TOXICOLOGICAL CHARACTERIZATION OF SODIUM METABISULFITE AND MITIGATION OF ITS EFFECTS BY STANDARDIZED *Ginkgo biloba* EXTRACT (EGb-761) IN A MOUSE MODEL

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DECLARATION

This dissertation is my original work and has not been presented in any other institution for a degree award or other qualification.

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DEDICATION

I dedicate this thesis to the Almighty God for the blessing of life and for keeping me strong and committed to the journey. Secondly I dedicate this thesis to my family for their unconditional support and finally I would like to dedicate this work to my friends who encouraged me throughout the journey.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
AST	Aspartate aminotransferase
CHD	Coronary heart disease
DSS	Dextran sulfate sodium
DTNB	Dinitrothiocyanatebenzene
EGb-761	Standardized *Ginkgo biloba*extract
ELISA	Enzyme-linked immunosorbent assay
GBPS	*Gingko biloba* polysaccharides
GGT	Gamma-glutamyl transferase
GSH	Reduced glutathione
H&E	Hematoxylin and eosin
HDL	High density lipoprotein
HGB	Hemoglobin
HRP	Horse radish peroxidase
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon-gamma
IL-10	Inter-leukin-10
iNOS	Inducible nitric oxide synthase
IPR	Institute of Primate Research
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion

MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MPV	Mean platelet volume
NF-Kb	Nuclear factor-kappa B
NO	Nitric oxide
PAD	Peripheral artery disease
PCT	Plateletcrit
PCV	Packed cell volume
PDW	Platelet distribution width
P-LCR	Platelet large cell ratio
PPM	Parts per million
RBC	Red blood cells
RDW-CV	Red cell distribution width coefficient of variation
RDW-SD	Red cell distribution width standard deviation
ROS	Reactive Oxygen Species
ROW	Relative organ weights
RT	Room temperature
$S0_2$	Sulphur dioxide
SMB	Sodium metabisulfite (SMB)
SO_3^{2-}	Sulfites
SO_4^{2-}	Sulfates
TBARS	Thiobarbituric acid reactive species
TNF-α	Tumor necrosis factor-α

WBC	White blood cells
WHO	World health organization

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ABSTRACT

Sodium metabisulfite (SMB), is a biocide and antioxidant agent generally used as a preservative in food and beverage industries, but can oxidize to harmful sulfite radicals. A standardized *Ginkgo biloba* (EGb-761) is well characterized, with 24% flavone glycosides (primarily quercetin, kaempferol and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B and C, and 2.6-3.2% bilobalide). Notably, Ginkgolide B and bilobalide account for about 0.8% and 3% of the total extract, respectively. EGb-761 has demonstrated potent antioxidant and anti-inflammatory activity, beneficial for the treatment of toxicants and diseases that exhibit oxidative stress and inflammation. The present study sought to investigate the putative ameliorative effects of EGb-761 against SMB-induced toxicity in mice. Thirty-two male Swiss white mice were randomized into control, SMB-treated, SMB + EGb-761-treated and EGb-761-treated groups. EGb-761 (100mg/kg/day) and SMB (98mg/kg/day) were administered by gastric gavage for 40 days. Body and relative organ weight, haematological profile, serum electrolytes and lipid profile, tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), IL-10, nitric oxide (NO),tissue malondialdehyde (MDA) and reduced (GSH) levels and organ damage and pathology have been estimated. Oral administration of EGb-761 restored SMB-induced decrease in body weight and prevented SMB-induced thrombocytopenia, leukocytosis and anaemia. Further, EGb-761-treatment protected against SMB-induced liver and kidney injury depicted by decreased serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, bilirubin, creatinine, urea, uric acid and albumin. Furthermore, EGb-761 treatment attenuated SMB-driven dyslipidemia and metabolic acidosis. Besides, EGb-761 supplementation abrogated SMB-driven oxidative stress as depicted by stabilized GSH levels in the brain, liver, kidney, spleen, heart and lungs. SMB induced a significant increase of tissue levels of MDA, NO, IFN-γ and TNF-α were abrogated by EGb-761 treatment. Histopathological analysis revealed that exposure to SMB resulted in liver and kidney damage. It was noted that EGb-761 nullified those adverse pathological lesions. In conclusion, these results demonstrate that oral administration of standardized *Ginkgo biloba*attenuated SMB-induced alteration of hematological parameters, metabolic acidosis, inflammatory responses, oxidative stress and organ damage. These findings provide a novel approach that can be optimized for preventing or treating exposure due to SMB toxicity.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Sodium metabisulfite (SMB) is an inorganic chemical widely used as a preservative in the food, beverage and pharmaceutical industries due to its ability to stop the growth of microorganisms and its antioxidant properties (Zhang *et al.,* 2015). It is a sulfating agent that reacts with water to release toxic sulphur dioxide ($S0_2$) when ingested or inhaled. Sulfites can be produced endogenously through the degradation of sulphur-containing amino acids such as cysteine and methionine or they can be obtained externally through food, drink, medicine or from the environment by inhalation of polluted air (El-Kadi *et al.,* 2014). The toxicity of sulfites is mitigated in vivo by sulfur oxidase that converts sulfites (SO_3^{2-}) to sulfates (SO_4^{2-}) (Cabre *et al.,* 1990). The established and acceptable daily intake of ingested sulfites expressed as $S0_2$ equivalents is 0.7 mg/kg body weight (Nair *et al.,* 2003). The toxic effects associated with SMB include male infertility (Zare *et al.,* 2019), pneumonitis (Sack *et al.,* 2023), increased lipid peroxidation (Elmas *et al.,* 2005), neurotoxicity (Noorafshan *et al.,* 2013), alterations in immunological, biochemical and hematological parameters (El-Kadi *et al.,* 2014), cytotoxic to cells and damage to the heart (Yoo *et al.,* 2018; Zhang *et al.,* 2015).

Usage of SMB within the recommended concentrations is usually safe but it becomes toxic when used in excess or due to prolonged exposure. At higher concentrations, SMB acts as a prooxidant (Elmas *et al.,* 2005) and prolonged exposure may lead to deleterious effects on biological systems causing organ damage such as hepatotoxicity, nephrotoxicity, coronary artery disease, brain edema and dementia (Sack *et al.,* 2023). The sulphates generated from sulphites are

oxidants that are converted to reactive oxygen species and other $S0_2$ free radicals responsible for various adverse effects (Vally & Misso, 2012).

From literature review, it is evident that the concentration of SMB is not regulated in most countries and most flesh food vendors have the temptation to use copious amounts in order to extend the self-life of food. In Kenya, however, this preservative is still in use in butcheries and supermarkets despite the health hazards it poses. Particularly, tests carried out by Kenya bureau of standards, revealed that over 98 mg/kg of sodium metabisulphite were found in the minced meat bought in a city supermarkets. By international standards, fresh meat is not supposed to contain any preservative. Therefore, there is a need for detailed studies to evaluate and characterize the negative physiological and biochemical process affected by SMB in humans to enable diagnostic and forensic determination of exposure.

Gingko biloba is a famous herbal medicinal plant that is cultivated because of its immense bioactive substances (Belwal *et al.,* 2019). Gingko leaf extracts (EGb) have wide applications in alternative medicine, complementary medicine, food and dietary supplements (Belwal *et al.,* 2019). The diverse EGb bioactive compounds include a variety of terpenoids, flavonoids, bioflavonoids, lignans and organic acids (Noor-E-Tabassum *et al.,* 2022; Xiong *et al.,* 2021; Biernacka *et al.,* 2023; shu *et al.,* 2020). A standardized *Ginkgo biloba* leaf extract (EGb 761) has been shown to contain multiple bioactive substances (Xie & Lu, 2022). This standardized extract is by far among the most commonly used herbal medicine (Noor-E-Tabassum *et al.,* 2022). Its composition is majorly flavonoids and terpenoids including 22-27% ginkgo flavonoids mainly quercetin, kaempferol, and isorhamnetin, 5-7% terpene lactones of 3-4% ginkgolides A, B, and C, 2.6 – 3.2% bilobalide and ginkgolic acid (<5ppm) (Smith *et al., 1*996). The EGb-761 is used for the treatment and management of neurological and cardiovascular diseases, its use has

shown beneficial results in Alzheimer, dementia and ischemic stroke (Cui *et al.,* 2023; Vellas *et al.,* 2012).

EGb-761 possess powerful antioxidant properties that neutralizes oxygen free radicals, the major cause of neurodegenerative diseases and aging (Olofunmilayo *et al.,* 2023). In addition, EGb-761 can mitigate against lipid peroxidation by acting as a free radical scavenger, and is able to reduce inflammation in diseases such as arthritis, irritable bowel syndrome, cancer and heart diseases (Mahitra *et al.,* 1995). This is achieved via the reduction of inflammation by inhibiting the transcription of genes responsible for inflammatory responses and histamine release (Olofunmilayo *et al.,* 2023). Besides, accumulating evidence suggests that *Ginkgo biloba* works by inhibiting inflammatory mediators like NO, TNF-α, and inducible nitric oxide synthase (iNOS) (Abdel-Emam & Abd-Eldayem, 2022; Wadsworth & Koop, 2001; Ilieva *et al.,* 2004). Further, ameliorative effects of EGb-761 against lead and fluoride-induced toxicity have been documented (Raju *et al.,* 2020; Amjad *et al.,* 2013). These previous findings informed our use of *Ginkgo biloba* in this study, given that organ damage, inflammation and oxidative stress are some of the features associated with SMB toxicity. Therefore, in the present study, the effects of EGb-761 treatment on various toxicities initiated by SMB exposure in mice were evaluated.

1.2 Problem Statement

Consumption of SMB contaminated food has undesirable health implications this is because its metabolic by-products are implicated in infertility, pneumonitis, oxidant damage, neurotoxicity alterations in immunological, biochemical and hematological parameters, cytotoxic to cells and damage to the heart (Yoo *et al.,* 2018; Zhang *et al.,* 2015; Zare *et al.,* 2019; Elmas *et al.,* 2005).

A survey done in Kenya in 2018 indicated that over 98 mg/kg of SMB residue was present in minced meat sold at various butcheries and supermarkets in Nairobi. These amounts are ten

times above the world health organization (WHO) recommendations. Despite the reported high dosages of SMB on meat, information on its toxicity and associated deleterious effects on human health remains scant. The negative physiological and biochemical processes affected by this dosage of SMB exposure have not been determined to enable diagnostic and forensic determination of exposure, as well as allow development of detoxification or treatment strategies. Due to induction of inflammatory responses, oxidative stress and alteration of physiological and biochemical parameters by SMB, it is imperative to assess if antioxidants and anti-inflammatory agents such as EGb-761 can ameliorate toxicity due to SMB. EGb-761 is a potent anti-inflammatory and anti-oxidative agent. However, the role of EGb-761 in ameliorating deleterious events due to SMB exposure remains elusive.

1.3 Justification

Consumption of high levels of SMB can lead to life-threatening effects since it has previously been associated with deleterious effects on biological systems such as organ damage i.e. hepatotoxicity, nephrotoxicity and edema in brain tissue. Understanding the impact of sodium metabisulfite driven toxicity in a mouse model is vital. Therefore, appropriate efforts must be instituted that are based on credible scientific evidence in regard to the adverse effects due to excess application of SMB on food staff.

Furthermore, the evaluation of the attenuative effects by EGb-761 which is an antioxidant, anti-inflammatory, anti-apoptotic and immuno-modulatory agent during SMB exposure is of critical importance. Data from this study will enable the generation of a toxicological profile due to SMB exposure and possible evidence of the use of EGb-761 as an ameliorating agent in SMB exposure. The toxicological profile can be used for immediate diagnosis for individuals exposed

to SMB for timely administration of the necessary therapy. The data will also help in the development of policy on the safe use of SMB and management of SMB exposure.

1.4. Research questions

1. What are the effects of sodium metabisulfite exposure on the host physiological, lipid and hematological profile?
2. What is the impact of sodium metabisulfite on oxidative events, organ damage and inflammation?
3. What is the impact of *Ginkgo biloba* on sodium metabisulfite-induced toxicity?

1.5 Objectives

1.5.1 General objective

To characterize toxicological effects of sodium metabisulfite and mitigation of its effects by standardized *Ginkgo biloba* extract in a mouse model

1.5.2 Specific objectives

1. To evaluate the impact of sodium metabisulfite driven alteration of host physiological, lipid profile and hematological changes
2. To decipher the putative effects of sodium metabisulfite driven oxidative events, organ damage and inflammation
3. To elucidate the impact of *Ginkgo biloba* against sodium metabisulfite driven toxicity

CHAPTER TWO

LITERATURE REVIEW

2.1 Sodium metabisulfite

Sodium metabisulfite (SMB) is an inorganic compound also known as sodium pyro-sulfite, di-sodium salt, di-sodium sulfite, sodium bisulfite or sodium sulfite anhydrous, and is the commonly produced salt of sulfurous acid (Elmas *et al*., 2005). It is crystalline and smells like a rotten egg or has a smell like that of sulfur. It is yellow in color and its chemical formula is $Na_2S_2O_5$ with a molar mass of 190.107 g/mol. It has a density of 1.48 g/cm^3, and a melting point of 170^{0}C but its decomposition starts at 150^{0}C.The salt is soluble in glycerol and partially soluble in ethanol. Its solubility in water increases with increasing temperature although when dissolved in water it produces bisulphate or sulfur dioxide gas (toxic) and this makes the aqueous solution acidic in nature (Zhang *et al.,* 2015)

2.2 Uses of sodium metabisulfite

2.2.1 Food preservative

Sodium metabisulfite is mainly used in food preservatives as it prevents the browning of food and also prevents the normal oxidation of microorganisms hence hindering the microorganism's reproduction in food (Zhang *et al.,* 2015). It is more effective against gram-negative than gram-positive bacteria and is more effective at low pH. Nevertheless, countries such as Australia and New –Zealand use the recommended dosage of 10mg/ kg of meat, while in the UK the SMB is only permitted in certain food staff but can cause significant loss of vitamins such as thiamin in food (Gerhard *et al.,* 2006). In the US the use of SMB was banned in 1986 by the US Food and Drug Authority as it was linked with 13 deaths and it's referred to as a silent killer (Vally & Misso, 2012).

In Kenya, SMB is hideously widely used in butcheries and supermarkets despite the related health hazards that it poses. It is mostly used in periods when business is low especially at the beginning of the year to aid in the preservation of meat for up to three months. It is usually mixed with water and sprinkled on meat to give it a deep red color and a fresh look to attract customers. Sodium metabisulfite is also used in snack crackers in dough modification to make them tender. Despite this known illegal application of SMB on meat, the detrimental effects associated with the consumption of such food staff especially in Kenya are yet to be investigated.

2.2.2 Fungicide and Antimicrobial

Sodium metabisulfite is used as an anti-fungal and anti-bacterial during the shipment of goods such as shoes and clothing. It is also used in plastic stickers and packaging to prevent fungal and bacterial growth (Ohara *et al.,* 2020). Moreover, it is applied in the destruction of cyanide in commercial gold cyanidation, prevents shrinking of garments after washing since it's added to textiles, and acts as a solvent during starch extraction from tubers and testing of sickle cell anemia in blood smears. It is also used in photographs and also in the removal of tree stamps by causing the degradation of lignin in stamps enhancing the removal of the stamps (Torabian *et al.,* 2017). Sodium metabisulfite is used as a sanitizer in juices and wines before fermentation as it kills all the harmful microbes in the juices and wines. It is added before fermentation to prevent it from killing the yeast that aids in fermentation. It is also used to sanitize equipment (Kamel *et al.*, 2015).

