toxic to tumour cells and pathogens microbes (Klebanoff et. al., 2013). Activities of this enzyme have been linked with inflammatory processes partly because it can oxidize different substances like phenols and anilines.

Myeloperoxidase has been shown to cause enhanced damaging effects oxidative and inflammatory in nature, to target tissue (Loria et. al., 2008). In addition, using immunohistochemical technique, it has been confirmed that the enzyme (myeloperoxidase) resides in human atherosclerotic lesions, whereas using mass spectrometry to analyse components of human atheroma and low-density lipoprotein (LDL) obtained from diseased arterial tissue, MPO oxidation products were recognized as major components of the tissue. Reports have shown that MPO catalytically oxidizes LDL *in vivo* and converts it into forms which are engulfed by macrophages. This leads to deposition of cholesterol and formation of foam cells. Furthermore, MPO capably utilizes nitric oxide as a physiologic substrate, which may enhance endothelial dysfunction (Abu-Soud*et al.,* 2001; Zhang*et al.,* 2001).

2.3.4 Nitric Oxide and Nitric Oxide Synthases

Nitric oxide (NO) is a metastable free radical synthesized by isoforms of nitric oxide synthase (NOS). There are three isoforms of this enzyme: neuronal (nNOS or NOS-1), and inducible (iNOS or NOS-2 and endothelial (eNOS or NOS-3) isoforms. They entirely catalyze the production of nitric oxide and L-citrulline from L-arginine. Two of the isoforms, NOS-1 and NOS-3, are calcium-dependent in action and produce low levels of nitric oxide gas (nM) which is vital for cell signaling. The iNOS, however is usually inactive, calcium-independent and generates high levels of the gas, which is known to be cytotoxic at high concentrations (usually μM levels) and worsens disease conditions. NOS-2 is induced by various cytokines and pathophysiologic agents like IFN-γ, TNF-α, IL-12, lipopolysaccharide (LPS) and bacterial endotoxin. Cofactors, such as calcium-calmodulin, tetrahydrobiopterin, NADPH, FAD AND FMN are vital requirements for proper functioning of all the NOS isoforms. It is noteworthy that NO can affect several stagess of the inflammatory cascade, starting from point of its expression to the stage at which leukocytes flux into the infected tissue (Laroux*et al.,* 2001). NOSs are the first examples of cytosolic mammalian CYP450s with both reductase and heme domains being part of the same polypeptide. These properties were found to be similar to the fatty acid mono-oxygenase characterized from Bacillus megatorium known to be a self-sufficient P-450 (Marletta, 1993).

In endothelial cells, NO can either be released as $NO^{\bullet}$ or bound to carrier molecules containing thiol (-SH) e.g. L-cysteine which stabilize it when released. In the smooth muscle cells and platelets, nitric oxide radicalactivates guanylate cyclase, raises the level of the intracellular messenger (cGMP) and decreases intracellular Ca^{2+} concentration, causing smooth muscles to relax thereafter and preventing platelets from aggregating. This effect is similar to that of prostacyclin (PGI_2), the endothelial vasodilator which activates adenyl-cyclase and thus raises the intracellular concentration of adenosyl monophosphate (AMP). Apart from being cytotoxic at high concentrations, NO* generated by cytokine induction interacts with other reactive products formed from macrophage activation. Cytotoxic effects of NO• in target cells include induction of metabolic dysfunctions that can be lethal to the target cells. Examples include inhibition of DNA synthesis, glycolysis, Krebs' cycle and respiratory chain; substantial loss of intracellular iron with altered ferritin and transferrin receptors function as well as altered mitochondrial enzymes which contain Fe-S group (Jerca*et al.,* 2002).

2.3.5 Prostaglandin-H Synthase (Cyclooxygenase*)* Expression and Activity

Cyclooxygenases (COX) are membrane-bound enzymes which catalyze the cyclization and peroxidation of di-homo-γ-linoleic (8c,11c,14c-eicosatrienoic acid), arachidonic (5c, 8c,11c,14c-eicosatetraenoic acid) and eicosapentaenoic (8c,11c,14c, 17c- eicosapentaenoic acid) acids to form prostaglandins PG_1, PG_2 and PG_3, respectively - with subscripts 1, 2 and 3 referring to their fatty acid sources (Smith, 1992).

2.3.5.1 Classes of prostaglandins

Prostaglandins are biological molecules which originated from seminal vesicles. There are different classes of prostaglandins depending on their fatty acid source, substituent groups on their carbons 9 and 11, and partition in different solvents (Ramwell*et al.,* 1977).

By nomenclature, there are ten specific prostaglandin molecular classes which have alphabet letters A-J assigned to them, being an indication of unique variations of functional groups attached to positions 9 and 11 of their cyclopentane rings. Among the PG classes, those derived from arachidonic acid are the most extensively distributed and highly characterized. They include PGE_2, PGD_2, PGI_2 and $PGF_{1\alpha}$ (where α and β refer to the spatial configuration of carbon 9 hydroxyl group. The functional groups of various classes of prostaglandins are shown in Figure 2.1 below (Gorman*et al.,* 1977; Smith and Murphy, 2002).

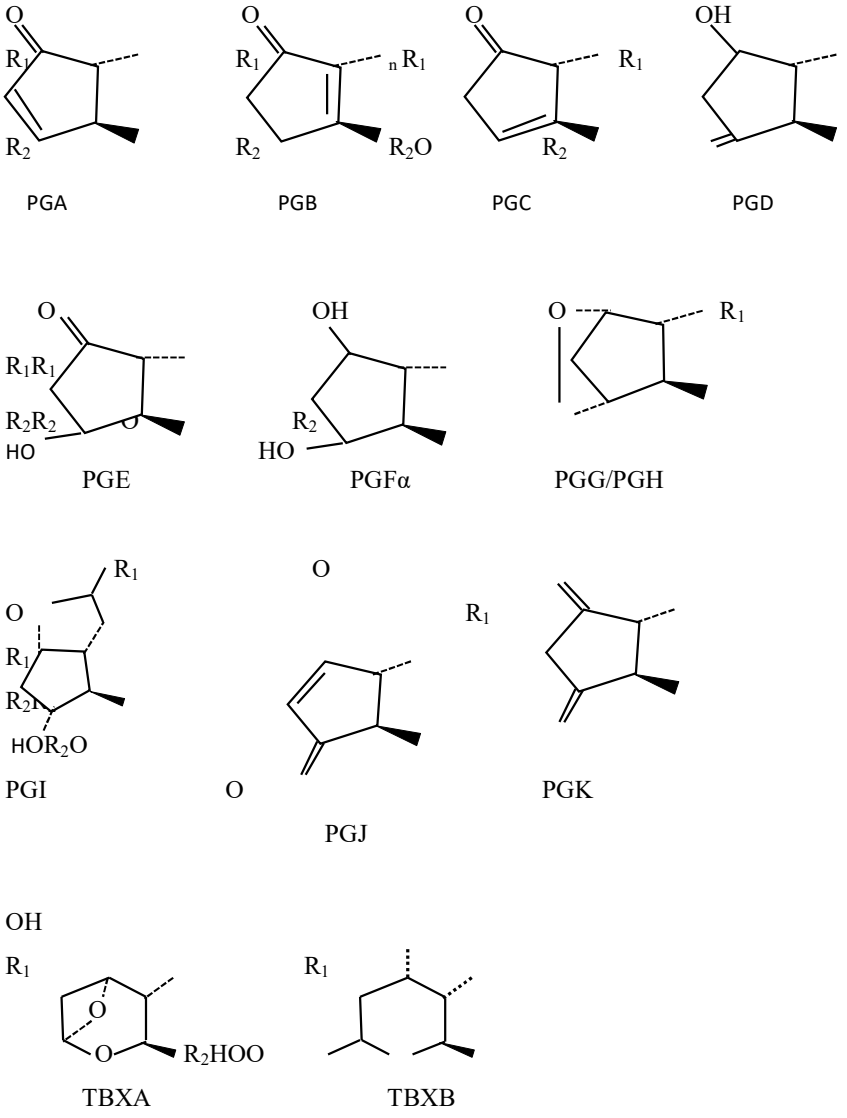


Figure 2.1: Structures of Prostaglandins

2.3.5.2 Synthesis of prostanoids

They are synthesized by the hydrolytic action of phospholipase A2 on membrane phospholipids (such as phosphatidyl inositol), by hydrolytic cleavage of arachidonic acid at sn2 carbon of the glycerol moiety of the phospholipid to form eicosatetraenoic acid. Cyclooxygenase enzyme converts eicosanoids to two forms of prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2), by its cyclooxygenase and peroxidase activities, respectively. The latter is then converted to other prostaglandins by other enzymes.

The main control points in the synthesis of prostaglandins which are also the target of most drugs include phospholipase A2 action and cyclooxygenase (Smith and Murphy, 2008).

2.3.5.3 Functions of prostanoids

Prostanoids are both autocrine and paracrine depending on whether they act respectively, on the originating cells or neighbouring cells. They normally harmonize the responses of these cells (i.e. the parent and neighboring cells), converting them into biosynthetic stimulus. This makes them hormone-like in action largely enhanced by G-protein-linked prostanoid receptors. Prostaglandins possess variable functions among which are protection of gastric mucosa; simulation of platelet aggregation which enhances its clotting function (mainly by thromboxanes); inhibition of platelet aggregation (by prostacyclins); maintenance of renal perfusion and inflammatory responses such as induction of inflammation and fever, mediation of pain signals, smooth muscle contraction (including uterus – mainly by PGF2α) and smooth muscle relaxation (- mainly by PGE series). Functions of prostaglandins depend on the cyclooxygenase isoforms from which they are produced (Smith and Murphy, 2008).

2.3.6 Pro- and Anti-Inflammatory Cytokines

Pro-inflammatory or inflammatory cytokines refer to signaling molecules released from immune cells and certain other cell types which stimulate inflammation while anti-inflammatory cytokines are a various immunoregulatory molecules that control response to proinflammatory cytokines (Zhang and An, 2007). Clinical reports of UC patients and studies on animal models reveal higher amount of proinflammatory molecules including interleukins -1β, -6, -8, -13, -23 and -33 together with TNF-α when compared with controls (Atreya and Neurath, 2005; Bamias*et al.,* 2011). These inflammatory proteins possess the potential to significantly

alter mucosal barrier function and intestinal permeability which under physiological conditions, are normally protected by tight junction proteins (Sartor, 2006).

The tight junction complex consists of trans-membrane proteins - occludin and claudins, with linker proteins - zonula occludens-1, all of which associate with the actin cytoskeleton. These are involved in effective regulation of membrane fluidity by means of paracellular transport while they inhibit transcellular movement (lateral diffusion of molecules between apical and basolateral plasma membrane domains).

In a physiological state, the innate immune system protects the GIT against pathogen invasion by releasing antimicrobial peptides such as β-defensins into intestinal lumen. These peptides have antibacterial and anti-viral activities which not only help in protecting the host mucosal epithelia and stem cells from virulent microorganisms but also maintain the amount and composition of commensal microbiota (Farombi*et al.,* 2013). Evidence abounds that submucosa of UC patients with active disease are exposed to a large number antigens resident in the luminal. This results from a breach in their mucosal barrier, with rearrangement and repression of some of these junctional proteins coupled with reduced level or malfunctioning of their intestinal β-defensins. (Han*et al.,* 2015; Weber*et al.,* 2008).

Up-regulation in the expressions and levels of inflammatory cytokines usually causes reduction in the amount and expression of anti-inflammatory gene products (Rani*et al.,* 2011; Xu*et al.,* 2014). These molecules can be determined quantitatively using conventional ELISA methods or by immuno-histochemical staining to detect their expressions (Farombi*et al.,* 2013).

2.3.7 Oxidative Stress and Lipid Peroxidation Markers

Oxidative stress refers to oxidative alteration of biological macromolecules - proteins, nucleic acids, and lipids, by reactive oxygen species. This results in injuries or disorders to various organs which become malfunctioning. In line with the 'free radical theory of aging', aggregation of a series of these injuries leads to age-related decline in biological functions, also known as 'senility'. Several scientific reports clearly demonstrate that reactive oxygen metabolites (ROM), namely superoxide anion ($O2^{-\cdot}$), Hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-), hydroxyl radical (OH^-), hypochlorous acid (HOCl) and peroxy radical (ROO^-), directly impair oxidizable biomolecules both reversibly and irreversibly. Superfluous formation of reactive species (ROS, RNS, nitric oxide and peroxynitrite anion coupled with ineffectiveness in scavenging them leads

to oxidative and nitrosative stress. These conditions are capable of inducing inflammatory responses which in turn can induce DNA damage, apoptosis or cancerogenic cell transformation, as well as modifications of cellular proteins and lipids. Thus, oxidative stress is potentially dangerous (Kruidenier and Verspaget, 2002) and is an implicating factor in several human disorders, IBDs inclusive (Piechota-Polanczyk and Fichna, 2014). It should be noted however, that some of these radicals possess some biological benefits when present at low concentration. There are recent reports confirming that free radicals are produced at low amount by some cells to mainly regulate the activities and functions of such cells (Birben*et al.,* 2012). Naito and colleagues reported on the roles of oxidative metabolites in modifying very reactive cysteine residues in various target proteins which are important in signal transmission and are capable of regulating the functions of important cellular components. Examples of such target molecules are thioredoxin-related proteins and tyrosine phosphatase (Naito*et al.,* 2010). To support the biological importance of their reactive metabolites, nitric oxide has also proven as an inhibitor of lipid peroxidation through 'chain breaking' mechanism (Hogg and Kalyanaraman, 1999).

Polyunsaturated fatty acids (PUFAs), which are major constituents of bio-membrane lipids and lipoproteins, form the major sites of attack by ROMs (most especially $OH^{\bullet}$). Lipid peroxidation is initiated by introduction of a hydroperoxy group into the fatty acyl ends of unsaturated fatty acids, immediately followed by a chain of reactions to primarily form toxic lipid hydro-peroxyl radicals and aldehyde derivatives examples of which include malondialdehyde and 4-hydroxynonenal. Secondary effects of lipid peroxidation results from accumulation of already formed fatty acyl radicals and aldehyde derivatives which affect membrane functions such as fluidity, permeability, signal transduction, transport as well as cellular homeostasis and metabolism Lipid peroxidation affects lipid-lipid and protein-lipid interactions and thus alters the structures and functions of biological membranes and lipoproteins. In addition, some of these radicals regulate cytokine production and are capable of recruiting acute and chronic inflammatory agents (neutrophils and lymphocytes) to target tissue (Catalá, 2009; Kruidenier and Verspaget, 2002; Piechota-Polanczyk and Fichna, 2014).

Assessment of extent of tissue damage by lipid peroxidation is possible through techniques such as spectroscopy and ELISA, which employ measurement of absorbance generated by coloured products formed from reaction of these species with assay components. Intensity of colour produced from this reaction is directly proportionate to absorbance read while absorbance

directly compares with extent of lipid damage. Examples of lipid peroxidation molecules include expired pentane or ethane, malondialdehyde (MDA), lipid hydroperoxides, isoprostanes and conjugated dienes (Del Rio*et al.,* 2005; Urso and Clarkson, 2003).

2.3.8 Antioxidant Markers

Antioxidants are endogenous and exogenous, enzymic and non-enzymatic substances which offer protection against oxidative impairment caused by reactive metabolites. Examples of endogenous antioxidants include beta-carotenoids, ascorbate, alpha-tocopherol, reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase. Exogenous antioxidants are normally obtained from dietary sources, dietary formulations (supplements) and drugs. Examples include carotenoids, polyphenols, transition metal-binding proteins (cerruloplasmin and transferrin), ubiquinol, alpha uric acid and trace elements/micro-nutrients such as selenium and zinc. These antioxidants are either lipophilic or hydrophilic and they possess different mechanisms by which they exert their antioxidant defense in biological cells. Such mechanisms include prevention of radical formation, scavenging of already formed radicals, repair of radical-damaged membrane macromolecules as well as recruitment of antioxidants to sites where radicals are already formed (by making use of signals generated for radical formation) (Elvington *et al*., 2015; Ighodaro and Akinloye, 2017).

2.3.9.1 Superoxide dismutase

Superoxide dismutase (SOD), enzyme commission number EC 1.15.1.1 is a first line defense antioxidant that destroys the highly reactive superoxide anion (which is capable of causing enzyme denaturation, lipid oxidation and DNA fragmentation), converting it to hydrogen peroxide (H_2O_2) and oxygen molecule. Although H_2O_2 is less reactive than superoxide itself, it is extremely toxic to cells, especially when its level exceeds the amount required for normal physiological function (Kruidenier and Verspaget, 2002).

$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$ HaberWeiss reaction

$H_2O_2 \longrightarrow O_2 + 2H_2O$ Catalase reaction

$H_2O_2 + Fe^{2+}_{(Cu^+)} \longrightarrow Fe^{3+}_{(Cu^{2+})} + OH^- + OH^*$ Fenton reaction

$H_2O_2 + H^+ + NADPH \longrightarrow 2H_2O + NADP^+$ Glutathione peroxidase reaction

Apart from scavenging superoxide anions, superoxide dismutase is also capable of protecting dehydratases against superoxide radical damage.

Superoxide dismutase has different isotypes each of which contain different transition metals and three-dimension structures on which their classification is based on. The trace metals (Cu/Zn, Fe, Mn and Ni) act as cofactors required for dismutation function of the enzyme. The isotypes of SOD are named after the cofactors they chelate.

SOD isoform	**Accronym**	**Location/site**
copper and zinc	(Cu-Zn)-SOD	Cytosol, chloroplasts and sometimes extracellular space
iron	(Fe)-SOD	Chloroplast
manganese	(Mn)-SOD	mitochondria and peroxisomes
nickel	(Ni)-SOD	Prokaryotes

Among the SOD isoforms, only Fe-SODs and Mn-SODs have substantially identical structures, sequences and metal-chelating residues at their catalytic site. Mechanism of superoxide anion ($O_2^{\cdot-}$) dismutation at the catalytic site of SOD occurs by successive redox reaction involving the transition metal ion, following Ping Pong mechanism of reaction (Farombi*et al.,* 2013).

2.3.9.2 Catalase

Catalase, EC 1.11.1.6, a peroxisomal enzyme is another 'first line' defense antioxidant enzyme which decomposes H_2O_2 to molecular oxygen and water, thus mitigating its toxic effects. In addition, catalase also acts as a peroxidase in the presence of other proton donors like short chain aliphatic acids (ethanol and methanol) as well as formic acid and phenol (Polavarapu*et al.,* 1998). It is a tetrameric haemin (ferric chloride heme)-enzyme made up of four identical subunits (60 kDa), with the chloride ion and Fe^{3+} respectively replacing the hydroxyl ion and Fe^{2+} present in heme protoporphyrin ring. Catalase is found in nearly all aerobic cells with four ferri-protoporphyrin groups per molecule, in tetrahedral arrangement, enclosing the Fe^{3+} at the core of the ring. This highly efficient antioxidant enzyme is not readily saturated by H_2O_2 at any given concentration, which is an indication that catalase has low Km for and exhibits constant rate of reaction in the presence of its substrate (Matés, 1999).

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{catalase} H_2O + ROH + A$$

Although aerobic cells normally require catalase to prevent lethal effect of oxidative stress, some reports have otherwise shown that presence of catalase in some transfected cells result in

adaptive response of such cells to oxidative stress, in which case the cells concerned become tolerant and insensitive to oxidative stress. This is brought about when transfected cells are enriched with catalase which helps to trap hydrogen peroxide in the medium, converting it into oxygen. This was reported to cause enhanced sensitivity of some cells to antibiotic drugs like bleomycin and Adriamycin and prevent drug-induced oxygen depletion (Makol*et al.,* 2011; Matés, 1999).

2.3.9.3 Glutathione peroxidase (GPx)

Glutathione peroxidase, of which the selenium-containing peroxidases are vital examples, represents another class of antioxidants with 'first-line' defense mechanism with ability to protect against cellular oxidative damage. These enzymes reduce a range of lipids and organic hydroperoxides, in addition to hydrogen peroxide (ROOH and H_2O_2). They require reduced glutathione (GSH) as cofactor in the peroxidation reaction. Glutathione peroxidase, is an 80 kDa protein having four polypeptide subunits, each of which has a selenocysteine (Sec) residue important for its catalytic activity (Arthur, 2001; Matés, 1999). Glutathione peroxidase can singly catalyze its peroxidative reaction efficiently while it can equally share its substrates with catalase. The reaction is as shown in the equation below:

$$ROOH + 2\,GSH \xrightarrow{GPx} ROH + GSSH + H_2O$$

At least five isozymes of GPx are identified in mammals all of which are ubiquitously expressed in organ-specific form. The isozymes with their functions and sites of expression include:

- GPx1 - found in cytosol and mitochondria of erythrocytes, kidney, and liver, reduces fatty acid hydroperoxides
- GPx2 (GPx-G1) - highly expressed in cytosol of gastrointestinal tract
- GPx3 (GPx-P) - extracellular surface of kidney
- GPx4 - localized in cytosol and the membrane fractions of renal epithelial cells and testes, reduces hydroperoxides of phospholipids, fatty acids and cholesterol from peroxidized membranes and oxidized lipoproteins
- GPx5 (selenium-independent) - expressed specifically in mouse epididymis (Matés, 1999).

2.3.9 Xenobiotic metabolizing Enzymes and Drug Efflux Transporters

2.3.9.1 The cytochrome P450 enzyme system

The cytochrome P-450 enzyme system (CYP450) majorly participates in transformation of endogenous and exogenous substances. It is crucial for lipophilic substances (drugs and chemical agents) to be bio-converted into less toxic more polar (hydrophilic) intermediates or products for total excretion from the system. Xenobiotic biotransformation takes place mainly in the liver and is composed of Phase I (e.g., oxidation, reduction and hydroxylation) and Phase II (e.g., conjugation- sulphation, glucuronidation, e.t.c.) reactions. Oxidation of lipophilic substances, in which an atom of oxygen is inserted into substrate to produce a polar hydroxyl group, represents the most common reaction in Phase I mechanism of drug biotransformation. Cytochrome P-450 group of enzymes play major role in this reaction and are also known to be the most important of all the enzymes in this phase. Apart from the liver, intestinal mucosa, kidney, lungs, placenta, brain, testis and ovary also contain xenobiotic biotransformation enzymes, although with variable levels and expressions. For instance, CYP450 in the proximal portion of small intestine are found to be more active than colonic isoforms. (Gavhane and Yadav, 2012).

Cytochrome P450s are hemoproteins, bound to membranes of endoplasmic reticulum and consisting of families, subfamilies and/or single enzymes which are capable of metabolizing many types of biological and chemical agents, for instance steroids, fatty acids, poisons as well as drugs (Thörn*et al.,* 2005).

Classification of CYP450 isozymes is based on the proportions their homologous amino acid sequence. Three among the various CYP450 gene families play principal roles in drug metabolism. These gene families are CYP1, CYP2 and CYP3. By definition, a particular CYP45O gene family is has over 40% amino acid sequence homology, whereas members of a subfamily have greater than 55% homologous sequence identity. By means of this classification, cytochrome P450 enzymes are assigned alphabet letters CYP signifying cytochrome P450. An Arabic numeral following the letters indicate the family, another letter signifying the subfamily (in cases where they are more than one) and lastly an Arabic numeral describing a singular gene within the subfamily. Examples include CYP1A2, CYP2C9 and CYP3A4.

CYP3A4 isoenzyme (isoform) is a significant subdivision of the cytochrome P450 family. It represents about 60% and 70% of all hepatic and intestinal CYP450s, respectively. CYP3A4

biotransforms several drugs. It is reported that extensive gastrointestinal metabolism (catalyzed by CYP3A4) results in poor oral bioavailability of many drugs (Hochman*et al.,* 2000).

2.3.9.2 Role of drug efflux proteins on drug absorption and bioavailability

Functionally, gastro-intestinal epithelium physically shields the GIT lumen from making contact with systemic circulation thus protecting cells of the mucosal immunological system against antigens present in the lumen. In order to keep this protective barrier intact, epithelial cells express numerous proteins many of which are transport proteins (for substrates), tight junction proteins and trefoil peptides. Mucosal inflammation is a resultant effect of any disturbance affecting the integrity of epithelial cell barrier. Apart from being protective, the epithelium facilitates trans-epithelial movement of vital nutrients coupled with efflux of potentially toxic substancesby proteins families of the ATP-binding cassette (ABC) transporter. P-glycoprotein is a drug efflux transporter among others (Hochman*et al.,* 2000).

The multidrug resistance gene (MDR1), codes for P-glycoprotein (P-Gp/ABCB1), which is a 170kDa multi-pass membrane protein with three N-glycosylation sites. It is an ATP-dependent efflux pump which prevents drugs from accumulating in multidrug resistant cells and also extrudes toxic agents out of normal cells. The multi-drug transporter, P-glycoprotein, is constitutively expressed on the apical region of intestinal epithelium (i.e. mucosal cells). In the liver and kidney respectively, its expression is localized in biliary canaliculi and brush border of proximal tubules. In these organs, it excretes structurally diverse substances into the gastrointestinal tract, the bile and urine, as the case may be (Blokzijl*et al.,* 2007; Thörn*et al.,* 2005).

Scientific claims show that altered function and/or expression of MDR1 makes significant impact on pathogenesis of inflammatory bowel disorders. Susceptibility genes for IBDs are reportedly located on chromosome 7, which is equally MDR1 gene locus. Additionally, Single Nucleotide Polymorphism (SNPs) in the human *MDR1* gene also associates MDR1 functional alteration with IBD(Blokzijl*et al.,* 2007).

2.3.9.3 CYP450 and drug efflux protein interactions

Although CYP450 and P-glycoprotein play different roles in absorption, two models provide evidence of synergistic relationship between these two proteins. The first model known as the circumstantial model proposed three factors as being responsible for this synergistic interaction. They include

(a) overlapping substrate specificity for both P-glycoprotein and CYP450s,
(b) similarities in the regulation of their genes and their distribution in various tissue and
(c) spatial juxtaposition of CYP3A4 to same location on apical membrane where P-glycoprotein is expressed. The second model however, provided direct evidence of synergistic interaction by using caco2 cells over-expressing high CYP3A4 activity, cultured in a medium containing 1α,25-di-OH vitamin D3 (di-OH vit D3) (Fisher*et al.,* 1999).

