NEUROPROTECTIVE EFFECTS OF *GREWIA carpinifolia* AGAINST VANADIUM-INDUCED TOXICITY IN MICE

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ABSTRACT

Nigeria is currently the second highest gas flaring country in the world with resultant environmental vanadium discharge. Vanadium toxicity is implicated in neurodegenerative changes via free radical production. The Blood-Brain Barrier permeability for synthetic antioxidants currently used in protecting the central nervous system in vanadium toxicity remains a challenge. *Grewia carpinifolia* (*GC*) possesses antioxidant property. This study was designed to investigate the protective activities of ethanol extracts of *GC* in vanadium-induced toxicity in mice.

Safe doses of *GC* leaf and stem extracts (FHI-109693) were assessed in 50 mice equally divided and administered 100, 200, 400 and 800 mg/Kg of each extract orally for 28 days using standard haematological, biochemical and histopathological methods. Distilled water served as control. Protective effects of each extract at 100 and 200 mg/Kg following vanadium toxicity in 120 mice (control, vanadium, extract-treated and standard-treated with α-tocopherol) were studied using behavioural tests (open field, hanging wire and Morris water maze), hepatic enzymes, haematological and histological parameters. *In vitro* antioxidant and lipid peroxidation activities were measured using ABTS, DPPH and TBARS assays. Pure bioactive compounds were isolated from leaf and stem extracts by TLC, open column chromatography, HPLC and characterised by NMR and MS. Brain Uptake Index (BUI) of each compound was determined. Neuroprotective and antioxidant activities of pure compounds following vanadium-induced toxicity were evaluated in a separate cohort of 72 mice equally divided; control, vanadium-treated, vanadium alongside compounds with high BUI and standard-treated groups by behavioural tests, antioxidant enzymes' activities (catalase, SOD, GPx, GSH), oxidative stress markers (MDA, NO and H_2O_2) measurements and immunohistochemical expression of Myelin Basic Protein (MBP) in the brain. Data were analysed using one-way ANOVA at $\alpha_{0.05}$.

There were no significant differences in haematological parameters at tested doses, however, significant increase in ALP (40.85±6.78 to 81.40±6.24 IU/L), congestion of hepatic sinusoids were observed at 800 mg/Kg following 28-day administration. The extracts at 200 mg/Kg increased line crossings (18.60±4.67 to 59.00±5.93), reduced rearing (73.25±7.23 to 10.00±1.82) in open field test and increased latent time on hanging wire (27.67±5.12 to 70.34±8.05 secs). It also decreased AST (81.20±10.06 to 45.00±7.07 IU/L), ALT (75.40±9.07 to 45.00±5.82 IU/L) levels, increased PCV (32.40±4.10 to 40.50±3.54%) and prevented disorganisation of Purkinje cells of the cerebral cortex caused by vanadium. The IC_{50} of ABTS, DPPH and TBARS by extracts were 0.32, 0.41, 0.21 mg/mL (leaf) and 1.98, 0.80, 0.30 mg/mL (stem), respectively. Bioactive compounds isolated were

β-spinasterol, dibutyl phthalate, β-sitosterol, benzoic acid butyl ester and stigmasterol with BUI of 70.6%, 3.5%, 76.5%, 1.1% and 87.0%, respectively. Concurrent administration of β-sitosterol and stigmasterol significantly attenuated spatial learning deficits caused by vanadium than α-tocopherol by reducing escape latency (20.66±2.13 to 37.19±4.63 secs), grooming, rearing and stretch-attend posture frequency. They also significantly increased activities of catalase (28.72±1.04 to 11.04±1.79 IU/mg protein), SOD (38.63±3.17 to 12.06±1.03 U/mg tissue), decreased oxidative stress markers and increased MBP expression.

β-sitosterol and stigmasterol isolated from *Grewia carpinifolia* crossed the Blood-Brain Barrier, exhibited potent antioxidant and neuroprotective activities. They are therefore potential candidates in treatment of vanadium toxicity.

Keywords: Vanadium toxicity, *Grewia carpinifolia*, β-sitosterol, Stigmasterol, Neuroprotective

Word Count: 498

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CERTIFICATION

We certify that this work was carried out by Olamide Elizabeth, Adebiyi in the Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan under our supervision.