2.3 Effects of sodium metabisulfite-induced toxicity

Sodium metabisulfite has been associated with many biological deleterious effects. An avalanche body of evidence has shown that exposure to SMB results in strong allergic reactions that lead to the triggering of asthma through the induction of broncho-constriction with concomitant

wheezing and coughing when inhaled (Shekarforoush *et al.,* 2015). Exposure to elevated levels of SMB has also been associated with male infertility as Sulphur dioxide levels increase in the testis of male mice, the lactate dehydrogenase activities increase hence reducing the protein levels, testosterone, and total number of spermatogonia, primary spermatocyte and spermatids (Shekarforoush *et al.,* 2015). Further, SMB causes molecular oxidation to form sulfite radicals which form lipid alkyl radicals when sulfites react with lipids (Yoo *et al.,* 2018). These radicals cause oxidative stress in the body which causes serious damage. Molecular oxidation leads to lipid peroxidation that causes tissue injuries apoptosis and necrosis and DNA damage leading to mutations hence can trigger cancer (Ercan *et al.,* 2015).

In addition, SMB has been associated with increased biochemical parameters such as urea, creatinine, calcium, uric acid and transaminases (Aslam, 2022). Besides, a decrease in immunoglobulin and the levels of hemoglobin, red blood cells and white blood cells have been reported (Elkadi *et al*., 2014). Increasing piece of evidence has also demonstrated that exposure to sodium metabisulfite causes physiological changes in the body such as splenomegaly, hepatomegaly, stomach inflammation and also kidney enlargement (Ercan *et al.,* 2015). It is very clear from the foregoing that the toxic effects of sulfite-free radicals toxic on neuronal cell lines are the major underlying causes of neurological diseases such as Alzheimer's and Parkinson's (Cabre *et al.,* 1990). Indeed, neuronal cell lines are associated with memory storage and once damaged they cause memory loss. Furthermore, SMB has also been associated with a decrease in body weight, an increase in the total protein concentration and lactate dehydrogenase activity in the bronchoalveolar lavage fluid and metaplasia of the squamous cell when inhaled (Lai *et al.,* 2018).

2.4. *Ginkgo biloba*

Ginkgo biloba is a native tree species in China. It's the last species of the order Ginkgoales which is referred to as the "living fossils. This tree has been used largely in traditional medicine as it was associated with neuroprotection effects amongst other therapeutic benefits. *Ginkgo biloba* leaf extract (*EGb-761*) (Fig. 2.1) was and is still widely used as an herbal supplement in traditional Chinese medicine. The tree is dioecious with separate sexes with some trees being male and others female. Fertilization of ginkgo seeds occurs through motile sperms like in ferns. Its seasonal fan-shaped leaves, and leathery leaf blade that is dissected with veins that are dichotomous. It has yellow seeds on a long stalk surrounded by sarcotesta and has a smell like that of rancid butter (Belwal *et al.,* 2019). Roasted seeds of Gingko are edible as traditional Chinese medicine. They are used in the management of diseases such as bronchitis, renal dysfunction, anti-inflammation and bladder disease (Strain *et al.,* 2019). Ginkgo has a large genome of about 10.6 billion nucleotide base pairs and this makes it have a considerable number of antimicrobial and other chemical defense mechanisms. Previous studies have reported *Ginkgo biloba* polysaccharides molecules to have different biological actions such as anti-oxidation, anti-inflammation, immunomodulation, anti-tumor and therapeutic values in cardiovascular and ischemic diseases. They have also been implicated in memory loss and cognitive disorders, cancer pain, metabolic syndrome, thrombotic disorders and sexual enhancement. A recent study has shown consistent antiviral activity (Ibrahim *et al.,* 2021).

Figure 2.4: Gingko biloba leaves

2.4.1 The standardized *Ginkgo biloba* leaf extract (EGb- 761)

The proven role of EGb-761 against cerebral and peripheral disorders of blood flow and cerebral atherosclerosis is well documented (Zeng *et al.,* 2018). The use of *Ginkgo biloba* as an herbal supplement especially due to its enrichment with chemical substances that are not known in other living things is well-accepted in literature (Smith *et al.,* 1996). EGb-761 is known to contain 6% terpene lactones (ginkgolides A, B and C, and bilobalide) and 24% flavones (isorhamnetin, kaempferol and primarily quercetin) being the largest group of active compounds (Smith *et al.,* 1996). Other constituents include glucose, D-glucaric, proanthocyanidins, organic acids rhamnose and gingolic acids (Goktaş *et al.,* 2008).

2.4.2 Pharmacological action of EGb-761

2.4.2.1 Antioxidant activity

The EGb-761 act as an antioxidant by directly scavenging the free radicals and thus neutralizing them in the cellular environment (Rahal *et al.,* 2014). Free radicals are highly reactive oxygen species that can react with biological molecules causing apoptosis and necrosis leading to tissue and organ injury. Oxidative stress is the major cause of neurodegenerative diseases and accelerated aging (Tewari *et al.,* 2017; Nowak *et al.,* 2021). The EGb-761 constituent's ginkgolides are known to provide inhibitory effects on lipid peroxidation (Derin *et al.,* 2006). It

also acts as an antagonist platelet aggregation factor, a factor that initiates the generation of reactive oxygen species flavonoids glycosides which reduces the formation of lipoprotein oxidation which is accompanied by the reduction in the formation of atherosclerosis and vascular injury (Kaur *et al.,* 2018). It also acts as a free radical scavenger (Zhou *et al.,* 2017).

Moreover, *Ginkgo biloba* is known to contain high levels of terpenoids, flavonoids, glycosides and terpene lactones which are compounds known for their strong antioxidant effects (Zhou *et al.,* 2017).

2.4.2.2 Anti-inflammation

The inflammation process is part of the body's natural response to injury or invasion by a foreign substance. The inflammatory responses include the production of various components such as pro-inflammatory cytokines and interleukins, which are recruited directly or indirectly to fight against the immunogens or to facilitate healing of the injured area. However, excessive inflammatory response can cause permanent damage to the body's tissues and DNA. A growing body of evidence has shown that ginkgo extract can reduce markers of inflammation in both human and animal cells in a variety of disease states including arthritis, irritable bowel syndrome, heart disease and stroke (Pizza *et al.,* 2019; Ilieva *et al.,* 2004). The extract reduces inflammation by inhibiting the transcription of genes responsible for inflammatory responses (Abdel-Salam *et al.,* 2004).

2.4.2.3 *Gingko biloba* and neuronal protection

Reduction of neuronal injury by EGb-761 extract has also been observed against cerebral ischemia, hypoxia, and animal models of amyotrophic lateral sclerosis (Cui *et al., 2023*; Vellas*et al.,* 2012). The neuroprotective impact of EGb-761 has been demonstrated to be mediated by ginkgolides (1-100 M in vitro or 50-100 mg/kg in vivo), bilobalides (25-100 M in vitro or 10

mg/kg in vivo), and, in some circumstances, flavonoids (25-100 g/ml in vitro or 40-100 mg/kg in vivo) (Singh *et al.,* 2019). Further, administration of EGb-761 in rat model with ischemic brain injury induced by middle cerebral artery occlusion (MCAO) improved neurological deficits through modulation of the proteins peroxiredoxin-2, protein phosphatase 2A subunit B, collapsing response mediator protein 2, adenosylhomocyteinase, pyruvate kinase isoenzyme, and isocitrate dehydrogenase has been observed (Sung *et al.,* 2012).

Administration of *EGb-761* in mouse model of AD attenuated the loss of synaptic structure proteins PSD-95, Munc18-1, and SNAP25, and inhibited β-secretase activity and Aβ aggregation. Notably, *EGb-761* extract protects against rat models of experimental autoimmune encephalomyelitis in bone marrow-derived mesenchymal stem cells (Mahitra *et al.,* 1995). In addition, EGb-761 provided neuroprotection by alleviating demyelination and axonal loss and reducing serum concentrations of the phosphorylated neuro-filament heavy chain, tumor necrosis factor-α and interferon-γ (Hao *et al.,* 2016).

2.4.2.4 Impact of EGb-761 in ulcerative colitis and Alzheimer's disease

Administration of EGb-761 in a dextran sulfate sodium (DSS) mouse model of ulcerative colitis showed that the EGb-761 abated inflammation in vitro by modulating the pro-inflammatory markers iNOS, Cox-2 and TNF-α, p53 and p53-phospho-serine 15 (Kotakadi *et al.,* 2008).

The EGb-761 has been reported to ameliorate the effects of Alzheimer's disease (AD), a most common progressive human neurodegenerative disease among the elderly worldwide (Cui *et al.,* 2023). The characteristic effect of AD dementia is the progressive death of brain cells resulting in a deterioration in cognitive ability and daily functioning (Breijyeh & Karaman, 2020). Many pathophysiological pathways, including aberrant protein metabolism, the development of amyloid plaques that harm neuronal cells, inflammatory reactions, and oxidative stress, have

been explored as potential causes of AD. Phyto-polyphenolic compounds have shown promise in the development of effective AD drugs and therapeutics. For instance, studies have shown that *Gingko biloba* extract protects against beta-amyloid plaques formation, the hallmark of AD, by obstructing Aβ-induced events including oxidative stress leading to ROS (Reactive Oxygen Species) production (Singh *et al.,* 2019).

2.4.2.5 EGb-761 in cardiovascular diseases

Cardiovascular diseases are a complex of many cardiovascular and heart-related diseases including endocarditis, rheumatic heart disease, coronary heart disease (CHD), cerebrovascular disease, peripheral artery disease (PAD), and aortic atherosclerosis (Lopez *et al.*,2022). In vitro, studies have demonstrated that EGb-761 can modify the liver's blood cholesterol levels by modulating the development of Apo lipoprotein B and LDL receptors (Noor-E-Tabassum *et al.,* 2022). In a study investigating the cardio protective effect of Gingko biloba extract, administration of *Gingko biloba* extract re-established autonomic imbalance in the heart by activating cholinergic signaling in a rat model of isoproterenol-induced cardiac hypertrophy (Mesquita *et al.,* 2017). However, a study investigating the potential of Gingko extract to prevent cardiovascular disease showed no significant effect of the extract on cardiovascular disease mortality and events (Kuller *et al.,* 2010). Therefore, there is a need to investigate further the roles played by individual components of EGb-761 to better understand its potential role in the management of various heart and cardiovascular diseases.

2.4.2.6 EGb-761 in hypertension and chronic kidney disease

Hypertension is one of the most significant causes of chronic kidney disease, a condition marked by a long-term decrease in kidney function and cumulative damage. End-stage renal disease, a progressive and irreversible loss of kidney function that is fatal without dialysis or kidney

transplantation, is further exacerbated by chronic kidney disease. Studies investigating the potential role of EGb-761 on hypertension showed that no significant difference exists between test and placebo groups of elderly individuals with hypertension (Brinkley *et al.,* 2010). However, when used as an adjuvant therapy against hypertensive nephropathy, as compared with using antihypertensive drugs alone, combined treatment of EGb-761 and dipyramidole with antihypertensive drugs decreases 24-hour urinary total protein, blood urea nitrogen and serum creatinine (Jialiken *et al.,* 2021).

2.4.2.7 Immunomodulatory effects of EGb-761

The inflammatory immune response is an excessive systemic immune response induced by inflammatory immune cells leading to undesirable downstream effects on cellular and organ integrity (Ferrero-Miliani *et al.,* 2007; Medzhitov, 2010). Immuno-modulators regulate immune function by ensuring that the immune responses are in check. Immunomodulation can either take natural or drug-induced forms. *Gingko biloba* polysaccharides have been examined against different diseases in animal models of disease and human disease to identify their role in modulating immune responses (Kaur *et al.,* 2018). *Gingko biloba* polysaccharides (GBPS) particularly exhibit enhanced immunological and anticancer effects (Massieu*et al.,* 2004). Ginkgolides have been shown to have anti-platelet-activating, anti-apoptotic, anti-oxidative, neurotrophic, and neuro-immunomodulatory properties in numerous studies by inhibition of Nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways (Liu *et al.,* 2023). In a study investigating immune stimulatory effects of *Gingko biloba* on oxidative stress and toxicity induced by the organophosphate insecticide diazinon, EGb-761was shown to decrease peroxidase activity, immunoglobulin activity, and lysozyme activity in fish exposed to diazinon (Hajirezaee *et al.,* 2019).

2.4.2.8 Protective role of EGb-761 against heavy metal toxicity

Ginkgolides have been shown to have neuro-immunomodulatory properties in numerous studies by inhibition of Nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways driven by toxicants (Li *et al.,* 2020). In a study investigating the immune-stimulatory effects of *Gingko biloba* on oxidative stress and toxicity induced by the organophosphate insecticide diazinon, EGb-761 was shown to decrease peroxidase activity, immunoglobulin activity, and lysozyme activity in fish exposed to diazinon (Hajirezaee *et al.*, 2019).

Contamination of food, water, and the environment by heavy metals has been an undesirable outcome of the industrialization process. Since the beginning of human activities such as mining, which started in pre-historic times, such metals have found their way closer to both human and animal living environments. Studies into the effects of heavy metal poisoning such as mercury, lead, chromium, cadmium, and arsenic have yielded important information on how to better avoid such contaminations. However, other sources of heavy metal poisoning include the use of old building materials still being used in water pipes. In Pakistan, for instance, one of the major causes of blood lead levels in children as of 2002 was leaded petrol (Kadir *et al.*, 2008). EGb-761 has previously been hypothesized to alleviate heavy metal poisoning. In animal models, it has been shown to alleviate lead poisoning (Amjad, 2020). Other studies have shown that EGb-761 can protect against mercury-induced oxidative damage in rats by mitigating oxidative stress in the brain, lung, liver, and kidney tissues (Amjad *et al.,* 2020). Furthermore, treatment with EGb-761 was observed to significantly reverse the cellular levels of GSH and malondialdehyde levels (Abdel-Emam & Abd-Eldayem, 2022).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical Statement

The experimental guidelines and procedures pertaining to the use of mice were strictly observed and approval was obtained from the Faculty Biosafety, Animal use and Ethics committee of the University of Nairobi. Humane endpoints such as respiratory distress, unarousable coma, decerebrate rigidity, pouting, convulsions, retinal haemorrhages, and dysconjugate gaze were observed throughout the study to lessen animal suffering. This research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, as stipulated in the Institutional Animal Care and Use Committee (IACUC) and the ethical review committee for the use of laboratory animals. All the protocols concerning the use of mice were sought and approved by the Faculty Biosafety, Animal use and Ethics committee of the University of Nairobi (REF: FVM BAUEC/2022/351).

3.2 Animals and experimental design

This study utilized female Swiss white mice. Six to eight weeks-old female Swiss white mice were purchased from Biotechnology Research institute-Muguga. The experimental work and design for this study was based on the rules and guidelines of animal husbandry (Kilkenny *et al.*, 2010).The present study employed one control (naïve) group of mice and three treatment groups. Briefly, thirty-two (32) healthy female Swiss albino mice (6-8 weeks old) were randomly allotted into the four groups; each group containing 8 mice. Group 1 served as control and received distilled water and mice pellets. Group II mice received 98 mg/kg/day of sodium metabisulfite (SMB). Group III mice received 100 mg/kg/day of standardized *Ginkgo biloba* (EGb-76) and 98 mg/kg/day of SMB. EGb-76 is a well characterized and standardized extract of *Ginkgo biloba*

leaves that contains 24% flavone glycosides (primarily quercetin, kaempferol and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B and C, and 2.6-3.2% bilobalide). Notably, ginkgolide B and bilobalide constitute approximately 0.8% and 3% of the total extract, respectively (Clostre, 1999). Group IV mice received 100 mg/kg/day of EGb-761 only. The mice were exposed to the treatments through oral administration using gastric gavage for 40 days. The animals were housed in sterile plastic cages under a controlled room temperature of 23-25℃ and a 12-hour light/dark cycle and allowed to acclimatize for one week before the start of the experiments. The mice were fed on pellets (Unga feeds, Kenya) and had access to clean water ad libitum.