2.3.9.4 Hormonal influence on CYP3A4 and MDR1 expressions

Sex-related differences are observed in the prevalence and progression of IBDs. There are inconsistent findings on incidence and severity of colitis in male and female patients and animal models. However, Bábíčková and co-workers proved that 17β-estradiol supplementation to ovariectomized female mice partially protected them and lessened the severity of colitis in relation to orchidectomized male mice which received testosterone supplementation (Bábíčková*et al.,* 2015). Expression of P-glycoprotein in adrenal gland is very strong, a site where it actively transports some steroid hormones (cortisol, aldosterone, and dexamethasone) out of the cell. Contrarily, progesterone inhibits P-glycoprotein expression thus reversing drug resistance in cells expressing this transporter. More also, Kim and Benet reported that estradiol induced P-glycoprotein expression and increased cytoplasmic concentration in ER^- positive MCF7 breast carcinoma cells (Kim and Benet, 2004). Hormones thus play major role in drug and xenobiotic absorption, bioavailability, elimination and clearance.

CYP450 genes are polymorphic and variations in their expression and activity have clinical consequences in adverse drug reactions and disease susceptibility.

Sex-dependent variance in expressions of xenobiotic drug metabolizing enzymes has been reported. Expression of CYP is inversely related to that of P-glycoprotein, an indication of their interaction in drug metabolic reactions. Women strongly express certain isoforms of CYP450 (mainly CYP3A4) in a higher proportion than men which is known to be a crucial factor in drug elimination and clearance. Contrarily, men express a higher fraction CYP1A2 which is believed to compensate for their low expression of CYP3A4 (Meibohm*et al.,* 2002; Wolbold*et al.,* 2003). In some human populations women often experience adverse drug reactions after taking some drugs which are substrate of CYP1A2 e.g. clozapine, theophylline and caffeine.

Evidence of gender-divergent and tissue-specific distribution of the 78 CYP450 mRNAs has been demonstrated using mice model. Apart from the liver, other organs like gastro-intestinal

tract, kidney, brain, lung, testes and ovaries exhibit gender differences in CYP450 expression and activity (Renaud*et al.,* 2011).

2.3.9.5 Glutathione-s-transferase

Glutathione-s-Transferases (GST) is a Phase II drug metabolizing enzyme which conjugates glutathione to numerous electrophilic compounds, to make them more hydrophilic and easier to eliminate from the system (Townsend and Tew, 2003). GSTs are categorized as members of two separate super-families and they include (i) the membrane-bound microsomal family and (ii) cytosolic family of enzymes. Major distinguishing feature between a microsomal and cytosolic GST is structural, microsomal GSTs undergo homo- and hetero-trimerization to form a single active site whereas cytosolic GSTs form dimers. Functionally, the microsomal GSTs exert a major effect on metabolism of prostaglandins and leukotrienes. Thirteen classes of GSTs are known but human cytosolic GSTs exhibit high polymorphism, six classes of these enzymes have been identified in humans and are accordingly named as alpha, mu, omega, pi, theta, and zeta, with symbols α, μ, ω, π, θ and ζ, respectively (McIlwain*et al.,* 2006; Townsend and Tew, 2003). It is credible that GSTs play two distinct roles (i) direct detoxification reaction, which aids development of resistance to drugs and (ii) inhibiting MAP kinase pathway. The relation between GSTs and the MAP kinase pathway explains why drugs used in many cases to select for resistance do not get conjugated with GSH, while some are not substrates for GSTs, hence are not metabolized by the enzyme (Townsend and Tew, 2003).

2.3.10 Non Enzymatic Antioxidants

2.3.10.1 Reduced glutathione (GSH)

Living cells require glutathione (the reduced form) for metabolism, differentiation, proliferation, and apoptosis. Thus, irregularities in its homeostasis are closely linked with etiology and/or development of several diseases. Examples are various forms of cancer, old age diseases, inflammatory, immunological, cardiovascular, and neuro-degenerative disorders. Glutathione can be synthesized by *de novo* and by salvage methods. Both pathways are important for the cellular environment to be kept in a reduced state. The protein, a dipeptide made up of three amino acids – glutamate, glycine and cysteine, functions a co-factor for many cytoplasmic enzymes. Equally, it is essential for post-translational modification of many cellular proteins (Townsend*et al.,* 2003).

Synthesis and degradation of GSH mainly occur in the liver, which incidentally, is the basic site for drug biotransformation reactions. The cytosol and extracellular space are respectively, the specific locations in cells where glutathione is synthesized and broken down. Therefore, the protein needs to be transported out of the cell in order to maintain its normal turnover. Secretion of glutathione across hepatic sinusoidal membrane into blood plasma and bile occurs at very high rates and from there, it is delivered into other tissues. Like the antioxidant enzymes, glutathione is required for transport and detoxification of reactive species in the liver (Ballatori*et al.,* 2009).
Defensive reaction of glutathione against the effects of toxic agents, including drugs, environmental pollutants and carcinogens, is widely described (Pompella*et al.,* 2003). The cysteine thiol is nucleophilic in reactions involving electrophilic species (both exogenous and endogenous). Therefore, GSH often attacks reactive oxygen species (ROS) in reactions which occur spontaneously or enzyme-catalyzed ones. Balance should be maintained in the expression of glutathione and the enzymes which require it as cofactor. This will help in regulating the level of ROS in the cell, taking note that these ROS at physiological concentration play significant roles in cell signaling events but are implicated in the pathology of many disorders affecting humans (Ribas*et al.,* 2014; Townsend*et al.,* 2003).

2.3.11 Assessment of Genetic Markers in Ulcerative Colitis:

There is usually reduced expression of apoptotic and inflammatory genes and their products (i.e. mRNA or their proteins) in ulcerative colitis. The list includes but not limited to MUC2 gene and anti-apoptotic proteins p53, p38, p21, NOD2, bax, bcl2, caspases, e.t.c., (Heazlewood*et al.,* 2008). There are also decreased expressions or activities of drug metabolizing enzymes (CYP450 isoforms e.g. CYP1,2 and 3) and drug efflux transport protein, such as p-glycoprotein (Mennigen*et al.,* 2009; Thörn*et al.,* 2005).

2.3.12 Immune and Hormonal Responses

Alterations have been reported in immunoglobulin levels in patients with colitis. High levels of serum IgA, IgG and IgM were reported in patients compared to control (MacDermott*et al.,* 1981; Rosekrans*et al.,* 1980).

2.4 MANAGEMENT OF ULCERATIVE COLITIS

Presently, there is no known cure for UC due to its multifactorial nature. However, there has been advancement in management therapies over the years. UC therapies include use of synthetic (orthodox) drugs, probiotics, dietary supplements, botanicals and flavonoids (Montrose*et al.,* 2010; Park*et al.,* 2012; Wadie*et al.,* 2012).

2.4.1 Conventional Treatment of UC

2.4.1.1 Aminosalicylates

They were the first set of synthetic drugs employed to maintain remission of mild to moderate conditions. Examples include sulfasalazine (azulfadine), 5-amino salicylic acid (5-ASA) or mesalamine, balsalazide, and olsalazine. These class of drugs possess anti-inflammatory effects with ability to inhibit interleukins -1 and -2, alongside NF-kB. Moreover, the drugs do alter chronic inflammatory agents (monocytes and lymphocytes). They possess antioxidant activity as well as potential to inhibit sulfide production (Hanauer, 2004). However, these drugs are only suitable for managing moderate (acute) form of colitis. There were accompanying adverse effects on prolonged duration and dosage which present with symptoms including headache, fever, nausea, rash, hepatitis, nephritis, pancreatitis agranulocytosis, as well as male infertility. The 'sulfa' part of the drug is also reported to interfere with absorption of folic acid, which explains why patients using sulfasalazine treatment do take vitamin supplement. On the other hand, 5-ASA medications which lack the sulfa moiety have lesser side effects associated with their usage but there were reported cases of diarrhea and abdominal pain (Dignass*et al.,* 2012). Aminosalicylates were later replaced with corticosteroids which were relevant for acute episodes.

2.4.1.2 Corticosteroids (Dignasset al., 2012)

Corticosteroids are synthetic derivatives of cortisol. They are also referred to as glucocorticoids or cortisones and they are known to possess immense anti-inflammatory effects. Examples include triamcinolone, prednisolone, methylprednisolone, hydrocortisone, and beclomethasone. They have potential inhibitory effects on arachidonic acid cascade, interferon-gamma and interleukins-1, -2, -4, -5, -6, and -8. Short and long-term side effects include oedema, sleep disturbance, mood swings, increase in appetite and body mass, increased cataract risk, moon

face, osteoporosis, myopathy, drug resistance, immune-related infective conditions and adrenal failure (Baron*et al.,* 1962).

2.4.1.3 Antibiotics

Case reports abound on ineffectiveness of antibiotic prescription for patients mild UC. Among the reasons could be lack of evidence of bacterial infection before prescribing antibiotics. Metronidazole, vancomycin, ciprofloxacin and tobramycin are examples of such antibiotics. Although studies have shown initial improvement on antibiotic usage, remission is not usually maintained on prolonged usage. This might result from development of more virulent strains that are antibiotic-resistant or by increased use of broad-spectrum antibiotics (Musher*et al.,* 2005).

2.4.1.4 Immunosuppressive Drugs, Heparin, Melatonin and Probiotics

Immune modulators such as cyclosporine (Lichtiger*et al.,* 1994), azathioprine and 6-mercaptopurine, were prescribed for patients on steroid therapy for severe colitis. These drugs demonstrated anti- inflammatory effects by selectively inhibiting immune responses mediated by T lymphocytes. Although there are reports on efficacy of immunosuppressive drugs, significant side effects are associated with prolonged usage, examples include paresthesia, hypocholesterolemia, seizures, hypertension and possibly nephrotoxicity on prolonged usage. Pancreatitis, fever, rashes, arthralgia, nausea, and diarrhea were also among side effects reported on usage. (Head and Jurenka, 2003).

Certain percentages of efficacy have been reported using various other treatment protocols such as heparin (Shen*et al.,* 2007), melatonin (Li*et al.,* 2005) , and probiotics (Kruis, 2004). However, none of these has a 100% success rate as patients still experience either short period of remission or undesirable side effects on prolonged usage. (Head and Jurenka, 2003).

2.4.2 Management of UC with dietary supplements and Botanicals

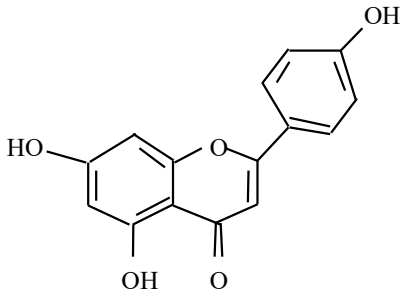
Despite the challenges of short duration of remission and ineffective therapy, researchers are relentlessl looking for agents that can proffer lasting solutions to the burden of ulcerative colitis and other secondary disorders connected with either treatment strategies or persistent UC. Some of the newly explored areas of research involve use of herbal preparations, elemental diet, elimination/hypoallergenic diet, natural dietary supplements, botanicals and flavonoids.

Scientific reports abound on efficacy of preclinical treatment protocols involving animal models and cell lines. These agents reportedly elicit their significant effects through mechanisms such as

improvement of disease activity indices, restoration of colonic morphology to (or near) normal compared with control, reduction in the levels and expressions of pro-inflammatory mediators of colitis, improvement in antioxidant status of the animals and many more. Of note are kolaviron (Farombi*et al.,* 2013), 6-gingerol (Ajayi*et al.,* 2015; Chang and Kuo, 2015), freeze-dried black raspberry (Montrose*et al.,* 2010), dietary kaempferol (Park*et al.,* 2012), neuropeptide-γ antisense oligonucleotide (Pang*et al.,* 2010), anti-TNF-α and zinc acetate (Barollo*et al.,* 2011), Ginkgo biloba, *Boswellia serrate,* quercetin, rutin, Punica granatum and plant sterols, (Awaad*et al.,* 2013; Low*et al.,* 2013).

2.5 APIGENIN:

2.5.1 Structure and Sources



Apigenin is also referred to as (i) 4', 5, 7-Trihydroxyflavone, (ii) 5, 7-Dihydroxy-2-(4-Hydroxyphenyl) 4H-1-benzopyran-4-one, (iii) C.I. Natural Yellow 1, (iv) Versulin, (v) Spigenin, (vi) Apigenol and (vii) Apigenine

Apigenin is a yellow crystalline solid flavone common in varieties of plants and herbs including celery (*Apium graueolens*) parsley (*Petroselinum cripspum*), thyme, peppermint, chamomile (*Matricaria chamomilla*), beach naupaka (*Scaevola sericea*), chili pepper (*Capsicum annuum*), Chinese violet (*Asystasia gangetica*), Rosemary (*Rosemarinus officinalis*), sweet bitter leaf (*Vernonia hymenoepis*), weed passion flower (*Passiflora foetida*), lemon, balm, thyme (*thymus vulgaris*), oregano (*Origanum vulgare*), lovage (*Levisticum officinale*), garden sage (*Salvia officinalis*), etc. (Głowacki*et al.,* 2016; Patel*et al.,* 2007; Yang*et al.,* 2008). The flavone is equally present in beer, red wine and Gingko Biloba memory herb. Its proportion in chamomile is approximately 0.8-1.2% apigenin by weight (Shukla and Gupta, 2010) while in some food and

herbal sources, it exists as its acylated and glucosyl derivatives (e.g. apigenin-7-O-glucoside (Tolstikova*et al.,* 2009).

2.5.2 Physicochemical Properties of Apigenin

It is a secondary plant metabolite, derived from the phenyl-propanoid unit: C6-C3. Natural apigenin is present principally in glycosylated form, with the tricyclic core structure linked by O- or C- glycosidic bond to a sugar moiety. Examples include apigenin-6-C-glucoside (isovitexin), apigenin-7-O-glucoside, apigenin7-O-neohesperidoside (rhoifolin), apigenin-8-C-glucoside, (vitexin), apiin and 8-C-arabinoside (Patel*et al.,* 2007). Pure apigenin has a low molecular weight of 270.24g/mole with a very high melting point of 345 to 350 °C. It does not directly solubilise in water unless it is first dissolved in other solvents like dilute potassium hydrochloride or Dimethyl Sulfoxide (Bevilacqua*et al.,* 2004). It is moderately soluble in hot alcohol but incompatible with strong oxidizing agents. However, water solubility increases in food bound apigenin containing glycosidic bonds, for instance apigenin-7-O-glucoside (Tolstikova*et al.,* 2009). The food-borne apigenin is known to be more stable at normal environmental conditions than the synthetic one. Chemicular apigenin should be kept in a dry and dark place at low temperature (-20˚C) to increase its shelf life to at least two years (Ross and Kasum, 2002).

2.5.3 Pharmacokinetic Properties of Apigenin

2.5.3.1 Absorption of apigenin

Dietary apigenin is present as a glycoside which, upon cleavage in the lumen of intestinal tract is thereafter absorbed and distributed as apigenin. Based on Pharmaceutics classification system (BCS), apigenin is a BCS II drug due to its low solubility and high intestinal permeability (Zhang*et al.,* 2012). This means that there is higher concentration of apigenin in the gastrointestinal epithelium than at other body tissues. It undergoes first pass effect/metabolism in the small intestine and later in the liver. It is first metabolized by Phase I drug metabolizing enzymes (CYP 450 isoforms) to luteolin (the major metabolite), scutellarein and isoscutellarein (hydroxylated metabolites) and rapidly conjugated through UDP glucuronosyltransferase to form glucuronide and sulfate conjugates which are released into serum (Gradolatto*et al.,* 2005; Gradolatto*et al.,* 2004). Different transport mechanisms are involved in the absorption of

apigenin in four segments of the intestine: dueodenum, jejunum, ileum and colon (Zhang*et al.,* 2012). According to these researchers, apigenin undergoes active transport in the duodenum and jejunum in a concentration- dependent mode but it is passively transported in the ileum and section of the colons in a concentration independent manner. Although apigenin is readily absorbed, its distribution to various organs is slow and this is one important factor reduces its bioavilability at the effector sites in addition to the first bypass effect. It is slowly eliminated which may lead to accumulation on prolonged usage, which was thought to be responsible for its ability to inhibit the growth and spread of epithelial cancers (Lefort and Blay, 2013).

Ingested apigenin is quickly metabolized via UDP glucuronosyltransferase and enters the serum in form of its glucuronide and sulfate conjugates. (Gradolatto*et al.,* 2005). In 2005, Gradolatto and co-workers orally administered a single dosage of radiolabelled apigenin to mature and immature rats of both sexes. Their report showed that apigenin had a half- life of 91.8 hours (> 3 days) with the radio-labelled [^{3}H] apigenin appearing 24 hours after ingestion but the baseline amount was regained after ten days of [^{3}H] apigenin admiiniastration. Various proportions of the radio-labelled apigenin was recovered from urine (51.1%), faeces (12.0%), blood (1.2%), the kidneys (0.4%), the intestine (9.4%) and the liver (1.2%) while the proportion regained from the rest of the body within 10 days was 24.8%. They reported further that apigenin metabolism was sex and age dependent. Their studies also showed that a higher proportion of sulphate conjugate was eliminated from mature male rats than glucuronide conjugate while the opposite ratio was recovered for mature female rats as well as immature male and female rats.

2.5.4 Biological Properties and Mechanism of Action of Apigenin

Apigenin possesses immense beneficial and health promoting effects. It has lower intrinsic toxicity and differential effects *in vivo* compared to other flavonoids with related structures. This finding was based on reports carried out on normal and cancer cells where apigenin displayed medicinal effects which include protective roles against oxidants, mutagens, carcinogens as well as inhibition of inflammation, cancer proliferation and progression (Birt*et al.,* 1986). Anti-inflammatory property of apigenin was demonstrated in LPS-activated RAW 264.7 mouse microphages, in which activities and expressions of inducible nitric oxide synthase-2 and cyclooxygenase-2 were suppressed. (Liang*et al.,* 1999). Apigenin K also showed anti-

inflammatory effect in DSS-induced rat colitis by reducing myeloperoxidase activity disease activity index and interleukin 1-beta (IL-1β) expression (Mascaraque*et al.,* 2015).

Another study showed that apigenin and other flavonoids present in the diet of Mediterranean postmenopausal women, help in cancer-prevention and treatment (Potentas*et al.,* 2015).

Apigenin was equally identified as the active component in the bark of *Cordia dichotoma* for traditional treatment of ulcerative colitis (UC) and colic pain (Ganjare*et al.,* 2011).

Apigenin is believed to offer protection against obesity by inhibiting NAD^+ase CD38 thus making NAD^+ available to activate sirtuins, (NAD+ dependent protein deactylases) (Escande*et al.,* 2013).

Apigenin and 2,4,5-Trimethoxycinnamic acid were found to cause reduction of immobility in mice in a behavioural despair test. In the study, swim stress-induced decline in the dopamine (DA) turnover in the amygdala and increase in the DA turnover in the hypothalamus of mice was reportedly reduced after intraperitoneal administered of two doses of apigenin (12.5 and 25 mg/kg body weight) (Nakazawa*et al.,* 2003).

Again, apigenin administered to male Swiss albino mice at 15 mg/kg body weight, significantly reduced mortality induced by gamma irradiation by protecting circulating leucocytes and the intestinal epithelium. It also enhanced antioxidant status by increasing activities of SOD, catalase, GPx and GSH whereas it suppressed lipid peroxidation. Equally, Red and white blood cell counts and haemoglobin concentration were increased. It was inferred that these effects led to increased blood cell production, reduced haemorhagic cell loss or leakage through capillary walls enhanced immune response against irradiation induced damage in the animals (Begum*et al.,* 2012).

It was demonstrated that apigenin had inhibitory effects on benzo[a]pyrene and 2-aminoanthracene mutagenesis in *Salmonella typhimurium* and ornithine decarboxylase activity in mouse epidermis (Birt*et al.,* 1986).

Apoptotic effect of apigenin was proven to be cell selective and dose-dependent in human cervical cell lines through oxidative stress induction via increased H_2O_2 generation, induction of reductive-oxidative impairment of mitochondria in addition to inhibition of cancer cell migration and invasion (Souza*et al.,* 2017). In another study, apigenin and some of its analogs was found to cause G2/M cell cycle arrest in human colon cancer cell lines (Wang*et al.,* 2004). Way and co-researchers demonstrated that apigenin induced apoptosis in HER2/neu-overexpressing breast

cancer cells, through mechanism involving release of cytochrome c (cyt. c) and activation of caspase 3 (Way*et al.,* 2005).

2.5.5 Interaction of Apigenin with Hormones

Apigenin has been shown to inhibit two enzymes (aromatase and 17β-hydroxyl steroid dehydrogenase) involved at different stages of testosterone synthesis. Inhibition of the latter was found to be peculiar, not only to apigenin but also three other tested flavonoids: chrysin, genistein and naringenin (Le Bail*et al.,* 2001). Equally, apigenin directly blocks signalling pathways involving thromboxane A2 (TBXA2) receptor and indirectly increases steroidogenic acute regulatory activity and cAMP-induced testosterone synthesis. It was observed in testicular leydig cells that apigenin inhibited TBXA2-COX_2-induced expression of DAX-1, a protein which normally represses a rate-determining step in the synthesis of steroidogenic acute regulatory (StAR) protein at the transcriptional level (Montrose*et al.,* 2010). Apigenin also inhibited proliferation in DU-125 and MDA-MB-231 breast cancer cells and activated the two subunits of yeast estrogen receptor (ERα and ERβ) at different concentrations. ERβ was activated at100nM while ERβ was activated at a greater concentration of 1μM (Mak*et al.,* 2006). Apigenin affects steroid hormones, being demonstrated in isolated human H295R adrenal cells, where 12.5μM of apigenin was able to reduce cortisol to 47.5% of the level of control. Apigenin was found to be significantly effective at concentrations greater or equal to 6μg/mL. **Cortisol** is a glucocorticoid (steroid hormone) produced from cholesterol by the zona fasciculate of adrenal cortex, and released in response to stress and low level of glucose in the blood. It is capable of suppressing the immune system and bone formation, while it important in of fat, protein, and carbohydrates metabolism. (Ohno*et al.,* 2002).

2.5.6 Interaction of polyphenols with gut microbiota and Cytochrome P450s

Gastrointestinal microorganisms (gut microbiota) perform important function in the metabolism, bioavailability and bioactivity of dietary polyphenols which leads to their structural biotransformation into metabolites with altered bioactivity profiles which may make them to become better substrates of CYP450s. The metabolic fate of polyphenols is largely dictated by their chemical structures and depends on several parameters such as their functional groups (i.e.,

benzene or flavone derivatives), molecular weight, stereo-structure, glycosylation, polymerization, and conjugation with other phenolics (Basheer and Kerem, 2015).

Studies using perfused rat liver microsomes showed that apigenin undergoes phase I hydroxylation reaction with formation of luteolin (3' hydroxylation), scutellarein (C6-hydroxylation) and isoscutellarein (C8 hydroxylation). However, luteolin was found to be a major product while the other two metabolites were minor products. Using different inducers and inhibitors of CYPs in liver microsomes of male and female SPF Wistar rats, Gradolatto *et al* showed that apigenin is metabolized by several CYP450s isoforms such as CYPs 2B, 2C, 2E1 and 3A but they concluded that there was only a slight involvement of CYP1A contrasting the previous report of Breinholt *et al.,* 2002. Apigenin and its metabolite – luteolin also undergo phase II conjugation by glucuronidation and sulfation but not by methylation (Gradolatto*et al.,* 2005).

Polyphenols have stimulatory or inhibitory effects on xenobiotic drug metabolizing enzymes and these have been shown to alter concentrations and pharmacokinetics of drugs. The inhibition of CYP450 can result in the accumulation of parent drug concentrations (i.e. CYP450 substrates) which can put a patient at increased risk for side effects and possible toxicity. However, inhibition of P450 enzymes by polyphenols may have a chemo-preventive effect in cases where metabolites are more toxic than the parent drugs. Chemo-preventive mechanisms of naturally occurring polyphenols may either be through repression of phase I or induction of phase II drug metabolizing enzymes which are responsible for the detoxification of carcinogens. Uridyl diphosphoglucuronosyl transferase (UDP-GT) and glutathione S-transferase are examples of such enzymes (Basheer and Kerem, 2015).

Kimura and colleagues from an *in vitro* experiment, using sixty polyphenols including apigenin, its dimer - amentoflavone and imperatorin showed that these flavones displayed mixed inhibition against CYP3A4 and CYP2C9. It was also stated that the inhibitory effects of polyphenolic inhibitory role on activity of human isoforms of these two enzymes is usually by a covalent interaction between the CYP3A4 molecule and the polyphenol, in which the enzyme becomes inactive. On the other hand, the polyphenol may reversibly bind with the enzyme, in this case, reversible inhibition occurs (Dresser*et al.,* 2000; Kimura*et al.,* 2010).

