EFFECTS OF SOME INDIGENOUS PLANT EXTRACTS ON CARBON TETRACHLORIDE-INDUCED HEPATIC FIBROSIS IN MALE WISTAR RATS

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CERTIFICATION

This is to certify that this study on **"Effects of some indigenous plant extracts on carbon tetrachloride-induced hepatic fibrosis in male Wistar rats"** was carried out by **Ukairo, Doris Ifeyinwa (Reg. No. 20094757798)** in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D) in the Department of Biochemistry of the Federal University of Technology, Owerri, Imo State.

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DEDICATION

This work is dedicated to the memory of my late father, Engr C.M.C. Iwunze, whose uttermost desire was for me to become a medical doctor. I wish to pass it on to my lovely children and all who aspire for greatness through diligence.

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TABLE OF CONTENTS

Title i

Certification ii

Dedication iii

Acknowledgement iv

Table of Contents v

List of Tables xiii

List of Figures xv

List of Plates xvi

Abstract xvii

CHAPTER ONE

1.0 Introduction 1

1.1 Background information 1

1.2 Problem statement 6

1.3 Aim and objectives of study 6

1.4 Justification of study 8

1.5 Scope of study 8

CHAPTER TWO

2.0 Literature review

2.1 Anatomy and physiology of the liver 9

2.2 Physiological functions of the liver 12

2.3 Pathogenesis of liver fibrosis 15

2.3.1 Initiation of hepatic stellate cell (HSC) activation 16

2.3.1 Perpetuation of hepatic stellate cells 18

2.4 Collagen 20

2.5 Types and causes of liver fibrosis 22

2.5.1 Acquired liver fibrosis 23

2.5.1.1 Alcoholic liver disease 23

2.5.1.2 Non-alcoholic liver fatty diseases 24

2.5.1.3 Cholestatic liver diseases 25

2.5.1.4 Chronic viral hepatitis 26

2.5.1.5 Fibrosis by chemical substances 27

2.6 Mechanism of CCl_4 induction of hepatic fibrosis 28

2.7 Role of ROS and oxidative stress in hepatic fibrosis 33

2.8 Detection of fibrosis 38

2.8.1 Biopsy 39

2.8.2 Non-invasive detection methods 40

2.9 Therapeutic strategies of liver fibrosis 42

2.9.1 Therapies to eliminate the etiological factors 42

2.9.2 Anti-inflammatory and immunosuppressive therapies 42

2.9.3 Suppression of activation and promotion of hepatic stellate cells (HSCs) apoptosis 43

2.9.4 Protection of liver function and promotion of hepatocyte regeneration 44

2.9.5 Gene therapy and targeted therapy 46

2.9.6 Complementary and alternative medicine 47

2.10 Antioxidants 49

2.10.1 The enzymic antioxidants 49

2.10.2 Non-enzymic antioxidants 50

2.10.2.1 Glutathione 50

2.10.2.2 Vitamin C 51

2.10.2.3 Vitamin E 52

2.11 Plant bioactive substances 53

2.11.1 Phenols 55

2.11.2 Flavonoids 55

2.11.3 Alkaloids 58

2.11.4 Tannins 59

2.11.5 Saponins 61

2.11.6 Oxalate 63

2.12 Natural sources of antioxidants and other bioactive substances 66

2.12.1 Silymarin 67

2.12.2 *Monodora myristica* 68

2.12.3 *Chromolaena odorata* 70

2.12.4 *Buccholzia coriacea* 71

2.12.5 *Sphenostylis stenocarpa* 73

2.13 Effect of processing on plant bioactive substances 74

2.13.1 Cooking 76

2.13.2 Drying 78

CHAPTER THREE

3.0 Materials and Methods 79

3.1 Materials 79

3.1.1 Glasswares and equipment 79

3.1.2 Reagents and chemicals 79

3.1.3 Collection of plant materials 80

3.1.4 Animals 81

3.2 Methods 81

3.2.1 Treatment of samples 81

3.2.2 Methanol extraction 82

3.2.3 Extract dosage selection 82

3.2.4 Experimental protocol 84

3.2.5 Induction of hepatic fibrosis 85

3.2.6 Collection of blood and tissue samples 85

3.2.7 Determination of proximate compositions 86

3.2.8 Qualitative analysis of phytochemicals 89

3.2.9 Quantitative analysis of phytochemicals 91

3.2.10 Determination of some antioxidants in pulverized plant samples 96

3.2.10.1Selenium analysis using Atomic Absorption Spectrophotometer 96

3.2.10.2 Determination of Ascorbic acid (Vitamin C) concentration 97

3.2.10.3 Determination of Vitamin E concentration 97

3.2.11 Free radical scavenging activities of the pulverized samples 98

3.2.11.1 Superoxide scavenging assay 99

3.2.11.2 Hydrogen peroxide (H_2O_2) radical scavenging assay 100

3.2.11.3 Hydroxyl radical scavenging assay 100

3.2.11.4 Nitric oxide scavenging assay 101

3.2.11.5 Ferric reducing ability 102

3.2.12 Haematological analyses 102

3.2.13 Analysis of biochemical parameters 104

3.2.13.1 Determination of aspartate amino transferase (AST) activity 104
3.2.13.2 Determination of alanine aminotransferase (ALT) activity 105
3.2.13.3 Determination of alkaline phosphatase (ALP) activity 106
3.2.13.4 Determination of total protein concentration 106
3.2.13.5 Determination of albumin concentration 107
3.2.13.6 Determination of creatinine concentration 108
3.2.13.7 Determination of urea concentration 109
3.2.13.8 Determination of chloride ion (Cl^-) concentration 109
3.2.13.9 Determination of potassium ion (K^+) concentration 110
3.2.13.10 Determination of carbon dioxide (CO_2) concentration 111
3.2.13.11 Determination of sodium ion (Na^+) concentration 112
3.2.13.12 Determination of Cholesterol (Chol) concentration 113
3.2.13.13 Determination of Triglycerides (TG) concentration 114
3.2.13.14 Determination of High density lipoprotein (HDL) concentration 115
3.2.13.15 Determination of lipid peroxidation index (LPO) 116
3.2.13.16 Determination of Superoxide dismutase (SOD) activity 117
3.2.13.17 Determination of Catalase (CAT) activity 117
3.2.13.18 Determination of lactate dehydrogenase activity (LDH) 118
3.2.14 Histopathological examination of liver and kidney tissues 119
3.2.15 Immunohistochemical determination of collagen expression 119
3.2.16 Statistical Analysis 121

CHAPTER FOUR

4.0 Results and Discussion 122
4.1 Results 122

4.1.1 Proximate compostions of raw and cooked pulverized samples of *S. stenocarpa, B. coriacea, M. myristica* and *C. odorata* 122
4.1.2 Qualitative phytochemical contents of the pulverized plant samples 123
4.1.3 Quantitative phytochemical contents of the pulverized plant samples 124
4.1.4 Antioxidant properties of the pulverized plant samples 125
4.1.5 Radical scavenging activities of pulverized samples 127
4.1.5.1 Superoxide radical scavenging assay 127
4.1.5.2 Hydrogen peroxide scavenging activity of the samples 128
4.1.5.3 Hydroxyl radical scavenging assay of the samples 129
4.1.5.4 Nitric oxide scavenging assay 130
4.1.5.5 Total reducing ability of the samples 131
4.1.6 Body weights of animals, clinical signs and mortality patterns 132
4.1.7 Relative liver organ weights of the rats 135
4.1.8 Effect of extracts on haematological indices 137
4.1.9 Effect of extracts on hepatic indices
4.1.9.1 Aspartate aminotransferase activity 143
4.1.9.2 Alanine aminotransferase activity 145
4.1.9.3 Alkaline phospatase activity 146
4.1.9.4 Total Protein, albumin and bilirubin concentrations 147
4.1.10 Effect of raw and cooked samples on renal functions of experimental animals 149
4.1.11 Effect of extracts on lipid profile 152
4.1.12 Effect of extracts on lipid peroxidation 156
4.1.13 Effect of extracts on catalase activity 158
4.1.14 Effect of extracts on Lactate Dehydrogenase (LDH) activity 160
4.1.15 Effect of plant extracts on kidney and liver histology 161
4.1.16 Effect of cooked extracts on collagen expression 180

4.2 Discussion 182

CHAPTER FIVE

5.0 Conclusion and Recommendations

5.1 Conclusion 212

5.2 Contribution to Knowledge 213

5.3 Recommendation for further studies 213

References 214

Appendices 241

LIST OF TABLES

Table 4.1 Proximate compostion of raw and cooked pulverized samples of *S. stenocarpa, B. coriacea, M. myristica* and *C. odorata* 122

Table 4.2 Qualitative phytochemical compositions of the samples 123

Table 4.3 Quantitative phytochemical compositions of the samples 124

Table 4.4 Antioxidant properties of pulverized plant samples 126

Table 4.5 Effects of extracts on serum total protein, albumin and bilirubin concentrations 148

Table 4.6 Effects of extracts on serum urea and creatinine concentrations 150

Table 4.7 Effect of extracts on serum electrolyte concentrations 151

LIST OF FIGURES

Figure 2.1	The anatomy of the liver	10
Figure 2.2	Structure of the lobule	11
Figure 2.3	Key concepts involved in the activation of hepatic stellate cells and pathogenesis of hepatic fibrosis	17
Figure 2.4	Biotransformation of carbon tetrachloride (CCl_4)	30
Figure 2.5	Mechanism of CCl_4 toxicity	31
Figure 2.6	Effects of reactive oxygen species	34
Figure 2.7	Scavenging mechanisms of flavonoids	57
Figure 2.8	chemical structure of tannic acid	60
Figure 2.9	Structure of saponin	62
Figure 2.10	Biosynthetic pathway of oxalate in plants	64
Figure 4.1	Superoxide radical scavenging activities of the samples	127
Figure 4.2	Hydrogen peroxide scavenging activities of the samples	128
Figure 4.3	Hydroxyl radical scavenging activities of the samples	129
Figure 4.4	Nitric oxide scavenging activities of the samples	130
Figure 4.5	Total reducing ability of the raw and cooked samples	131
Figure 4.6	Body weights of animals	134
Figure 4.7	Relative liver organ weights of rats	136
Figure 4.8	Effect of extracts on haemoglobin concentrations of the experimental animals	139
Figure 4.9	Red blood cell counts of the animals	140
Figure 4.10	White blood cell counts of the animals	141
Figure 4.11	Platelet counts of the animals	142
Figure 4.12	Effect of extracts on AST activities of intoxicated and non-intoxicated animals	144
Figure 4.13	Effect of extracts on ALT activities of the rats	145

Figure 4.14 Effect of extracts on ALP activities 146
Figure 4.15 Total cholesterol concentrations of intoxicated and non-intoxicated animals 153
Figure 4.16 Effect of extracts on triacylglycerol concentrations 154
Figure 4.17 Effects of extracts on high density lipoprotein 155
Figure 4.18 Malondialdehyde Concentrations of intoxicated and non-intoxicated animal groups 157
Figure 4.19 Effect of extracts on catalase activities of animals 159
Figure 4.20 Effect of extracts on lactate dehydrogenase (LDH) activity 160

LIST OF PLATES

Plate 4.1- 4.9	Effect of extracts on kidney histology	162
Plate 4.10 - 4.18	Effect of extracts on liver histology	171
Plate 4.19 - 4.20	Effect of extracts on collagen expression of liver	180

ABSTRACT

Plant-derived drugs and antioxidants are widely used in the management of chronic liver diseases and cirrhosis. Methanol extracts of raw (R) and cooked (C) samples of *Sphenostylis stenocarpa* (SS), *Chromolaena odorata* (CO), *Buccholzia coriacea* (BC) *and Monodora myristica* (MM), at doses of 250 mg and 500 mg per kilogram body weight of male Wistar rats were evaluated for their effects on carbon tetrachloride (CCl_4)-induced hepatic fibrosis. One hundred and sixty (160) rats (155 ± 20 g) were distributed into twenty groups of eight rats each; representing normal control, olive oil control, negative control, positive control (silymarin) and various groups treated with raw and cooked extracts of the samples at the stated doses (RSS_{250}, RSS_{500}, CSS_{250}, CSS_{500}, RCO_{250}, $CCO_{250,}$ etc.). The extract-treated groups were pre-treated with their respective extracts twice a week for four weeks before the introduction of toxicant. All the groups except normal and olive oil controls were intraperitoneally injected with 1 mg/kg of CCl_4/olive oil (1:1 v/v) mixture twice a week for four weeks with continued extract-treatment. Liver and kidney damage were assessed using various biochemical parameters, such as the enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phospatase (ALP), lactate dehydrogenase (LDH) activities, as well as bilirubin, urea, creatinine, electrolytes and malondialdehyde (MDA) concentrations. In addition, CCl_4-induced pathological changes were evaluated by histopathological studies and the expression of collagen as evidence of induced fibrosis confirmed by immunohistochemistry. Treatment with methanol extracts of SS, BC and MM especially at low concentrations significantly ($P<0.05$) ameliorated the effects of CCl_4 intoxication by reducing hepatic enzymes and LDH activities and as well increased other parameters. The effects of the extracts were attributed to the phytochemical compositions of the plants. The findings provided evidence to show that methanol extracts of SS, BC and MM at low concentrations possessed antifibrotic and hepatoprotective properties against CCl_4-induced liver injury in male Wistar rats through their antioxidant and anti-inflammatory activities.

Keywords: Hepatic fibrosis, indigenous plants, antioxidants, immunohistochemistry, CCl_4

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND INFORMATION

Liver fibrosis is an important public health concern with significant morbidity and mortality worldwide (Chakraborty *et al.*, 2012; Seki and Brenner, 2015). It is a common pathologic process for almost all chronic liver diseases which results in liver cirrhosis (Liu *et al.*, 1998). Liver fibrosis and cirrhosis are the main risk factors of hepatocellular carcinoma; the third major cause of death from cancer worldwide (Bolarin and Azinge, 2007; Chakraborty *et al.*, 2012).The study of Nwokediuko *et al.* (2013) at the University of Nigeria Teaching Hospital, Ozalla, Enugu State, revealed that liver cirrhosis and primary liver cancer accounted for two thirds of liver diseases in hospitalised patients.

Liver injury occurs in response to a variety of insults caused by alcohol, viral hepatitis, steatosis, insulin resistance, autoimmune disease, excessive deposition of iron or copper on liver and congenital abnormalities (Chakraborty *et al.*, 2012), drugs (Sreya and Revathi, 2015), xenobiotics and oxidative stress (Jaeschke *et al.*, 2002). Carbon tetrachloride (CCl_4)-induced liver injury is one of the well established systems of xenobiotic-induced hepatotoxicity and a common

experimental model for the study of hepatotoxic and hepato-protective properties of drugs or plant extracts (Abdou *et al.*, 2012).

An overactive wound healing process in response to injury, results in hepatic fibrosis (Friedman, 2003). Pathogenesis of liver fibrosis is complex and varies between the different kinds of hepatic injuries. Usually, after an acute liver damage, parenchymal cells regenerate and replace necrotic and apoptotic cells (Odena *et al.*, 2015). This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). When the injury persists, eventually the regenerative response fails and the hepatocytes are substituted by abundant ECM comprising mainly of collagen, fibronectin, elastin, laminin, and proteoglycans (Odena *et al.*, 2015). Hepatic stellate cells (HSC) are the primary and main sources of ECM (Reeves and Friedman, 2002; Seki and Brenner, 2015).

Hepatic stellate cells are quiescent cells rich in vitamin A. Upon liver injury, HSC `get activated and lose the vitamin A, then take on a myofibroblastic phenotype and synthesize pro-inflammatory cytokines (Friedman, 2008a). The persistent "activated" state of the fibroblasts lead to the excessive accumulation of ECM, predominantly fibrillar collagens type I and III, which results in the disruption of normal tissue functions (Fritz, 2008). The activated myofibroblasts also contribute to the chemotaxis of leukocytes as well as their own chemotaxis through the

production of chemokines and cytokines such as Monocyte Chemotactic Protein-1 (MCP-1), Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor (TGF) (Iredale, 2001). The imbalance between the production and degradation of ECM has been implicated as the critical factor in the fibrogenesis of liver fibrosis (Geng *et al.*, 2005; Bolarin and Azinge, 2007), hence it is regarded as the target cells of anti-fibrotic therapy (Li and Wang, 2009).

In chronic liver diseases, apoptosis is a prominent feature and correlates with the worsening fibrosis (Jaeschke *et al.*, 2002). Apoptosis is an active physiological process, highly regulated and a conserved form of cell suicide that is involved in maintaining tissue homeostasis and health by counter-balancing cell proliferation and eliminating damaged and/or aged cells (Guicciardi and Gores, 2010). These apoptotic bodies are recognised, engulfed, and degraded by professional phagocytes, innate immune cells, and HSC (Chakraborty *et al.*, 2012). Excessive apoptosis stimulates inflammation, resulting in the production of pro-inflammatory cytokines and reactive oxygen species by innate immune cells (Grivennikov *et al.*, 2010). Increased free radical production causes DNA damage and lipid peroxidation, increasing the severity of the disease by causing oxidative stress-induced hepatocytic damage (French *et al.*, 1993). Lipid peroxidation products are emerging as important stimuli to ECM production; their effects may be amplified by loss of antioxidant capacity of stellate cells as they get activated (Natori *et al.*,

2001). These important insights have provoked efforts to use antioxidants as therapeutic agents for hepatic fibrosis.

Other therapeutic interventions in liver fibrosis include, the elimination of primary diseases, immunomodulation, suppression of hepatocyte inflammation, prevention of death and damage of hepatocytes, inhibition of over-secretion and accumulation of ECM proteins, promotion of ECM degradation, improvement of microcirculation and metabolism of liver, reduction of complications (Feng *et al.*, 2009), inhibition of HSC activation, suppressing activation and promoting apoptosis of HSC and promotion of hepatocyte regeneration (Zhou *et al.*, 2014). There is yet no standard and reliable modern treatment for liver fibrosis, although it is known that reducing liver injury events, such as interruption of alcohol intake or successful treatment of viral hepatitis, contributes to the control of the process (Abdou *et al.*, 2012). Nevertheless, these actions do not seem to be sufficient, in the vast majority of patients, to avoid progression to cirrhosis (Abdou *et al.*, 2012). Currently, medicinal plants enjoy a growing popularity as anti-fibrotic agents, supported by their safety, cost-effectiveness, versatility (Duval *et al.*, 2015), and physiological functions (Geng *et al.*, 2005). A number of medicinal preparations in *Ayurveda* are recommended for the treatment of liver disorders (Abdou *et al.*, 2012). There are several research reports on the hepato-protective activities of many plant extracts. Ethanol extracts of celery, *Chrysophyllum albidum*, and

Pyrenacantha staudtii as well as aqueous extract of *Cnidoscolus aconitifolius* have been reported to exhibit hepato-protective effect against, or ameliorate CCl_4-induced liver damage in male albino rats (Saba *et al.*, 2010; Abdou *et al.*, 2012).

Hence, these traditional medicinal plants; *Monodora myristica*, *Chromolaena odorata, Sphenostylis stenocarpa* and *Buchholzia coriacea* were selected based on their local use and reports on their bioactive components, especially antioxidant components. Several studies on these plants revealed the presence of phenols, flavonoids, alkaloids, saponins and in some cases, tannins. Flavonoids possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombic, antiviral and anti-carcinogenic activities (Bohm *et al.*, 1998; Middleton *et al*., 2000). Saponins are reported to exhibit hypolipidemic, anticancer and anticytotoxic activities among others (Podolak *et al.*, 2010). Tannins modulate immunoresponse in addition to its antimicrobial and antioxidative properties (Chung *et al.*, 1998; Min *et al.*, 2015). Despite the numerous beneficial components of the selected plants, little or no research has been carried out to elucidate the effects of the plants on carbon tetrachloride – induced hepatic fibrosis.

1.2 PROBLEM STATEMENT

Liver fibrosis is an important public health concern with significant morbidity and mortality worldwide (Chakraborty *et al.*, 2012; Seki and Brenner, 2015). Presently, there is no standard and effective pharmaceutical intervention for liver fibrosis. Many drugs that show potent anti-fibrotic activities *in vitro* often show only minor effects *in vivo* because of insufficient concentrations of drugs at the target cells and their adverse effects on other non-target cells (Li and Wang, 2009; Abdou *et al.*, 2012).

1.3 AIM AND OBJECTIVES

OVERALL AIM

This study was aimed at evaluating the anti-fibrotic and hepatoprotective effects of methanol extracts of *Monodora myristica, Chromolaena odorata* and *Buchholzia coriacea* on carbon tetrachloride induced hepatic fibrosis in male Wistar rats, consequent to exclusion of possible organ-toxic effects of the selected plants.

SPECIFIC OBJECTIVES

The objectives of this study were to:

1 Evaluate the proximate (moisture, protein, fats, crude fibre, total ash, and carbohydrate) and nutritive compositions (selenium, vitamin C and vitamin E) contents of the raw and cooked samples of the plants

2 Ascertain the qualitative and quantitative compositions of the phytochemicals (total phenol, tannins, alkaloids, flavonoids, saponins and oxalates) of the raw and cooked samples.

3 Determine the radical (superoxide, hydrogen peroxide, hydroxyl and nitric oxide) scavenging activities and ferric reducing power samples.

4 Evaluate the organ (liver and kidney) effects of the methanol extracts of the samples on male Wistar rats.

5 Assess the liver and kidney status after carbon tetrachloride intoxication, through analyses of serum markers (alkaline phosphotase, aspartate and alanine aminotransferases, total protein, bilirubin, urea, creatinine and electrolytes), other parameters (catalase and lactate dehydrogenase activities malondialdehyde, platelet, WBC) and histological examination of the tissues.

6 Ascertain the anti-fibrotic effect of the extracts by immunohistochemical evaluation of collagen expression in the liver tissue.

7 Propose the likely mechanism by which the methanol extracts exert their therapeutic actions against carbon tetrachloride damage.

8 To decipher the mechanism of action of the plant extracts.

1.4 JUSTIFICATION OF STUDY

Complementary and alternative medicines (CAM) are documented to be effective in the treatment of liver diseases, such as antagonizing fibrosis, steatosis, and hepatitis, and in protecting the liver cell (Guan and He, 2013). Similarly, several plant-derived antioxidants, such as silymarin, baicalin, beicalein, quercetin, apigenin, have been shown to interfere with liver fibrogenesis (Wei *et al.*, 2015). Consequently, positive effects of the selected indigenous plants on the inhibition or regression of HSC activation and proliferation will immensely reduce the great challenges posed by fibrosis and related diseases.

1.5 SCOPE OF STUDY

The study covered preliminary studies on the selected plants which include the proximate compositions, some phytochemical contents and antioxidant properties of the plants. It also focused on the organ (liver and kidney) toxicity effects of the methanolic extracts of the plants on male albino Wistar rats, which were determined prior to the anti-fibrotic effects of the plants on CCl_4-induced hepatotoxicity. Effect of processing (cooking) on the plant properties was also assessed.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANATOMY AND PHYSIOLOGY OF THE LIVER

The liver is structurally and functionally complex and has been considered second only to brain in its complexity. The liver is the largest gland in the body and the second largest organ after the skin measuring roughly 1.5kg and constituting 2.5% of the total body weight of an adult (Si-Tayeb *et al.*, 2010). It is a wedge-shaped organ located at the upper right quadrant of the abdomen, below the diaphragm (Mounika *et al*., 2017). The liver consists of very soft, pinkish-brown tissues encapsulated by a connective tissue known as Glisson's capsule which is further covered and reinforced by the peritoneum of the abdominal cavity (Juza and Pauli, 2014). The liver is a multilobed organ consisting of two main lobes (the larger right lobe and smaller left lobe) separated by a band of tissue called the falciform ligament or broad ligament, which helps to attach the liver to the diaphragm (Fig 1.0).

The liver arises from the foregut endoderm of an embryo (Si-Tayeb *et al.*, 2010), believed to be in response to the fibroblast growth factor (FGF-1 and FGF-2) produced by the mesoderm of the developing heart. Several developmental

pathways regulate further growth and differentiation of the hepatic parenchyma as well as its vascularisation.

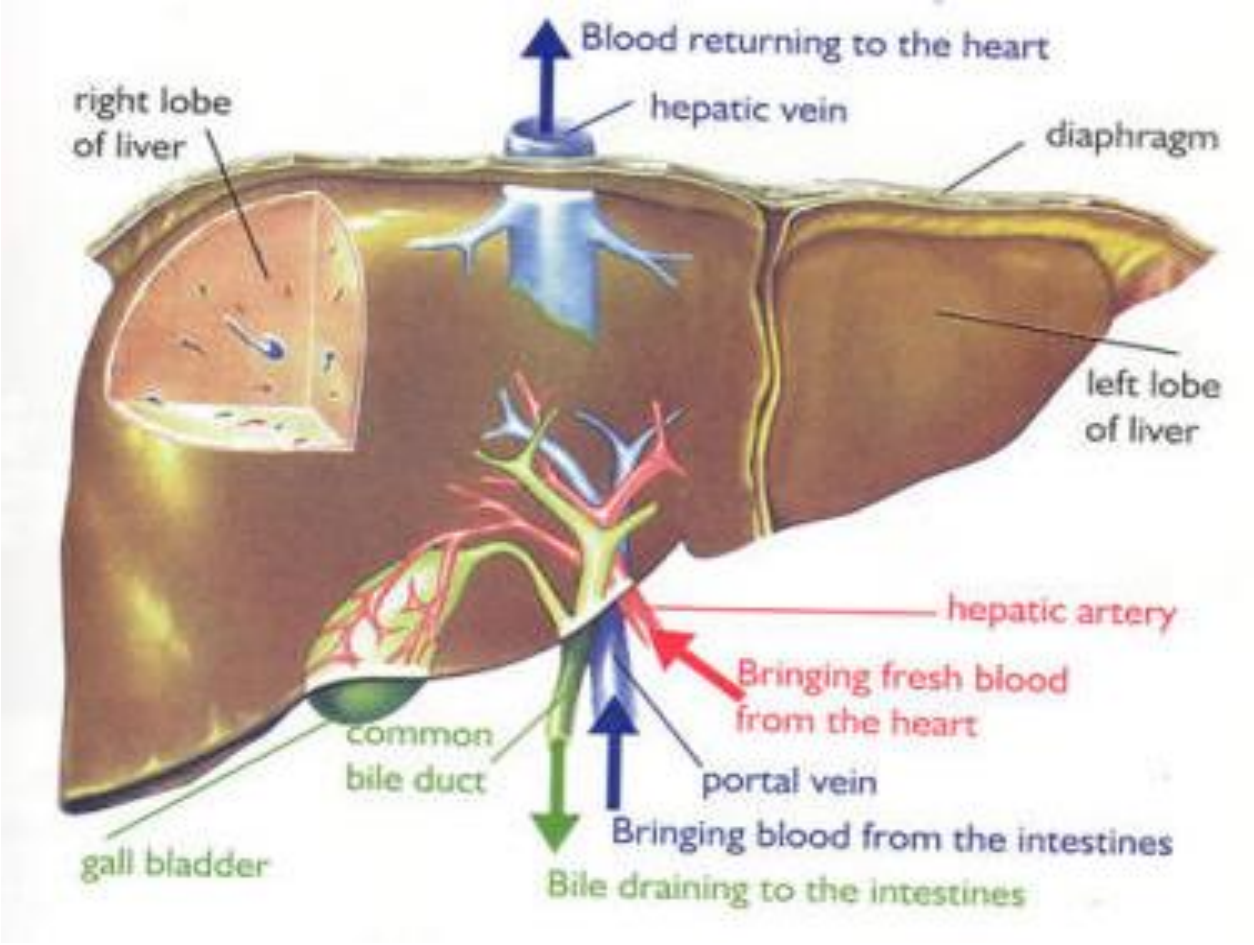


Fig. 2.1: The anatomy of the liver (Baltazar, 2013)

The parenchyma cell of the liver, the hepatocyte (70-85% of the liver volume) is responsible for the metabolic and exocrine functions of the liver and most often, it is the target cell of hepatotoxicity. Hepat ocytes are structurally arranged in cords that are lined by a specialized sinusoidal endothelium that is fenestrated and

lacks basement membrane (Braet and Wisse, 2002). This facilitates free exchange of blood solutes with the sub-endothelial membrane of the hepatocytes. Hepatocytes are connected to each other by gap junctions and tight junctions; the latter forming bile canaliculi which serve as channels for bile flow from the hepatocytes to the portal ducts that ultimately merge to form the main bile duct of the liver, the hepatic duct (Fig 2.2). This joins the cystic duct, which leads from the gallbladder, to form the common bile duct, which drains bile into the duodenum.

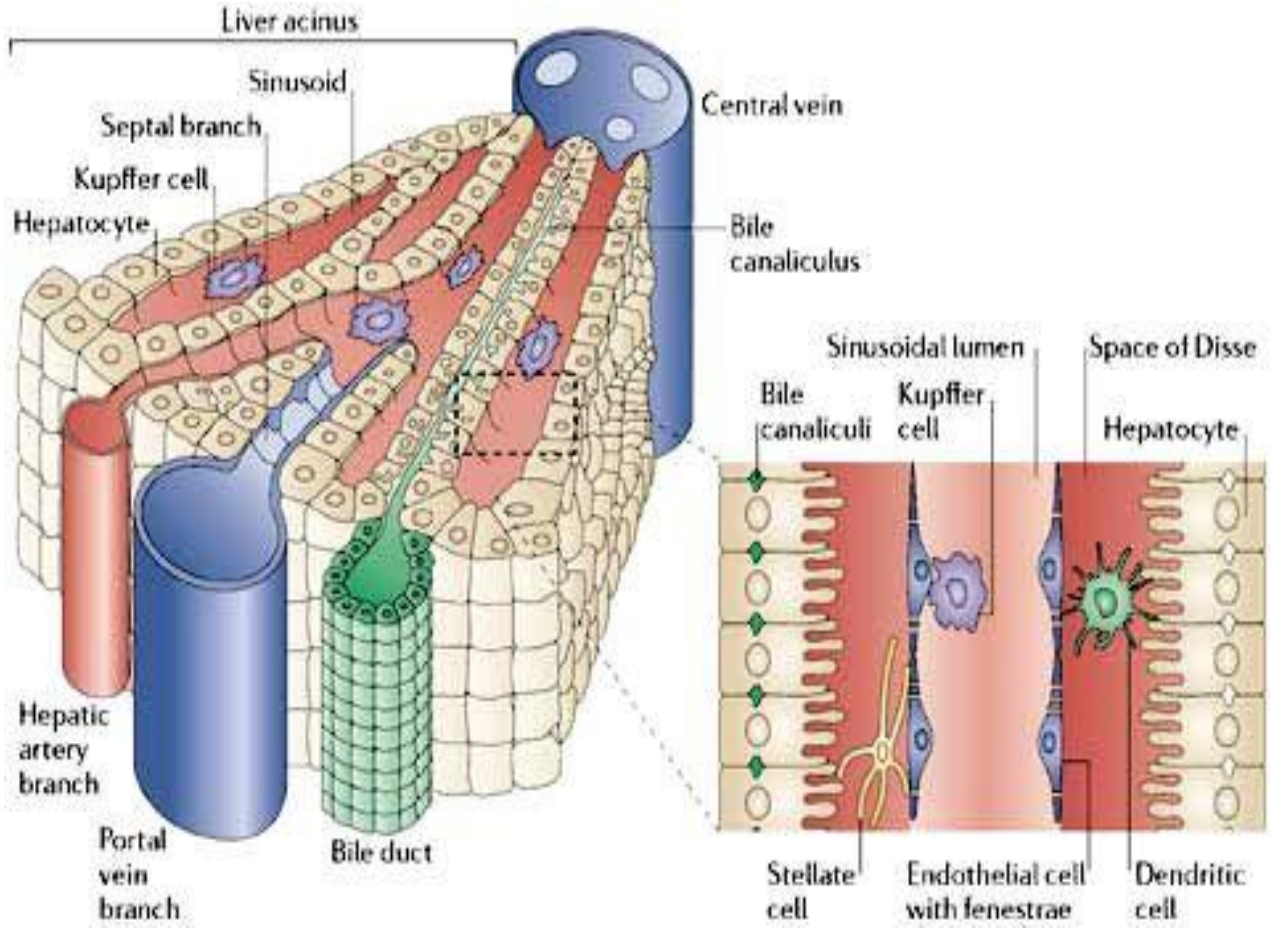


Fig. 2.2: Structure of the lobule (Adams and Eksteen, 2006)

Residing along the intravascular surface of the hepatic sinusoids are Kupffer cells (fig 2.2) which are phagocytic cells that share common lineage with monocytes derived from the bone marrow (Klein *et al.*, 2007). The non-parenchymal cells which constitute 40% of the total number of liver cells and 6.5% of its volume (Kmiec, 2001) also include natural-killer cells (NK), natural killer T-cells (NK-T) and stellate cells. Hepatic stellate cells (sometimes called Ito cells) are pericytes residing in the peri-sinusoidal space (space of Disse) between the basolateral surface of the hepatocytes and the abluminal surface of the sinusoidal endothelial cells (Senoo, 2004). They are quiescent cells, rich in vitamin A and represent 5-8% of the total number of liver cells.

2.2 PHYSIOLOGICAL FUNCTIONS OF THE LIVER

The liver performs many important functions in the body. These include the followings:

- **Metabolism**

The hepatocytes of the liver are tasked with many vital metabolic roles that support the cells of the body. The liver plays a central role in the homeostasis of carbohydrate, lipid, and protein metabolism of an organism. It is the main target of insulin and glucagon signaling and contributes to blood glucose balance by regulating glycogenesis and gluconeogenesis in the hepatocytes (Bugianesi *et al.*,

2005). The liver is the sole organ of fatty acids biosynthesis by *de novo* mechanism and the main site of fatty acid oxidation together with the muscle (Fabbrini *et al.*, 2010). The liver also synthesizes non-essential amino acids from other amino acids, glucose and fatty acids. The enzymes alanine and aspartate aminotransferases convert amino acids that are in abundance to others that are needed by the body (Dorcas and Solomon, 2014).

- **Detoxification**

Every substance absorbed from the gastrointestinal tract passes through the liver (first pass). The liver monitors the contents and removes many potential toxic substances such as drugs, toxins and metabolic products (ammonia, alcohol, bilirubin) by binding reversibly to inactivate, chemically modifying the compounds for excretion or metabolising them to useful substances (Jaeschke *et al.*, 2002). In order to keep hormone levels within homeostatic limits, the liver also metabolizes and removes from circulation hormones produced by the glands (Genes, 1977).

- **Storage**

The liver provides storage for many essential nutrients, vitamins, and minerals obtained from blood passing through the hepatic portal system. Glucose is transported into hepatocytes under the influence of the hormone insulin and stored as the polysaccharide, glycogen (Bugianesi *et al.*, 2005). Hepatocytes also absorb

and store fatty acids from digested triacylglycerols (Bemeur *et al.*, 2010). The storage of these nutrients allow the liver to maintain the homeostasis of blood glucose. The liver also stores vitamins (A, D, E, K, and B_{12}) and the minerals such as iron and copper in order to provide a constant supply of these essential substances to the tissues of the body (Dorcas and Solomon, 2014).

- **Biosynthesis**

The liver is responsible for the production of several vital protein components of blood plasma such as clotting factors (prothrombin, fibrinogen), globins and albumins. Albumins maintain the isotonic environment of the blood thus preventing excess gain or loss of water in body fluids (Salawu *et al.*, 2010**)**. The deamination of amino acids leads to the production of toxic ammonia, which is rapidly converted to less harmful urea. The biosynthesized urea is then transported through the blood to the kidneys, where it is excreted (Noori *et al.*, 2014).

- **Immunity**

The liver functions as an organ of the immune system through the activities of the Kupffer cells that line the sinusoids. Kupffer cells are a type of fixed macrophage that form part of the mononuclear phagocyte system along with macrophages in the spleen and lymph nodes. Kupffer cells play an important role by capturing and digesting bacteria, fungi, parasites, worn-out blood cells, and cellular debris. The

large volume of blood passing through the hepatic portal system and the liver allows Kupffer cells to clean large volumes of blood very quickly (Klein *et al.*, 2007).

2.3 PATHOGENESIS OF LIVER FIBROSIS

Liver fibrosis is defined as the building up of excessive amount of extracellular matrix, also known as scar tissue in the liver parenchyma (Baranova *et al.*, 2011). The process of fibrogenesis is a component of the normal healing response to various kinds of injury. Liver injury of any etiology will ultimately lead to activation of HSCs (Moreira, 2007). Stellate cell "activation" refers to the conversion or trans-differentiation of a resting vitamin A-rich cell to one that is proliferating, fibrogenic, and contractile (Moreira, 2007; Friedman, 2008a). Stellate cell activation represents a continuum, such that early changes in cellular phenotype may be distinct from those occurring with progressive injury and activation in terms of growth characteristics, response to soluble mediators, inflammatory signaling, and apoptotic potential (Friedman, 2008a). Activation of hepatic fibrosis consists of two major phases: initiation and perpetuation (Friedman, 2008a).

2.3.1 Initiation of hepatic stellate cell activation

Initiation refers to early paracrine-mediated changes in gene expression and phenotype that render the cells responsive to other cytokines and stimuli (Friedman, 2008a). Following persistent liver injury, there is paracrine stimulation by all neighboring cell types, including sinusoidal endothelium, Kupffer cells, hepatocytes, platelets, and leukocytes. Through the production of cellular fibronectin and conversion of transforming growth factor (TGF) to profibrogenic form, the endothelial cells participate in the activation of HSCs. The activation and infiltration of the resident macrophage in the liver (kupffer cells), stimulates matrix synthesis, cell proliferation, and release of retinoids by stellate cells through the expression of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL) 1β and IL-6, and several chemoattractant molecules such as monocyte chemotactic protein 1 (MCP-1) (López-Navarrete *et al.*, 2011) (Fig 3.0).

Kupffer cells also synthesize anti-inflammatory profibrogenic factors, such as transforming growth factor beta 1 (TGF-β1) capable of activating the hepatic stellate cells (HSC). Kupffer cells are important source of reactive oxygen species and also produce nitric oxide (NO), which can counterbalance the stimulatory effects of ROS by reducing stellate cell proliferation and contractility (Friedman,

2008b; López-Navarrete *et al.*, 2011). Under conditions of inflammatory liver diseases, hepatocytes serve as potent source of fibrogenic lipid peroxides; platelet provide growth factors which include platelet-derived growth factor (PDGF), TGF-beta 1, and epidermal growth factor (EGF); leukocytes release compounds that modulate stellate cell behavior while activated neutrophils are an important source of ROS, which have a direct stimulatory effect of over 3 fold increase on stellate cell collagen synthesis (Friedman, 2008b).

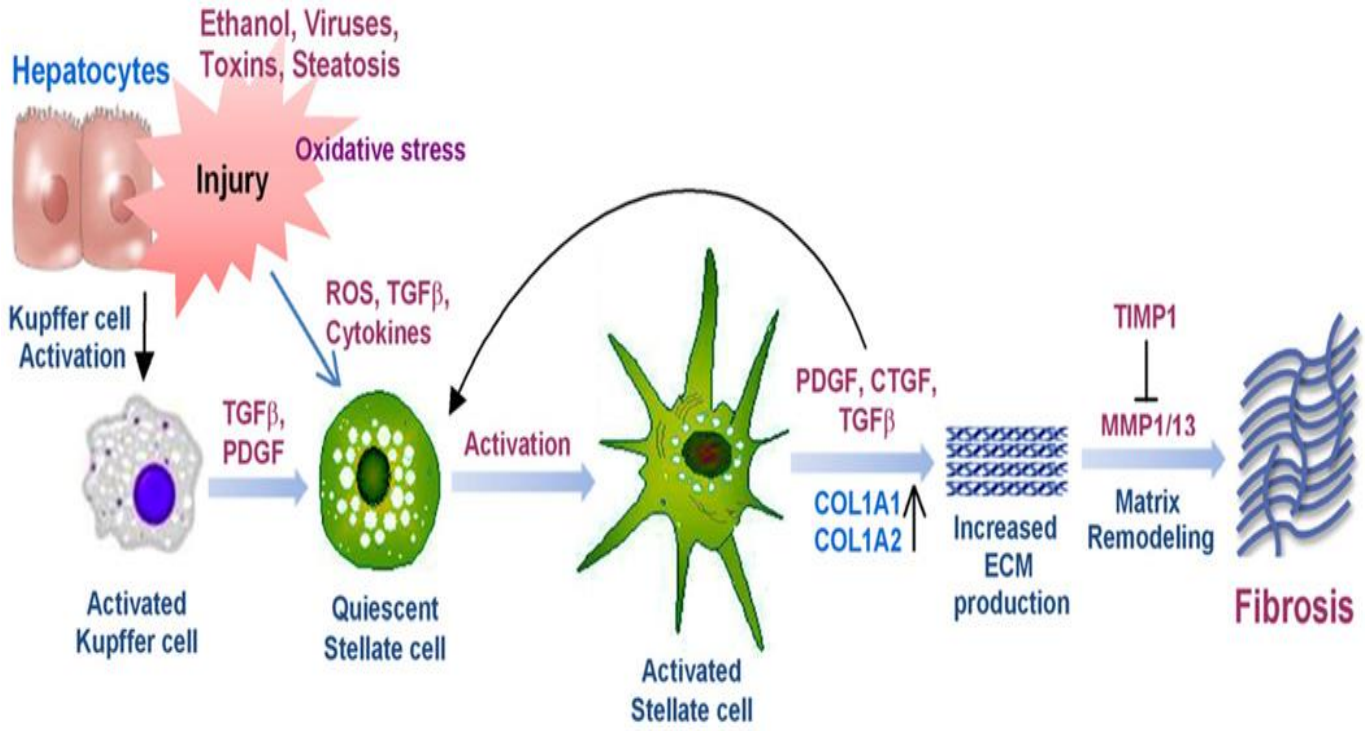


Fig 2.3: Key concepts involved in the activation of hepatic stellate cells and pathogenesis of hepatic fibrosis (Mormone *et al.*, 2011).

2.3.2 Perpetuation of HSCs

Perpetuation of stellate cell activation results from the effects of these stimuli on maintaining the activated phenotype and generating fibrosis (Friedman, 2008b). It involves at least seven discrete changes in cell behavior: retinoid loss, proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation and WBC chemoattractant/cytokine release. The net effect of these changes is to increase accumulation of extracellular matrix (Friedman, 2008b).

PDGF is the most potent stellate cell mitogen identified. Induction of PDGF receptors early in stellate cell activation increases responsiveness to this potent mitogen (Yamada *et al.*, 1997). The binding of the PDGF to the PDGF cell surface receptor activates signalling cascades that lead to the activation of MAPK-JNK, Ras-MEK-ERK and FAK pathways involved in HSC proliferation which signals through P13-kinase-ATK-p70S6 kinase pathway (Gabele *et al.*, 2003). Other compounds with mitogenic activity in stellate cells and a potential role in fibrogenesis include vascular endothelial cell growth factor (VEGF), thrombin and its receptor, EGF, TGF-α, keratinocyte growth factor, and bFGF (Friedman, 2008b).

Fibrogenesis is a result of imbalance between ECM synthesis and degradation; hence a net increase in the synthesis and deposition of extracellular matrix proteins

accompanied with an increase in tissue inhibitor of matrix metalloproteinase (TIMP) expression that inhibits collagenase activity. Several mediators such as retinoids, interleukin-1b, TGF-b, tumor necrosis factor, and acetaldehyde can influence the fibrogenic response of HSCs. However, transforming growth factor – beta (TGF-b) is the most potent fibrogenic cytokine known for the HSC (Gabele *et al.*, 2003). Hyperglycemia and hyperinsulinemia upregulates the TGF-b -dependent stimulation of connective tissue growth factor (CTGF/CCN2), a potent fibrogenic signal towards stellate cells (Paradis *et al.*, 2001; Gao and Brigstock, 2004).

Activated HSCs secrete inflammatory chemokines which promote the migration of fibrogenic cells to the site of injury, thereby enhancing fibrogenesis through increased cell number and amplified inflammation (Lee and Friedman, 2011). In a research study by Yang *et al.* (2003), stimulations of HSCs with PDGF-BB, TGF-beta1, and epithelial growth factor (EGF) resulted in an increase in their migratory capacity and up-regulated matrix metalloproteinase (MMP)-2 activity. Matrix metalloproteinase-1 (MMP-1) is the main protease that can degrade type I collagen, the principal ECM in fibrotic liver. Regulation of matrix metalloproteinase activity occurs at many levels, among which is the inactivation by binding to tissue inhibitors of metalloproteinases (TIMPs). The sustained production of TIMP-1 and TIMP-2 during liver injury by stellate cells could inhibit the activity of interstitial collagenases, leading to reduced degradation of the

accumulating matrix, a major determinant in progressive fibrosis (Yang *et al.*, 2003).

Activated HSCs also express the cytoskeleton protein, α-smooth muscle actin (α-SMA), which confers increased contractile potential on cells and various connective tissue proteins including collagen types I, III, and IV, thus, being capable of impeding portal blood flow by constricting individual sinusoids as well as the entire fibrotic liver. Endothelin-1 (ET-1) and nitric oxide are major counter-regulators controlling stellate cell contractility, in addition to a growing list of additional mediators including angiotensinogen II, eicosanoids, atrial natriuretic peptide, somatostatin and carbon monoxide, among others (Friedman, 2008a). A balance between ET-1 and NO is assumed to regulate the contractile activities of HSC, wherein ET-1 is the key contractile stimulus of HSC while NO is an antagonist of ET-1 produced by HSC, Kupffer cells, and liver endothelial cells (Ahmad and Ahmad, 2012). Stellate cell contraction has also been reported to be mediated by Ca^{++} dependent and independent mechanisms (Rockey, 2013).

2.4 COLLAGEN

Collagen and other extracellular matrix (ECM) components are deposited as the liver generates a wound healing response to encapsulate injury. Collagen, an insoluble fibrous protein is the most abundant components of the ECM and many

types of soft tissues (Halper and Kjaer, 2014), constituting 25% of the total protein mass in mammals (Alberts *et al.*, 2002). There are at least 16 types of collagens, but 80 – 90 percent of the collagens in the body consists of types I, II, and III. These collagen molecules pack together to form long thin *fibrils* of similar structure (Lodish *et al.*, 2000). It is present in scar tissue, the end product when tissue heals by repair. Collagen found in tendons, skin, arterial walls, cornea, the endomysium surrounding muscle fibers, fibrocartilage, and the organic part of bones and teeth (Shoulders and Raines, 2009).

In normal liver, HSCs express only trace amounts of type 1 collagen. The persistent "activated" state of the fibroblasts lead to excessive accumulation of ECM, predominantly fibrillar collagens type I and III, which results in the disruption of the normal tissue functions (Fritz, 2008). Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory, and fibrogenic properties. Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation. Collagen synthesis in HSCs is regulated at the transcriptional and post-transcriptional levels. Increased collagen mRNA stability mediates the increased collagen synthesis in activated HSCs. In these cells, posttranscriptional regulation of collagen is governed by sequences in the 3′

untranslated region via the RNA-binding protein αCP2 as well as a stem-loop structure in the 5′ end of collagen mRNA (Bataller and Brenner, 2005).

Inflammatory cells, either lymphocytes or polymorphonuclear cells, activate HSCs to secrete collagen (Bataller and Brenner, 2005). Procollagen precursors released by fibrogenic cells are processed by procollagen peptidases. Removal of the bulky propeptides allows the formation of collagen fibrils in the extracellular space. Thus, circulating propeptide levels should reflect *de novo* synthesis and deposition of collagen, i.e. fibrogenesis. On the other hand, action of matrix metalloproteinases (MMPs) is expected to generate fragments of already deposited matrix proteins, the levels of which should reflect matrix dissolution, i.e. fibrolysis (Schuppan *et al.*, 2003). Tissue inhibitors of the metalloproteinases are also expressed to counter-regulate the degradation process. They may also induce expression of the anti-apoptotic protein, Bcl-2, and thereby enhance survival of hepatic stellate cells (Czaja, 2014). Maturation of the collagen matrix depends mainly on lysyl oxidases that cross-link the collagen fibrils and increase the resistance to degradation (Czaja, 2014)

2.5 TYPES AND CAUSES OF LIVER FIBROSIS

Liver fibrosis is the common pathological pathway of liver damage arising from a wide variety of chronic liver diseases which could be hereditary or acquired. The

common causes are alcoholic liver damage (ALD), non-alcholic fatty liver damage (NAFLD), chronic viral hepatitis. Others include cholestatics, autoimmune hepatitis, and parasitic infections like schistosomiasis; these have been shown to trigger advanced liver fibrosis and portal hypertension (Anderson and Chung, 2007). Metabolic disorders such as hemochromatosis (excessive absorption and accumulation of iron in tissues and organs) and Wilson's disease or hepatolenticular degeneration (a genetic disorder leading to copper accumulation in the liver) are typically accompanied by chronic hepatitis and fibrosis (Mormone *et al.*, 2011)

2.5.1 Acquired Liver Fibrosis

Acquired liver fibrosis may result from the action of a number of pathogenic factors and toxic exposures which may work separately or in combination with each other to produce cumulative effects (Wang *et al.*, 2012).

2.5.1.1 Alcoholic Liver Disease

Excessive and chronic alcohol consumption is an important causative factor of liver fibrosis and cirrhosis. Two pro-fibrotic agents, acetaldehyde and reactive oxygen species (ROS) are produced during the process of ethanol metabolism in the hepatocytes (Baranova *et al.*, 2011). Acetaldehyde, the first metabolite of

ethanol, upregulated transcription of collagen I directly as well as indirectly by increasing the synthesis of transforming growth factor-beta 1 (TGF-β1) (Purohit and Brenner, 2006). Reactive oxygen species sensitize HSCs to various pro-inflammatory factors and elicit the production of inflammatory mediators that contribute to the fibrotic changes in the liver. The phagocytosis of alcohol-induced hepatocyte apoptotic bodies by HSCs and Kupffer cells as a normal repair process can result to increased expression of TGF-β1 and subsequent HSC activation (Purohit and Brenner, 2006). Kupffer cells may contribute to the activation of HSCs by releasing ROS and TGF- β1. Innate immunity which usually suppresses hepatic fibrosis by killing activated HSCs and blocking TGF-β1 signaling may be overwhelmed. In patients infected with hepatitis C virus (HCV), alcohol may promote hepatic fibrosis by suppressing innate immunity (Purohit and Brenner, 2006).

2.5.1.2 Non-alcoholic Fatty Liver Diseases (NAFLD)

Non-alcoholic fatty liver damage (NAFLD and its subtype, Non-Alcoholic Steatohepatitis or NASH, are wide range of conditions caused by a build up of fat within the liver. They are usually associated with metabolic syndrome (MS) or its components such as obesity, type-2 diabetes (DM), dyslipidemia, and insulin resistance (Baranova *et al.*, 2011). The spectrum of NAFLD is a continuum

ranging from simple steatosis to NASH, fibrosis and finally cirrhosis (Hassan *et al.*, 2014). The mechanism of NAFLD is not yet elucidated (Hassan *et al.*, 2014). The development of steatosis in the liver has been related in the 'first hit' hypothesis of Day and James (1998) to an enhanced production of long-chain fatty acids with impaired hepatic mitochondrial β-oxidation, enhanced synthesis and secretion of triglycerides in hepatocytes in addition to lack of very low-density lipoprotein (VLDL)synthesis. A key-defining feature of the NASH is the presence of inflammation marked by changes in several inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and Interleukin-6 (IL-6) which have been linked to both insulin resistance and progression to liver damage (Day and James, 1998; Sabio *et al.*, 2008; Feldstein, 2010).

2.5.1.3 Cholestatic Liver Diseases

Cholestasis is another well-known cause of liver fibrosis (Baranova *et al.*, 2011). Cholestasis represents the consequence of impaired bile formation and decrease in bile, characterized by the appearance of jaundice, fatigue, pruritus, and/or complications of cirrhosis (McGill and Kwiatkowsk, 1998; Jüngst *et al.*, 2013). Cholestasis triggers the proliferation of the cholangiocyte lining of the intrahepatic and extrahepatic bile duct systems through autocrine and paracrine signaling factors (Hirschfield *et al.*, 2010). The proliferating bile duct epithelia upregulates

integrin αvβ6 which in turn promotes fibrogenesis via adhesion to fibronectin and autocrine/paracrine activation of TGF-β1 (Patsenker *et al.*, 2008). In response to inflammation, cholangiocytes secrete cytokines and chemokines (e.g, tumor necrosis factor α, IL-1, or interferon gamma) that recruit and activate immune cells, including T cells, macrophages, and natural killer (NK) cells. Human cholangiocytes constitutively express and secrete chemotactic agents for neutrophils, monocytes, and T cells, including IL-8, IL-6, and MCP-1; under basal conditions, cholangiocytes express low levels of lymphocyte adhesion molecules (Hirschfield *et al.*, 2010)

2.5.1.4 Chronic Viral Hepatitis

Worldwide, hepatitis B and C infections represent the primary cause of liver fibrosis. Viruses pose an important risk for the development of liver fibrosis. McCaughan and George (2004), in a study involving 260 patients with chronic hepatitis C infection, reported that both the extent and rate of progression of hepatic fibrosis were associated with insulin resistance (IR) resulting to portal, other than lobular inflammation. Thus they stated that "TNF may be the key molecular link between inflammation, steatosis, and fibrosis in chronic HCV infection". Most likely, the pathogenesis is multifactorial as it involves a combination of both viral and host-specific factors, including oxidative stress,

hepatic steatosis, increased iron stores, and increased rate of hepatocyte apoptosis, under the pressure of the viral proteins and viral replication (McCaughan and George, 2004). HCV proteins appear to affect both lipid accumulation and degradation, with the consequent disruption of the normal process of lipid compartmentalization and metabolism, skewing towards ROS production (Cederbaum, 2010). In the case of HBV infection, studies have shown that the X protein of HBV (HBx) directly induces TGF-β secretion by hepatocytes and thus, contributes to the paracrine activation of HSC's (Cederbaum, 2010).