3.3 Preparation of sodium metabisulfite and *Ginkgo biloba*

Sodium metabisulfite 98 mg/kg/day (Sigma Aldrich, St Louis, MO) and standardized *Ginkgo biloba* extract (EGb 761) (eCRATER USA) were prepared fresh daily by dissolving them in sterile distilled water. The choice of 100 mg/kg/day dosage of EGb-761was based on previous studies that showed potentiation of protective effects against lead-induced toxicity (Asiwa *et al.*, 2023; Yallapragada & Velaga, 2015; Asiwa *et al.*, 2022). The treatments were administered for 40 days orally by a gavage.

3.4 Determination of the body weights and relative organ weights (ROW)

The live body weights of animals from each experimental group were measured after every three days throughout the experimental period. Subsequently, the relative organ weight of each individual organ was determined by dividing the organ weight by the final body weight and then expressed as a percentage. The body and organ weights measurements were done using an analytical electronic balance (Mettler PM34, DoltaRange®).

3.5 Euthanization of mice and sample collection and preparation

After 40 days post-treatment mice were sacrificed through euthanization with ketamine (50 mg/ml) and xylazine (100 mg/ml) (Merck KGaA, Darmstadt, Germany) in a ratio of 4:1 through intramuscular injection. Humane endpoints were monitored to limit suffering. A sick animal with clear health problems like unarousable coma, retinal haemorrhages, dysconjugate gaze, pouting, decerebrate rigidity, respiratory distress and convulsions prior to treatment and infection were euthanized by cervical dislocation under isoflurane. Blood samples were collected intra-cardially from individual mice and placed in heparinized tubes for complete hemogram analysis and for biochemical analysis, blood was collected in sterile Eppendorf tubes. To obtain serum, blood in the Eppendorf tubes was left to settle for one hour at normal room temperature and centrifuged at 10000 rpm at 4°C for 5mins (Centurion Scientific Ltd. K240R, UK). Mice were perfused with sterile PBS buffer after which spleens, kidneys, livers, lungs, hearts, and brains were harvested and placed in Eppendorf tubes that were under dry ice. Snap-frozen whole brains, kidneys, heart, lungs, spleen, and liver were homogenized on ice-cold water (4ºC) in 0.5 ml of 0.25 M sucrose, 5 m MHepes-Tris, pH 7.4, with protease inhibitor cocktail to a final concentration of 10% (w/v).

3.6 Hematological determination, electrolytes, liver and kidney function and lipid profile analysis

Blood was collected through cardiac puncture and placed in EDTA tubes at the end of the treatment period to analyze the hematological parameters for each experimental mouse. This was done using hematology autoanalyzer (Sysmex XS 1000i Hematology Analyzer, WA, USA). The parameters generated from the hemogram include; Total Red Blood Cell count and red cell indices, Total White Blood cell and differential count, and total platelet count and related thrombocytic parameters. For serum analysis, blood samples were left to settle at room

temperature for 1 hour and centrifuged at 10000 rpm at 4^0C for 5mins. Serum (supernatant) collected was stored at -20^0C.

Serum levels of liver enzyme markers: Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), direct and total bilirubin, creatinine, urea, uric acid and albumin. For lipid profile, the levels of total cholesterol, high density lipoprotein (HDL) and triglycerides were estimated. Furthermore, electrolytes (sodium, potassium and chloride), total proteins and immunoglobulins were assayed using an automatic analyzer (Integra 400 plus analyzer, Roche Diagnostics).

3.7 The Cytokine assays

Serum levels of pro-inflammatory cytokines TNF-α and IFN-γ, and IL-10 anti-inflammatory cytokine were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (Thermo Fischer Scientific, California, USA). The ELISA-kits were used according to the manufacturer's detailed protocol. Briefly, Corning Costar 9018 ELISA plates were coated with 50µl/well of capture antibody in coating buffer. The plates were sealed with para-film and incubated overnight at 4°C. The wells were then aspirated and washed three times with 250 µl/well wash buffer. In order to increase effectiveness of the washes, the plates were allowed to soak for about one minute. The plates were blotted on absorbent papers in order to remove any residual buffer. The wells were blocked with 200 µl/well of 1X Assay diluent then the plates were incubated at room temperature for 1 h. Standards were prepared by diluting the standard antibody with 1X assay diluent and then a two-fold serial dilution was performed of the top standards to make the standard curve for a total of 8 points. Then 100 µl/of the samples was added to the appropriate wells. The plates were sealed and then incubated at room temperature (RT) for 2 h (or overnight at 4°C for maximal sensitivity). The plates were further washed 3-5 times, after which

approximately 100µl/well of detection antibody was added to each micro-plate well followed by incubating the plate at room temperature for 1 h. The plate was washed 3-5 times by aspiration. After aspiration, Avidin-HRP was added; followed by incubating the plate at RT for another 30 min and then washed. Finally, the substrate solution was added to each well plate, and then the plate was incubated at room temperature for 15 min which was the sufficient time that was required for the reaction to occur. The reaction was stopped by additional of 50 µl of stop Solution (Conc H_2SO_4). The ELISA optical reader (Multiskan ex-355, Thermo Electron Corporation, Waltham, Massachusetts, USA) was used to measure the absorbance that was set at 450nm

3.8 Quantification of nitric oxide in plasma using Griess assay

The blood was collected intra-cardially after 40 days post-treatment. Nitric oxide (NO) was quantified as nitrite. Nitrates were reduced to nitrites by enzymatic conversion by nitrate reductase (SIGMA). The level of nitrites was determined by the Griess method. Briefly, Griess reagent was prepared by mixing equal volumes of components A (N-(1-naphthyl) ethylenedi-amine) and B (sulfanilic acid). Approximately 10 µl of freshly prepared Griess reagent was then mixed with 75 µl of sample/Aqua dest. Blank or standard (serial dilution 10 - 1 µM) and 65 µl of Aqua dest, to a total volume of 150 µl. The mix was incubated for 30 min in the dark. NO production was then quantified by measuring colour change at 548nm.

3.9 Assessment of malondialdehyde (MDA) levels

To determine lipid peroxidation levels in murine brains, liver, serum, kidney, spleen, heart and lungs, malondialdehyde levels were measured by assays of thiobarbituric acid reactive species (TBARS) (Draper and Hadley, 1990). The organs were homogenized in cold phosphate buffer, pH 7.4 with BHT (final concentration 0.2%). Briefly, the organs homogenate samples (0.5 ml)

were mixed with equal volume of thiobarbituric acid 0.67% (Sigma Chemical, St. Louis, MO) and then heated at 92-96^{o}C for 30 min. Thiobarbituric acid reactive species production was then quantified at 535nm using a spectrometer.

3.10 Reduced glutathione (GSH) assay

Glutathione standard of 200 µmol/l was prepared in 0.5% sulphosalicylic acid (SSA). The standard control was prepared by two-fold serial dilution of 0.5% SSA in wells A1-12. This was done by dispensing 100µl of GSH standard with 0.5% SSA in A1-12. Then, a volume of 100µl of 0.5% SSA was transferred to A1 and mixed well. Next, 100µl of A1 contents was drawn and transferred to A2. This process was done up to well A12 and the remaining 100µl drawn from A12 well discarded. Then, a volume of 50µl of brain/liver/kidney/lungs/heart and spleen tissue homogenates was mixed with 50µl sulphosalicylic acid (5% w/v) and 0.25mM EDTA and centrifuged at 10000 rpm. The GSH standard (25µl) was then loaded in the first two columns of the plate, specifically wells B–H. Then, 25µl of the sample was loaded to the remaining wells. One hundred microliter of the substrate; the Elman's reagent (DNTB) was then added to each well. Absorbance was measured at 405nm using a multi-detection microtitre plate reader (Bio-Tek Synergy HT).

3.11 Histopathological analysis of the liver, brain and kidney

The Liver, brain and kidney samples were rinsed in phosphate-buffered saline, placed in a fixative (10% formalin) and then stored at room temperature until further analysis. The tissues were processed by dehydration in ascending grades (50%, 70%, 90%, 95% and 100%) of alcohol and embedded in paraffin wax in the automatic tissue processor. Sections of 5µm thickness were cut using a HM310 rotary microtome and mounted on Mayer's egg albumin-coated glass slides. Each section was de-waxed in two changes of xylene for two minutes, then rehydrated through descending grades of alcohol for thirty minutes (100%, 95%, 90%, 70%, and 50%) and further washed in tap water. Water was gradually removed from the tissues to prevent sudden shrinkage and rapture of the cells. The sections were then stained with hematoxylin and then stained with

eosin (1% for 2min). The sections were then dehydrated in ascending grades of alcohol for thirty minutes (70%, 80%, 95%, and 100%), cleared in three changes of xylene and mounted in DPX and examined microscopically (400X).

3.12 Statistical Analysis

Statistical analysis was done using the GraphPad Prism software package (Version 5.0). One-way ANOVA was done to compare the treatment groups with controls. For internal comparisons, Turkey's post hoc test was used. The results were given as a mean ± with significance set at $p<0.05$.

CHAPTER FOUR

RESULTS

3.1. Effects of SMB and EGb-761on body weight and organ weights

There was a progressive a progressive increase in the live mean weight across all the groups of mice up to 18 days post-treatment. However, an intermediary decrease in body weight was observed in mice orally administered with SMB relative to other groups of mice (Fig. 3.1). Notably, this decrease in general body weight was reversed by EGb-761 administration.

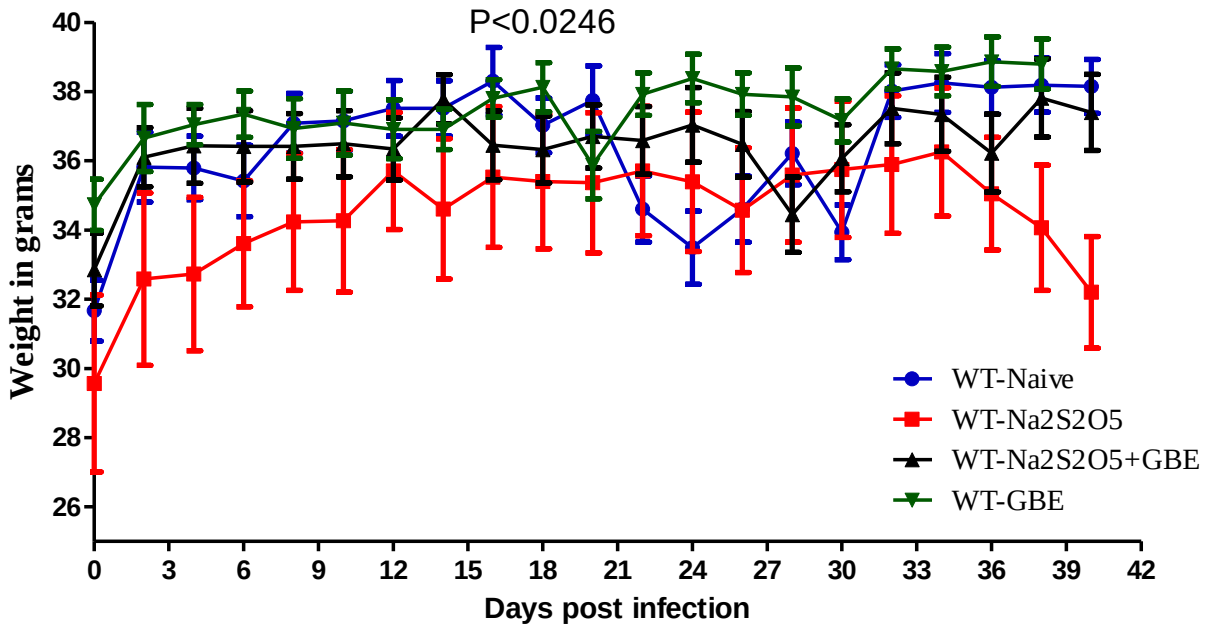

Figure 5.1: The effects of *Ginkgo biloba* on sodium metabisulfite driven change in the general body weight. Change in body weight was analysed using one-way ANOVA with Tukey's test for group comparisons.

3.2. Effects of SMB and EGb-761on relative organ weights

In the present study, SMB administration did not demonstrate any adverse effects on the relative organ weights, since the relative organ weights for the liver, spleen, lungs, kidney, brain, and heart were comparable across all the treatment groups (Fig. 3.2A-F).

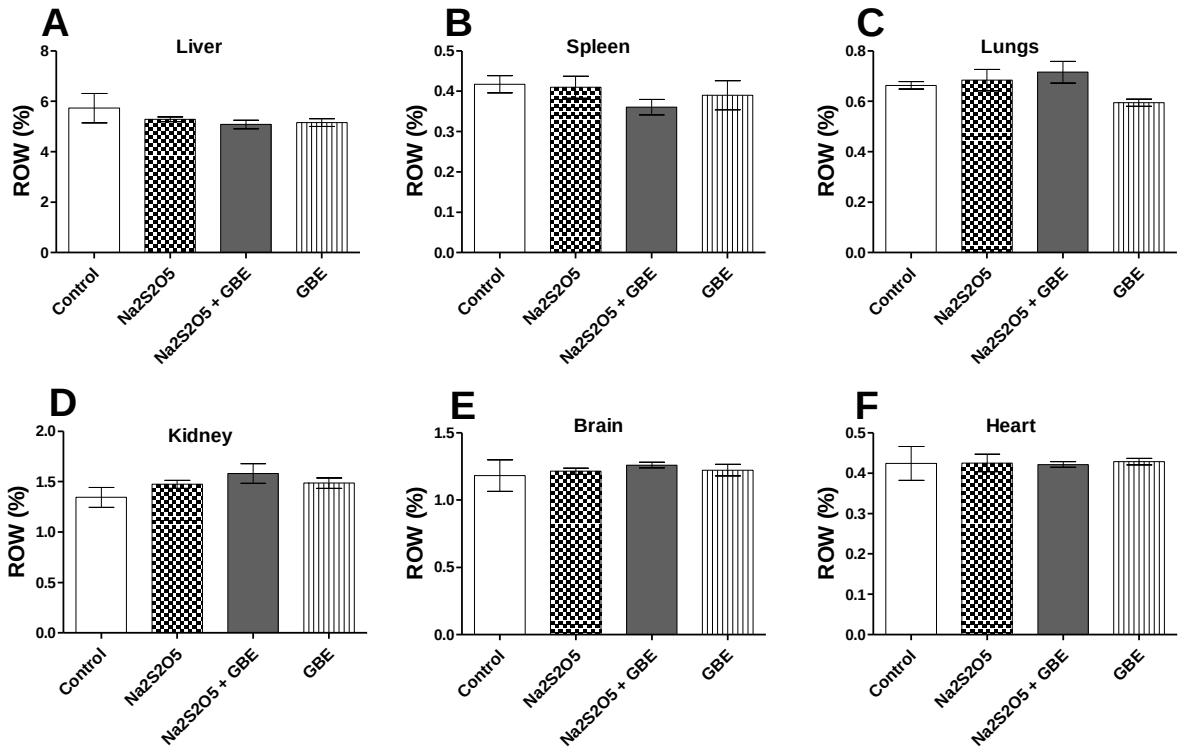

Figure 6.2: The effects of *Ginkgo biloba* on sodium metabisulfite driven change in the relative organ weight. Change in the relative organ weight was analysed using one-way ANOVA with Tukey's test for group comparisons.