Lipophilicity, stereo-structure, molecular weight, number of hydroxyl groups at specific positions like C-5,C-7 and C-4' as well as double bonded oxygen in the oxane ring of apigenin

and some other flavones), are notable features responsible for their inhibitory effect on CYP3A4 and these are beneficial by enhancing oral bioavailability of certain drugs e.g. midazolam and paclitasel (Kimura*et al.,* 2010).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Chemicals

Apigenin (Santacruz Biotechnology, USA); dextran sulphate sodium (TDB consultancy, Sweden); L-Glutathione (reduced), 5,5'-Dithiobis(2-nitrobenzoic acid) - DTNB, 1 chloro-2,4-dinitrobenzene(CDNB), Bovine serum albumin, epinephrine (adrenaline), xylenol orange (Sigma Aldrich Co., 3050 Spruce Street, St. Louis, U.S.A.); antibodies for cyclooxygenase-2, inducible nitric oxide synthase (iNOS), cytochrome P450 isoform (CYP3A4), and p-glycoprotein were bought from Elabscience, Texas, U.S.A.; Thiobarbituric acid (TBA) was purchased from BDH Laboratory Supplies, England; sodium trioxocarbonateIV (Na_2CO_3) anhydrous, Trichloro-acetic acid(TCA) and sulphanilamide were purchased from JHD – Guangdong Guanghua Sci-Tech Co. Ltd., Shantou, Guangdong, China; sodium hydrogen trixocarbonate IV ($NaHCO_3$), N-1-naphthyl ethlenediaminedihydrochloride, phosphoric acid, D-sorbitol, o-dianisidine hydrochloride, and diammonium iron(II) sulphate were procured from LOBA Chemie Pvt Ltd., India; dimethyl sulphoxide (DMSO) was purchased from Guangshou Jinhuada Chemical Reagent Company Ltd., China; sulphosalicyclic acid, sodium dihydrogen tetraoxophosphate (v) NaH_2PO_4, disodium hydrogen tetraoxophosphate (v) - Na_2HPO_4, potassium dihydrogen tetraoxophosphate (v) – KH_2PO_4, dipotassium hydrogen tetraoxophosphate (v) K_2HPO_4, potassium iodide, sodium hydroxide (NaOH), distilled water, tetraoxosulphate(vi) acid (H_2SO_4), hydrochloric acid, copper(II)tetraoxosuphate(VI).pentahydrate ($CuSO_4.5H_2O$), potassium-sodium tartrate, potassium iodide (KI), hydrogen peroxide (H_2O_2), sodium azide (NaN_3), tetraoxosulphate(VI) acid (H_2SO_4), formalin, ethanol, paraffin wax, xylene and other reagents used were of analytical grade.

3.2 Apparatus/Laboratory Equipment

Syringe, oral feeding needle (oral cannula), cotton wool, nose masks, hand gloves, micropipettes, micropipette tips, dissecting sets, cuvettes, timing device, foil paper, bench and cold centrifuges, digital weighing balance, eppendorff tubes, measuring cylinder, homogenizer, refrigerator/freezer andUV-Visible spectrophotometer.

3.3 ANIMALS USED

A total of sixty-four (64) male and female *Balb/c* mice weighing 22-26 g and 16-23 g, respectively were employed in this experiment. They were equally separated into four male and four female groups of eight mice each and kept in plastic cages, with adequate ventilation, in the animal house of the Department of Biochemistry, University of Ibadan, Ibadan, following animal ethical protocols for experimental animals The mice were fed with starter mash from Ladokun Feeds and had free access to drinking water. They were left to acclimatise for one week under standard laboratory conditions for animal care, with 12-hour light-dark cycle before commencement of drug administration.

3.4 EXPERIMENTAL DESIGN

Apigenin was dissolved in 1.5% DMSO (dissolved in normal saline) while dextran sulphate sodium (DSS) was dissolved in drinking water of the mice. All animals daily received 1.5% DMSO orally (diluted DMSO) and in addition received either 10mg/kg body weight apigenin (dissolved in DMSO) or 5% DSS or both while Control animals received only the diluted DMSO (as shown in Table 3.1).

The mice were thereafter sacrificed after overnight fasting (but with access to drinking water) and their peritoneum was opened up and their colon and liver were removed, rinsed in phosphate buffered saline (pH 7.4) and weighed. Portions of liver lobes were cut whereas 1cm length ofsigmoidal colon was cut and fixed in 10%neutral buffered formalin (pH 7.2) for histological assessment. The remaining portions were homogenized in potassium phosphate buffer (pH 7.4), centrifuged at 10,000 g for 15 minutes, using TG-16 cold centrifuge, then kept frozen until used.

TABLE 3.1: ANIMAL GROUPING AND TREATMENT PROTOCOL

Group Number/Name	Substance Administered/Dosage	Duration (Days)
1 – Control	1.5% DMSO (dissolved in normal saline (Diluted DMSO)	10
2 – API	10mg/kg body weight apigenin (dissolved in diluted DMSO)	10
3 – DSS	1.5% DMSO (dissolved in normal saline (Diluted DMSO)	10
	5% dextran sulphate sodium dissolved in drinking and replaced every two days.	7 (4^{th} to 10^{th} day)
4 - API+DSS	10mg/kg body weight apigenin (dissolved in diluted DMSO)	10
	5% dextran sulphate sodium (DSS) dissolved in drinking and replaced every two days.	7 (4^{th} to 10^{th} day)

3.5 DETERMINATION OF BIOCHEMICAL PARAMETERS

3.5.1 Total Protein Determination (Gornall*et al.,* 1949)

Protein concentrations of colon and liver post-mitochondrial fractions were determined using modified method of Biuret's test (Gornall *et al*., 1949).

Principle

Addition of potassium iodide to Biuret reagent which is composed of copper(II) tetraoxosulphate(VI) ($CUSO_4$) salt and potassium-sodium tartrate dissolved in sodium hydrxide, inhibits formation copper(I)oxide (Cu_2O), which is a precipitated form of copper(II) ions (Cu^{2+}). At alkaline pH, copper(II)ions, CU^{2+} present in the Biuret reagent form purple/violet coloured coordination complexes with peptides. The complex so formed maximally absorbs at 540nm and the colour intensity is shown to be equivalent to the amount of amino acids in the sample being tested.

Procedure for BSA Standard Curve for Total ProteinDetermination

20mg/ml stock solution of Bovine serum albumin (BSA) was prepared from which several dilutions were made. 0.2 ml each the serially diluted BSA standard solution was added to 1.8ml of Biuret in test tubes and gently mixed. The mixture was left standing on laboratory bench at room temperature for 20 min after which absorbance was spectrophotometrically at 550 nm against a blank of 0.2 ml of distilled water in place of BSA standard. Readings were taken within 10 minutes after incubation. The procedure was carried out in duplicate and a curve of mean absorbance against protein concentration was plotted.

Procedure for Total Protein Determination in Samples

0.2 ml volume of each test sample was added to 1.8ml of Biuret reagent and mixed. The procedure for protein standard was followed and the total protein present in each sample was obtained by from the BSA standard graph earlier plotted, taking care to multiply the result by dilution factor.

3.5.2 Determination of Catalase Activity (Aebi, 1984)

Principle

This protocol is used to assess the amount of H_2O_2 split by catalase present in tested samples at 240nm. Although H_2O_2 does nothave absorbance peak at this wavelength, the reaction follows Beer-Lambert's law from which extinction coefficient of H_2O_2 can be determined, using the equation $A=\varepsilon cl$. where A, ε, c and l refer to absorbance, extinction coefficient, concentration and path length, respectively. As H_2O_2 is being split by catalase, the a

bsorbance decreases and when catalase in the sample is used up, absorbance begins to rise. Decrease in absorbance per second is therefore used to determine catalase activity (Noble and Gibson, 1970)

Procedure

2.95 ml of the 19 mM hydrogen peroxide solution was measured into a 1 cm quartz cuvette using graded pipettes while sample addition followed immediately. The cuvette was covered and rapidly but gently mixed. Change in absorbance was recorded every thirty seconds for at least two minutes, depending on the organ one is working with.

Catalase activity was calculated using the formula

$$\text{Catalase activity} = \frac{(\text{Change in absorbance/min}) \times \text{volume of assay mixture} \times \text{dilution factor}}{\text{extinction coefficient of H2O2} \times \text{volume of sample}}$$

$= (\Delta abs/min \times \text{volume of assay mixture}) \times (\text{dilution factor})/(\varepsilon \times \text{volume of sample})$

3.5.3 Assessment of Lipid Peroxidation (Heath and Packer, 1968)

Peroxidation of membrane lipids was assessed through measurement of peroxidation product (malondialdehyde) present in the samples.

Principle

Extent of lipid peroxidation is quantified by measuring absorbance of pink-coloured complex formed between malondialdehyde and 2-thiobarbituric acid at acidic pH. The complex absorbs maximally at 532nm. Change in absorbance at 532nm and 600nm can be calculated to determine extent of lipid peroxidation.

Procedure

0.5% TBA was diluted in 20% TCA (1:1). An aliquot of 0.5 ml each of post-mitochondrial fractions of colon and liver and 1.5 ml of this mixture (TBA+TCA) were added to glass test tubes incubated in water bath at 95^{o}C for 25 minutes to develop the coloured complex. The tubes

were cooled on ice to stop the reaction and centrifuged at 3,000 rpm for 10 min. The clear supernatant was collected and absorbance measured at 532 nm and 600nm. Absorbance values at 600nm were deducted from the values at 532nm and MDA content for each sample was calculated following Beer- Lambert law and using an extinction coefficient (ε) =155mM^{-1}cm^{-1}. Results were expressed as nanomols MDA produced/mg protein

3.5.4 Estimation of Reduced Glutathione Level (Beutler*et al.*, 1963)

Principle

When 5,5′–dithiobis-2-nitrobenzoic acid (DTNB), also called Ellman's reagent is added to sulphur-containing compounds which includes L- reduced glutathione, a fairly stable yellowish product is produced which absorbs maximally at 412 nm wavelength.

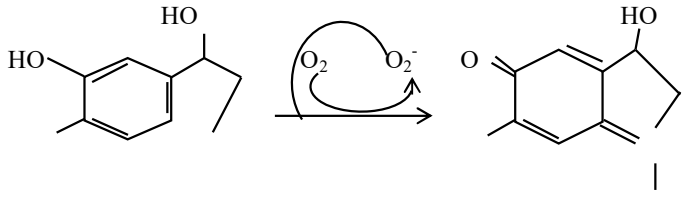
Procedure for determination of GSH in samples

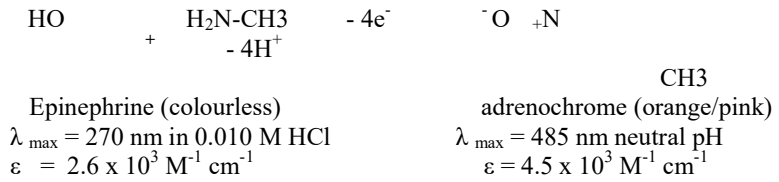
Aliquots of each sample was deprotonated by mixing of equal amount of both sample and sulphosalicylic acid in sample bottles. The precipitate was vortexed and centrifuged at 4000g for five minutes and of the supernatant was picked and 0.5ml of it was mixed with 1.5ml of DTNB while absorbance was read at 412 nm. Stock solution of reduced glutathione was serially diluted in 0.1M phosphate buffer and mixed with DTNB (as for sample) and was used to generate values for standard graph from which glutathione concentrations were extrapolated.

3.5.5 Determination of Superoxide Dismutase Activity (Misra and Fridovich, 1972)

Principle

This protocol assesses inhibition of autoxidation of epinephrine/adrenaline at alkaline condition (pH 10.2) to adrenochrome, by superoxide dismutase present in tested samples. Adrenochrome (oxidized form of adrenaline) is formed directly by exposure to air (oxygen) and indirectly when superoxide is converted into oxygen. Presence of superoxide anions (O^{2-}) increases rate of adrenochrome formation, which is indicated by orange/pink compound which absorbs at 480nm. Superoxide dismutase slows down rate of disappearance of adrenaline/adrenochrome formation. Oxidation of epinephrine is shown below:





Epinephrine (colourless) — adrenochrome (orange/pink)

λ_{max} = 270 nm in 0.010 M HCl — λ_{max} = 485 nm neutral pH

$\varepsilon = 2.6 \times 10^3\ M^{-1}\ cm^{-1}$ — $\varepsilon = 4.5 \times 10^3\ M^{-1}\ cm^{-1}$

Procedure for determining SOD activity in sample

100µl of sample was added to a cuvette containing 1.25 ml of 0.05M carbonate buffer (pH 10.2) and 150µl of epinephrine and gently mixed by inverting the cuvette. Change in absorbance was recorded at thirty seconds interval for three minutes at 480 nm wavelength. Distilled water was used instead of sample to obtain blank reading.

Percentage inhibition by SOD and unit of SOD activity were obtained from absorbance readings using the following equations:

Percentage inhibition (%I) $= \frac{Vo - Vsod}{Vo} \times 100\%$,

where Vo = change in absorbance/min for blank and Vsod

= change in absorbance/min for sample

SOD activity $= \frac{\text{(Change in absorbance/min) x volume of assay mixture x dilution factor}}{\text{extinction coefficient of adrenochrome x volume of sample}}$

= (Δabs/min x volume of assay mixture x dilution factor)/(ε x volume of sample),

where unit of SOD activity is defined as the quantity of SOD needed to inhibit oxidation of the epinephrine (SOD_{50}) by half.

Specific SOD activity = SOD activity/mg protein, where $\varepsilon = 4.5 \times 10^3\ M^{-1} cm^{-1}$

3.5.6 Determination of Hydrogen Peroxide Concentration (Wolff, 1994)

Principle

According to this methodology, a bluish-violet complex is produced when iron(III) reacts with xylenol orange. This product absorbs maxuimally at 560nm wavelength. Iron (II) present in ferrous ammonium sulphate is oxidized into iron(III) by H_2O_2 in the sample. Iron(III) yield is increased by formation of hydroxyl radical (OH^-) from sorbitol, which also amplifies formation of the coloured complex.

Procedure for Hydrogen peroxide standard

Stock hydrogen peroxide (30%) was diluted in distilled water, following which 1.9 ml of FOX1 reagent was added to 100 µl of the serially diluted stock H_2O_2. The mixture was vortexed

andincubated at room temperature for 30 minutes afterwhich the absorbance recorded at 560 nm wavelength against reagent blank.

Procedure for samples

Same steps for standard were followed for samples, with 100 µl of sample replacing the standard, but in addition, the whole mixtures were centrifuged at 3,000g for 5 minutes prior to incubation at room temperature. From the standard curve, concentration of H_2O_2 in each sample was determined.

3.5.7 Estimation of Glutathione S-Transferase Activity (Habig*et al.,* 1974)

Principle

The principle of this protocol centres on the fact that glutathione S-transferase reacts actively in the presence of two substrates: reduced L-glutathione and 1-chloro-2,4-dinitrobenzene (CDNB). These substrates form conjugation complex which absorbs maximally at 340nm. Increase in absorbance at this wavelength is directly related to activity of the enzyme.

Procedure

20mM CDNB (100 µl) was put in a glass cuvette to which 20 µl of 0.1M reduced glutathione, 1.86 ml of 0.1 M phosphate buffer (pH 6.5), and 20 µl of distilled water were added. Sample was used in place of distilled water. Change in absorbance was recorded at thirty seconds interval for three minutes for samples and blank at 340 nm.

GST activity was calculated as indicated in the equation below:

$$\text{GST activity} = \frac{\text{(Change in absorbance/min) x volume of assay mixture x dilution factor}}{\text{extinction coefficient of adrenochrome x volume of sample}}$$

$$= (\Delta A340nm/min \times \text{volume of assay mixture}) \times (\text{dilution factor})/(\varepsilon \times \text{volume of sample}),$$

where ε = extinction coefficient = $9.6mM^{-1}cm^{-1}$

SpecificGST activity = GST activity/mg protein

3.5.8 Assay for Glutathione Peroxidase (GPx) Activity: (Rotruck*et al.,* 1973)

Measurement of the activity of glutathione peroxidase (GPx) was based on the modified method of Rotruck and colleagues (1973).

Principle

Formation ofH_2O_2-L-glutathione conjugation complex is catalyzed by the enzyme Glutathione peroxidase. The reaction is allowed to run for a fixed period of time (usually three minutes) before it is terminated. Some of the glutathione present in the whole reaction vial is consumed in the process. The amount of glutathione remaining is determined by reactingthe assay mixture with Ellman's reagent and the absorbance is read at 412nm wavelength. GPx activity is expressed in proportion to the amount of GSH consumed and the GSH consumed is then used as a measure of enzyme activity.

Procedure

0.1 ml of NaN3, 0.2 ml of GSH, 0.1 ml of H_2O_2 and 0.5 ml of sample were added in that order to 0.5 ml of phosphate buffer in a test tube. The whole mixture was incubated in water bath at 37˚C for three minutes and 0.5 ml of TCA was thereafter added. The mixture centrifuged for five minutes at 3,000g. The supernatant was removed. 2 ml of K_2HPO_4 and 1 ml of DTNB were added to 1 ml of supernatant and the absorbance was recorded at 412nm against a reagent blank (in which distilled water replaced the sample).

GPx activity was determined using the equations below:

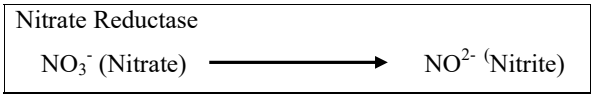
GSH consumed = initial GSH amount (129.39 µg) – GSH remaining (µg/ml × 4 ml)

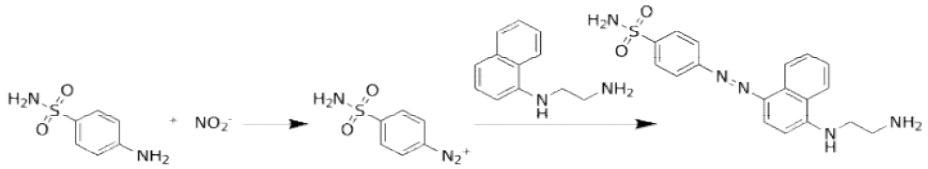
Specific activity of GPx = GSH consumed/mg protein = µg GSH/mg protein

3.5.9 Determination of Nitrite Ion Concentration (NO_2^-) - (Tsikas, 2007)

Principle of the Assay

Nitric oxide is unstable, so it is indirectly measured through its metabolites: nitrites and nitrates. Nitrate is in turn enzymatically converted into nitrite (an azo dye) by nitrate reductase. Nitrite is determined using Griess's test, which is a two-step reaction in which nitrite ion reacts with sulfanilamide to form a diazonium ion. The latter reacts with N-(1-naphthyl) ethylenediamine to form a chromophoric azo-derivative which absorbs light between 540-570 nm wavelengths.





Step 1 Sulfanilamide + Nitrite ⟶ diazonium ion

Step 2: Diazonium ion + N-(1-naphthyl)ethylenediamine ⟶ purple azo compound

Procedure

Griess reagent and serially diluted standard as well as sample were mixed in separate test tubes, incubated in the dark for twenty minutes at room temperature and the absorbance reading was taken at 546nm wavelength. Nitrite concentrations were extrapolated from sodium nitrite standard curve.

3.5.10 Estimation of Myeloperoxidase (MPO) Activity (Bradley*et al.*, 1982)

Principle

This methodology works on the fact that myeloperoxidase catalyzes formation of hypochlorous acid from hydrogen peroxide and chloride anion or non-chlorine halide equivalent).

Procedure:

1.25ml of reagent (2) and 250µl of reagent (3) above were added to 35µl of sample in a cuvette and mixed gently. Change in absorbance was recorded at thirty seconds interval for up to five minutes at 460nm depending on the organ used. Myeloperoxidase activity was calculated as indicated by the equation below:

Calculation

$$\text{Myeloperoxidase activity} = \frac{(\textbf{Change}\text{ in absorbance/min}) \times \text{volume of assay mixture} \times \textbf{dilution}\text{ factor}}{\text{extinction coefficient of adrenochrome} \times \text{volume of }\textbf{sample}}$$

= (ΔA340nm/min x (volume of assay mixture) x (dilution factor)/(ε x volume of sample)

where ε = extinction coefficient = 11.3 mM^{-1} cm^{-1}

Unit of myeloperoxidase activity is defined as the amount of enzyme which causes an increase in absorbance of 0.001 per minute.

Specific activity of myeloperoxidase = Myeloperoxidase activity/mg protein or mmol H2O2 split/mg protein

3.5.11 Haematoxylin and Eosin Staining Technique (Clifton, 2011)

Principle

The oxidation product of haematoxylin is haematin which is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The *in situ* oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate.

Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions, and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the overstaining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution (acid alcohol). This process is called "differentiation" and it is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei. Full cellular detail is obtained by counterstaining with the eosin mixture.

3.5.11.1 Protocol for Histology (Sloaui and Fiette, 2011)

The tissues were fixed in 10% formalin. They were processed (i.e dehydrate in ascending grades of alcohol, clear in xylene, and impregnate in molten paraffin wax) in Automatic tissue processor. The tissues were then embedded in paraffin wax using Embedding system (Leica EG 1160). They were thereafter sectioned with microtome at 4microns. The sections were then floated on water using water bath at 45 degree Celsius and then picked on frosted end slide. The slides were fixed on hot plate for about thirty minutes. The sections were then stained with Haematoxylin and Eosin.

3.5.11.2 Protocol for Haematoxylin and Eosin Staining:

i. Slides containing paraffin sections were placed in a slide holder.

ii. The sections were deparaffinized and rehydrated by placing in xylene, thrice (3 minutes each); 100% ethanol, thrice (3 minutes each); then once in 95% ethanol, 80% ethanol and deionized water for 3 minutes, 3 minutes and 5 minutes, respectively. Excess water was wiped from slide holder, oxidized particles was also wiped from hematoxylin before staining.

iii. Haematoxylin staining: The slide was dipped once in Erlich's hematoxylin for 5 minutes and rinsed in deionized water once, for 5 minutes to allow stain to develop. They were rinsed in

running tap water, differentiated with 0.5% acid alcohol for 1-2seconds, rinsed again in running tap water, then rinsed in Scott's tap water substitute. They were further rinsed in tap water and stained with eosin for 2 minutes and dehydrated, cleared and mounted in DPX.

3.5.12 Immunohistochemical Staining Technique (Ramos-Vara, 2017)

Principle

Immunohistochemistry (IHC), or immunohistochemical staining, is a technique which employs antibodies to detect antigens in cells within a tissue section. This application is used to locate specific antigens in tissue sections with labeled antibodies based on antigen-antibody interactions. The immune reactive products can be visualized by a marker including fluorescent dyes, enzymes, radioactive elements or colloidal gold.

Immunohistochemical protocol for formalin-fixed, paraffin-embedded tissue sections

i. Slides were deparaffinized twice in xylene for 2 times, 5 min each. Slides were transferred to 100% alcohol, twice, 3 minutes each, then transferred through 95% twice, 70% once alcohols respectively, for 3 minutes each. They were rinsed with Wash Buffer twice, 5 minutes each. Antigen retrieval was performed to unmask the antigenic epitope.

N.B. The most commonly used antigen retrieval is a citrate and EDTA buffer method. To Preheat, Pour 300 ml of 10 mM citrate pH 6.0 /EDTA pH 9.0 buffer, into the staining container and incubate it at 95-100°C for 5 min. Arrange the slides in a staining hanger and dip in the preheated buffer, incubate for 10-20min in the water bath. (Optimal incubation time should be determined by user). Remove the staining container to room temperature and allow the slides to cool in the retrieval buffer for 20 min.

ii. After retrieval, slides were rinsed with Wash buffer twice, 5 minutes each. Blocking buffer (e.g. 10% fetal bovine serum in PBS or 3% H_2O_2) was added onto the sections of the slideswith incubation in a humidified chamber at room temperature for 15minutes. The blocking buffer was drained from the slides and they were washed in wash buffer. 130 µl of appropriately diluted primary antibody was applied to the sections on the slides and they were incubated in a humidified chamber at room temperature for 1 hour. The slides were washed with Wash buffer twice, 5 minutes each.

iii. 130 µl of suitably diluted biotinylated secondary antibody (using the antibody dilution buffer) was applied to the sections on the slides and incubated in a humidified chamber at room temperature for 15 min. The slides were washed with wash buffer twice, 5 minutes each.

iv. 130 µl of appropriately diluted HRP conjugates (using the antibody dilution buffer) was applied to the sections on the slides and incubated in a humidified chamber (protected from light) at room temperature for 15 minutes. The slides were washed with wash buffer twice, 5 minutes each.

v. 130 µl of DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% H_2O_2 in PBS) was applied to the sections on the slides to reveal the color of antibody staining. The color was allowed to develop for less than 5 minutes until the desired color intensity was reached. Slides were washed with phosphate buffered saline thrice, 2 minutes each.

vi. The slides were counterstained by immersing in Hematoxylin for 10-20seconds, rinsed in running tap water for 10 minutes. The tissue slides were dehydrated in increasing concentrations of alcohol (95%, 95%, 100% and 100%), 5 minutes each. They were cleared thrice in xylene and covered with coverslips using mounting solution. The mounted slides can be stored at room temperature permanently.

vii. The colour of the antibody staining in the tissue sections were observed under light microscope at 40, 100 and 400 magnifications.

3.5.13 Statistical Analysis

GraphPad Prism 6 Software (USA) was used to analyse results of biochemical assays. All values were expressed in terms of their mean ± standard error (Mean±SEM) using analysis of variance(ANOVA), followed by Tukey, Dunnett or Bonferroni tests. Probability less than 0.05 ($P < 0.05$) were taken to be significant.

CHAPTER FOUR
RESULTS

4.1 Summary of Results

Results obtained from this study are shown in Tables 4.1to 4.4 and Figures 4.1 to 4.9. Histological results are represented in Plates 4.1 to 4.4 while immunohistochemical results are shown in plates 4.5 to 4.20

4.2 Biochemical Results

Physical symptoms of ulcerative colitis such as decrease in body mass, bloody diarrhea and rectal bleeding were observed from the second day of administering 5% DSS to the mice in DSS group and the symptoms became more severe from the third day till the animals were eventually sacrificed. Tables 4.1 to 4.3 represent role of apigenin on change in body mass, colon length and percentage relative liver weights of themice used in this study.

Table 4.1 represents change in body weights from 1st to 3rd day (i.e. prior to DSS administration) and from the 3rd to the 10th day (i.e. after DSS administration).

There was initial reduction in body weight caused by apigenin pretreatment (in API and API+DSS, $p< 0.05$) prior to DSS administration. However, this was reversed by the last day of the study as apigenin caused significant increase in body weight in API (both sexes) and API+DSS (female) groups, ($p<0.05$). Disease activity index which includes scores of stool consistency, rectal bleeding, excessive weight loss and shortened colon length ((Tables 4.1and 4.2) were more pronounced among the male than the female animals in DSS-administered groups. Apigenin caused increased colon lengths in API+DSS groups of both sexes although not to a significant extent. API group was not significantly different from Control.

The female animals had reduced absolute liver weights in relation to the male mice but the results obtained for percentage liver weights relative to their body weights in addition to total protein concentrations did not differ significantly across all the groups.