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DEDICATION

This dissertation is dedicated to God Almighty; my Alpha and Omega.

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ABBREVATIONS

ABC: Avidin-biotin complex

ABTS: 2, 2-azinobis-3-ethyl- benzothiazoline-6-sulfonic acid radical scavenging activity

ACGIH: American Conference of Governmental Industrial Hygenists

AChE: Acetylcholinesterase

ACUREC-UI: Animal care and use research ethics committee, University of Ibadan

ALB: Albumin

ALP: alkaline phosphatase

ALT: Alanine Aminotransferase

ANOVA: Analysis of variance

AST: Aspartate aminotransferase

Aβ: amyloid-β peptide

BBB: Blood-brain barrier

BMVEC: Brain micro-vascular endothelial cells

BUI: Brain Uptake Index

BUN: Blood urea nitrogen

CNS: Central Nervous System

CSF: Cerebrospinal fluid

DAB: 3, 3'- diaminobenzidine

DEPT: Distortionless enhancement by polarization transfer

dH_2O: distilled water

DPPH: 1, 1-diphenyl-2-picryl hydroxyl radical

DTNB: $5'$, $5'$ – dithiobis, 2-nitrobenzoic acid

FRAP: Ferric Reducing antioxidant power

G6PDH: glucose-6-phosphate dehydrogenase

G-CSF: Granulocyte colony stimulating factor

GGT: Gamma glutamyl transferase

GM-CSF: Granulocyte-macrophage colony stimulating factor

HPLC: High performance liquid chromatography

IC- inhibitory concentration

IR: Infra red

LD: Lethal dose

MAP: Mitogen activated protein

MBP: Myelin basic protein

MCH: Mean corpuscular haemoglobin

MCHC: Mean corpuscular haemoglobin concentration

MCV: Mean corpuscular volume

MDA: malondialdehyde

MS: Mass spectrometer

NMR: Nuclear magnetic Resonance

NMR: Nuclear magnetic Resonance

NSAIDS: Non-steroidal anti-inflammatory drugs

NTP: The National Toxicology Program

OEL: Occupational exposure limit

PBS: Phosphate buffer saline

PCs: Phytochelatins

PCV: Packed cell volume

RBC: Red blood cell

ROS: Reactive oxygen species

SOD: Superoxide dismutase

T TP: Time to platform

TBARS: Thiobarbituric acid reactive substances assay

TLC: thin layer chromatography

TP: Total protein

TPTZ: tripyridyltriazine

UV: ultra violet

WBC: White blood cell

WHO: World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

Vanadium is a trace element and distributed widely in nature. Power and heat-producing plants using fossil fuels (petroleum, coal, oil) cause the most widespread discharge of vanadium into the environment. The incidence of exposure to toxic levels of vanadium to living organisms has been an increasing concern (Plunkett, 1987). Environmental exposure occurs via inhalation in the surrounding area of metallurgical plants or through consumption of contaminated foods (Barceloux & Barceloux, 1999; IARC 2006), and recently from massive oil burning as seen in Arabian Gulf (Haider *et al*, 1998), the Niger-Delta region of Nigeria (Igado *et al.,* 2008) and the Gulf of Mexico (Avila-Costa *et al.,* 2006). The mangrove forest of the Niger-Delta of Nigeria, covering about 70,000 km^2 of wetlands (the largest in Africa and the third largest in the world) with a population of about 20 million has been the centre of constant exploration for oil by many international oil companies (Twumasi and Merem, 2010). These exploratory activities often lead to gas flaring and oil spillage, impacting negatively on the aquatic and terrestrial habitats as well as animal and human health (Kadafa, 2012).

Vanadium compounds have been reported to cause toxic effects by most routes of exposure in most species (Garcia *et al.,* 2005). Disposition of vanadium in specific tissues may be involved in the pathogenesis of certain neurological disorders and cardiovascular diseases (Venkataraman and Sudha, 2005). It has been documented to damage cell membrane, affecting several physiological processes via the production of free radical has also been documented (Haider and El-Fakhri, 1991).