3.3. The effects of SMB and EGb-761 on red blood cells, packed cell volume and hemoglobin

Exposure of mice to SMB led to a significant reduction in the red blood cells (RBC), hemoglobin (HGB), and packed cell volume (PCV) (Fig. 3.3A-C), an indication of anemia. However, this suppression of RBC, HGB, and PCV levels was reversed by EGb-761 administration.

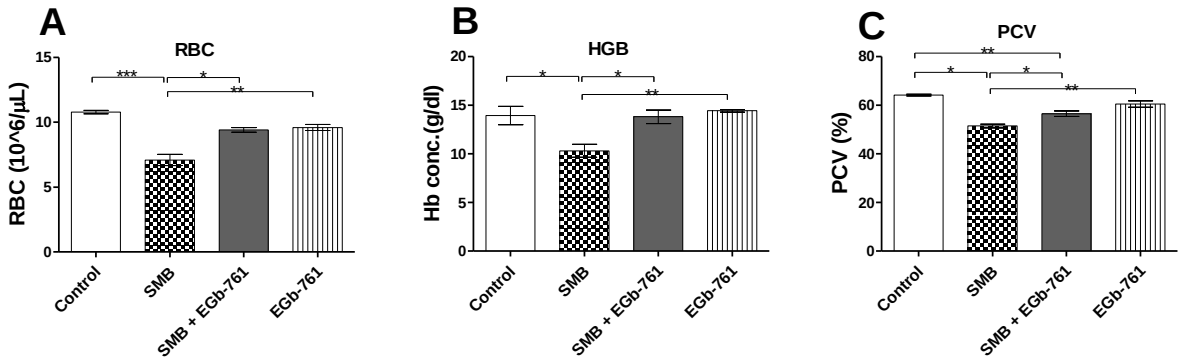

Figure 3.3: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on PCV, RBCs, and HGB in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.4. The effects of SMB and EGb-761 on red cell indices

The findings from the current study reveal a significant decrease in the levels of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in the SMB treated group of mice. The values of these parameters were reversed in the EGb-761-treated groups (Fig. 3.4A-C). On the contrary, the levels of red cell distribution width standard deviation (RDW-SD) and red cell distribution width coefficient of variation (RDW-CV) were comparable across all the treatment groups (Fig. 3.4D-E).

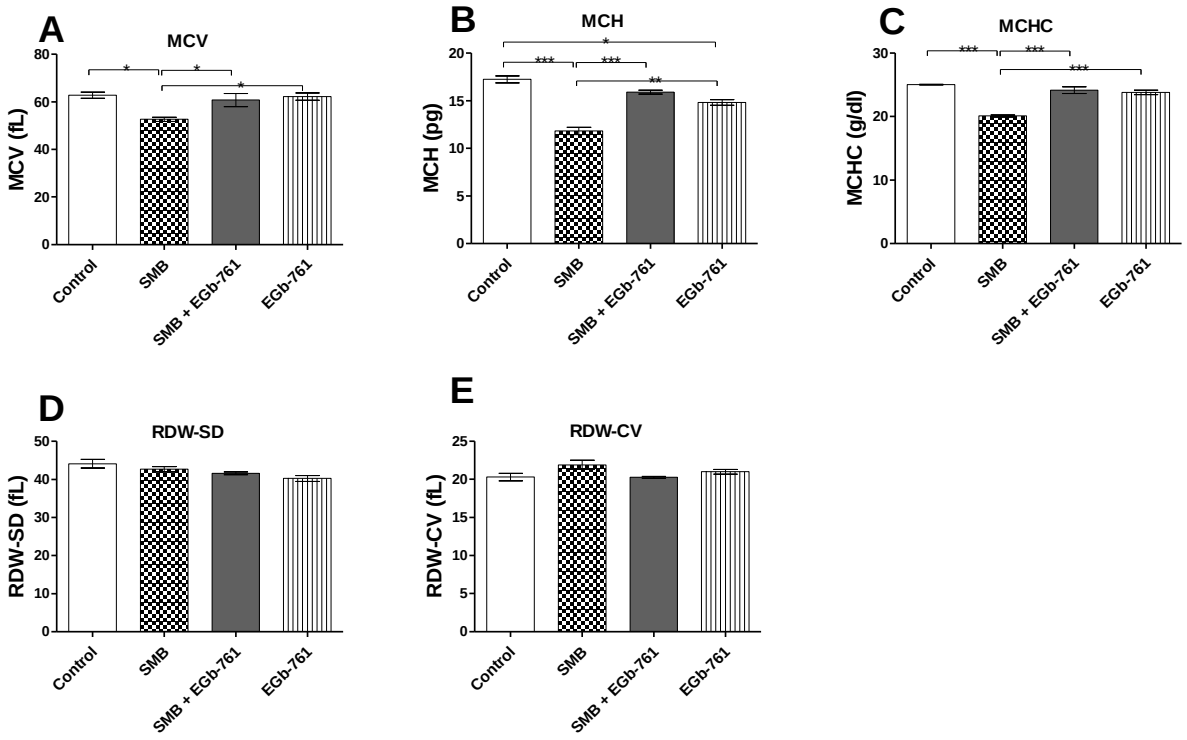

Figure 3.4: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on red blood cell indices in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.5. Effects of SMB and *Ginkgo biloba* on white blood cells and its sub-types

Exposure to SMB resulted in a significant increase in the levels of total white blood cells count (WBC) relative to those in the control group (Fig. 3.5A). Remarkably, administration of EGb-761 significantly restored SMB-driven leukocytosis. The results of WBC sub-types further confirmed that exposure to SMB resulted in a significant reduction in neutrophils (Fig. 3.5B), which were upregulated in the EGb-761-treated mice. Further, the levels of monocytes (Fig. 3.5C), basophil (Fig. 3.5D), and lymphocytes (Fig. 3.5E) were significantly elevated following exposure to SMB, EGb-761-blocked such SMB-driven elevation change. On the contrary, there

was no statistical significant difference in the levels of eosinophils across all the treatment groups (Fig. 3.5F).

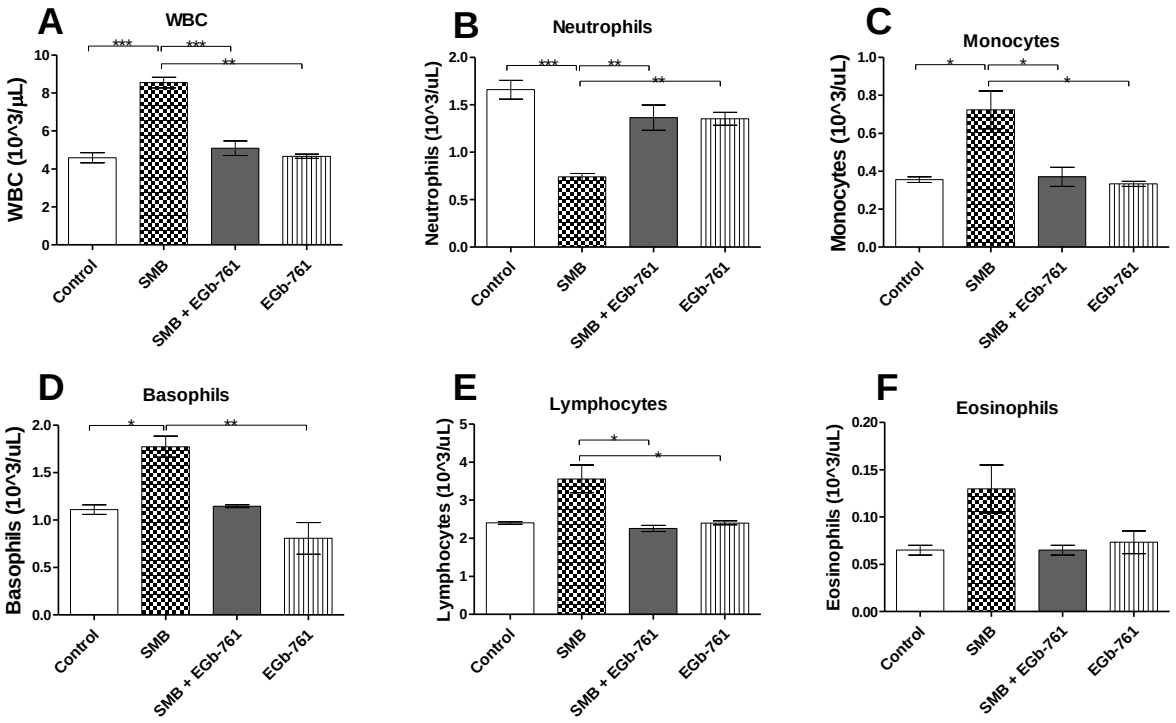

Figure 3.5: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on WBC and sub-types in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.6. Effects of sodium metabisulfite and *Ginkgo biloba* on platelets and its indices

Exposure of mice to SMB led to a significant decrease in platelet levels when compared to the control group (Fig. 3.6A), denoting thrombocytopenia which was restored by EGb-761 administration. An analysis of the platelet indices showed SMB-driven down-regulation of the mean platelet volume (MPV) (Fig. 3.6B), platelet large cell ratio (P-LCR) (Fig. 3.6C), platelet distribution width (PDW) (Fig. 3.6D), such changes were not present in the EGb-761 treated mice. The levels of plateletcrit (PCT) were unaffected by the treatments (Fig. 3.6E).

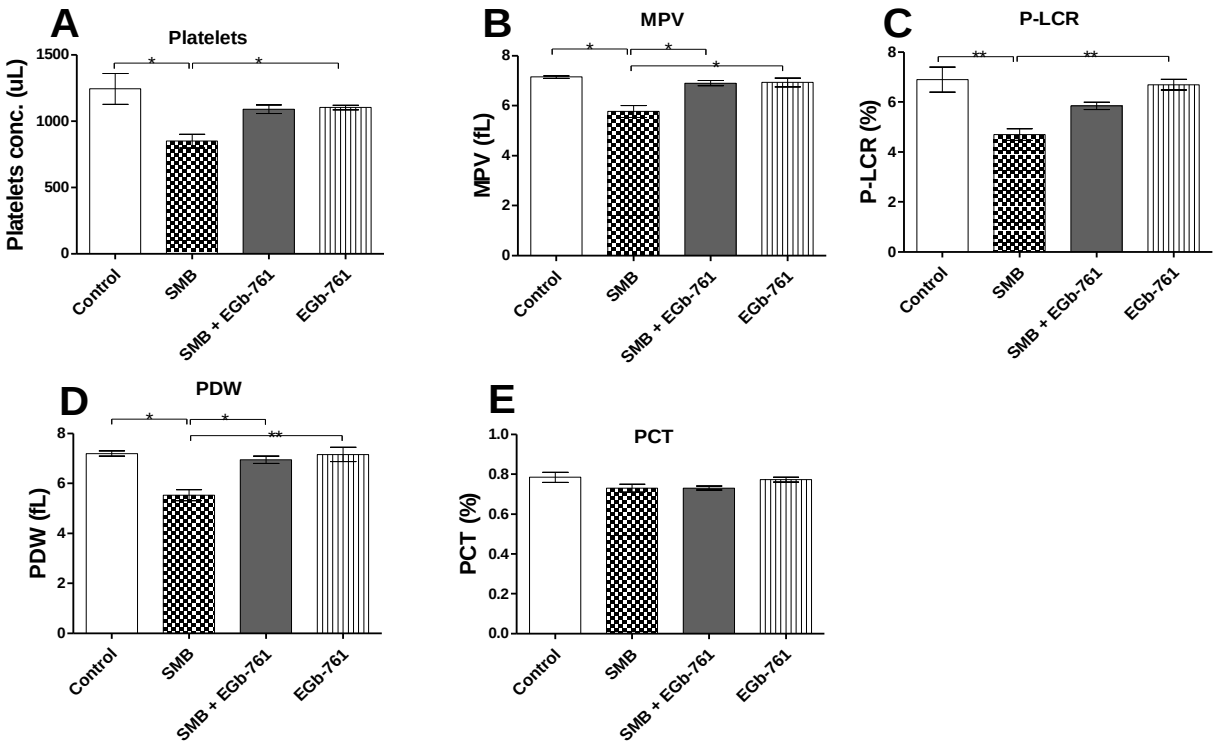

Figure 3.6: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on platelets and platelet sub-types in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, **$p<0.01$.

3.7. Effects of sodium metabisulfite and *Ginkgo biloba* on serum lipid levels

The SMB-administered mice showed a significant increase in the total cholesterol and triglyceride levels when compared to the control group (Fig. 3.7A-B respectively). Notably, the administration of EGb-761 significantly attenuated the SMB-induced increase in the total cholesterol and triglycerides. In contrast, the levels of high-density lipoprotein (HDL) were significantly decreased in SMB-treated mice when compared to control. Evidently, treatment with EGb-761 stabilized lipid levels across the board (Fig. 3.7C).

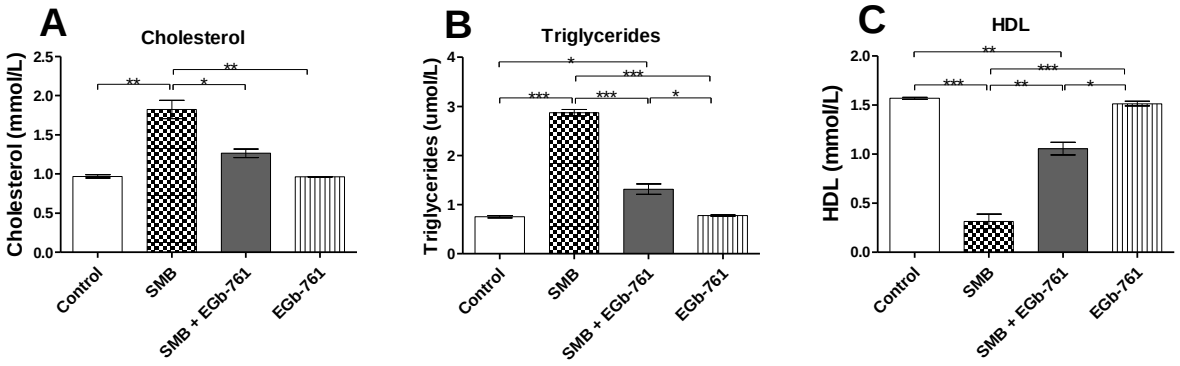

Figure 3.7: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on lipid profile. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.8. Effects of SMB and *Ginkgo biloba* on liver function

Serum activities of ALT, AST, and ALP were significantly increased in the SMB-treated group compared to the control (Fig. 3.8A-C), indicative of active liver injury. Intriguingly, administration with EGb-761 protected mice against SMB-induced liver damage.