2.5.1.5 Fibrosis by Chemical Substances

Several well-established chemical substances have been identified that induce liver inflammation and fibrogenesis (Liedtke *et al.*, 2013). These substances include thioacetamide (TAA), carbon tetrachloride (CCl_4), hepatocarcinogen dimethynitrosamine (DMN) **(**Liedtke *et al.*, 2013; Mormone *et al.*, 2011), dimethylformamide (DMF), dimethylacetamide (DMA), trichloroethylene (TCE), tetrachloroethylene, xylene, toluene, and chloroform (Malaguarnera *et al.*, 2012). Factors such as specie differences, nutritional condition, genetic factors, interaction with medications in use, alcohol abuse and interaction, and age affect the the hepatotoxicity of the chemical substances (Malaguarnera *et al.*, 2012). The main pathogenic mechanisms responsible for functional damage caused by solvents are:

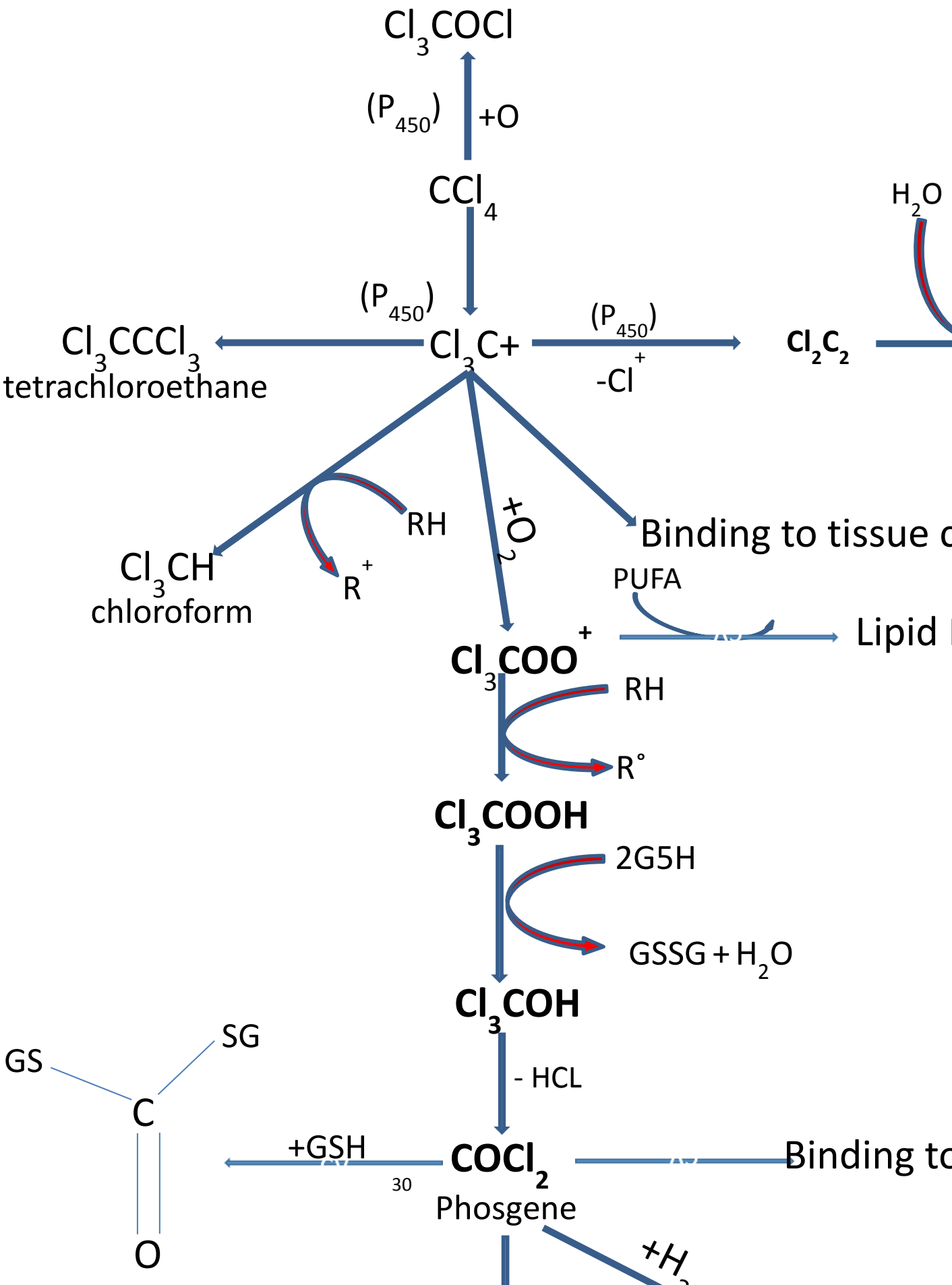
inflammation, dysfunction of cytochrome P450, mitochondrial dysfunction and oxidative stress (Malaguarnera *et al.*, 2012).

2.6 MECHANISM OF CCL_4 INDUCTION OF HEPATIC FIBROSIS

Carbon tetrachloride is one of the most powerful substances well-known for hepatotoxicity and widely used in scientific research to assess liver damage and hepatoprotective agents (Jeong and Park, 1998). Carbon tetrachloride (CCl_4) is a small lipophilic molecule that spreads easily in the lipid compartments of the body and is metabolized in the liver. Effects such as fatty degeneration, fibrosis, hepatocellular apoptosis and carcinogenicity have been associated with CCl_4 toxicity (Saba *et al.*, 2010).

Its mechanism of toxicity requires a hepatic microsomal CYP450. Among the several isoforms of CYP450 that metabolize CCl_4 and many other chemical substances such as acetaminophen, organic alcohols, benzene, N-nitrosodimethylamine, etc. CYP2E1 inducible by ethanol has been the main focus (Jeong and Park, 1998). The bio-activation of CYP450 following CCl_4 administration, produces a free radical specie, trichloromethyl ($^{\cdot}CCl_3$), which binds to cellular molecules such as nucleic acids, protein and lipids, thereby impairing crucial cellular processes with pathological resultant outcome such as fatty degeneration from impaired lipid metabolism and initiation of cancer due to

alterations in the DNA (Saba *et al.*, 2010). $^{\cdot}CCl_3$ can also react with oxygen to form trichloromethylperoxy radical ($^{\cdot}OOCCl_3$), a highly reactive specie that initiates the chain reaction of lipid peroxidation leading to the destruction of polyunsaturated fatty acids (PUFA) and alteration of the permeabilities of the mitochondrial, endoplasmic reticulum and plasma membrane (Fig 2.4). This culminates to loss of calcium, hence disruption in calcium homeostasis; a decrease of mitochondrial membrane potential causing release of proapoptotic factors resulting in apoptosis (Saba *et al.*, 2010). Trichloromethyl radical can also react covalently with sulfhydryl groups of glutathione and protein thiols to initiate lipid peroxidation (Jeong and Park, 1998). These lipids then damage the membranes of organelles and liver cells causing the swelling and necrosis of hepatocytes (Moreira *et al.*, 2014) which in turn results in the release of cytosolic enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) (Singh *et al.*, 1998).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(P450)
+O
CCl4
H2O
(P450)
Cl3CCCl3
tetrachloroethane
Cl3C+
(P450)
-Cl+
Cl2C2
RH
R+
+O2
Cl3CH
chloroform
Binding to tissue c
PUFA
Cl3COO+
Lipid
RH
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Cl3COOH
2G5H
GSSG + H2O
Cl3COH
- HCL
GS
SG
C
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30
COCl2
Phosgene
Binding to
+H2

Fig 2.4: Biotransformation of Carbon tetrachloride (McGregor and Lang, 1996)

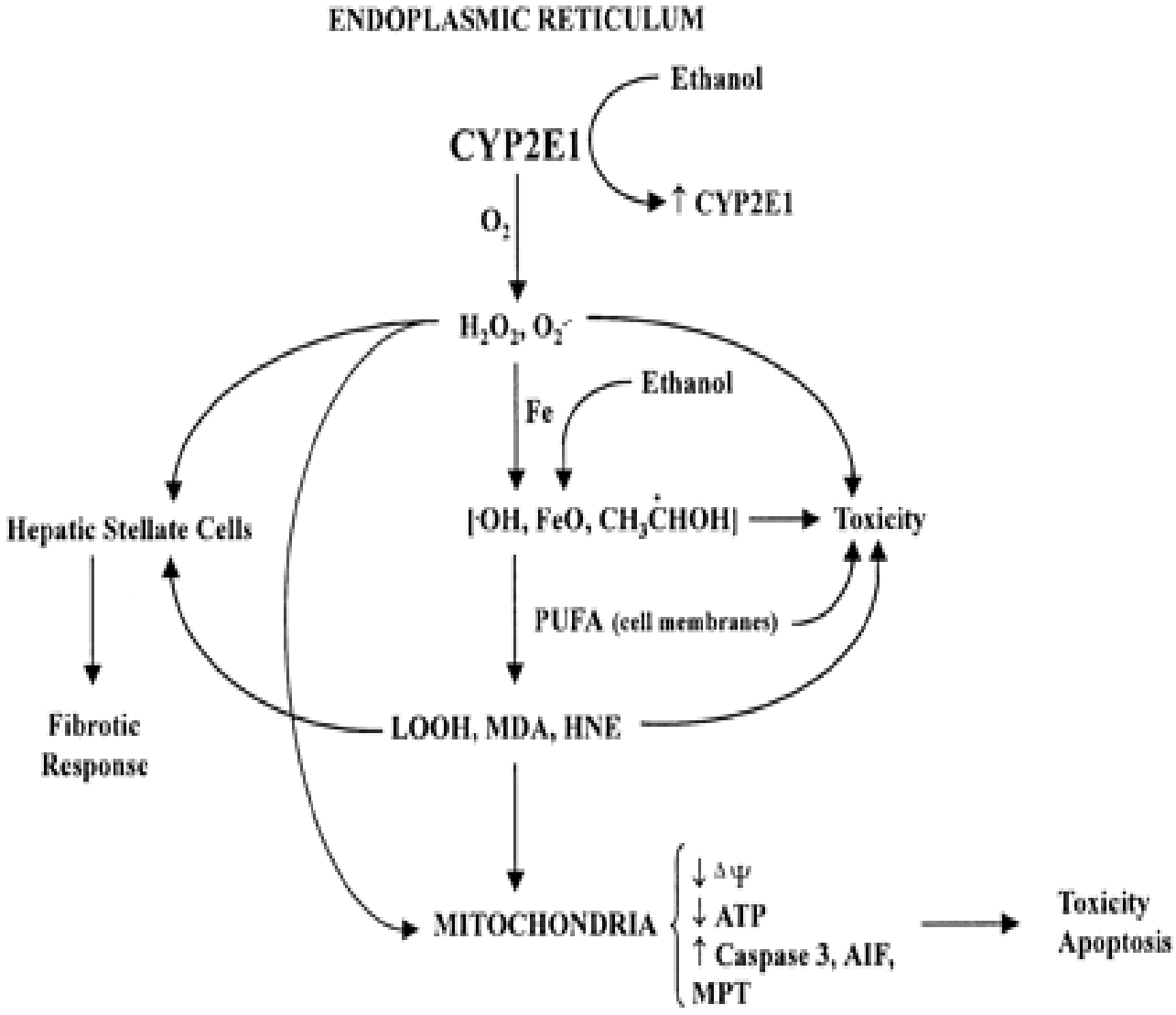


Fig. 2.5: Mechanism of CCl_4 Toxicity (Brattin *et al.*, 1985)

CYP2E1, a loosely coupled enzyme, also generates reactive oxygen species such as superoxide radical and hydrogen peroxide during its catalytic cycle. In the presence of iron, which is increased after ethanol treatment (Fig 2.5), more

powerful oxidants including hydroxyl radical, ferryl species, and 1-hydroxyethyl radical are produced (Brattin *et al.*, 1985). These various oxidants can promote toxicity by protein oxidation and enzyme inactivation, lipid peroxidation and production of reactive lipid aldehydes, such as malondialdehyde and 4-hydroxynonenal. Some CYP2E1-derived reactive oxygen species, e.g., H_2O_2, LOOH, MDA, HNE, are diffusible and may exit hepatocytes and enter other liver cell types, such as stellate cells, and stimulate these cells to produce collagen and elicit a fibrotic response (Brattin *et al.*, 1985).

CCl_4 intoxication has been implicated in the induction and promotion of hepatocytes necrosis, inflammation and hepatic fibrogenesis, antioxidant disorder and oxidative stress (Abdel-Ghaffar, 2013). Several experiments have shown that a single injection of CCl_4 causes hepatocyte necrosis in zone 3 of the liver lobule, and repeated treatments over long periods induce chronic liver diseases (e.g., fibrosis, cirrhosis, and cancer) (Malaguarnera *et al.*, 2012; Ki *et al.*, 2013). Abdel-Ghaffar (2013) reported a significant reduction in the hepatic antioxidants' defense markers {reduced glutathione (GSH), glutathione S -transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT)} and significant elevation of oxidative stress biomarker (malondialdehyde) in CCl_4 intoxicated rats.

The most common approach to toxin-induced experimental liver fibrosis is the periodic administration of carbon tetrachloride (CCl_4) in mice or rats. In mice, typically, 0.5 to 2 ml/kg body weight of CCl_4 (diluted in corn oil) is injected intraperitoneally (i.p.) two to three times per week, resulting in robust and highly reproducible liver fibrosis between 4 and 6 weeks of treatment (Liedtke *et al.*, 2013)**.**

2.7 ROLE OF ROS AND OXIDATIVE STRESS IN HEPATIC FIBROSIS

Oxidative stress is a redox imbalance between pro-oxidants and antioxidants in favour of pro-oxidants, leading to different responses depending on the level of pro-oxidants and the duration of the exposure (Videla, 2009). The imbalance could be as a result of lack of antioxidant capacity due to disturbances in production and distribution or by an over-abundance of reactive oxygen species (ROS) from other factors (Ha *et al.*, 2010). Various forms of ROS include free radicals, such as superoxide anion radical ($O_2^{\cdot\circ}$), hydroxyl radical ($^{\circ}OH$) and non-free radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) (Huda-faujan *et al.*, 2009).

ROS alterations in different signaling pathways may modulate gene expression, cell metabolism, cell adhesion, cell cycle and cell death, in addition to the activation of cellular signaling pathways, such as mediated by mitogen-activated protein kinase (MAPK) (Ha *et al.*, 2010).

Reactive oxygen species contribute to the hepatic fibrosis from various kinds of liver injuries, including alcohol abuse, HCV infection, iron overload, and chronic cholestasis (Paik *et al.*, 2014).

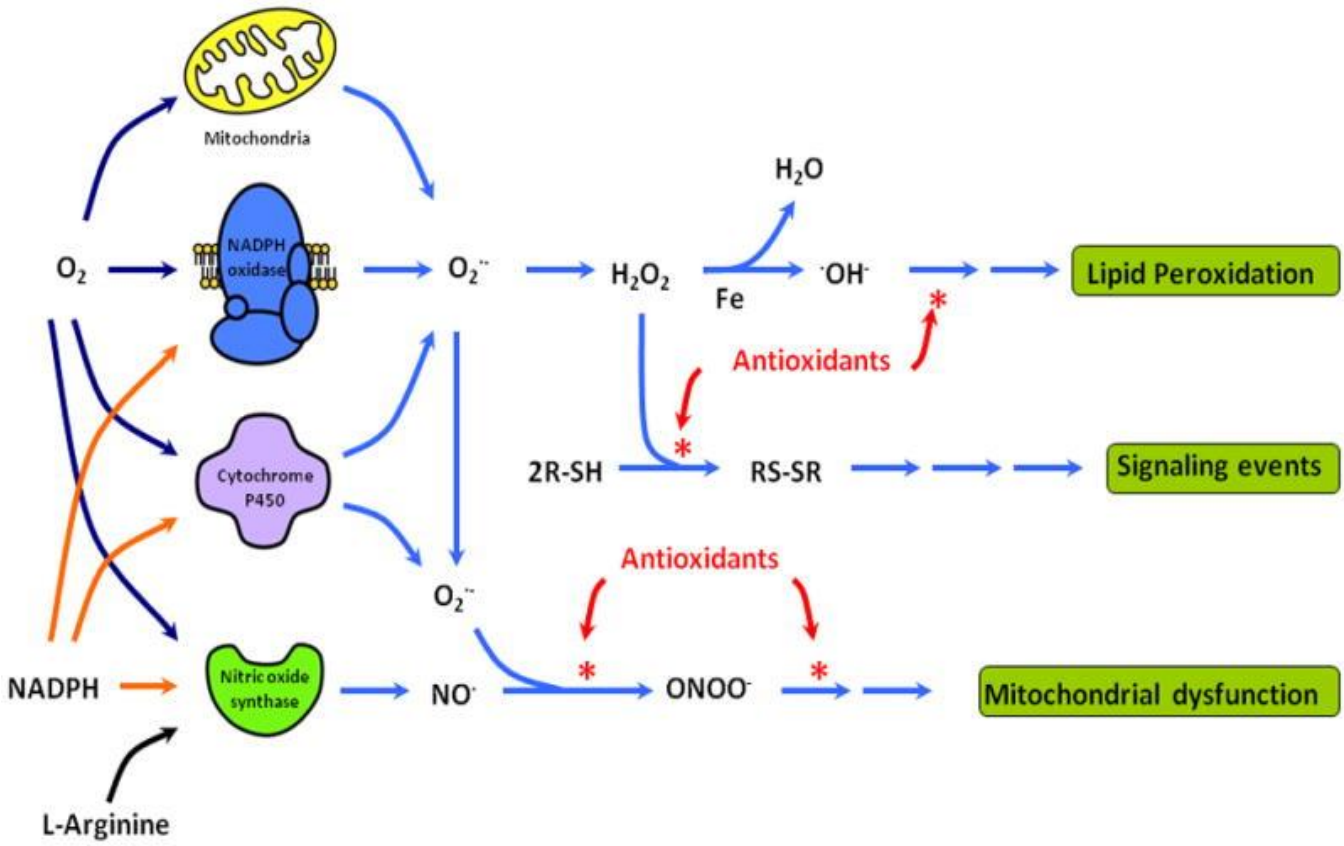


Fig. 2.6: Effects of Reactive oxygen species (Singal *et al.*, 2011)

Oxidative stress begins with the generation of reactive oxygen species (ROS) as part of normal cellular function (Singal *et al.*, 2011), involving the mitochondrial respiratory chain, cytochrome P450 (CYP) family members, peroxisomes, xanthine oxidase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases

(Paik *et al.*, 2014). These processes initially generate superoxide which is sequentially reduced to form hydrogen peroxide, hydroxyl radical, and ultimately water in the presence of antioxidants (Signal *et al.*, 2011). Superoxide ($^{\cdot}O_2^-$) ions are regarded as the major cause of the cellular oxidative damage that may underlie degradative diseases and aging; they are generated in large numbers in the mitochondria from complexes I and III of the electron transport chain and get dismutated to H_2O_2 (Keshari *et al.*, 2015).

$$^{\circ}O_2^- + {}^{\circ}O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2$$

The hydrogen peroxide formed reacts with a reduced heavy metal ion (Fe^{++} or Cu^+, usually) to form hydroxyl radical ($^{\circ}OH$) in a reaction called Fenton reaction (Keshari *et al.*, 2015).

$$Fe^{++} + H_2O_2 \Rightarrow Fe^{+++} + {}^{\circ}OH + :OH^- \text{ (Fenton Reaction)}$$

Fenton reaction may be the most dangerous because it can occur in the cell nucleus and lead to DNA damage. The oxidized iron (Fe^{+++}) can also catalyze the Haber-Weiss Reaction between superoxide and hydrogen peroxide to produce more hydroxyl radicals:

$$^{\circ}O_2^- + H_2O_2 \Rightarrow O_2 + {}^{\circ}OH + :OH^- \text{ (Haber-Weiss Reaction)}$$

Metal ions can also react with ascorbate (Vitamin C) to produce singlet oxygen ($^1O_2$) from normal triplet oxygen ($^3O_2$):

These reactive intermediates, however, interact with other molecules to form secondary reactive oxygen species, such as lipid peroxidation products, peroxynitrite, sulfenic acid and disulfides (Singal *et al.*, 2011). The hydroxyl radical can react with lipid molecules (LH) in membranes to produce lipid molecule radicals (alkyl = $^{\cdot}L$) which can react directly with oxygen (autoxidation) in a self-propagating chain reaction forming lipid peroxides (LOO°) and lipid hydroperoxides (LOOH).

$$^{\circ}OH + LH \Rightarrow {}^{\circ}L + H_2O$$

$$^{\circ}L + O_2 \Rightarrow LOO^{\circ}$$

$$LOO^{\circ} + LH \Rightarrow LOOH + {}^{\circ}L$$

The lipid hydroperoxides (LOOH) can promote a Fenton reaction:

$$Fe^{++} + LOOH + H^{+} \Rightarrow Fe^{+++} + {}^{\circ}OL + H_2O$$

ROS production by each of these sources can be stimulated by cytokines, inflammation, viral proteins and other mechanisms (Martin-Dominguez *et al.*, 2015). During liver inflammation due to paracrine signals from immune cells consequent to injury, ROS-sensitive cytokines get activated and contribute to HSC activation and responsiveness to PGDF and TGF-β (Ha *et al.*, 2010). Through

their signaling pathway, the potent mitogen (PGDF) and fibrogenic factor (TGF-β) release more ROS in the system, thus inducing more stress due to oxidation and enhancing fibrosis. Platelet growth development factor (PGDF) has been implicated in the accumulation of hydrogen peroxide while TGF-β is noted to stimulate ROS production and reduce glutathione concentration in hepatic fibrosis (Ha *et al.*, 2010).

Oxidative stress in Kupffer cells is known to initiate the formation of liver fibrosis in many diseases (Bonepally *et al.*, 2013). Correlations have been made between hepatic peroxidative injury and enhanced Kupffer cell cytokine production during fibrosis resulting from bile duct ligation in rats. Leukocytes that are recruited to the liver during injury join with Kupffer cells in producing compounds that modulate stellate cell behavior. Neutrophils are an important source of ROS, which have a direct stimulatory effect on stellate cell collagen synthesis (Friedman, 2008b). While fibrosis is reversible in its initial stages, progressive fibrosis can lead to cirrhosis. Increasing evidence also suggests that even early stages of cirrhosis may be reversible (Friedman, 2008b). In the liver, this healing process normally involves the recruitment of immune and/or inflammatory cells to the site of injury, secretion of extracellular matrix (ECM) proteins, reorganization of the ECM and possible regeneration of the hepatic tissue. When the damage to the liver is chronic, excess fibrous connective tissue accumulates (Bataller and Brenner,

2005). With time, this process eventually distorts the normal parenchymal structure of the liver and impairs its function. As chronic liver disease progresses, hepatic fibrosis is accompanied by the formation of septae and nodules that intervene with the portal blood flow, leading to hypertension and formation of distinctive cirrhotic architecture. At all stages of the fibrogenesis, the stress exerted on the liver parenchyma is exemplified by subsequent activation of the immune system accompanied by increased levels of certain cytokines and growth factors, which augment fibrogenesis. In proinflammatory fibrotic microenvironment, constant stimulation of hepatocellular regeneration could predispose to the development of hepatocellular carcinoma (HCC). However disruptive, hepatic fibrosis even early cirrhosis can be reversed by suppression of the fibrotic response (Baranova *et al.*, 2011; Odena *et al.*, 2015).

2.8 DETECTION OF FIBROSIS

Detection and quantification of hepatic fibrosis represent a long standing challenge in hepatology. Accurate assessment of liver fibrosis is necessary to make therapeutic decisions, determine prognosis and to follow-up disease progression (Papastergiou *et al.*, 2012).

Biopsy and non-invasive tests can be applied in the detection of significant hepatic fibrosis or cirrhosis (Papastergiou *et al.*, 2012)

2.8.1 Biopsy

For the past 50 years, liver biopsy has been considered to be the gold standard for staging of liver fibrosis. This technique allows physicians to obtain diagnostic information not only on fibrosis but also on many other liver injuring processes such as inflammation, necrosis, steatosis and hepatic deposits of iron or copper. However, increasing awareness of several drawbacks of liver biopsy has repeatedly questioned its accuracy and value in clinical practice (Papastergiou *et al.*, 2012). In addition to variable accessibility, high cost, sampling errors and inaccuracy due to inter- and intra-observer variability of pathologic interpretations (Baranova *et al.*, 2011), liver biopsy samples are an extremely small (1/50000) part of the liver and therefore sampling error can occur even with optimal specimens (Papastergiou *et al.*, 2012). Liver biopsy is an invasive procedure with a small but significant risk of procedure-related morbidity and mortality, with pain and hypotension as the most frequent complications and intraperitoneal bleeding and injury to the biliary system as the most serious complications. Due to these limitations, consideration of liver biopsy as the "gold standard" has declined to "best available" standard, and has been challenged by the recent increasing availability and validation of noninvasive methods to assess liver fibrosis (Baranova *et al.*, 2011; Papastergiou *et al.*, 2012)

2.9.2 Non-invasive detection methods

The non-invasive methods of detecting fibrosis have been grouped into Serum biomarkers and imaging. The serum biomarkers are classified further into two, viz;

1. Direct biomarkers or Class I fibrosis markers

2. Indirect biomarkers or Class II fibrosis markers

Direct markers: This method directly correlates with the liver matrix produced by the hepatic stellate cells during ECM turnover in the fibrotic process (Fallatah, 2014). The most studied direct markers are hyaluronic acid (HA), YKL-40, laminin, fibronectin, alpha-2-macroglobulin, procollagen type I carboxy terminal peptide (PICP), procollagen type III amino-terminal peptide (PIIINP), N-terminal propeptide of type II collagen, metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs) and transforming growth factor-b1 (TGF-b1) (Schiavon *et al.*, 2014).

The activation of HSCs and deposition of the ECM in liver fibrosis lead to alterations in liver microstructure that are reflected by an increase in the liver stiffness and changes in the blood flow. Imaging techniques like Fibroscan, ARFI and MRI allow the bedside assessment of liver stiffness (Lai and Afdhal, 2011). The advent of hepatic elastography allows the measurement of the rigidity of liver

parenchyma and offers the same advantages as ultrasound scan in that it is a non-invasive procedure that allows evaluating a large portion of the parenchyma and can be employed repeatedly throughout cirrhotic patient follow up. The main advantages of elastography, however, include the determination of a numeric mean for some measurements and the fact that it is less dependent on the operator than an ultrasound exam, thereby allowing its use by healthcare professionals without the need for vast experience (Medeiros *et al*., 2014).

Indirect markers: This method reflects changes in liver functions and molecules released into the blood due to liver inflammation, but they do not correlate with ECM turnover (Baranova *et al.*, 2011). The most commonly studied indirect markers of fibrosis caused by HCV infection include aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, gamma-glutamyltransferase (GGT), bilirubin, haptoglobin, apolipoprotein A1, and alpha-2-macroglobulin. The combination of indirect markers are usually devised and modelled from retrospective studies as a rule for non-invasive fibrosis markers (Schiavon *et al.*, 2014). The detection of fibrosis using Serum biomarkers have the advantages of wide availability, reproducibility, cost effectiveness and validity but limited in discriminating between intermediate stages of fibrosis (Schiavon *et al.*, 2014).

2.9 THERAPEUTIC STRATEGIES OF LIVER FIBROSIS

Recent understanding of the process of hepatic fibrogenesis has revealed that the process is dynamic and reversible. Animal and clinical evidence has confirmed that any degree of fibrosis and even cirrhosis are potentially reversible by reasonable therapeutic strategies (Zhou *et al.*, 2014). The therapeutic strategies for liver fibrosis include the following.

2.9.1 Therapies to eliminate the etiological factors

Removing the etiological factors is the most direct and perhaps most effective method of treating liver fibrosis. As such, treatments against HBV and HCV infections, abstinence from alcohol abuse, weight and blood lipid control, chelation of overloaded iron and copper are considered potentially effective (Zhou *et al.*, 2014).

2.9.2 Anti-inflammatory and immunosuppressive therapies

Hepatic fibrosis is commonly preceded by chronic inflammation and persistence of this inflammation has been associated with progressive hepatic fibrosis and the development of cirrhosis (Czaja, 2014). Therefore, prompt and sustained suppression of inflammatory activity by eliminating the etiological agent (virus) or dampening the immune response (lymphocytic proliferation and infiltration) can

halt and even reverse the fibrotic process, especially for fibrosis and cirrhosis resulting from viral hepatitis, autoimmune hepatitis, and primary sclerosing cholangitis (Czaja, 2014; Zhou *et al.*, 2014).

The anti-inflammatory drug celecoxib and antioxidative agents, taurine and vitamin E have shown some degree of anti-fibrotic effect. Likewise, glucocorticoids, corticosteroids, azathioprine, myciphenolate mofetil, prednisone, colchicines and rapamycin appear to exert anti-inflammatory, antifibrotic and immunomodulatory effects, and therefore may potentially be useful in the treatment of liver fibrosis (Makol *et al.*, 2011; Zhou *et al.*, 2014; Czaja, 2014). Recent review on the protective action of medicinal plants has highlighted anti-inflammatory properties as an important common anti-fibrotic mechanism of medicinal plants (Duval *et al.*, 2015).

2.9.3 Suppression of activation and promotion of HSCs apoptosis

Activation of HSC plays critical role in the initiation and progresion of fibrosis, hence inhibition of HSC activation and the induction of selective apoptosis or necrosis of activated HSCs, or reverse trans-differentiation of activated HSCs into the quiescent phenotype is an attractive therapeutic approach for liver fibrosis (Purohit and Brenner, 2006). Inactivation of HSCs can be achieved by inhibiting the TGF-β1 signaling pathway and PDGF-B, and activated HSCs can be removed

by inducing these cells to undergo apoptosis (Zhou *et al.*, 2014). Some cytokines and growth factors such as insulin-like growth factor-1, IFN-α and IFN-γ have been found to induce apoptosis of HSCs (Zhou *et al.*, 2014). Inhibitors of IκB kinase has also been shown to promote apoptosis of HSCs and exert antifibrotic effect. 18α-Glycyrrhizin (GL) has been demonstrated to suppress the activation of HSCs and induce the apoptosis of HSCs by blocking the translocation of NF-kappaB into the nucleus (Qu *et al.*, 2012). The results of Son *et al.* (2013) demonstrated that synthesized ethyl 6-(5-(phenylsulfonamido) pyridin-3-yl) imidazo[1,2-a]pyridine-3-carboxylate (HS-173), a new imidazo [1,2-a]pyridine derivative ameliorated liver fibrosis *in vitro* and *in vivo* by promoting HSC apoptosis and inhibiting the expression of fibrotic mediators by blocking the PI3K/Akt pathway *in vitro* and *in vivo.* Other pharmacological agents such as gliotoxin, sulfasalazine, benzodiazepine ligands, curcumin and tanshinone I have been explored for their effects in inducing HSC apoptosis (Zhou *et al.*, 2014).

2.9.4 Protection of liver function and promotion of hepatocyte regeneration

Hepatocyte apoptosis is a common event in liver injury and contributes to fibrogenesis and development of cirrhosis. Hence, preventing the hepatocytes from undergoing apoptosis and promoting hepatocytes regeneration can be useful therapeutic strategies for liver fibrosis and cirrhosis (Zhou *et al.*, 2014). The ability

of the liver to regenerate is crucial to protect liver function after injury and during chronic disease. Increases in hepatocyte growth factor (HGF- an antifibrotic growth factor that induces apoptosis in HSCs) in liver sinusoidal endothelial cells (LSECs) are thought to drive liver regeneration (Wang *et al.*, 2012). The infusion of cells such as bone marrow-derived cells and mesenchymal cells that can differentiate into hepatocyte-like cells and stimulate proliferation of hepatocytes by secreting some growth factors such as HGF, have been reported as a potentially effective method for the treatment of liver cirrhosis (Wang *et al.*, 2012). Furthermore, HGF-overexpressing human umbilical-cord-blood-derived mesenchymal stem cells have shown promising therapeutic effects on liver fibrosis (Zhou *et al.*, 2014).

The hepatoprotective agent silymarin has been widely used in the management of chronic liver diseases and cirrhosis (Kabel, 2014). Ursodeoxycholic acid and tauroursodeoxycholic acid have shown protective effects against hepatocyte organelle injury, and have been confirmed as effective agents for the treatment of primary sclerosing cholangitis. Calcium channel blockers (*e.g.*, verapamil) also show hepatoprotective and antifibrotic effects by stabilizing the hepatic cellular membrane and lowering the portal vein pressure (Zhou *et al.*, 2014).

2.9.5 Gene therapy and targeted therapy

Liver-based gene therapy has been used to down-regulate specific genes, block the expression of deleterious genes, deliver therapeutic genes, prevent allograft rejection and augment liver regeneration (Salazar-Montes *et al.*, 2015). Several critical genes implicated in the pathogenesis of liver cirrhosis such as TGF-β, PDGF-β, CTGF, and TIMP have been investigated as therapeutic targets for liver cirrhosis. Recently, miRNA has been found to play a regulatory role in the pathogenesis of liver fibrosis and cirrhosis through regulating the expression of profibrotic or antifibrotic genes, and influencing the proliferation and activation of HSCs. As such, miRNA-based therapy can potentially be useful for the treatment of liver fibrosis (Zhou *et al.*, 2014). Viral and non-viral vectors have been used, with viral vectors proving to be more efficient (Salazar-Montes *et al.*, 2015). An adenoviral vector carrying a modified cDNA coding for human pro-MMP-1 administered into established liver fibrotic rats significantly attenuated the lesion by inducing MMP-1 expression in the liver and degrading fibrillar collagens (Salazar-Montes *et al.*, 2015). Intravenous injections of naked HGF expression plasmid in to a bile duct ligated mice showed remarkable amelioration of the hepatic fibrotic lesions, as demonstrated by reduced α-smooth muscle actin (αSMA) expression, attenuated deposition of type I and type III collagen, and

normalized total hydroxyproline content. HGF also suppressed transforming growth factor-β1 (TGF-β1) expression (Xia *et al.*, 2006).

Stem-cell transplantation seems to be a more promising alternative approach. Bone marrow deriven cells have great power of regeneration and may develop into specific cellular phenotypes with different functions. These stem cells play an active role in liver repair and hepatic regeneration. Using markers such as aldehyde dehydrogenase, it has been demonstrated that these migrated stem cells fuse with the host hepatocytes or liver cells and help to generate fresh cell types (Ahmad and Ahmad, 2012).

2.9.6 Complementary and alternative medicine

Herbal extracts as complementary and alternative medicines (CAM) are effective in the treatment of liver diseases, such as antagonizing fibrosis, steatosis, and hepatitis viruses, and in protecting the liver cell. There is a long history in the use of traditional Chinese medicine which is considered a complementary or an alternative medical system in most Western countries, for the treatment of liver diseases (Guan and He, 2013). A recent investigation from Saudi Arabia on the prevalence of alternative medicine in liver disease patients, demonstrated common usage (over half of the patients) and general positive attitude towards alternative and complementary medicine (Al-Zahim *et al.*, 2013). A research study from

Korea on the incidence of herbal medicine-induced adverse effects on liver functions concluded that herbs are rather safe when used alone, but the risk of adverse reactions may increase when herbs and conventional drugs are used together (Jeong *et al.*, 2012).

Proposed action mechanisms of the anti-fibrotic Chinese medicines (pure compounds, composite and combinational therapies) include inhibition of viral replication, immunomodulation, antioxidant and anti-inflammation (*Salvia miltiorrhiza*, *Panax notoginseng*, *Gingko bilobia*), regulation of cytokines, collagen metabolism and inhibition of HSC (*Salvia miltiorrhiza*,), anti-apoptosis in hepatocyte and induction of apoptosis in HSC (Feng *et al.*, 2009). Oxidative stress is considered the main pathogenic mechanism of liver fibrosis and the most promising anti-fibrotic therapies that have been evaluated in humans with chronic liver disease have been the antioxidants (Czaja, 2014). Several plant-drived antioxidants, such as silymarin, baicalin, beicalein, quercetin, apigenin, have been shown to interfere with liver fibrogenesis (Wei *et al.*, 2015). The antioxidants components(polyphenols, flavonoids or structurally related compounds) of Pomegranate peels and seeds have demonstrated protective effects against liver fibrosis induced by CCl_4 , and its mechanisms might be associated with antioxidant activity, the ability to decrease the level of TGF-β1 and inhibition of collagen synthesis (Wei *et al.*, 2015).

2.10 Antioxidants

Antioxidants are substances when present at low concentrations compared to those of the oxidizable substrate, significantly delay or inhibit oxidation of the substrate (Halliwell and Gutteridge, 1995). Antioxidants could delay, prevent or remove oxidative damage to a target molecule either by directly scavenging free radicals or inhibiting their production or indirectly up-regulating antioxidant defences against free radicals (Halliwell 2007). The detoxification of ROS is a major prerequisite of aerobic life and could be done by enyzmic or non-enzymic antioxidants (Videla, 2009; Lu *et al.*, 2010).

2.10.1 Enzymic antioxidants

These include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Ha *et al.*, 2010; Ojiako *et al.*, 2015); Superoxide dismutase is the most abundant antioxidant enzyme in animals, especially in the liver compartment. It detoxifies the superoxide ion by converting it to H_2O_2 and oxygen;

$$M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$$

$$M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2.$$

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

Catalase and the GSH peroxidase system, peroxiredoxins, eliminate hydrogen peroxide (H_2O_2) before the Fenton reaction can create the strongest oxidant ·OH. Catalase also exhibit peroxidatic activity by promoting the interactions of H_2O_2 with hydrogen donors to produce one molecule of water and an oxidized donor (Ha *et al.*, 2010). Similarly, glutathione peroxidase inactivates cellular peroxides (Ha *et al.*, 2010; Lu *et al.*, 2010).

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ *(catalase)*

$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$ *(glutathione peroxidase)*

2.10.2 Non-enzymic antioxidants

The non-enzymic antioxidants, include ascorbic acid, tocopherol, uric acid, glutathione (GSH), bilirubin and some phytochemicals, such as alkaloids, phenols, flavonoids, carotenoids, steroids and thiol compounds (Ha *et al.*, 2010; Lu *et al.*, 2010; Khan, 2012).

2.10.2.1 Glutathione

Glutathione is a tripeptide composed of the amino acids cysteine, glycine and glutamic acid complements, the antioxidant enzymes in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death by donating a reducing equivalent (H^+) to unstable molecules such as reactive oxygen species (Dunning *et al.*, 2013).

2.10.2.2 Vitamin C

Vitamin C (ascorbic acid) is a major water-soluble, chain-breaking antioxidant capable of scavenging essentially all physiologically relevant free radicals (Ipsen *et al.*, 2014), such as singlet oxygen, superoxide, hydroxyl, water soluble peroxyl radical and hypochlorous acid (Adikwu and Deo, 2013). It potentiates the activities of free radical scavengers, superoxide dimutase, catalase and glutathione peroxidase, thereby preventing microsomal lipid peroxidation, liver fibrosis, liver necrosis and hepatic inflammation. It is reported to be an excellent source of electrons, therefore can donate electrons to free radicals and reducing equivalents in multiple enzymatic reactions, of which its role in proline and lysine hydroxylation during collagen synthesis is probably most widely known (Singal *et al.*, 2011; Adikwu and Deo, 2013). Vitamin C is an important free radical scavenger in extracellular fluids, trapping radicals and protecting biomembranes from peroxide damage. Vitamin C is believed to decrease lipid peroxidation either directly or indirectly by regenerating vitamin E (Adikwu and Deo, 2013). Due to its delocalized structure, vitamin C ($AscH^-$) turns to a very stable radical after electron donation ($Asc^{-\cdot}$), but is readily regenerated with NADH or NADPH-dependent reductases (Lu *et al.*, 2010). The hepatoprotective effect of vitamin C is attributed to its antioxidant property and the effect is reported to increase when co-administered with other antioxidants (Ha *et al.*, 2010; Adikwu and Deo, 2013).

Vitamin C is an essential nutrient, thus must be acquired through dietary means (Ha *et al.*, 2010).

2.10.2.3 Vitamin E

Vitamin E which is almost 90% α-tocopherol in human tissues, is a fat-soluble antioxidant mostly found in the lipid phase of membranes (Ha *et al.*, 2010; Singal *et al.*, 2011). It serves as an antioxidant by complexing with unpaired electrons thus stabilizing these free radical compounds and preventing lipid peroxidation. Vitamin E (TOH) in the lipid phase, is similar to vit C in the aqueous phase and will directly react with or neutralize hydroxyl, alkoxyl and lipid peroxyl and form water, alcohol and lipid hydroperoxides, respectively. Vit E itself becomes a phenyl radical which can be neutralized and regenerated by vit C (Lu *et al.*, 2010). Latest studies have shown that vitamin C and vitamin E have synergistic antioxidant effect (Li *et al.*, 2014). Some observed effects of vitamin E include, a decrease in production of tumour necrosis factor in alcoholic hepatitis and prevention of hepatic stellate cell (HSC) activation in chronic hepatitis C (Singal *et al.*, 2011).

Of the various available antioxidants, strong evidence exists that several agents mediate their action primarily based on changes in ROS and redox state of the cell. These agents include vitamins E, C and silymarin (Singal *et al.*, 2011). Vitamin E

is noted to block the production of ROS during fat oxidation (Ha *et al.*, 2010). Several study reports have indicated a correlation in the significant reduction of serum level of vit E with increase in oxidative stress products, which correspond to the extent or degree of liver damage (Ha *et al.*, 2010).

2.11 Plant bioactive substances

Bioactive compounds are compounds or chemical substances produced by plants (phytochemicals) with pharmacological or toxicological effects in man and animals (Bernhoft, 2010). They include a broad diversity of structures and functionalities in any food or part of food that provide further health benefits than those attributed to macronutrients and micronutrients. They are also useful in the prevention and treatment of diseases (Hasler and Blumberg, 1999; Lee *et al.*, 2007). The typical bioactive compounds in plants are produced as secondary metabolite and appear to be randomly synthesized within the plants besides the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development, such as carbohydrates, amino acids, proteins and lipids. They can be regarded as products of biochemical "side tracks" in the plant cells that are not needed for daily functioning of the plant (Bernhoft, 2010). Bioactive substances give plants colour, flavor, and smell and are part of a plant's natural defense system (Ibrahim and Fagbohun, 2012). For example, flavonoids protect plants against free radicals

generated during photosynthesis; terpenoids may attract pollinators or seed dispersers, or inhibit competing plants; alkaloids usually ward off herbivorous animals or insect attacks (phytoalexins) (Bernhoft, 2010). Other secondary metabolites function as cellular signalling molecules or have other functions in the plants (Bernhoft, 2010).

Some of these chemicals produced by plants for their own defence among other biological functions, restrict or reduce the optimal utilization of some nutrients (proteins, vitamins, and minerals) by animals and other organisms (Gemede and Ratta, 2014). They can also interfere with metabolic processes so that growth and bioavailability of nutrients in mammals are negatively influenced (Champ, 2002). Thus, they are regarded as 'antinutritional factors' or 'antinutrients'. Antinutrients have been shown to be deleterious to health or evidently advantageous to human and animal health if consumed at appropriate amounts (Gemede and Ratta, 2014). According to Champ (2002), antinutrients can be classified into the followings:

(a) Enzyme inhibitors e.g. lectins and tannins

(b) Antivitamins e.g.phytates and glucosinolates

(c) Mineral-binding agents e.g. oxalates

(d) Goitrogens

2.11.1 Phenols

Polyphenols are natural aromatic hydroxylated organic compounds commonly found in fruits and vegetables, tea, red wine, honey, cocoa, beans and many food sources that form a significant portion of our diet (Li *et al*., 2014). Plant phenols are derived from the shikimate pathway and phenylpropanoid metabolism, following the phosphoenolpyruvate →phenylalanine → cinnamate → 4-coumarate course, leading to chalcone, flavanone, dihydroflavonol, anthocyanin, etc (Robards and Antolovich, 1997). Phenolic derivatives represent the largest group of plant secondary metabolites synthesized probably as a result of anti-oxidative strategies adapted in evolution by respirative organisms to provide defense against microbial attacks and herbivorous predators (Bennick, 2002). Polyphenols with multiple hydroxyl groups can effectively remove free radicals including O_2^- and singlet oxygen to execute healthcare functions. Cocoa polyphenols have been reported to significantly decrease the level of oxidative stress in alcoholic fatty liver in a model mice study (Li *et al.*, 2014).

2.11.2 Flavonoids

Flavonoids are polyphenolic compounds synthesized by phenylpropanoid pathway in plants in response to microbial infections (Kumar and Pandey, 2013). Flavonoids in food are generally responsible for colour, taste, prevention of fat

oxidation and protection of vitamins and enzymes (Kumar and Pandey, 2013). Fruits and vegetables are the main source of flavonoids and they give high potent antioxidants which could reduce incidence of chronic inflammatory diseases or cancer (Chataikun and Chiabchalard, 2013). Their basic chemical structure consists of two benzene rings bound by a three-atom heterocyclic carbon chain. The oxidation of the structure generates several families of flavonoids (flavones, flavones, flavanones, anthocyanins, flavanols, and isoflavones). Chemical modifications of each family can lead to more than 5000 individual compounds with different properties (Vargas-Mendoza *et al.*, 2014). The bioavailability, metabolism and biological activity of flavonoids depend upon the configuration and total number of hydroxyl groups' substitution of functional groups about their nuclear structure (Kumar and Pandey, 2013). The functional hydroxyl groups mediates their antioxidants effects by donating hydrogen or electron to free radicals, thereby stabilizing them and giving rise to a relatively stable flavonoid radical (Kumar and Pandey, 2013; Li *et al.*, 2014).

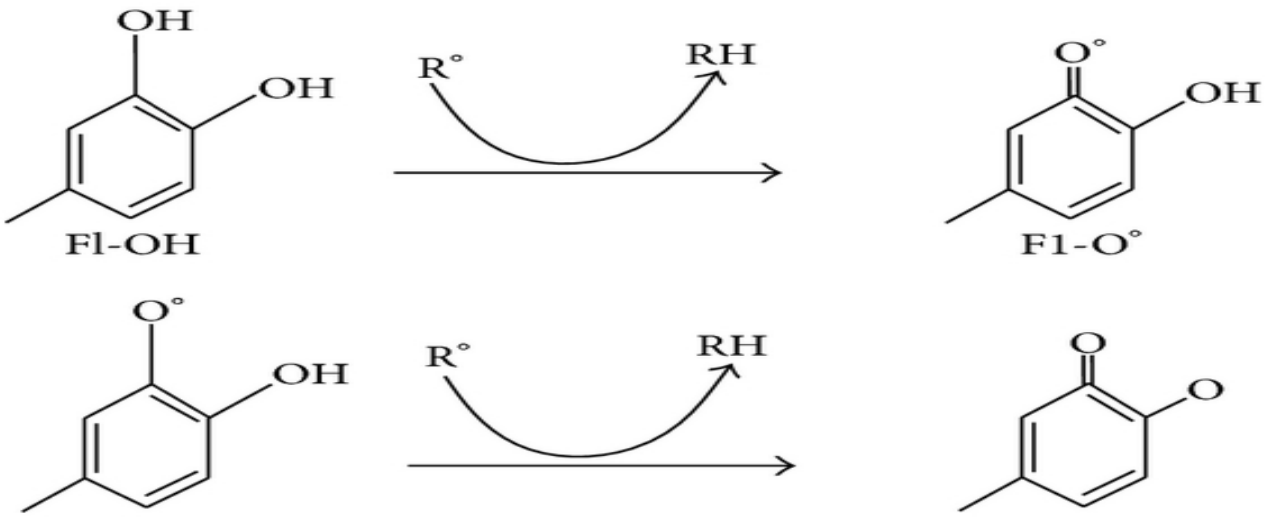


Fig 2.7: Scavenging of ROS (R˚) by flavonoids (Fl-OH) (Kumar and Pandey, 2013)

Mechanisms of antioxidant actions of flavonoid include; suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation, scavenging of ROS, protection of or increased antioxidant defences. Flavonoids have been referred to as nature's biological response modifiers because of their ability to modify the body's reaction to allergies, viruses and carcinogens (Madziga *et al.*, 2010). Many flavonoids are shown to have antioxidant activity, free radical scavenging activity, coronary heart disease prevention, anti-inflammatory, anti-allergic and anti-cancer activities, while some exhibit anti-microbial activities (Yamamoto and Gaynor, 2001).

2.11.3 Alkaloids

The term alkaloid is derived from “alkaline” and used to describe highly diverse group of compounds that contain nitrogen in a heterocyclic ring (Roberts and Wink, 1999; Mahajan *et al*., 2011). They are widely distributed in higher plants, such as those belonging to Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae (Lu *et al.*, 2012). Alkaloids accumulate in the vacuoles and their concentration increases just prior to seed formation and drops when the seed is ripe (Baumann and Waldhauser, 1996). They are stored in sites other than the site of synthesis (e.g nicotine is synthesized in the tobacco roots but is translocated and stored in the leaves) and some are modified at the storage sites. Solutions of alkaloids are intensely bitter (Verma *et al.*, 1995). These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities (Verma *et al.*, 1995).

Lu *et al.* (2012) in their review article reported that several alkaloids isolated from natural herbs exhibit anti-proliferation and anti-metastasis effects on various types of cancers both *in vitro* and *in vivo*. Vinblastine, and camptothecin, which have been developed into chemotherapeutic drugs exhibit anticancer effects. Other biological activities of alkaloids include; the relieving action of ephedrine in

asthma and the analgesic action of morphine (Lu *et al.*, 2012). *In vitro* and *in vivo* hepatoprotective effects of the alkaloid fraction of the methanolic extract of *Hygrophila auriculata* leaves administered to CCl_4-induced toxicity in freshly isolated rat hepatocytes, HepG2 cells and animal models have been reported (Raj *et al.*, 2010). Similar outcome is documented for the alkaloid fraction of methanol extract of *Solanum pseudocapsicum* leaves (Vijiyan *et al.*, 2003) and hydroalcoholic extract of the aerial part of *Cajanus cajan* (Singh *et al.*, 2011).

2.11.4 Tannins

Tannins are water-soluble, polyphenolic compounds of high molecular weight, that form complexes with proteins and various other organic compounds including carbohydrates, gelatin and alkaloids (Fig 2.8) (Akiyama *et al.*, 2001; Elgailani and Ishak, 2014). They are colourless and non-crystalline substances which form astringent colloidal solutions in water (Elgailani and Ishak, 2014**).** Tannins are divided into hydrolysable tannins and non-hydrolysable (condensed) tannins; the former are esters of phenolic acids (gallic acid or hexahydroxydiphenic acid) and a polyol, usually glucose, while the latter are polyhydroxyflavan-3-ols (also known as pro-anthocyanidins) such as catechin or epicatechin (Min *et al.*, 2015).

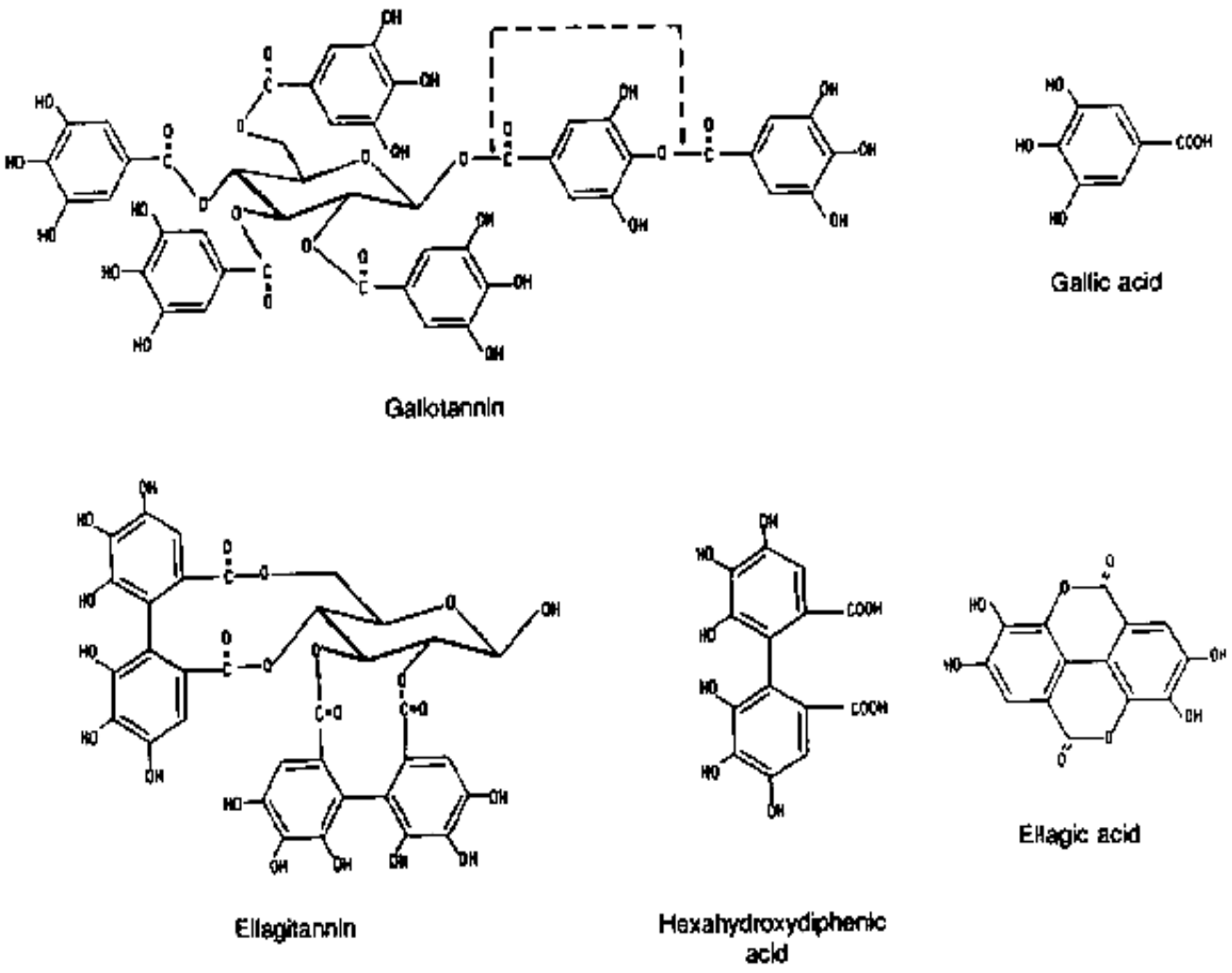


Fig 2.8: Chemical structures of some tannins (Ashok and Upadhyaya, 2012)

Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or ellagitannins (Akiyama *et al.*, 2001; Min *et al.*, 2015). Active substances that have phenolic groups in their structure have great pharmacological potential (Siqueira *et al.*, 2012). The antimicrobial activities of tannins are well documented (Chung *et al.*, 1998; Akiyama *et al.*, 2001; Min *et al.*, 2015). The

different mechanisms proposed so far to explain tannin antimicrobial activity include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation. A further mechanism involving iron deprivation is proposed (Scalbert, 1991). Many tannin molecules have also been reported to exhibit anti-mutagenic and anti-carcinogenic potentials; these effects have been related to tanins antioxidative property, which is important in protecting cellular oxidative damage, including lipid peroxidation. The generation of superoxide radicals was reported to be inhibited by tannins and related compounds (Chung *et al.*, 1998). However, incidences of certain cancers, such as esophageal cancer, have been reported to be related to consumption of tannins-rich foods such as betel nuts and herbal teas, suggesting that tannins might be carcinogenic. Tannins have also been reported to exert other physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses (Chung *et al.*, 1998). The dosage and kind of tannins used are critical to these effects (Chung *et al.*, 1998).