In addition, interaction with environmental pollutants has been reported as a leading contributing factor in the development of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Guglielmotto *et al*. 2010; Caudle *et al*. 2012; Moulton and Yang 2012). The incidence of neurodegenerative diseases, like Alzheimer's and Parkinson's diseases, have increased due to higher life expectancy, reaching epidemic proportions in all industrialised countries and becoming an important socioeconomic problem (Melo *et al*., 2011). Vanadium toxicity is characterised, among other cellular pathologies, by mitochondrial dysfunction resulting in production of reactive oxygen species (ROS), and the toxicity is related to oxidative stress (Bonda *et al.,* 2010; Gandhi *et al*, 2012). Therefore, the

Central Nervous System (CNS) is especially sensitive to free radical oxidative damage due to its high oxygen consumption ratio, a high amount of polyunsaturated fatty acid side chains, a rich content of phospholipids, easily oxidisable, and high levels of iron, which can catalyse oxidative reactions and contribute to an increase in production of free radicals (Halliwall, 1992, Floyd, 1999). ROS induced damage is further enhanced by a low content of antioxidant defences in the brain that are even more depleted following neurodegenerative conditions. This oxidative damage occurs due to an imbalance between ROS production and antioxidant cell defences (Wang *et al.,* 2010; Gandhi *et al.,* 2012; Feng *et al.,* 2012). The elevation of ROS production further leads to mitochondrial dysfunction, causing an increase in free radical production and an exacerbation of the oxidative stress cycle (Gandhi *et al.,* 2012). Normal mitochondrial respiration produces molecular oxygen, but in the presence of defects on the electron transport chain the molecular oxygen is increased and promotes deficits in several enzymes in charge of reducing this molecular oxygen (Feng *et al.,* 2012), converting the mitochondria into the main producers of ROS. This free radical damage by vanadium consequently leads to tremor, CNS depression and various behavioural alterations (Soazo and Garcia, 2007).

In view of this, several studies have been demonstrated to target the use of chemical substances to reduce the effect of vanadium toxicity in living organisms. Hill (1979) had earlier reported that oral intake of ascorbic acid reduces the toxicity of high levels vanadium in chicks. El-Shaari *et al.* (2002), also concluded that ascorbic acid reduces sensorimotor dysfunctions and axonal degeneration caused by vanadium neurotoxicity and α-tocopherol has also been reported to protect the brain against vanadium-induced free radical injury (Halliwell and Gutteridge, 1986; Olopade *et al.,* 2011).

Some medicinal plants have also been reported to offer protection following heavy metal toxicity (Gohari *et al.,* 2011), apparently due to their phytochemicals which are mostly polyphenol and flavonoids (Hasani-Ranjbar, *et al.,* 2009). For instance, *Ginkgo biloba* (EGb 761) is known for its excellent antioxidant properties that restrict brain damage and neurodegeneration (Yang *et al.,* 2001) following lead-induced toxicity.
Leaf from ethanol extract of *Spondias mombin* have recently been demonstrated to have an ameliorative effect in experimental rats following arsenic-induced toxicity (Ola Davies *et al.,* 2013). These natural sources of antioxidants are believed to provide potentially safer, effective and cheaper antioxidants (Mundhe *et al.,* 2011; Amit *et al.,* 2012; Rajamurugan *et al.,* 2013).

Most species in the genus, *Grewia* have been reported to have antioxidant properties, and are used in the treatment of various disorders in man and animsals (Goyal, 2012). Triterpenoids, steroids, glycosides, flavones, lignans, phenolics, alkaloids, lactones, anthocyanins, flavones, and organic acids have been isolated from various species of this genus (Morton, 1997; Onwuliri *et al*, 2006).

Grewia carpinifolia is a member of Malvaceae family (previously belonging to Tiliacaea family) widely distributed within the Northern, middle belt of Nigeria, some African countries and in tropical regions of the world. Various parts of the plant are used in food and medicine. In Nigeria, the stem is used for the treatment of ulcerated tongue, colic, wounds, cholera and dysentery (Muazu *et al.,* 2008). However, extensive search of the literature showed a relative dearth of report on scientific studies of *Grewia carpinifolia* against metal toxicity.

To this end, the present study was designed to investigate if a nutritional strategy like co-administration of ethanol extract of *G. carpinifolia* leaf could ameliorate vanadium-induced neurotoxicity.