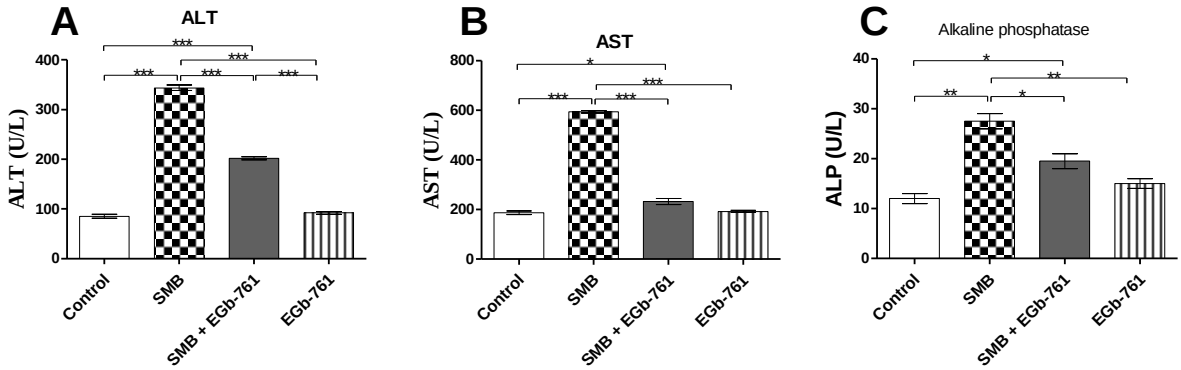

Figure 3.8: Effect of sodium metabisulfite and/or *Ginkgo biloba* on the levels of liver enzymes. Mean comparison procedures were done with one-way ANOVA with Tukey multiple

comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.9. Effects of SMB and *Ginkgo biloba* on hepatic levels of bilirubin and gamma-glutamyltransferase

In addition, our results indicated the serum levels of direct bilirubin and total bilirubin activities were significantly increased in mice exposed to SMB, which were reduced in the EGb-761-treated group (Fig. 3.9A-B). On the other hand, hepatic gamma-glutamyltransferase (GGT) was comparable in all the treated groups (Fig. 3.9C).

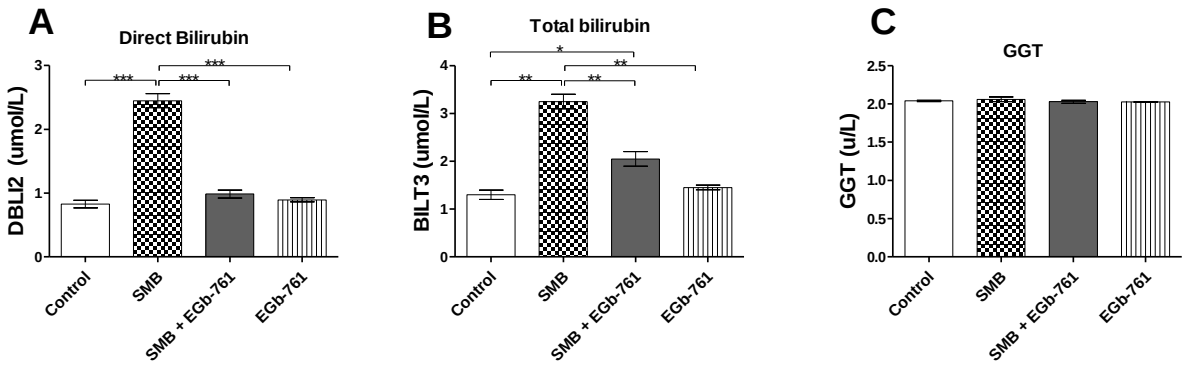

Figure 3.9: Effect of sodium metabisulfite and/or *Ginkgo biloba* on the levels of bilirubin and GGT. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.10. The impact of SMB and *Ginkgo biloba* kidney function

Exposure of mice to SMB caused a significant increase in the serum levels of creatinine, urea, and uric acid in comparison to the controls (Fig. 3.10A-C). These heightened levels of creatinine, urea, and uric acid were reduced by treatment with EGb-761. Conversely, SMB caused a significant decrease in serum albumin levels; such changes were nullified by EGb-761 (Fig. 3.10D).

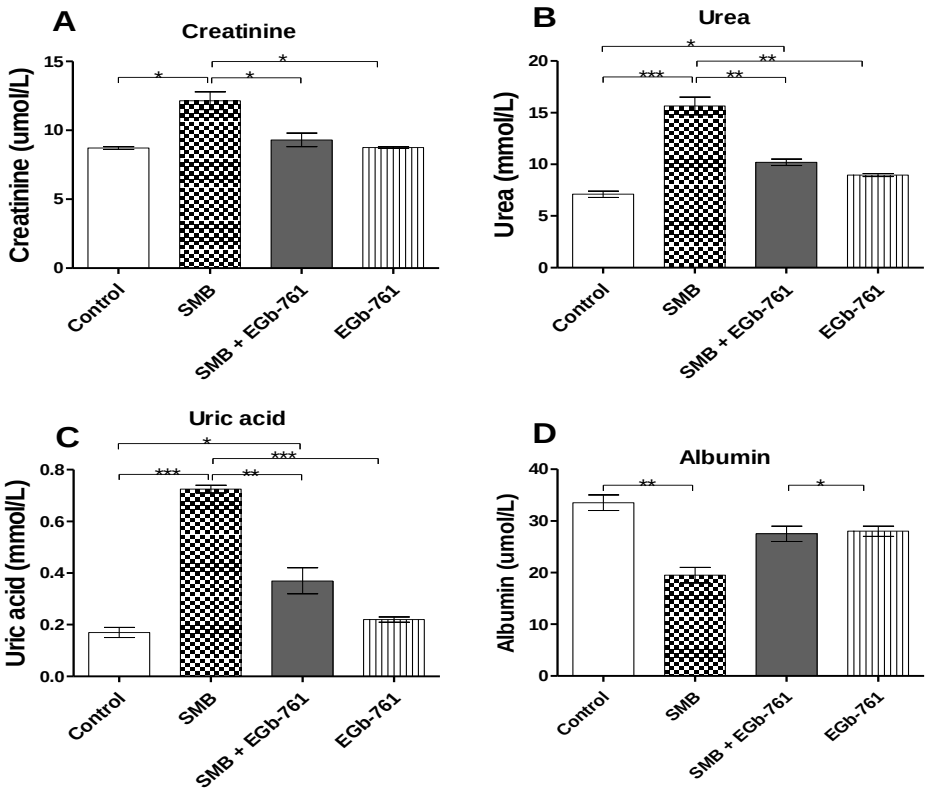

Figure 3.10: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of creatinine, urea, uric acid and albumin. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.11. The impact of sodium metabisulfite and *Ginkgo biloba* on potassium, sodium and chloride ions

Exposure to SMB resulted in a significant decrease in the serum levels of potassium, sodium, and chloride ions (Fig. 3.11A-C respectively), indicative of SMB-driven active metabolic acidosis. In the presence of EGb-761, this phenomenon was alleviated.

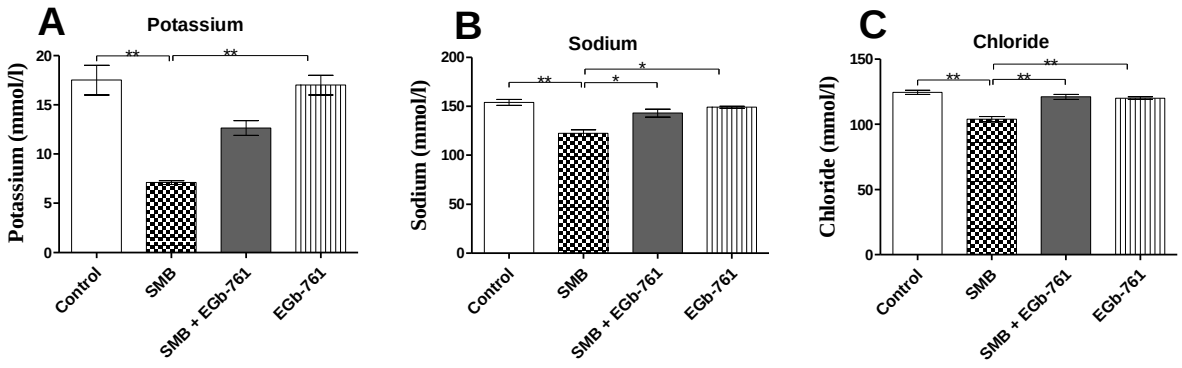

Figure 3.11: The effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of serum electrolytes. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, **$p<0.01$.

3.12. The impact of sodium metabisulfite and *Ginkgo biloba* on malondialdehyde levels

Exposure to SMB resulted in a significant increase in the levels of malondialdehyde (MDA) in the liver, brain, spleen, and lungs relative to the control (Fig. 3.12A-D respectively); depicting SMB-driven lipid peroxidation. Treatment with EGb-761 was able to alleviate this SMB-driven augmentation of MDA levels.

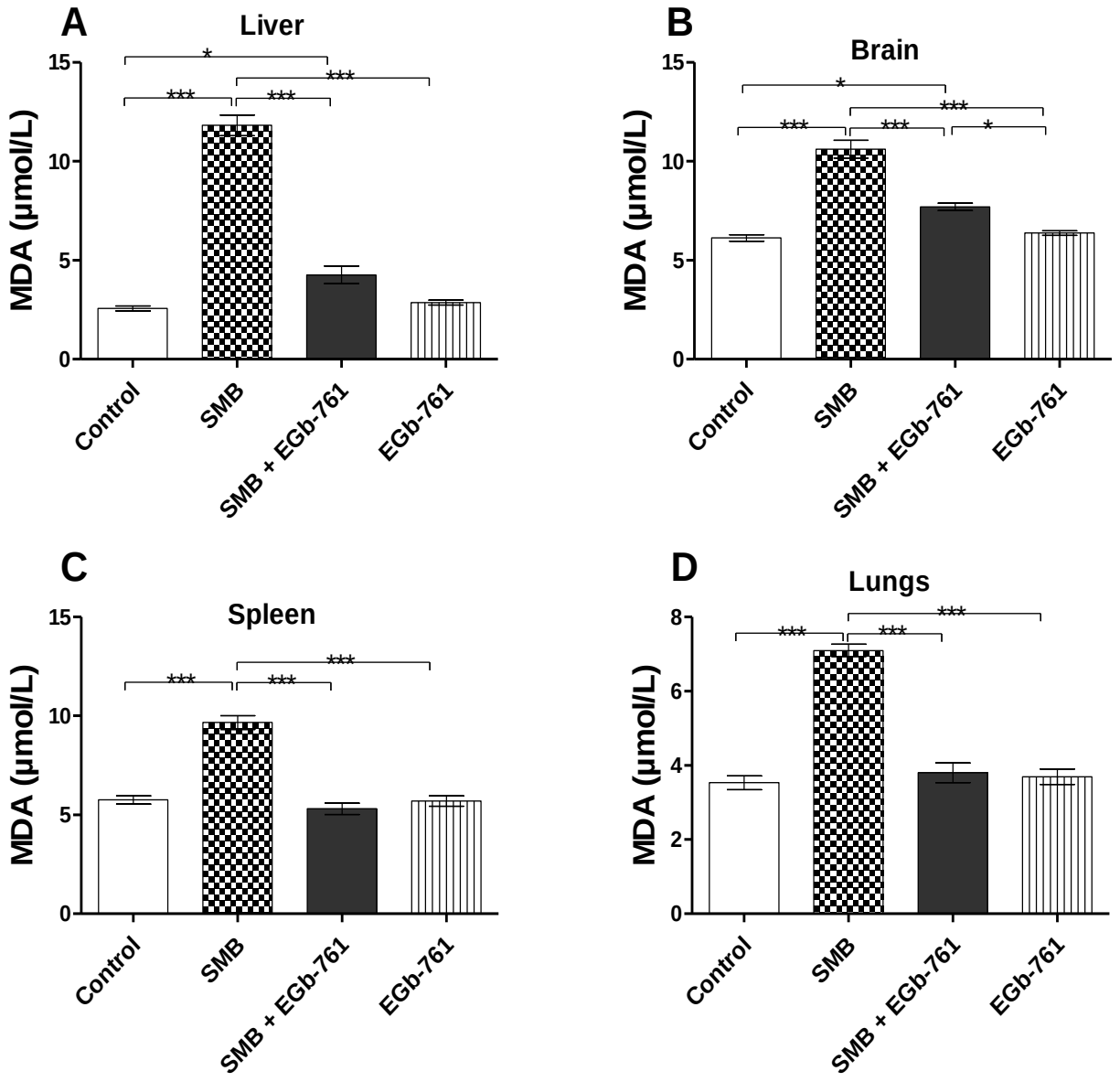

Figure 3.12: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of malondialdehyde. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, ***$p<0.001$.

3.13. The impact of sodium metabisulfite and *Ginkgo biloba* on serum, heart and kidney malondialdehyde levels

Exposure to SMB resulted in a significant increase in the levels of malondialdehyde (MDA) in the kidney and serum relative to the control (Fig. 3.13A-B respectively); depicting SMB-driven lipid peroxidation. Treatment with EGb-761 was able to alleviate this SMB-driven augmentation of MDA levels. In stark contrast, MDA levels in the heart were comparable for the normal control and mice that were administered with SMB and EGb-761 (Fig. 3.13C).

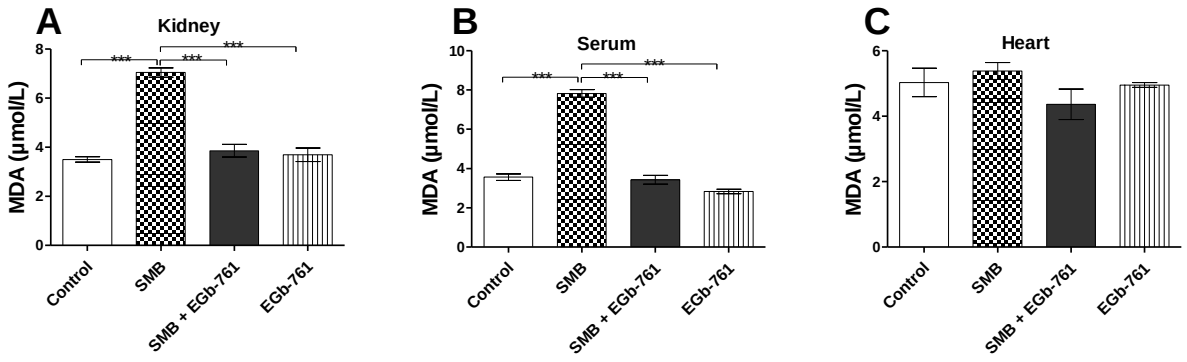

Figure 3.13: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of MDA. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, ***$p<0.001$.

3.14. Effects of sodium metabisulfite and *Ginkgo biloba* on the levels of reduced glutathione

The principal component analysis of reduced glutathione (GSH) revealed that exposure to SMB resulted in a significant depletion of liver, brain, and heart GSH levels when compared to the control group (Fig. 3.14A-C). Treatment of mice with EGb-761 significantly restored the levels of both hepatic and brain GSH levels. Furthermore, the results from the present study revealed that exposure to SMB caused a significant increase in the cellular GSH levels in the spleen,

which was restored in the presence of EGb-761 (Fig. 3.14D), reflecting the ameliorative effect of EGb-761 against SMB-induced oxidative stress in these organs.