2.11.6 Saponins

Saponins are structurally complex amphipatic glycosides of steroids and triterpenoids that are widely produced in higher plants and also found in certain

marine organisms such as starfish and sea cucumbers (Podolak *et al*., 2010; Faizal and Geelen, 2013). The term "saponin is" derived from the latin word *'sapo'* meaning soap, because they have surfactant properties, forming stable soap-like foam upon shaking in water (Faizal and Geelen, 2013). Saponins are water-soluble, high molecular weight compounds, which like glycosides give aglycones called sapogenin on hydrolysis (Hostettmann and Marston, 1995).

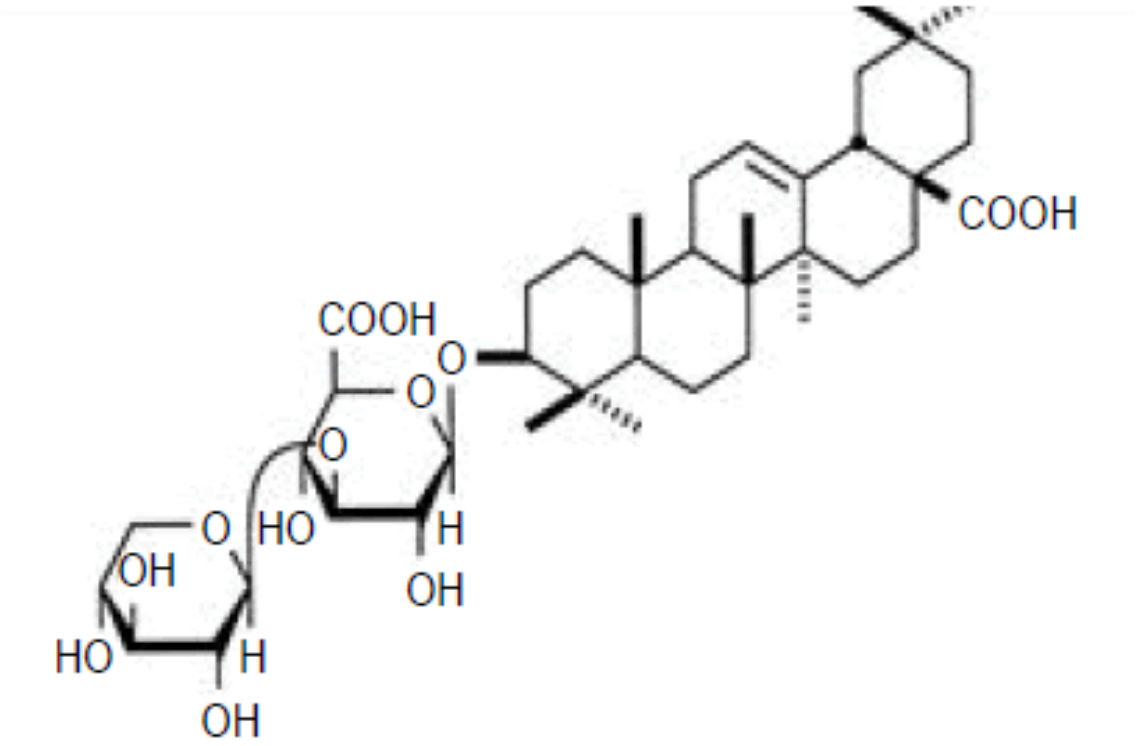


Fig 2.9: Structure of triterpene saponin (Kilinc *et al*., 2016)

The presence of saponins has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anti-cancer properties (Man *et al.*, 2010).

Saponins are also important therapeutically as they are shown to have hypolipidemic, anticancer and cytotoxic activities (Podolak *et al.*, 2010) as well as hepatoprotective effects. The study of Zhang *et al.* (2014) indicated that saponins from *Rhizoma panacis majoris* exerted beneficial hepatoprotective effects on the CCl_4-induced hepatic fibrosis, mainly by enhancing liver tissue antioxidant capacity, reducing the lipid peroxidation of hepatocyte membranes, and alleviating hepatic fibrosis and hepatic cell death. Similar protective effect by *Taraphochlamys affinis* has been attributed to the capacity of the isolated total saponins on reducing oxidative stress, suppressing inflammatory responses and improving drug-metabolizing enzyme activity in liver (Huang *et al.*, 2012).

Saponins possess bitter and acrid taste, besides causing irritation to mucous membranes and heamolysis of red blood cells, which seems to result from its ability to form complexes with cell membrane cholesterol leading to pore formation, cell permeability, and also alterations in the negatively charged carbohydrate portions on the cell surface (Podolak *et al.*, 2010).

2.11.7 Oxalate

Plants utilize oxalate primarily for ion regulation and secondarily for defence (Penniston, 2014). Oxalate is synthesized via the incomplete oxidation of carbohydrates (Fig 3.0) (Stritweiser *et al.*, 1976). It can also be produced through

the oxidation of glycolate and glyoxylate and in some cases ascorbic acid (Penniston, 2014).

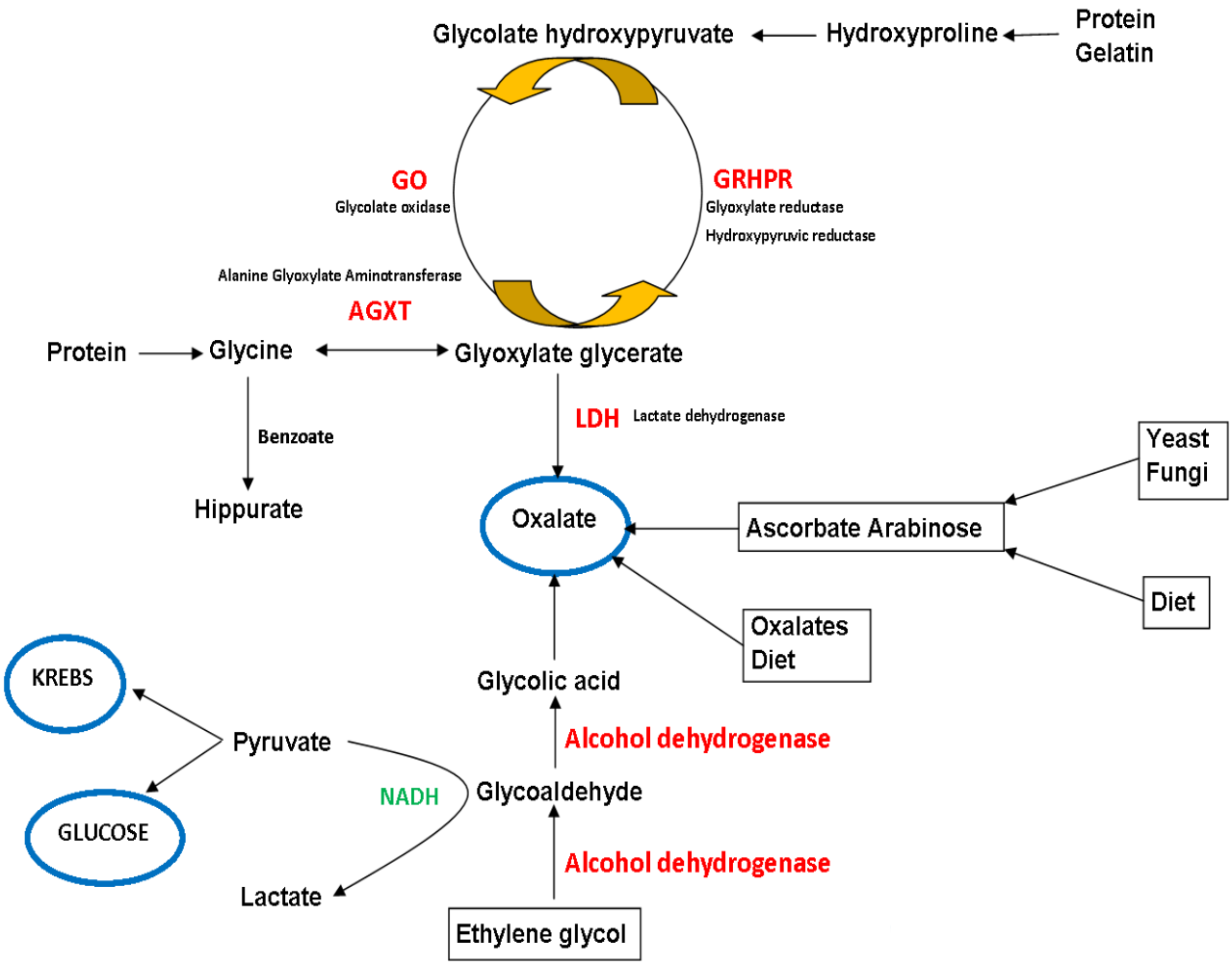


Fig 3.0: Biosynthetic pathway of oxalate in plants

The presence of oxalates in food is known to impact acrid taste and exert caustic effect and irritations to the intestinal tract. Certain plants such as spinach, fat hen, root and leaves of rhubarb, sorrel, black peper, cocoa and several oxalis species

have high amounts of oxalate (Streitweiser *et al.*, 1976).The bioavailability and oxalate content of plants represent a potential health hazard to both humans and livestock, through its effect on renal function among other effects (Penniston, 2014).

Though a phytochemical, it is not known for any health benefit to humans. In fact, it is commonly referred to as an "anti-nutrient" because the conjugated anion (C_2O_4 –oxalate ion) binds with calcium, magnesium, zinc, iron, and other cations in the gastrointestinal tract and reduce their absorption, potentially leading to mineral deficiency (Penniston, 2014). Complex formation with divalent cations results to minute crystals but may form larger kidney stones. Chronic oxalate ingestion in animals has been associated with renal fibrosis and failure (Hagler and Robert, 1973). Oxalate has also been associated with urolithiasis (Bele and Hajdinjak, 2012) and oxalosis (Hagler and Robert, 1973) in humans and animals. Furthemore, it is increasingly gaining attention in other medical conditions such as pancreatitis/exocrine pancreatic insufficiency, autism, vulvar pain, and in cases of coronary atherosclerosis (Penniston, 2014).

One possible approach to prevent renal stone recurrence and other oxalate effects is to decrease the consumption of oxalate-rich foods and more recent is the

microbiological approach involving a substrate-specific, oxalate-degrading and gut-resistant bacterium *Oxalobacter formigenes* (Ivanovski and Drüeke, 2013).

2.12 Natural sources of antioxidants and other bioactive substances

Synthetic antioxidants have been widely used in food industry to retard lipid peroxidation, but not preferred for pharmacological use due to its toxicological concern (Lu *et al.*, 2010). According to WHO, more than 80% of the world population rely on traditional medicines for their primary health care needs (Ibrahim and Fagbohun, 2012). The basis for the treatment and cure of numerous endemic diseases in Nigeria by traditional medical practitioners is the use of medicinal plants from different botanical sources (Sofowora *et al.*, 2013). The medicinal value of plants lies in the bioactive components or phytochemicals that produce a definite physiologic action on the human body (Ibrahim and Fagbohun, 2012). A whole range of plants derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating diseases are now being described as functional foods, nutriceuticals and nutraceuticals (Ibrahim and Fagbohun, 2012).

Edible and non-edible plants are potential sources of natural bioactive compounds such as antioxidants and secondary metabolites or organic compounds that play important roles in the defensive system (Huda-Faujan, 2009; Chatatikun and

Chiabchalard, 2013). Natural antioxidants from foods are regarded the first choice of antioxidants because of their crucial role in the prevention and adjunctive treatment of diseases with no adverse reactions to human health (Li *et al.*, 2014). More interests have been focused on identifying plant extracts as dietary antioxidant supplements. Most of these natural antioxidants; phenolics (phenols and polyphenols), flavonoids, vitamins (vit A, C and E) and carotenoids (β-carotene, lycopene and astaxanthin) are from fruits, vegetables, spices, grains and herbs such as gingseng, curcuma, gingko, rosemary, green tea, grape, ginger and garlic (Lu *et al.*, 2010; Li *et al.*, 2014).

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Sasidharan *et al.*, 2011).

2.12.1 Silymarin

Silymarin has been used worldwide for many years as a complementary alternative medicine for hepatic diseases (Vargas-Mendoza *et al.*, 2014). It is a standardized plant extract from *Silybum marianum* commonly known as milk thistle of the family *Asteraceae* (Boigk *et al.*, 1997). It contains the flavonoid taxifolin and more than seven isomeric flavonolignans including; silibinin (60%), isosilibinin, silydianin and silychristin (Boigk *et al.*, 1997; Vargas-Mendoza *et al.*, 2014).

Silymarin is regarded an effective antioxidant due to its ability to inhibit free radical production from the metabolism of xenobiotics such as ethanol, acetaminophen and CCl_4 (Vargas- Mendoza *et al.*, 2014). It has also been reported to enhance hepatic GSH generation by elevating cysteine availability by inducing cysteine synthesis while inhibiting its catabolism to taurine (Kwon *et al.*, 2013). Silymarin also interfers with the expression of the cell cycle regulators and proteins involved in apoptosis; for instance silybin inhibited the kinase activity of mitogen-activated protein kinase (MAPK) in melanoma cells (Vargas-Mendoza *et al.*, 2014). Boigk *et al*. (1997) reported that silymarin administered orally to adult Wistar rats ameliorated the accumulation of collagen in early and advanced biliary fibrosis, secondary to complete bile duct occlusion.

Although silymarin is considered to be ideal for the treatment of liver fibrosis, the effectiveness of its oral administration has been questioned due to its poor solubility, low bioavailability and low half-life, hence requires daily administration to achieve its effects (Bonepally *et al.*, 2013).

2.12.2 *Monodora myristica*

Monodora myristica also known as calabash nutmeg is a perennial edible plant of the Annonaceae family (Agriga and Siwela, 2017). It is a climbing tree with many seeded berry fruits that grows well in the evergreen forests of West Africa, native

to Jamaica, but prevalent in Southern part of Nigeria as ‘ehuru’ or ‘ehiri’ and known as ‘ariwo’ by the Yorubas (Ojiako *et al.*, 2010). Investigations reveal that almost every part of the tree has economic importance (Okafor, 1987). However, the most economically important part is the seed which is embedded in a white sweet smelling pulp of the sub-spherical fruit. It has been observed that an average of 119 – 122 seeds can be found in one fruit (Burubai, 2008). Phytochemical screening revealed that *Monodora myristica* is rich in flavonoids, saponins and steroids, but with very little antinutrients like cyanogenic glycosides, tannins, oxalates and phytates (Middleton *et al.*, 2000; Ekeanyanwu and Etienajirhevwe, 2012).The seeds and seed coats are used as spice for seasoning food and as topical medication for wounds and headaches (Ojiako *et al.*, 2010). The pulverized kernel is used to prepare pepper soup as stimulant to relieve constipation and control passive uterine hemorrhage in women immediately after parturition (Akinwunmi and Oyedapo, 2013). The kernel is a popular condiment used as a flavouring agent, and has been found to exhibit anti-hemorrhagic, anti-diurectic and anti-septic properties (Okafor, 1987; Udeala, 2000; Iwu, 2002).

Monodora myristica also possess antioxidant and cholesterol-lowering properties.The study of Erukainure *et al.* (2012) elucidated high antioxidant activities of *Monodora myristica in vitro*. Ethanolic extract of the flavonoid fraction of *Monodora myristica* seed was found to exhibit a dose dependent

protective effect against free radical induced haemolysis of red blood cells with maximum protection of 37.00 ± 0.13 and 46.23 ± 0.41% inhibition of lipid peroxidation and free radical generation in liver homogenate, signifying antioxidant properties (Akinwunmi and Oyedapo, 2013). Treatment with *M. myristica* elicited a significant reduction in serum aminotransferases, TC, TG and LDL-C levels, with concomitant increase in HDL-C of hypercholesterolemic rats (Onyenibe *et al.*, 2015).

2.12.3 *Chromolaena odorata* (L.)

Chromolaena odorata (Linn) is a perennial herb belonging to the flowering shrub of the sunflower family, *Asteraceae* (Chakraborty *et al.*, 2011; Pandith *et al.*, 2013). A common plant known as Siam weed, 'Elizabeth', 'Independence leaf', 'Awolowo' and 'Enugu plantation weed' among the Igbos of the South-Eastern Nigeria (Alisi *et al.*, 2011). The proximate analysis of dried pulverized *C. odorata* leaf contained ash (10.5%), crude fat (10.75%), fibre (14.9%), moisture (15%), crude protein (18.37%) and carbohydrate (30.48%), while the presence of saponins, phenols and tannins were observed in the aqueous and ethanolic leaf extracts (Vijayaraghavan *et al.*, 2017). The boiled roots of *Chromolaena odorata* are used for urinary retention in traditional Yucatan healing (Chakraborty *et al.*, 2011). The fresh leaves or decoction are exploited extensively in West and Central

African ethnopharmacy for the treatment of a wide range of conditions; hemostatic and wound healing (Pandith *et al.*, 2013), sore throat and cold, leach and poison bites, burns an scald, skin infection, dendo-alveolitis (Alisi *et al.*, 2011), malaria, rheumatism, gonorrhoea, ulcers and hematuria (Chakraborty *et al.*, 2011). Methanol leaf extracts of *Chromoaena odorata* has been demonstrated to have nitric oxide scavenging ability (Alisi and Onyeze, 2008), anti-microbial effect (Alisi *et al.*, 2011), while the ethanolic extract exhibited hypoglycaemic, anti-microbial and antioxidant activities (Mbajiuka *et al.*, 2014). Phenolic compounds from *Chromolaena odorata* have been shown to protect the fibroblasts and keratinocytes of cultured skin cells from oxidative damage (Phan *et al.*, 2001).

2.12.4 *Buchholzia coriacea*

Buchholzia coriacea is a forest tree with large, glossy leaves and conspicuous cream white flowers in racemes at the end of the branches. The plant is easily recognized by the compound pinnate leaves and the long narrow angular fruits containing large, usually aligned seeds (Ibrahim and Fagboun, 2013). *B. coriacea* is a perennial plant of the family *Capparaceae* and commonly called ‘wonderful Kola’ due to the popular use of the seeds in traditional medicine (Nwaehujor *et al.*, 2012). Its local names in Nigeria include ‘uwuro’ (Yoruba), ‘esson bossi’ (Central Africa), ‘Uke’ (Ibo), ‘ovu’ (Bini), and ‘Aponmu’ (Akure) (Ibrahim and Fagboun,

2013). The seeds, covered in purple aril, are either cooked or eaten raw and is known to have a sharp pungent taste (Nwaehujor *et al.*, 2012; Ibrahim and Fagboun, 2013). The proximate analysis showed that the seeds contain moisture (1.30%), crude fat (2.30%), crude protein (13.34%), ash content (6.6%), crude fibre (2.19%), carbohydrate (75.43%), while the phytochemicals detected in the ethanol and methanol extracts respectively were alkaloids (3.16 and 3.32%), glycosides (2.16 and 2.46 %), saponin (2.10 and 2.23%), steroids (0.14 and 0.16%), tannin (6.46 and 6.73%), flavonoids (0.68 and 0.79%), terpenes (0.22 and 0.16%), reducing sugars (1.14 and 1.71%) and phenol (1.83 and 1.26%) (Ibrahim and Fagbohun, 2013). Mbata *et al.* (2009) also reported the presence of alkaloids, anthraquinone, cardiac glycosides, flavonoids, resins and saponin in the seed of *B. coriacea*.

Wonderful kola is regarded as brain food and memory promoter; and known worldwide as ‘memory nut’, because it enhances the memory and facilitates learning absolutely (Ibrahim and Fagbohun, 2013). It is documented to strengthen the nervous system, act as blood cleanser, stop migraine. It is effectively applied in the treatment of menstrual problems, hypertension and prevention of premature aging (Ibrahim and Fagbohun, 2013). Traditionally, *B. coriacea* seed is used in treating diabetes, hypertension, rheumatism, cold, cough and catarrh. Other experimental applications include respiratory disorders, dysmenorrhoea, ulcer,

fibroid, chest pains, hypoglycaemia, hypercholesterolemia and as anti-helminthics (Amaechi, 2009; Adisa *et al.*, 2010).

The plant is also reported to possess anti-plasmodial properties; usually the ground seeds were routinely mixed with palm oil and taken orally as treatment for malaria (Nwaehujor *et al.*, 2012). Research results by Ayoola *et al.* (2011) indicated that *B. coriacea* extract is a potent anti-inflammatory agent at a low dosage and could be a substitute for aspirin. The methanol extract of the seed has been demonstrated to exhibit antimicrobial activity (Mbata *et al.*, 2009), anti-diabetic and antioxidant properties (Nwaehujor *et al.*, 2012).

2.12.5 *Sphenostylis stenocarpa*

Sphenostylis stenocarpa (*Fabaceae*) or African yam bean as it is commonly called, is an herbaceous leguminous plant occurring throughout tropical Africa (Porter and Doyle, 1992). Scientific information on *S. stenocarpa* is scanty when compared to other major food legumes such as cowpea, soy beans due to its under utilization, low exploitation and cultivation. Its low acceptability has been associated with the antinutritional contents (such as phytate, trypsin inhibitor, cyanogenic glucosides and saponin) and the hard seed testa which requires several hours of boiling. However, soaking for several hours and fermentation has been shown to improve its digestibility (Betsche *et al.*, 2005). The seeds have high protein content ranging

from 21.0 to 29.0% with about 50% carbohydrate mainly as starch (Ajibola *et al.*, 2011). Study results of Ajibola *et al.* (2011) showed potential use of African yam bean protein hydroxylate (APH) and its membrane fractions as antioxidants in the management of oxidative stress-related metabolic disorders and in the prevention of lipid oxidation in food products. The acute toxicity (LD50) study of the methanol extract showed no toxicity up to 5000 mg/kg body weight. Treatment of carbon-tetrachloride induced liver damage in *Wistar* rats using the methanol extract of *S. stenocarpa* showed some dose dependent antioxidant and non-hepatotoxic properties of the seed (Okonkwo *et al.*, 2013).

2.13 EFFECT OF PROCESSING ON PLANT BIOACTIVE SUBSTANCES

Consumption of natural, fresh plant products rich in bioactive substances (phytochemicals and antioxidants) has been reported to overcome some of the degenerative diseases that affect humans (Alothman *et al.*, 2009). However, fresh foods especially pulses and legumes contain high anti-nutritional factors which can lead to adverse effects and acute toxicity at high consumption, hence limiting their utilization (Champ, 2002; Fagbemi *et al.*, 2005). Most bioactive health promoting substances are unstable during processing and storage. They undergo various chemical reactions such as oxidation, hydrolysis, thermal degradation and Maillard reactions; the result being a change and reduction in their bioactivity (Lee *et al.*,

2007). In order to harness the full potential of some foods, physical and chemical methods are employed to reduce or remove anti-nutrional factors. These methods include soaking, cooking, germination, fermentation, selective extraction, irradiation and enzymatic treatment. A combination of two or more methods is considered to be more effective (Hefnawy, 2011).

Most of the previous investigations in the literature on the antioxidants of fruits, vegetables, and grains have shown that food-processing operations reduced the antioxidants of processed foods, especially vitamin C, which is also the usual consumer perception. But, in the last decade some articles in the literature reported that the evaluation of nutritional quality of processed fruits and vegetables do not only depend on the quantity of vitamin C but should include analyses of other antioxidant phytochemicals and antioxidant activity (Nayak *et al.*, 2015). Most importantly, analysis also depends on the condition, type, and mechanism of assays used (Nayak *et al.*, 2015), as well as, the structure of food matrix, and the chemical nature of the specific compound (Palermo *et al.*, 2014).

Thermal processing of foods is extremely beneficial leading to inactivation of food-borne pathogens, natural toxins or other detrimental constituents, prolonged shelf-life, improved digestibility and bioavailability of nutrients, improved palatability, taste, texture and flavour and enhanced functional properties, including augmented antioxidants and other defense reactivity or increased

antimicrobial effectiveness and overall quality (Lee and Ho, 2002). Thermal processing can bring some unintentional and undesired consequences, such as loss of certain nutrients, formation of toxic compounds (acrylamide, furan or acrolein), or of compounds with negative effects on flavour perception, texture or colour (Lee and Ho, 2002; Boekel *et al.*, 2010).

2.13.1 Cooking

Cooking ensures food safety because of destruction of microorganisms and the inactivation of antinutritional factors, growth inhibitors and hemagglutinins. It also increases the palatability and bioavailability of nutrients and antioxidants (Thi and Hwang, 2015). A common perception is that thermally cooked foods have lower nutritional value than fresh foods because of the loss of vitamin C and particular physiochemical characteristics (Hwang *et al.*, 2012). But some research studies show contrary discoveries for some plants. Thermal processing was found to increase the total antioxidant activity of tomato and sweet corn (Nayak *et al.*, 2015). Boiling of fresh broccoli and brussel sprout caused an increase in carotenoids and polyphenols respectively, whereas, a decrease of almost all other phytochemicals was observed in fresh and frozen foods. Steaming caused a release of polyphenols in both fresh and frozen samples, while microwaving was the best cooking method to maintaining the colour of both fresh and frozen vegetables and also obtain a good retention of glucosinolates (Pellegrini *et al.*, 2010). Antioxidant

activity determined by 2,2-diphenyl-1-picrylhydrazy (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, reducing power, hydroxyl and superoxide anion radical scavenging assays showed a higher level of antioxidant activities in steamed pods than the uncooked (Thi and Hwang, 2015). In most cases, boiled vegetable extracts showed high antioxidant activity, this indicates that valuable phytochemicals remains in water during cooking process (Mitic *et al.*, 2013). Hwang *et al.* (2012) suggest that cooking increases antioxidant activities by liberating antioxidant compounds from insoluble portions of foods. Analyses of the antinutrient composition of processed (boiled and roasted) and unprocessed *S. stenocarpa* registered significant reduction in the levels of hydrogen cyanide, trypsin inhibitor, phytate, oxalate, and tannins compared to the unprocessed (Ndidi *et al.*, 2014). Cooking treatments caused a decrease in trypsin inhibitor, tannins and phytic acid of lentils (Hefnawy, 2011).

Cooking induces changes in phytochemicals through two opposite phenomena: thermal degradation, which reduces their concentration, and matrix softening effect, which increases the extractability of phytochemicals, resulting in a higher concentration with respect to the raw material (Palermo *et al.*, 2014).

2.14.2 Drying

Thermal methods are known to cause a decline in the total phenolic content compared to non thermal methods. This was evident in a study where the total phenolic and flavonoid content of the processed ginger increased with reducing temperature used in processing, that is, from oven drying, sun drying, and freeze drying (Offei-Oknye *et al.*, 2015). However, drying at low oven temperature (60ºC-80ºC) exerted some beneficial effects on the nutritional compositions; it inactivated the anti-nutritional factors and increased nutrient availability of the plant. Practical investigation by Famurewa *et al.* (2014) showed that the anti nutritional content of the heat treated cocoyam fairly decreased with increased temperature. According to Famurewa *et al.* (2014), cooking, boiling, baking and oven drying at temperature as high as 105ºC for sufficient time, inactivated anti-nutritional factors and removed acridity. In the study carried out by Sommano *et al.* (2013), heat processing stabilized the bioactive contents and increased the level of lycopene and betacarotene in Australian bush food products, though the vitamin C content was minimized. Processing techniques (boiling, fermentation, germination and roasting) reduced the antinutritional factors in the seeds of fluted pumpkin, cashew nuts and breadnuts and improved their *in vitro* protein digestibility (IVPD), when compared with the raw dried (50˚C) seed flours (Fagbemi *et al.*, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Glasswares and equipment

Atomic absorption spectrophotometer (AAS; Buck Scientific 211 VGT, model 2010/2011, England), UV/VIS Spectrophotometer (Spectrolab 755S: 320 – 1100nm; CETIL instrument, UK), bench centrifuge (Gallenkamp), electro-thermostastic water cabinet (Gallenkamp), muffle furnance (Gallenkamp England), thermostatic air circulating oven (Gallenkamp) and rotary evaporator (Gallenkamp), heating mantle and hot plate/magnetic stirrer (DERDHT instrument, Germany), Kjeldal distillation apparatus (TETATOR instrument, Germany), vaccum pump (Edwards instrument Co, England), pH meter (HANNA instrument, England), electronic analytical balance (XS 104; 0.0001g), separating funnel, desiccators, volumetric flask (50 – 1000 ml), beakers (250 – 1000 ml), dissecting set, porcelain crucibles, syringes (1 – 20 ml), test tubes and boiling tubes.

3.1.2 Reagents and chemicals

2-deoxyribose, copper sulphate ($CUSO_4$), ammonium hydroxide (NH_4OH), boric acid, nitro blue tetrazolium, methanol, ethanol, butanol, acetic acid (glacial), formalin, hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH), potassium iodide, sodium sulphate (Na_2SO_4), tetraoxosulphate (VI) acid (H_2SO_4), thiobarbaturic acid

(TBA) and ethylene diamine tetracetic acid (EDTA) (were products of BDH chemicals Ltd Poole England). Dichloromethane, diethyl ether, ascorbic acid, butylated hydroxy toluene (BHT), epinephrine, ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, gallic acid, Griess reagent, phosphate buffer and potassium ferrocyanide (were products of Sigma-Aldrich USA). Hydrochloric acid, indicators (methyl red), iodine, petroleum ether, lead acetate, phenazine methosulfate solution (PMS), sodium chloride, sodium carbonate buffer, sodium nitroprusside (SNP) and trichloroacetic acid (TCA) (were manufactured by May & Baker, Nigeria). Normal saline (Juhel Nigeria Ltd, Awka, Anambra state), Olive oil (Goya; product of Ekulo International Ltd), carbon tetrachloride (Kermel chemical reagent Co. Ltd China) and silymarin (Alexandria pharmaceutical Co, Egypt).

3.1.3 Collection of plant materials

Seeds of *S. stenocarpa, M. myristica and B. coriacea were* purchased from the local markets (Relief and Obazu-Mbieri markets) located within Owerri metropolis, Imo State. The leaves of *C. odorata* were obtained from the surrounding garden in Amakohia early in the morning after the dew has dissipated. The plants *S. stenocarpa, M. myristica, B. coriacea* and *C. odorata* were identified and authenticated by a professional taxonomists, Dr. Mbagwu with voulcher numbers as follows; IMSUH 429, IMSUH 430 and IMSUH 431, which were thereafter deposited at Imo State University herbarium.

3.1.4 Animals

One hundred and sixty (160) adult male Wistar rats (155 ± 20 g) were used for the experiment. They were obtained from Chris Research Farms, Awka, Anambra State, and were housed at room temperature (25 ± 3˚C) and 12h light/dark cycle in well ventilated and hygienic animal house. The animals were allowed to acclimatize for one week and maintained *ad libitum* on clean water and grower's mash (product of Vital Feeds, Grand Cereals Ltd. Jos, Plateau State). All animals were treated in accordance with the recommendations of National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH, 1985).

3.2 METHODS

3.2.1 Treatment of samples

The edible portion of each sample was cleaned and washed under running water then divided into two groups: raw (R) and cooked (C). The Cooked samples were prepared by submerging in boiling water and allowed to cook till soft before drying. Seeds of *B. coriacea, S. stenocarpa, M. myristica* were cooked for 3h, 1h 30 min and 10 min respectively, while the leaves of *C. odorata* were submerged in boiling water for 5 min. The raw and cooked samples were then dried in the oven at 60˚C till they attained constant weights. The dried samples were ground into flour and packaged in air-tight containers with corresponding labels.

3.2.2 Methanol extraction

Methanol extraction of the samples was carried out according to the method of Huda-Faujan *et al.* (2009). The pulverized parts of the different plants were measured out and soaked with methanol in the ratio of 1 g of sample to 6 ml of methanol for 3 days with constant shaking before filtering with Whatman No 1 filter paper. The methanol was completely removed using vacuum evaporator set at 50°C resulting in viscous masses. Then the crude extracts were weighed and stored at -4°C for analysis (See Appendix II).

3.2.3 Extract dosage selection

- **Acute studies**

This was based on Lorke's modified method (Salawu *et al.*, 2009). Seventy two (72) adult male rats were randomized into 24 groups of three rats each and administered 10 mg, 100 mg and 1000 mg of the different extracts per kilogram body weight. The rats were kept under the same conditions and observed for signs of toxicity (paw-licking, stretching and respiratory distress) and mortality for the first critical 4h and thereafter daily for 7 days.

Within the period of examination, there were no signs of paw-licking, stretching, respiratory distress, dizziness or weakness and no death was recorded in all the groups. All the test animals gained weight. Hence, selected plant concentrations below 1000 mg/kg were regarded safe for the test animals. Based on the acute

studies 100 mg, 250 mg and 500 mg of plant extracts were chosen for sub-chronic studies.

- **Sub-chronic Studies (Pilot studies)**

Seventy five (75) adult male albino rats were purchased and acclimitized for five days and separated into groups of three rats each. Groups A1, A2 and A3 were orally administered 100 mg, 250 mg and 500 mg of methanol extracts of raw *Sphenostylis stenocarpa* per kilogram weight of the animal, groups A4, A5 and A6 were given 100 mg, 250 mg and 500 mg of the cooked portion of *Sphenostylis stenocarpa* twice a week for four weeks. Similarly, sub-groups of B, C and D were orally fed raw and cooked extracts of *C. odorata, B. coriacea and M. myristica* respectively. Group E was administered 1 ml of normal saline and maintained as control. After twenty eight days, the test animals were sacrificed; blood samples and organs were collected and assessed for the effect of the plant extracts on the animals.

In comparison with the normal contol, the results showed generally non significant ($P>0.5$) effect on the liver parameters of the animals administered 100 mg/kg of the respective extracts. The histopathological examination (x400 H&E) of the liver tissues of the animals administered 100 mg/kg of the plant extracts showed typical morphology (binucleated hepatocytes, prominient nuclei and intact sinusoid) in line with that of control.

Based on the outcome of acute and pilot studies, the concentrations; 250mg and 500 mg per kilogram weight of animal were adopted for further studies.

3.2.4 Experimental protocol

The male Wistar rats were grouped (eight rats per group) based on weight. The difference in weight within each group was ± 7.0 g. The 20 groups comprised:

Group A - Normal control, received 1 ml of normal saline only

Group B - Olive oil control, received 1 ml/kg of olive oil (vehicle for CCl_4 and solvent for *M. myristica* and *C. odorata*).

Group C - Negative control, received 1ml/kg CCl_4/olive oil mixture

Group D - Positive control, received 100 mg/kg body weight of silymarin

Group E - *S. stenocarpa* (E_{R250}, E_{R500}, E_{C250} and E_{C500})

Group F - *C. odorata* (F_{R250}, F_{R500}, F_{C250} and F_{C500})

Group G - *B. coriacea* (G_{R250}, G_{R500}, G_{C250} and G_{C500})

Group H - *M. myristica* (H_{R250}, H_{R500}, H_{C250} and H_{C500})

Groups E-H consist of four subgroups of eight rats each, fed with either 250 mg/kg or 500 mg/kg body weight of the cooked (C) or raw (R) methanolic extract by intubation for eight (8) weeks. Similarly, groups A and B were given normal saline and olive oil respectively, for the same duration. After 28 days of feeding, 3 rats were withdrawn from Groups A, B and E to H for sub-chronic toxicity studies. Subsequently, all the groups except groups A and B were administered with CCl_4 twice a week for four weeks, concurrently with the extract on alternate days.

Silymarin (100 mg/kg body weight) was administered daily after two weeks of CCl_4 introduction to group D for two weeks.

3.2.5 Induction of hepatic fibrosis

Hepatic fibrosis was induced in the rats with carbon tetrachloride (CCl_4) solution following the method of Abdou *et al.* (2012) with some modifications. Olive oil was used in place of corn oil. The experimental animals in groups C to H were intraperitoneally injected with 1 ml/kg of CCl_4/olive oil mixture (1:1v/v) twice a week for 4 weeks.

3.2.6 Collection of blood and tissue samples

The animals were all sacrificed 12 h after the last extract was fed, by cervical dislocation under anesthesia using dichloromethane (CH_2Cl_2) vapour. The blood sample was collected by cardiac puncture and a portion dispensed into ethylene diamine tetraacetic acid (EDTA) container for haematological analyses and lipid profile. The remaining portion of blood was dispensed into plain vial, allowed to clot and centrifuged for 10 minutes at 3000×g. The clear serum was collected in clean vial for various analyses. The liver and kidneys of each rat were excised and washed in normal saline, blot dried using filter paper and weighed. Some portions of the liver and kidneys were fixed in 10% formalin for histological analyses.

3.2.7 Determination of proximate compositions

- **Total moisture content**

The moisture contents of the pulverized plant samples were determined by gravimetric method of Association of Official Analytical Chemists (AOAC, 2000). The different samples (5g each) were weighed with previously weighed glass petri-dish and dried in an air-circulating oven at 105˚C till a constant dry weight was obtained. The moisture content was determined based on weight differences.

$$\text{Moisture (\%)} = \frac{\text{Wt. of original sample} - \text{Wt. of dried sample}}{\text{Wt. of original sample}} \times 100$$

- **Determination of protein content**

The Kjeldahl distillation method described by AOAC (2000) was employed. It measures protein indirectly by conversion factor, which is equivalent to the nitrogen content per gram of protein.

Principle: Food is digested with strong acid so that it releases nitrogen in the form of ammonium sulphate which can be determined by a suitable nitrogen titration technique.

$$\text{Organic } N_2 \text{ (food)} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + CO_2 + H_2O \text{ (Digestion)}$$

$$(NH_4)_2SO_4 + NaOH \longrightarrow 2NH_3 + Na_2SO_4 + H_2O \text{ (Neutralization)}$$

$NH_3 + H_2SO_4 \longrightarrow NH_4^+ + SO_4^{2-}$

$H_2SO_4 + NaOH \longrightarrow Na_2SO_4 + H_2O$ (Titration)

Procedure: The sample (2.0 g) was placed into a 500 ml Kjeldahl digestion flask containing anti-bumping chips (glass beads). Two tablets of $CuSO_4$ and Na_2SO_4 mixture were added to the flask to serve as catalyst, followed by the addition of 35 ml concentrated H_2SO_4. The test flask alongside a control were fitted properly to the Kjeldahl set-up and digested until transparent greenish-blue colouration was obtained. The digest was diluted to 200 ml using distilled water.

50 ml of the digest were transferred into a Kjeldahl/distillation flask, while 50 ml of 0.1 M H_2SO_4 solution were added into the receiving flask under the condenser and 2 drops of methyl red indicator added. The tip of the condenser was allowed to fully dip into the 0.1 M H_2SO_4 solution. As soon as the ammonium sulphate started distilling, the flask was lowered to avoid a sulk back. An aliquot (50 ml) of 40% NaOH was added and the plug quickly replaced. The mixture was distilled till about 70 ml of the distillate was collected. The distillate including the blank was titrated with 0.1 M NaOH to an orange end point. The percentage nitrogen concentration was determined using the expression:

$$\% \text{ Nitrogen} = (V_b - V_s) \times 1.4 \times \frac{200}{50} \times \frac{100}{W} \times \frac{1}{1000}$$

Where, V_b = Volume (ml) of acid required to titrate the blank

V_s = Volume (ml) of acid required to titrate sample

1.4= mg nitrogen equivalent of 1.0 ml of 0.1N H_2SO_4 solution

200 = Total volume of diluted digest used in distillation

50 = Volume of diluted digest used in distillation

1000 = Scaling factor to convert millgram to gram

W=Weight of sample (g).

Percentage crude protein in the sample was calculated from the % Nitrogen as

% crude protein = % N x F

Where, F (conversion factor) is equivalent to 6.25

- **Determination of crude fat content**

The fat contents of the ground samples were determined by solvent extraction method as described by AOAC (2000). The samples (2.0g) were weighed into labelled thimbles plugged with cotton wool and placed in a soxhlet extractor. Dried round bottom flasks (250 ml) were weighed correspondingly and 150 ml of petroleum ether (b.p 60/80˚C) poured in. The Soxhlet apparatus was assembled and and extraction was allowed to go on for 12 h. The thimble was removed with care and petroleum ether distilled into a receiving bottle for re-use. The flasks were heated in a hot air oven (70˚C) until free of petroleum ether, then cooled in desiccators and weighed.

Crude fat (%) = [(Wt. of fat / Wt. of original sample) × 100]

- **Determination of ash content**

Ash content was determined by furnace incineration method according to the procedure of Association of Official Analytical Chemists (AOAC, 1990). Some crucibles were thoroughly washed, dried in hot air circulation oven and weighed (W_1). A portion of the sample flour was placed into a previously weighed porcelain crucible, which was weighed (W_2) and incinerated in a muffle furnace at 550°C until the residue turned greyish-white (within 5 - 6h). The crucible with content was cooled in a desiccator and weighed (W_3).

$$\% \text{ Ash content} = [\text{Wt. of ash / Wt. of sample}) \times 100]$$

$$= \frac{W_3 - W_1}{W_2 - W_1} \times \frac{100}{1}$$

3.2.8 Qualitative analysis of phytochemicals

A portion of the sample (20 g) was placed in a beaker and 100 ml of water added to it. The mixture was vigourously shaken intermittently for 2h in order for extraction to take place. The mixture was filtered and the filtrate was used to carry out the qualitative analyses of the samples.

- **Saponins**

The presence of saponin in the sample was detected using the frothing test method (Singh *et al.*, 2012). An aliquot (1 ml) of the extract was diluted with 4 ml of water. The mixture was shaken vigorously for 1 min and then observed on standing for stable froth, which persisted on boiling.

- **Tannins**

The presence of tannins in the samples was determined using the ferric chloride test in accordance to the procedure of Singh *et al*. (2012). Two drops of 1 % $FeCl_3$ were added to 2 ml of the filtrate and observed for the formation of bluish-black colouration which indicated the presence of tannins.

- **Flavonoids**

The sodium hydroxide test of Trease and Evans (1989) was used to detect the presence of flavonoids. An aliquot (1.0 ml) of the filtered extract was introduced into a test tube and few drops of 10 % NaOH solution added. Formation of intense yellow colouration indicated the presence of flavonoids. The extract was also treated with 1.0 ml of 5% lead acetate. Formation of yellow precipitate indicated flavonoid

- **Anthocyanins**

The method of Lalitha and Jayanthi (2012) was adopted. An aliquot (2 ml) of the extract was treated with 2 ml of 2 M NaOH and observed for the formation of blue green colouration.

- **Alkaloid determination**

In accordance to the method of Singh *et al.* (2012), each plant extract (2 ml) was shaken and warmed with 2 ml of 2% H_2SO_4 for two minutes on a steam bath and filtered. Few drops of Wagner's reagent (prepared by dissolving 27 g of iodine and 2 g of potassium iodide in 5 ml of water and the volume made up to 100 ml with distilled water) was added to the filtrate and observed for the presence of reddish-brown precipitate which indicated the presence of alkaloids.

3.2.9 Quantitative analysis of phytochemicals

- **Determination of total phenol content**

Total phenolic contents were determined by the Folin-Ciocalteau method (Ebrahimzaded *et al.*, 2010). The sample (200 mg) was extracted with 10 ml of methanol and the mixture filtered. The filtrate (0.3 ml) was mixed with 1.5 ml of Folin Ciocalteu reagent (1:10 diluted with distilled water) for 5 min and 1.2 ml of 7.5 % Na_2CO_3 solution added. The mixture was allowed to stand for 30 min and the absorbance read at 765 nm. A calibration curve was prepared with 0, 10, 20,

30, 40 and 50 mg/ml solutions of gallic acid in methanol-water mixture (50:50 v/v). Total phenol values were expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound (see Appendix II).

- **Tannin determination**

The method of Van-Burden and Robinton (1981) was used in the determination of tannins present in the pulverized samples. The sample (200 mg) was placed in a 100 ml conical flask, to which 10 ml of methanol was added and gently shaken at intervals for 1 h. The mixture was filtered and 0.1 ml of the filtrate placed in a test tube. Similarly, 0.1 ml of water, as well as 10, 20, 30, 40 and 50 µg/ml of tannic acid solutions was added in different test tubes to serve as blank and working solutions for calibration curve. This was followed by the addition of 1.0 ml each of 0.01 M $FeCl_3$, 0.01 M HCl and 0.0008 M potassium ferrocyanide to all the test tubes and their absorbance read in a spectrophotometer at 720 nm wavelength without delay. Tannin values were expressed in terms of tannic acid equivalent (mg/g of dry mass), which is a common reference compound (see Appendix II).

$$\text{mg/g Tannin} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{\text{Absorbance of standard}}{1}$$

- **Flavonoid determination**

Flavonoid content was determined using the method of Boham and Kocipai-Abyzan (1994). The sample (2 g, W_1) was placed in a 250 ml beaker and 100 ml of

80% methanol added to it at room temperature. The beaker was placed on a magnetic stirrer for 3 h and the mixture filtered using Whatmann No 42 filter paper (125 mm). The residue was re-extracted twice with fresh 100 ml of 80 % methanol as before. The combined filtrate was later transferred into a pre-weighed beaker (W_2), evaporated to dryness over a water bath set at 80˚C. The beaker with the extracted flavonoid was weighed again (W_3).

$$\% \text{ Flavonoid} = \frac{W_3 - W_2}{W_1} \times \frac{100}{1}$$

- **Alkaloid determination**

The method described by Obadoni and Ochuko (2001) was adopted for the quantitative analysis of alkaloids in the plants. The sample (5 g) was weighed (W_1) into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added, covered and allowed to stand overnight. The mixture was filtered and the filtrate concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the cooled concentrate until precipitation was complete. The reaction mixture was allowed to settle and the precipitate collected by filtration using pre-weighed filter paper (W_2) dried at 70˚C. The residue was dried at 70˚C with the filter paper and weighed (W_3).

$$\% \text{ Alkaloid} = \frac{W_3 - W_2}{W_1} \times \frac{100}{1}$$

- **Saponin determination**

Saponin content was determined using the method of Nahapetian and Bassiri (1975). The plant sample (2.0 g) was dispersed in 200 ml of 20 % ethanol. The suspension was heated over a hot water bath set at 55ºC for 4 h with continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml of 20 % ethanol. The combined extracts were reduced to 40 ml over water bath set at 90ºC. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added to the aqueous layer to extract the saponin. The butanol layer was collected and washed with 10 ml of 5 % aqueous sodium chloride. The saponin in butanol layer (upper) was collected in a weighted beaker and evaporated to dryness.

$$\text{Saponins (\%)} = \frac{\{(\text{Weight of saponin + beaker}) - (\text{weight of beaker})\} \times 100}{\text{Weight of sample}}$$

- **Determination of oxalate**

The AOAC (1990) analytical method was used to determine the oxalate content of the powdered samples. In the acidic solution, purple potassium permanganate reacts with oxalate ions to produce carbon dioxide, water and nearly colourless

manganese (II) as shown below. Excess permanganate gives a pink colour which signifies complete reaction with oxalate

$$5C_2O_4^{2-}{}_{(aq)} + 2MnO_4^{-}{}_{(aq)} + 16H^{+}{}_{(aq)} \rightarrow 10CO_{2(g)} + 8H_20_{(l)} + 2Mn^{2+}{}_{(aq)}$$

The sample (5 g) was measured out and in 100 ml Erlenmeyer flask, and 20 ml of 0.3 M HCl added. The mixture was placed on a magnetic stirrer at 50˚C for 1h, and then filtered. The process was repeated twice and the filtrate made up to 100 ml. Aliquots (20 ml) of the filtrate was mixed with 3-5 drops of phenolphthalein indicator and drops of ammonium hydroxide solution till the reaction mixture was alkaline. Glacial ethanoic acid was then added in drops till the pink colouration disappeared. To the reaction mixture was added 5 ml of 5% calcium chloride, which was allowed to stand for 3h, then centriguged at 300 rpm for 15 min. The residue was collected and washed thrice with hot water using the centrifugation technique. The washed residue was dissolved in 5 ml of 3.0M H_2SO_4 by warming at 70˚C. The resulting solution was titrated with freshly prepared 0.01 M potassium permanganate solution till pink stable colouration was formed. The percentage oxalate contents of the plants were determined as below;

$$M_aV_a = M_bV_b$$

Where M_a = molarity of oxalate solution

V_a = volume of oxalate solution

M_b = molarity of potassium permanganate solution

V_b = volume of potassium permanganate solution

g/l of oxalate (represented as X) = molarity x molar mass

$$\% \text{ oxalate} = \frac{X}{1} \times \frac{20_a}{1000} \times \frac{100_a}{20_b} \times \frac{100_b}{5}$$

Where X = weight of oxalate obtained from molarity and molar mass

20_a = volume of extract aliquot taken for analysis

1000 = reference volume for mola concentration

100_a = total volume of extract

20_b = volume of extract aliquot taken for analysis

100_b = scaling factor to convert to percentage

5 = weight of sample taken for analysis

3.2.10 Determination of some antioxidants in pulverized plant samples

3.2.10.1 Selenium analysis using atomic absorption spectrophotometer (AAS)

Principle: The technique of AAS is based on the quantification of radiation absorbed at specific wavelength by free atoms of the analyte (Dakashev *et al*., 2012).

Procedure: Water digestion of each sample for selenium analysis was carried out by digesting 0.10 g of sample in 250 ml conical flask containing 25 ml of concentrated nitric acid and 10 ml of concentrated sulphuric acid. The mixture was heated on a hot plate until the volume remained about 7-12 ml. The digest was filtered using Whatman No 1 filter paper and the filtrate added to 50 ml of distilled

water in 100 ml volumetric flask. The beaker was rinsed several times to ensure that all the materials were transferred and the solution made up to 100 ml mark with distilled water. Diluted digests were stored in a plastic container for AAS analysis. The digest was thoroughly mixed by shaking, then transferred into a 250 ml glass beaker and aspirated into the oxidizing air-acetylene flame and the sensitivity for 1% absorption observed. Selenium concentration was determined by tracing the value of the absorbance on a calibration curve prepared using five concentrations of the standard (Appendix II)

3.2.10.2 Determination of Ascorbic acid (Vitamin C)

Principle: The iodine titrimetric method described by Yebio *et al*. (2015) was used for ascorbic acid determination. Under aqueous condition, vitamin C can undergo redox reaction with iodine. The mixture of iodate ion (IO_3^-) and iodide (I^-) produces triiodide which oxidizes vitamin C to dehydroascorbic acid. The triiodide gets converted to iodide ion which reacts with starch suspension (indicator) to produce a blue-black colouration.

$$KIO_{3(aq)} + 5I^-_{(aq)} + 6H^+_{(aq)} \longrightarrow 3I_{2(aq)} + 3H_2O_{(aq)} + K^+$$

$$C_6H_8O_{6(aq)} + I_{2(aq)} \longrightarrow C_6H_6O_{6(aq)} + 2I^-_{(aq)} + 2H^+$$

Procedure: The pulverized sample (0.2 g) was dissolved in 100 ml of distilled water, incubated for 3 h at room temperature with regular shaking, then filtered to

extract the ascorbic acid. Ten drops of 1 % starch were added and thoroughly mixed with 20 ml of the filtrate and titrated against iodide solution (containing 2.0 g KI, 0.11g KIO_3 and 12 ml of 3M H_2SO_4 in 1000 ml of distilled water) to pale blue-black end point and the titration value (consumed iodide solution) was recorded.

$$\text{Vitamin C content of sample (mg/100g)} = \frac{V_{t(sample)} \times C_{vitC(SS)}}{V_{t(SS)}}$$

Where $V_{t(sample)}$ is titration volume of samples; $C_{VitC(SS)}$ is the concentration of Vitamin C in standard solution, and $V_{t(SS)}$ is the average titration volume of standard solution.

3.2.10.3 Determination of vitamin E

This was determined by the colorimetric method of Kirk and Sawyer (1991). Homogenisation and hydrolysis of the sample were carried out by mixing 1.0 g of sample with 10 ml of ethanoic sulphuric acid.The mixture was boiled gently under reflux for 30 min and cooled in a dessicator. It was transferred to a separating funnel and treated with 3×30 ml diethyl ether; recovering ether layer each time. The ether extract was evaporated to dryness at room temperature. The dried extract was dissolved in 10 ml of pure ethanol, 1 ml of the dissolved extract and equal volume of standard were transferred to separate test tubes. After continuous addition of 5 ml of absolute alcohol and 1 ml of concentrated nitric acid solution,

the mixtures were allowed to stand for 5 min and respective absorbance measured in a spectrophotometer at 410 nm with a blank reagent at zero.

$$\text{Concentration of Vitamin E} = \frac{\text{Abs of sample}}{\text{Abs of standard}} \times \text{Conc of standard}$$

3.2.11 Free radical scavenging activities of the pulverized samples

3.2.11.1 Superoxide scavenging assay

Principle: The activity was evaluated using the nitro blue tetrazolium (NBT) reduction method given by Nishikimi *et al.* (1972). The $O_2^{-\cdot}$ generated from methosulfate can reduce NBT to form blue formazan which has absorbance at 500 nm. Antioxidants scavenge $O_2^{-\cdot}$, thereby inhibiting the NBT reduction.