1.1 Justification for the study

Oil spillage as well as intense rainfall and flooding have been reported to be indirectly responsible for the enhanced concentrations of vanadium via its ingestion. In addition, the availability of petroleum hydrocarbons may increase its activities of biodegradation on site, the physicochemical properties of the soils and inherent mobility of the metal. Therefore, there is a need to study plants that may offer some protection against the effects of this metal in Nigeria known for oil spillage and gas flaring.

In view of the facts that vanadium has been shown to induce various CNS toxicities through oxidative stress mechanisms, the antioxidant properties of phytochemical constituents of plants may offer protection against oxidative stress in tissues there is, therefore, the need to study plants that may offer some protection against the toxic effects of vanadium in a country such as Nigeria where about 61% of its citizen are impoverished and cannot readily afford the costs of conventional drugs (NBS, 2014).

1.2 Aim of study

The aim of the present study was to investigate the neuro-protective roles of *Grewia carpinifolia* in mice following acute vanadium toxicity.

1.3. Objectives of the study

The study was aimed at:

i. Acute and sub chronic toxicological evaluation of the leaf and stem of *Grewia carpinifolia*
ii. Investigating the protective efficacy of crude extracts from the stem and leaf of *Grewia carpinifolia* and its isolated fractions on vanadium induced damages on some organ systems of rat.
iii. Evaluating the *in vivo and in vitro* antioxidant property of the leaf and stem of *Grewia carpinifolia*
iv. Carrying out bioactivity guided fractionation of the leaf and stem of *Grewia carpinifolia* to obtain the potent fraction for antioxidant activity.
v. Chemical elucidation of the crude extract of the leaf and stem extracts of *Grewia carpinifolia* to identify, isolate and purify its constituents.
vi. Examining the protective effects of *G. carpinifolia* pure compounds on vanadium induced neurotoxicity

1.4. Study Hypotheses

i. Ho_1: The leaf and stem of *Grewia carpinifolia* are not toxic to rats
ii. Ho_2: The extract of the leaf and stem of *Grewia carpinifolia* cannot ameliorate or protect against behavioural impairment and other damages induced by vanadium
iii. Ho_3: The extract of the leaf and stem of *Grewia carpinifolia* do not have any antioxidant property.
iv. Ho_4: The extract of the leaf and stem of *Grewia carpinifolia* cannot cross the blood brain barrier.

Null Hypothesis 1: To accept the hypotheses?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HEAVY METAL TOXICITY

Metals are substances with high electrical conductivity, malleability, and lustre, which voluntarily lose their electrons to form cations. Heavy metals are naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water. They are generally referred to as those metals which possess a specific density of more than 5 g/cm^3 and adversely affect the environment and living organisms (Järup, 2003). Their multiple industrial, domestic, agricultural, medical and technological applications have led to their wide distribution in the environment (Khlifi and Hamza-Chaffai, 2010). These metals are quintessential to maintain various biochemical and physiological functions in living organisms when in very low concentrations; however they become noxious when they exceed certain threshold concentrations. Although these heavy metals have many adverse health effects and last for a long period of time in the environment, exposure to them continues and it is on the increase in many parts of the world (Nagajyoti *et al.*, 2010; Mahmoud *et al.,* 2011; Jaishankar *et al.*, 2013). They are abundant in water, air and soil. They are present in virtually every area of modern consumerism-from construction materials to cosmetics, medicines to processed foods, fuel sources to harmful agents, appliances to personal care products. It is very difficult for anyone to avoid exposure to any of the many harmful heavy metals that are so prevalent in the environment.

Even though they are naturally occurring elements found throughout the earth's crust, most environmental contamination and exposure result from anthropogenic activities such as mining or smelting operations, industrial production and use, as well as domestic and agricultural use of metals and metal-containing compounds (Shallari *et al*., 1998; Goyer, 2000; Herawati *et al.,*. 2000; He *et al.,* 2005). Environmental contamination can also occur through metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, sediment re-suspension and metal evaporation from water resources to soil and ground water (Nriagu, 1989). Natural phenomena such as weathering and volcanic eruptions have also been reported to significantly contribute to heavy metal pollution (Nriagu, 1988; Fergusson, 1990; Bradl, 2002). Industrial sources include metal processing in refineries, coal burning in power plants, petroleum combustion, nuclear power stations and high tension

lines, plastics, textiles, microelectronics, wood preservation and paper processing plants (Pacyna, 1996; Arruti *et al.*, 2010; Sträter *et al.*, 2010)

Due to their high degree of toxicity, arsenic, cadmium, chromium, copper, nickel, zinc, lead, vanadium and mercury rank among the priority metals that are of public health significance to animals and human population at large (Lambert *et al.*, 2000). These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure.