Table 4.1: Effect of Apigenin on DSS-induced reduction in body weight

Parameters	Change in body weight (1^{st} -3^{rd} Day)		Change in body weight (4^{th} – 10^{th} Day)	
SEX	MALE	FEMALE	MALE	FEMALE
CONTROL	0.97±0.19	-0.004±0.28	0.90±0.43 *	-1.835±0.4 *
API	-1.96±0.42 $^{a, \beta}$	-0.099±0.09	2.21±0.37 $^{\beta, \dagger}$	-0.656±0.32 †
DSS	0.76±0.13 $^{b, \gamma}$	0.324±0.27	-2.83±0.39 $^{a,b, \gamma}$	-1.473±0.35
API+DSS	0.23±0.17 $^{b,\#, \varepsilon}$	-1.830±0.5 $^{c,\#, \mu}$	-3.81±0.55 $^{a,b,\pi, \varepsilon}$	0.348±0.72 $^{d,\gamma,\mu, \pi}$

Values are Mean±SEM (n=8); groups indicated by superscripts a,b,c are significantly different from male Control, API and DSS, respectively; d is significantly different from female DSS; groups with same symbols (*, $^{\beta, \dagger, \gamma, \#, \varepsilon, \mu}$ and $^{\pi}$) are significantly different from each other ($P < 0.05$)

Group names: Control = diluted DMSO alone; API = apigenin only; DSS = DSS only; API+DSS =

apigenin and DSS

Table 4.2: Effect of Apigenin on Colon Length, Colon Weigth:Length Ratio and Disease Activity Index (DAI)

	SEX	CONTROL	API	DSS	API+DSS
(cm)	Male	10.08±0.39	10.49±0.55	7.33±0.33[a, b]	7.75±0.33 [a, b]
	Female	10.13±0.44	10.35±0.35	7.91±0.42[d, e]	8.10±0.45 [d, e]
(g/cm)	Male	0.064±0.007	0.073±0.004	0.071±0.006	0.057±0.004 [#]
	Female	0.064±0.005	0.068±0.003	0.076±0.004	0.078±0.006 [#]
Activity Index	Male	0.00±0.00	0.00±0.00	9.63±0.32 [a, b]	5.63±0.60 [a, b, c, #]
	Female	1.13±0.44	0.25±0.16	8.38±0.60[a, b]	1.13±0.72 [c, #]

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Values are Mean±SEM (n=8); groups indicated by superscripts a, b, d and e are significantly different from male Control and API, female Control and API, respectively; groups symbol [#] are significantly different from each other ($P < 0.05$).

Group names: Control = diluted DMSO alone; API = apigenin only; DSS = DSS only; API+DSS =

apigenin and DSS

Table 4.3: Effect of Apigenin on Absolute and Percentage Relative Liver Weight

PARAMETERS	Absolute Liver Weight (g)		Percentage Relative Liver Weight (%)		Percentage Survival (%) (n=8)	
SEX	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
CONTROL	1.07±0.06	0.86±0.11	4.32±0.09	4.33±0.54	100	100
API	1.07±0.07†	0.77±0.03†	4.37±0.14	4.04±0.16	100	100
DSS	1.05±0.05	0.91±0.08	4.94±0.29	4.59±0.26	50	100
API+DSS	0.98±0.09	0.99±0.04	4.70±0.27	4.65±0.11	75	100

Values are Mean±SEM (n=8); the difference between the values indicated by the symbol † are significant ($P < 0.05$)

Group names: Control = diluted DMSO alone; API = apigenin only; DSS = DSS only; API+DSS = apigenin and DSS

Table 4.4: Total Protein Concentrations of Colon and Liver Post Mitochondrial Fractions

Parameters/Organ	Total Protein Concentration (mg/ml)			
	Colon		Liver	
SEX	MALE	FEMALE	MALE	FEMALE
CONTROL	0.19±0.03	0.13±0.02	29.57±1.75	23.26±1.82
API	0.23±0.03	0.27±0.03	25.74±2.32	23.90±1.71
DSS	0.50±0.08^{a,b}	0.36±0.05^{d}	25.63±2.09	20.40±1.37
API+DSS	0.41±0.06^{a}	0.32±0.02^{d}	27.88±1.72	27.68±2.85

Values are Mean±SEM; the values in the liver were not significantly different from one another Group names: Control = diluted DMSO alone; API = apigenin only; DSS = DSS only; API+DSS = apigenin and DSS

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Figures 4.1 to 4.3 represent role of apigenin on oxidative stress markers and antioxidant enzymes in colon and liver post-mitochondrial fractions.

Figure 4.1a shows that hydrogen peroxide concentration in the colon increased significantly ($P < 0.05$) in male than female mice that took only DSS. Pretreatment with apigenin significantly reduced H_2O_2 concentration in API+DSS male and female mice to levels that were comparable with Control. In the liver of male mice, there was no observed significant difference in H_2O_2 concentration but female DSS had significantly higher H_2O_2 than female Control and male DSS (Figure 4.1b).

Similarly, lipid peroxidation (LPO) in the colon which was significantly elevated by DSS administration was significantly reduced by apigenin pretreatment in the male mice (figure 4.2a) while in the female groups, any significant difference was not observed. When sexes were compared, female DSS had lower LPO than male DSS while an opposite effect was observed in the liver (Figure 4.2b). Female DSS group had significantly elevated LPO than male DSS and female API+DSS.

Figure 4.3 shows that apigenin raised reduced glutathione (GSH) level significantly in the colon of male and female API+DSS groups, above the depleted levels observed in DSS groups ($P <0.05$). Equally, apigenin exerted similar effect in the male mice liver, but the difference observed in the female mice was not up to a significant extent. Reduced glutathione levels were higher in API+DSS male than female in both colon (significant, $P < 0.05$) and liver (not significant)

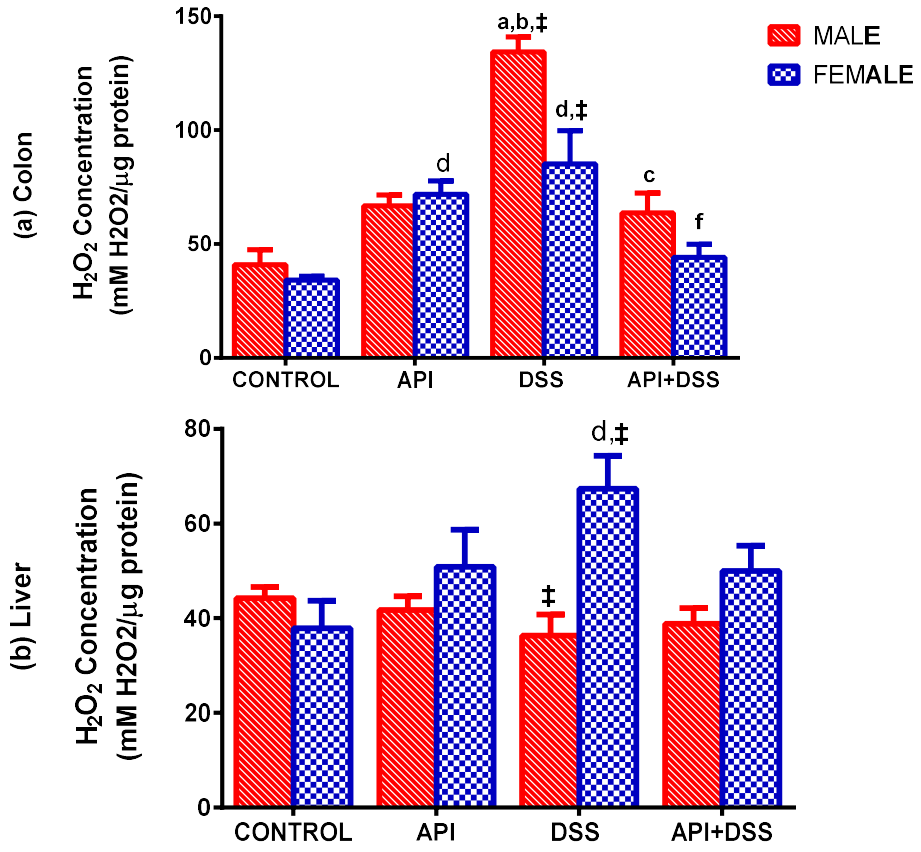

Figure 4.1:Hydrogen Peroxide (H_2O_2) Concentrations in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

a,b,c d and f mean the groups are significantly different from male Control, API, DSS, and female Control and DSS, respectively ($P< 0.05$)

‡ indicates male DSS group was higher than female in the Colon but female DSS was higher in the liver ($P <0.05$)

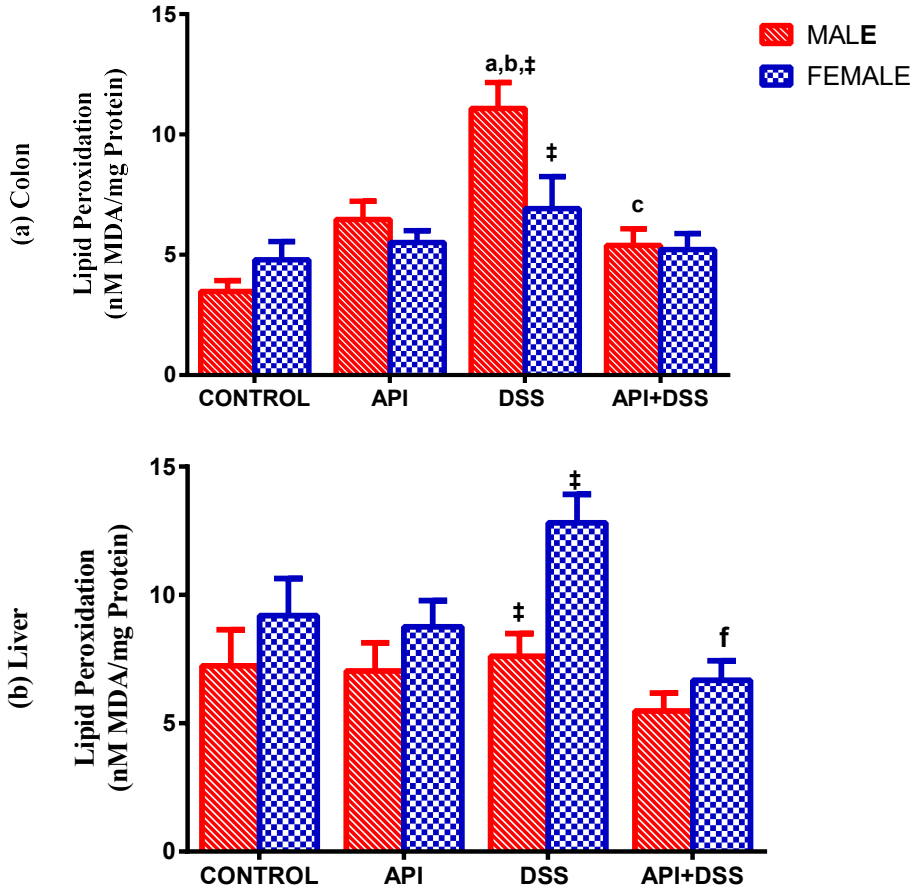

Figure 4.2:Lipid Peroxidation in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

a,b,c and f mean the groups indicated are significantly different from male (Control, API and DSS) and female DSS, respectively ($P < 0.05$)

‡ indicates male DSS group was higher than female in the Colon but female DSS was higher in the liver ($P < 0.05$)

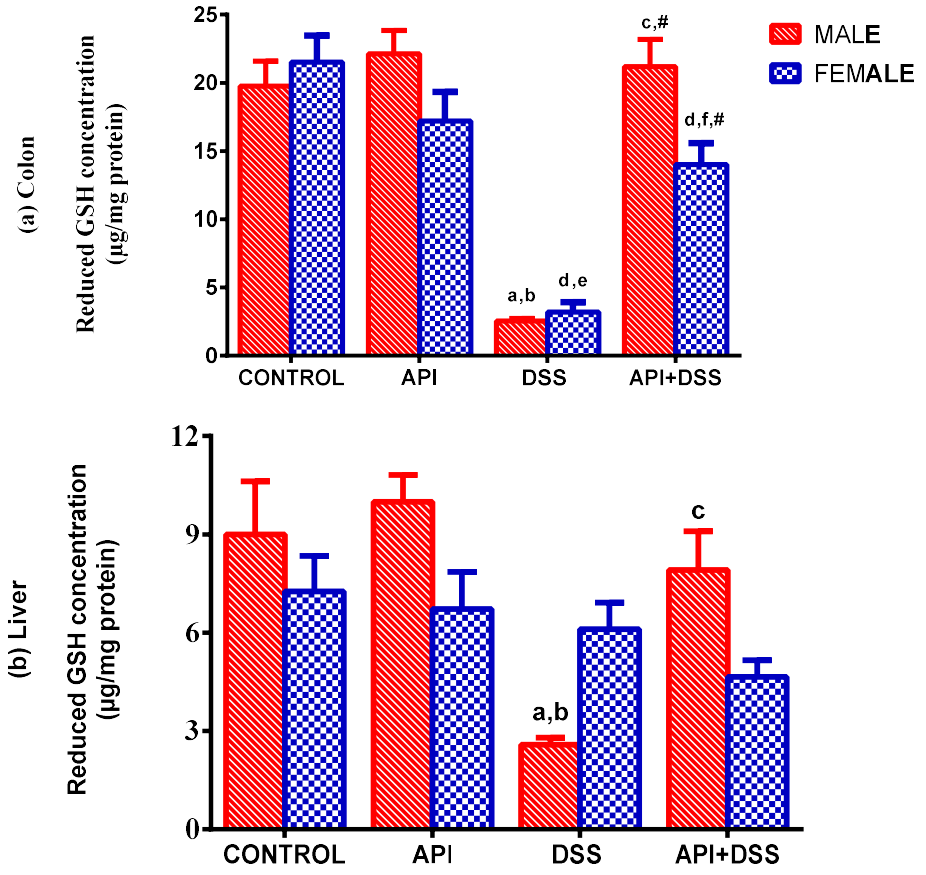

Figure 4.3: Reduced glutathione (GSH) Concentrations in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin With DSS (API+DSS)

a,b,c, and d, e, f indicate the groups were significantly different from male (Control, API, API+DSS) and female (Control, API and API+DSS), respectively ($P < 0.05$).

# indicates male API+DSS group is higher than female in the Colon ($P < 0.05$).

Figures 4.4 to 4.7 show effects of DSS on antioxidant parameters. In figure 4.4, DSS caused significant reduction in SOD activity in the colon whereas pretreatment of API+DSS group with apigenin increased SOD activity above DSS group but comparable with Control and API groups. The effect seen in the liver was not significant (Figure 4.4).

Figure 4.5a shows significant decrease in catalase activity in the colon as a result of DSS administration (DSS and API+DSS groups). Also, the male mice in the two groups had significantly reduced catalase activities when compared with their female counterparts. DSS administration did not significantly alter catalase activities in the liver but female Control had significantly higher catalase activity than male control (Figure 4.5b).

No significant difference was observed in Glutathione peroxidase (GPx) activities across the groups in both colon (Figure 4.6a) but in the liver, female Control had significantly higher GPx activity than male control (Figure 4.6b)

Figure 4.7 shows influence of DSS on GST activity. In the colon, GST activities of DSS and API+DSS groups significantly reduced compared with Control and API ($P < 0.05$) and females were higher than the males. API+DSS female had higher activity than DSS female (although not significant). In the liver, male and female API had significantly higher GST activity than other groups ($P< 0.05$) while female API+DSS was significantly low than female API ($P < 0.05$)

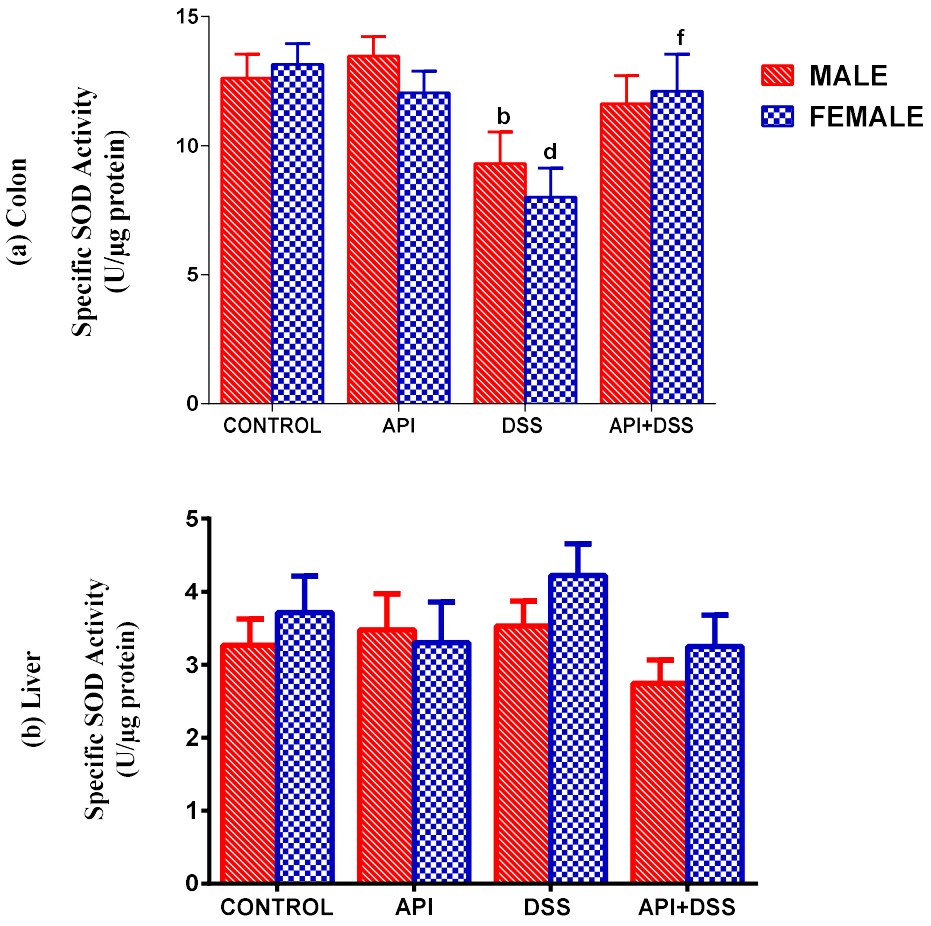

Figure 4.4: Specific SOD Activities in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

Values are expressed as Mean±SEM.

b, d and f indicate the groups are significantly different from Male API, female Control and female DSS,respectively ($P < 0.05$)

No significant difference was observed in the liver

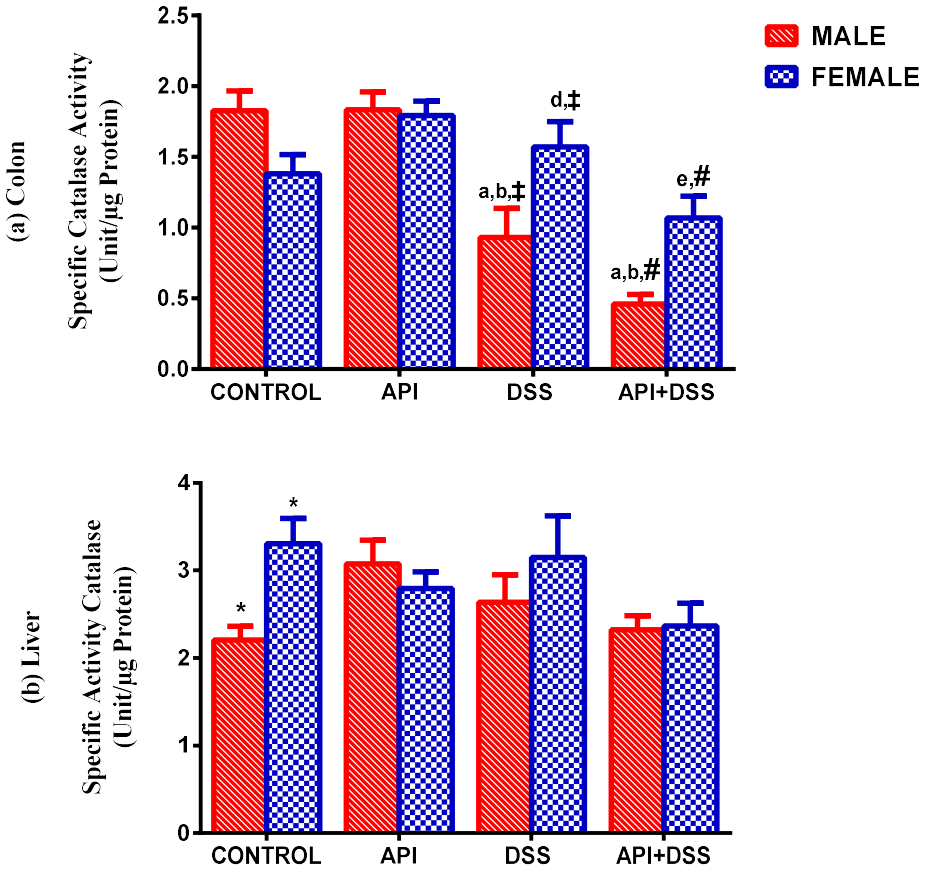

Figure 4.5: Specific Catalase Activities in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

Values are expressed as Mean±SEM.

a, b,d and e indicate the groups were significantly different from male Control and API and female Control and API, respectively ($P < 0.05$)

*, ‡ and # indicate differences between male and female Control, DSS and API+DSS groups, respectfully ($P < 0.05$)

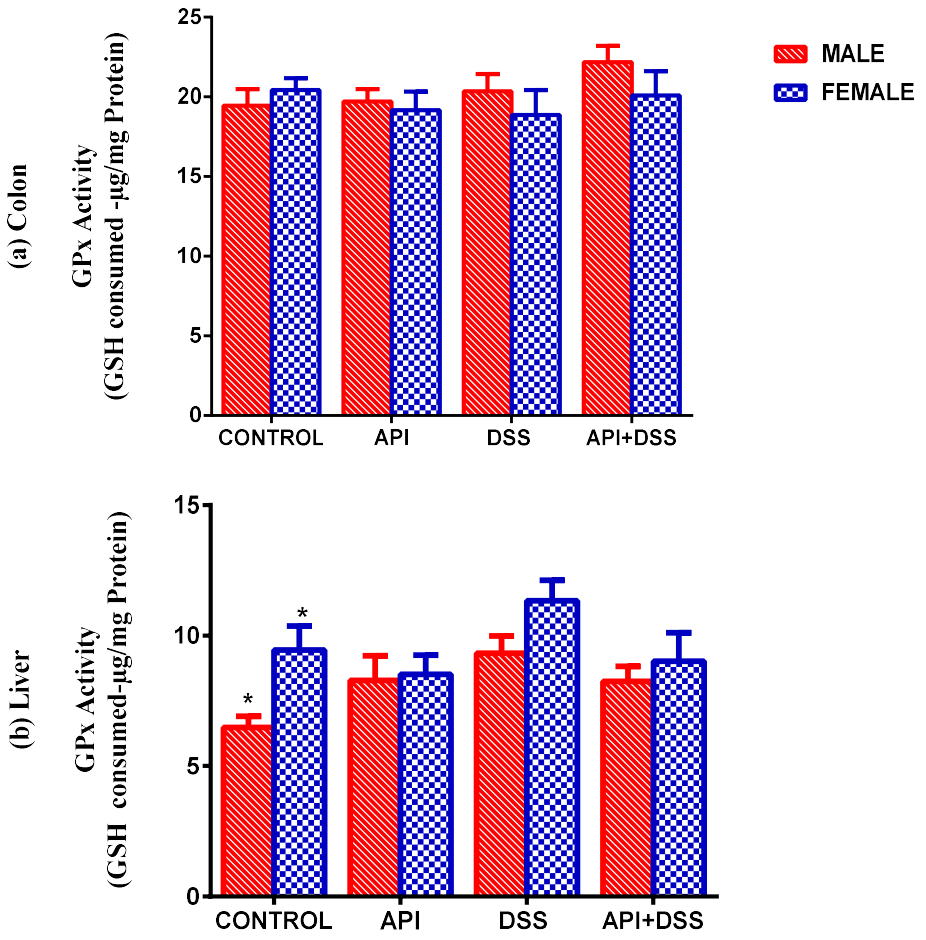

Figure 4.6: Specific Glutathione Peroxidase Activities (GPx) in Colon and Liver Post-Mitochondrial Fractions of *Balb/c* mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

Values are expressed as Mean±SEM.

.* indicates Female Control group was significantly higher than male in the Liver ($P < 0.05$).

No significant difference was observed in other groups.

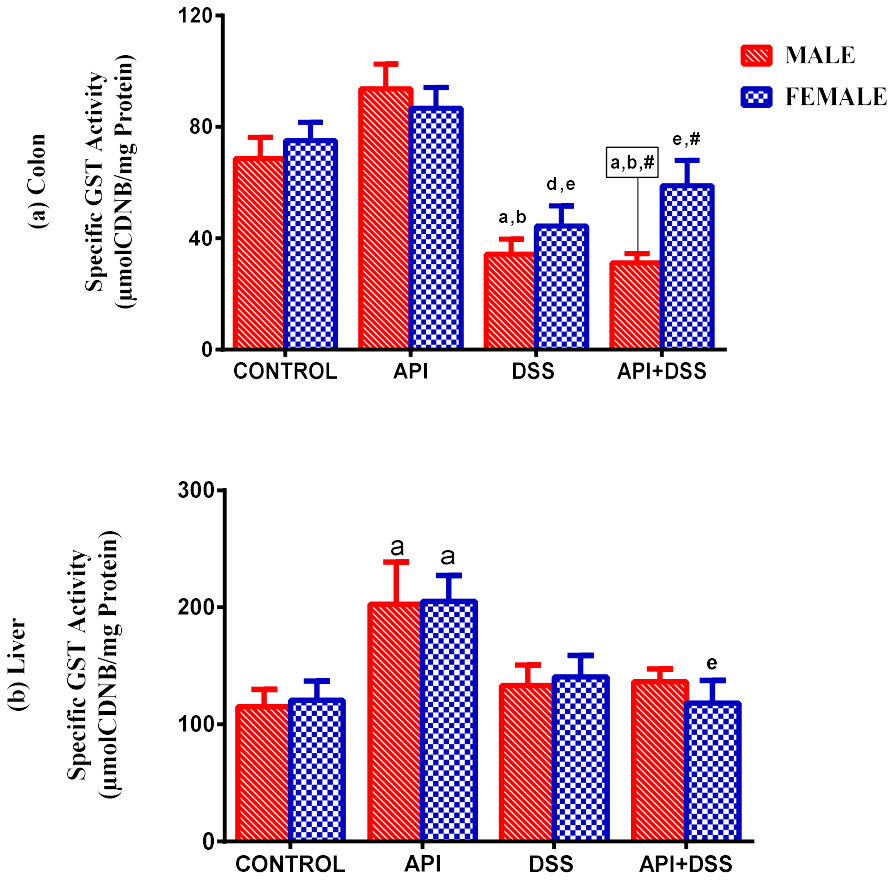

Figure 4.7: Specific Glutathion-S-Transferase Activities (GST) in Colon and Liver Post-Mitochondrial Fractions of *Balb/c* mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

a,b,d and e indicate the groups were significantly different from male Control and API and female Control and API, respectively; # means API+DSS female was significantly higher than male group ($P < 0.05$).