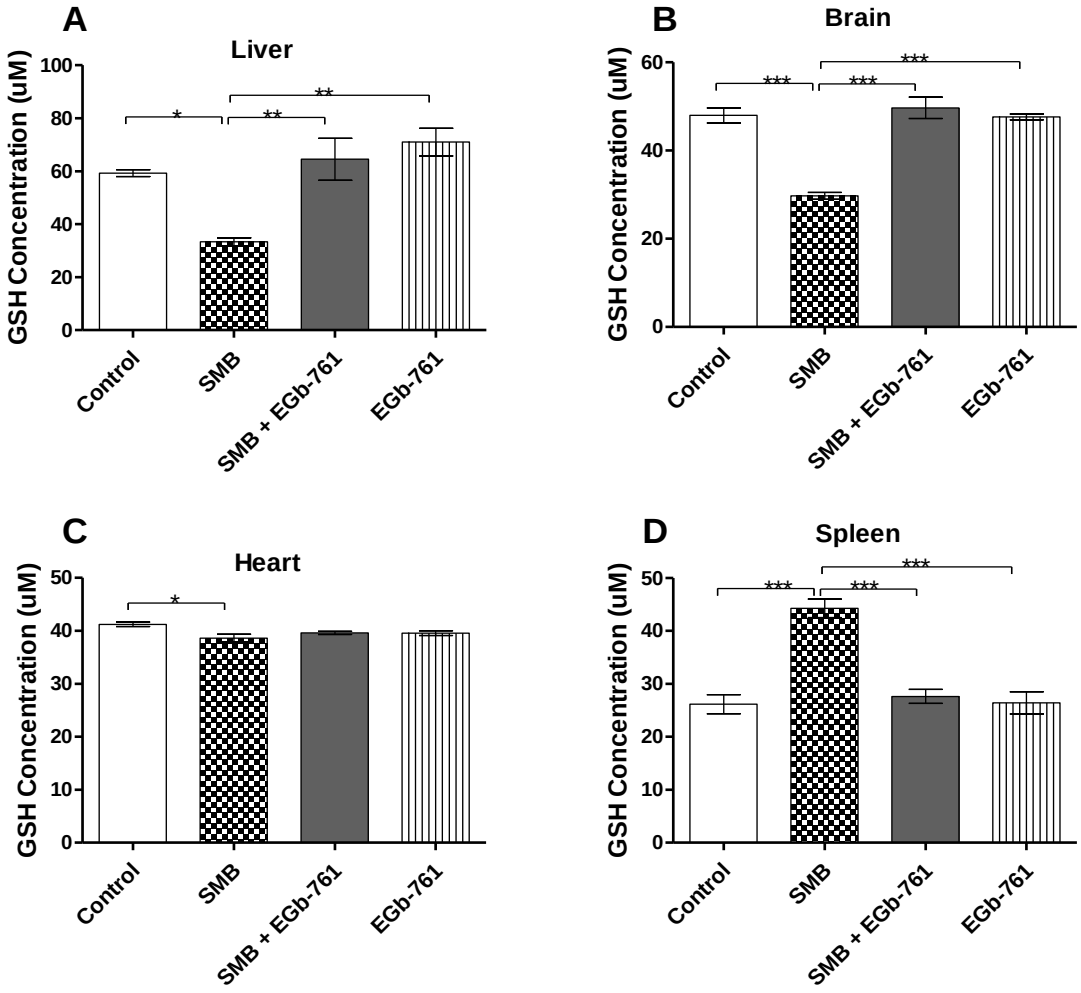

Figure 3.14: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of cellular reduced glutathione concentration in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.15. Effects of sodium metabisulfite and *Ginkgo biloba* on GSH levels of lungs and kidney

The results from the present study revealed that exposure to SMB caused a significant increase in the cellular GSH levels in the lungs and kidney, which was restored in the presence of EGb-761 (Fig. 3.15A-B), reflecting the protective effect of EGb-761 against SMB-driven oxidative stress in these organs.

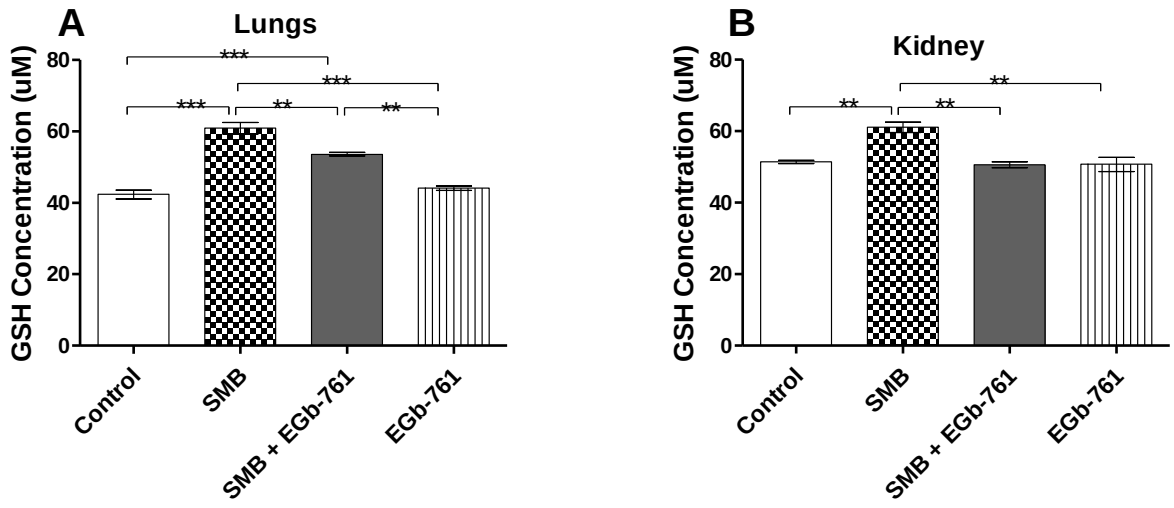

Figure 3.15: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of cellular reduced glutathione concentration in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.16. Effects of sodium metabisulfite and *Ginkgo biloba* on nitric oxide levels

The levels of NO were significantly increased upon exposure of mice to SMB when compared to the control (Fig. 3.16). Administration of EGb-761 decreased the SMB -induced increase of serum NO levels.

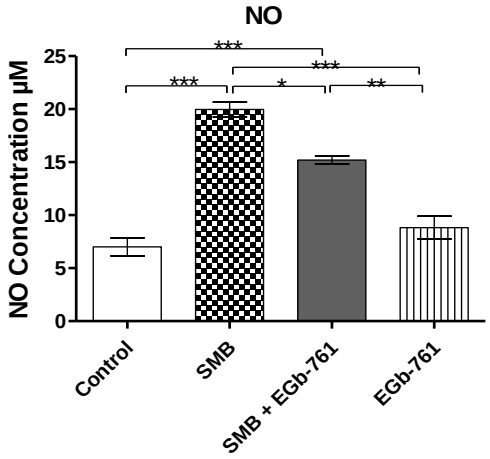

Figure 3.16: Effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of nitric oxide in mice. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.17. Effects of sodium metabisulfite and *Ginkgo biloba* on cytokine levels

Exposure to SMB caused a significant elevation of the pro-inflammatory cytokine tumor necrotic factor-alpha (TNF-α) and interferon-gamma (IFN-γ) (Fig. 3.17A-B), indicative of inflammatory responses. Notably, this increase in pro-inflammatory cytokines was diminished in the presence of EGb-761. The serum levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were comparable in all treated and control groups of mice (Fig. 3.17C). An analysis of the ratios of the pro-inflammatory cytokines versus the anti-inflammatory cytokines TNF-α:IL-10 and IFN-γ-IL-10, revealed that the ratios were significantly higher in SMB exposed group of mice; which were reduced in the presence of EGb-761 (Fig. 3.17D-E).

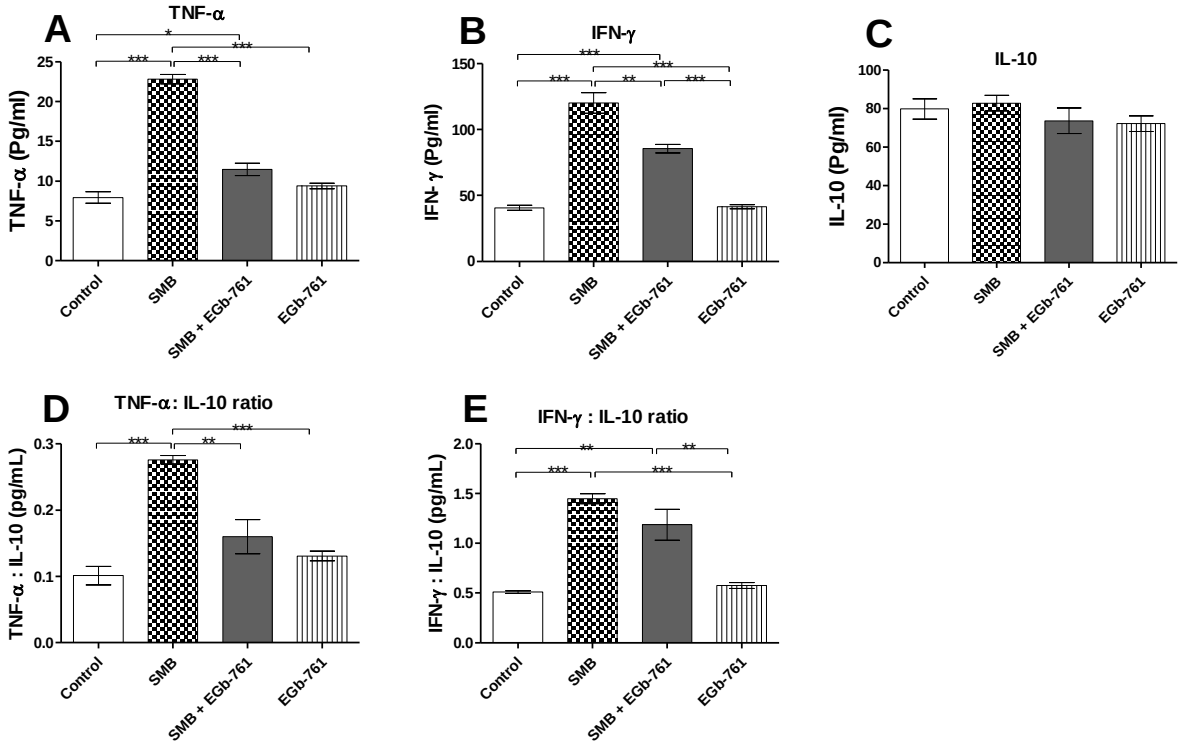

Figure 3.17: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of the cytokines. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01, ***p<0.001.

3.18. Effects of sodium metabisulfite and *Ginkgo biloba* on total immunoglobulins and protein levels

Exposure to SMB caused a significant elevation of total immunoglobulins (Fig. 3.18A), suggestive of induction of immune responses. Notably, this increase in total immunoglobulins was restored in the presence of EGb-761. Further, the serum levels of total proteins were significantly elevated in SMB treated mice relative to the control groups of mice (Fig. 3.18B). Administration of EGb-761 stabilized the SMB -induced increase of serum total protein levels.

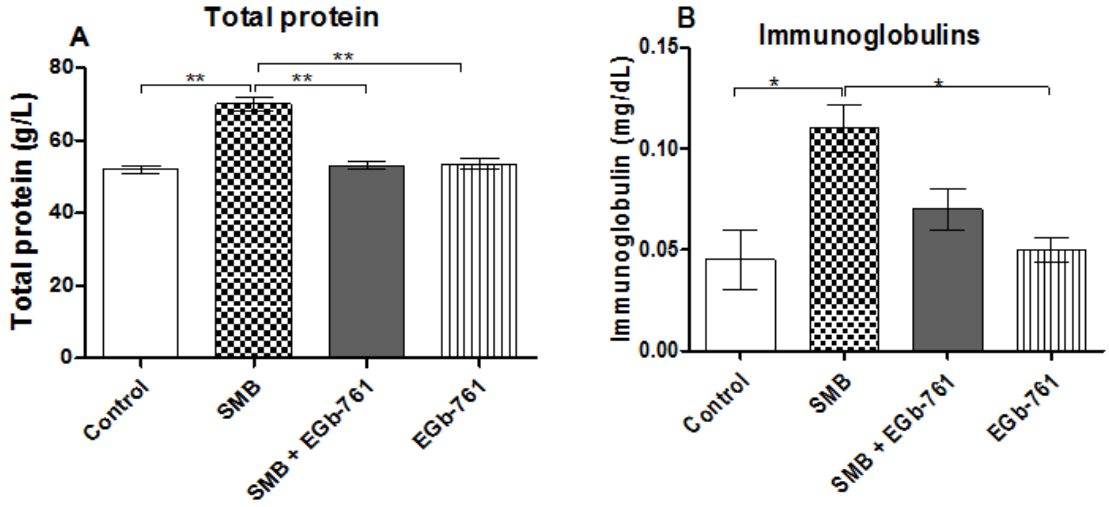

Figure 3.18: Comparison of the effect of sodium metabisulfite and/or *Ginkgo biloba* administration on the levels of immunoglobulins and total proteins. Mean comparison procedures were done with one-way ANOVA with Tukey multiple comparison post hoc test. The results are expressed as ± SEM. The indicated level of significance *p<0.05, **p<0.01.

3.19. Histopathological analysis of the effects of *Ginkgo biloba* on mice liver tissues following sodium metabisulfite exposure

Earlier experiments in this study already demonstrated that EGb-761 prevented SMB-induced liver damage. To corroborate the results, this study further performed a histopathological examination of the liver tissues. Mice treated with SMB had visible hepatic injury demonstrated by diffuse fatty infiltration and hepatocyte necrosis (Fig. 3.19). Oral administration of EGb-761 appeared to protect mice from SMB-induced liver injury.

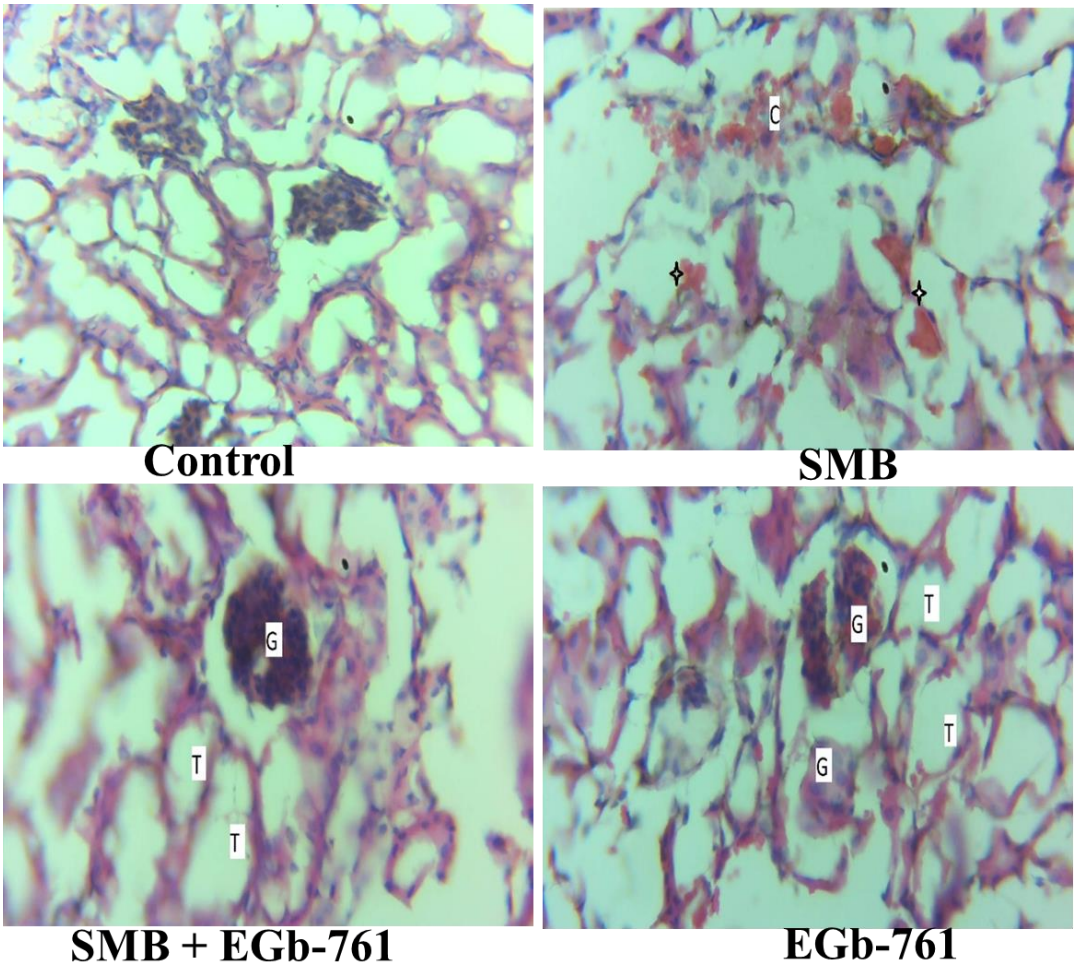

Figure 3.19: Effects of EGb-761 on mice liver tissues upon exposure to SMB. Normal liver tissue from the control group showing normal tissue with Hepatocyte vacuolation (arrow head); Liver section from SMB group showing diffuse fatty infiltration, hepatocyte necrosis (arrow), Liver section from mice exposed to SMB and EGb-761 showing diffuse fatty infiltration (arrow) and Liver section from mice exposed to EGb-761 showing normal architecture with hepatocyte vacuolation (arrow head). Histological examination was done using Hematoxylin and Eosin staining. Magnification: x400.