Although these metals have crucial biological functions in plants and animals, sometimes their chemical coordination and oxidation-reduction properties have given them an additional benefit so that they can escape control mechanisms such as homeostasis, transport, compartmentalization and binding to required cell constituents. These metals bind with protein sites which are not specific for them by displacing original metals from their natural binding sites causing malfunctioning of cells and ultimately toxicity. Previous research has found that oxidative deterioration of biological macromolecules is primarily due to binding of heavy metals to the DNA and nuclear proteins (Flora *et al.*, 2008).

2.2 Properties and Uses of Vanadium

Vanadium is a chemical element of the first series of transition metals with complex aqueous geochemistry (Bycakowski and Kulkarni, 1992). Vanadium (V) is a natural element in the earth. It is an odourless, white to grey metal which is often found as crystals. Vanadium is hard, corrosion-resistant and readily forms alloys, when combined with other metals such as oxygen, sodium, sulphur or chloride. These properties have made its industrial use very popular (Amado *et al.*, 1993; Barceloux and Barceloux, 1999). Vanadium is also used as a catalyst in the production of tetra-oxo sulphate (VI) acid, in converting organic compounds into plastic, photo development, creating artificial yellow pigments, and in the production of ceramics (Barceloux and Barceloux, 1999). Natural vanadium consists of two isotopes, ^{50} V and ^{51}V, the former being slightly radioactive. Seven other radioisotopes of the element have been synthesized (Crans, 1994). Vanadium oxide (vanadium bound to oxygen) is a synthetic form that is often used industrially in making steel, rubber, plastics and ceramics (Chan *et al.*, 2009).

2.2.1 Relevance of Vanadium to Public Health

Vanadium is the 22nd most abundant element in the earth's crust with an average concentration of 100 ppm. It has a very complex chemistry and different states of protonation and conformations can occur simultaneously in equilibrium in vanadate solutions (Chasteen, 1983; Amado *et al.,* 1993; Crans, 1994). It exists in oxidation states, ranging from 2^- to 5^+ with 3^+, 4^+, and 5^+ being the most common oxidation states. The vanadium (III) species are unstable at physiological pH and in the presence of oxygen. Vanadium (IV) is easily oxidised to vanadium (V) under physiological conditions and vanadium (V) species are found as vanadate anions. For example, the pentavalent V_2O_5 has been reported to be more than 5 times as toxic as trivalent V_2O_3. Vanadium is released to the environment by continental dust, marine aerosols, volcanic emissions, and the combustion of coal and petroleum crude oils. It is naturally released into water and soil as a result of weathering of rock and soil erosion. Ambient air concentrations of vanadium are low, with urban areas having higher concentrations. Vanadium is ubiquitous in the biosphere, resulting in detectable trace levels in most living organisms (McNeilly *et al.,* 2004).

Vanadium is found in many petroleum products. It occurs naturally in fuel oils and coal (Hazardous Substance Database, 2006). It is also a by-product of petroleum refining (Bunting, 2006). The extraction of vanadium from petroleum ash is a possible future source of the element (Bunting, 2006). Exposure to vanadium is also common in oil-fired boiler electricity generating plants, petrochemical, steel and mining industries and it can be found in groundwater, rocks, soils, coal and oil deposits (Stock, 1960; Kustin *et al.,* 1983; Goyer, 1993). Vanadium is the most abundant trace metal in petroleum samples and can be found in concentrations reaching 1500 $mgkg^{-1}$, depending on the source of the crude oil (Amorim *et al.,* 2007). Vanadium accumulates in the soil, groundwater, and plants that may be consumed by animals and humans (Pyrzynska and Weirzbicki, 2004).
Vanadium residence time in the environment is inversely related to the particle size. In water, vanadium is converted from trivalent forms to pentavalent forms.