Procedure: The reaction mixture consisted of 1 ml of 156 μM NBT solution and sample solution at different concentrations (200 – 800 μg/ml). The reaction was started by adding 100 μl of 60 μM phenazine methosulfate solution (PMS) in phosphate buffer (pH 7.4) to the reaction mixture followed by incubation at 25˚C for 5 min and the absorbance read at 500 nm against blank. Ascorbic acid was used as the standard.

$$\text{Superoxide scavenging activity (\%)} = \frac{\text{Abs (control)} - \text{Abs(test)}}{\text{Abs (control)}} \times 100$$

Abs = Absorbance

3.2.11.2 Hydrogen peroxide (H_2O_2) radical scavenging assay

The ability of the plant samples to scavenge hydrogen peroxide was determined according to the method given by Ruch *et al.* (1989). A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (pH 7.4). Extract (200 – 800 µg/ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of the hydrogen peroxide solution at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound.

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{\text{Abs (control)} - \text{Abs(test)}}{\text{Abs (control)}} \times 100$$

Abs = Absorbance

3.2.11.3 Hydroxyl radical scavenging activity

Principle: The scavenging ability for hydroxyl radicals was determined by the method of Basha *et al.* (2011) based on the degradation of 2-deoxyribose by the ·OH to give a reactive specie malondialdehyde, which forms an adduct with thiobarbituric acid (TBA). The adduct MDA-TBA has an absorption at 532 nm that can be assayed spectrophotometrically.

Procedure: Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled

deionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of 2-deoxyribose, 1.0 ml of plant extract (200 – 800 µg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37˚C for 1 h. About 1.0 ml portion of the mixture was mixed with 1.0ml of 0.5% TBA and 1.0 ml of 10% trichloroacetic acid (TCA) to develop the pink chromogen, measured at 532 nm.

$$\text{Hydroxyl scavenging activity (\%)} = \frac{\text{Abs(control)} - \text{Abs(test)}}{\text{Abs(control)}} \times 100$$

Abs = Absorbance

3.2.11.4 Nitric oxide scavenging activity

Principle: Nitric oxide scavenging activity was determined using Griess reagent in accordance to the modified method of Basha *et al.* (2011). Sodium nitroprusside decompose in aqueous solution at physiological pH (7.2) to produce NO which reacts with oxygen under aerobic conditions to produce stable products (nitrate and nitrite); the quantities of which can be determined using Griess reagent.

Procedure:

1ml of 5 mM sodium nitroprusside (SNP) dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was added to 4 ml of sample extract at various concentrations (200 – 800 µg/ml). The mixture was incubated at 27ºC for 2 h. An aliquot (2 ml) of the incubated solution was withdrawn and diluted with 1.2 ml of Griess reagent (1 %

sulfanilamide in 5 % H_3PO_4 and 0.1 % naphthylethylenediamine dihydrochloride). The absorbance of the chomophore was read immediately at 550 nm and compared with that of standard, butylated hydroxyltoluene (BHT).

$$\text{Nitric oxide scavenging activity (\%)} = \frac{\text{Abs (control)} - \text{Abs(test)}}{\text{Abs (control)}} \times 100$$

Abs = Absorbance

3.2.11.5 Ferric reducing ability

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Different concentrations of the extract (200 – 800 µg/ml) in 1.0 ml of deionised water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1 %). The mixture was incubated at 50°C for 20 min and later 2.5 ml of trichloroacetic acid (10 %) was added to the mixture and centrifuged at 3000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 0.5 ml of 0.1% $FeCl_3$ and the absorbance measured at 700 nm

3.2.12 Haematological analyses

The complete blood count (CBC) is commonly performed on an automated hematology analyzer, usually in less than one minute using well mixed whole blood that is added to an EDTA container to prevent clotting. A CBC is a group of tests used to quantify the number of red blood cells (RBC), White blood cells (WBC), and platelets (PLT), measure the haemoglobin (Hb) content of RBCs,

determine the percentage and absolute number of the five white blood cell types, and identify early and abnormal blood cells (Buttarello and Plebani, 2008).

Principle: White blood cells, red blood cells and platelets are determined based on the Coulter principle of detection and measurement of changes in electrical resistance (impedance) produced by cells as they traverse a small aperture, while haemoglobin concentration (Hb) is measured using the cyanmethemoglobin method. In the presence of a haemolyzing agent containing cyanide, lysed RBC release haemoglobin which react with cyanide to form cyanmethemoglobin capable of absorbing green light at 540 nm (Yamao, 2015).

Procedure: A small sample (1 ml) of the blood was aspirated into a chamber (the WBC counting bath) and treated with electrically conductive diluents such as isotonic saline. The diluted blood sample was split into two parts, one for counting RBCs and platelets and the other for counting WBCs. The RBC portion was transferred to the RBC/platelet counting bath where it was diluted further. The other portion remained in the WBC bath and a detergent (lysing agent) was added to destroy (hemolyze) the red blood cells. A small portion of the diluted fluid in each bath was allowed to flow past a small aperture. An electrical current was generated in each aperture by two electrodes, one on the inside and the other on the outside of the aperture. The passage of a cell through the aperture caused

displacements in the electrolytes (saline), resulting to impedance in the flow of current. This produced a voltage pulse, the magnitude of which is proportional to the size of the cell. The electronic instrument counter is adjusted to discriminate voltage pulses produced by different cells. These adjustments are called thresholds. For example, the threshold for counting RBC is equivalent to a cell volume of 36 femtoliters or higher and pulses that are equivalent to volumes of 2–20 femtoliters are counted as platelets (Constantino, 2011)

The hemoglobin concentration is measured optically using the solution in the WBC bath. The lysing agent contains potassium cyanide that reacts with the hemoglobin to form cyanmethemoglobin. The optical density of the cyanmethemoglobin is proportional to hemoglobin concentration (Yamao, 2015).

3.2.13 Analysis of biochemical parameters

3.2.13.1 Determination of aspartate aminotransferase (AST) activity

Principle: The assay is based on the coupling of oxaloacetic acid formed by the enzyme (AST) with 2, 4-dinitrophenylhydrazine (DNP) to form oxaloacetate-hydrazone that absorbs at 540 nm (Schmidt and Schmidt, 1963). AST catalyses the reaction below:

L-aspartate + α-ketoglutarate ⟶ Oxaloacetate + L-glutamate

Reagent 1 (0.5 ml) of Randox (USA) assay kit, containing phosphate buffer (100 mmol/l, pH 7.4), L-aspartate (100 mmol/l) and α-ketoglutarate (2 mmol/l) was added to each of the test tubes containing 0.1 ml serum and the blank, mixed and incubated for 30 min at 37˚C. An aliquot (0.5 ml) of reagent 2 (2 mmol/l of 2, 4-dinitrophenyl hydrazine) was added to all the test tubes and allowed to stand for 20 min at 37˚C, followed by the addition of 5ml of NaOH (2 mmol/l). The absorbance of the sample against the sample blank was read at 540 nm after 5 min and the corresponding activity extrapolated from the standard table (See Appendix II).

3.2.13.2 Determination of alanine aminotransferase (ALT) activity

Principle: The transfer of amino group of alanine to α- ketoglutarate by ALT produces pyruvate which is coupled to DNP to form pyruvate hydrazone, which concentration can be monitored at 540 nm (Schmidt and Schmidt, 1963). ALT catalyses the reaction below:

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}$$

Procedure: Assay procedure and reagents are similar to that of AST except that the reagent 1 of ALT contained L-alanine (200 mmol/l) in place of L-aspartate (100 mmol/l). The absorbance of the sample against the sample blank was also read at 540 nm and the corresponding activity extrapolated from a standard table (See Appendix II).

3.2.13.3 Determination of alkaline phosphatase (ALP) activity

Principle: The assay is based on the action of alkaline phosphatase (ALP) on sodium thymolphthalein monophosphate. The addition of alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically at 590 nm (Tietz, 1976).

Procedure: The assay kit was purchased from Teco Diagnostics Ltd, USA. An aliquot (0.25 ml) of ALP substrate (sodium thymolphthalein monophosphate solution) was dispensed into labeled test tubes and equilibrated to 37˚C for 3 min and 0.25 ml of standard, control and serum were added to their respective tubes. The test tubes were mixed gently and incubated at 37˚C for 10 min. An aliquot (1.25 ml) of ALP colour developer (0.1M NaOH and 0.1M Na_2CO_3) was added into all the test tubes, mixed well and read at 590 nm.

$$\text{ALP activity (IU/L)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Value of standard (IU/L)}$$

3.2.13.4 Determination of total protein concentration

Principle: Cupric ions interact with protein peptide bonds resulting in the formation of a coloured complex (Tietz, 1995).

Procedure: Total protein concentration of the samples was determined using kit obtained from Randox Diagnostics Ltd, USA. An aliquot (0.01 ml) of distilled water (reagent blank), standard, sample blank and serum samples were dispensed into their respective test tubes and 0.5 ml of Biuret reagent (containing 100, 16, 15 and 6 mmol/l of NaOH, Na-K-tartarate, potassiunm iodide and cupric sulphate, respectively) was added to all test tubes and mixed. The reaction mixture was incubated for 30 min at room temperature and read at 540 nm

$$\text{Total protein concentration (g/l)} = \frac{\text{Absorbance of Sample} \times \text{Standard conc (g/l)}}{\text{Absorbance of Standard}}$$

3.2.13.5 Determination of albumin concentration

Principle: This assay is based on the quantitative binding of serum albumin to the indicator $3,3^{1},5,5^{1}$- tetrabromo cresol sulphonephthalein (bromocresol green, BCG) to form a complex which absorbs at 630 nm (Grant, 1987).

Procedure: The products of Randox Diagnostics Ltd, USA were used. Aliquots (0.01 ml) of distilled water (reagent blank), standard and serum samples were dispensed into their appropriately labeled test tubes and mixed with 3.0 ml of BCG reagent. The reaction mixture was incubated at room temperature for 5 min and read at 630 nm. Absorbance of the sample and standard were measured against the reagent blank.

$$\text{Albumin concentration (g/l)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Standard conc. (g/l)}$$

3.2.13.6 Determination of creatinine concentration

Principle: The method of serum creatinine determination is based on Jaffe's reaction. Creatinine reacts with picric acid in alkaline solution to form an orange coloured complex that is proportional to the creatinine concentration in the sample (Glick *et al*., 1986).

Procedure: The commercial kit purchased from Randox Ltd was used. Aliquot (1.0 ml) of the working reagent {35 mM picric acid and 0.32M NaOH (v/v)} was added to well labeled test tubes and 0.1 ml each of distilled water (reagent blank), standard and serum samples dispensed into their appropriate test tubes. The reaction mixture was mixed and the absorbance (A_1) of the standard and the samples read after 30 sec. Two minutes later, the absorbance (A_2) of the standard and samples were taken again. The change in absorbance ($A_2 - A_1$) was noted and used for calculation.

$$\text{Concentration of Creatinine} = \frac{\Delta Abs_{Sample}}{\Delta Abs_{Standard}} \times \text{Standard conc (mg/dl)}$$

Where ΔAbs = change in absorbance

3.2.13.7 Determination of urea concentration

Principle: In the presence of urease (Randox Ltd), urea is hydrolysed to ammonia which is measured photometrically at 550 nm by Berthelot's reaction (Patton and Crouch, 1977).

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$2NH_3 + \text{Hypochlorite} + \text{Phenol} \longrightarrow \text{Indophenol (blue compound)}$$

Procedure: 10 µl each of distilled water (blank), standard and serum samples were dispensed into different labelled test tubes and mixed with 100 µl of reagent 1 solution (116 mmol/l EDTA, 6 mmol/l sodium nitroprusside and 1 g/l of urease). The reaction mixture was mixed gently and incubated for 10 min at 37˚C. An aliquot (2.5 ml) each of reagent 2 (120 mmol/l phenol) and reagent 3 (27 mmol/l sodium hypochlorite and 0.14 N NaOH) were added to all the test tubes, then mixed and incubated at 37˚C for 15 min and the absorbance read.

$$\text{Concentration of Urea} = \frac{\text{Absorbance of Sample} \times \text{Standard conc (mg/dl)}}{\text{Absorbance of standard}}$$

3.2.13.8 Determination of chloride ion (Cl^-) concentration

Principle: Chloride ions form soluble, non-ionized compound with mercuric ions and will displace thiocyanate ions from non-ionized mercuric thiocyanate (Teco

Diagnostics, USA). The released thiocyanate ions react with ferric ions to form a coloured complex that absorbs light at 480 nm (Tietz, 1976). The intensity of the colour produced is directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$

$3SCN^- + Fe^{3+} \longrightarrow Fe(SCN)_3$ (red complex)

Procedure: An aliquot (1.5 ml) of chloride reagent (containing 0.058 mM mercuric nitrate, 1.75 mM mercuric thiocyanate, 0.74 mM mercuric chloride and 22.3 mM ferric nitrate) was drawn with pipette and dispensed into appropriately labeled test tubes and 10µl of the blank, calibrator (100 mEq/L of NaCl) and samples added to their respective tubes. The solutions were mixed and incubated at room temperature for 5 min and absorbance readings of the test tubes were taken at 500 nm.

$$\text{Concentration of Chloride ion (mEq/L)} = \frac{\text{Abs. of Sample}}{\text{Abs. of calibrator}} \times \text{Conc. of calibrator}$$

Abs = Absorbance

3.2.13.9 Determination of potassium ion (K^+) concentration

Principle: Potassium concentration is measured based on the turbidity of colloidal suspension formed in a reaction involving sodium tetraphenylboron (Tietz, 1976).

Procedure: An aliquot (1.0 ml) of potassium reagent (containing 2.1 mM sodium

tetraphenylboron) was drawn with pipette and dispensed into all the test tubes and 10 µl of the blank, standard (4 mEq/L) and serum added to their respective tubes. The solutions were mixed and incubated at room temperature for 3 min and absorbance readings of the test tubes were taken at 500 nm. The blank was used to zero the spectrophotometer.

$$\text{Conc. of potassium ion (mEq/L)} = \frac{\text{Abs of Sample}}{\text{Abs. of Standard}} \times \text{Conc of standard}$$

Conc = Concentration

Abs = Absorbance

3.2.13.10 Determination of carbon dioxide (CO_2) concentration

Principle: Phosphoenol pyruvate carboxylase (PEPC) catalyzes the reaction between phosphoenol pyruvate and carbon dioxide (bicarbonate) to form oxaloacetate and phosphate ion. Oxaloacetate is reduced to malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ by the enzyme malate dehydrogenase (MDH). This results in a decrease in absorbance at 340 nm that is directly proportional to CO_2 concentration of the sample (Forrester *et al.*, 1976).

$$\text{Phosphoenol pyruvate} + H_2CO_3 \xrightarrow{\text{PEPC}} \text{Oxaloacetate} + H_2PO_4$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

Procedure: Aliquot (1.0 ml) of CO_2 reagent (containing PEP, MDH, PEPC, sodium oxalate and buffer pH 7.0) was added into appropriately labeled test tubes consisting of blank, standard and serum specimens. The tubes were incubated for 3 min at 37˚C and 5 µl of water, standard and sample to their respectively labelled tubes. The tubes were mixed gently by inversion and the absorbance read after 5 min at 340 nm.

$$CO_2 \text{ concentration} = \frac{\text{Abs. of blank - Abs. of Sample}}{\text{Abs. of blank - Abs. of Standard}} \times \text{Conc. of standard}$$

Abs = Absorbance

3.2.13.11 Determination of sodium ion (Na^+) concentration

Principle: The assay is based on the modified method of Maruna (1958), in which sodium is precipitated as the triple salt; sodium magnesium uranyl acetate when the excess uranium reacts with ferrocyanide to produce a chomophore whose absorbance varies inversely as the concentration of sodium in the test specimen (Teco Diagnostics, USA).

Procedure: An aliquot (1.0 ml) of fiiltrate reagent (containing uranyl acetate and magnesium actetate in ethyl alcohol) was dispensed into well labeled comprising blank, standard, control and various serum sample test tubes, followed by the addition of 50µl of distilled water, sodium standard and the different serum to as

the appropriate test tubes. The test tubes were vigorously shaken continuously for 3 min and centrifuged at 1,500 g for 10 min. An aliquot (50 µl) of the supernatant fluids were carefully taken and dispensed into another set of test tubes (corresponding to the former labels) containing 1.0 ml of acid reagent (acetic acid). An aliquot (50 µl) of sodium colour reagent (containing potassium ferrocyanide) was added to all the test tubes, then mixed and the absorbance read at 550 nm using distilled water to zero the spectrophotometer.

$$\text{Conc, of sodium (mEq/L)} = \frac{\text{Abs. of blank - Abs. of Sample}}{\text{Abs. of blank - Abs. of Standard}} \times \text{Conc. of std.}$$

3.2.13.12 Determination of cholesterol (Chol) concentration

Principle: Cholesterol is determined after the enzymatic hydrolysis and oxidation of cholesteryl ester. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Allain *et al*., 1974).

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidise}} \text{Quinoneimine} + H_2O$$

Procedure: 1000 µl of cholesterol reagent (containing 4-aminoantipyrine, phenol, peroxidase, cholesterol esterase and cholesterol oxidase) was drawn with pipette

and dispensed into all the test tubes and 10 µl of the blank, standard and serum added to their respective tubes. The solutions were mixed and incubated at room temperature for 10 min and absorbance readings of the test tubes taken at 500 nm.

$$\text{Conc. of Cholesterol in sample} = \frac{\text{Abs. of Sample} \times \text{Conc. of standard (mg/dl)}}{\text{Abs. of Standard}}$$

Abs = Absorbance

3.2.13.13 Determination of triglyceride (TG) concentration

Principle: Triglycerides are determined after the enzymatic hydrolysis with lipases. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminophenazone in the presence of chlorophenol and peroxidase (Fossati and Prencipe, 1982).

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipases}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase (GK)}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol -3-phosphate} + O_2 \xrightarrow{\text{Glycerol-3-P oxidase (GPO)}} \text{Dihydroxyacetone} + \text{phosphate} + 2H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4chlorophenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + HCl + 4H_2O$$

Procedure: An aliquot (10 µl) each of distilled water (blank), standard and serum samples were dispensed into well labeled test tubes and mixed with 1000 µl of triglycerides reagent solution (4-aminophenazone, 4-chlorophenol, ATP, buffer

pH 7.6, magnesium ions, GK, GPO and peroxidase). The reaction mixture was mixed gently and incubated for 5 min at 37˚C and the absorbance read at 500 nm.

$$\text{Triglyceride concentration} = \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Standard}}} \times \text{Standard conc (mg/dl)}$$

Abs = Absorbance

3.2.13.14 Determination of high density lipoprotein (HDL) concentration

Principle: The chylomicrons, low density lipoproteins (LDL) and very low density lipoprotein (VLDL) of serum are precipitated by phosphotungstic acid and magnesium ions (Grillo *et al*., 1981). After centrifugation, HDLs are in the supernatant and similar to cholesterol, can be enzymatically measured.

Procedure: An aliquot (150 µl) of HDL reagent was added to previously labelled test tubes containing 150 µl of the different serum samples. The solutions were mixed and allowed to stand at room temperature for 10 min. Thereafter, the mixture centrifuged at 4000 g for 10 min and 50 µl of the supernatants were transferred into corresponding test tubes. The supernatants alongside 50 µl HDL standard and distilled water in different test tubes were mixed with 1000 µl of cholesterol reagent, then mixed and incubated for 5 min at 37˚C. The absorbance

readings of the reaction mixtures were taken at 500 nm. The blank was used to zero the spectrophotometer.

$$\text{HDL cholesterol concentration (mg/dl)} = \frac{\text{Abs. of Sample}}{\text{Abs. of Standard}} \times N \times 2$$

Where, 2 = dilution factor of the sample

Abs = Absorbance

N = standard concentration (50 mg/dl)

3.2.13.15 Determination of lipid peroxidation (LPO) index

Lipid peroxidation was estimated based on the formation of thiobarbituric acid reactive substances (TBARs) in accordance with the method of Usoh *et al.* (2005). Malondialdehyde (MDA) which is an index of LPO was calculated with extinction coefficient of 1.5×10^5 $M^{-1}cm^{-1}$. An aliquot (2.0 ml) of the reaction mixture containing equal volume (1:1:1) of 0.25 M HCl, 0.37% TBA and 15% trichloroacetic acid (TCA) was mixed with 0.1 ml of the serum and boiled for 15 min. The mixture was centrifuged and the absorbance of the coloured layer read at 530 nm.

$$\text{Concentration of MDA} = A/\varepsilon l$$

Where A = Absorbance

ε = Molar extinction coefficient

l = Path length of the light-absorbing samples (cm)

3.2.13.16 Determination of Superoxide dismutase (SOD) activity

The method of Ogbunugafor *et al.* (2012) was adopted. The method is based on the reduction in autoxidation of epinephrine. The oxidation of epinephrine under alkaline pH indirectly generates Superoxide radical (O_2^-) and pink coloured adrenochrome. SOD reacts with O_2^-, thus reducing the rate of formation of adrenochrome. The reaction mixture (3ml) contained 2.95 ml of Na_2CO_3 buffer (0.05M, pH 10.2), 0.02 ml of serum and 0.03 ml of substrate (epinephrine) in 0.005N HCl used to initiate reaction. The reference contained equal substrate and buffer but 0.02 ml of distilled water. An extinction coefficient for epinephine at 480 nm of 4020 $M^{-1}cm^{-1}$ was used in calculating activity.

$$\text{Activity of SOD} = A/\varepsilon l$$

Where A = Absorbance

ε = Molar extinction coefficient

l = Path length of the light-absorbing samples (cm)

3.2.13.17 Determination of Catalase (CAT) activity

Serum catalase activity was determined according to the method of Ogbunugafor *et al.* (2012). The assay is based on the action of catalase on hydrogen peroxide to form the catalase-hydrogen peroxide complex. The reaction mixture contained 0.1 ml of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H_2O_2. An

extinction coefficient for H_2O_2 at 240 nm of 40 $M^{-1}cm^{-1}$ as described by Aebi (1984) was used for the calculation.

Catalase activity = A/εl

Where A = Absorbance

ε = Molar extinction coefficient

l = Path length of the light-absorbing samples (cm)

3.2.13.18 Determination of Lactate dehydrogenase (LDH) activity

Principle: LDH catalyses the conversion of pyruvate to lactate with simultaneous oxidation of NADH (reduced form) to NAD^+ (nicotinamide adenine dinucleotide) (Pan Diagnostics Ltd, UK). The rate of decrease in absorbance due to the formation of NAD^+ is measured at 340 nm and is proportional to the LDH activity in the sample (Kaplan and Lavernel, 1983).

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

Procedure: 10 µl of distilled water (blank) and serum specimens were dispensed into well labeled test tubes and 1000 µl of working reagent (pyruvate buffer and NADH) added to all the test tubes. Absorbance of the mixture was taken after 60 min and repeated readings taken within 120 min after every 30 sec. The absorbance change per minute (ΔA/minute) was noted and activity determined as:

LDH activity (IU/L) = ΔA/minute × kinetic factor.

Where, Kinetic factor (K) = 16030

3.2.14 Histopathological examination of the liver and kidney tissues

The liver and kidney tissues fixed in formalin were processed according to the method of Baker and Silverton (1985). The fixed tissues were cut into small pieces (about 3-5 mm thick and 1 cm long) and refixed in 10% formalin prior to processing. The tissues were thereafter dehydrated in an ascending series of ethanol (70%, 80%, 90% and 100%), followed by clearing through changes (thrice) in xylene for 1 hr 30mins each. The tissues were embedded in paraffin, sectioned transversally at 4-6 µm and mounted on glass slides. The section was de-paraffinzed with xylene and rehydrated in descending series of ethanol. It was rinsed in water and stained in heamatoxylin for 5 min. Thereafter, it was quickly dipped in 1% acid alcohol for 3 seconds to de-stain and rinsed in water. Excess water from the slide was blotted out and counterstained with eosin for 2 min. After rinsing with water, the section was dehydrated in ascending grades of ethanol, cleared in xylene. A xylene based coverslip was placed on the slide and mounted on a di-n-butyl phthalate in xylene (DPX – mounting medium) and viewed under a microscope.

3.2.15 Immunohistochemical determination of collagen expression

The anti-fibrotic effect of methanol extracts of SS, MM, CO and BC were confirmed by immunohistochemical staining for collagen expression.

Principle: Immunohistochemistry (IHC) refers to the process of detecting antigens in tissue sections based on the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vera, 2011).

Method: The immunohistochemical technique of Matos *et al.* (2010) was adopted, using cytokeratin cocktail AE1 & AE3 kit manufactured by Bio SB, USA. Cytokeratin AE1/AE3 is a mixture of two different clones of anti-cytokeratin monoclonal antibodies, AE1 and AE3 capable of detecting certain high and low molecular weight keratins (Miller, 2003). Similar to the histopathological protocol, the liver tissues were dehydrated with alcohol, de-alcoholed with xylene, embedded in paraffin and sectioned. Paraffin sections were deparaffinised in xylene and rehydrated in ethanol and water. The sections were incubated for 10 minutes in 0.1 % H_2O_2 thus avoiding endogenous peroxidase activity. After washing with phosphate-buffered solution (PBS), the sections were incubated in immunoDNA retriever containing ethylene diamine tetraacetic acid (EDTA). The set up was immersed for 30 minutes in a water bath set at 95˚C and later cooled to room temperature. In order to avoid the background activity, sections were incubated in 10 % normal mouse serum for 20 minutes and subsequently with the primary antibodies and mouse secondary antibody for 30 minutes at room temperature. The sections were then washed in PBS, stained with diaminobenzidine tetrahydrochloride solution (DAB), and counter-stained with

hematoxylin. The negative controls were made for each primary antibody by labeling the tissues only with secondary antibody and staining with DAB.

3.2.16 Statistical Analysis

The data obtained were expressed as mean ± standard deviation (SD) and statistical analysis was carried out by two-way ANOVA using GraphPad Prism software, version 5.3. Probability was set at $P < 0.05$.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.0 RESULTS

4.1.1 Proximate compositions of samples

The results of proximate composition of the raw and cooked samples revealed high percentage contents of carbohydrates in the raw and cooked samples of *S. stenocarpa, C. odorata* and *B. coriacea* compared to that of *M. myristica* which contained significantly ($P<0.05$) high lipid. The crude protein content of *S. stenocarpa* was significantly ($P<0.05$) the highest while that of *cooked M. myristica* was the lowest. The moisture and ash contents of the leaves of *C. odorata* was significant ($P<0.05$) the highest among the plants sampled. Generally, boiling reduced the protein, lipid and ash contents of the samples and increased the water and carbohydrate contents of the sampled plant materials (Table 4.1).

Table 4.1: Proximate compostions of raw and cooked pulverized samples of *S. stenocarpa, B. coriacea, M. myristica* and *C. odorata*

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Carbohydrate (%)
*S, stenocarpa**	8.89 ± 0.05^{a}	21.20 ± 0.35^{a}	7.38 ± 0.24^{a}	3.38 ± 0.07^{a}	59.15 ± 0.49^{a}
*C. odorata**	10.77 ± 0.02^{b}	11.07 ± 0.08^{b}	9.36 ± 0.09^{b}	8.53 ± 0.09^{b}	60.27 ± 0.22^{b}
*B. coriacea**	7.14 ± 0.09^{c}	12.96 ± 0.15^{c}	4.05 ± 0.10^{c}	3.20 ± 0.27^{a}	72.65 ± 0.31^{c}
*M. myristica**	6.62 ± 0.03^{d}	11.97 ± 0.14^{d}	33.69 ± 1.82^{d}	3.82 ± 0.04^{c}	43.90 ± 1.89^{d}
S, stenocarpa	10.38 ± 0.08^{e}	19.19 ± 0.08^{e}	6.83 ± 0.20^{e}	2.42 ± 0.06^{d}	61.17 ± 0.27^{e}
C. odorata	12.64 ± 0.06^{f}	10.48 ± 0.06^{b}	8.74 ± 0.13^{f}	8.14 ± 0.04^{e}	60.25 ± 0.06^{b}
B. coriacea	9.76 ± 0.11^{g}	9.25 ± 0.07^{f}	3.77 ± 0.08^{g}	1.88 ± 0.05^{f}	75.34 ± 0.15^{f}
M. myristica	9.51 ± 0.04^{h}	8.94 ± 0.14^{f}	29.03 ± 1.05^{h}	3.37 ± 0.06^{a}	49.15 ± 3.88^{g}

Values are mean ± SD of triplicate determinations. Values with different superscript on the same column are statistically different ($P<0.05$). Superscript* indicates raw samples.

4.1.2 Qualitative screening for phytochemical contents

The results of the qualitative phytochemical screening of the pulverized plant samples showed the presence of alkaloids, flavonoids, tannins and saponins in all the sampled plant materials. Anthocyanin was not detected in cooked *S. stenocarpa*. Moderate concentrations of alkaloids, flavonoids, anthocyanidins and tannins were observed in the raw and cooked samples of CO while the other plants had low concentrations. Moderate concentrations of saponin observed in raw SS and BC were reduced to low concentrations in their cooked samples (Table 4.2)

Table 4.2: Qualitative phytochemical compositions of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica*

Sample	Alkaloids	Flavonoids	Anthocyanin	Tannins	Saponins
SS*	+	+	+	+	++
CO*	+	++	++	++	++
BC*	+	+	+	+	++
MM*	+	+	+	+	+
SS	+	+	-	+	+
CO	+	++	++	++	+
BC	+	+	+	+	+
MM	+	+	+	+	+

++ = Present in moderate concentration, + = Present in low concentration, - = Absent, Values are mean ± SD (n = 3). SS = *S. stenocarpa*, CO = *C. odorata*, BC = *B. coriacea*, MM = *Monodora myristica.* Superscript* indicate raw samples.

4.1.3 Quantitative analyses for phytochemical contents of samples

The results of the quantitative analyses of the phytochemical contents of the raw and cooked samples revealed highest concentration of total phenol expressed in mg GAE/g of dry plant material, flavonoids, saponins and oxalates in raw *C. odorata*, while *M. myristica* had the highest content of tannin and alkaloid. However, the tannin content of *M. myristica* was not significantly ($P>0.05$) higher than that of raw *C. odorata*. Cooking significantly ($P<0.05$) reduced the tannin, alkaloid, flavonoid, saponin and oxalate contents of all the samples; with the oxalate content of *C. odorata* being the least affected. Except for *C. odorata*, the total phenolic contents of the other samples were also significantly ($P<0.05$) reduced (Table 4.3).

Table 4.3: Quantitative phytochemical compositions of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica*

Sample	Total Phenolic (mg GAE/g)	Tannin (mg/g)	Flavonoids (%)	Alkaloids (%)	Saponins (%)	Oxalate (%)
SS*	1.00 ± 0.03^{a}	8.10 ± 0.12^{a}	6.33 ± 0.29^{a}	4.83 ± 0.29^{a}	2.83 ± 0.29^{a}	14.50 ± 0.05^{a}
CO*	7.97 ± 0.20^{c}	12.63 ± 0.62^{c}	21.00 ± 1.00^{b}	16.50 ± 0.50^{b}	4.33 ± 0.29^{b}	14.99 ± 0.11^{b}
BC*	3.02 ± 0.03^{d}	7.39 ± 0.43^{a}	6.33 ± 0.29^{a}	7.00 ± 0.87^{c}	2.33 ± 0.29^{ac}	7.51 ± 0.02^{c}
MM*	4.04 ± 0.01^{f}	13.28 ± 0.27^{c}	14.17 ± 0.29^{c}	31.33 ± 0.29^{d}	1.67 ± 0.28^{c}	4.13 ± 0.08^{d}
SS	0.26 ± 0.04^{b}	0.52 ± 0.01^{b}	3.17 ± 0.29^{e}	2.17 ± 0.29^{f}	2.33 ± 0.29^{ac}	5.96 ± 0.04^{e}
CO	7.90 ± 0.09^{c}	10.42 ± 0.18^{d}	12.33 ± 0.29^{d}	7.33 ± 0.29^{c}	1.00 ± 0.01^{d}	13.75 ± 0.19^{f}
BC	0.59 ± 0.03^{e}	0.46 ± 0.12^{b}	1.33 ± 0.29^{f}	1.50 ± 0.01^{f}	1.67 ± 0.29^{c}	1.28 ± 0.02^{g}
MM	3.12 ± 0.19^{d}	10.62 ± 0.47^{d}	6.50 ± 0.01^{a}	20.00 ± 0.50^{e}	0.33 ± 0.09^{e}	4.62 ± 0.07^{h}

Values are mean ± SD (n = 3). Values with different superscript letters per column are statistically significant ($p<0.05$). SS = *S. stenocarpa,* CO = *C. odorata,* BC = *B. coriacea,* MM = *Monodora myristica.* Superscript* indicates raw samples. GAE = Gallic acid equivalent.

4.1.4 Antioxidant properties

The vitamin C and E contents of the raw *C. odorata* leaf were observed to be the highest compared to the seeds of SS, MM and BC. The vitamin C and E contents of the four selected plants were markedly reduced to low values after cooking and this effect was more pronounced with *C. odorata*. Generally, low values (< 1.6mg/l) of selenium (Se) were observed in the raw and cooked samples and the highest value was obtained from cooked *C. odorata* (CCO). Increased selenium content was observed in CO and BC, while the contents of SS and MM decreased after cooking.The catalase activity of *C. odorata* and *B. coriacea* were significantly ($P<0.05$) higher and the values significantly ($P<0.05$) different from those of the cooked samples. Raw SS (RSS) and raw MM (RMM) exhibited low catalase activities while the cooked samples had no and negligible activities, respectively. Similarly, SOD activity was observed in raw CO and MM and the activities reduced in their cooked samples (Table 4.4).

Table 4.4: Antioxidant properties of pulverized samples of raw and cooked *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica*

Sample	Vitamin C (mg/100g)	Vitamin E (µg/100g)	Selenium (mg/l)	CAT ($x10^{-2}$) IU	SOD($x10^{-4}$) IU
SS*	356.50 ± 6.50[a]	10.62 ± 0.15[a]	1.29 ± 0.00[a]	0.04 ± 0.01[a]	0.37 ± 0.04[c]
CO*	581.25 ± 4.36[b]	43.49 ± 0.32[b]	1.09 ± 0.01[b]	0.54 ± 0.14[b]	2.10 ± 0.12[a]
BC*	89.13 ± 3.28[c]	4.36 ± 0.18[c]	0.65 ± 0.01[c]	0.47 ± 0.15[b]	0.00 ± 0.00
MM*	193.75 ± 2.10[d]	3.85 ± 0.27[c]	1.39 ± 0.01[d]	0.20 ± 0.02[c]	1.11 ± 0.04[b]
SS	73.63 ± 6.33[e]	9.85 ± 0.31[a]	0.99 ± 0.03[e]	0.00 ± 0.00	0.00 ± 0.00
CO	220.88 ± 3.17[f]	38.56 ± 0.09[d]	1.46 ± 0.03[f]	0.14 ± 0.03[c]	0.62 ± 0.12[d]
BC	85.25 ± 5.67[c]	3.54 ± 0.45[c]	0.98 ± 0.04[e]	0.09 ± 0.02[c]	0.00 ± 0.00
MM	170.50 ± 8.29[g]	1.67 ± 0.18[e]	1.33 ± 0.01[g]	0.08 ± 0.02[c]	0.86 ± 0.04[d]

Values are mean ± standard deviations of triplicate determinations. Values with different superscript letters per column are statistically significant ($p<0.05$). SS = *S. stenocarpa*, CO = *C. odorata*, BC = *B. coriacea*, MM = *Monodora myristica.* Superscript* indicates raw samples.

4.1.5 Radical scavenging activities of pulverized samples

4.1.5.1Superoxide radical scavenging activity

Percentage inhibition of superoxide radical generation by all the samples increased in dose-dependent manner. Higher activities were observed in the raw samples except in *M. myristica*. At concentrations of 400 and 800 µg/ml, the superoxide scavenging activity of most of the samples were higher than standard (ascorbic acid). Raw *B. coriacea* and Cooked *M.myristica* recorded the highest activity while cooked *C. odorata* had the lowest activity among all the sampled plants (Fig. 4.1).

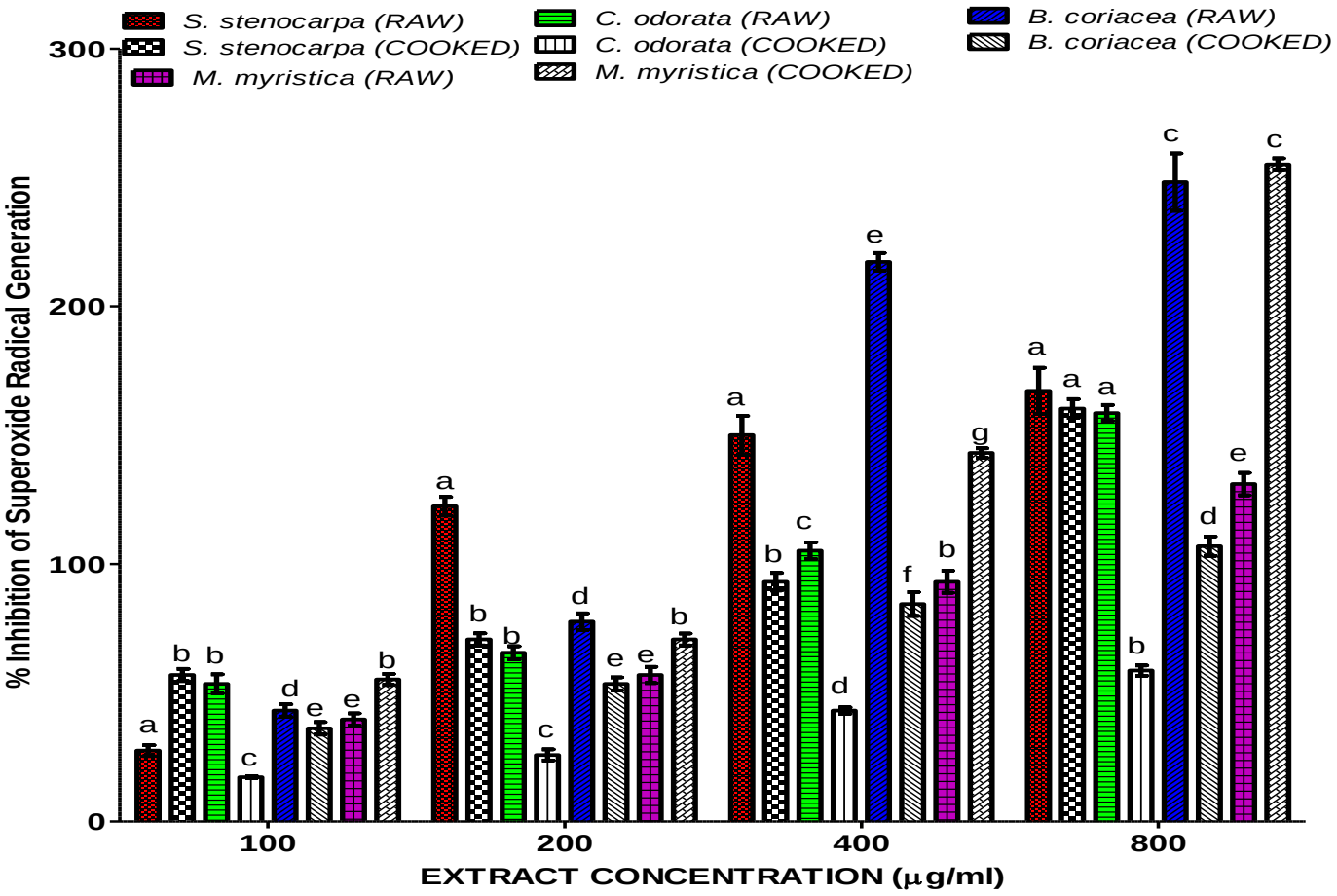


Fig 4.1: Superoxide radical scavenging activities of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica* calculated based on vitamin C control (800µg/ml of absorbance 0.058±0.1)

4.1.5.2 Hydrogen peroxide scavenging activity of the samples.

Raw and cooked samples of *S. stenocarpa*, *B. coriacea* and *C. odorata* samples were capable of reacting with H_2O_2 in concentration- dependent manner but raw *M. myristica* exhibited highest activity at 200 µg/ml, while its cooked sample showed highest activity at 800 µg/ml. The raw samples showed higher activities than the cooked samples**,** except at 400 and 800 µg/ml in cooked samples of *C. odorata* (Fig. 4.2).

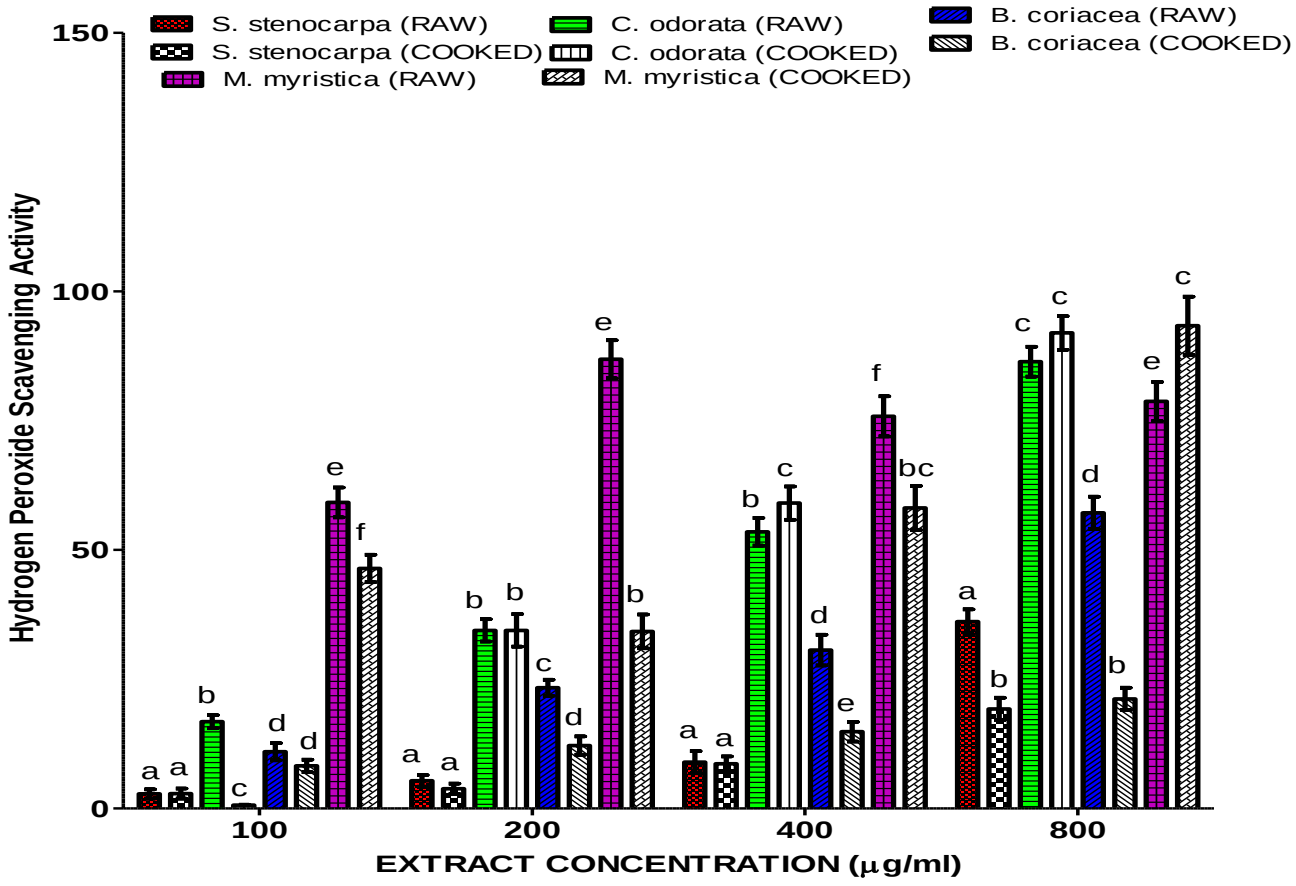


Fig 4.2: Hydrogen peroxide scavenging activities of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica* calculated based on vitamin C control (800µg/ml with absorbance of 1.495±0.03)

4.1.5.3 Hydroxyl radical scavenging activity of the samples.

Hydroxyl radical scavenging investigation revealed concentration-dependent increase in activity of all the samples. At 800 µg/ml, the activities of RSS, RMM and RBC were significantly ($P \leq 0.05$) higher than that of standard (ascorbic acid). Decrease in percentage inhibition activity was observed in the cooked samples except *C. odorata* which had less cooking time (Fig. 4.3).

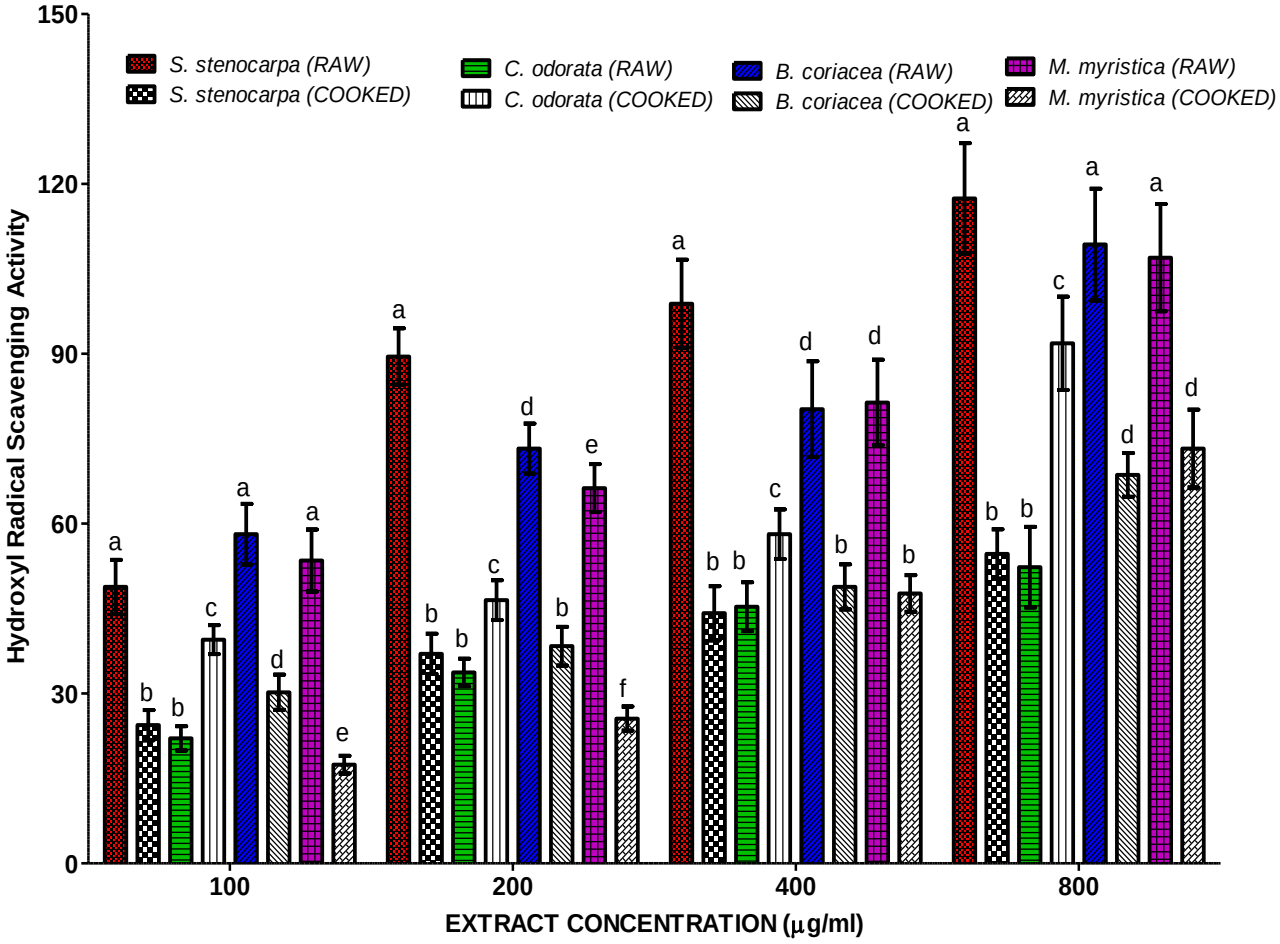


Fig 4.3: Hydroxyl radical scavenging activities of the raw and cooked samples calculated based on vitamin C control (800µg/ml of absorbance 0.086±0.01)

4.1.5.4 Nitric oxide scavenging activity of the samples.

Slight increases in nitric oxide scavenging activities with increase in concentrations were observed in *B.coriacea, C. odorata* and *M. myristica*. Raw and cooked *S. stenocarpa* (SS) exhibited very high significant (P≤0.05) increase in activity at 400 µg/ml and the increase was not significantly altered at 800 µg/ml. Cooked SS showed highest activity among all the samples (Fig. 4.4).

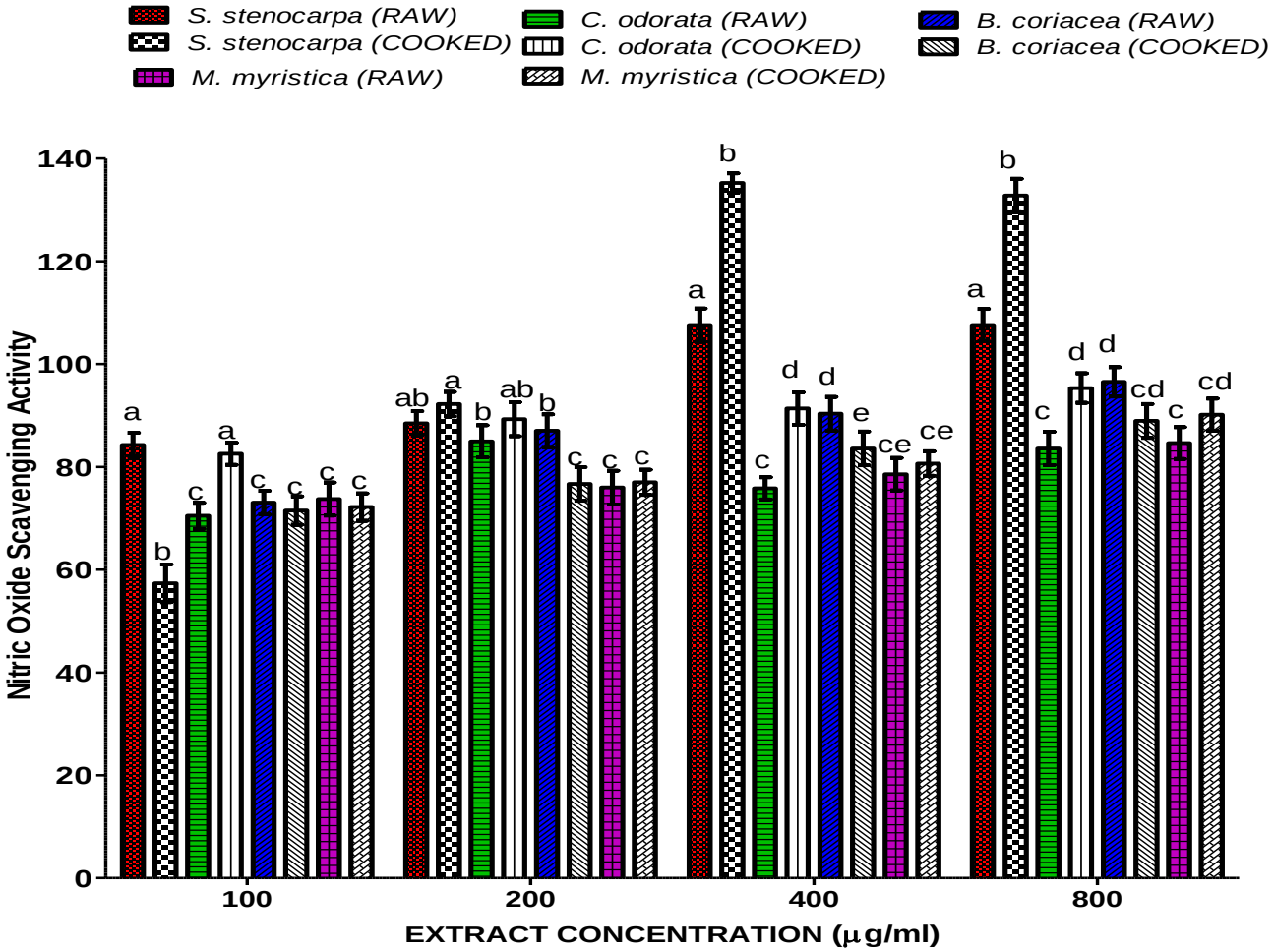


Fig 4.4: Nitric oxide scavenging activities of raw and cooked samples of *S. stenocarpa, C. odorata*, *B. coriacea* and *M. myristica* calculated based on butylated hydroxyltoluene control (800µg/ml of absorbance 0.579±.05)

4.1.5.5 Total reducing ability of the samples.

The raw samples exhibited higher reducing power than cooked samples at lower concentrations (100 - 200 µg/ml) except for MM, while those of cooked samples improved with increase in extract concentration (Fig. 4.5).