Figures 4.8 and 4.9 show effects of DSS and apigenin on inflammatory markers (myeloperoxidase activity and nitrite concentration).

In the colon the male DSS and API+DSS groups had elevated MPO activities which were significantly different from their female counterparts as well as from Control and API groups ($P < 0.05$). Control female mice had significantly increased MPO activity than Control male, DSS female and AI+DSS female ($P < 0.05$).

In the male liver (figure 4.8b), MPO activity significantly increased in male API+DSS than male DSS ($P < 0.05$) and its female counterpart (API+DSS female)

Male DSS and API+DSS groups had significantly elevated colonic nitrite ion concentrations than their female counterparts as well as male Control and API groups ($P < 0.05$). In the liver API+DSS male was significantly lower than control, other groups were not significantly different.

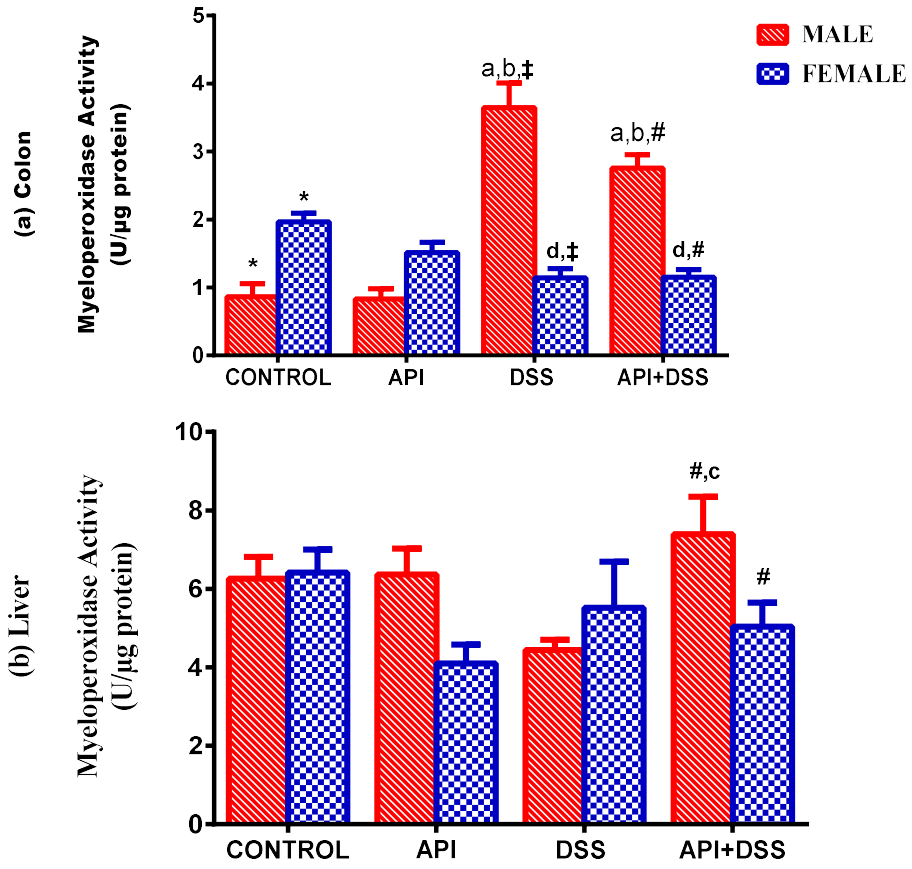

Figure 4.8: Myeloperoxidase (MPO) Activities in Colon and Liver Post-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

a,b,c and d mean the groups indicated are significantly different from male Control and API and DSS and female Control ($P < 0.05$), respectively

‡ and # indicate male DSS and API+DSS groups, respectively were significantly higher than their female counterparts ($P < 0.05$)

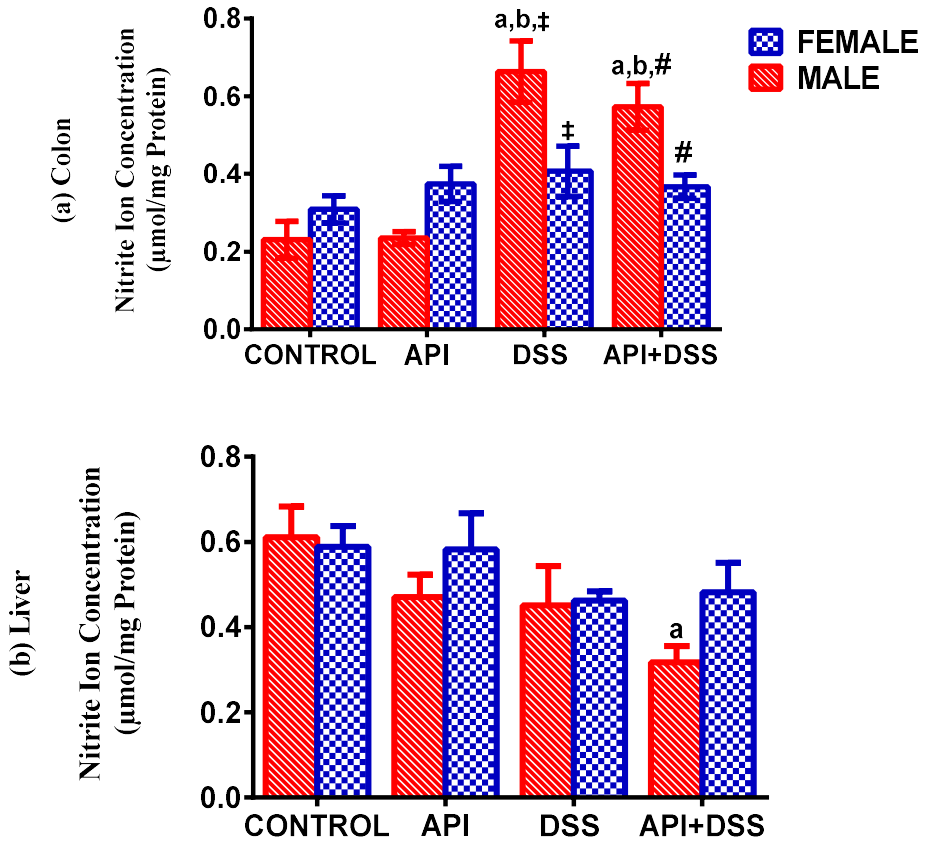

Figure 4.9: Nitrite ion Concentration in Colon and LiverPost-Mitochondrial Fractions of *Balb/c*Mice Administered Diluted DMSO (Control) and Treated with Apigenin (API), DSS only (DSS) and Apigenin with DSS (API+DSS)

a and b denote that the groups indicated were significantly different from male Control and APIin colon and in liver

‡ and # indicate that male DSS and API+DSS groups, respectively were significantly higher than their female counterparts($P < 0.05$)

4.3 Histological Assessment of Colon and Hepatic Sections

Plates 4.1 to 4.4 show microscopic images histological assessment of formalin-fixed paraffin-embedded colon and hepatic sections of selected male and female mice in the four experimental groups. Colon section of male mice showed extensive mucosal erosion in DSS group while API+DSS group pretreated with apigenin had mild erosion with mild infiltration by acute inflammatory cells (Plates 4.1 c-d). Histological plates of female DSS and API+DSS showed mild mucosal erosion (Plates 4.2 c-d). Normal morphology of the colon was observed in Control and API groups of both sexes (Plates 4.1 a and b and 4.2 a and b). In plates 4.3 and 4.4, normal hepatic morphology was observed in all the groups.

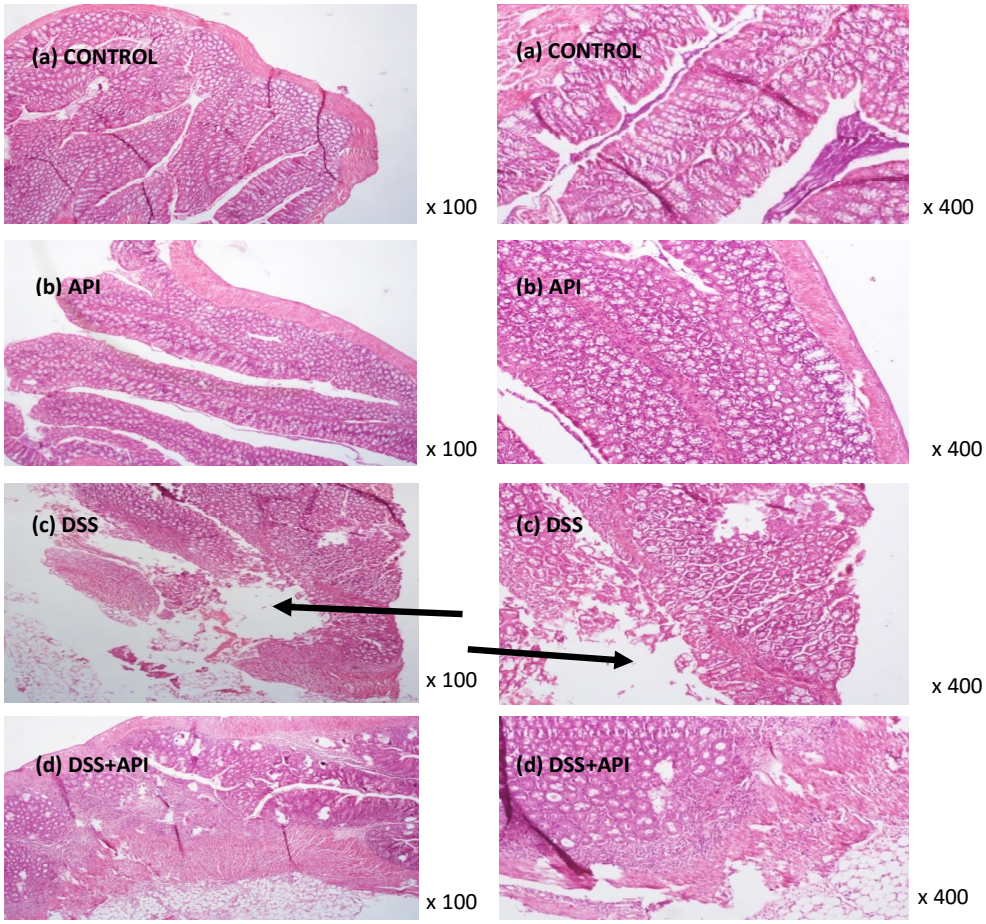

Plate 4.1

(a) Photomicrographs of section of the colon of untreated male mice showing normal morphology with mucin-containing goblet cells in the mucosa

(b) Photomicrographs of section of the colon of male mice treated with apigenin showing normal colon architecture

(c) Photomicrographs of section of the colon of male mice exposed to DSS showing mucosal erosion

(d) Photomicrographs of section of the colon of male mice exposed to DSS and apigenin showing mild mucosal inflammation with neutrophilic infiltration

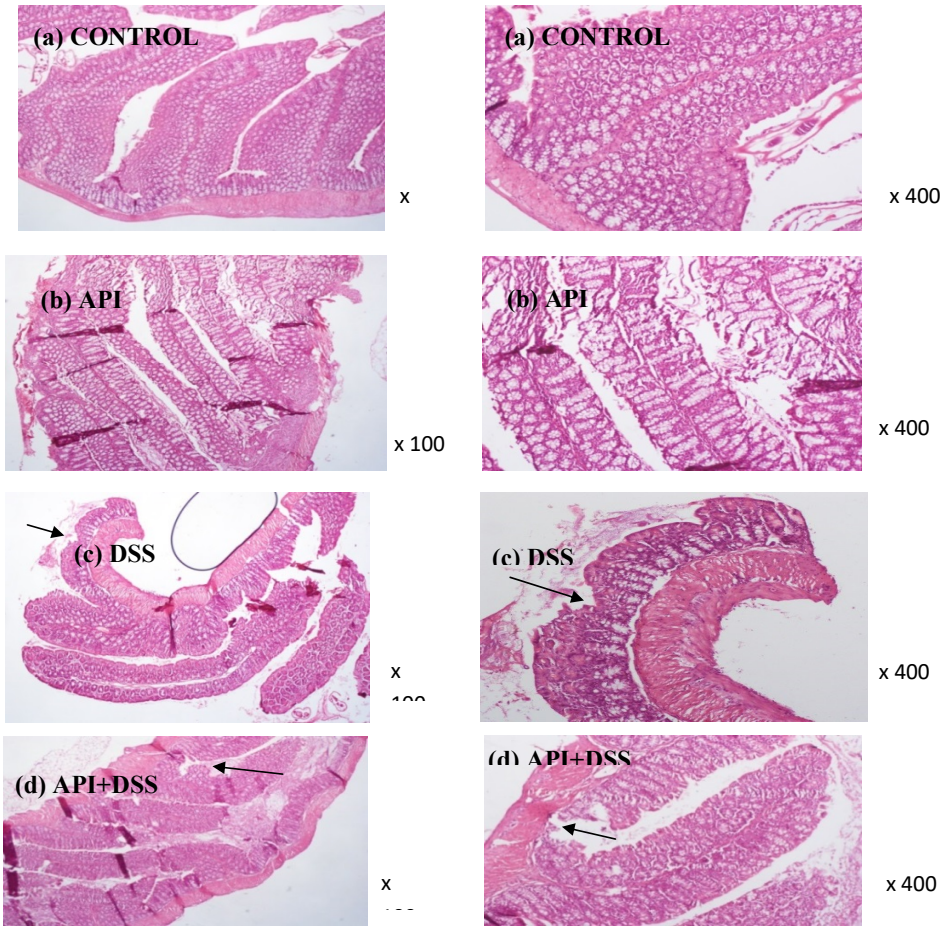

Plate 4.2

(a) Photomicrographs of section of the colon of untreated female mice showing normal colon architecture

(b) Photomicrographs of section of the colon of female mice treated with apigenin showing normal colon morphology

(c) Photomicrographs of section of the colon of female mice exposed to DSS showing mild mucosal erosion

(d) Photomicrographs of section of the colon of female mice exposed to DSS and treated with apigenin showing mild erosion of the mucosa with mild infiltration by neutrophils

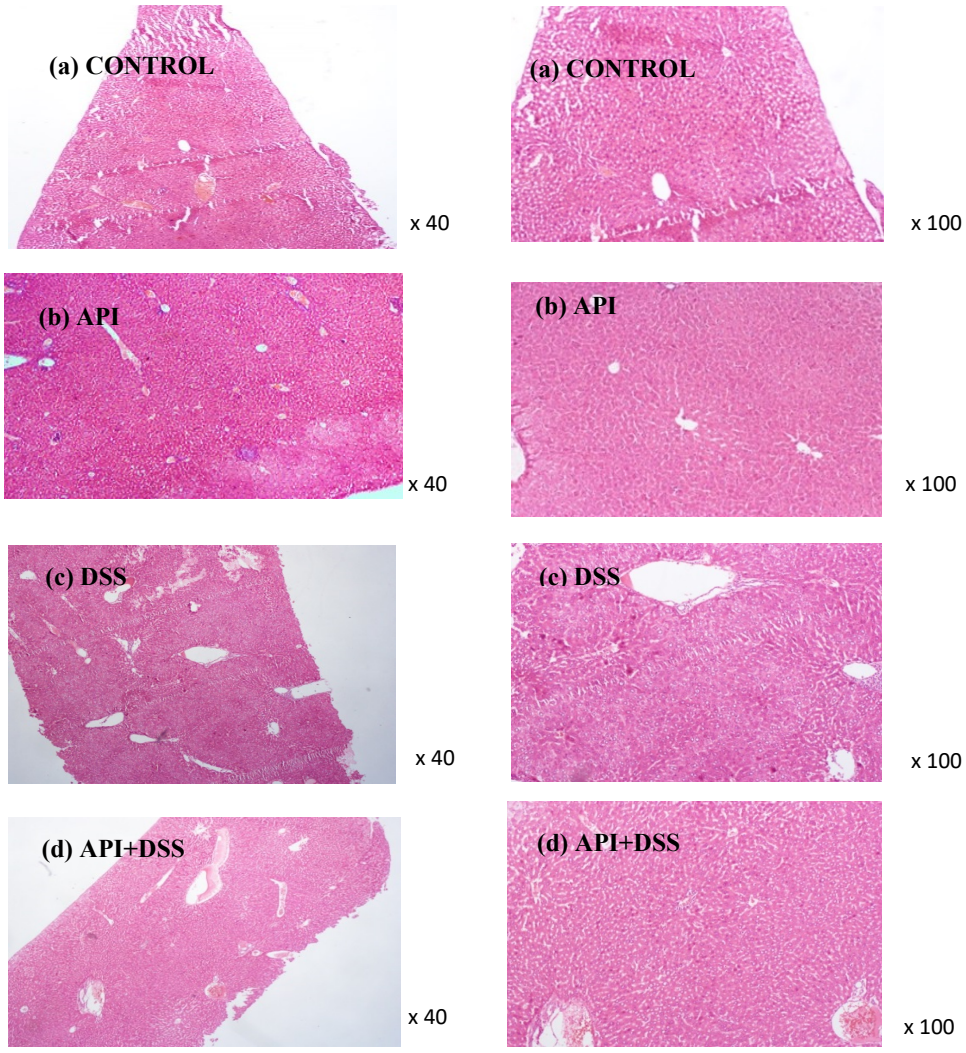

Plate 4.3

(a) Photomicrographs of hepatic section of untreated male mice showing no lesion

(b) Photomicrographs of hepatic section of male mice treated with apigenin showing no lesion

(c) Photomicrographs of hepatic section of male mice exposed to DSS showing no lesion

(d) Photomicrographs of hepatic section of male mice exposed to DSS and apigenin showing no lesion

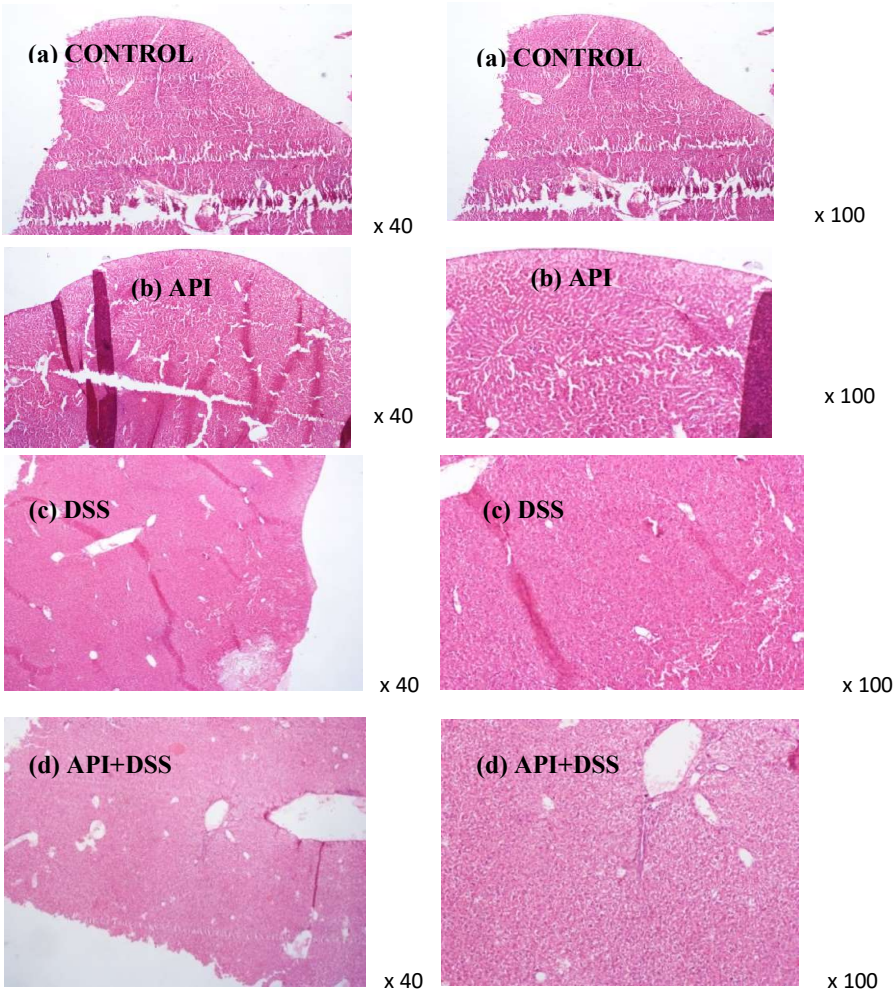

Plate 4.4

(a) Photomicrographs of hepatic section of untreated female mice showing no lesion

(b) Photomicrographs of hepatic section of female mice treated with apigenin showing no lesion

(c) Photomicrographs of hepatic section of female mice exposed to DSS showing no lesion

(d) Photomicrographs of hepatic section of female mice exposed to DSS and treated with apigenin showing no lesion

4.4 Results of Immunological-Histological Chemistry (IHC)

Plates 4.5- 4.20 show photomicrographs of immunohistochemical staining for expressions of four membrane-bound enzymes (iNOS, COX-2, CYP3A4 and P-glycoprotein), in colonic and hepatic sections. Expressions were evidenced by brown staining of the colonic mucosa as well as primary cells of the liver such as Kupffer and parenchymal cells. Expressions (positive staining) were graded as weak, moderate or strong based on staining intensity or negative if there was no staining.

Inducible nitric oxide synthase (iNOS) was strongly expressed in male and female DSS (plates 4.5c and 4.6c) and weakly expressed in male Control and API groups(Plates 4.5 a and b). Negative membrane staining was observed for iNOS in other groups. (Plates 4.5d, 4.6 a, b and d)

In the hepatic section, there were weak membrane stainings for iNOS in male API+DSS (Plate 4.7d), and female Control and DSS (Plates 4.8a and c). Others were negative.

Similar trend was observed for COX-2 in section of the colon (Plates 4.9 and 4.10). Only male DSS (Plate 4.9c) showed strong membrane staining, other groups were negative.

Immunohistochemical profiling for CYP3A4 in section of the colons showed negative staining for Control (male and female) but moderate, strong and weak stainings for API, DSS and API+DSS male, respectively and negative, weak and strong staining in the female. Hepatic sections showed weak, strong, moderate and negative staining for male Control, API, DSS and API+DSS, respectively and strong, weak, strong and strong staining for the female.

Immunohistochemical profiling for P-glycoprotein (mdr-1/ABCB1) showed weak staining for DSS female colon and male hepatic sections, intense staining for API+DSS female colon and moderate staining for API female hepatic section. Other groups showed negative staining.