In most parts of the globe food is the primary route of exposure for the general population; foods with the highest vanadium content include ground parsley, freeze-dried spinach, wild mushrooms, and oysters. Vanadium in food is mainly ingested as VO_2^+ (vanadyl, $V4^+$) or HVO_4^2 (vanadate, $V5^+$). Humans and animals are potentially exposed to a variety of vanadium compounds, the most common being vanadium pentoxide, sodium metavanadate,

sodium orthovanadate, vanadyl sulphate, and ammonium metavanadate. Production of vanadium in the 1970's was approximately 35,000 tons throughout the world (Haider *et al.,* 1998), but as at 2011 vanadium-containing fossil fuels are alleged to emit approximately 65,000 tons of the metal into the atmosphere in Nigeria alone (National Oceanic and Atmospheric Administration, NOAA, 2011) via gas flare. Gas flaring is the burning of natural gas and petroleum hydrocarbons in flare stacks by upstream oil companies in oil fields during operations (Ubani and Onyejekwe, 2013). It is the singular and most common source of global warming and contributes to emissions of carbon monoxide, nitrogen (ii) oxide and methane which have the propensity of causing environmental pollution and ecological disturbances or destruction (Ubani and Onyejekwe, 2013). Thus, occupational exposure occurs mainly during the industrial production and use of vanadate in oil fields (WHO, 1990). Figure 2.1 shows top five gas flaring countries in the world (NOAA, 2011), with Nigeria ranking as second.

Although there is evidence to suggest that vanadium is an essential nutrient, a functional role for vanadium in living organisms has not been established; increases in abortion rates and decreased milk production have been observed in vanadium-deprived goats. Vanadium mimics insulin and stimulates cell proliferation and differentiation. In animal models, particularly streptozotocin-induced diabetes in rats, vanadium has been shown to normalise blood glucose and lipid levels and improve insulin sensitivity. Though these roles have been discussed at length in animals and humans there is no definitive information to name it as an essential element (WHO, 1988).

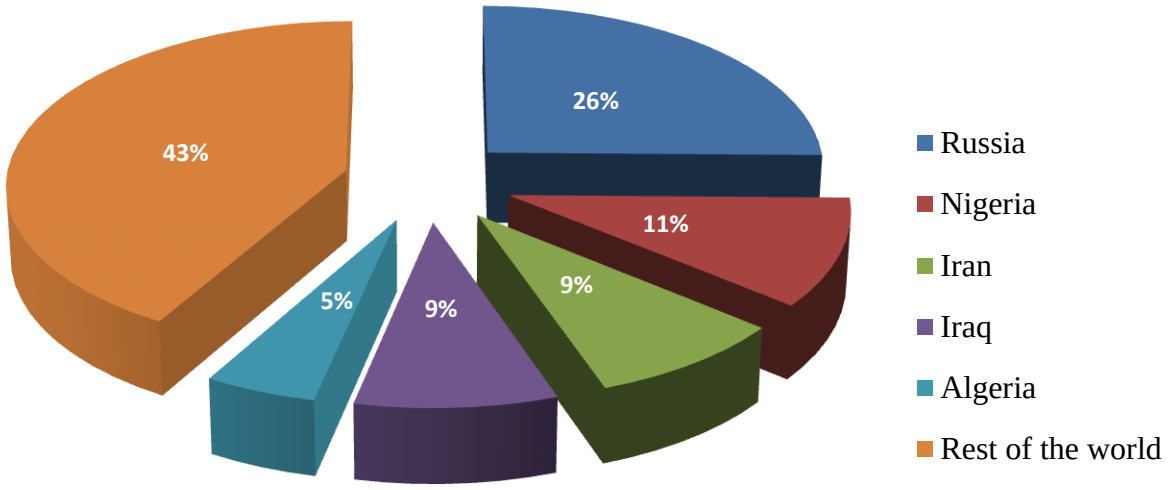


Figure 2.1: Top five gas flaring countries (National Oceanic and Atmospheric Administration, NOAA, 2011)

2.2.2 Health Effects of Vanadium

Analysing the toxic effects of vanadium is complicated because the toxicity is highly influenced by its oxidation state and solubility, as well as many other intrinsic and extrinsic factors (Keller *et al*., 1997).