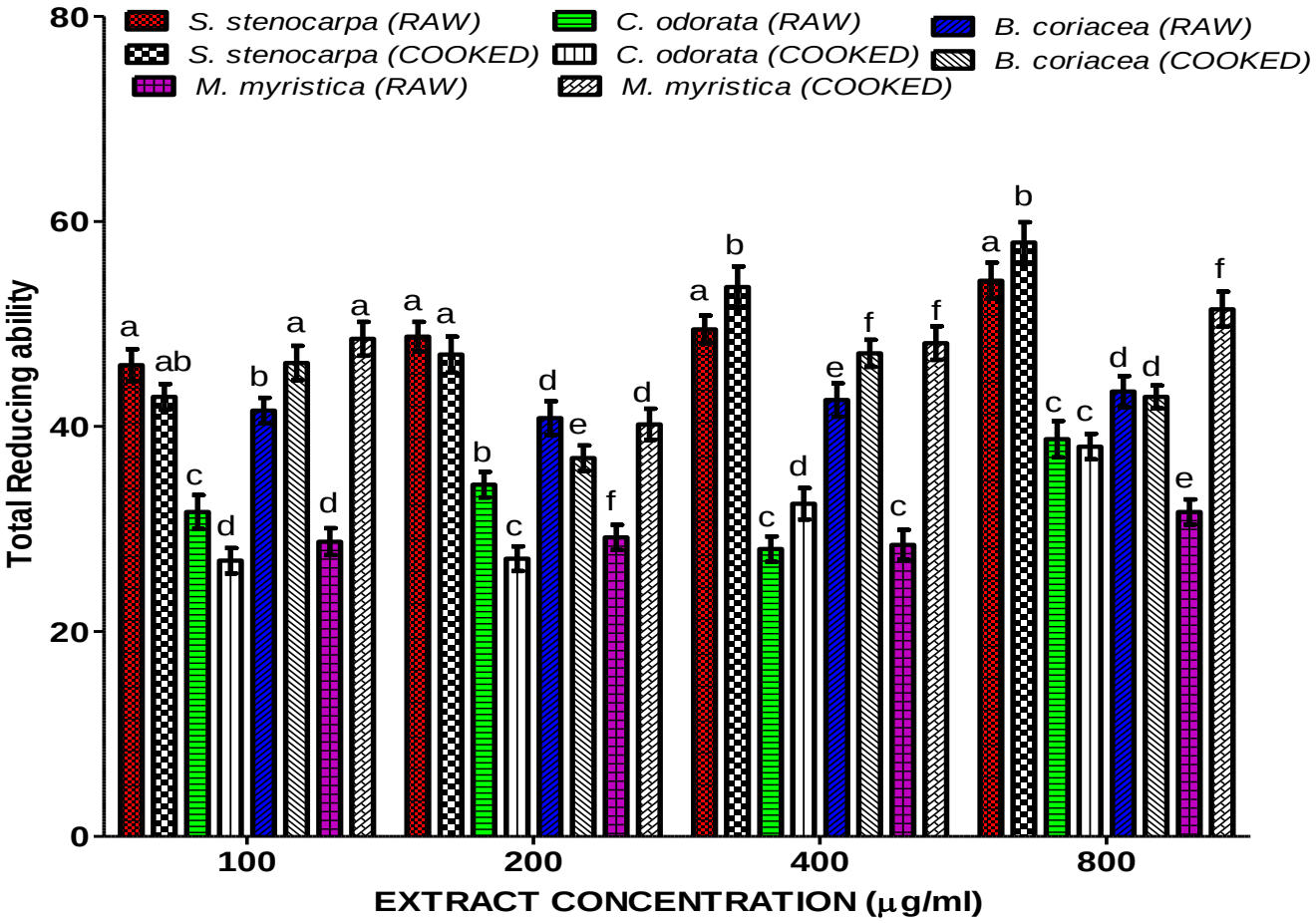


Fig 4.5: Total reducing ability of raw and cooked samples of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica* calculated based on vitamin C control (800µg/ml of absorbance 0.97±0.03)

4.1.6 Body weights of animals, clinical signs and mortality patterns

No remarkable behavioural changes and death were observed in all dose levels of all the extracts, within seven days of intraperitoneal acute toxicity. Hence concentrations below 1000 mg/kg body weight of animals were regarded safe for all the extracts.

The growth rate curve showed a gradual and steady body weight increase in the normal (Grp A) and olive oil (Grp B) control throughout the 8 weeks of laboratory study. The dose-dependent increase in the body weights of animals fed with extracts prepared in normal saline (*S.stenocarpa* and *B.coriacea*) was more than *M. myristica* and *C. odorata* that were prepared in olive oil when compared with their controls. RSS_{500} group had the highest increase in body weight. A decrease was observed in the body weights of the animals a week after intoxication. Thereafter, there were fluctuations in the body weights of the treated groups; the silymarin-treated group (Grp D) and most of the cooked extract-treated groups (CCO_{500}, CSS_{250}, CSS_{500}, CMM_{250} and CMM_{500}) recovered healthwise after the 6^{th} week (See Fig. 4.6).

During the eight weeks of treatment, mortalities of 25% were recorded for the group that received 250 mg/kg of RSS (RSS_{250}) within 48 hours of CCl_4 intoxication (fifth week) and 37.5% for group CCO_{500} within the first week. No

adverse clinical manifestations were observed within four weeks of pre-treatment with extracts but mild sedative effects, yellow skin colouration, weight loss and black stool were observed few days after CCl_4 intoxication. At sacrifice, the liver excised from Group C (the negative control) showed pale brown, spotty openings with high fatty liver. Similarly, the CCl_4-intoxicated groups had varying degree of paleness and fatty liver. The groups that were treated with raw samples presented higher degrees of alteration; of particular interest is the group that received 500 mg/kg of raw MM extract (RMM_{500}). In this group, the liver had moderate fatty deposits but one of the rats with the highest weight loss had multiple growths, which when investigated were masses of fat deposits (Appendix III).

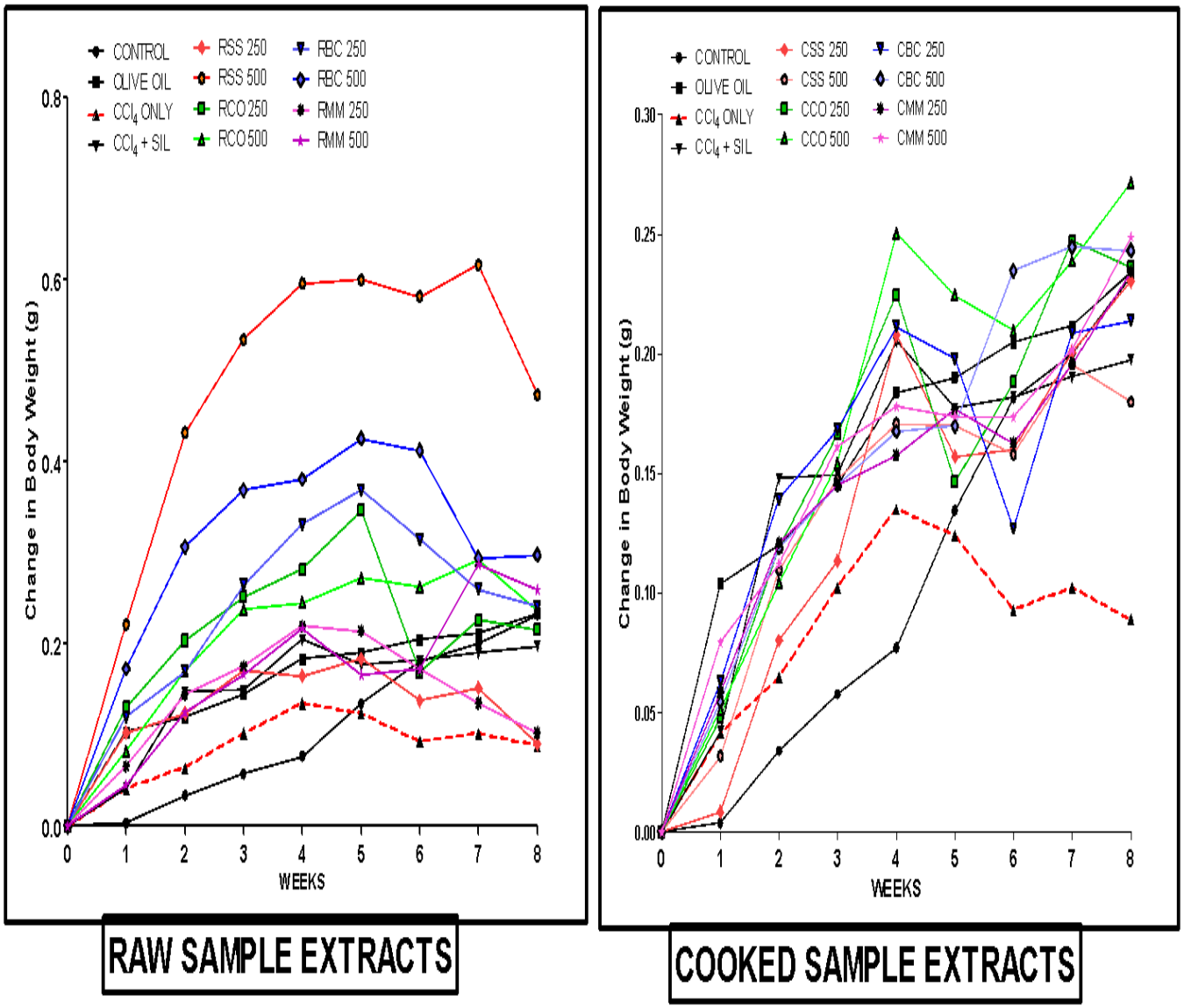


Fig 4.6: Change in body weights of animal groups intoxicated with CCl_4 and treated with 250 and 500 mg methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* CCl_4 - carbon tetrachloride; CCl_4 + SIL - carbon tetrachloride + Silymarin; RSS - Raw *S. stenocarpa;* RCO - Raw *C. odorata;* RBC - Raw *B. coriacea*; RMM - Raw *M. myristica*; CSS - Cooked *S. stenocarpa;* CCO - Cooked *C. odorata;* CBC - Cooked *B. coriacea* and CMM - Cooked *M. myristica*.

4.1.7 Relative liver organ weights of the rats

The plant extracts elicited slight increases in the liver weights of the non-intoxicated animals except for those fed with *M. myristica* and 500 mg/kg of raw and cooked *C. odorata*. CCl_4-intoxication significantly ($P<0.05$) increased the liver weights in comparison with the controls. Treatment of the intoxicated animals with raw plant extracts caused significant ($P<0.05$) decrease in liver weights with increase in extract dosage while the reverse was the case for cooked samples. (See Fig. 4.7)

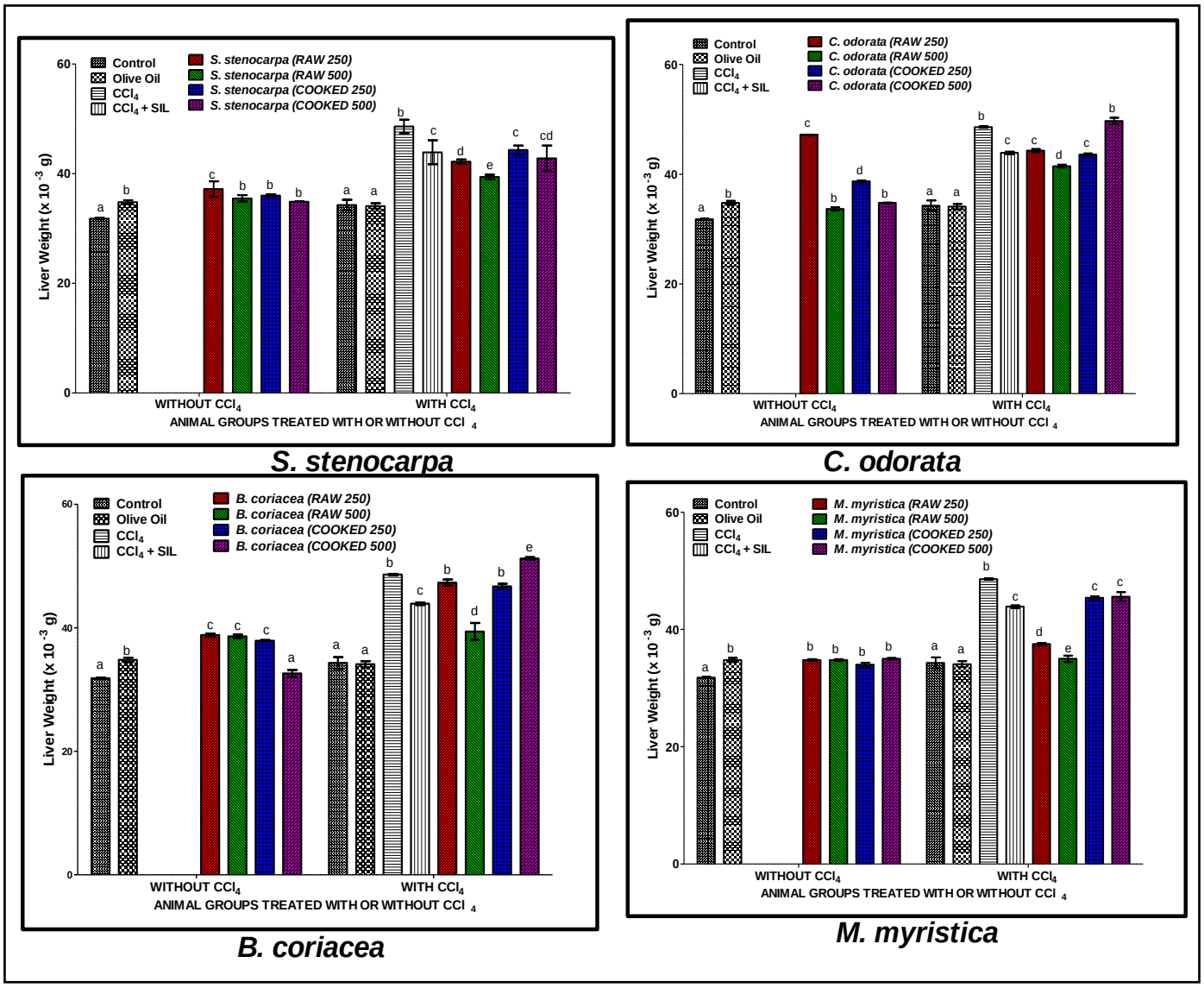


Fig 4.7: Liver weight of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica*. Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.8 Effects of extracts on haematological indices

Generally, the various plants extracts did not have significant ($P>0.05$) effect on the haemoglobin (Hb) concentrations of animals that were not exposed to CCl_4 in comparison with the normal and olive oil controls; except the group that received 250 mg/kg cooked *B. coriacea*. Silymarin, raw and cooked extracts of the plants at both concentrations significantly ($P<0.05$) increased the Hb concentrations of the animals in comparison with the CCl_4-treated groups. Treatment of the CCl_4-intoxicated groups with raw samples of the plants at both concentrations improved the Hb concentrations to values significantly ($P>0.05$) similar to the normal saline control, unlike the cooked samples (Fig 4.8).

Dose-dependent decreases were observed in the red blood cell (RBC) count of animals treated with raw and cooked *B. coricea* and *C. odorata* while the reverse was the case with *M. myristica* and *S. stenocarpa*. CCl_4 intoxication significantly ($P<0.05$) reduced the RBCs of all the induced liver fibrotic animals. Treatment with raw and cooked extracts of the plants increased the RBCs of the animals in comparison with the CCl_4 group except for the groups treated with 500 mg/kg RBC, 250 mg/kg CBC, 250 mg/kg RMM and 500 mg/kg CMM (Fig 4.9).

There was a dose-dependent increase in the white blood cell (WBC) counts of animals treated with raw and cooked extracts of the raw and Intoxication with CCl_4 significantly ($P<0.05$) increased the WBC counts in comparison with the normal saline and olive oil controls. Treatment with silymarin and the various extracts except high concentrations of RSS and RBC as well as 250 mg/kg CCO significantly ($P<0.05$) reduced the WBCs of the intoxicated animals (Fig 4.10).

There were concentration-dependent increases in the platelet counts of animals treated with raw and cooked plants extracts except in cooked samples of *B. coriacea* and *S. stenocarpa* that showed dose-dependent decrease in platelet counts. CCl_4-intoxication significantly ($P<0.05$) reduced the platelet counts in comparison with the controls. Treatment of CCl_4-intoxicated animals with samples of the raw plant extracts as well as the 500 mg of the cooked samples significantly ($P<0.05$) improved the platelet counts (Fig 4.11).

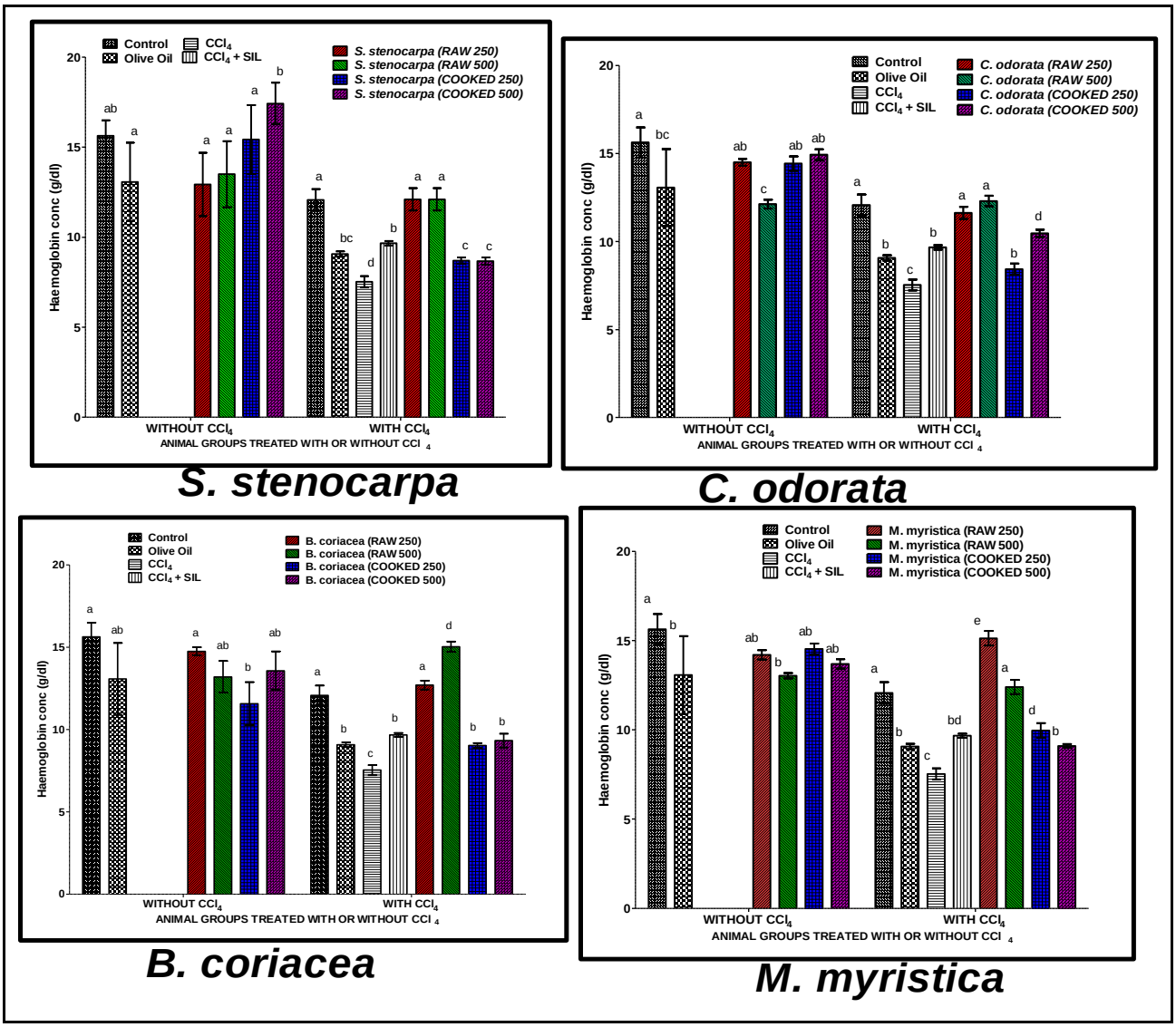


Fig 4.8: Haemoglobin concentrations of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

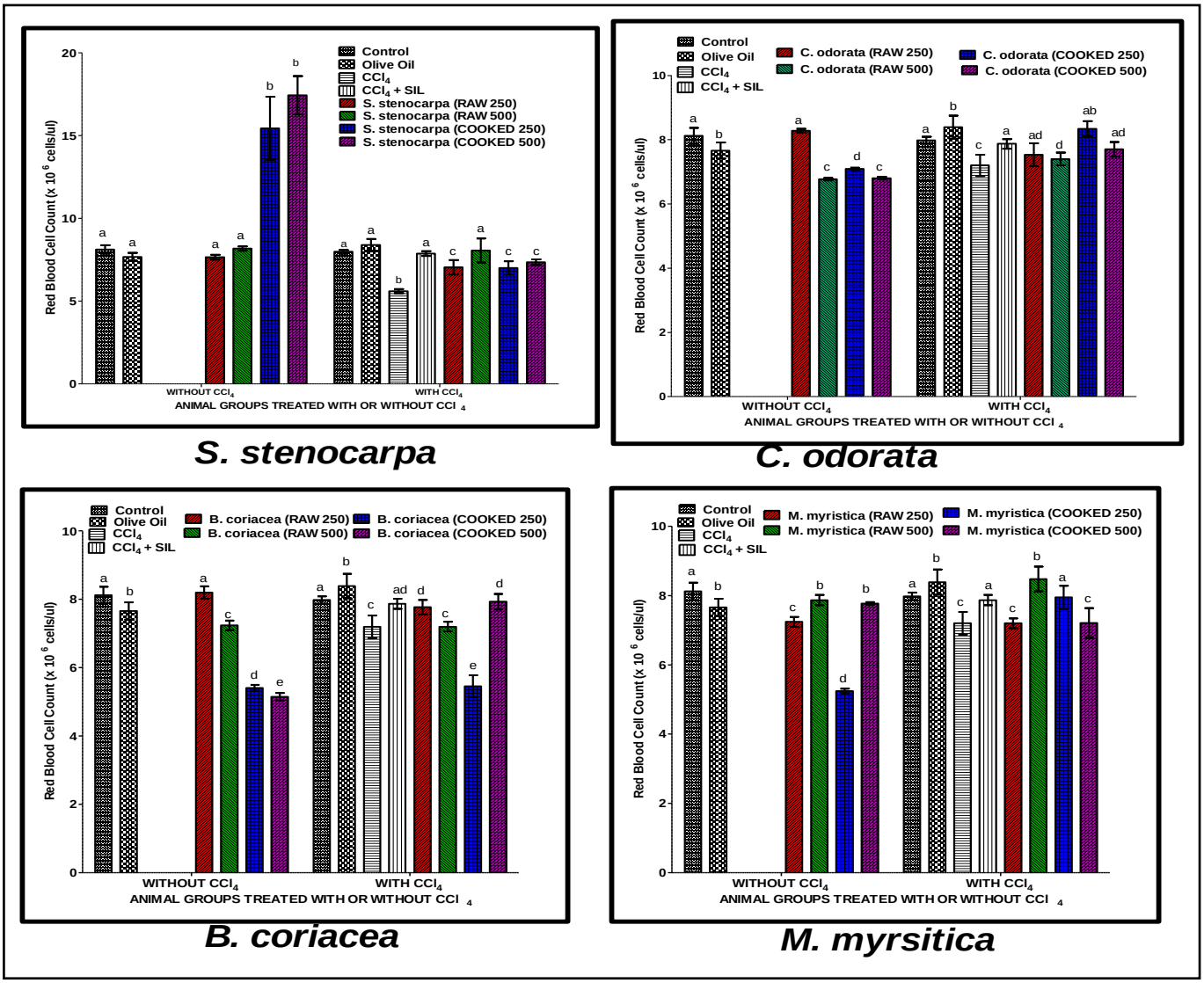


Fig 4.9: Red blood cell count of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

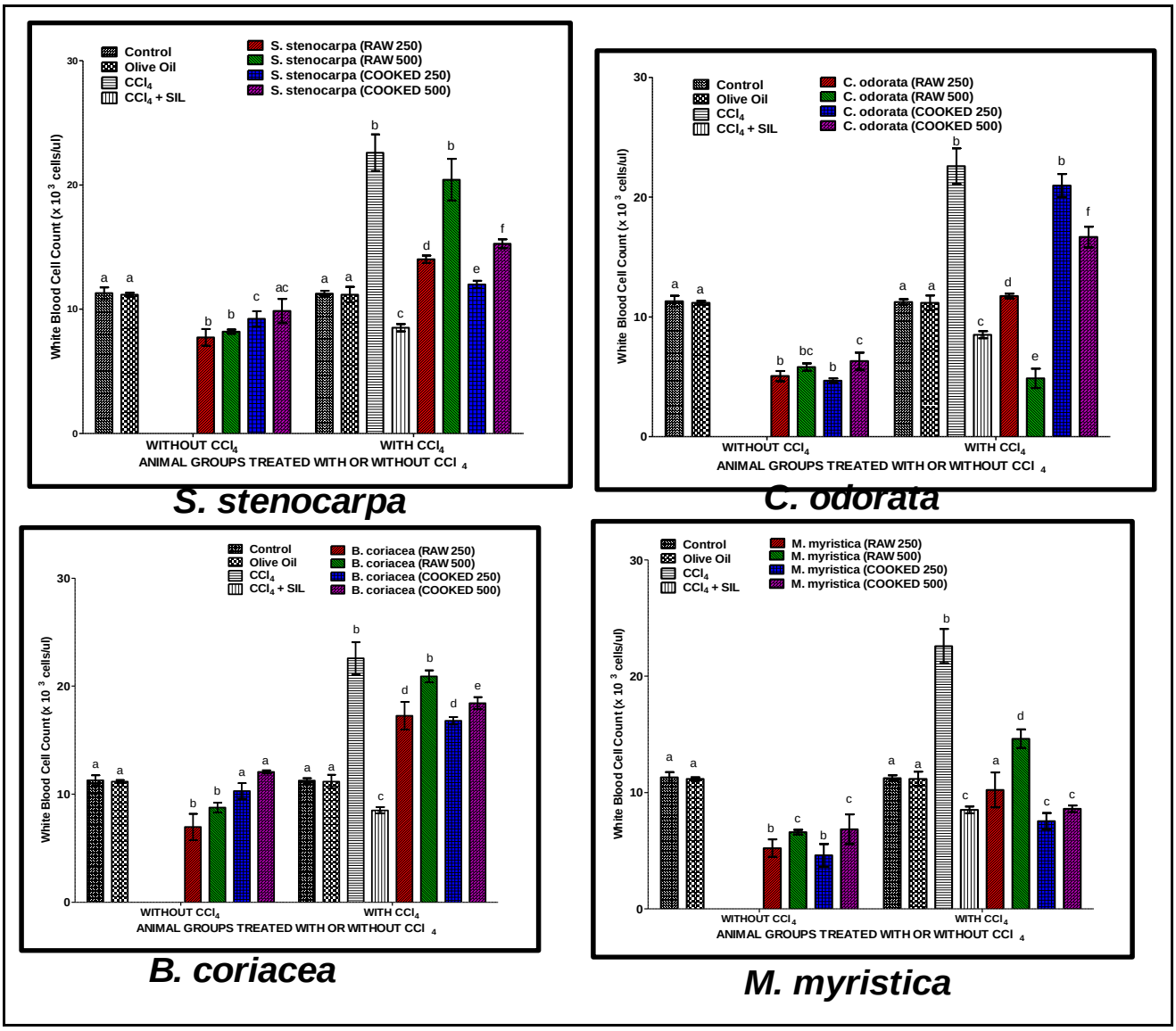


Fig 4.10: White blood cell count of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata*, *B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

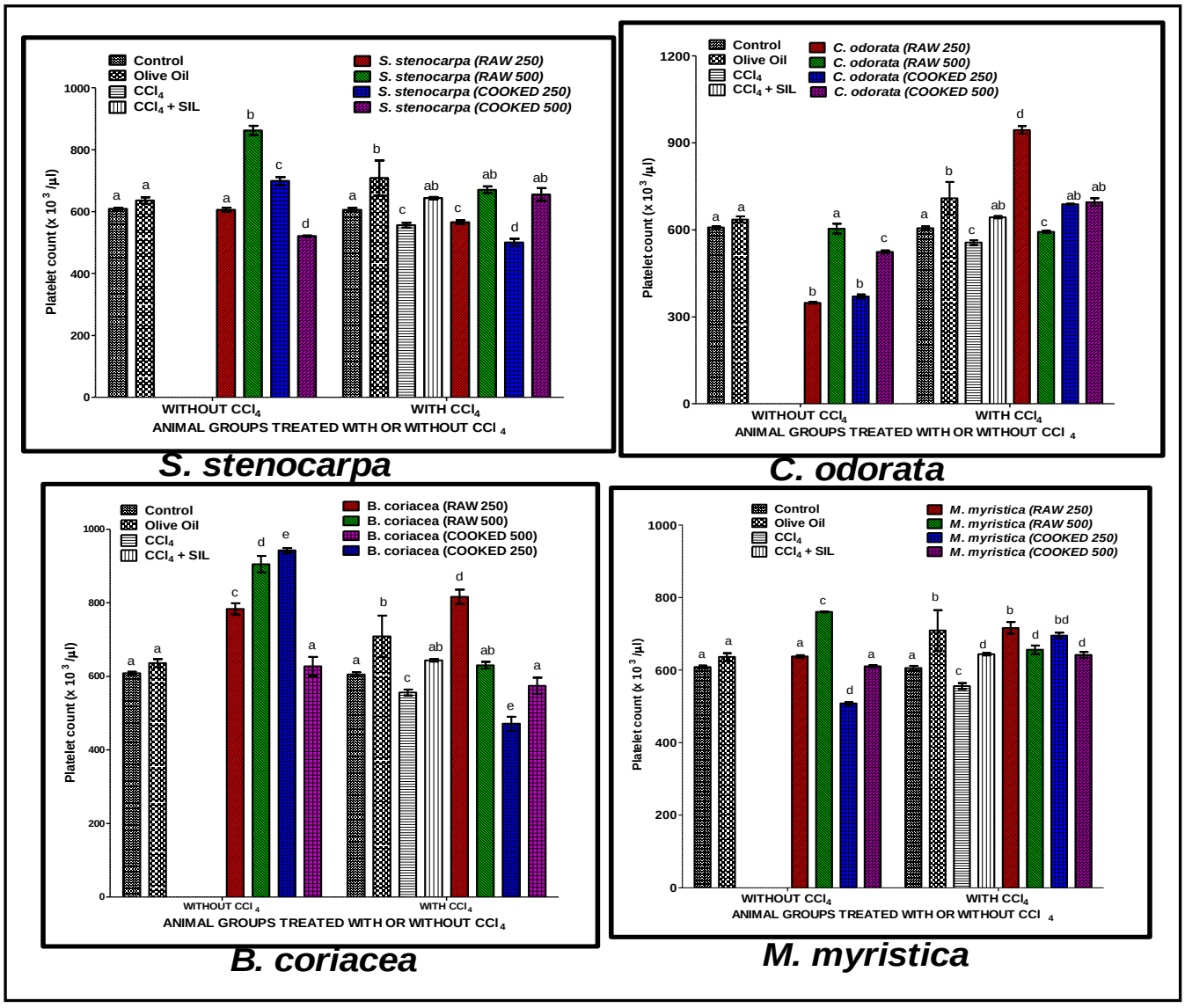


Fig 4.11: Platelet count of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.9 Effect of Extracts on Hepatic Indices

4.1.9.1 Aspartate aminotransferase activity

There was dose-dependent significant ($P<0.05$) increase in the AST activity of the animal groups treated with raw plant extracts in comparison with NS control except those treated with raw *Stenorcapa*. CCl_4 intoxication increased significantly ($P<0.05$) the AST activities of the animals in comparison with NS and olive oil controls. Treatment of the CCl_4-intoxicated animals with plant extracts reduced significantly the AST activity but not to the extent of the values of the controls (Fig. 4.12).

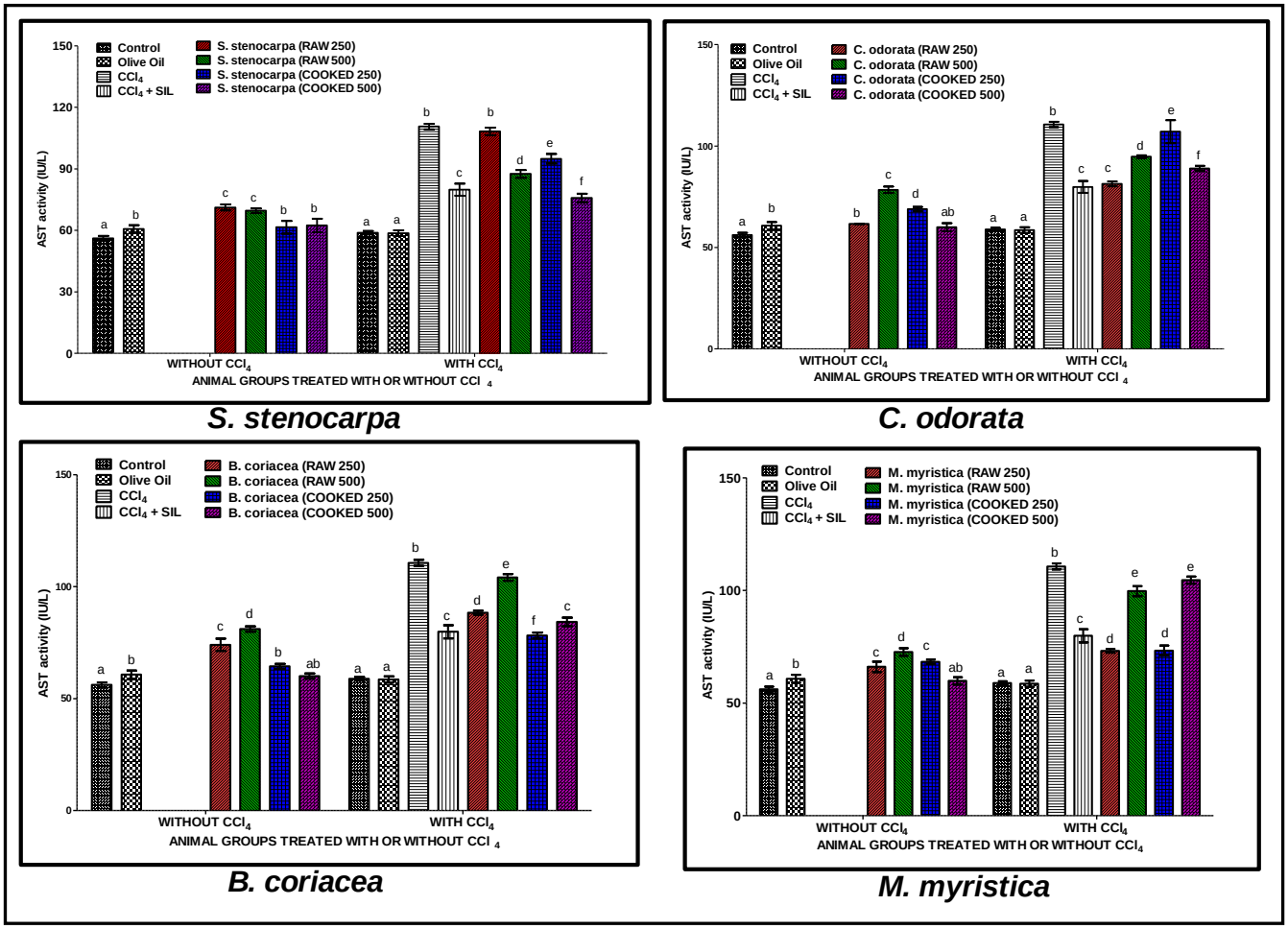


Fig 4.12: Aspartate aminotransferase (AST) activity of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.9.2 Alanine aminotransferase activity

Generally, treatment of the animals with plant extracts slightly increased ALT activities of the animals in a dose –dependent manner compared to the controls. As observed in AST activity, CCl_4-intoxication significantly increased the ALT activities of the animals which were significantly ($P<0.05$) reduced with plants extract treatment (Fig. 4.13).

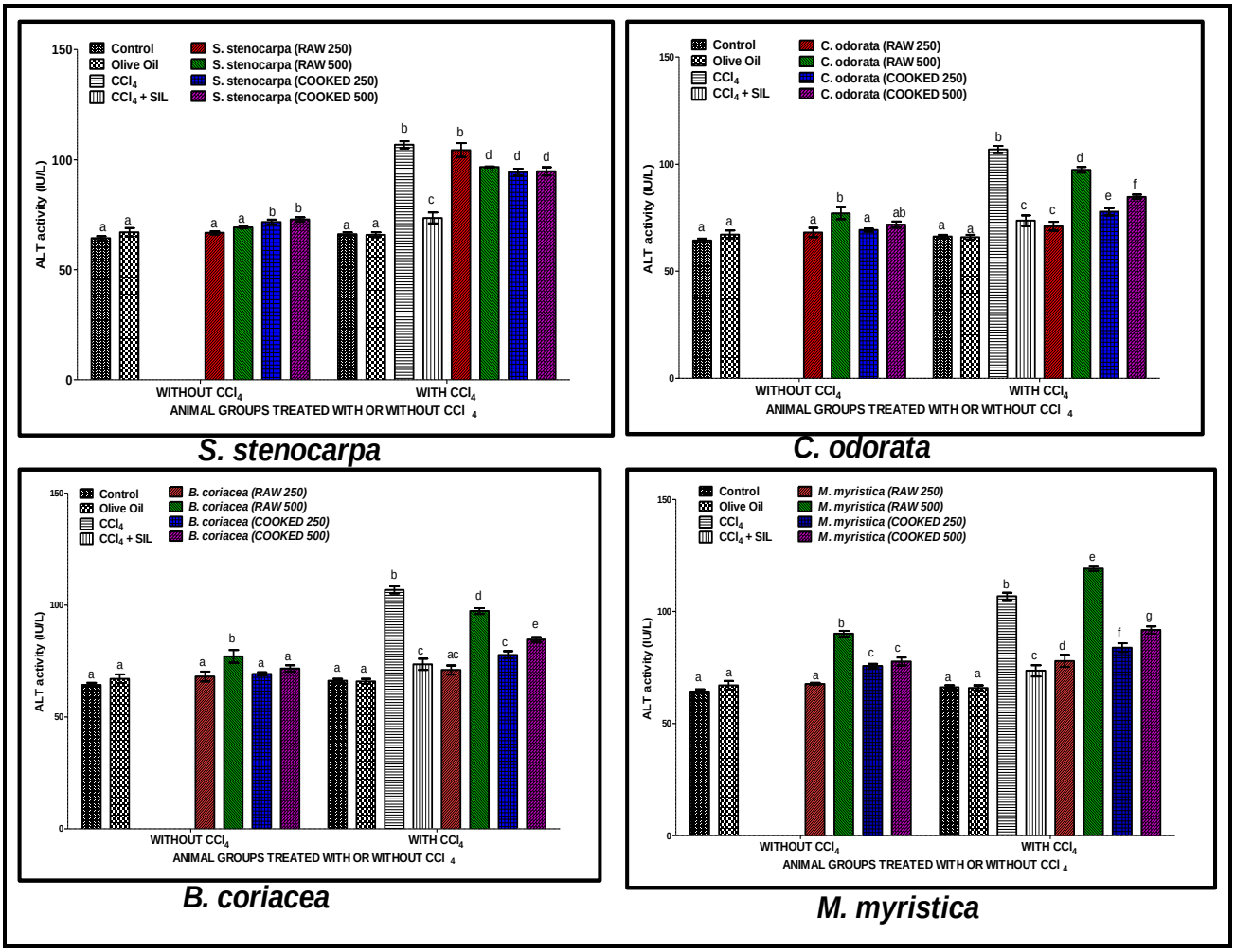


Fig 4.13: Alanine Aminotransferase (ALT) Activity of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.9.3 Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was significantly ($p<0.05$) increased in the animal groups treated with raw and cooked plant extracts in comparison with the NS control. On the other hand, intoxication with CCl_4 and treatment with 500 mg/kg b.wt of all the raw plant extracts significantly increased ($p<0.05$) the ALP activity. The cooked plants significantly reduced ($p<0.05$) the effect of the CCl_4-intoxication on ALP activity (Fig. 4.14).

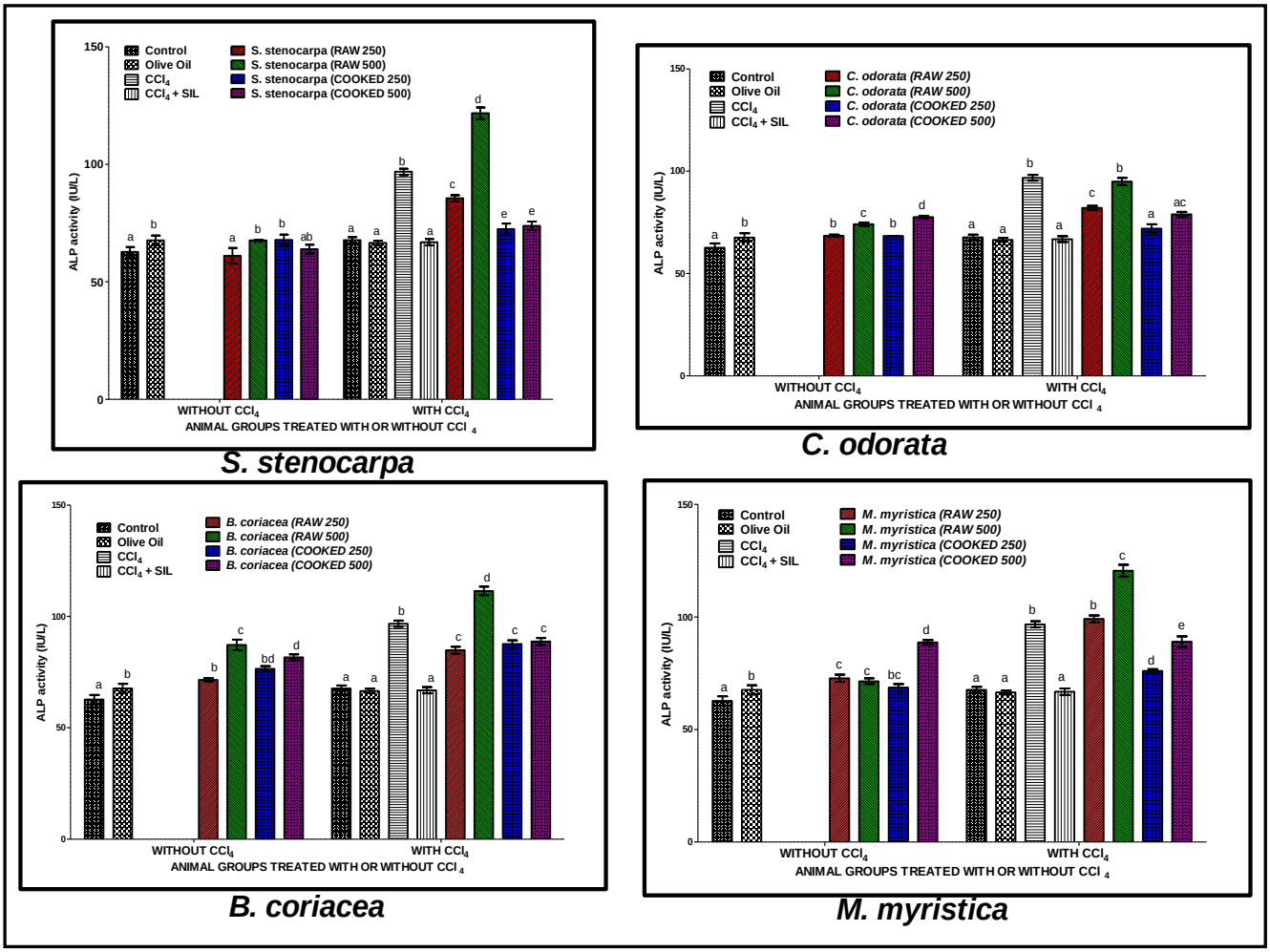


Fig 4.14: Alkaline Phospatase (ALP) Activity of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.9.4 Total protein, albumin and bilirubin concentrations

There was a significant increase ($P<0.05$) in the total protein (TP) concentration and dose-dependent increase in albumin concentration of animals treated with the various plants extracts. No significant ($P>0.05$) differences were observed in the bilirubin concentrations of the plants extract groups in comparison with the controls. CCl_4-intoxication significantly ($P<0.05$) reduced the TP and albumin concentrations and as well significantly ($P<0.05$) increased the bilirubin concentration in comparison with the controls. Treatment with silymarin, raw and cooked extracts of the plants significantly ($P<0.05$) reversed the effects of CCl_4-intoxication on TP, albumin and bilirubin (Table 4.5).

Table 4.5: Effects of extracts on total protein, albumin and bilirubin concentrations of CCl_4 non- intoxicated and intoxicated animals

	Total protein	$^{\Lambda}$Total protein	Albumin	$^{\Lambda}$Albumin	Bilirubin	$^{\Lambda}$Bilirubin
Normal control	7.55 ± 0.09	7.63 ± 0.09	3.74 ± 0.03	3.95 ± 0.08	0.67 ± 0.09	0.87 ± 0.08
Olive Oil	6.91 ± 0.16^{a}	7.27 ± 0.07^{a}	3.46 ± 0.07^{a}	3.61 ± 0.10^{a}	0.67 ± 0.05	0.85 ± 0.07
CCl4 only		5.74 ± 0.25^{ab}		2.30 ± 0.12^{ab}		1.69 ± 0.12^{ab}
CCl4 + Silymarin		6.54 ± 0.27^{abc}		3.21 ± 0.08^{abc}		1.04 ± 0.05^{abc}
RSS 250	8.10 ± 0.04^{ab}	7.65 ± 0.12^{bcd}	4.21 ± 0.01^{ab}	3.68 ± 0.06^{acd}	0.74 ± 0.06	1.47 ± 0.09^{abd}
RSS 500	8.25 ± 0.30^{ab}	7.47 ± 0.38^{cd}	3.61 ± 0.04^{a}	4.23 ± 0.05^{abcd}	0.66 ± 0.04	1.16 ± 0.05^{abcd}
CSS 250	7.62 ± 0.05^{b}	7.26 ± 0.12^{acd}	3.90 ± 0.07^{b}	3.31 ± 0.16^{abc}	0.72 ± 0.06	1.06 ± 0.06^{abc}
CSS 500	7.80 ± 0.09^{b}	7.32 ± 0.12^{acd}	4.57 ± 0.02^{ab}	3.66 ± 0.06^{acd}	0.68 ± 0.09	0.94 ± 0.06^{c}
RCO 250	7.00 ± 0.21^{a}	6.46 ± 0.33^{abc}	3.42 ± 0.06^{a}	3.58 ± 0.04^{acd}	0.80 ± 0.08	1.57 ± 0.03^{abd}
RCO 500	7.95 ± 0.24^{ab}	6.95 ± 0.14^{abc}	3.68 ± 0.04^{b}	4.25 ± 0.08^{abcd}	0.64 ± 0.08	1.17 ± 0.03^{abcd}
CCO 250	6.61 ± 0.18^{ab}	6.56 ± 0.27^{abc}	3.72 ± 0.06^{b}	3.22 ± 0.09^{abc}	0.72 ± 0.07	1.20 ± 0.03^{abcd}
CCO 500	7.63 ± 0.09^{b}	6.52 ± 0.23^{abc}	4.04 ± 0.11^{ab}	3.43 ± 0.07^{abcd}	0.67 ± 0.10	0.97 ± 0.07^{c}
RBC 250	8.43 ± 0.10^{ab}	6.48 ± 0.27^{abc}	4.04 ± 0.11^{ab}	3.84 ± 0.11^{cd}	0.64 ± 0.02	1.11 ± 0.06^{abc}
RBC 500	7.36 ± 0.25^{b}	5.91 ± 0.14^{abd}	4.37 ± 0.05^{ab}	3.40 ± 0.10^{abcd}	0.60 ± 0.03	1.33 ± 0.06^{abcd}
CBC 250	7.54 ± 0.08^{b}	7.20 ± 0.19^{acd}	3.75 ± 0.07^{b}	3.42 ± 0.09^{abcd}	0.68 ± 0.04	0.99 ± 0.03^{c}
CBC 500	7.50 ± 0.19^{b}	6.99 ± 0.26^{acd}	3.81 ± 0.05^{b}	3.53 ± 0.06^{acd}	0.63 ± 0.04	1.14 ± 0.04^{abc}
RMM 250	7.50 ± 0.18^{b}	7.89 ± 0.11^{bcd}	3.75 ± 0.07^{b}	3.44 ± 0.12^{abcd}	0.64 ± 0.07	1.18 ± 0.03^{abcd}
RMM 500	7.02 ± 0.14^{a}	5.73 ± 0.45^{abd}	3.96 ± 0.17^{ab}	3.47 ± 0.11^{abcd}	0.61 ± 0.05	1.49 ± 0.08^{abd}
CMM 250	7.47 ± 0.22^{b}	6.83 ± 0.16^{abc}	3.86 ± 0.14^{b}	3.24 ± 0.21^{abc}	0.67 ± 0.05	1.01 ± 0.04^{c}
CMM 500	7.49 ± 0.23^{b}	6.95 ± 0.10^{abc}	3.98 ± 0.16^{ab}	3.62 ± 0.03^{acd}	0.77 ± 0.05	1.15 ± 0.02^{abc}

KEYS: Values are mean ± standard deviations. [a] Values per column are statistically significant ($P<0.05$) from the Normal control value; [b] values different from Olive Oil group; [c] values different from CCl_4 only; [d] values different from CCl_4 + Silymarin group. The symbol $^{\Lambda}$ represent intoxicated groups.

4.1.10 Effects of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica* on renal functions of experimental animals

Pre-treatment with plant extracts and the extract vehicle (olive oil) significantly increased ($P<0.05$) the urea concentrations of the animals in comparison with the normal saline group. The urea concentration of the CCl_4-intoxicated animal group was significantly raised ($P<0.05$) in comparison with the control, silymarin, raw and cooked plant extracts. Treatment with the plant extracts did not significantly ($P>0.05$) affect creatinine concentrations. However, intoxication with CCl_4 significantly increased ($P<0.05$) serum creatinine concentrations which were significantly ($P<0.05$) reduced by treatments with plants extracts (Table 4.6).

CCl_4-intoxication significantly increased ($P<0.05$) the potassium, chloride, sodium and bicarbonate concentrations in serum (Table 4.7). Treatment with plants extracts in the presence of CCl_4-intoxication caused significant dose-dependent reductions in K^+, Na^+, Cl^- and HCO_3^- concentrations of the animals in comparison with the CCl_4-intoxicated groups.

Table 4.6: Effects of methanol extracts of raw and cooked samples of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica* on serum urea and creatinine concentrations of experimental animals

	Urea	^Λ^Urea	Creatinine	^Λ^Creatinine
Normal control	22.67 ± 1.00	23.71 ± 0.55	0.48 ± 0.02	0.51 ± 0.01
Olive Oil	27.21 ± 0.11[a]	27.25 ± 0.34[a]	0.50 ± 0.01	0.49 ± 0.02
CCl4 only	-	37.77 ± 0.19[ab]	-	0.81 ± 0.06[ab]
CCl4 + Silymarin	-	27.56 ± 0.10[ac]	-	0.54 ± 0.06[c]
RSS 250	27.37 ± 0.23[a]	31.81 ± 0.20[abcd]	0.49 ± 0.01	0.53 ± 0.01[c]
RSS 500	33.67 ± 0.17[ab]	33.64 ± 0.19[abcd]	0.50 ± 0.02	0.65 ± 0.04[abc]
CSS 250	26.23 ± 1.07[a]	30.75 ± 0.70[ac]	0.46 ± 0.02	0.55 ± 0.09[c]
CSS 500	28.75 ± 0.01[a]	37.64 ± 0.17[abd]	0.62 ± 0.13[ab]	0.59 ± 0.11[c]
RCO 250	30.54 ± 0.10[ab]	30.85 ± 0.48[ac]	0.50 ± 0.03	0.54 ± 0.06[c]
RCO 500	32.03 ± 0.37[ab]	34.60 ± 0.40[abcd]	0.49 ± 0.01	0.53 ± 0.04[c]
CCO 250	32.25 ± 0.37[ab]	33.89 ± 0.13[abcd]	0.45 ± 0.01[b]	0.63 ± 0.07[c]
CCO 500	30.80 ± 0.18[ab]	35.98 ± 0.18[abd]	0.56 ± 0.13[ab]	0.53 ± 0.13[c]
RBC 250	23.85 ± 0.40[b]	27.51 ± 0.15[ac]	0.50 ± 0.01	0.53 ± 0.03[c]
RBC 500	27.16 ± 0.23[a]	30.13 ± 0.35[ac]	0.51 ± 0.02	0.54 ± 0.05[c]
CBC 250	26.29 ± 0.33[a]	32.61 ± 0.23[abcd]	0.49 ± 0.01	0.54 ± 0.03[c]
CBC 500	31.18 ± 0.36[ab]	28.96 ± 0.44[ac]	0.53 ± 0.08	0.52 ± 0.10[c]
RMM 250	30.40 ± 0.20[ab]	32.75 ± 0.13[abcd]	0.49 ± 0.01	0.54 ± 0.04[c]
RMM 500	34.53 ± 0.31[ab]	35.05 ± 0.32[abcd]	0.58 ± 0.02[ab]	0.65 ± 0.03[abc]
CMM 250	27.90 ± 0.44[a]	30.21 ± 0.44[ac]	0.53 ± 0.09	0.51 ± 0.06[c]
CMM 500	32.71 ± 0.15[ab]	33.18 ± 0.18[abcd]	0.66 ± 0.13[ab]	0.61 ± 0.10[c]

KEYS: Values are mean ± standard deviations. [a] Values per column are statistically significant ($p<0.05$) from the Normal control value; [b] values different from Olive Oil group; [c] values different from CCl_4 only; [d] values different from CCl_4 + Silymarin group. The symbol [Λ] represents intoxicated groups.