3.20. Histopathological analysis of the effects of *Ginkgo biloba* on mice kidney tissues following sodium metabisulfite exposure

Histological examination on the kidney tissue of mice administered with SMB revealed multifocal vascular congestion and interstitial hemorrhages (Fig. 3.20). Treatment with EGb-761 attenuated SMB-induced renal injury.

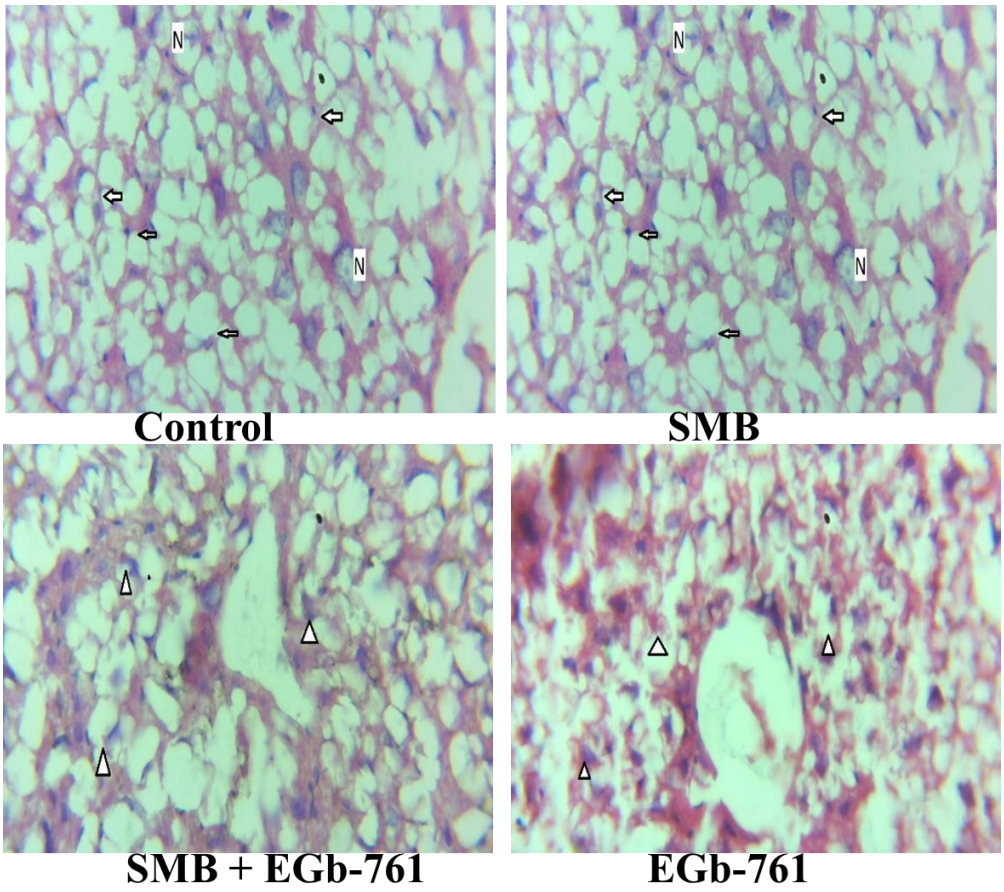

Figure 3.20: the effect of sodium metabisulfite and/or *Ginkgo biloba* on the liver tissue. Normal kidney tissue from the control group; Kidney section from SMB group showing multifocal vascular congestion (C) and interstitial hemorrhages (star), Kidney section from mice exposed to SMM and EGb-761 showing normal kidney tissue and (d) Normal kidney tissue from the EGb-761 group of mice. Histological examination was done using Hematoxylin and Eosin staining. Magnification: x400.

3.21. Histopathological analysis of the effects of *Ginkgo biloba* on mice brain tissues following sodium metabisulfite exposure

Brain sections from the control and treated groups had normal brain cellular architecture (Fig. 3.21).

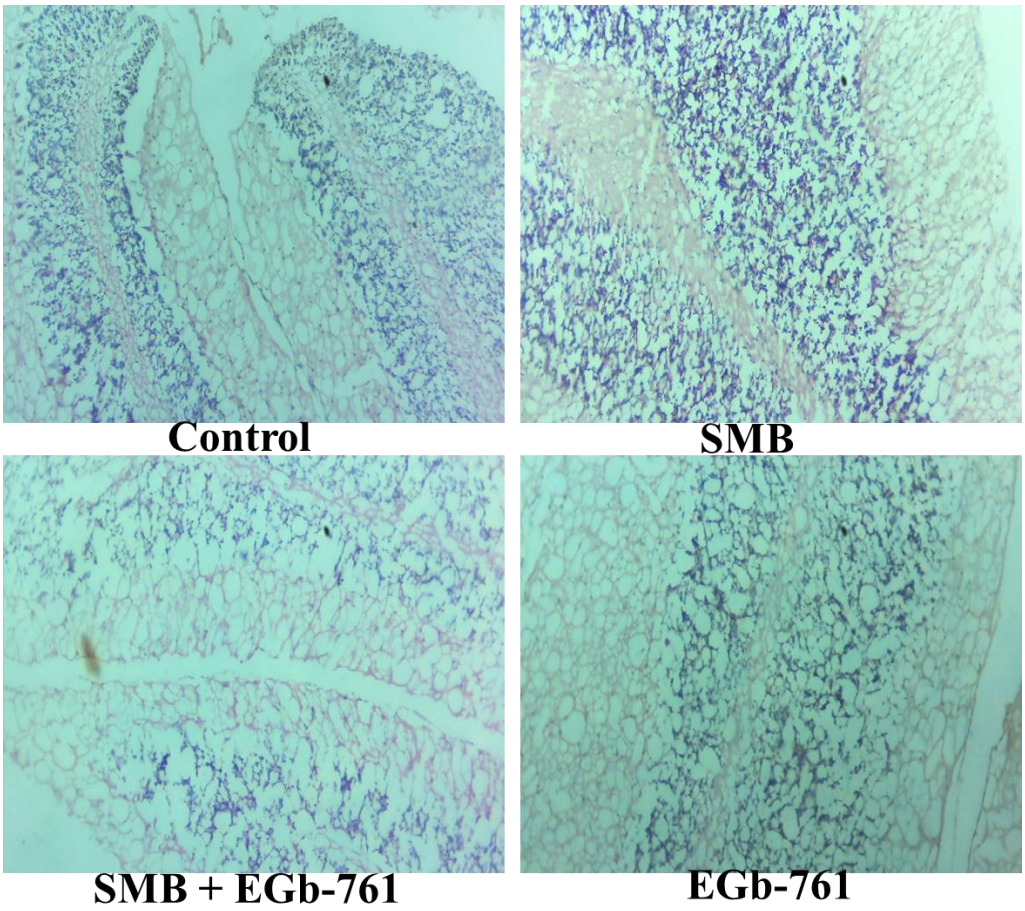

Figure 3.21: The figure shows the effect of sodium metabisulfite and/or *Ginkgo biloba* on the brain tissue. Normal brain tissue of mice from control group and treatment groups. Histological examination was done using Hematoxylin and Eosin staining. Magnification: x400.

CHAPTER FIVE

DISCUSSION

5.1 The effect of EGb-761 supplementation against SMB driven alteration of host physiological and hematological alterations

Food preservatives are widely employed to circumvent food contamination due to microbial growth or undesirable chemical variations in packaged and stored food (Leyva *et al.,* 2017). In the face of the well-known application of these preservatives in the beverage and food industry, the extent of their detrimental and toxic impact requires scrutiny. Sodium metabisulfite (SMB) is commonly used as a preservative in food processing and consumer products to combat the growth of microorganisms (Zhang *et al.,* 2015). Excessive consumption of SMB, either through higher dosages or prolonged usage, causes many undesirable toxic and adverse effects (Ercan *et al.,* 2010).

It is increasingly evident that overuse of food additives used as preservatives can significantly increase the development of human diseases (Mepham, 2011). *Ginkgo biloba* leave extract is a potent antioxidant and anti-inflammatory agent. Besides this, it has been shown to have immunomodulatory effects as well as offer protection against various drug-induced organ pathologies. In this study, the role of standardized *Gingko biloba* leaf extract (EGb-761) in ameliorating sodium metabisulfite (SMB)-induced toxicities was evaluated. From our study, SMB induced hematotoxicity, oxidative stress and disrupted immune function in mice. It was noted that most of these negative effects were markedly attenuated in the presence of EGb-761.

In this study, exposure to SMB-induced weight loss in mice relative to the control. Similar findings have been evident before in rats fed on high doses of SMB (El-Kadi *et al.,* 2014; Aslam, 2022), as well as pigs and rabbits (Til *et al.,* 1972; Miyata *et al.,* 1990). SMB-induced changes in

feeding behavior contributed to the loss of weight. Furthermore, the weight loss following SMB exposure could be due to toxicological effects of SMB (Yoo *et al.,* 2018). Administration of EGb-761significantly attenuated the SMB-induced weight loss. It is plausible to assume that the lipolysis-inducing property of EGb-761 may have contributed to this outcome as shown in a previous study (Hirata *et al.,* 2019).

Interference with production of blood cells by chemical toxins is a common phenomenon with serious health implications. There was clear evidence of SMB-driven decrease in the levels of RBCs, HGB, PCV and the red cell indices, suggestive of anaemia. These results corroborate a recently conducted study by Aslam [2022]; which showed that sulphites provoked a significant production of ROS that resulted in oxidative damage of the RBC membrane. In addition, RBC damage can be linked to lysis or feasible shrinkage of erythrocytes in blood (Hezbullah *et al.,* 2016). The decline in the frequency of PCV may be associated with the reduction in the size of RBCs and the drop in the rate of synthesis of hemoglobin, which in turn controls the development and maturation of RBCs. Notably, MCH levels provides an indication of the actual content of hemoglobin in the RBC cytoplasm. Hence, MCV and MCHC levels are dependent upon the content of RBCs (Grings *et al.,* 2016). Possibly the decrease in MCHC could be associated with the toxic effects of SMB in the bone marrow impairing its ability to produce hemoglobin at a requisite rate. Such effects on the hematopoiesis, would affect synthesis and production of all blood cells, consequently affecting transport of oxygen and immune function.

In the current study, it was demonstrated that EGb-761 reversed the SMB-induced anemia, indicative of a beneficial modulatory effect of EGb-761 on the hematopoietic system. These outcomes may be ascribed to the suppressive effect of SMB on the host hematopoiesis system. *Ginkgo biloba* has demonstrated a robust capacity to impede lipid peroxidation of RBC

membranes, glutathione depletion and methaemoglobin development (He *et al.,* 2009). It is therefore plausible that these effects of EGb-761 may be played a fundamental role, and perhaps protected the RBC from SMB-induced oxidant driven damage.

White Blood Cells, also known as leukocytes are highly versatile and play a critical role in coordinating and shaping the immune response. Any chemical driven changes on WBCs would have a detrimental impact on immunity. In this study, exposure to SMB significantly increased the levels of WBCs, lymphocytes, basophils and monocytes. Such findings had previously been shown by El-Kadi et al. (2014). From this study SMB induced leukocytosis, perhaps due to stimulation of the lymphoproliferative responses by sulphites. A remarkable SMB-driven depletion of Neutrophils was noted. Such suppression of neutrophils has the potential to predispose individuals to bacterial infections. Moreover, monocytosis and a significant increase in levels of basophils that was observed in mice exposed to SMB in the present study, may pre-dispose to inflammation-related ailments, given that effector basophils monocytes are implicated in inflammatory responses (Das *et al.,* 2015; Masamoto *et al.,* 2009). Remarkably, the findings from this study demonstrated that treatment with EGb-761 can alleviate these detrimental effects due to its ability to stabilize WBCs levels in the presence of SMB. The mechanism by which *Ginkgo biloba* ameliorates SMB-driven derangement of WBC and its subtypes could be multi-factorial perhaps due to its anti-oxidant and anti-inflammatory activities (Abdel-Emam & Abd-Eldayem, 2022).

Platelets in tandem with coagulation factors are indispensable during the thrombosis and hemostasis processes. Further, platelets are involved in the inflammatory response and wound healing (Budak *et al.,* 2016). Therefore, a change in the content of platelet levels will critically interfere with these vital physiological and biochemical processes and presents a higher threat for

patients on blood thinning drugs (Mwaeni *et al.,* 2021). To this end, exposure to SMB significantly suppressed platelet levels as well as MPV, P-LCR and PDW, a clear indication of thrombocytopenia. From this study, treatment with EGb-761 significantly attenuated SMB-driven thrombocytopenia. These findings suggest that EGb-761 may have a modulatory role on the thrombocytosis and hemostasis process. This phenomenon warrants further inquiry. Collectively, the results demonstrate that SMB negatively affected the hematopoietic processes. Importantly, EGb-761 supplementation reversed the SMB-induced hematotoxicity.

5.2 The impact of EGb-761 administration against SMB driven alteration of lipid profile

Lipid metabolism plays a critical role as an important source of macromolecular structures for the cell as well as a source of cellular energy. Consequently, alteration in lipid play a significant part in several pathophysiological disorders (Lee *et al.,* 2003). In the current study, exposure to SMB resulted in a significant elevation of total cholesterol and triglycerides with a concomitant decrease in the levels of high-density lipoproteins. This implies that people suffering from metabolic disorders and who are constantly exposed to SMB may aggravate the development of severe forms of the disease. In the presence of EGb-761, lipids metabolism was stabilized, demonstrating a possible modulatory role of *Ginkgo biloba* in lipid metabolism (Hirata *et al.,* 2019). In a previous study, exposure to SMB was shown to influence lipid metabolism through increased release of free fatty acids (FFA) into the plasma (Elmas *et al.,* 2005); usually accompanied with inhibition of the enzyme lipase resulting in severe hypertriglyceridemia and hypercholesterolemia (Elmas *et al.,* 2005).