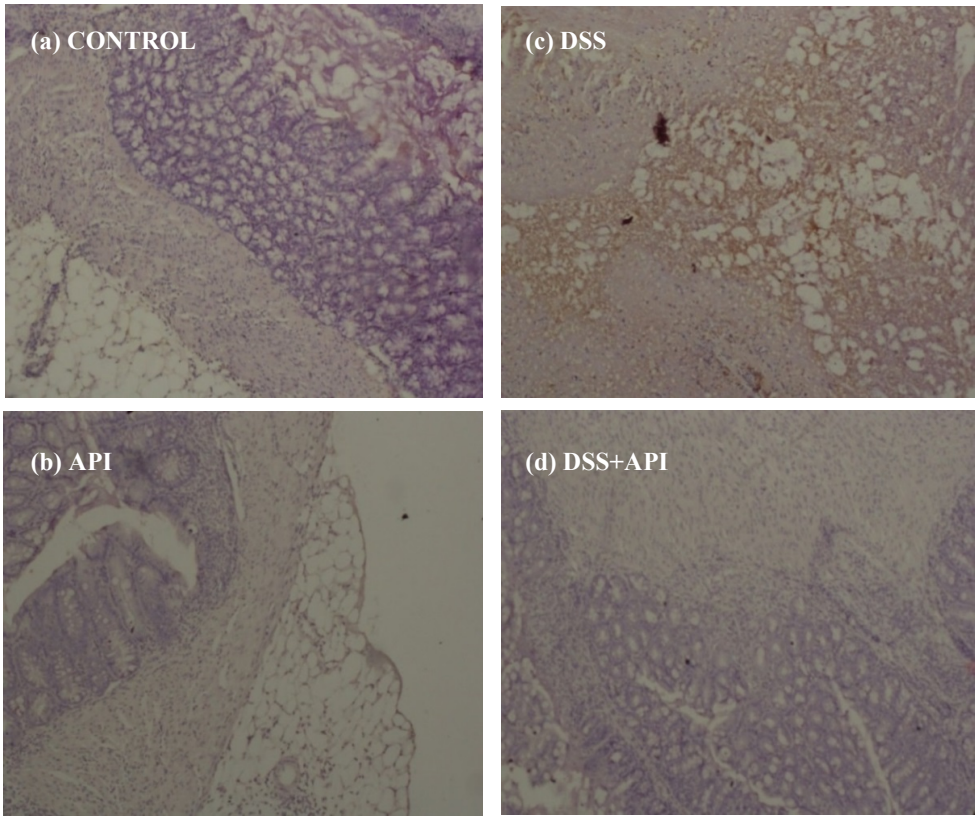

Plate 4.5Photomicrographs of Immunohistochemical Staining for Inducible Nitric Oxide synthase (iNOS) Expression in the Section of the colon of Male Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated male mice showing no expression of iNOS (negative)

(b) Photomicrograph of section of the colon of male mice treated with apigenin showing no expression of iNOS (negative)

(c) Photomicrograph of section of the colon of male mice exposed to DSS showing strong (positive) expression of iNOS as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of male mice exposed to DSS and apigenin showing no expression of iNOS (negative)

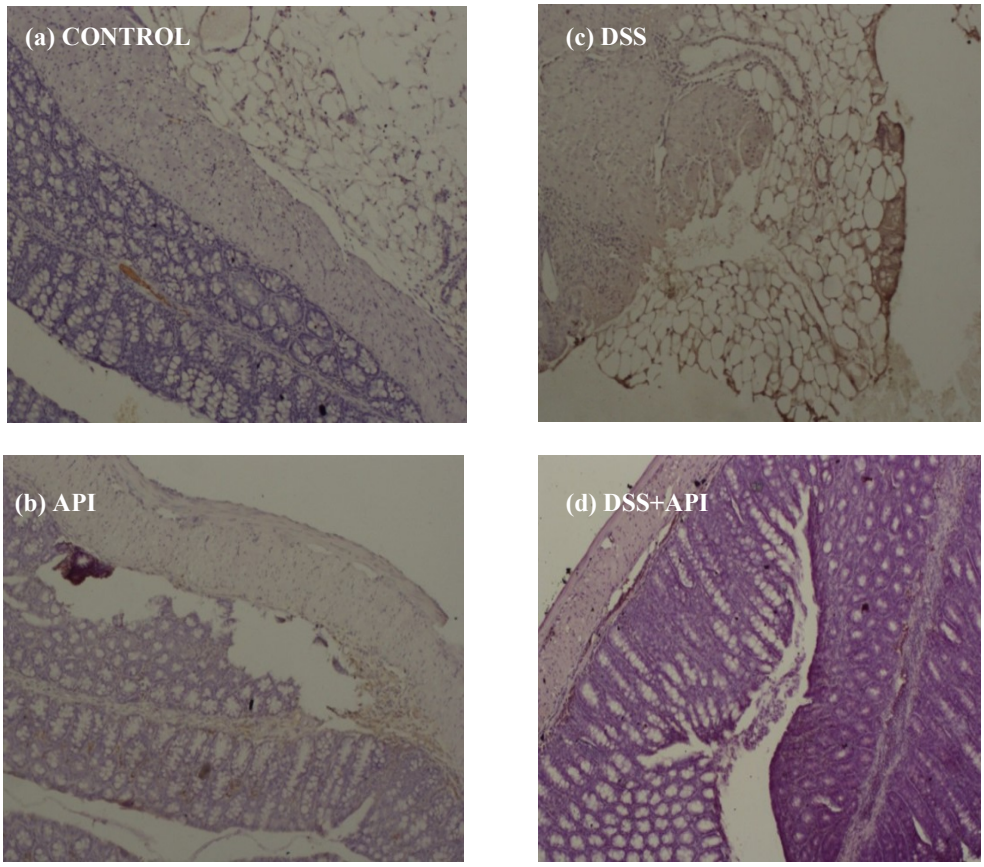

Plate 4.6Photomicrographs of Immunohistochemical Staining for Inducible Nitric Oxide synthase (iNOS) Expression in the Section of the colon of Female Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated female mice showing no expression of iNOS (negative)

(b) Photomicrograph of section of the colon of female mice treated with apigenin showing no expression of iNOS (negative)

(c) Photomicrograph of section of the colon of female mice exposed to DSS showing strong (positive) expression of iNOS as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of female mice exposed to DSS and apigenin showing no expression of iNOS (negative)

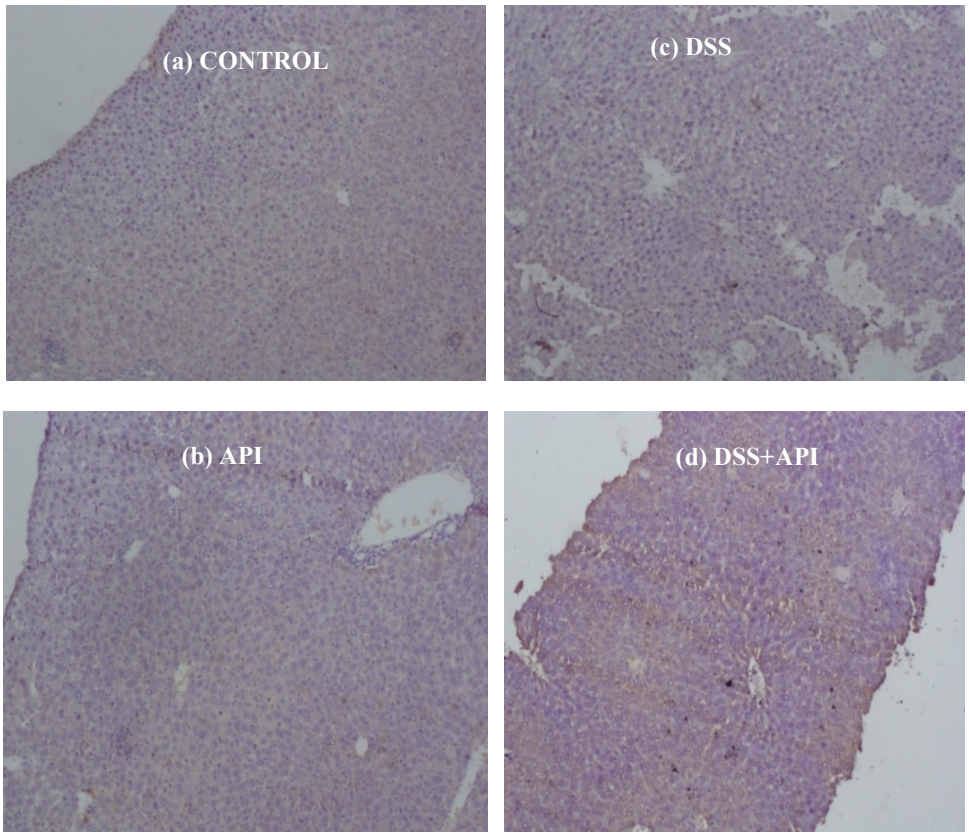

Plate 4.7Photomicrographs of Immunohistochemical Staining for Inducible Nitric Oxide synthase (iNOS) Expression in the Hepatic section of Male Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated male mice showing no expression of iNOS (negative)

(b) Photomicrograph of hepatic section of male mice treated with apigenin showing no expression of iNOS (negative)

(c) Photomicrograph of hepatic section of male mice exposed to DSS showing no expression of iNOS (negative)

(d) Photomicrograph of hepatic section of male mice exposed to DSS and apigenin showing weak expression of iNOS as evidenced by brown staining

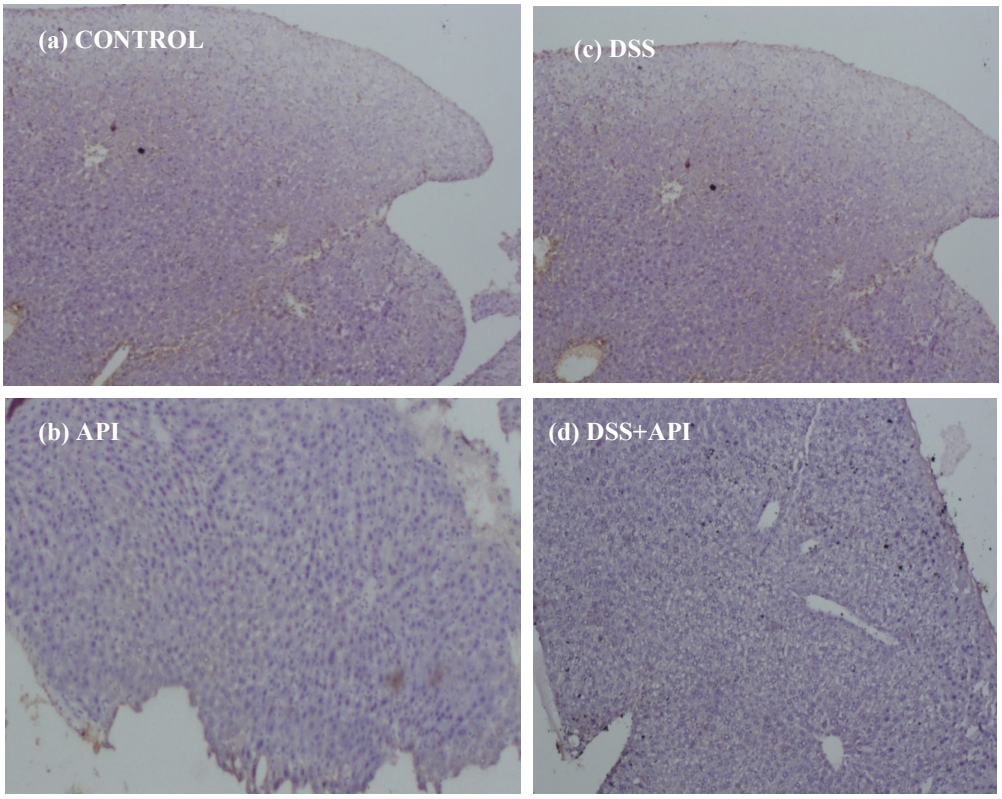

Plate 4.8Photomicrographs of Immunohistochemical Staining for Inducible Nitric Oxide synthase (iNOS) Expression in the Hepatic section of Female Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated female mice showing weak expression of iNOS as evidenced by brown staining

(b) Photomicrograph of hepatic section of female mice treated with apigenin showing no expression of iNOS (negative)

(c) Photomicrograph of hepatic section of female mice exposed to DSS showing weak expression of iNOS as evidenced by brown membrane staining

(d) Photomicrograph of hepatic section of female mice exposed to DSS and apigenin showing no expression of iNOS (negative)

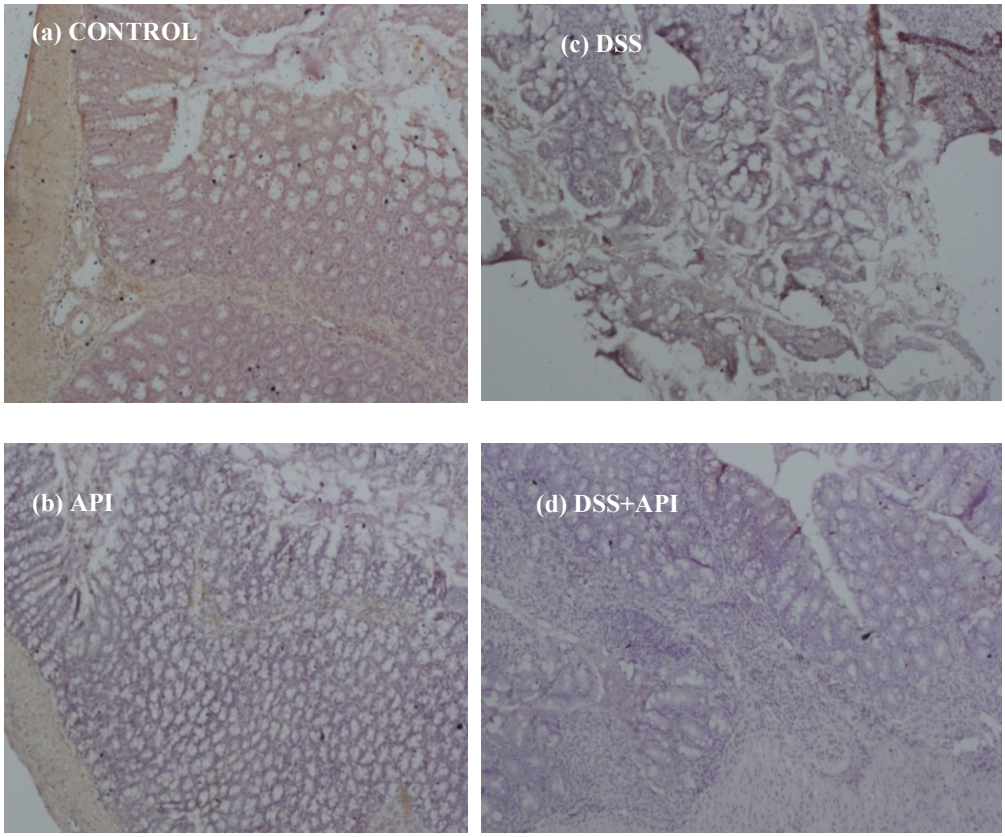

Plate 4.9Photomicrographs of Immunohistochemical Staining for Cyclooxygenase-2 (COX-2) Expression in the Section of the colon of Male Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated male mice showing no expression of COX-2 (negative)

(b) Photomicrograph of section of the colon of male mice treated with apigenin showing no expression of COX-2 (negative)

(c) Photomicrograph of section of the colon of male mice exposed to DSS showing strong expression of COX-2 (positive) as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of male mice exposed to DSS and apigenin showing no expression of COX-2 (negative)

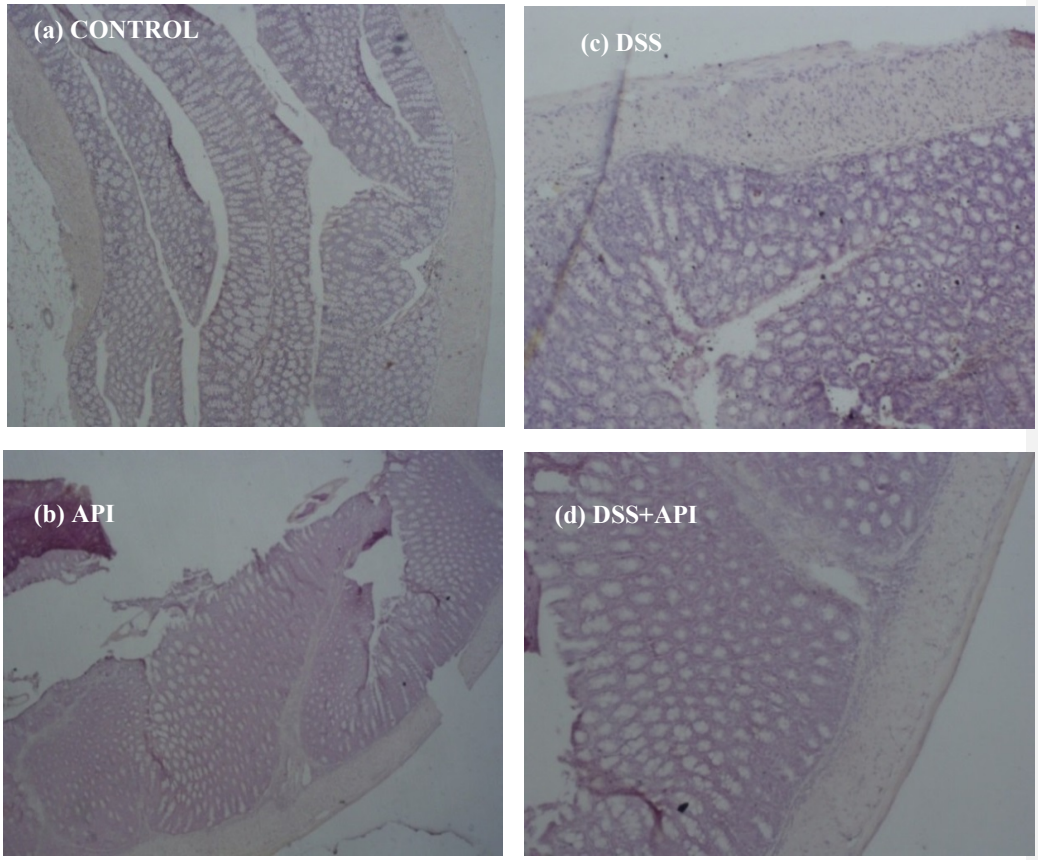

Plate 4.10 Photomicrographs of Immunohistochemical Staining for Cyclooxygenase-2 (COX-2) Expression in the Section of the colon of Female Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated female mice showing no expression of COX-2 (negative)

(b) Photomicrograph of section of the colon of female mice treated with apigenin showing no expression of COX-2 (negative)

(c) Photomicrograph of section of the colon of female mice exposed to DSS showing strong expression of COX-2 (positive) as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of female mice exposed to DSS and apigenin showing no expression of COX-2 (negative)

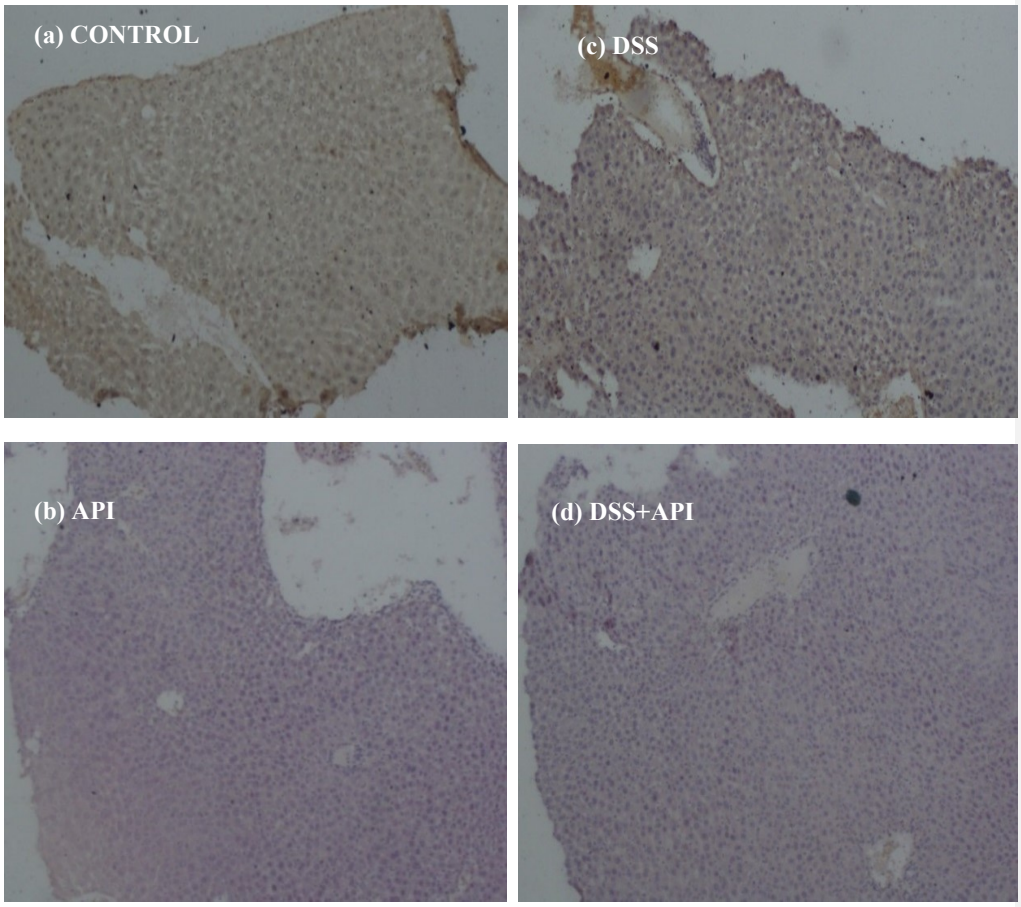

Plate 4.11Photomicrographs of Immunohistochemical Staining for Cyclooxygenase-2 (COX-2) Expression in the Hepatic section of Male Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated male mice showing no expression of COX-2 (negative)

(b) Photomicrograph of hepatic section of male mice treated with apigenin showing no expression of COX-2 (negative)

(c) Photomicrograph of hepatic section of male mice exposed to DSS showing no expression of COX-2 (negative) staining

(d) Photomicrograph of hepatic section of male mice exposed to DSS and apigeninshowing no expression of COX-2 in the mucosa (negative)

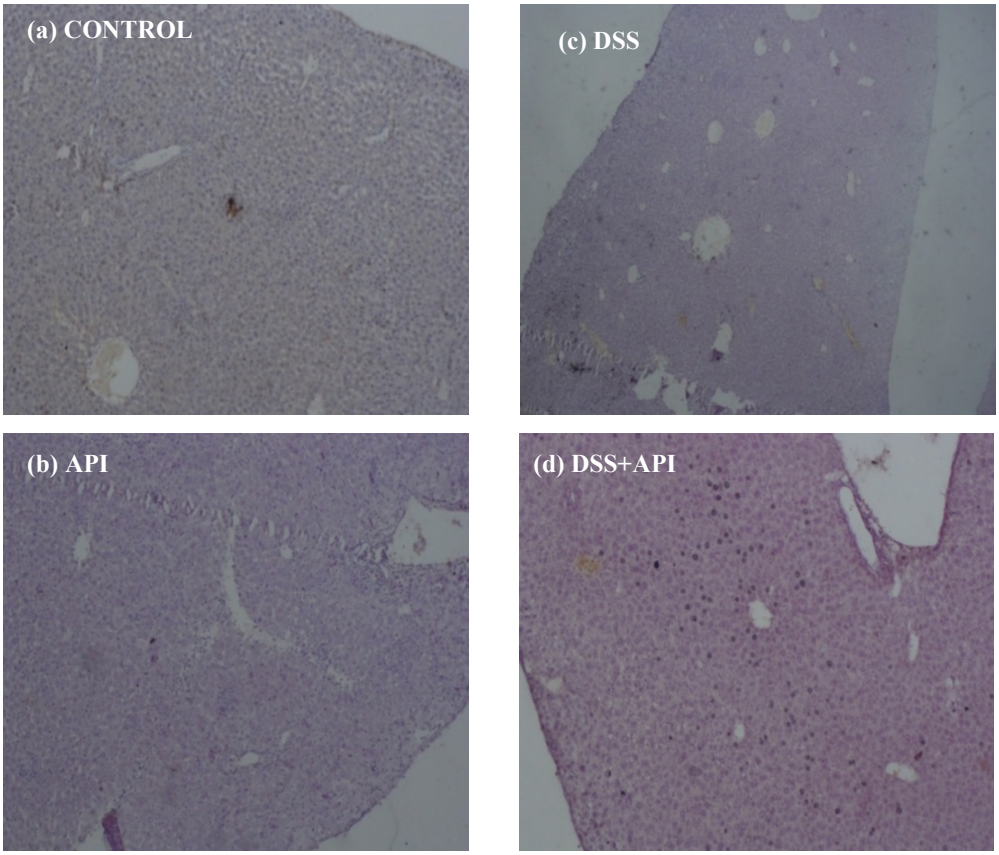

Plate 4.12Photomicrographs of Immunohistochemical Staining for Cyclooxygenase-2 (COX-2) Expression in the Hepatic section of Female Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated female mice showing no expression of COX-2 (negative)

(b) Photomicrograph of hepatic section of female mice treated with apigenin showing no expression of COX-2 (negative)

(c) Photomicrograph of hepatic section of female mice exposed to DSS showing no expression ofCOX-2 (negative)

(d) Photomicrograph of hepatic section of female mice exposed to DSS and apigenin showing no expression of COX-2 in the mucosa (negative)

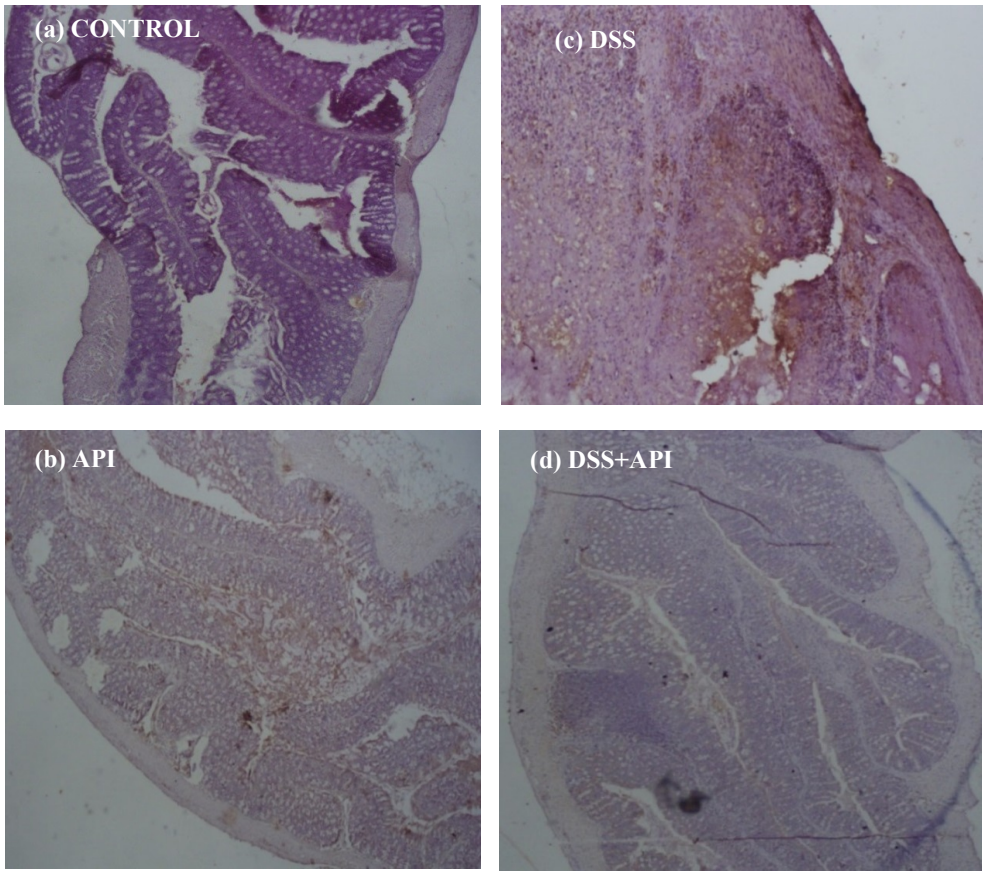

Plate 4.13Photomicrographs of Immunohistochemical Staining for Cytochrome P450 (CYP3A4) Expression in the Section of the colon of Male Mice (Magnification x 40)

(a) Photomicrograph of section of the colon of untreated male mice showing no expression of CYP3A4 (negative)

(b) Photomicrograph of section of the colon of male mice treated with apigenin showing moderate expression of CYP3A4 as evidenced by brown membrane staining