Table 4.7: Effect of raw and cooked extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica* on serum electrolyte concentrations of CCl_4 non- intoxicated and intoxicated rats

	Na^+	$^{\Lambda}Na^+$	K^+	$^{\Lambda}K^+$	Cl^-	$^{\Lambda}Cl^-$	HCO_3^{2-}	$^{\Lambda}HCO_3^{2-}$
Normal control	147.60 ± 2.36	156.89 ± 2.26	4.57 ± 0.12	4.24 ± 0.10	95.69 ± 1.14	96.45 ± 1.06	28.76 ± 1.07	26.05 ± 0.32
Olive Oil	151.74 ± 3.83	152.43 ± 3.60	4.09 ± 0.01	3.48 ± 0.40	93.44 ± 0.28	91.70 ± 1.26	25.06 ± 0.98	27.01 ± 0.41
CCl4 only	-	195.68 ± 3.25^{ab}	-	7.28 ± 0.25^{ab}	-	140.00 ± 0.99^{ab}	-	42.39 ± 1.45^{ab}
CCl4 + Silymarin	-	158.24 ± 4.33^{c}	-	5.39 ± 0.21^{bc}	-	118.87 ± 1.73^{abc}	-	29.71 ± 0.65^{c}
RSS 250	155.23 ± 6.11	174.19 ± 5.44^{abcd}	4.50 ± 0.10	4.55 ± 0.02^{bc}	87.43 ± 0.77^{ab}	107.15 ± 0.90^{bc}	27.68 ± 1.49	29.89 ± 1.32^{c}
RSS 500	154.48 ± 5.50	190.68 ± 3.10^{abd}	5.00 ± 0.12^{b}	5.50 ± 0.36^{abc}	99.82 ± 0.33^{ab}	135.73 ± 0.83^{abd}	29.50 ± 0.68	34.39 ± 2.01^{abc}
CSS 250	ND	171.08 ± 3.53^{bc}	4.22 ± 0.20	5.19 ± 0.47^{abc}	113.14 ± 1.03^{ab}	123.93 ± 0.70^{abc}	28.16 ± 0.36	30.78 ± 0.56^{c}
CSS 500	ND	188.11 ± 3.87^{abd}	4.72 ± 0.31^{b}	6.05 ± 0.24^{ab}	124.85 ± 0.91^{ab}	143.19 ± 0.45^{abd}	26.82 ± 0.86	35.85 ± 0.32^{abcd}
RCO 250	170.23 ± 1.12^{ab}	168.78 ± 8.72^{c}	4.56 ± 0.20	4.61 ± 0.22^{bc}	101.74 ± 1.94^{ab}	110.93 ± 1.18^{bc}	26.39 ± 0.98	31.90 ± 1.32^{c}
RCO 500	157.80 ± 1.46^{a}	193.51 ± 2.31^{abd}	4.58 ± 0.11	4.77 ± 0.17^{bc}	94.81 ± 0.43	98.49 ± 1.74^{cd}	28.81 ± 0.35	30.76 ± 0.88^{c}
CCO 250	ND	158.65 ± 9.03^{c}	5.42 ± 0.25^{ab}	5.25 ± 0.25^{abc}	101.52 ± 0.52^{ab}	129.79 ± 0.67^{abcd}	27.50 ± 0.65	31.20 ± 0.29^{c}
CCO 500	ND	173.65 ± 1.83^{abcd}	5.31 ± 0.08^{ab}	5.37 ± 0.39^{abc}	106.01 ± 0.59^{ab}	124.18 ± 1.16^{abc}	25.07 ± 0.74	25.20 ± 0.42^{cd}
RBC 250	145.94 ± 3.01	158.51 ± 4.27^{c}	4.75 ± 0.33^{b}	4.12 ± 0.12^{cd}	90.67 ± 1.31	110.60 ± 2.18^{bc}	27.43 ± 1.73	30.08 ± 1.16^{c}
RBC 500	144.20 ± 4.38	175.41 ± 5.13^{abcd}	4.90 ± 0.22^{b}	4.25 ± 0.11^{cd}	101.10 ± 2.62^{ab}	110.55 ± 1.67^{bc}	31.14 ± 1.46	28.80 ± 0.33^{c}
CBC 250	ND	182.57 ± 8.56^{abcd}	4.30 ± 0.51	5.63 ± 0.22^{abc}	95.17 ± 0.75	130.25 ± 5.42^{abcd}	28.52 ± 0.55	33.82 ± 0.89^{abc}
CBC 500	ND	166.62 ± 7.51^{c}	4.40 ± 0.27	5.69 ± 0.33^{abc}	110.89 ± 1.35^{ab}	110.74 ± 1.32^{bc}	30.20 ± 0.74	30.98 ± 0.76^{c}
RMM 250	152.90 ± 2.23	151.35 ± 5.29^{c}	5.45 ± 0.32^{ab}	4.26 ± 0.13^{bc}	97.38 ± 1.51	116.34 ± 1.37^{bc}	30.50 ± 1.26	31.08 ± 0.35^{c}
RMM 500	173.72 ± 2.83^{ab}	183.92 ± 4.14^{abd}	6.83 ± 0.23^{ab}	5.35 ± 0.24^{abc}	91.51 ± 1.54	129.09 ± 1.80^{abcd}	32.25 ± 0.20^{b}	36.66 ± 0.73^{abcd}
CMM 250	ND	161.76 ± 3.06^{c}	4.50 ± 0.41	5.35 ± 0.22^{abc}	92.76 ± 1.18	114.10 ± 0.93^{bc}	27.42 ± 0.28	29.80 ± 0.70^{c}
CMM 500	ND	168.38 ± 3.77^{abc}	5.51 ± 0.38^{ab}	6.36 ± 0.24^{abd}	127.52 ± 1.09^{ab}	151.07 ± 0.74^{abcd}	37.07 ± 0.64^{ab}	33.86 ± 0.27^{abc}

KEYS: Values are mean ± standard deviations. [a] Values per column are statistically significant ($p<0.05$) from the Normal control value; [b] values different from Olive Oil group; [c] values different from CCl_4 only; [d] values different from CCl_4 + Silymarin group. The symbol $^{\Lambda}$ represents intoxicated groups.

4.1.11 Effect of Extracts of on Lipid Profile

There was a dose-dependent increase in the total cholesterol (TC) concentration of the animals treated with *S. stenocarpa* (SS), *B. coriacea* (BC) and *M. myristica* (MM) while the reverse was the case for those treated with *C. odorata* (CO). CCl_4-intoxication increased significantly ($P<0.05$) the TC concentration in comparison with the controls. Treatment with the plants extracts especially SS and MM reduced TC concentration significantly ($P<0.05$) in a dose-dependent manner more than silymarin (Fig. 4.15).

Triacylglyerol (TG) concentrations of the plant-extract fed groups varied in a dose-dependent manner with the controls. CCl_4-intoxication significantly ($P<0.05$) increased the TG concentration in comparison with all the other groups. Treatment with silymarin and the plant extracts reduced the TG concentration in a dose-dependent manner in comparison with CCl_4 intoxicated group (Fig. 4.16).

The high density lipoprotein (HDL) concentration of the animals significantly ($P<0.05$) increased in a dose-dependent manner in the plant-extract fed groups except for CO where there were no significant ($P>0.05$) difference in the HDL concentrations of animals treated with the cooked extracts. Intoxication with CCl_4 and treatment with silymarin significantly ($P<0.05$) reduced the HDL concentrations in comparison with the control groups and the plant-extract treated groups with the exception of those exposed to extract doses of cooked CO (Fig. 4.16).

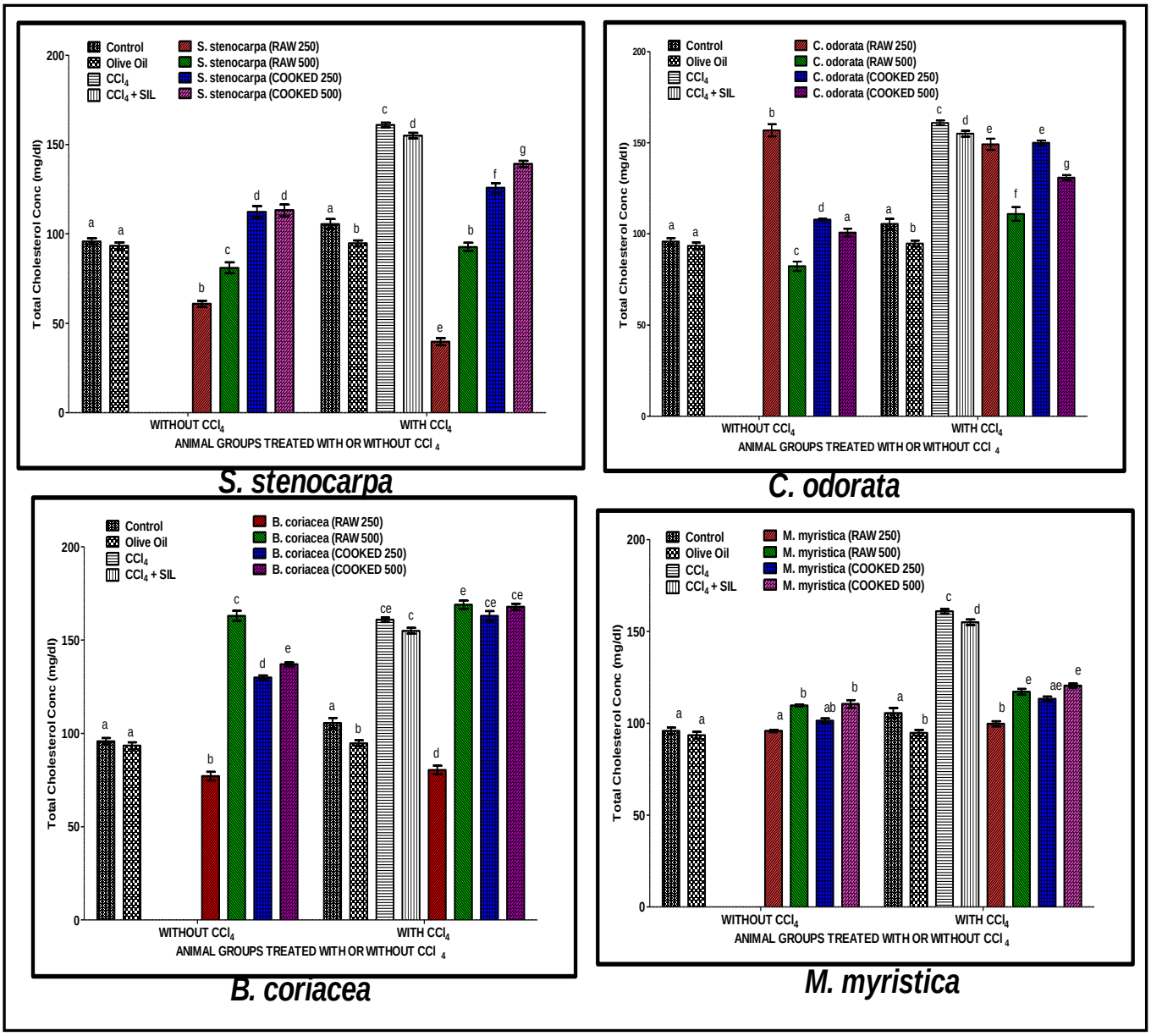


Fig 4.15: Total Cholesterol Concentration of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

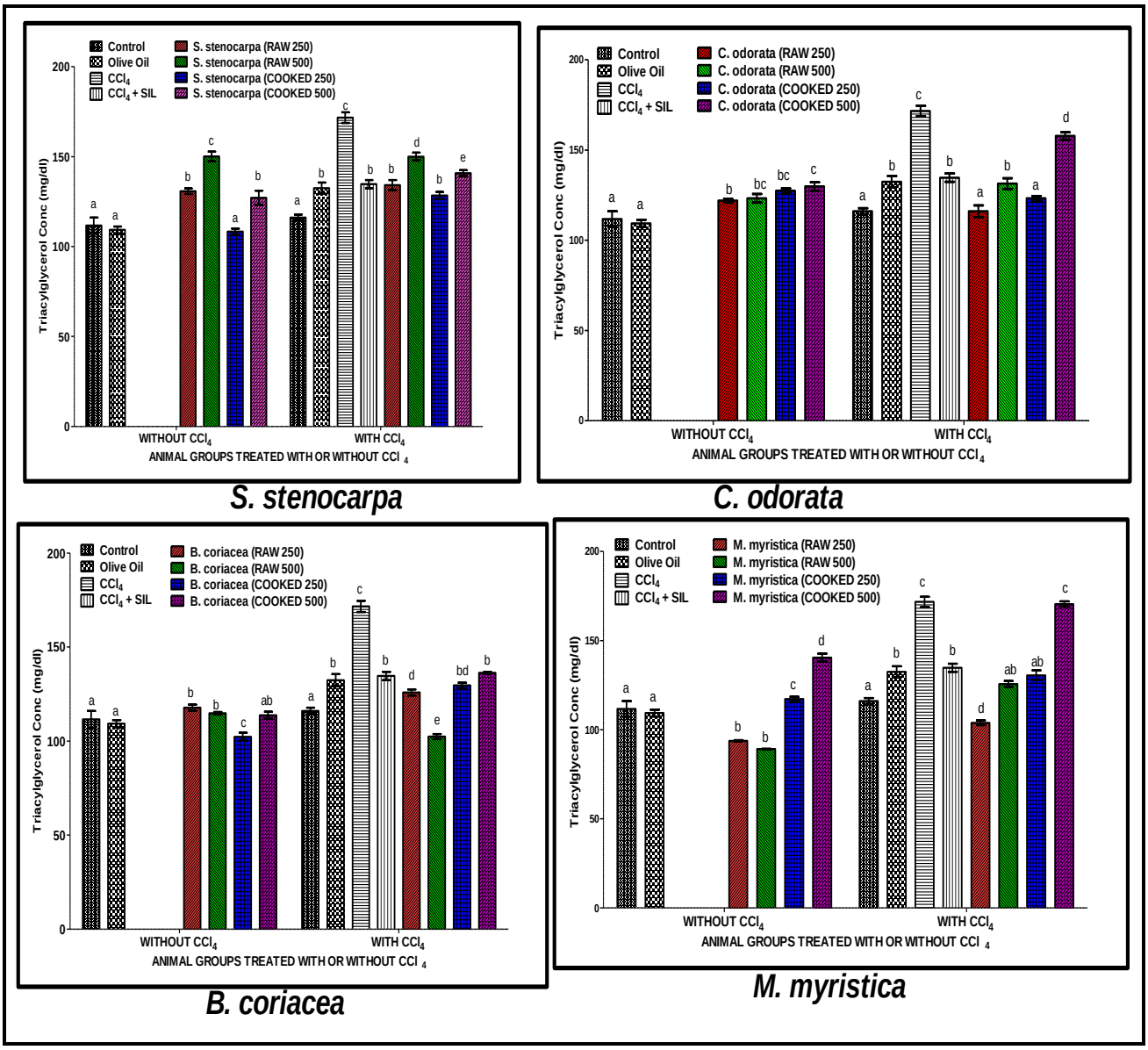


Fig 4.16: Triacylglycerol Concentration of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

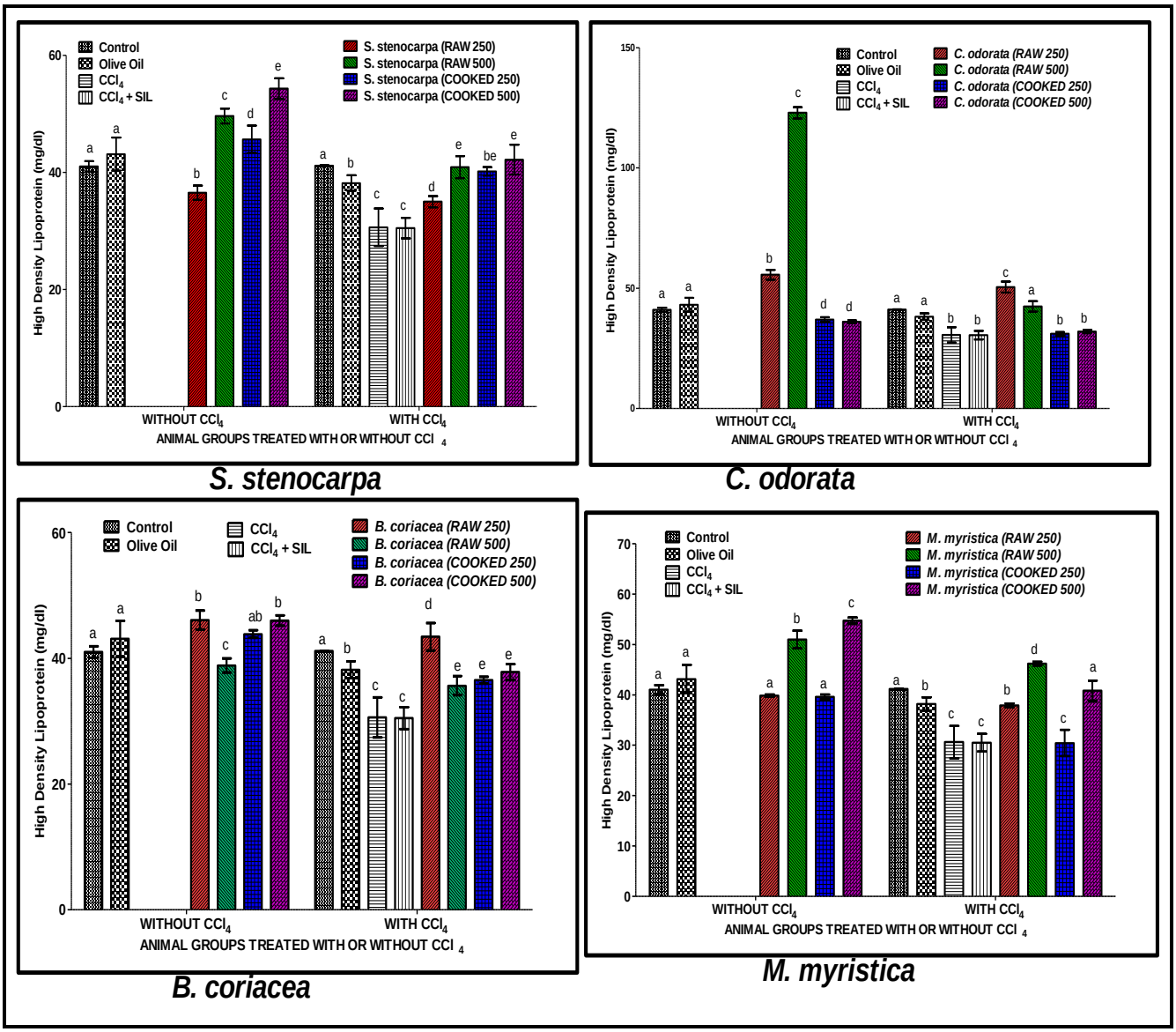


Fig 4.17: High Density Lipoprotein Concentration of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.12 Effect of Extracts on Lipid peroxidation (LPO)

The results of the effect of extracts on the malondialdehyde concentration, an index of LPO showed non-significant ($P>0.05$) increases in the malondialdehyde (MDA) concentrations of animals treated with the various extracts in comparison with the controls, except those that received 250 mg of raw and cooked *C. odorata* as well as 500 mg *B. coriacea*. MDA concentration was significantly increased by CCl_4-intoxication. However, treatment with the various plants extracts as well as silymarin reduced the observed increase in MDA due to CCl_4 intoxication to values comparable to those of controls (See Fig. 4.18).

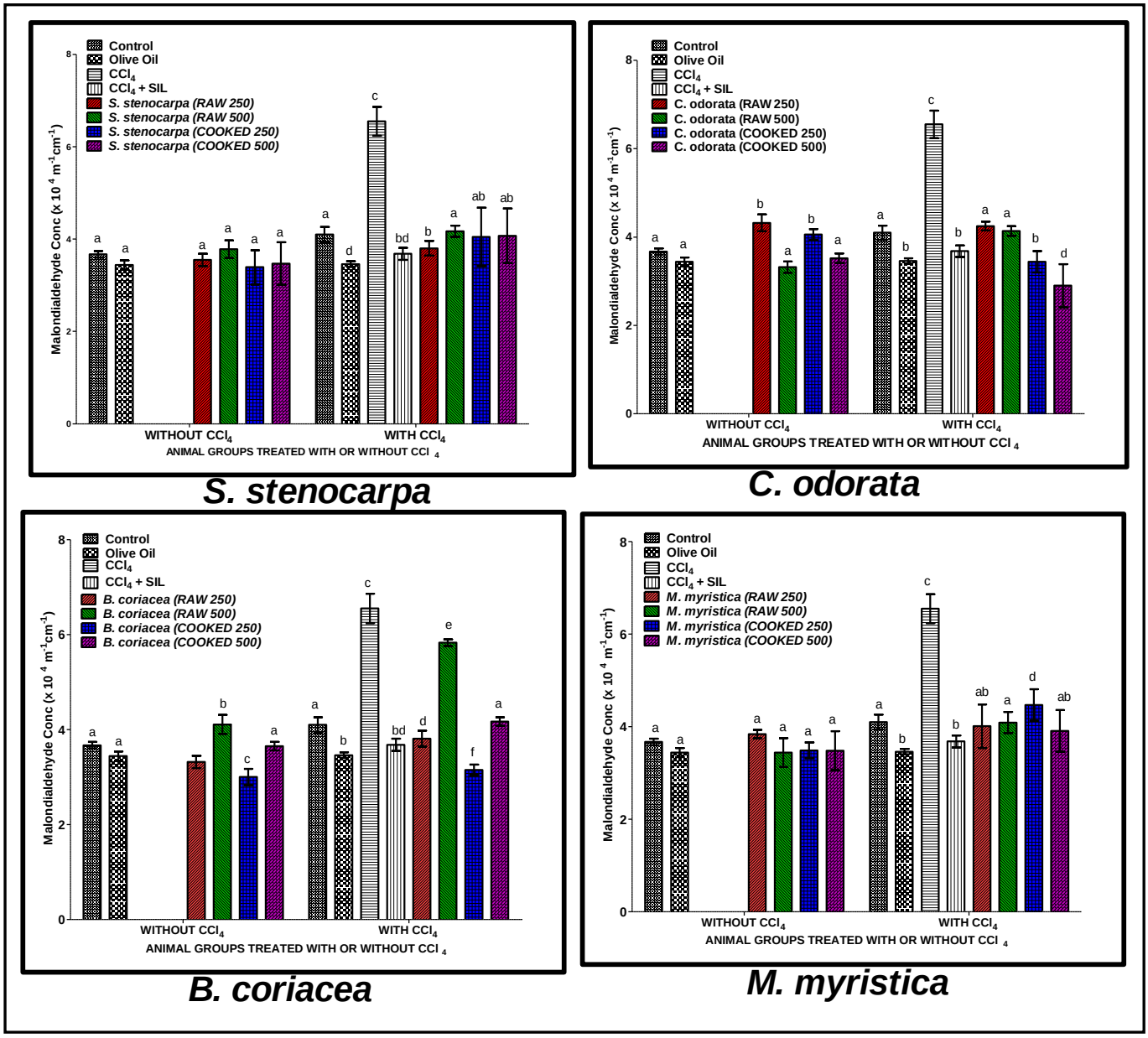


Fig 4.18: Malondialdehyde Concentration of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica*. Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.13 Effect of extracts on catalase activity

Catalase activity was significantly ($P<0.05$) improved in most of the extract-fed animals without CCl_4-intoxication when compared with the NS control except the groups that received 500 mg of cooked SS and 250 mg of cooked CO which were reduced. CCl_4-intoxication reduced significantly ($P<0.05$) the catalase activities of the animals. Treatment with silymarin, raw and cooked extracts of SS as well as the 250 mg of cooked extracts of the other plants increased significantly ($P<0.05$) the catalase activity in comparison with CCl_4-intoxicated group (Fig. 4.19).

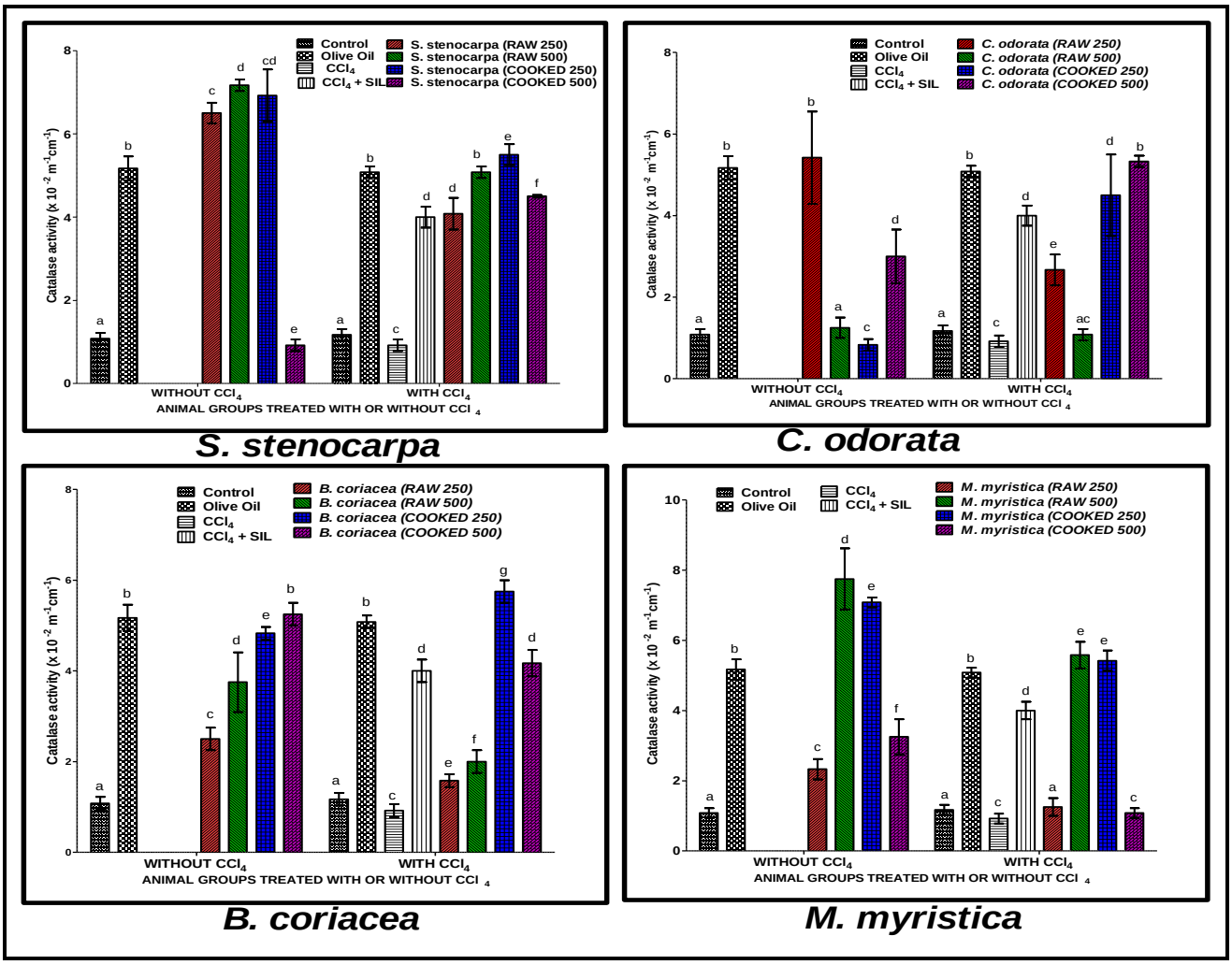


Fig 4.19: Catalase Activity of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.14 Effect of extracts on lactate dehydrogenase (LDH) activity

Treatment with the raw plant extracts of SS and CO increased LDH activity more than their cooked samples and the controls, while the LDH activity of animals exposed to raw and cooked BC showed dose-dependent variations. Similarly, raw and cooked plant extracts reduced significantly in a dose-dependent manner the observed increase in LDH activity of animals intoxicated with CCl_4 (Fig. 4.20).

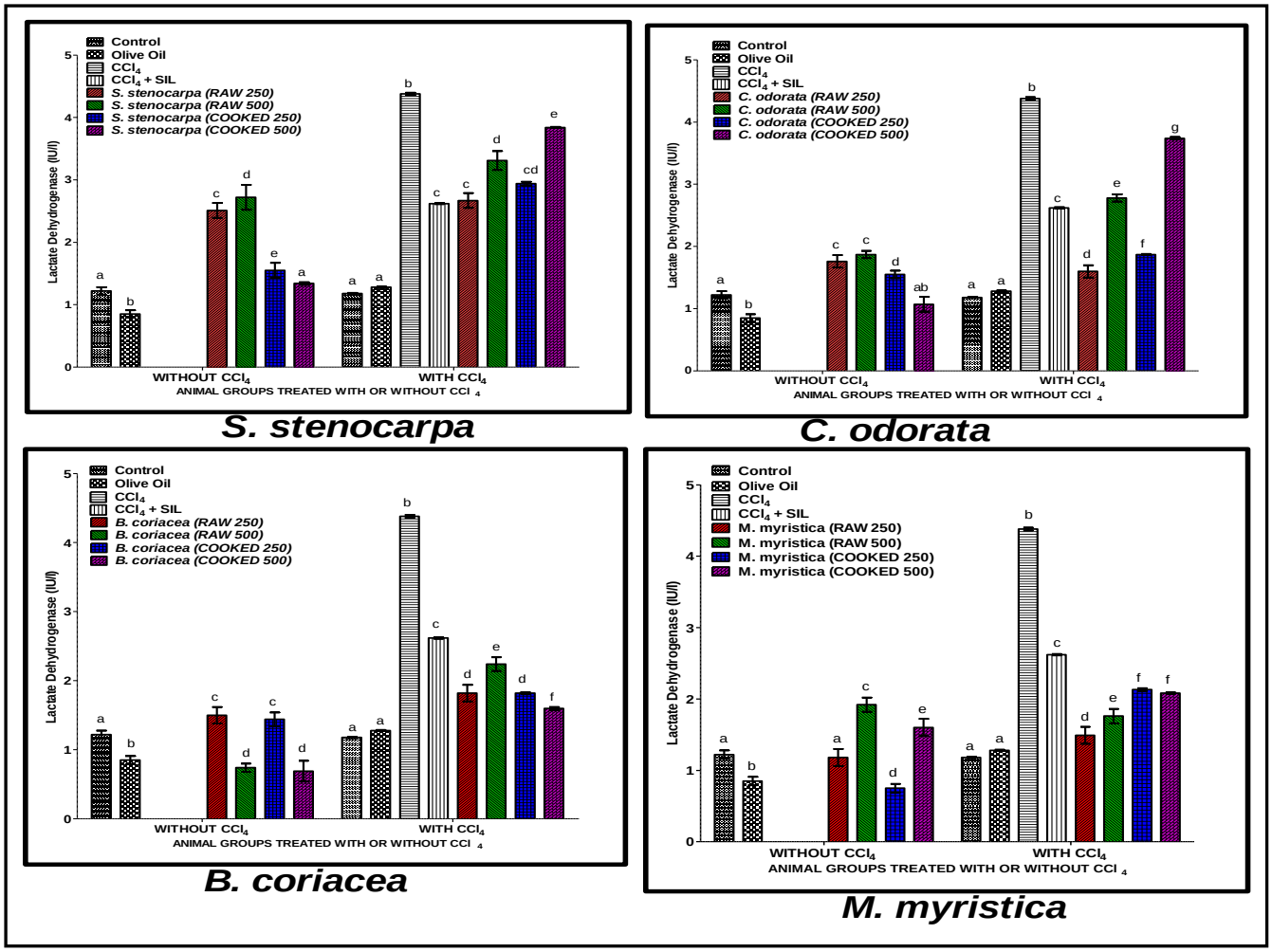


Fig 4.20: Lactate Dehydrogenase Activity of CCl_4 administered animal groups treated with raw and cooked methanol extracts of *S. stenocarpa, C. odorata, B. coriacea* and *M. myristica.* Bars are mean ± standard deviation. Bars with different letters per block of treatment (with or without CCl_4) per plant are statistically significant ($p<0.05$).

4.1.15 Effect of plant extracts on kidney and liver histology

Liver and kidney tissues were subjected to Hematoxylin and eosin (H & E) staining in order to evaluate the degree of tissue injury. The histological examination of the tissue sections of the normal control revealed normal architecture of kidney (intact glomeruli, tubules, interstitum and blood vessels) and liver (binucleated hepatocytes, prominent nuclei and sinusoids), while the negative control showed extensive changes in kidney and liver morphology**.** The kidney sections of rats that received olive oil as well as non-intoxicated rats that received low dose (250 mg/kg) of raw and cooked extracts of *S. stenocarpa* (SS), *M. myristica* (MM) and *B. coriacea* (BC) showed normal histological appearance. At both concentrations (250 and 500 mg/kg per body weight) of the extracts, the liver showed mild to moderate alterations. The alterations seen in the liver and kidney tissues included necrosis, degeneration and inflammatory responses. The introduction of CCl_4 caused significant increase in the changes observed in the liver and kidney tissues and mild alterations in the unaffected groups that were intoxicated. Significant alleviation in CCl_4-induced tissue injury was observed with treatments involving silymarin and extracts of SS, MM and BC. At both concentrations (250 and 500 mg/kg) of *C. odorata* (CO), severe alterations including vacuolations, lymphatic infiltration, vascular congestion, hemorrhagic necrosis and loosed cortex were observed (See plates 4.1 -4.6).

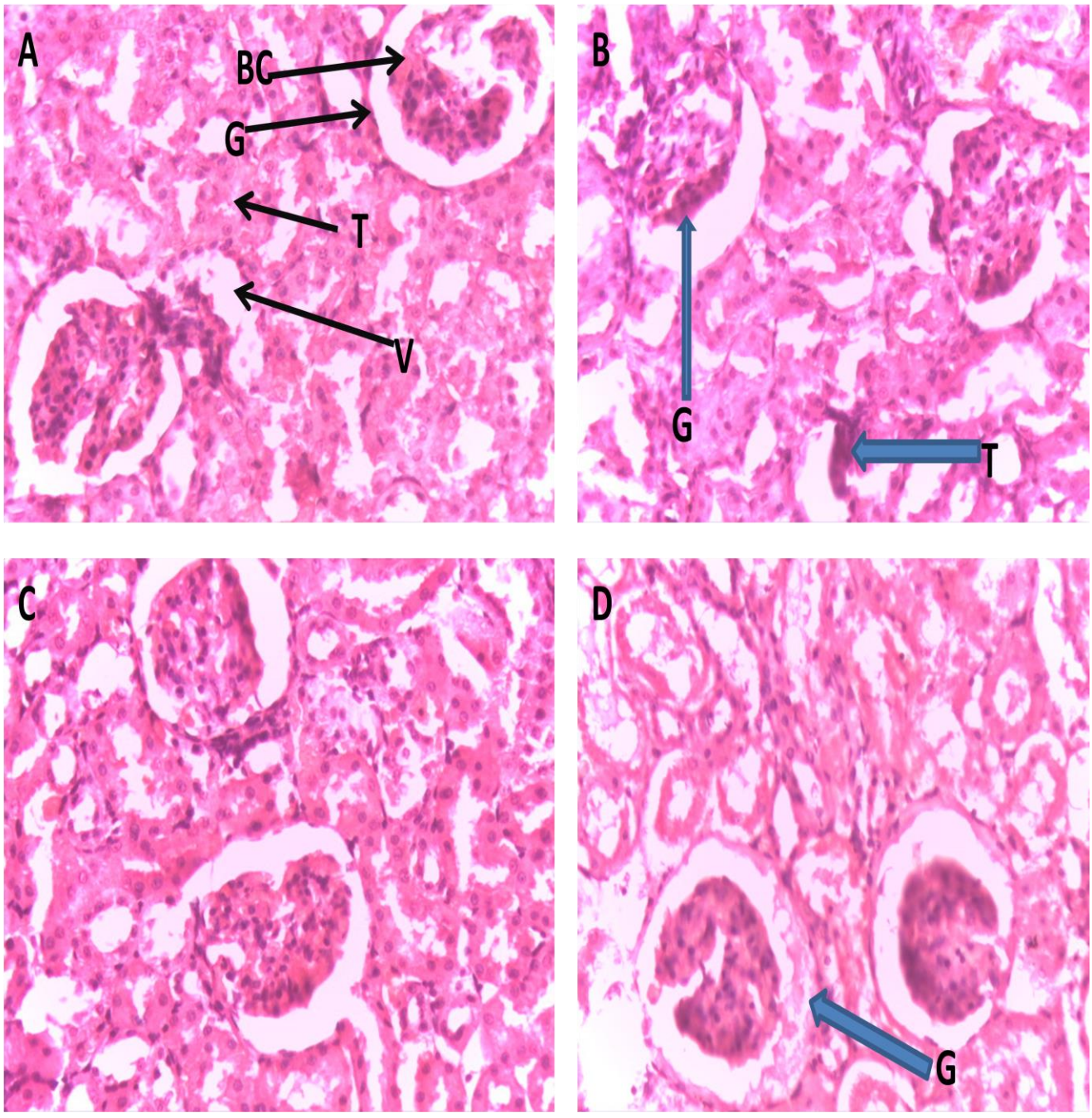


Plate 4.1: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with; A- Normal saline, B – CCl_4 only, C – Olive oil, D – Silymarin. BC=Bowman's capsule, G=Glomerulus, T= Tubules, V=Veins

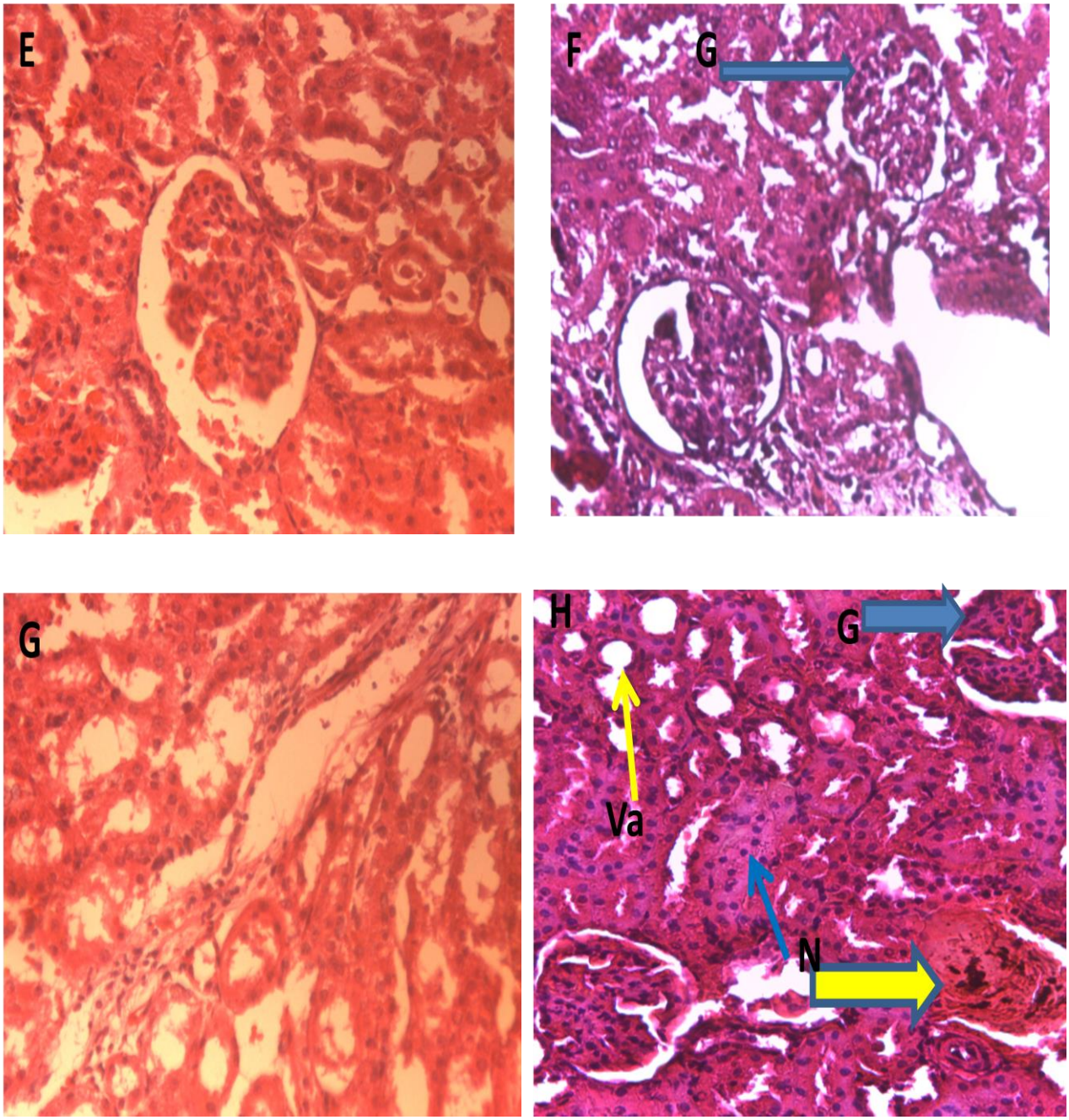


Plate 4.2: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with raw *Sphenostylis stenocarpa*(RSS); E – RSS_{250}, F – $^{\Lambda}RSS_{250}$, G– RSS_{500}, H – $^{\Lambda}RSS_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

G = glomerulus, Va = vacualation, N = necrosis

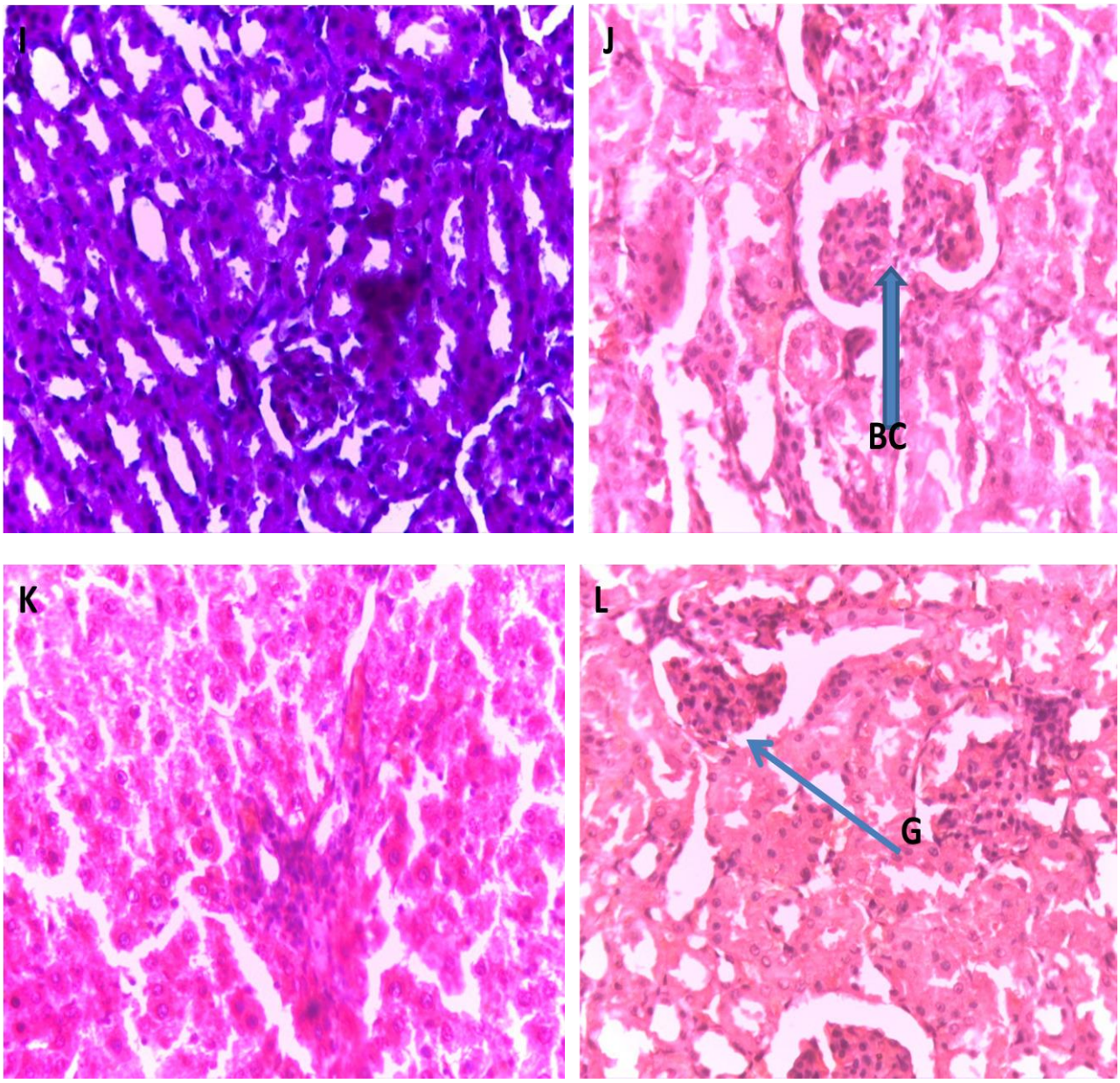


Plate 4.3: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with cooked *Sphenostylis stenocarpa* (RSS); I – CSS_{250}, J – $^{\Lambda}CSS_{250}$, K– CSS_{500}, L – $^{\Lambda}CSS_{500}$.
$^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

BC= Bowman's capsule G=Glomerulus, Va=Vacuolation

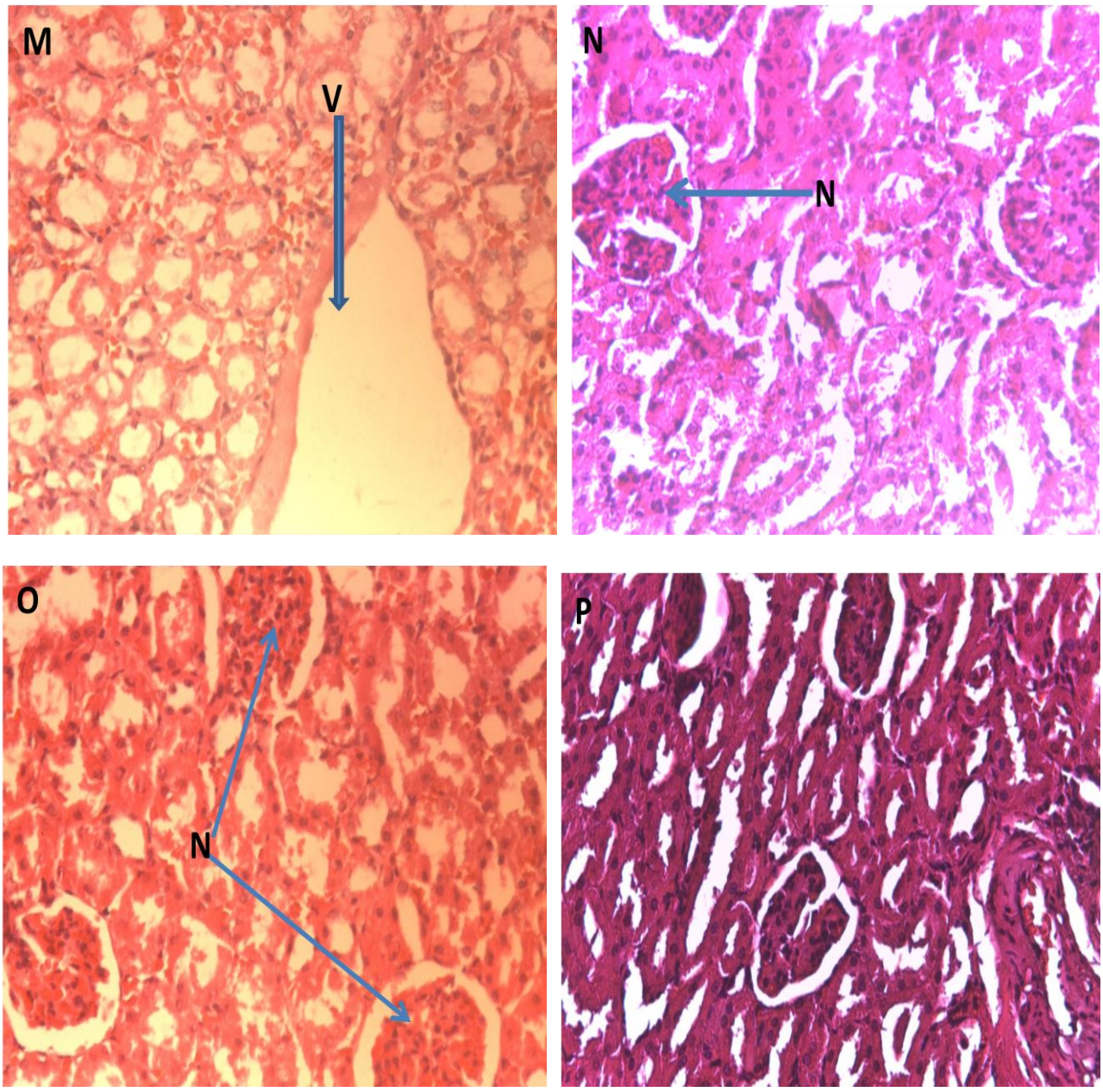


Plate 4.4: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with raw *Chromolaena odorata* (RCO); M – RCO_{250}, N – $^{\Lambda}RCO_{250}$, O– RCO_{500}, L – $^{\Lambda}RCO_{500}$.
$^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

BC= Bowman's capsule G=Glomerulus, V=Vein, N=Necrotic Glomeruli

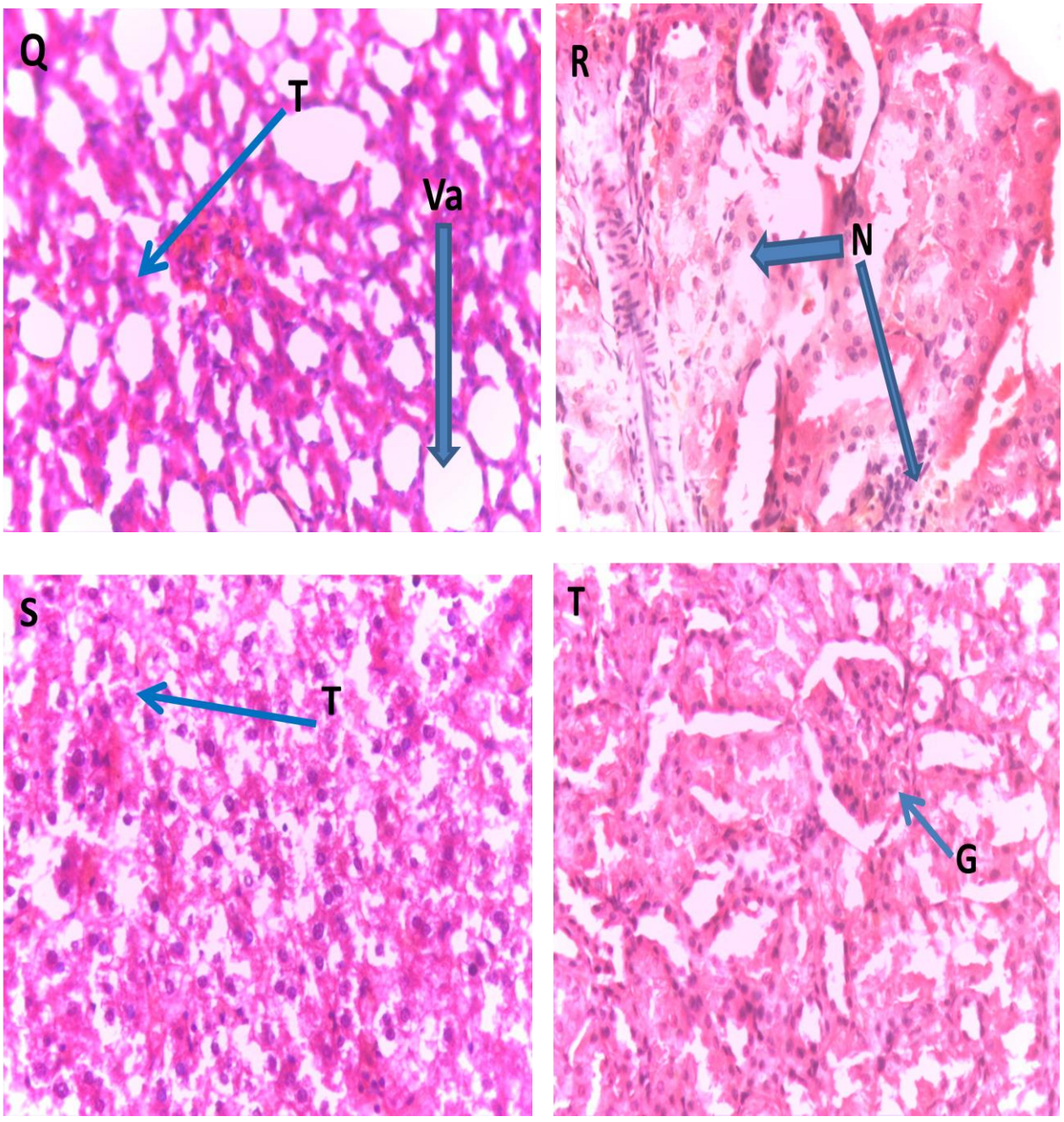


Plate 4.5: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with cooked *Chromolaena odorata* (CCO); Q – CCO_{250}, R – $^{\Lambda}CCO_{250}$, S – CCO_{500}, T – $^{\Lambda}CCO_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

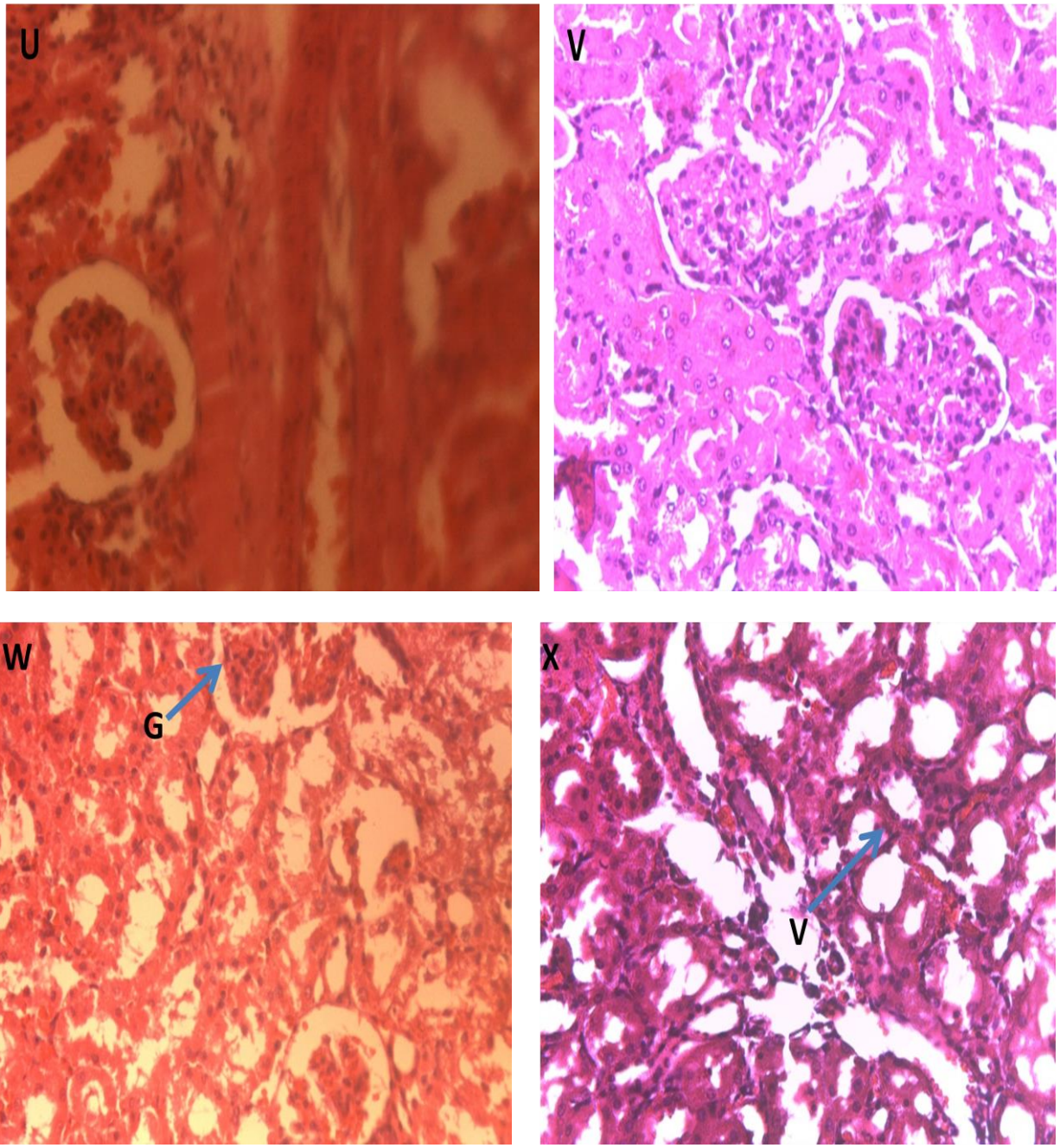


Plate 4.6: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with raw *Bulccholzia coriacea* (RBC); U – RBC_{250}, V – $^{\Lambda}RBC_{250,}$ W– RBC_{500}, S – $^{\Lambda}RBC_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

BC= Bowman's capsule G=Glomerulus, V=Vein, N=Necrotic Glomeruli

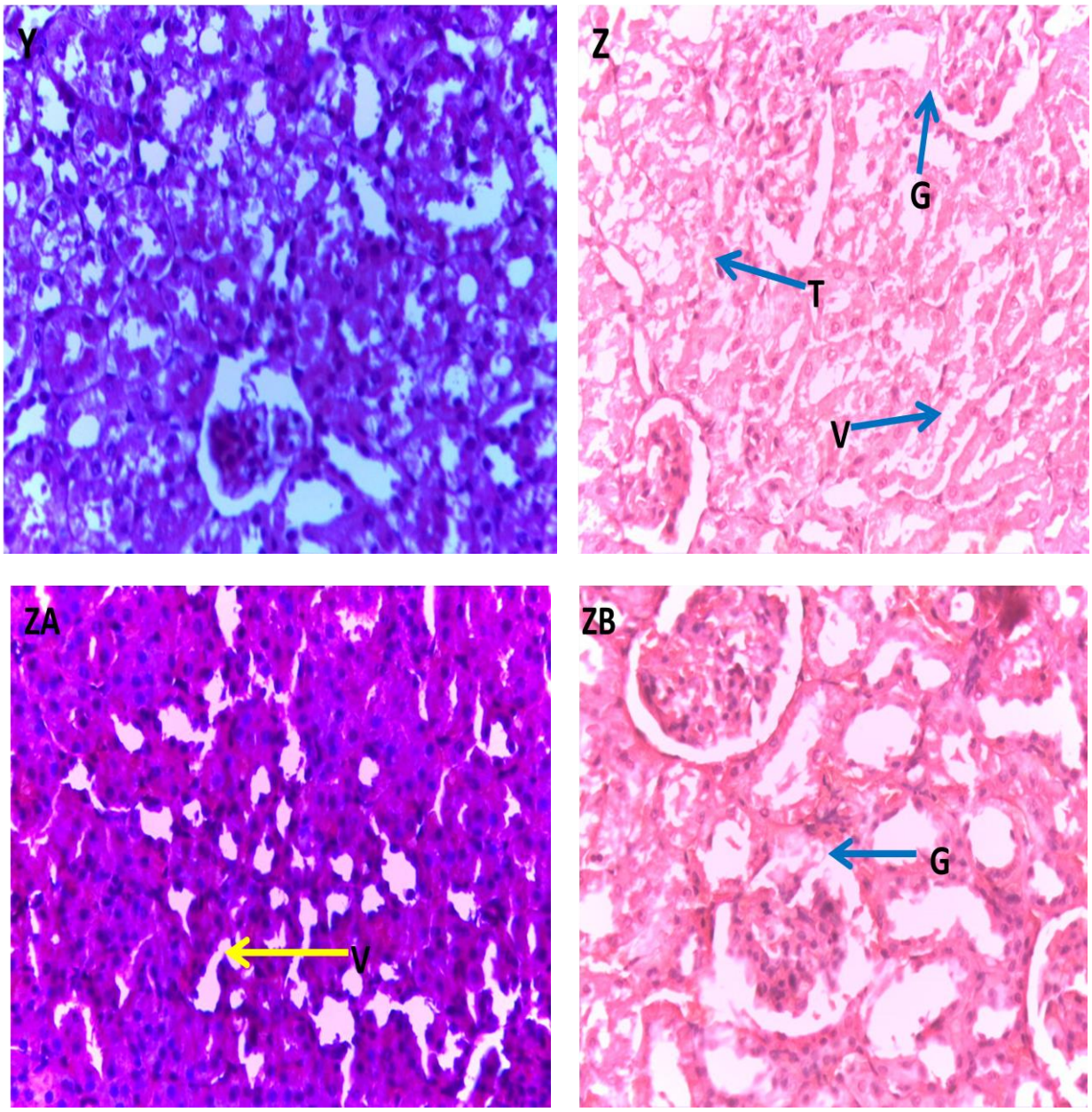


Plate 4.7: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with cooked *Bulccholzia coriacea* (CBC); Y – CBC_{250}, Z – $^{\Lambda}CBC_{250, ZA}$ – CBC_{500}, ZB – $^{\Lambda}CBC_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts.