5.3 The impact of oral administration of EGb-761 against SMB driven kidney, liver damage and metabolic acidosis

Sodium metabisulfite has been directly linked with severe liver damage in several studies (Aslam, 2022; El-Kadi *et al.,* 2014). In this study, liver enzymes that are important metric indexes of liver injury were measured to evaluate whether exposure to SMB affected liver function; and if administration of EGb-761 protected from SMB-driven liver damage. Herein, it can be reported, that exposure to SMB significantly increased serum AST, ALT and ALP, denoting liver damage. SMB-driven liver injury has been demonstrated in a prior study (Aslam, 2022). The levels of these enzymes are elevated and released into the plasma under hepatocellular membrane stress, depicting liver injury (El-Kadi *et al.,* 2014). It is noteworthy that in the presence of EGb-761 the SMB-driven elevation of liver enzymes was abrogated.

Bilirubin is a by-product released following prompt destruction of the RBC. Heightened levels of bilirubin and its buildup in the hepatocellular environment result in inflammation and organ injury (Kalakonda & John, 2018). SMB-induced significant elevation of bilirubin. Notably, SMB-driven elevation of bilirubin was attenuated by oral administration of EGb-761. Since bilirubin is a product of RBC breakdown, the results point to a possible novel protective effect on the liver and RBCs.

Additional investigations determined the integrity of renal function in the presence of SMB and EGb-761. Creatinine and urea are important markers of kidney function, and their upsurge or reduction mirrors a dysfunction of the kidney (Thadhani *et al.,* 1996).Indeed, the breakdown of liver protein compounds has been implicated in the intensification of urea and creatinine levels in animal models (El Kadi *et al.,* 2014). These increased levels of urea and creatinine may be linked to the kidney injury that was observed in this study as revealed by a significant increase

of serum creatinine and urea levels in mice exposed to SMB. The observed upsurge in serum levels of uric acid may be attributed to the reduction in urinary excretion of the metabolites. Notably, it can be revealed that SMB-driven kidney injury was attenuated by administration of EGb-761; inferring protection against nephrotoxicity. This result is in harmony with the previous work where *Ginkgo biloba* was observed to have a renoprotective effect against cisplastin-induced nephrotoxicity and renal damage due to ischemic reperfusion (Okuyan *et al.,* 2012; Song *et al.,* 2013; Sener *et al.,* 2005).

Uric acid is the end product of purine degradation (Borghi *et al.,* 2020). Hyperuricemia has been implicated as a driving force behind cognitive impairment, cardiovascular maladies, and oxidative stress (Tian *et al.,* 2021). In the present study, mice exposed to SMB had significantly high uric acid levels, which is in agreement with prior studies (Aslam, 2022). Augmented levels of uric acid are associated with increased production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Busso and So, 2010; Gallego-Delgado *et al.,* 2014). Thus, SMB-driven elevation of uric acids may in part, contribute to inflammation of the kidney and liver, which will directly exacerbate the pathophysiology of these organs. This study reports that administration of EGb-761, significantly reduced these elevated levels of uric acid. This modulation of uric acid by EGb-761may attenuate SMB-driven toxicity and inflammation of the kidney and liver.

Serum albumin is a crucial protein with vital physiological role and antioxidant activities (Chien *et al.,* 2017). It is produced in the liver and can be used as a biomarker for early liver impairment and chronic liver disease (Das *et al.,* 2019). In the current study, serum albumin levels in mice exposed to SMB and *Ginkgo biloba* was assessed. Exposure of mice to SMB resulted in a significant decrease in the serum levels of serum albumin, indicative of liver impairment. It was

clear that, EGb-761treatment of SMB-exposed mice significantly increased the levels of serum albumin, demonstrating protection against liver injury.

Metabolic acidosis is a common condition characterized by a fall in pH by several toxins (Emmett, 2022). Additionally, metabolic acidosis is usually an indication of a serious pathological states. Thus understanding how exposure to toxicants like SMB disrupts the physiological pH buffering system is important. A significant sodium metabisulfite-driven metabolic acidosis was noted in the present study as demonstrated by a significant decrease in serum levels of potassium, sodium and chloride ions. Metabolic acidosis often arises, partly when there is the acceleration of movement of sodium ions into the cell in response to severe intracellular acidosis with the potential for cell dysfunction. Notably, SMB-driven metabolic acidosis was prevented by EGb-761 administration. This observation may be ascribed to its role in the maintenance of the membrane ultrastructure against lethal effects associated with the generation of free radicals as well as protection against modulation of enzymatic systems and ionic pumps (Clostre, 1986). Nevertheless, the mechanisms by which EGb-761 regulates metabolic acidosis merit further investigations.

5.4 The impact of EGb-761 administration against SMB driven oxidative events and inflammation

Oxidative stress is a phenomenon that is known to underlie or even aggravate the pathogenesis of several disease processes including but not limited to cancer, atherosclerosis, neurodegenerative diseases hypertension, diabetes mellitus, cardiovascular disease, atherosclerosis, reproductive system diseases, and aging (Kruk *et al.,* 2019). Moreover, elevated levels of lipid peroxides resulting from augmented production of free radicals may be important molecular mechanisms for sodium metabisulfite-associated deleterious effects (Rahal *et al.,* 2014). The uncontrolled

oxidation of sulphite into sulphite-free radicals may trigger sulphite-driven lipid peroxidation (Derin *et al.,* 2006). Additionally, uncontrolled lipid peroxidation may drive the production of malondialdehyde (MDA). MDA is a critical marker of lipid peroxidation (CipakGasparovic *et al.,* 2017). In the current study, exposure of mice to SMB led to an increase in tissue and serum MDA, indicating presence of lipid peroxidation. Remarkably, the administration of EGb-761 attenuated an SMB-driven increase in MDA levels, a protective effect that can be attributed to its antioxidant properties. These results are in line with published data (Kaur *et al.,* 2018; Abdel-Emam & Abd-Eldayem, 2022), that demonstrated the ability of EGb-761to scavenge free radicals with concomitant reduction in MDA associated with lipid peroxidation.

Indeed, oxidative stress is known to cause damage to important cellular biomolecules (Duygu *et al.,* 2012). Besides, accumulating evidence has shown that due to its sulphites and its derivatives , SMB can cause oxidative stress as a result of sulfite oxidation and DNA damage in vital organs like the liver, brain, lung, and spleen (Chiarani *et al.,* 2008; Bai and Meng, 2005; Gordon *et al.,* 2004).

In the physiological environment, cells cope with excessive ROS using a highly versatile and potent endogenous antioxidant enzymes consisting of GSH, superoxide dismutase (SOD), glutathione peroxidase and catalases. Depletion of these important antioxidant systems elicits elevation of lethal ROS, thus causing oxidative stress. Consequently, levels of anti-oxidant enzymes such as GSH are very good indicators of oxidative stress. In agreement with the earlier findings (Ozturk *et al.,* 2019), SMB administration significantly depleted GSH levels in the liver, brain and heart with elevation of GSH being observed in the spleen, lungs and kidney, indicative of oxidative stress. Depletion of GSH is a clear indication of overwhelming and lethal oxidative

stress levels; whereas is characteristically associated with an initial response to rising levels of oxidative stress (Oula *et al.,* 2023; Ngatuni *et al.,* 2022; Kennedy *et al.,* 2020).

Ginkgo biloba has been proposed as an antioxidant agent in numerous studies (Rojas *et al.,* 2016; Nowak *et al.,* 2017; Tewari *et al.,* 2017; Cefali *et al.,* 2019). Recently it has been shown to exert its effect directly by scavenging ROS or elevating the expression of genes encoding antioxidant enzymes (Nowak *et al.,* 2021). Moreover, both in vitro and in vivo studies have shown that the antioxidant property of *Ginkgo biloba* is associated with its flavonoid components, like kaempferol and quercetin that suppress ROS (Xin *et al.,* 2000; Smith and Luo, 2003). The antioxidant activity of EGb-761 using GSH levels following exposure of mice to SMB was also investigated. Remarkably, this is the first study demonstrating that EGb-761 administration resulted in the assuaging oxidative stress by SMB in vital organs such as brain, liver, kidney, lungs, spleen and heart.

The induction of nitric oxide synthase (iNOS) leads to the elevation of nitric oxide (NO), leading to the inhibition of the respiratory chain and a reduction in ATP formation (Barker *et al.,* 1996). Besides, the excessive production of NO is the hallmark of different pathological disorders (Pacher *et al.,* 2007). Specifically, NO facilitate generation of lethal reactive metabolite, peroxynitrite ($ONOO^-$) (Alvarez & Radi, 2003), which nitrate vital lipids, nucleic acids, proteins and/or enzymes in the physiological environment of vital organs, altering their structure and rendering them dysfunctional. Moreover, NO-mediated inflammatory processes and oxidative stress events have also been outlined (Sharma *et al.,* 2007; Lubos *et al.,* 2008). Given that many toxic chemicals induce inflammation and oxidative stress, the identification of novel compounds that are good candidates for the downregulation of inflammatory mediators is of great significance. It was observed from the present study that exposure of mice to SMB resulted in a

significant increase in the serum levels of NO. Remarkably, EGb-761 nullified SMB-induced elevation of NO. Overwhelming evidence has demonstrated that *Ginkgo biloba* protects cells from NO-induced neurotoxicity and a number of inflammatory mediators (Massieu *et al.,* 2004; Eckert *et al.,* 2005; Wadsworth *et al.,* 2001). Thus, the protective ability of EGb-761against SMB, noted in the current study, may be associated with its proven anti-inflammatory and anti-oxidant activities (Kaur *et al.,* 2018).

The body gets rid of detrimental stimuli such as toxic compounds and invading pathogens by mounting strong immune responses (Ferrero-Miliani *et al.,* 2007; Medzhitov, 2010). Exposure to SMB has been demonstrated to enhance the pyroptosis process which ultimately results in increased amounts of pro-inflammatory cytokines IL-1β and IL-18 (Liu *et al.,* 2023). Impairment of these processes due to continuous exposure to sodium metabisulfite may cause chronic inflammation. This study revealed that exposure to SMB resulted in a significant increase of serum TNF-α and IFN-γ, indicative of active SMB-induced inflammation. In the presence of EGb-761, SMB-induced elevation of these pro-inflammatory cytokines was abrogated. It is well documented that a balance between anti-inflammatory and pro-inflammatory cytokines defines the inflammatory state of the cellular environment. Thus, determining the ratio between the pro and anti-inflammatory cytokines may help to determine the degree of inflammatory status due to SMB exposure. Furthermore, the current study demonstrate a noticeable imbalance of pro-inflammatory and anti-inflammatory cytokines in an SMB-administered group of mice that reflects aggravated inflammation. Additionally, the anti-inflammatory effects of EGb-761 treatment were also confirmed by a stable balance between pro-inflammatory and anti-inflammatory cytokines, once again showing the anti-inflammatory action of EGb-761. The anti-inflammatory properties of *Ginkgo biloba* have been proven in several studies (Abdel-Emam &

Abd-Eldayem, 2022; Pizza *et al.,* 2019; Ilieva *et al.,* 2004). Accordingly, it has been shown that administration of *Ginkgo biloba* plays an important role in the resolution of inflammation through the reduction of tumor necrosis factor (TNF-α) and interleukin 1β (IL-1β), while enhancing the level of anti-inflammatory cytokine interleukin 10 (IL-10) (Yang *et al.,* 2013). Its anti-inflammatory properties are attributed to various flavone glycosides and terpenoids contained in.

Analysis of total serum immunoglobulins revealed that exposure of mice to SMB resulted in a significant elevation of these antibodies. Though the exact mechanisms by which SMB toxicity induces humoral immune response resulting in the secretion of immunoglobulins remains scanty, nevertheless, sulphites-induced allergies are associated with the induction of IgE (Vally & Misso, 2012). In the current study, there was clear evidence that treatment with EGb-761 restored SMB-induced elevation of these immunoglobulins to almost that of the control. Further, treatment with EGb-761 normalized the levels of SMB-induced elevation of total proteins. The extent of toxicity by SMB-driven induction of humoral immune response is still unknown and warrants further investigation.

5.5 The ameliorative impact of EGb-761 supplementation against SMB driven organ pathological lesions

Additionally, this study established the histopathological effects of SMB exposure and the effect of EGb-761 on liver, kidney and brain sections. In the liver, exposure to SMB resulted in aggravated pathological lesions characterized by diffuse fatty infiltration and hepatocyte necrosis. The outcome further corroborates findings from previous studies following exposure to SMB (Elmas *et al.,* 2005). Intriguingly in the presence of EGb-761, the SMB-induced hepatic necrosis was abrogated. The role of EGb-761 in ameliorating SMB-driven liver pathology may

be somewhat ascribed to its role as a potent antioxidant and anti-inflammatory agent (Abdel-Emam &Abd-Eldayem, 2022; Cefali *et al.,* 2019; Kaur *et al.,* 2018). Notably, oxidative stress and inflammation are some of the vital mechanisms by which SMB initiates its toxicity (Rahal *et al.,* 2014; Liu *et al.,* 2023.

Scrutiny on the kidney tissue sections demonstrated multifocal vascular congestion and interstitial hemorrhages due to SMB exposure. Earlier studies have attributed inflammatory mediators such as hyperuriceamia and urea accumulation as the driving force behind kidney pathology due to SMB-driven toxicity (El-Kadi *et al.,* 2014). Intriguingly, treatment with EGb-761 protected mice from SMB-induced renal injury. Further, histological evaluation of the brain tissues revealed no effects following SMB exposure. The normal brain architecture observed in the present study following exposure of mice to SMB perhaps may be due to the inability of sulphites to penetrate the brain barrier. Nevertheless, this observation merits further investigation.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The present study demonstrates for the first time that oral administration of standardized *Ginkgo biloba* (EGb-761) attenuated SMB-induced alteration of hematological parameters, metabolic acidosis, inflammatory responses, oxidative stress and organ damage. Arguably, because exposure to SMB results in varied detrimental effects, these findings have significant and immediate clinical implications.

6.2 Recommendations

The following recommendations are drawn from the current study:

1. There is a need to further elucidate the apparent molecular processes responsible for the ability of EGb-761 to confer protection from SMB toxicity in mice
2. Further studies should be done to determine the residue levels of SMB in various vital organs
3. In this study, EGb-761 was observed to have a modulatory role on the thrombocytosis and hemostasis process. This phenomenon warrants further inquiry.
4. The mechanisms by which EGb-761 regulates metabolic acidosis merit further investigations.

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APPENDICES

Appendix I: Ethical clearance certificate

UNIVERSITY OF NAIROBI
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REF: FVM BAUEC/2022/351

Nancy Wambui Wairimu,
Dept. of Biochemistry & Biotechnology
Technical University of Kenya
09/03/2022

Dear Nancy,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

"Toxicological characterization of sodium metabisulfite and mitigation of its effects by standardized *Ginkgo biloba* extract (EGb-761) in a mouse model".

We refer to your MSc. proposal submitted to our committee for review and your application letter dated 22nd February 2022. We have reviewed your application for ethical clearance for the study. The number of mice and protocols used to evaluate organ physiological, biochemical, immunopathological, inflammation and oxidative stress parameters meets the minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Dr. Catherine Kaluwa, Ph.D
Chairperson, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi

Appendix II: Plagiarism report

Nancy Wambui

ORIGINALITY REPORT

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