(c) Photomicrograph of section of the colon of male mice exposed to DSS showing strong expression of CYP3A4 as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of male mice exposed to DSS and apigenin showing weak expression of CYP3A4

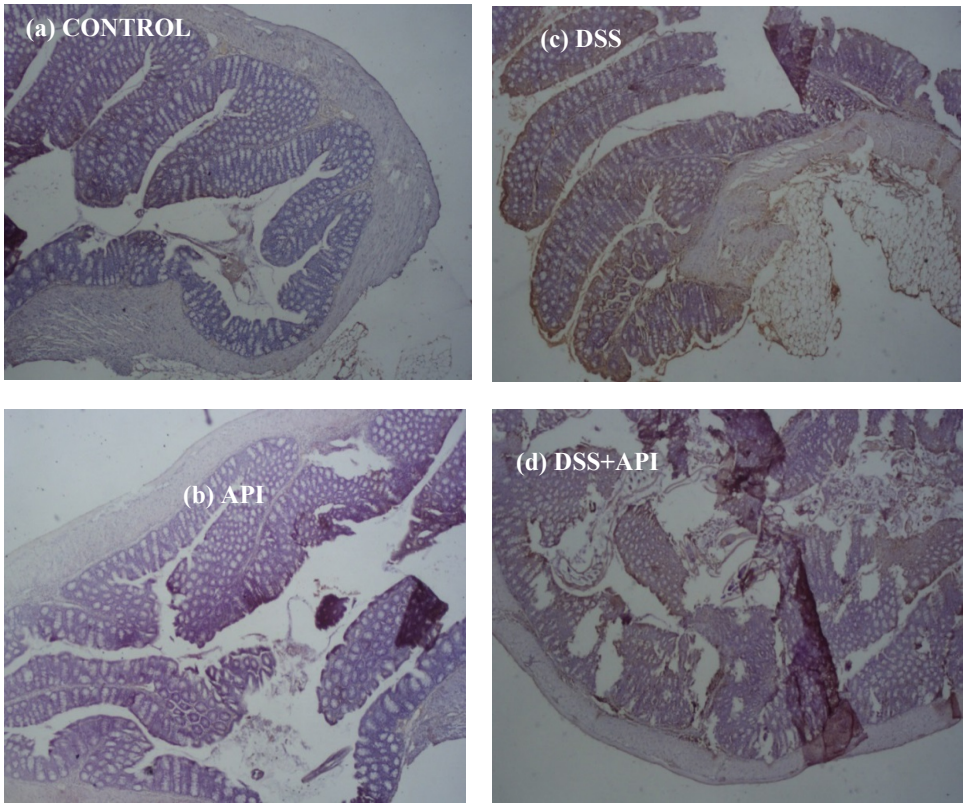

Plate 4.14Photomicrographs of Immunohistochemical Staining for Cytochrome P450 (CYP3A4) Expression in the Section of the colon of Female Mice (Magnification x 40)

(a) Photomicrograph of section of the colon of untreated female mice showing no expression of CYP3A4 (negative)

(b) Photomicrograph of section of the colon of female mice treated with apigenin showing no expression of CYP3A4 (negative)

(c) Photomicrograph of section of the colon of female mice exposed to DSS showing strong expression of CYP3A4 as evidenced by the intensely brown membrane staining

(d) Photomicrograph of section of the colon of female mice exposed to DSS and apigenin showing moderate expression of CYP3A4 in the mucosa

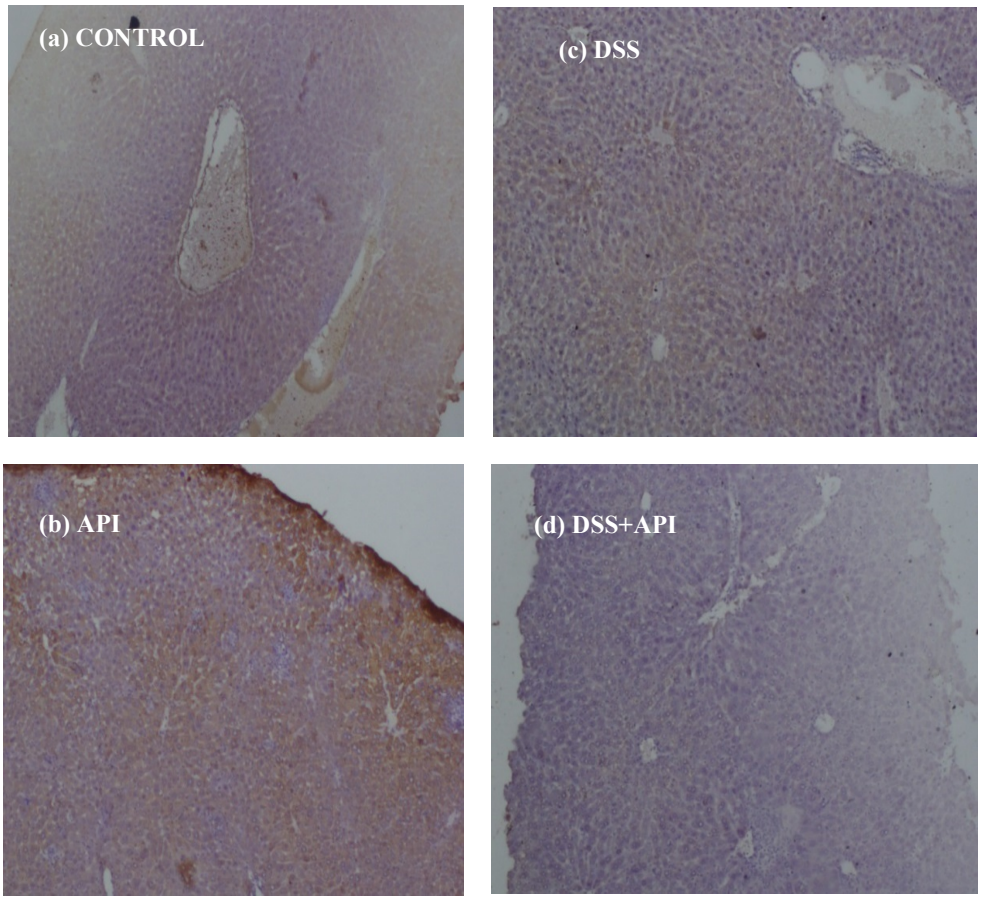

Plate 4.15Photomicrographs of Immunohistochemical Staining for Cytochrome P450 (CYP3A4) Expression in the Hepatic section of Male Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated male mice showing weak expression of CYP3A4

(b) Photomicrograph of hepatic section of male mice treated with apigenin showing strong expression of CYP3A4 as evidenced by intensely brown staining

(c) Photomicrograph of hepatic section of male mice exposed to DSS showing moderate expression of CYP3A4

(d) Photomicrograph of hepatic section of male mice exposed to DSS and apigenin showing no expression of CYP3A4 (negative)

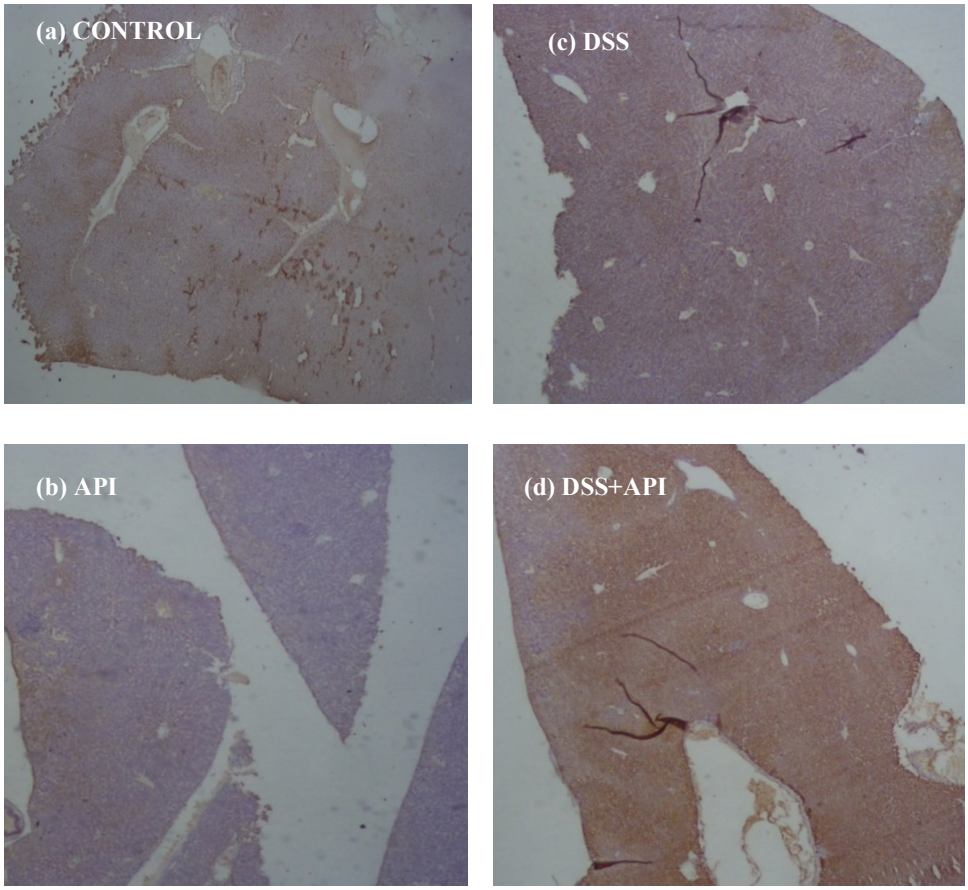

Plate 4.16 Photomicrographs of Immunohistochemical Staining for Cytochrome P450 (CYP3A4) Expression in the Hepatic section of Female Mice (Magnification x 40)

(a) Photomicrograph of hepatic section of untreated female mice showing strong expression of CYP3A4 as evidenced by intense brown staining

(b) Photomicrograph of hepatic section of female mice treated with apigenin showing weak expression of CYP3A4

(c) Photomicrograph of hepatic section of female mice exposed to DSS showing strong expression of CYP3A4 as evidenced by intensely brown staining

(d) Photomicrograph of hepatic section of female mice exposed to DSS and apigenin showing strong expression of CYP3A4 as evidenced by intensely brown staining

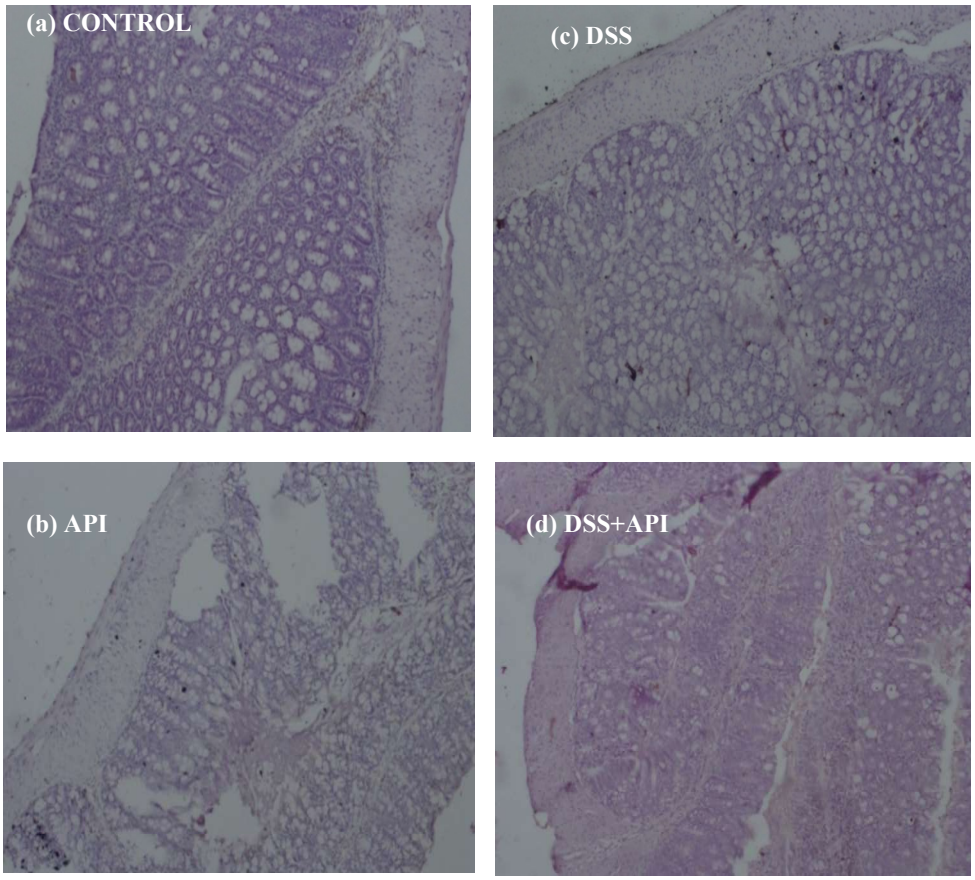

Plate 4.17 Photomicrographs of Immunohistochemical Staining for P-glycoprotein Expression in the Section of the colon of Male Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated male mice showing no expression of P-glycoprotein

(b) Photomicrograph of section of the colon of male mice treated with apigenin showing no expression of P-glycoprotein

(c) Photomicrograph of section of the colon of male mice exposed to DSS showing no expression of P-glycoprotein

(d) Photomicrograph of section of the colon of male mice exposed to DSS and apigenin showing no expression of P-glycoprotein

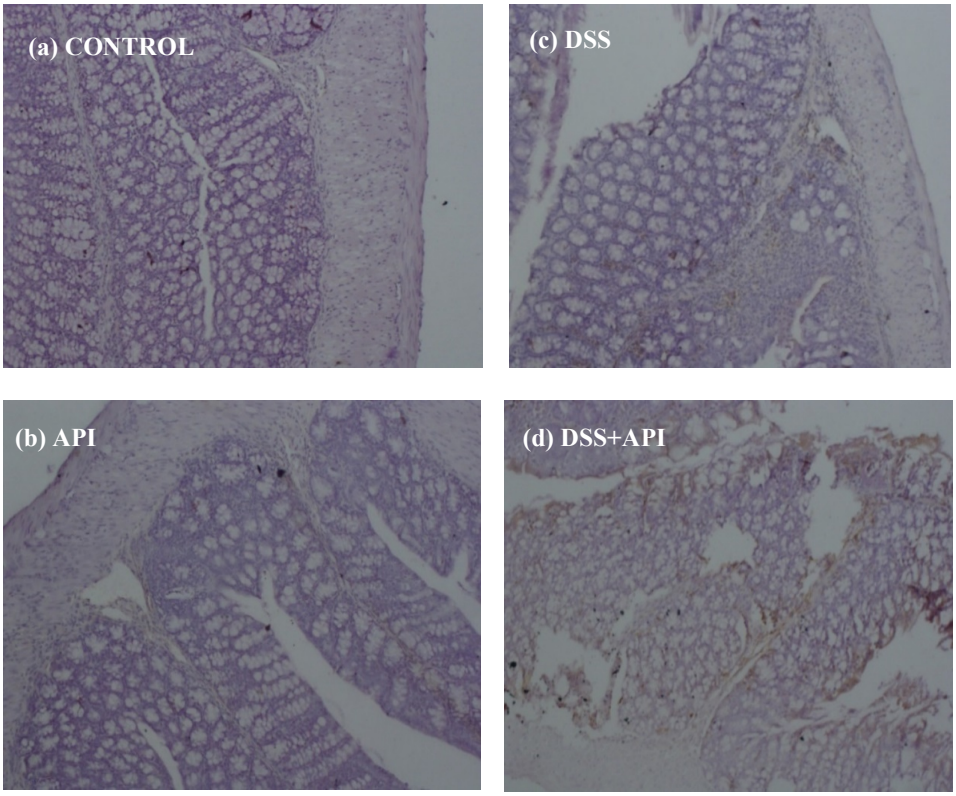

Plate 4.18 Photomicrographs of Immunohistochemical Staining for P-glycoprotein Expression in the Section of the colon of Female Mice (Magnification x 100)

(a) Photomicrograph of section of the colon of untreated female mice showing no expression of P-glycoprotein

(b) Photomicrograph of section of the colon of male mice treated with apigenin showing no expression of P-glycoprotein

(c) Photomicrograph of section of the colon of female mice exposed to DSS showing weak expression of P-glycoprotein

(d) Photomicrograph of section of the colon of female mice exposed to DSS and apigenin showing strong expression of P-glycoprotein as evidenced by intensely brown membrane staining

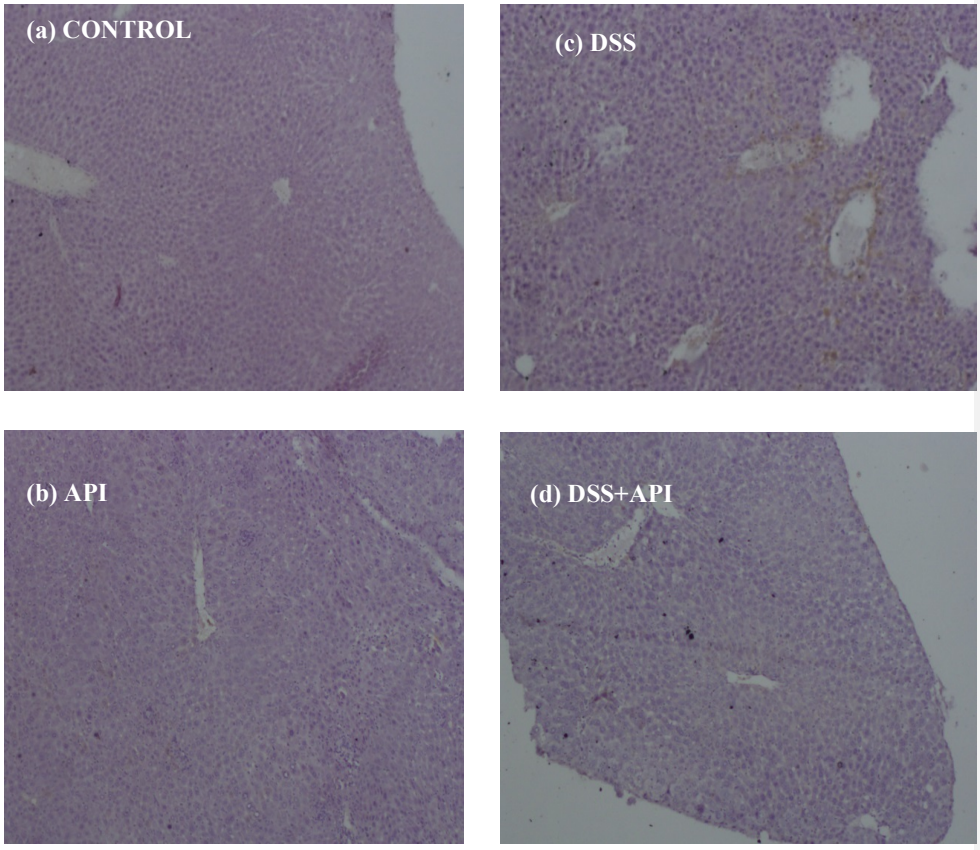

Plate 4.19 Photomicrographs of Immunohistochemical Staining for P-glycoprotein (P-GP) Expression in the Hepatic section of Male Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated male mice showing no expression of P-lycoprotein

(b) Photomicrograph of hepatic section of male mice treated with apigenin showing no expression of P-glycoprotein

(c) Photomicrograph of hepatic section of female mice exposed to DSS showing weak expression of P-glycoprotein

(d) Photomicrograph of hepatic section of female mice exposed to DSS and apigenin showing no expression of P-glycoprotein

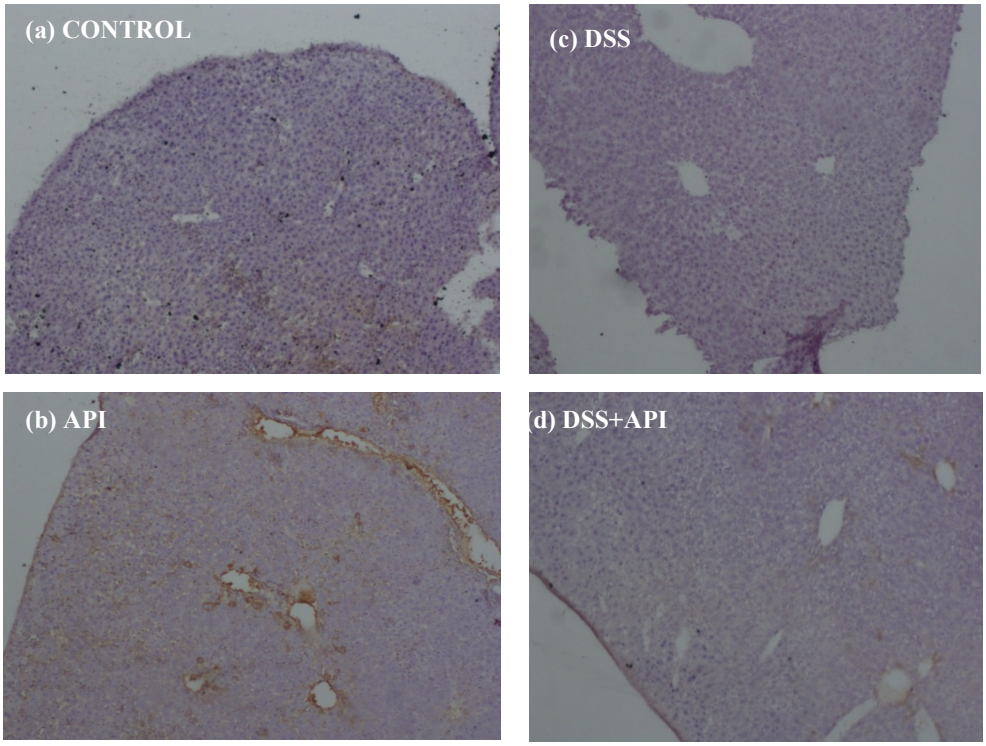

Plate 4.20Photomicrographs of Immunohistochemical Staining for P-glycoprotein (P-GP) Expression in the Hepatic section of Female Mice (Magnification x 100)

(a) Photomicrograph of hepatic section of untreated female mice showing no expression of P-glycoprotein

(b) Photomicrograph of hepatic section of female mice treated with apigenin showing moderate expression of P-glycoprotein

(c) Photomicrograph of hepatic section of female mice exposed to DSS showing no expression of P-glycoprotein

(d) Photomicrograph of hepatic section of female mice exposed to DSS and apigenin showing no expression of P-glycoprotein

CHAPTER FIVE

DISCUSSION, CONCLUSION AND CONTRIBUTION TO KNOWLEDGE

5.1 DISCUSSION

Disease activity indices (DAI) of ulcerative colitis result from inflammation and oxidative stress which have inter-related mechanisms that can be traced to molecular level and clinical symptoms which manifest based on time-course and disease severity. Onset and severity of DSS-induced ulcerative colitis in animal models vary significantly based on the species of animals used as well as the proportion (percentage) and molecular weight of DSS administered (Elvington*et al.,* 2015; Håkansson*et al.,* 2009). Administration of 3% to 5% DSS is known to cause acute ulcerative colitis in different strains of mice while DIA ranging from loss of body mass to occult blood in stool, rectal bleeding and shortened colon length usually manifest within seven days. (Farombi*et al.,* 2013; Farombi*et al.,* 2016). Significant loss in body mass with rectal bleeding and weakness observed in the mice from the second day following DSS administration aligns with previous studies and these DIA severely increased with continual DSS administration till the animals were sacrificed. There are conflicting reports on sexual influence on susceptibility to ulcerative colitis. While higher incidence were reported among males by some researchers (Hou*et al.,* 2013), lower incidence were reported by others and yet certain finding showed no variation in susceptibility to colitis in males and females. According to them, other factors such as environment and life style were likely implicating factors responsible for differences observed among studied population (Low*et al.,* 2013). However, results from this study which showed higher and more severe disease activity indices among the male mice administered only DSS relative to the female mice and this is in accordance with reports that claim higher incidence of colitis among male patients and in male animal models(Hou*et al.,* 2013).

Significant loss in body weight recorded in this study possibly resulted from severe watery and bloody diarrhoea caused by DSS administration.

DSS is degraded in the colon to its carbohydrate and sulphate moieties. Gut microflora make use of the carbohydrate conjugate to generate energy through fermentation while the sulphate anion is broken down by sulphur-reducing bacteria in the colon to generate hydrogen sulphide which is a bacterial toxin in colonic mucosa. Low amount of sulphate is physiologically important,

especially for formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) required for synthesis of chondroitin sulfate and cerebroside sulfate (important sulfur-containing compounds). However, high concentration of sulphates leads to increased amount of sulphur-reducing bacteria and diminish the number of methanogens which are capable of increasing both intestinal transit time and absorption capacity of the bowel. Hydrogen sulphide is implicated in diarrhea by its cleavage of polymeric mucin structure which causes enhanced epithelial permeability and damage to or loss of barrier function (Pitcher and Cummings, 1996).

When there is an uncontrolled imbalance between the absorptive and secretory mechanism in the intestinal tract, diarrhoea usually results, coupled with excessive fluid loss in the faeces. Pathophysiology of diarrhoea involves four basic mechanisms which are inter-related and include (i) mal-absorption of nutrients or ingestion of un-absorbable nutrients, (ii) excessive secretion of water from intestinal crypt into the lumen (by inducing activity of adenyl cyclase to increase cAMP production), (iii) inflammatory or infectious diarrhoea which occurs when epithelium is already damaged and has lost its absorptive properties. This causes serum and blood to flow into the lumen and (iv) deranged motility due to accelerated transit time which also leads to mal-absorption of nutrients. DSS administration is known to cause disruption of mucosal epithelium which is notably involved in absorption of nutrients, salt and water from intestinal lumen (both small and large bowel), thereby leading to diarrhoea with mechanisms relating to the afore-mentioned (Field, 2003; Kantheshet *al.,* 2013; Peuhkuriet *al.,* 2010).

Intestinal mucosa is a complex multi-layer system that separates the intestinal milieu from the lumen in order to foster symbiotic existence between the host and the gut microflora. Inflammation disrupts mucosal barrier with gradual loss of intestinal permeability and cellular homeostasis. Symbiotic microflora are exposed and become parasitic and infectious, triggering the immune response through activation of white blood cells and secretion of inflammatory mediators and cytokines (Field, 2003).