BC= Bowman's capsule G=Glomerulus, T= Tubule

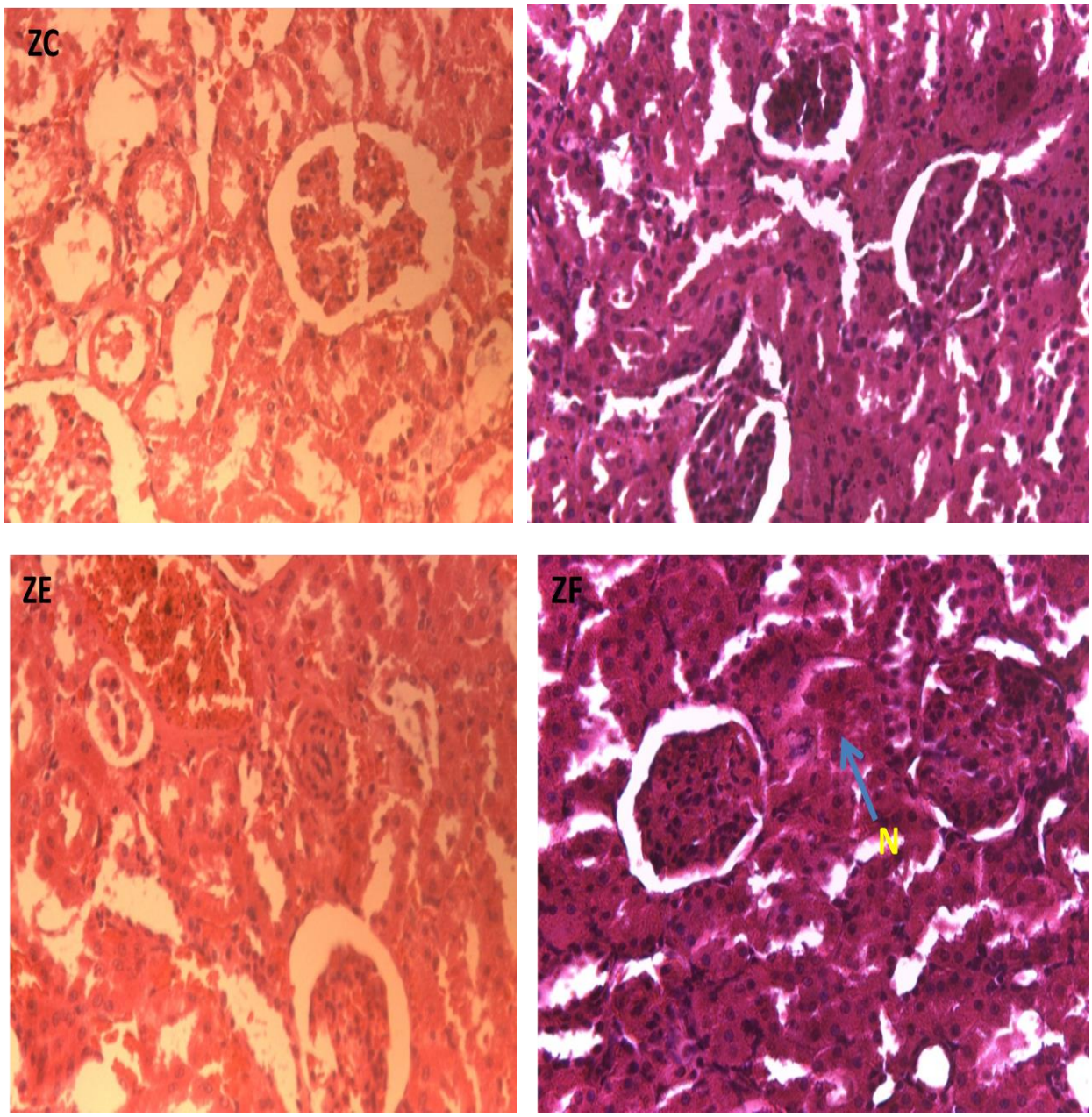


Plate 4.8: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with raw *Monodora myristica* (RMM); ZC – RMM_{250}, ZD – $^{\Lambda}RMM_{250}$, ZE – RMM_{500}, ZF – $^{\Lambda}RMM_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. N = Necrosis

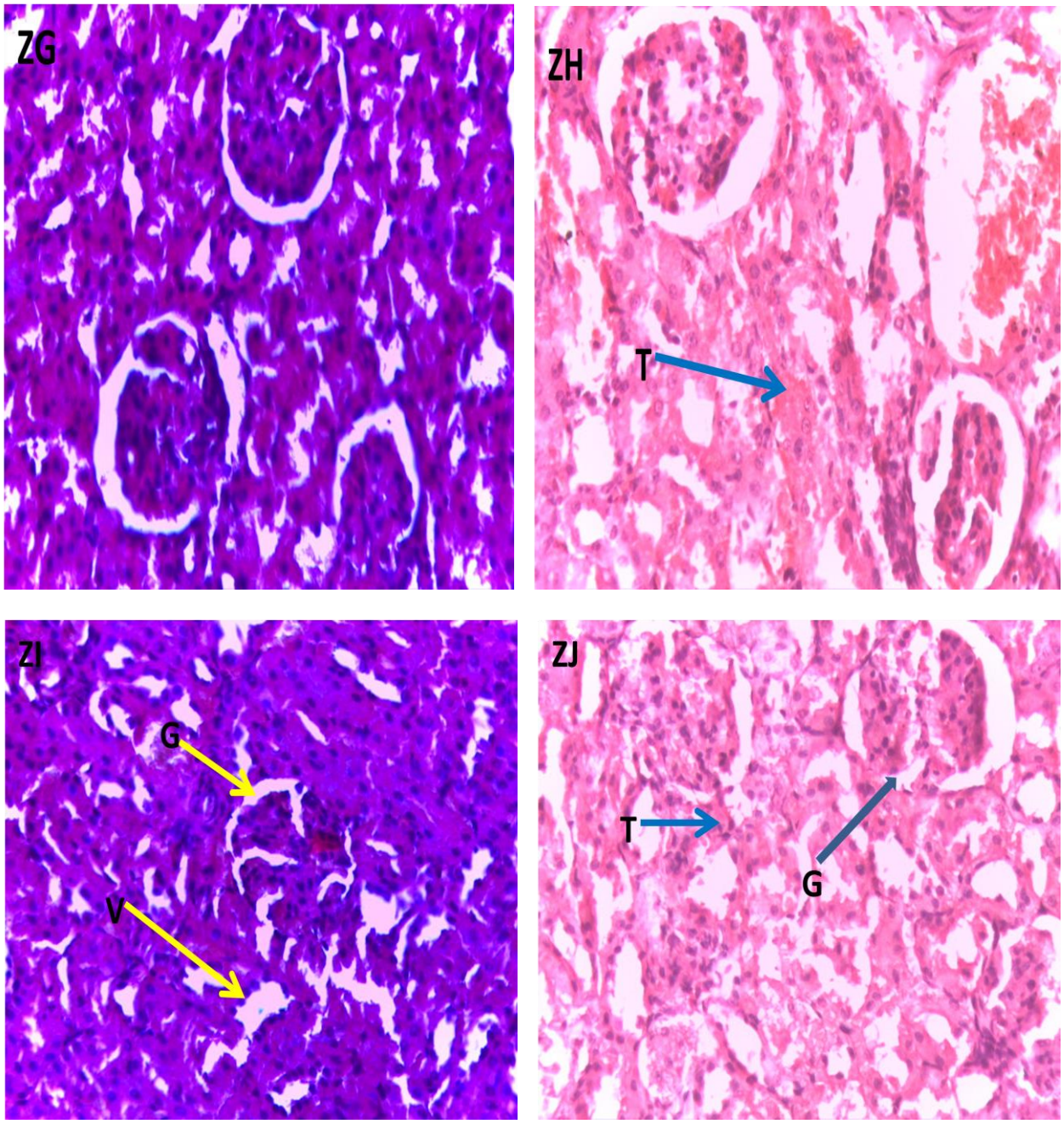


Plate 4.9: Representative photomicrographs (×400 H&E) of kidney tissues of animals treated with cooked *Monodora myristica* (RMM); ZG – CMM_{250}, ZH – $^{\Lambda}CMM_{250}$, ZI – CMM_{500}, ZJ – $^{\Lambda}CMM_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. BC= Bowman's capsule G=Glomerulus, T= Tubule, V= Veins

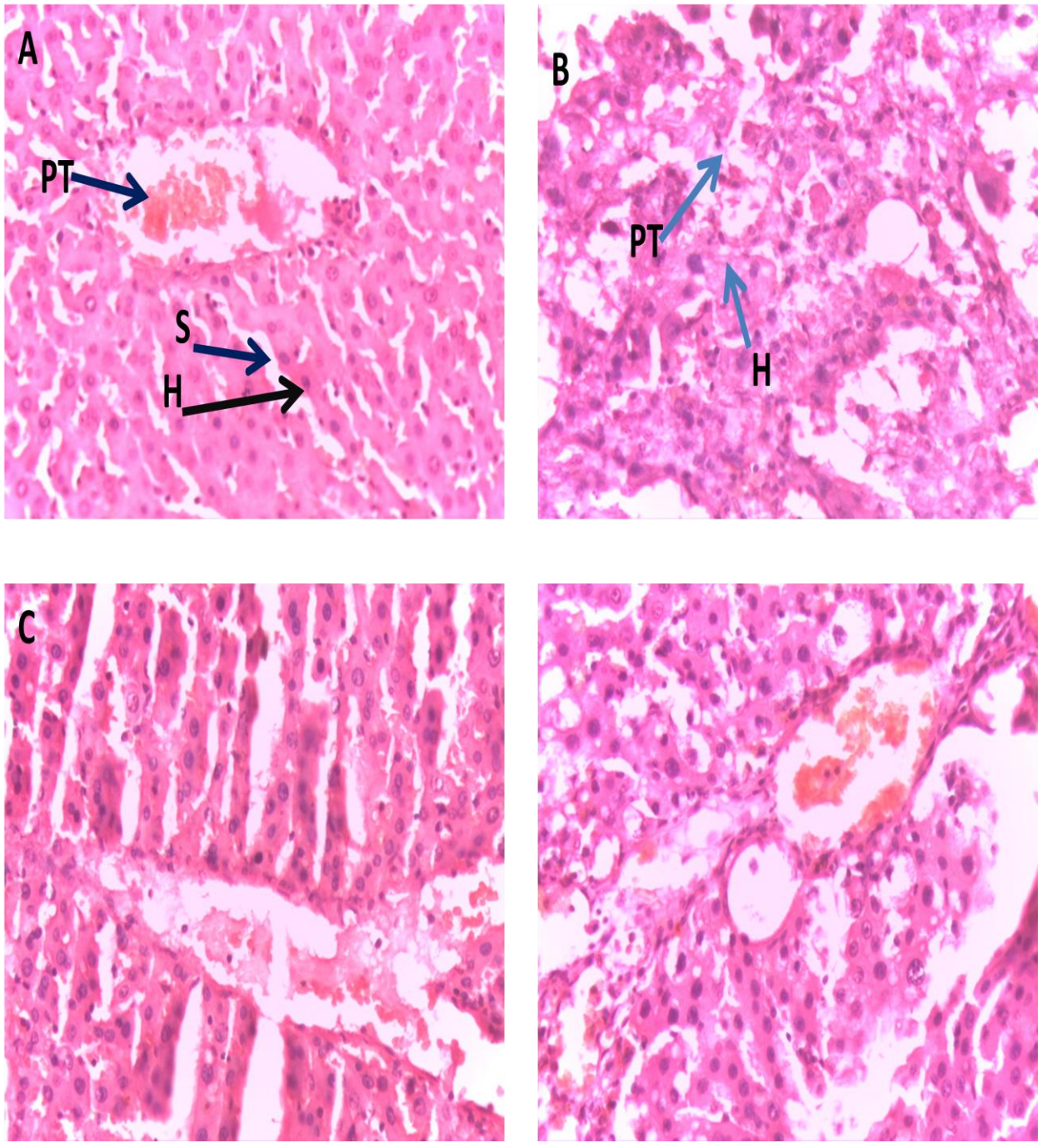


Plate 4.10: Representative photomicrographs (×400 H & E) of liver tissues of animals treated with the followings; A- Normal saline, B – CCl_4/oliveoil mixture, C – Olive oil, D – Silymarin. PT= Portal tract, S = Sinusoid, H= Hepatocyte

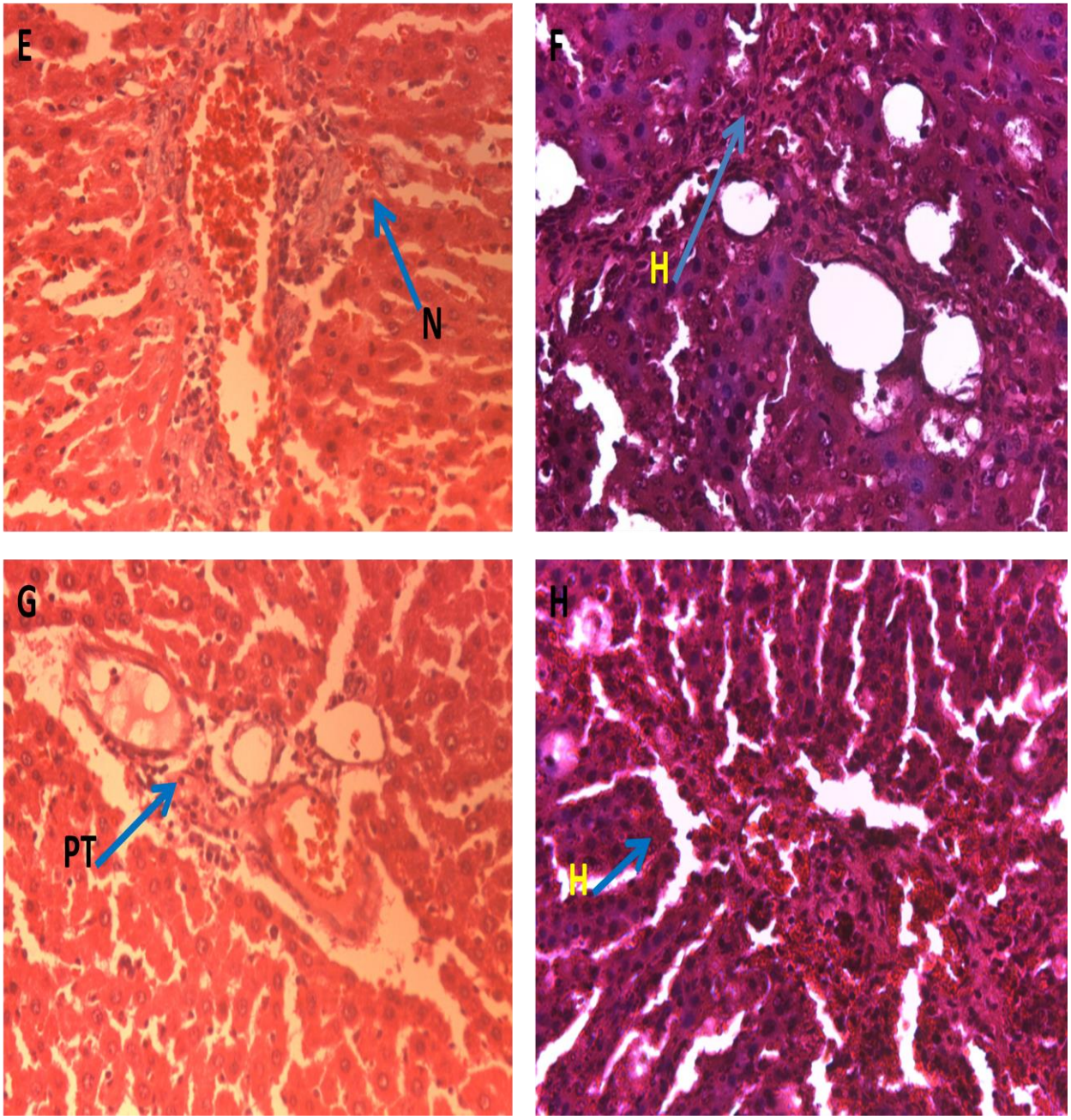


Plate 4.11: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with raw *Sphenostylis stenocarpa* (RSS); E – RSS_{250}, F – $^{\Lambda}RSS_{250}$, G– RSS_{500}, H – $^{\Lambda}RSS_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts PT= Portal tract, S = Sinusoid, H= Hepatocyte

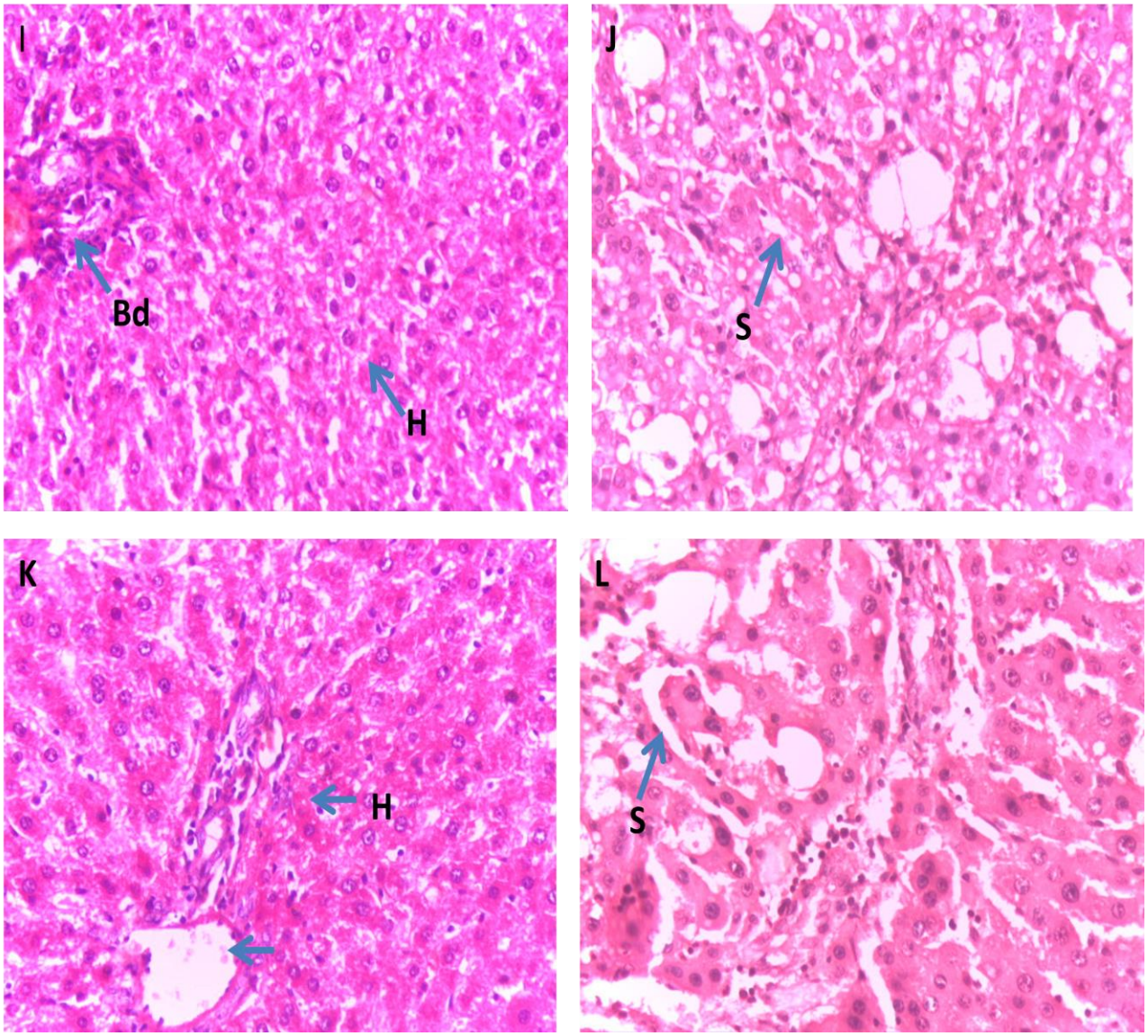


Plate 4.12: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with cooked *Sphenostylis stenocarpa* (RSS); I – CSS_{250}, J – $^{\Lambda}CSS_{250}$, K– CSS_{500}, L – $^{\Lambda}CSS_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. PT= Portal tract, S = Sinusoid, H= Hepatocyte

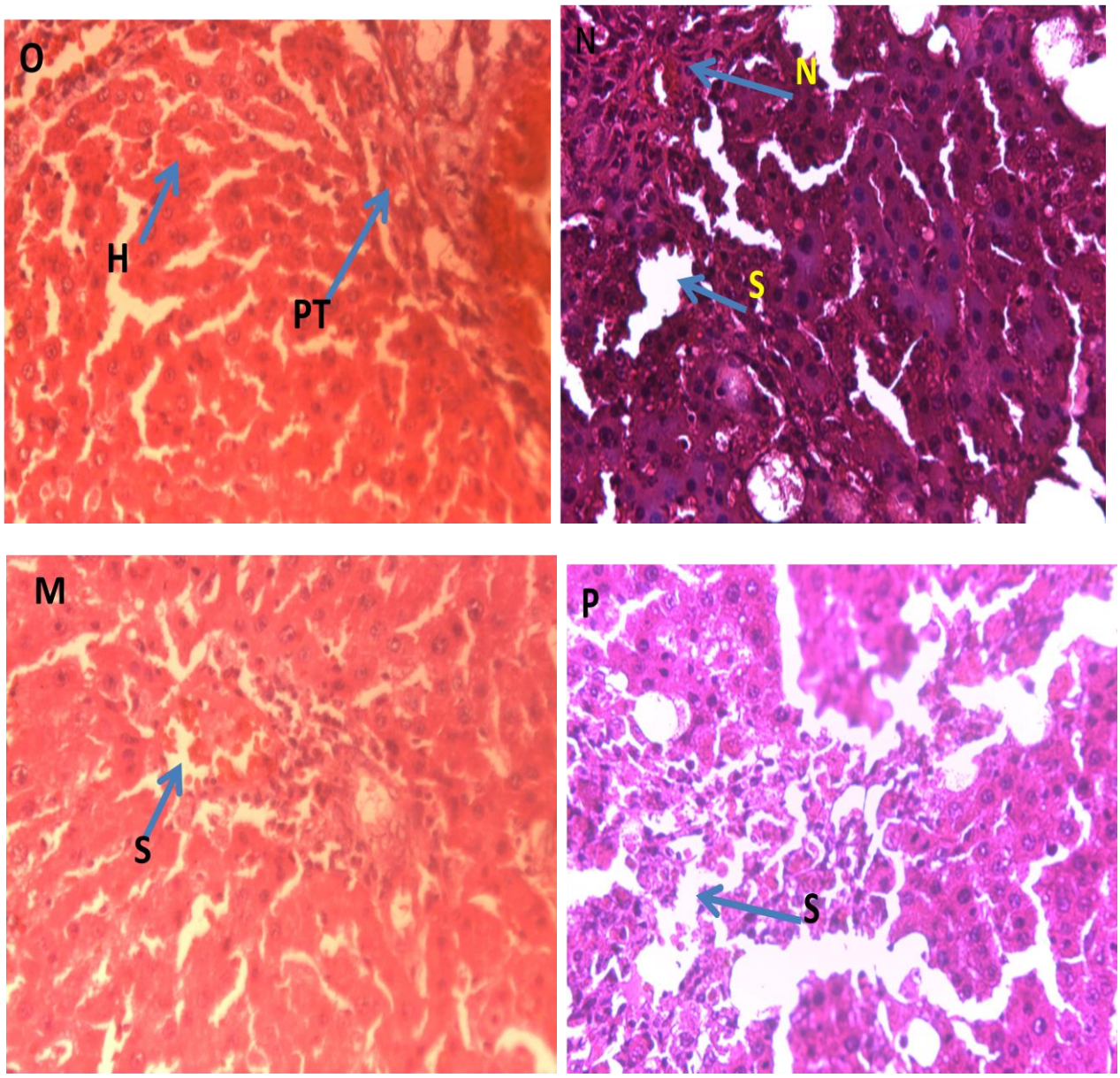


Plate 4.13: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with raw *Chromolaena odorata* (RCO); M – RCO_{250}, N – $^{\Lambda}RCO_{250}$, O– RCO_{500}, P – $^{\Lambda}RCO_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. PT= Portal tract, S = Sinusoid, H= Hepatocyte

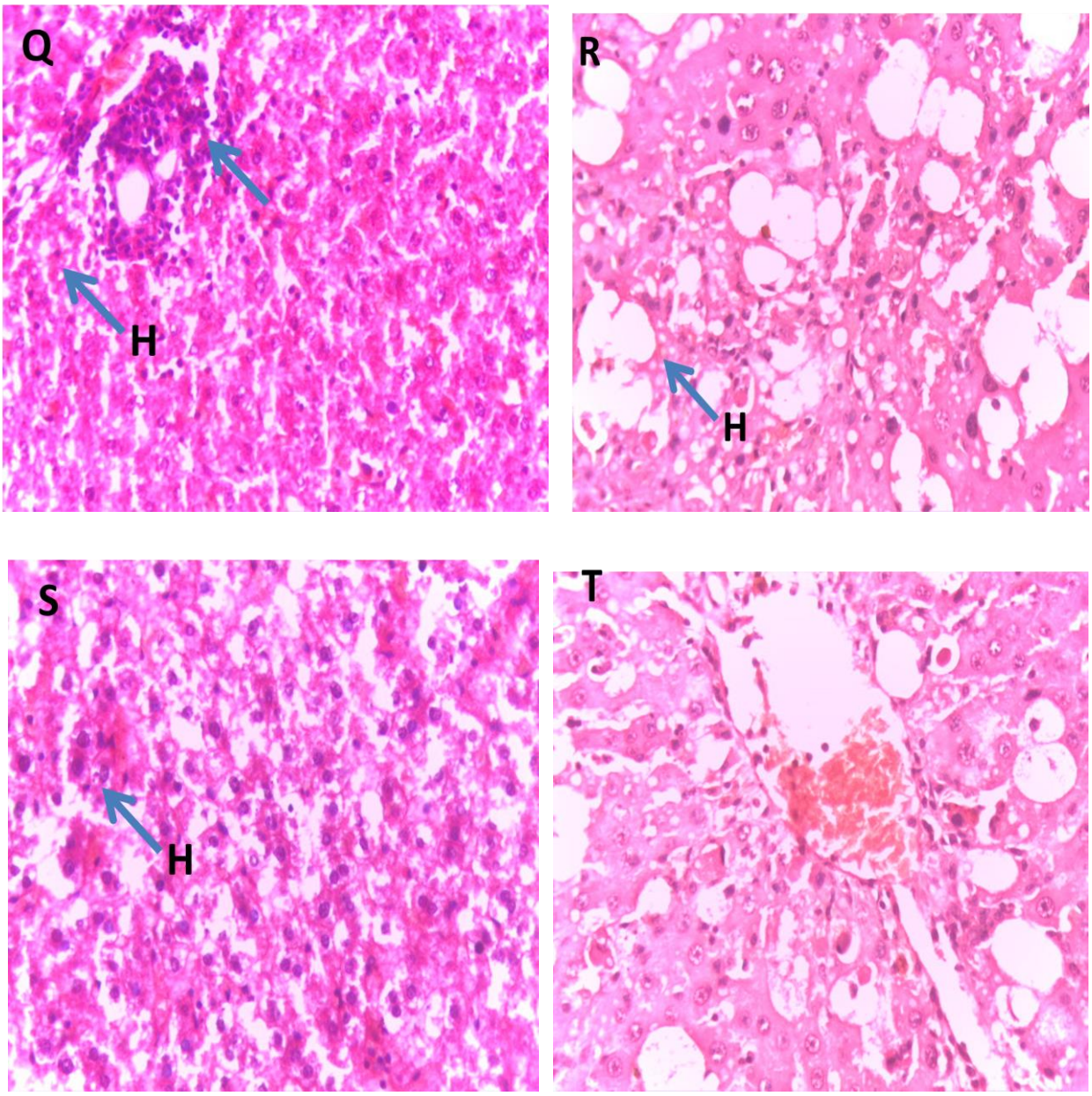


Plate 4.14: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with cooked *Chromolaena odorata* (CCO); Q – CCO_{250}, R – $^{\Lambda}CCO_{250}$, S – CCO_{500}, T – $^{\Lambda}CCO_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. PT= Portal tract, S = Sinusoid, H= Hepatocyte

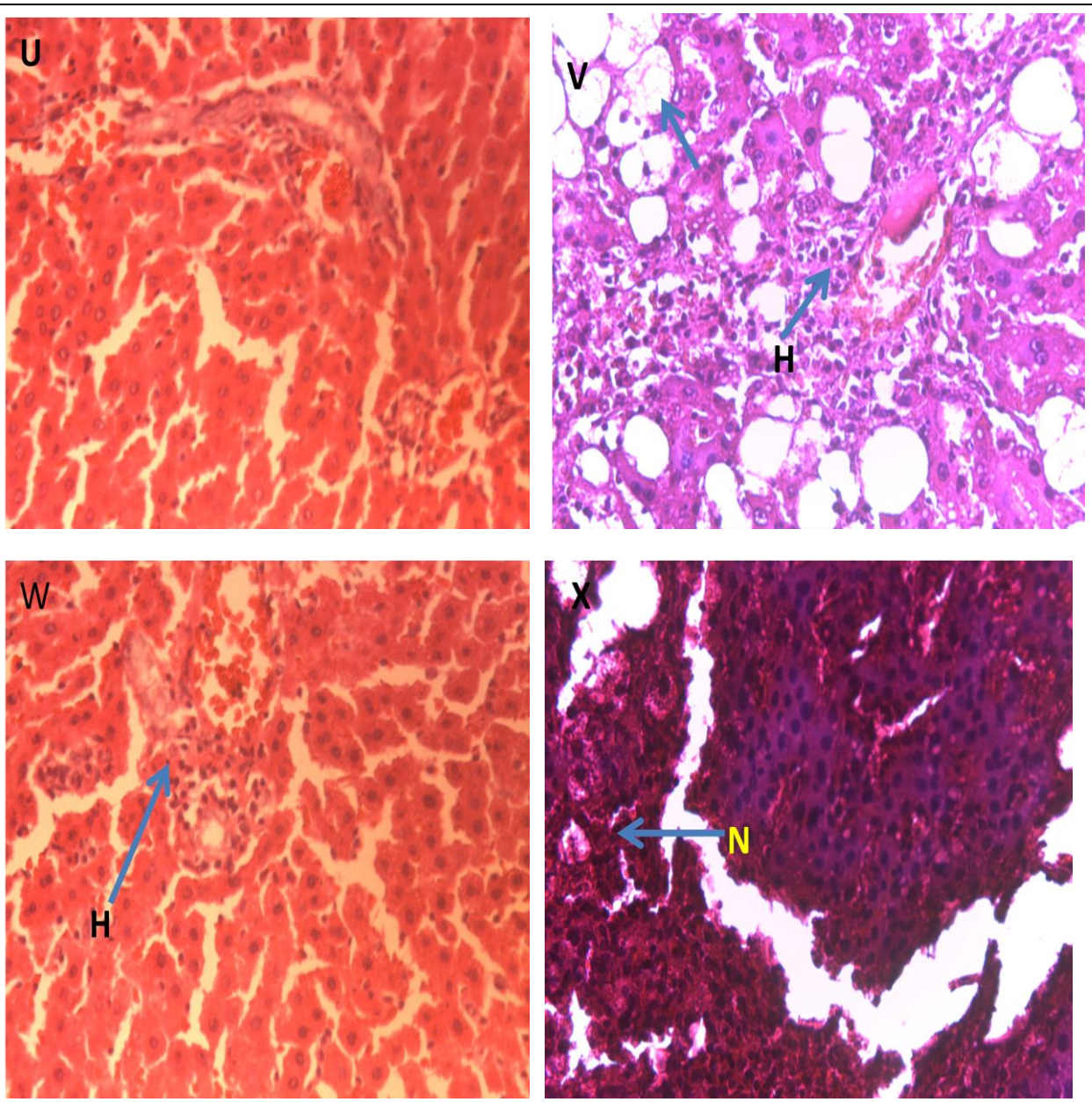


Plate 4.15: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with raw *Bulccholzia coriacea* (RBC); U – RBC_{250}, V – $^{\Lambda}RBC_{250}$, W– RBC_{500}, S – $^{\Lambda}RBC_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. PT= Portal tract, S = Sinusoid, H= Hepatocyte

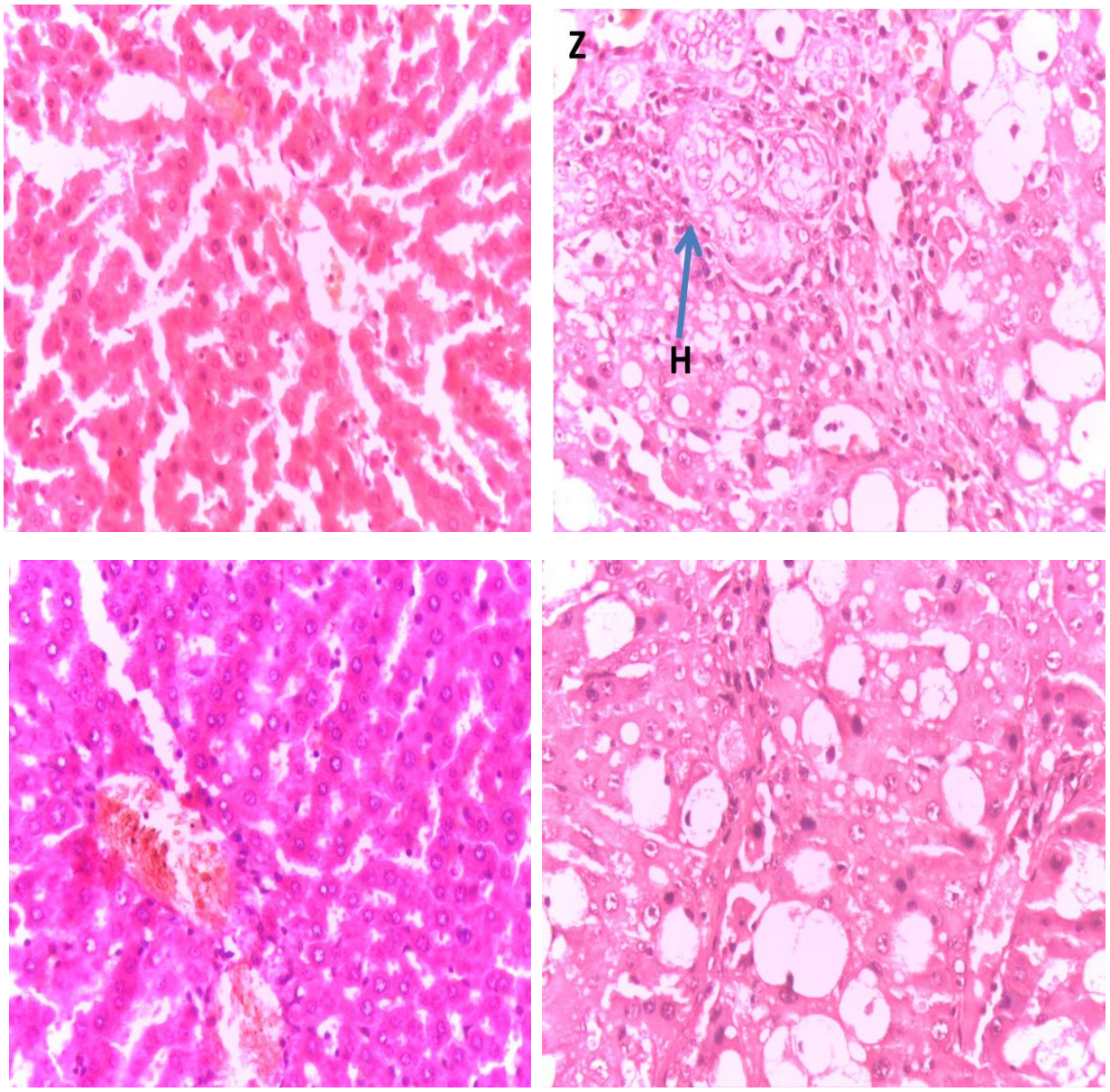


Plate 4.16: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with cooked *Bulccholzia coriacea* (CBC); Y – CBC_{250}, Z – $^{\Lambda}CBC_{250, ZA}$ – CBC_{500}, ZB – $^{\Lambda}CBC_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. H = Hepatocyte

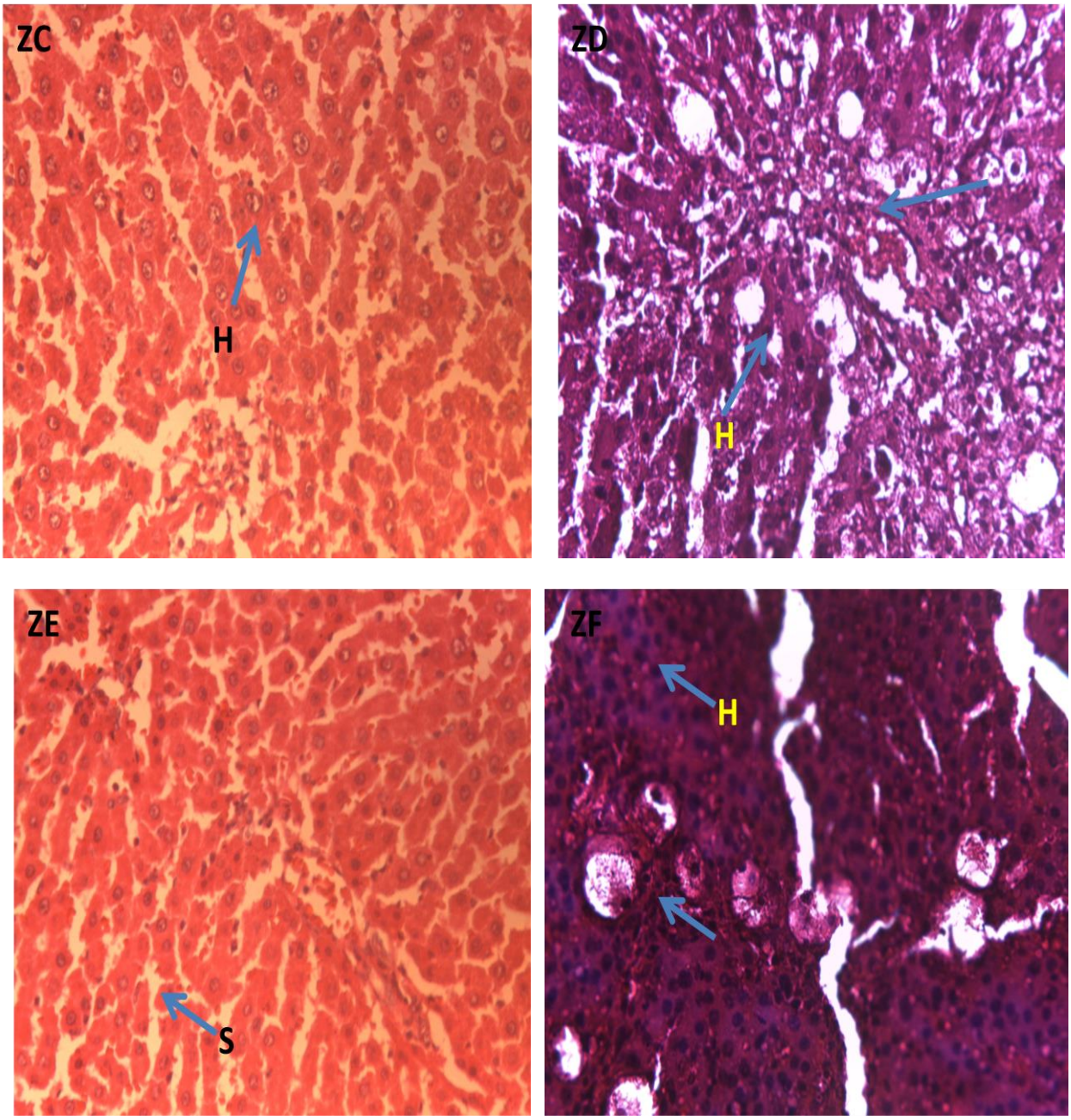


Plate 4.17: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with raw *Monodora myristica* (RMM); ZC – RMM_{250}, ZD – $^{\Lambda}RMM_{250}$, ZE – RMM_{500}, ZF – $^{\Lambda}RMM_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. PT= Portal tract, S = Sinusoid, H= Hepatocyte

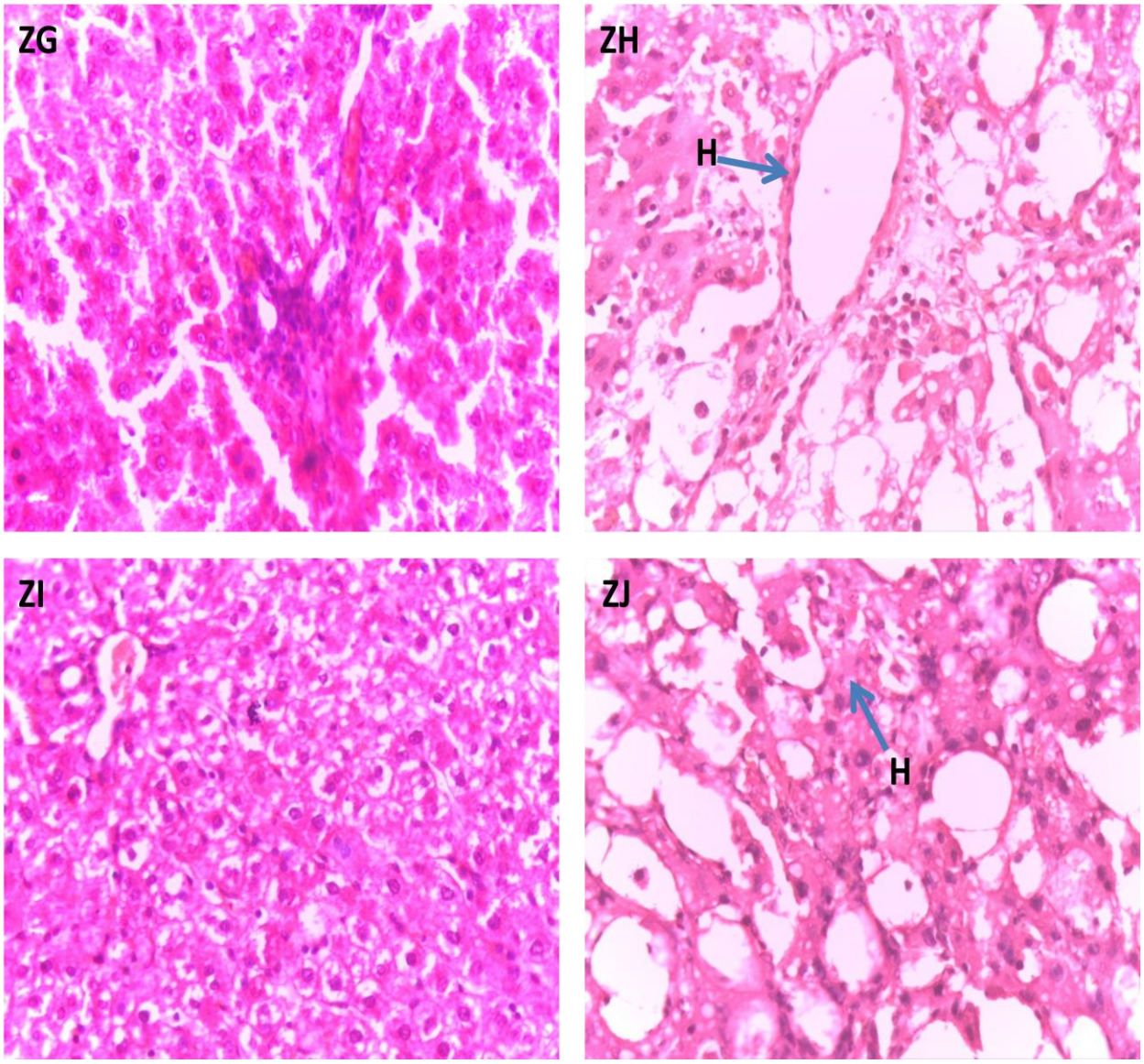


Plate 4.18: Representative photomicrographs (×400 H&E) of liver tissues of animals treated with cooked *Monodora myristica* (RMM); ZG – CMM_{250}, ZH – $^{\Lambda}CMM_{250}$, ZI – CMM_{500}, ZJ – $^{\Lambda}CMM_{500}$. $^{\Lambda}$ represent CCl_4 intoxicated groups, subscripts indicate concentrations of extracts. BC= Bowman's capsule G=Glomerulus, T= Tubule, V= Veins

4.1.17 Effects of cooked extracts on collagen expression

The results of the immunohistochemical analyses of hepatic tissues of intoxicated rats treated with cooked plant extracts are presented below;

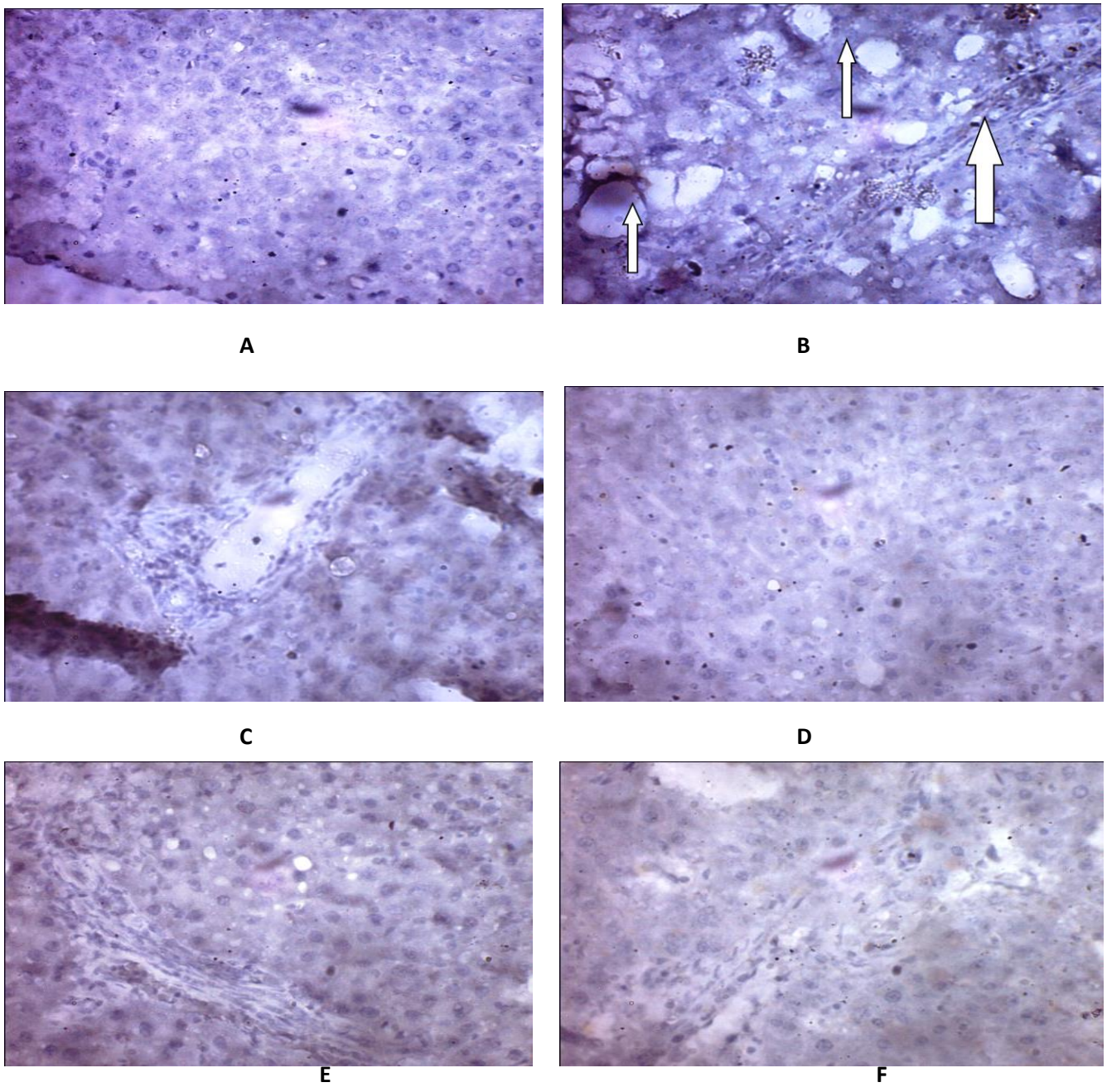
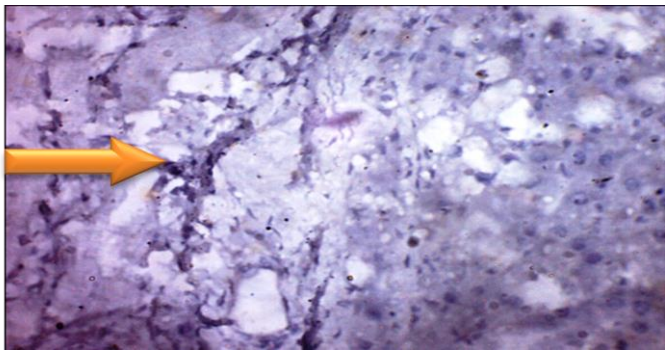
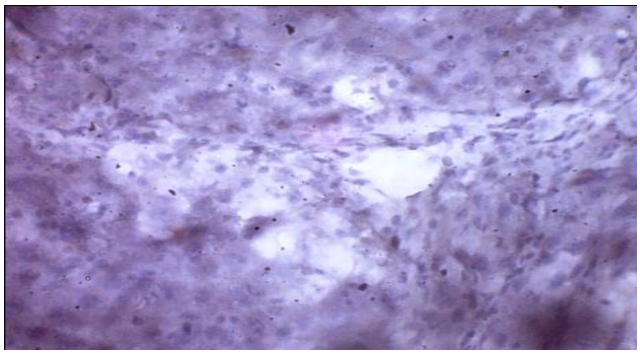


Plate 4.19: The immunohistochemical examinations of the representative liver tissues reveal the followings: A (Normal control) - no collagen fibres were evident in the normal undisturbed liver parenchyma. B (CCl_4) - small dark brown stained lines of collagen deposition within the liver parenchyma and the portal tracts, liver cell death was more evident than in the haematoxylin and eosin stains. C (Olive oil), D (Silymarin), E (SS_{250}) and F (SS_{500}) - No fibrosis was seen.