Inflammatory diarrhea can result in either secretory or absorptive diarrhea through secretion of reactive oxygen species by leukocytes which can destroy epithelial cells in the brush-boarder. Disrupted mucosal cells are often replaced by immature ones lacking transporters and enzymes necessary for absorbing luminal nutrients (Peuhkuriet *al.,* 2010).

Diarrhoea caused by DSS administration usually progresses from occult blood in stool to watery and finally bloody diarrhoea resulting from the afore-mentioned major mechanisms. Shortened

and thickened colon length resulting from DSS administration can equally lead to reduced transit time and mal-absorption of nutrients which possibly caused the significant weight loss observed.

DSS-induced diarrhea observed in this study was possibly inflammatory absorptive and deranged due to inflammation confirmed by increased MPO activity and nitric oxide concentration, increased expressions of iNOS and COX2 and erosion of the colonic mucosa. DSs is also known to destroy vascular endothelial cells and shortened and thickened colon length observed after DSS administration might have caused reduced GI transit time, leading to deranged diarrhea. Therefore, weight loss observed in all the mice that received DSS was inevitable due to inability of damaged intestinal epithelial cells to absorb essential nutrients and salts which resulted in starvation and dehydration (osmosis) and eventually death.

Sex hormones and intestinal smooth muscle are known to influence transit time for food and digestive products along the gastro-intestinal tract. When levels of sex hormones fluctuate, steroid receptors located on the GIT sense this and necessary symptoms (sensations) are generated. Reports have shown that high levels of estradiol and progesterone reduce GI transit time while ovariectomy and low levels of these hormones increase the transit time. Levels of these hormones usually vary based on the stage of menstrual cycle (i.e. follicular, luteal or menstrual state) an animal or human is. Their levels rise at the middle of follicular stage and become very low at the late luteal stage (Bharadwaj*et al.,* 2015). In this study, pre-pubertal mice were used when hormonal effect was minimal. Hence, diarrhoea and rectal bleeding observed might have been solely due to DSS administration without hormonal influence while mild diarrhoea, longer colon length and minimal weight loss observed in the female when compared with the male mice were likely to be due to less susceptibility of the female mice to DSS-induced ulcerative colitis which supports previous claims by Hou and co-workers (Hou*et al.,* 2013).

Recently, apigenin and some hydroalcoholic and hexane extracts of *Dracocephalum kotschyi* Boiss were shown to lessen frequency of diarrhea while they increased intestinal transit time in mice administered with castor oil and magnessum sulphate Apigenin is also known as a potent anti-inflammatory agent capable of down-regulating the expressions of inflammatory molecules and cytokines such as NF-kB family proteins (e.g. TNF-alpha, IL-1beta, IL-6), iNOS, prostaglandin H-synthase (COX-2), myeloperoxidase, among others (Begum*et al.,* 2012; Liang*et al.,* 1999; Mascaraque*et al.,* 2015). Similarly in this study, apigenin reduced the severity of DSS-induced colitis in the mice by (a) improving GIT barrier function as seen in less severe and

slower onset of diarrhoea, (b) reducing inflammation based on reduced activity of colonic myeloperoxidase and nitric oxide concentration which was confirmed by mild erosion of mucosal epithelium observed on histological plates and by (c) reducing oxidative stress caused by reactive oxygen species as evidenced by lower H_2O_2 concentration and lipid peroxidation. Longer colon length recorded in DSS groups pretreated with apigenin might be responsible for mild diarrhoea in the mice compared with groups that received DSS alone.

Existing reports on effect of apigenin on body weight and food intake are contradictory. Some studies showed anti-obesity effect of apigenin while in another study, no significant effect of apigenin was observed on body weight changes. However, most of these reports agree that apigenin can maintain body weight by regulating cholesterol metabolism and protecting vascular endothelial cells from oxidative damage and inflammation (Jung*et al.,* 2016; Zhang*et al.,* 2017). Similarly in this study, apigenin was possibly regulated body weight changes through mechanisms already mentioned i.e. by preventing oxidative damage and inflammation as well as by improving GIT barrier function thereby enhancing digestion and absorption of nutrients.

The sex and organ –dependent differential expressions of the drug efflux transporter (p-glycoprotein) and phase I drug metabolizing enzyme were equally responsible for the differences observed in susceptibility to colitis and responses to apigenin treatment. Sex differences observed in the way apigenin elicited its protective function were inevitable as previous studies have shown that apigenin is differentially metabolized in male and female animals with production of sulphate and glucuronide conjugates in varying proportions. After administering a single oral dose of apigenin, administered to mature and immature rats of both sexes, the mature male rats reportedly produced higher proportion of sulphate conjugate than glucuronide conjugate while immature male and female rats as well as mature female rats produced the inverse proportions of the conjugates (Gradolatto*et al.,* 2005). It can thus be inferred that apigenin protects the GIT from DSS-induced epithelial damage by scavenging carbohydrate and sulphate metabolites of DSS, thereby preventing their conversion by gut microflora into toxic products such as sulphites and hydrogen sulphide. It is also possible that apigenin protected against DSS-induced intestinal barrier dysfunction through other mechansims not examined here (e.g. by protecting tight junction proteins such as claudins, cadherins, zonua occludens, e.t.c.)

5.2 Conclusion

Ulcerative colitis is known as a multifactorial inflammatory bowel disorder which majorly affects the colon and maternal inherited gene factors have been implicated in its development. Although controversies exist concerning influence of sex in susceptibility to this disorder but the results obtained from this study obviously showed that male mice were more readily susceptible to DSS-induced ulcerative colitis than their female counterparts. Evidence for this was based on severity of DAI such as loss of appetite, loss of weight, watery and bloody diarrhea as well as number of death recorded among the male mice compared to the female mice which resulted in termination of the experiment after seven days of DSS administration.

Apigenin, which occurs naturally in many vegetables and fruits, has many scientifically proven medicinal properties in experimental animals and cell lines which qualify it for preclinical trial. In this study, apigenin was able to demonstrate immense protective role against DSS-induced oxidative damage and inflammation in both sexes of mice used with evidence-based results such as reduction in the extent of lipid peroxidation, hydrogen peroxide concentration, myeloperoxidase activity and nitric oxide concentration. In addition, there was improvement in antioxidant status through elevation of SOD activity and reduced glutathione concentration in both sexes and organs. Its sex and organ specific effects were also confirmed through expressions of multidrug efflux protein (P-glycoprotein) and phase I drug metabolizing enzyme (CYP3A4) and some of the biochemical results.

Although ulcerative colitis was more severe in the male mice than the female ones, apigenin still offered better protective effects on the male animals, especially in the colon.

5.3 Contributions to Knowledge

From this study, it was discovered that

- the onset of ulcerative colitis was earlier and more severe in the male than female mice.
- inducible nitric oxide, cyclooxygenase-2, cytochrome P450 and P-glycoprotein were differentially expressed in colon and liver in sex-dependent manner.
- apigenin treatment reduced ulcerative colitis-induced mortality in male mice.
- apigenin offered protection against inflammation and oxidative stress through anti-inflammatory and antioxidative mechanisms

- enhanced expression of P-glycoprote by apigenin treatment also protected the female mice against DSS-induced ulcerative colitis

5.4 Recommendation:

Diets rich in fruits and vegetables such as apples, onions, oranges, lettuce, garlic and grape may ameliorate ulcerative colitis.

Further research should be carried out to further clarify other mechanisms of apigenin action.

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APPENDIX

APPENDIX 1A: PREPARATION OF REAGENTS

Determination of Total Protein

Reagents

1. **0.2M Sodium Hydroxide (NaOH):** 1.6 g of NaOH was dissolved in 200ml of distilled water.
2. **Biuret reagent:** 0.6 g of copper(II)tetraoxosulphate(VI)pentahydrate (CuSO4. 5H2O), 1.8 g of sodium potassium tartrate and 1 g of Potassium iodide were stepwisely dissolved in solution (1) above, with constant stirring to dissolve all particles.
3. **Bovine Serum Albumin (BSA) stock solution:** 20 mg of BSA was dissolved in 4 ml of distilled water to make a stock solution of 5 mg/ml.

Determination of Catalase Activity

Reagents

1. **0.05M Phosphate buffer (pH 7.4):** 8.7g of dipotassium hydrogen phosphate (K_2HPO_4) and 6.8g of potassium dihydrogen phosphate (KH_2PO_4) were separately dissolved in 1L of distilled water and the resulting solutions were added to a beaker in the proportion 4:1, respectively. and the pH adjusted to 7.4.
2. **19mM Hydrogen peroxide:** 194 µl of 30% H_2O_2 was weighed and dissolved in 100 ml of 0.05 M phosphate buffer at neutral pH of 7.4.

Specific catalase activity = catalase activity/mg protein where ε=0.0436 $mM^{-1}cm^{-1}$

Assessment of Lipid Peroxidation

Reagents

1. **20% Trichloroacetic acid (TCA):** 6.0 g of TCA was dissolved in diluted in 30ml of distilled water.
2. **0.1 M Hydrochloric acid (HCl):** 26µl of concentrated HCl (36.5-38%) was added to 30ml of distilled water in a glass beaker.
3. **0.5% Thiobarbituric acid (TBA):** 0.15g of TBA was dissolved in solution (2). Dissolution was aided by stirring in a hot water bath (50˚C).

Estimation of Reduced Glutathione (GSH) Level

Reagents

1. **0.1M Phosphate buffer (pH 7.4):** 17.4g of dipotassium hydrogen phosphate (K_2HPO_4) and 13.6g of potassium dihydrogen phosphate (KH_2PO_4) were separately dissolved in 1L of distilled water. The solutions were mixed in proportions 4:1, respectively with the pH adjusted to 7.4.
2. **GSH stock solution:** 0.4mg of reduced L-glutathione was dissolved in 100ml of solution (1) above
3. **Ellman's Reagent**: 80 mg of Ellman's was measured into 200ml of solution (1)
4. **4% Sulphosalicylic Acid:** 0.8 g of sulphosalicylic acid was weighed and dissolved in 20 ml of distilled water.

Preparation of GSH standard

GSH Stock (µl)	Phosphate Buffer (µl)	Ellman's Reagent	GSH Conc. (µg/ml)
15.6	484.4	1.5	6.25
31.2	468.8	1.5	12.5
62.5	437.5	1.5	25
125	375	1.5	50
250	250	1.5	100
375	125	1.5	150
500	0.0	**1.5**	**200**

Determination of Superoxide Dismutase Activity

Reagents

1. 0.05M Carbonate buffer (pH 10.2): 2.512 g of Na2CO3.10H2O and 0.941 g of NaHCO3 were weighed and dissolved in 400 ml of distilled water (pH 10.2).

2. 0.01M Epinephrine

0.0916g of epinephrine was measured and dissolved in 50ml of acidified water. (acidified water was prepared by adding 18.5µl of concentrated HCl (37%) to distilled water and making up the volume to 105ml).

Determination of Hydrogen Peroxide (H_2O_2) Concentration

Reagents

1. **100 µM Xylenol orange (MW 760.6):** 15.2 mg of xylenol orange was dissolved in 20 ml of distilled water.
2. **100mM Sorbitol:** 3.644 g of sorbitol was dissolved in 20 ml of distilled water.
3. **25mM Sulphuric acid:** 280µl of H2SO4 was added to distilled water and made up to 100 ml.
4. **250µM Ammonium ferrous sulfate (MW 392.14):** 19.6 mg of ammonium ferrous sulfate was dissolved in 100 ml of 25 mM H2SO4.
5. **100µM Hydrogen peroxide stock:** 15 µl of 30% H2O2 was added to distilled water and the volume made up to 25 ml. 500 µl of the resulting solution was taken and made up to 25 ml.

   200ml FOX1 reagent = 20 ml xylenol orange + 20 ml sorbitol + 100 ml AFS + 60 ml distilled water

Estimation of Glutathione S-Transferase (GST)Activity

Reagents

1. **20mM 1-Chloro-2,4-dinitrobenzene (MW= 202.55g/mol):** 80.80 mg of 1-chloro-2,4-dinitrobenzene (CDNB) was added to 20 ml of absolute ethanol.
2. **0.1M Phosphate buffer (pH 6.5):** 22.822 g of dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) and 13.6g of potassium dihydrogen phosphate (KH_2PO_4) were each added to 1L of distilled water. The solutions were mixed in ratio 3:7, respectively and the pH adjusted to 6.5.
3. **0.1M Reduced Glutathione (MW=307.32 g/mol):** 61.464 mg of L-glutathione was added to 2 ml of solution (2) above.

Assessment of Glutathione Peroxidase (GPx) Activity

Reagents

1. **10 mM Sodium azide (NaN_3):** 3.25 mg of NaN_3 was added to 50 ml of distilled water.
2. **0.1M Phosphate buffer (pH 7.4):** 17.4g of dipotassium hydrogen phosphate (K_2HPO_4) and 13.6g of potassium dihydrogen phosphate (KH_2PO_4) were each weighed and dissolved in 1L of distilled water in separate beakers. The salt solutions were mixed in proportion 4:1, respectively and pH adjusted to 7.4.
3. **4mM Reduced glutathione (GSH):** 10 ml of solution (2) above was added to 12.3 mg of GSH.
4. **2.5mM Hydrogen peroxide**: 14 µl of 30% hydrogen peroxide was added to distilled water and the volume was made up to 50 ml.
5. **10% Trichloroacetic acid (TCA):** 2 g of TCA was added to distilled water to make a total volume of 20ml.
6. **0.3M Dipotassium hydrogen orthophosphate (K_2HPO_4):** 60ml of distilled water was used to dissolve 4.11g of K2HPO4.3H2O.
7. **5,5'-Dithiobis(2-nitrobenzoic acid/Ellman's reagent:** 40.0mg of Ellman's reagent was dissolved in 100 ml of reagent (2) above.

Determination of Nitrite Concentration (NO_2^-)

Reagents:

1. **5% phosphoric acid (H_3PO_4):** 5.88ml of 85% H_3PO_4 was added to distilled water and the volume was made up to 100ml.
2. **1% sulfanilamide(MW 172.202g/mol)**: 1 g of sulfanilamide was dissolved in 99ml of reagent (1).
3. **0.1%*n*-(1-naphthyl) ethylenediamine dihydrochloride (NED) (MW 259.174g/mol):** 0.1 g of in distilled water NED was dissolved in distilled water and the volume was made up to 100ml.
4. **Griess reagent**: Equal volume of reagents (2) and (3) were mixed in equal proportion.
5. Sodium nitrite salt ($NaNO_2$): 4mg/ml (58mM) of $NaNO_2$ stock was serially diluted to detection limits of the spectrophotometer and used for the nitrite standard curve.

Estimation of Myeloperoxidase (MPO) Activity

Reagents

1. **50mM phosphate buffer pH 6.0:** 8.7g/L of KH_2PO_4 and 6.8045g/L of KH_2PO_4 prepared by dissolving in distilled water and mixed in volume ratio 13.3 : 86.8, respectively. The pH of final mixture was adjusted to 6.0.
2. **0.5mM O-dianisidine dihydrochloride (317.21g/mol):** 100ml of reagent (1) was used in dissolving 0.167mg of o- dianisidine dihydrochloride.
3. **0.005% H_2O_2:** This was prepared by dissolving 16.7 µl of 30% H_2O_2 in distilled water to make a final volume of 100ml.

Preparation of 10% Phosphate (Neutral) Buffered Formalin for Histology

100ml of 37% Commercial Formaldehyde was added to 900ml of distilled water. Then, 4 g of Sodium dihydrogen trioxophosphate (V) and 6.5 g of disodium hydrogen trioxophosphate (V) were dissolved in the mixture and stored at room temperature. (The pH was adjusted to 7.2, before making the final volume up to 1L).

Appendix 1b: Table showing Body weights of mice (g)

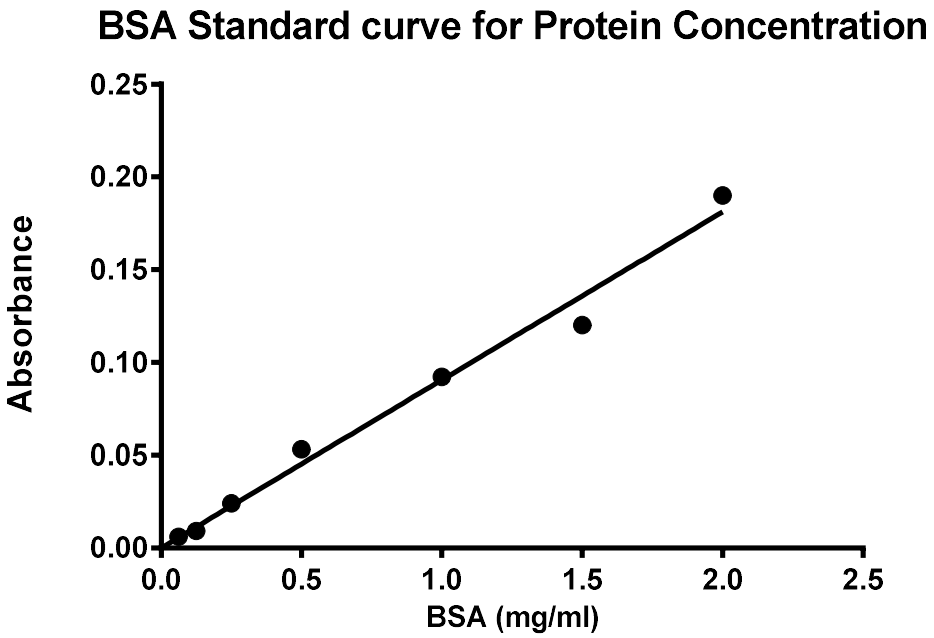
	1st Day		3rd Day		10th Day	
Group / Sex	**MALE**	**FEMALE**	**MALE**	**FEMALE**	**MALE**	**FEMALE**
Control	22.83±0.75	21.66±0.99	23.80±0.72	21.65±0.84	24.70±1.0.5	19.82±0.91
API	24.10±0.83	19.89±0.66	22.14±0.99	19.79±0.66	24.35±1.09	19.13±0.59
DSS	24.29±0.72	20.77±0.62	25.05±0.64	21.09±0.46	22.22±0.83	19.62±0.63
API +DSS	24.47±0.67	22.70±0.56	24.69±0.63	20.87±0.60	20.89±0.92	21.22±0.50

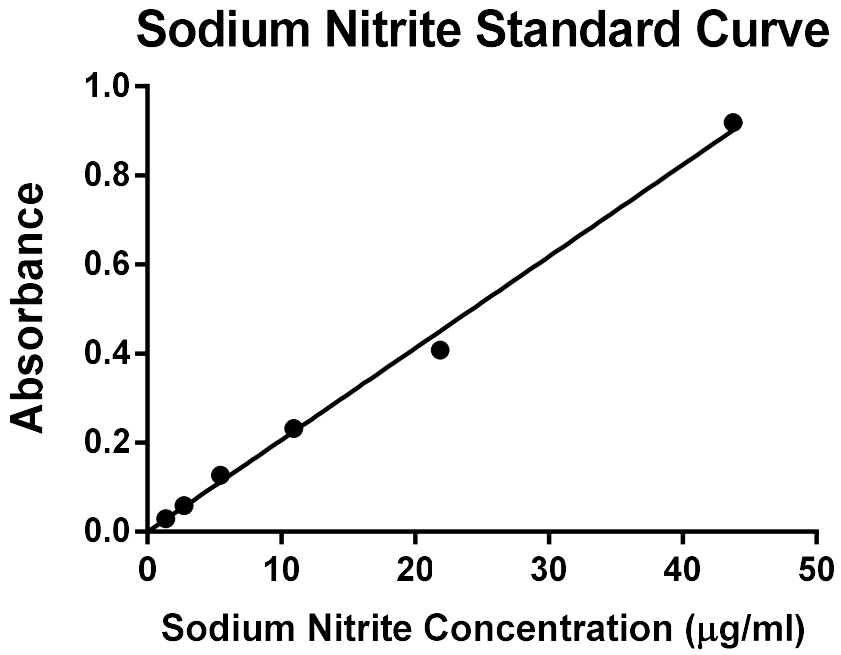
Appendix 1c: Table of Results of biochemical assays

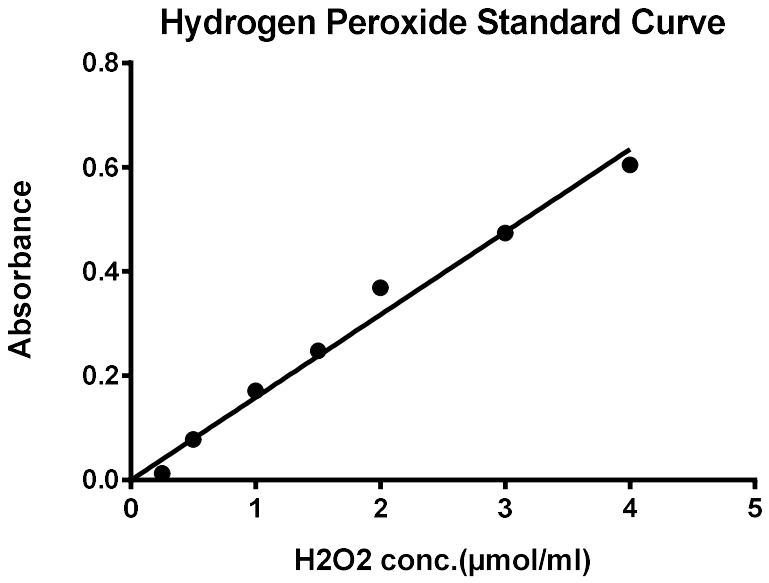
PARAMETERS	SEX	CONTROL	APIGENIN	DSS	API+DSS
Catalase activity	MALE	2.21±0.16 *	3.07±0.27	2.64±0.31	2.32±0.31
	FEMALE	3.30±0.29 *	2.79±1.90	3.15±0.47	2.37±0.26
SOD Activity	MALE	3.27±0.36	3.48±0.50	3.53±0.34	2.75±0.32
	FEMALE	3.71±0.50	3.30±0.56	4.22±0.44	3.25±0.43
GST activity	MALE	115.32±14.63	202.69±36.05 [a]	133.46±17.20	136.53±10.87
	FEMALE	120.78±16.43	205.21±22.11 [a]	140.58±18.32	118.35±19.303 [e]
GSH conc./mg protein	MALE	9.00±1.62	9.99±0.82	2.59±0.21 [a,b]	7.92±1.18 [c]
	FEMALE	7.26±1.10	6.72±1.13	6.11±0.81	4.65±0.51
PARAMETERS	SEX	CONTROL	APIGENIN	DSS	API+DSS
GPx activity	MALE	6.48±0.43	8.28±0.94	9.32±0.66	8.25±0.58
	FEMALE	9.45±0.91	8.51±0.74	11.34±0.79	9.02±1.10
PARA-METERS	SEX	CONTROL	APIGENIN	DSS	API+DSS
Lipid peroxi-dation/100 mg protein	MALE	7.23±1.42	7.03±1.11	7.60±0.88‡	5.47±0.71
	FEMALE	9.19±1.46	8.75±1.02	12.82±1.09‡	6.67±0.76 [f]
H_2O_2mM/ mg	MALE	44.21±2.36	41.71±2.98	36.31±4.48‡	38.81±3.32
	FEMALE	39.91±5.78	50.86±7.85	67.39±6.91[d,‡]	50.02±5.36

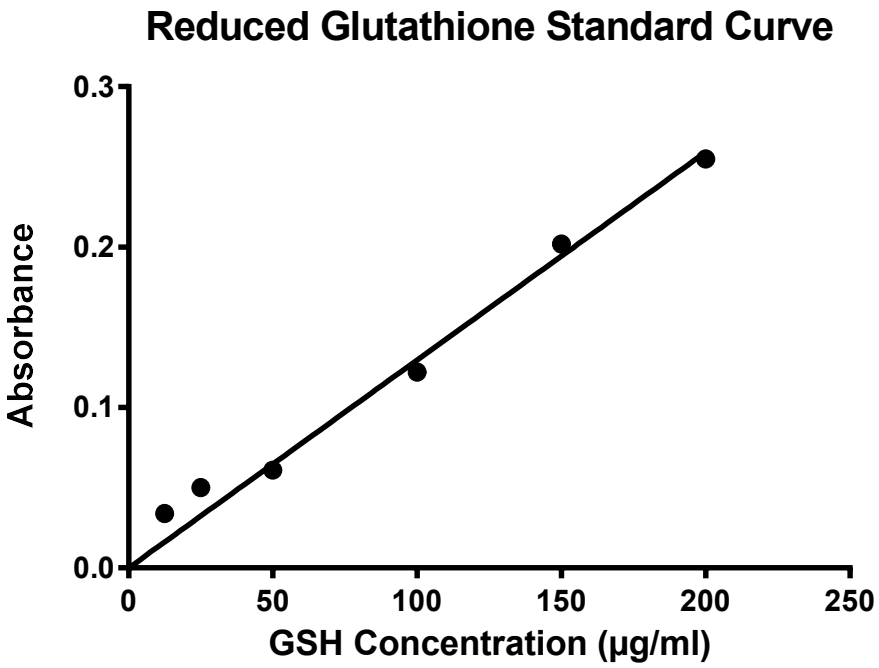
protein					
Myeloper-oxidase (U/µg protein)	MALE	6.26±0.58	6.36±0.67	4.44±0.26	7.39±0.96 [c,#]
	FEMALE	6.41±0.59	4.10±0.48	5.51±1.18	5.04±0.61 [#]
Nitrite ion conc.	MALE	0.61±0.07	0.47±0.05	0.45±0.09	0.31±0.04[a]
	FEMALE	0.59±0.05	0.58±0.08	0.46±0.02	0.48±0.07

Appendix 1d: Graphs of standards used for biochemical assays




Sodium Nitrite Standard Curve
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Sodium Nitrite Concentration (μg/ml)


Hydrogen Peroxide Standard Curve
Absorbance
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
H2O2 conc.(µmol/ml)


Reduced Glutathione Standard Curve
Absorbance
0.3
0.2
0.1
0.0
0
50
100
150
200
250
GSH Concentration (µg/ml)