G



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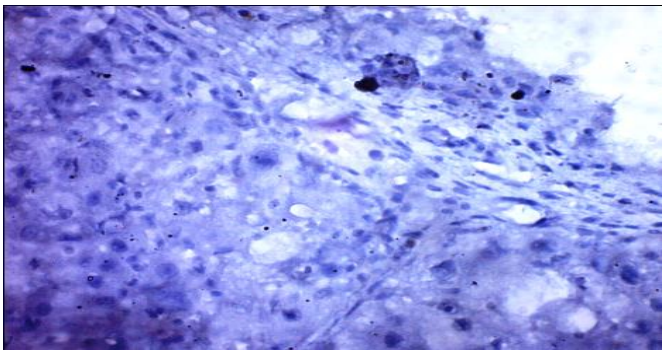

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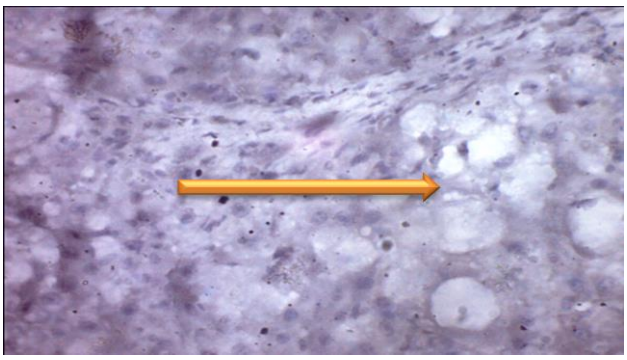

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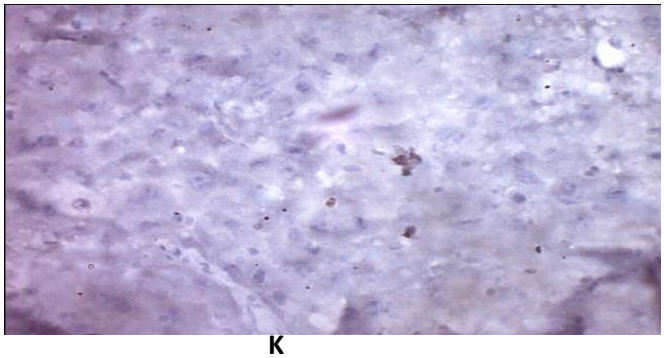
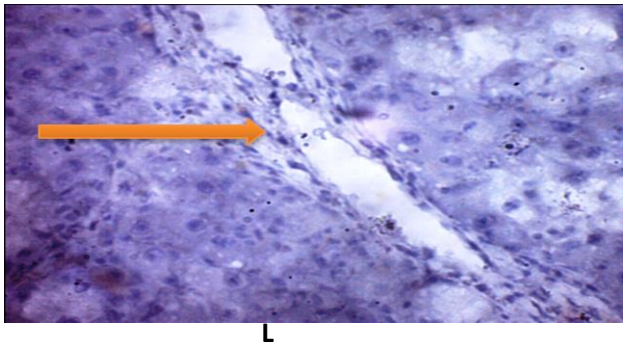


K



L

Plate 4.20: Immunohistochemical profile of representative liver tissues of the various groups shows the following: G (CO_{250}) - Dark line of significant small discontinuous bundles of collagen deposition. H (CO_{500}) - severe necrosis with no evident of cirrhosis. I (BC_{250}) - Bundles of non confluent dark staining fibroblasts laying down fresh collagen fibres. J (BC_{500}) - Severe fatty change, no collagen fibres seen. K (MM_{250}) - no evidence of cirrhosis. L (MM_{500}) Mild deposition of collagen fibres especially in the portal tracts region.

4.2 Discussion

The chemical characteristics of a food material which include the nutritive and bioactive compositions determine its quality as diet, effective adjunct, supplement or fortifying agent. Proximate analyses (Table 4.1) of the raw (R) and cooked (C) pulverized samples of *S. stenocarpa* (SS), *C. odorata* (CO), *B. coriacea* (BC) and *M. myristica* (MM) showed that they contained high amounts of carbohydrates except *M. myristica*. Carbohydrates play important roles in the provision of energy and in many other biochemical reactions (Mayes, 2000). Carbohydrates may serve as substrates for the production of aromatic amino acids and phenolic compounds through the Shikimic acid pathway and this may confer high phenolic and antioxidant potentials on the plants (Bhattacharje *et al.*, 2013). The lipid content of raw and cooked *M. myristica* is comparable to the value (27.77±2.59%) obtained by Ekeanyanwu *et al.* (2010).

Fat promotes the absorption of fat-soluble vitamins and contributes to the energy value without adding to the bulk of the diet (Ekeanyanwu *et al.*, 2010). However, excess intake of crude fat has some well-established health implications especially for the overweight (Oko *et al.*, 2012). The consumption of excess fats has been recognized as the most important dietary factor aiding increased level of cholesterol; a major risk factor related to the development of atherosclerosis and

coronary heart disease (CHD) (Bhattacharjee *et al*., 2013). Cooking decreased the lipid contents of all the investigated samples. This may be attributed to lipid loss in the water used for boiling. The lower fat contents of *C. odorata*, *S. stenocarpa* and *B. coriacea* compared to *M. myristica* are of added advantage, especially to adults and obese individuals who need to avoid fat accumulation and arterioscleriosis; invariably reducing the risk of coronary heart diseases, hypertension and other related health risks.

The protein contents of the investigated plants indicated that consumption of these plants can contribute to the formation of hormones, enzymes and immune molecules which control varieties of body functions such as growth, repair, defense and maintenance (Bhattacharjee *et al.*, 2013). Proteins also play important roles in body processes such as water balancing, nutrient transportation and muscle contraction (Bhattacharjee *et al*., 2013). In addition, plant proteins play roles in the organoleptic properties of food and may be useful as preferred options to animal proteins for diabetics, as the later tend to be high in saturated fats (Bhattacharjee *et al.*, 2013). The high concentration of protein in the raw seeds of *S. stenocarpa* was within the range 21.0 to 29.0% reported by Ajibola *et al.* (2011). Popularly consumed legumes such as chicken pea (*Cicer anetinum*), green pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*) and Lentil (*Lens culinarus*) have protein content that range from 18.5-21.9% except for

soybean that contains an average of 38% crude protein for the raw grains (Nwaoguikpe *et al.*, 2011). The protein content of *S. stenocarpa* is quite close to the recommended daily allowance (RDA) for children which ranges from 23.0-36.0 g per day (Ekeanyanwu *et al.*, 2010). The reduction in the protein contents of the plants after boiling may be due to leaching, denaturation or solubilization of some nitrogenous compounds during cooking (Nwaoguikpe *et al.*, 2011). The rich protein content of *S. stenocarpa* and its moderate carbohydrate compositions offer high nutritional benefits to the overweight and obese individuals. Studies have shown that high protein and moderate carbohydrate diet increases energy expenditure (Astrup *et al.*, 2015).

The total ash content of a food is directly proportional to the concentrations of inorganic elements present in it (Palma *et al.*, 2015). This implies that samples with high percentage ash content are expected to possess high concentrations of various mineral elements. These minerals have the advantage to speed up metabolic processes and improve growth and development (Bello *et al.*, 2008). Cooking caused a reduction in the total ash content of the samples, especially in *B. coriacea* which required the longest cooking time.

Water storage within plant tissues implies succulence and moisture content determines the storage period and rate of microbial action of a plant product

(Atwell *et al.*, 1999). Low moisture content enables plant products to be stored for longer periods due to reduced rate of metabolic activities and microbial actions (Ramaswamy, 2015; Rawat, 2015). Cooking prior to drying increased the moisture content of the samples, which may be due to water absorption during boiling.

The appreciable level of carbohydrates, lipids, protein and ash contents in the plant seeds and leaves investigated confirmed that the selected samples are good sources of nutrients and have high dietary values.

Phytochemical analysis is very useful in the evaluation of some biologically active components of medicinal and edible plants. The results of the qualitative (Table 4.2) and quantitative (Table 4.3) analyses of the raw and cooked extracts of the pulverized samples of *S. sphenostylis, C. odorata*, *B. coriacea* and *M. myristica* revealed the presence of secondary metabolites, such as flavonoids, phenolics, alkaloids, saponins and tannins, which might be responsible for their therapeutic effects. Phenolic compounds are regarded as antioxidants as well as anti-inflammatory, anti-microbial, anti-angionic, anti-allergic and anti-cancer agents (Hossain *et al.*, 2013). Appreciable levels of phenolic compounds in the selected plants indicated that they are potential good sources of antioxidants that can prevent oxidative cell damage. The total phenolic content of the leaf extract of *C.*

odorata was the highest and the least affected by cooking compared to the seeds of *S. stenocarpa, B. coriacea* and *M. myristica*. The reduction in the phenolic contents corresponded to the time of cooking; the total phenolic content of leaves of *C. odorata*, which had the least boiling time of five minutes, was not significantly ($P>0.05$) reduced (< 1%), *M. myristica* which was boiled for ten minutes was reduced by 22.7%, while *S. stenocarpa* and *B. coriacea* were reduced by 73% and 80.5% after boiling for 1.5h and 3h respectively.

Tannins are phenolic compounds and are regarded as primary antioxidants or free radical scavengers (Hossain *et al.*, 2013). They are reported to hasten the healing of wounds and inflamed mucous membranes (Okwu and Josiah, 2006), through lowering serum lipid level, modulation of immune responses and acceleration of blood clotting, (Chung *et al.*, 1998). Tannin-rich forages are also known to increase protein uptake and reduce gastrointestinal infections in grazing ruminants (Katiki *et al.*, 2013). The antimicrobial and antihelminthic activities of tannins are also well documented (Akiyama *et al.*, 2001; Katiki *et al.*, 2013; Min *et al.*, 2015).

Flavonoids are also polyphenolic compounds with high potent free radical scavenging activity, anti-inflammatory, anti-allergic and anti-cancer activities (Yamamoto and Gaynor, 2001; Chataikun and Chiabchalard, 2013). Flavonoids mediate their effects by donating hydrogen or electron to free radicals, hence their

biological activities depend on their configuration and total number of hydroxyl groups about their nuclear structure (Kumar and Pandey, 2013; Li *et al*., 2014). The highest content of flavonoids in *C. odorata* may be due to the plant part used. Fruits and vegetables are known as the main sources of flavonoids (Chataikun and Chiabchalard, 2013). Similarly, cooking caused a reduction in the flavonoid content of all the selected samples. This may be due to thermal effect on the structural configuration of the different flavonoid subgroups present in the plants, as observed in the qualitative test, where anthocyanin was absent in cooked *S. stenocarpa*. According to Lee and Ho (2002), most bioactive health promoting substances are unstable during processing; they tend to undergo reactions such as oxidation, hydrolysis, thermal degradation and maillard reactions which may result to a change and reduction in their bioactivity.

Alkaloids serve as anti-proliferative and anti-metastatic agents (Lu *et al.*, 2012), as well as powerful analgesics and anti-spasmodic agent (Shoaib *et al.*, 2016). Hepatoprotective effects of alkaloid fraction of methanol extract of *Hygrophilia auriculata* leaves has been reported (Raj *et al*., 2010). The higher concentrations of alkaloids in *M. myristica* and *C. odorata* support their use as medicinal plants and may add to the hepaoprotective and anti-fibrotic effects of the plants.

Saponin has the properties of precipitating and coagulating red blood cells, hemolysis, binding cholesterol, and bitterness (Eleazu *et al.*, 2012). Steroidal saponins are used in the commercial production of sex hormones for clinical use (Sarker and Nahar, 2007). The moderate amounts of saponin in all the plants investigated suggest that they may not be deleterious to the consumer. Though poisonous, saponins are reported to be therapeutically important as they are shown to have hypolipidemic and anticancer activities (Sarker and Nahar, 2007).

High values of oxalate observed in raw *S. stenocarpa* and raw and cooked *C. odorata* are suggestive of potential health hazard to humans and livestock. Oxalates are anti-nutrients which possess bitter and acrid taste besides causing irritations to the mucous membrane and binding to minerals, thereby reducing their absorption (Doughari, 2012). Formation of complexes with divalent cations results to minute crystals but may form larger kidney stones. Thus, chronic oxalate ingestion in animals has been associated with renal fibrosis and failure (Hagler and Robert, 1973). Cooking had no significant effect on the oxalate content of *C. odorata*, contrary to the observation made in *S. stenorcapa*, *M. myristica* and *B. coriacea*. This could be attributed to the cooking time. The oxalate contents of *B. coriacea*, *S. stenorcapa*, *M. myristica* and *C. odorata*, were reduced by 82.96%,

58.9%, 45.3% and 8.27%, respectively. Thus, cooking reduced the oxalate contents of the plants which is desirable to forestall potential health hazard.

Antioxidants are substances that delay, prevent or remove oxidative damage to a target molecule either by directly scavenging free radicals or inhibiting their production or indirectly up-regulating antioxidant defenses against free radicals (Halliwell, 2007). They potentiate the activities of free radical scavengers, thereby preventing microsomal lipid peroxidation, liver fibrosis, liver necrosis and hepatic inflammation (Adikwu and Deo, 2013). Ascorbic acid is one of the most effective naturally occurring antioxidants that are known to inhibit lipid peroxidation (Veeru *et al.*, 2009; Talukder *et al.*, 2013). It scavenges all relevant free radicals and chelates heavy metal ions (Veeru *et al.*, 2009; Ipsen *et al.*, 2014). Ascorbic acid is also known to play important roles in enzymatic reactions. Its role as a cofactor in proline and lysine hydroxylation is regarded a major regulator of collagen biosynthesis (Singal *et al*., 2011; Adikwu and Deo, 2013). The quantitative determination of ascorbic acid in the raw and cooked samples (Table 4.4) showed that the plants except *B. coriacea* are good sources of vitamin C. The highest quantity of ascorbic acid obtained in the leaves of raw *C. odorata* when compared to the seeds of *S. stenocarpa, B. coriacea* and *M. myristica* may be related to the nature and part of the plant materials used. Cooking reduced the ascorbic contents of *S. stenocarpa*, *C. odorata*, *M. myristica* and *B. coriacea* by 79.6%, 62%, 12%

and 4.35%, respectively. The result is in line with the report of Hwang *et al.* (2012) and Mitic *et al.* (2013). The ascorbic acid level of raw *B. coriacea* which was significantly lower than other samples and was not significantly ($P<0.05$) affected by processing though it underwent the longest cooking period of 3h is an indication of very low ascorbic acid content. Deficiency in ascorbic acid could result to the weakening of the endothelial wall of the capillaries leading to clinical manifestations such as scurvy, haemorrhage from mucous membrane of the mouth and gastrointestinal tract, anaemia and pains in the joints (Veeru *et al.,* 2009).

Similarly, the highest content of vitamin E observed in raw *C.odorata* may be as a result of the plant part used. Vitamin E serves as antioxidant by reacting with and stabilizing free radicals such as hydroxyl, alkoxyl and lipid peroxyl. It forms a pheny radical which can be neutralized and its activity regenerated by vitamin C (Lu *et al.,* 2010). Cooking caused 30.6 %, 24.2%, 10.97% and 7.08% reduction in the vitamin E content of *M. myristica*, *B. coriacea*, *C. odorata* and *S. stenocarpa,* respectively. Compared to the vitamin C content, the effect of cooking was mild on vitamin E and did not correspond with processing time. This fat-soluble vitamin is absorbed from the gut with the aid of bile salts and transported in the blood stream via chylomicrons to the various tissues (Mayes, 2000). Its role is attributed to its

ability, as antioxidant, to quench free radicals and reduce the rate of lipid peroxidation in the liver (Ismail *et al.*, 2009)

Diet represents the principal route of selenium (Se) intake and levels in food reflect the soil Se concentration (Sieprawska *et al.*, 2015). In general, fruits and vegetables contain low values, while legumes and nuts serve as good sources of Se; notably is the Brazil nuts (543.5mg Se) from the Amazon region which is the recorded richest known food source of Se (Stockler-Pinto *et al.*, 2014). The appreciable contents of Se in the selected plants (Table 4.4), though below that of the Brazilian nuts, are indication that these plants can serve as good sources of selenium. Selenium (Se) is believed to exert a number of beneficial health effects while deficiency can cause reproductive disorders in humans and animals (Mehdi *et al.*, 2013). Selenium is known primarily for its functions in redox homeostasis and is recognized as a promising cancer chemo-preventive agent (Peng *et al.*, 2016). It is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. The best known biological role of Se is attributed to its presence in glutathione peroxidase (GPx), which is one of the enzymes that protect membrane lipids and other cellular and extracellular components from oxidative damage (Stockler-Pinto *et al.*, 2014). Selenium also stimulates antibody formation and activity of helper T cells, cytotoxic T cells and Natural Killer (NK) cells

(Mehdi *et al.*, 2013). The reason for the reduced Se contents of SS and MM as well as the increased Se content observed in CO and BC could not be ascertained in this study.

Changes in physiology of the seeds are related to the integrity of their cell membranes, which in turn, depend on the nature of the enzymes and structural proteins of various species (Marques *et al.*, 2014). Under normal physiological conditions, the oxidative stress produced is fought by a complex antioxidant defense system, related to increased production and activation of enzymes that catalyze the conversion of hydrogen peroxide (H_2O_2) into water (H_2O), thereby protecting cells from oxidative damage. During the process of seed storage deterioration, there is a decrease in enzyme activity by its progressive inactivation or reduction and stoppage of its synthesis (Marques *et al.*, 2014). Catalase (EC1.11.1.6) activity is considered useful in the determination of seed viability and quality (Ayse *et al.*, 2012). The presence of multiple isoforms of catalase has been reported for a number of plant sources such as spinach, mustard, maize, sunflower, castor bean, etc (Tayefi-Nasrabadi *et al.*, 2011). Low catalase activity generally observed might be due to the effects of boiling and drying temperatures applied in this study. Catalase is reportedly inactivated at temperatures above 40˚C, and has optimum temperature of 30˚C (Dincer and Aydemir, 2001). Catalase isolated from *Agaricus bisporius was* found to retain 26.98 units/ml of its activity after being

heated at 10 - 30˚C for 60 min (Susmitha *et al.*, 2013). The thermal instability of catalase became evident in the cooked samples where the catalase activity was completely inactivated as in SS or might be considered negligible as in CO, BC and MM. Similar to catalase, the SOD activities of the pulverized samples were quite low. SOD is also a heat-sensitive enzyme (Sharma *et al.*, 2014). Maximum activity at 30˚C has been reported for SOD isolated from *Amaranthus spinosus;* with temperature stability lying between 20 - 30˚C (Sharma *et al.*, 2014).

Dietary components that can scavenge ROS/RNS to stop radical chain reactions are considered as primary or chain-breaking antioxidants, while those that can inhibit the reactive oxidants from being formed (e.g metal chelators) are called secondary or preventive antioxidants (Basha *et al.*, 2011). Various forms of ROS such as superoxide anion radical ($O_2^{\cdot}$), hydroxyl radical (OH) and non-free radical species such as hydrogen peroxide (H_2O_2) (Huda-faujan *et al.*, 2009) and nitric oxide (NO) can contribute to hepatic fibrosis (Paik *et al.*, 2014). Superoxide radical is a precursor to more reactive oxygen species and contributes to tissue damage and various diseases (Hussein *et al.*, 2011). Superoxide anion radicals are produced endogenously by flavoenzymes such as xanthine oxidase and can be eliminated by superoxide dismutase (Basha *et al.*, 2011). The superoxide radical scavenging activities increased in a dose-dependent manner in all the samples (Fig 4.1). At

high concentrations of 400µg/ml and 800µg/ml, most of the extracts showed higher activities than the standard ascorbic acid. Except for CCO, the plant extracts showed strong superoxide scavenging activity. This property is of great importance in preventing the generation of more oxygen species as well as radical chain reactions that could lead to tissue damage and fibrosis.

Hydrogen peroxide is an intermediate during endogenous oxidative metabolism. Though it has only a weak activity to initiate lipid peroxidation, it has the potential to mediate free radical formation such as highly reactive hydroxyl radical through Fenton reaction (Hussein *et al.*, 2011). Fig 4.2 shows that the raw and cooked samples of SS, BC and CO were capable of scavenging H_2O_2 radicals in concentration-dependent manner but RMM exhibited highest activity at 200 µg/ml indicating high activity at low concentration. The cooked samples of CO and MM had higher activities at high concentrations of 400 and 800µg/ml.

Hydroxyl radicals are the most reactive chemical species known. They induce some oxidative damage to biomolecules, such as proteins and nucleic acids (Hussein *et al.*, 2011). Concentration-dependent inhibition activities were observed in the investigated samples (Fig 4.3). The raw samples of SS, MM and BC exhibited higher radical scavenging activity than their cooked samples and at 800 µg/ml, their activities were higher than the standard ascorbic acid. This implied

that prolonged cooking may have destroyed some antioxidant components of the plants. The CCO which had the least cooking time showed increased hydroxyl radical scavenging activity.

Nitric oxide (NO), being a potent pleiotropic mediator in physiological processes and a diffusible free radical in pathological conditions, reacts with superoxide anion to form a potentially cytotoxic molecule, the 'peroxynitrite ($ONOO^-$)', a very strong oxidant (Basha *et al.*, 2011). The main route of damage is the nitration or hydroxylation of aromatic compounds, particularly tyrosine. Under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid (Basha *et al.*, 2011). This is responsible for oxidative damage of proteins in living systems (Basha *et al.*, 2011). The data (Fig 4.4) show that the extracts exhibited high NO scavenging activity and the activity increased with increase in extract concentration. The NO scavenging activity of the extracts was similar to that of the reference standard butylated hydroxyltoluene (BHT). At concentrations of 400µg/ml and 800µg/ml, the activities of raw and cooked *S. stenocarpa* were higher than the value of BHT.

The abilities of the sample extracts to transform Fe (III) to Fe (II) were between low to moderate compared with the synthetic antioxidant (BHT) (Fig4.5). Lower reducing abilities were observed in the cooked samples than the raw samples

except CMM which may be as a result of the different types of phenolic compounds present and the effect of processing methods on the phenol-types. For instance, *C. odorata* (CO) which had the highest phenolic content among the raw and cooked samples recorded the least reducing power. The ability of plant extracts to reduce Fe (III) has been attributed to hydrogen donation from phenolic compounds and the position of the hydroxyl group of phenolic compounds (Huda-Faujan *et al.*, 2009).

The high antioxidant activities of the crude extracts relative to the standard antioxidants attested to the popular use of preparations from *B. coriacea, M. myristica*and *C. odorata* in the local treatment of different health problems by people of diverse background.

Body and organ weight changes serve as sensitive indicators of the general health status of animals (Harlita *et al.*, 2016). The increase in the body weights of the extract-fed groups, which progressed more than the controls (Fig 4.6) till the introduction of CCl_4 at the end of the 4^{th} week, could be as a result of the extra proteins and other useful nutrients and biological molecules that aid food absorption that were obtained from the extracts. The raw samples were observed to contain high amounts of protein (Table 4.1), particularly *S. stenocarpa*. At

higher concentration of extracts, more proteins would be present. This may account for the better growth of the raw extract-treated groups and highest growth rate seen in the groups fed with 500 mg/kg RSS. The reduced growth rate with increase in concentrations of RCO and RMM could be as a result of the anti-nutrient contents of the extracts. High content of oxalates was observed in the raw extracts of *C. odorata* (RCO).

The fluctuations in weight gain of the extract-treated groups after the introduction of CCl_4 which corresponded with marked increase in the weight of the liver (Fig 4.7) indicated the negative effect of the toxin (CCl_4) on the internal organs of the animals, and probably the general discomfort which led to a low feeding rate. The increase and reduction in growth rate of the animals was an indication of recuperation or regeneration of internal organs before a succession of another CCl_4 dose which caused a relapse. The administration of a foreign chemical compound may bring about significant changes in the metabolic transformation and concentration of biomolecules, enzymes and even metabolic pathways (Shama *et al.*, 2013). Usually after an acute liver damage, parenchymal cells regenerate and replace the necrotic and apoptotic cells. When the injury persists, the regenerative response fails and the hepatocytes are substituted by abundant extracellular matrix (ECM) (Reeves and Friedman, 2002; Seki and Brenner, 2015). The ability of the liver to regenerate is crucial to protect liver function after injury and during chronic

disease (Wang *et al.*, 2012). Silymarin-treated group and many of the groups treated with cooked extracts recovered from weight loss after the 2nd week of CCl_4 intoxication. This could be attributed to regenerative response and built up resistance against the toxin due to biologically active substances present in the extracts.

Assessment of hematological parameters can be used to determine the extent of deleterious effect of foreign compounds including plant extracts on blood (Shama *et al.*, 2013). The observed non-significant ($P>0.05$) effect of plant extracts on the Hb concentrations of animals that were not exposed to CCl_4 except for slight reductions by 250 mg/kg b.wt of CBC (Fig 4.8) were indications that these extracts did not affect oxygen transportation of the animals. Higher red blood cell counts recorded for animals that were fed with raw and cooked extracts of SS and MM (Fig 4.9) may be due to intermediates or products in their metabolism that could have stimulated the kidney to form and secrete erythropoietin, the humoral regulator of RBC production (Shama *et al.*, 2013). The observed concentration-dependent increase in the platelet counts of the non-intoxicated animals fed with plant extracts except those of cooked BC and SS (Fig 4.11) are indications that the plant extracts have the potential to stimulate the biosynthesis of clotting factors. Therefore, they may be useful in the treatment of haemorrhage (Salawu *et al.*, 2010). The fresh leaves or decoction of RCO are exploited extensively in West and

Central African ethnopharmacy for haemostasis and wound healing (Pandith *et al*., 2013). However, an increase in platelet counts (thrombocytosis), which is usually attributed to a myelo-proliferative disorder (primary) or reactive process (secondary), may result to heart attack, stroke or increased danger of thrombosis and haemorrhage (Gregg and Goldschmidt-Clermont, 2003).

Similar to the report of Saba *et al.* (2010), CCl_4-intoxication caused significant ($P<0.05$) reductions in the Hb, RBC and platelet counts with increased WBC counts (Figs 4.8 – 4.11). Normally, CCl_4 administration produces oxidants that promote inflammation and toxicity by protein oxidation and enzyme inactivation (Brattin *et al.*, 1985). The stress-induced damage due to continued administration of CCl_4 could account for the reductive effect on Hb concentration, RBC and platelet counts, as well as the high WBC counts. Neutrophilia as a result of influence of stress hormones, cortisol and catecholamine has been reported to occur in CCl_4-intoxicated rats (Saba *et al.*, 2010). Treatment with raw and cooked extracts of the plants improved the Hb concentrations, RBC and platelet counts, thereby revealing the potentials of these extracts to reduce the toxicity effect of CCl_4. Higher improved effect of the raw extracts on the Hb concentrations to values similar to those of the control groups showed that the raw samples contain more biologically active molecules that are capable of activating the bone marrow. The marked increased in the WBC counts of the intoxicated rats are indications of

inflammatory response to CCl_4 toxin. The reductive effects in the WBC counts of the groups treated with silymarin and the various extracts showed that the extracts possess some antioxidant properties that can protect against inflammation. Dose dependent increase in WBC counts of animals treated with RSS and RBC may indicate toxicity with increase in concentrations of the extracts while the increase observed with 250 mg/kg CCO treatment could be attributed to individual differences (Bardo *et al.*, 2013).

Liver damage is characterized by elevated activities of liver enzymes in the blood (Akanbi, 2015). The liver enzymes found within the tissues of the organ leak into the bloodstream following cellular necrosis and increased cell membrane permeability and are used as diagnostic measure of liver damage (AbouGabal *et al.*, 2015). Usually, about 80% of AST is found in the mitochondria and appears in higher concentrations in a number of tissues (liver, kidneys, heart and pancreas), whereas ALT is localized primarily in the cytosol of hepatocytes and is considered a more sensitive marker of hepatocellular damage than AST (Thapa and Walia, 2007). Serum ALP on the other hand serves as a check for possible liver dysfunction, though it is a marker for bone disease, since the mucosal cells that line the billiary tract and gall bladder release ALP into the blood stream during intrahepatic and extrahepatic cholestasis (biliary obstruction) or malfunctions

(Salawu *et al.*, 2010**).** The dose-dependent increase in AST, ALT and ALP activities observed in the non-intoxicated animals administered raw extracts (Figs 4.12 – 4.14), showed that the continuous administration of these extracts at high doses may result to hepatotoxicity in the animals. The extracts did not affect bilirubin levels but increased the total protein and albumin concentrations of the non-intoxicated animals (Table 4.4). These may indicate that the liver functions were not adversely affected by the extracts. The increase in the enzyme activities, bilirubin concentrations as well as reductions in the protein and albumin concentrations of the intoxicated animals reflect impaired liver functions, which could be as a result of possible damage to the hepatocytes induced by CCl_4 (Anosike *et al.*, 2008). Reversals of the CCl_4 effects on the liver parameters of the intoxicated groups treated with silymarin, raw and cooked extracts of the samples suggested amelioration of liver damage and protective effect of the drug and extracts against CCl_4-induced toxicity. It may also be noted that the extracts increased the protein synthetic function of the liver in cases of injury resulting in enhanced haemostasis, prevention of ascites and deposition of fats in the liver (Salawu *et al.*, 2010).

The increase in ALP activities of all the intoxicated groups administered 500 mg/kg b.wt of the raw extracts, when compared to the controls, may be as a result

of high concentration of harmful substances or anti-nutritional factors which were not destroyed during the cooking process that could have caused biliary obstruction or malfunction. The reduction in ALP activities of the animals treated with cooked extracts shows that no possible cholestasis occurred at the dose levels tested.

The kidney is the primary organ for clearance and excretion of non-volatile waste products of metabolism, as well as a major regulator of electrolyte and water balance in the body (Dabla, 2010). The study showed that treatment with plant extracts and the extract vehicle (olive oil) increased the urea concentrations without affecting the creatinine concentrations of the non-intoxicated animals in comparison with the normal saline (Table 4.5). Urea is a by-product of protein metabolism, while creatinine is a product of creatine metabolism in the muscle (Dabla, 2010). Normally, creatinine is completely filtered by the kidney, so an alteration in the blood concentration can be a strong indication of renal health. Thus, the increase in the blood urea nitrogen (BUN) without a corresponding rise in the creatinine may be indicative of increased breakdown of protein, dehydration or low renal perfusion rate (Noori *et al.*, 2014). The elevations in the serum urea, creatinine, sodium (Na^+), potassium (K^+), chloride(Cl^-) and bicarbonate concentrations (Table 4.6) following intoxication, was an indication of renal dysfunction and nephrotoxicity by CCl_4. Oral treatment with the methanol extracts

twice a week conferred nephroprotection in the CCl_4 exposed rats in a dose-dependent manner. The marked elevation of serum Na^+ concentration may be as a result of excessive loss of Na^+ from intracellular fluids. It may also be attributed to increased production of aldosterone and other mineralocorticoids which in turn increase the reabsorption of Na^+ through the stimulation of Na^+/H^+ exchanger (Yakubu *et al.*, 2003). The concommitant increase in serum K^+ concentration with increase in Na^+ suggests a possible adverse effect on the sodium pump that maintains the low extracellular concentration of potassium (Yakubu *et al.*, 2003).

It has been suggested that oxidative stress can have profound effects on body haemodynamics and renal function (Natarajan *et al.*, 2006). Literature has shown that medicinal plants with nephroprotective properties mediate renal protection via antioxidant and/or free radical scavenging activities (Naggayi *et al.*, 2015). Treatment with antioxidants such as vitamin E has been reported to prevent renal dysfunction and normalize mean arterial pressure, renal blood flow, glomerular filtration rate (GFR), and sodium and water retention in animals (Natarajan *et al.*, 2006). It is possible that the protective effect of the methanol extracts mediated their nephroprotection through antioxidant and free radical scavenging activities.

The reductions in plasma concentrations of cholesterol and triacylglycerol as well as increase in the HDL cholesterol of the intoxicated groups treated with extracts

(except the HDL cholesterol concentrations of the animals treated with cooked CO) when compared to negative and positive controls (Figs 4.15 - 4.17) revealed the lipidaemic protective effect of the extracts against degenerative diseases. Various studies have indicated that serum levels of cholesterol are strongly related to coronary atherosclerosis and increased risk of coronary heart disease (Ojiako and Nwanjo, 2009). This is because high amounts of cholesterol and other saturated fatty acids in blood tend to cause the accumulation of lipids in walls of the arteries thereby causing a narrowing of the blood vessels and a resultant effect on blood pressure (Ogunka-Nnoka and Igwe, 2013). Clinical studies in humans have shown that lowering levels of serum cholesterol with diet or drugs decreases the incidence of coronary heart disease (Ojiako and Nwanjo, 2009). High concentrations of HDL cholesterol have been reported to be inversely related to the incidence of coronary heart disease and may foster the removal of cholesterol from peripheral tissues to the liver for catabolism and excretion (Bako *et al.*, 2014). This observation could be attributed to the phytochemical content of the plant extracts. Saponin, tannin and flavonoid fractions are known to lower serum lipid levels (Chung *et al.*, 1998; Nwankpa *et al.*, 2012; Ibegbulem *et al.*, 2013). Hypolipidaemic properties of *Monodora myristica* (Oyenibe *et al.*, 2015), *Buchholzia coriacea* (Olaiya and Omelaka, 2013) and *Chromolaena odorata* (Nwankpa *et al.*, 2012) have been reported.

The concentration of plasma MDA, an index of lipid peroxidation (LPO), was determined in this study in order to evaluate whether the repeated oral exposure to raw and cooked extracts of SS, MM, BC and CO were capable of pre-disposing the experimental animals to oxidative stress, and consequent effect of CCl_4 intoxication.The significant increase observed in the non-intoxicated rats administered 250 mg raw and cooked extracts of CO as well as those administered 500 mg of raw BC (Fig 4.18) were indications of occurrence of oxidations that could have lead to imbalance in the antioxidant/free radical ratio in the animals. The elevation of MDA levels in the negative control (CCl_4-olive oil mixture only) after 28 days of CCl_4 intoxication when compared to normal control, showed that CCl_4 intoxication enhanced the level of lipid peroxidation. Several reports showed that CCl_4 triggers a cascade of oxidative and lipid peroxidation reactions, which lead to cell injury; including chronic liver injury with resultant liver fibrosis (Wei *et al.*, 2015). However, treatment with silymarin and methanol extracts of the plants significantly ($P<0.05$) reduced the MDA levels of the treated rats indicating their protective effect against lipid oxidation (Zhang *et al.*, 2013).

The decrease in the level of catalase (CAT) activity in the intoxicated rats especially the negative control compared to the non-intoxicated groups (Fig 4.19),

may be attributed to the need for increased free radical scavenging activities of the antioxidant enzymes in order to protect the animals against oxidative damage caused by CCl_4. On the other hand, the improvement in catalase activity by treatment with 250 mg of the different cooked extracts may be indications of reduced oxidative stress in the animals. Reduction in CAT may be due to inflammation which caused increase in generation of intracellular H_2O_2, thereby enhancing oxidative stress. CAT is a major free radical scavenging enzymes noted to be reduced in a number of pathophysiological processes and diseases (Ganie *et al.*, 2011). SOD converts superoxide radical which is highly reactive to H_2O_2 while catalase eliminates the H_2O_2 before it gets converted to the strongest oxidant ·OH via the Fenton reaction (Ha *et al.*, 2010). Thus, activation of catalase by the administration of methanol extracts of SS, MM, BC and CO to normal rats showed that through their free radical scavenging activity, they can exert beneficial action against physiological alterations caused by the presence of superoxide, hydrogen peroxide and hydroxyl radicals. Oyenibe *et al.* (2015) reported significant increase in CAT activity of male Wistar rats administered aqueous *Monodora myristica* extract, which ameliorated oxidative stress induced by hypercholesterolemia due to its polyphenol content. Many investigations have shown that CCl_4 intoxication results in changes in expression level and consequently decreases the levels of antioxidant enzymes in liver tissue.

Lactate dehydrogenase is a tetrameric enzyme involved in the conversion of lactate to pyruvate for energy production under anaerobic conditions (Valvona *et al.*, 2016). It is present in all cells of the body with highest concentration in heart, liver, muscle, kidney, lung and erythrocytes but excess LDH activity which could result to acidosis represents increased anaerobic metabolism due to tissue hypoperfusion and reflects extent of tissue injury (Drent *et al.*, 1996; Zein *et al.*, 2004). Serum LDH activity is elevated in a number of clinical conditions and is useful in monitoring tissue damage and disruption (Drent *et al.*, 1996), such as muscle damage or necrosis, hemolysis, liver disease, renal tubular necrosis, pyelonephritis, and malignant neoplasia (Anadon *et al.*, 2014). The increase in LDH activity observed in the serum of non-intoxicated animals fed with raw plant extracts of SS and CO more than their corresponding cooked samples showed that methanol extracts of these plants caused tissue disruptions in the animals. Elevation in the LDH activity of all the CCl_4 intoxicated rats (Fig 4.20) indicated that repeated injection of CCl_4 twice a week for 28 days elicited tissue damage that resulted to increased expression of LDH. However, raw and cooked extracts of the plants reduced the expression of LDH to varying degrees in dose-dependent manners. The MM and BC were found to have had better reductive effects on LDH activity than silymarin signifying that MM and BC have high protective effect against tissue damage.

Histological examination is the golden standard for evaluating treatment-related pathological changes in tissues and organs (Salawu *et al.*, 2009). The mild to severe alterations in the kidney and liver tissues of toxicated and non-toxicated rats suggested varying degrees of disturbance in physiological parameters of the tissues. Necrosis, lymphatic infiltration and inflammatory response are common non-specific protective immune responses to tissue injury (Imig and Ryan, 2013). Hence the dose-dependent immune responses seen histologically with extracts-fed non-intoxicated rats were confirmation that the extracts were capable of eliciting deleterious inflammatory effects on the kidneys and liver at higher concentrations. The increased immune response and tissue alterations presented by the animals after intoxication were indication of inflammatory damage by CCl_4 among other likely mechanism of toxicity not elucidated in this study. Vascular congestions, hemorrhagic necrosis, interwoven tubules and loose cortex seen in the kidney tissues of rats treated with raw and cooked extracts of CO were indications of excessive inflammation. According to the report of Afsar *et al*. (2015), inflammation may promote and be a consequence of renal congestion. It may also cause vascular dysfunction via endothelial activation and enhanced arterial stiffness, reduced myocardial contractility and increased myocardial cell death. These may contribute to progressive renal dysfunction, fibrosis and increased endothelial permeability, thus facilitating fluid extravasation. The result obtained

in this study agree with those of previous studies suggesting that inflammation is one of the mechanisms of CCl_4 induced hepatic injury, which present signs such as severe hepatocellular degeneration, necrosis and mononuclear cellular infiltration (Abdel-Ghaffar, 2013). However, silymarin and methanol extracts of SS, MM, BC (both concentrations) and higher concentration of CO ameliorated the effect of CCl_4. The liver is capable of regenerating damaged tissues and liver function may not be impaired early following an insult from a toxicant (Salawu *et al.*, 2009). The liver tissue of rats treated with CBC 500 mg/kg b.wt had features of slight regenerative changes, suggesting that cooked BC was capable of promoting hepatocyte regeneration; a useful therapeutic strategy for liver fibrosis and cirrhosis (Zhou *et al.*, 2014). The ability of the liver to regenerate is crucial to protection of liver function after injury and during chronic disease. Increases in hepatocyte growth factor (HGF, an antifibrotic growth factor that induces apoptosis in hepatic stllate cells) in liver sinusoidal endothelial cells (LSECs) is thought to drive liver regeneration (Wang *et al.*, 2012).

Collagen is a key marker of liver fibrosis and CCl_4-induced liver fibrosis is a well established model of hepatotoxicity (Malaguarnera *et al.*, 2012; Ki *et al.*, 2013; Abdel-Ghaffar, 2013). The marked expression of collagen fibres in the negative control showed that intraperitoneal injection of CCl_4-olive oil mixture induced

liver cirrhosis. Treatment with the different extracts (except CCO at 250 mg/kg b.wt) inhibited collagen accumulation in the process of fibrosis. Excessive accumulation of collagen and other extracellular matrix (ECM) is encountered as a result of increased expression of Transforming Growth Factor (TGF-β) by activated HSC (Fritz, 2008). The over expression of TGF-β, has been correlated with the degree of fibrosis in both animal models and human diseases (Wei *et al.*, 2015). During liver inflammation due to paracrine signals from immune cells consequent to injury, ROS-sensitive cytokines get activated and contribute to hepatic stallate cell (HSC) activation and responsiveness to Platelet Derived Growth Factor (PDGF) and TGF-β (Ha *et al.*, 2010). The potent mitogen-PGDF and fibrogenic factor-TGF-β through their signaling pathway release more ROS in the system, thus inducing more stress due to oxidation, enhancing fibrosis (Ha *et al.*, 2010). Reduced or non accumulation of collagen in the silymarin and cooked extract-treated groups (except CCO at 250 mg/kg b.wt) indicated that methanol extracts of SS, MM and BC inhibited trans-activation of HSC, thus antagonizing significantly the ability of CCl_4 to increase levels of TGF-β and PDGF (Ki *et al.*, 2013). The accumulation of collagen in rats treated with 250 mg/kg b.wt of cooked CO showed that the plant extract may contain some toxic biologically active components which were not destroyed within the short period of cooking.

The data obtained in this study is in agreement with the report of Malaguarnera *et al.* (2012) which stated that the main pathogenic mechanisms responsible for functional damage caused by solvents such as CCl_4 are inflammation, dysfunctional cytochrome P450 and oxidative stress. Therefore, anti-fibrotic and hepatoprotective effects of the extracts involved mechanisms like anti-inflammatory reactions, immunosuppressive activities, inhibition of fibrotic gene expression and protection of hepatocyte regeneration.

The safety and effectiveness of drugs as well as plants interventions in human subjects can only be speculated from careful synthesis of data from animal studies (Ioannidis, 2012). Exposure of animals to chemical substance allows researchers to extrapolate the possible human response to the same substance (Ioannidis, 2012). Based on the high degree of genomic and physiological similarities between rats and humans, rats play an important role as model organisms in preclinical drug development and biomarker discovery (Blais *et al.*, 2017). In *vitro* and in *vivo* results obtained from this study provide useful information for risk assessment and therapeutic model.

CHAPTER FIVE

CONCLUSION AND RECOMMEMDATION

5.0 CONCLUSION

Evidence provided in this study indicated the induction of fibrosis within 28 days of repeated administration of CCl_4. This resulted in increased activities of hepatic marker enzymes and LDH, as well as increased serum concentrations of MDA, bilirubin, urea, creatinine and electrolytes with concomitant reduction in the total protein, albumin and catalase activity. Concomitant treatment with silymarin and methanol extracts of *S. stenocarpa*, *B. coriacea* and *M. myristica* especially at low concentration, ameliorated most of the altered biochemical variables induced by CCl_4 intoxication. The possible mechanisms underlying their protective actions were inferred to be antioxidation and anti-inflammation. These actions were attributed to the phytochemical components of the extracts. Though the cooking process applied in this study reduced most of the nutritive and bioactive components of the samples, it was of added advantage in destroying the anti-nutritional factors suspected to be the reason for the adverse effect of the extracts of *C. odorata*. Hence, this study is a proof to support the potentials of *S. stenocarpa*, *B. Coriacea* and *M. Myristica* seeds as anti-fibrotic and hepato-protective agents.

5.2 CONTRIBUTION TO KNOWLEDGE

i) The study showed that methanol seed extracts of *S. stenocarpa*, *B. coriacea* and *M. myristica* possess anti-fibrotic and hepato-protective properties.

ii) The study also showed that raw *C. odorata* exhibited toxic effect despite high compositions of antioxidants and bioactive substances, which may be due to anti-nutritional factors present.

5.3 RECOMMENDATIONS FOR FURTHER STUDIES

i) It is suggested that fractionation of bioactive principles with antioxidant properties be extracted from the raw extracts for more clinical studies.

ii) Further studies to unveil the molecular mechanisms through which the extracts elicit their anti-fibrotic and hepato-protective effects will be essential for the development of effective therapeutic approaches.

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APPENDICES

APPENDIX 1

Images of plant materials used

Monodora myristica

Chromolaena odorata

Buchholzia coriacea

Sphenostylis stenocarpa

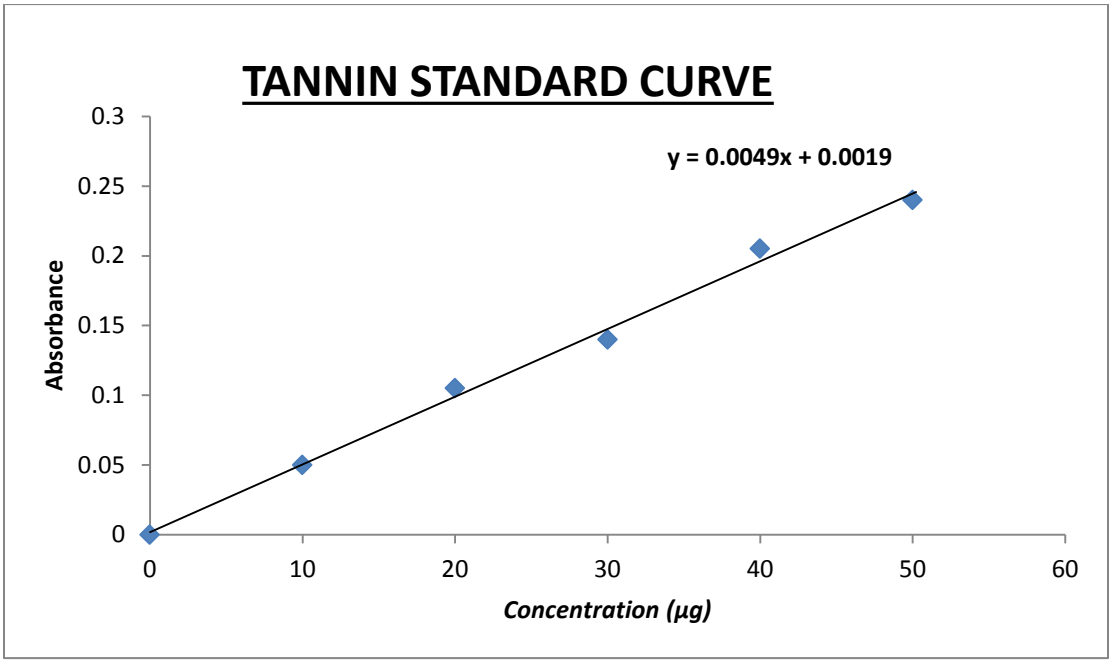
Weight of samples and Percentage yield of extracts

S/no	Plant sample	Weight of sample (g)	Percentage yield of extract
1.	*S. stenocarpa**	400	16.55
2.	*S. stenocarpa*	400	6.88
3.	*C. odorata**	200	15.17
4.	*C. odorata*	200	18.71
5.	*B.coriacea**	400	15.25
6.	*B. coriacea*	400	8.24
7.	*M. myristica**	400	31.68
8.	*M. myristica*	400	22.0

* indicate raw samples

APPENDIX II

Graphs



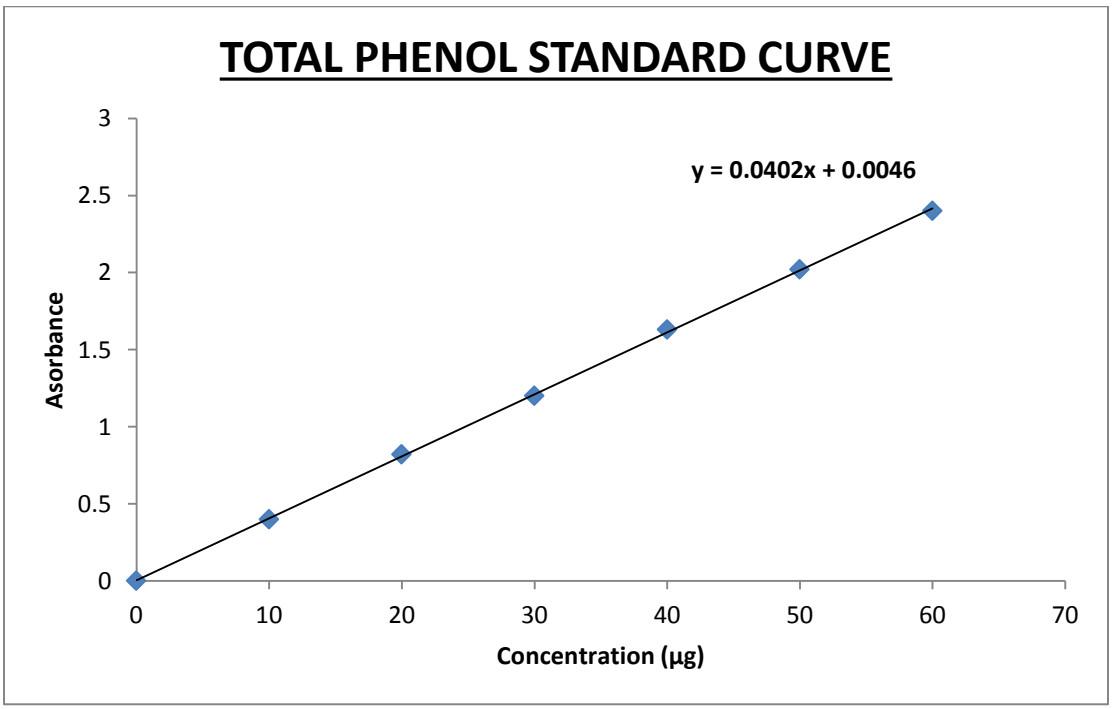


Table for calculation of aspartate aminotransferase (AST) activity

Absorbance	U/I	Absorbance	U/I
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

Table for calculation of alanine aminotransferase (ALT) activity

Absorbance	U/I	Absorbance	U/I
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

APPENDIX III