Experimental and human studies on vanadium have indicated a number of environmental effects that this metal may potentially have on health. Other sources of exposure to vanadium include drinking contaminated water and food (Wright and Belitz, 2010). Levels higher than 100 µg/L in water is toxic: this has led to regulation of vanadium in drinking water (Wright and Belitz, 2010; Crebelli and Leopardi, 2012). High levels of vanadate in drinking water have been shown to elicit some genotoxicity in animal studies (Crebelli & Leopardi, 2012). Dietary intake between 10-60 µg/day is considered safe (Barceloux and Barceloux, 1999).

Toxic symptoms in humans and animals have also been reported following inhalation of air contaminated with vanadium (Barceloux and Barceloux, 1999). Due to its wide/varying valence state and solubility experimental studies in humans and laboratory animals have shown that it exerts potent toxic effects on a wide variety of biological systems. Early toxic effects from vanadium exposure were reported in the 1940's (Williams, 1952). The CNS, respiratory tract, the gastro-intestinal tract and haematological system are the primary targets of toxicity.

2.2.2.1 Central Nervous System and Neurocognitive Effects of Vanadium

The Central Nervous System (CNS)

The CNS, being rich in polyunsaturated fatty acid side chains, high oxygen tension and poor in antioxidant capacity, is vulnerable to free radical damage (Halliwall, 1992). Vanadium is widely recognised in its different forms as a potentially neurotoxic environmental pollutant, because it causes the inhibition of certain enzymes in animals, which has several neurological effects. Vanadium toxicity in man may lead to tremor and CNS depression (Berman, 1980).

Sasi *et al.* (1994) had demonstrated that exposure of rats to vanadium perturbed the lipid and protein metabolism with concomitant stimulation of occurrence of lipid peroxidation in discrete brain regions. It was speculated that vanadium damages the cell membrane via free radical injury. In addition, vanadium crosses the blood-brain barrier (Berman, 1980),

inducing alterations in neurochemical substances of the brain (Witkowska and Brzezinski, 1979).

Barth *et al.,* (2002), examined a group of 49 male workers exposed to vanadium. Behaviours measured included attention, visuospatial functioning, visuomotor functioning, reaction time, short-term memory, and prefrontal functioning. The control group included workers of a steel production plant that were not exposed to vanadium. Serum vanadium levels with a range from 0 - 5 µg/L and from 0 - 40 µg/L were found. Vanadium exposed subjects displayed decreased ability in visuospatial skills as opposed to the control (unexposed) group suggesting that occupational vanadium exposure may lead to a variety of neurobehavioural impairments as well as attention deficits (Barth *et al.,* 2002).

Li *et al.* (2013) collected neurobehavioural data on vanadium-exposed workers from a steel and iron industry in China. It was observed that the amount of years spent at the facility led to an overall decline in neurobehavioural function, suggesting a slow-occurring change in cognition, and memory. Auditory tests indicated that when counting tones, exposed workers made significantly more errors than controls (Li *et al.,* 2013). This study indicated that CNS functioning was affected by vanadium exposure. Furthermore, vanadium exposure exerts adverse effects on emotions, coordination, short-term memory, and reaction speeds.

In addition to its CNS toxicity in humans several studies have also investigated the effects of vanadium in neurocognitive functioning in animals. The use of rodents allows for more controlled studies to be conducted. For instance, exposure can be precisely controlled and virtually any type of toxic effect can be measured. In one of such study, Sanchez, *et al.* (1998), reported a reduction in weight gained following vanadium administration in for eight weeks.In regards to tissue content, vanadium was found in the liver, kidneys, and muscles. As dosages increased, the amount of vanadium present in these tissues increased. Behavioural results showed that vanadium treated rats exhibited decreased locomotion compared to non-treated rats. Vanadium administration was further found to negatively affect avoidance performance.

Haider *et al.,* (1998) also administered 1.5 mg of vanadium per kg^{-1} body weight intraperitoneally (i.p.) for 12 days and found that rats had difficulty in walking and moving their hind limbs in addition to, convulsions, muscular problems, difficulty breathing, lack of coordination, inactivity, and diarrhea. Body weight was also reduced significantly by 7.7%. Weight of the brain was also significantly lower compared to the control (saline) group. A

higher skeletal uptake of vanadium and longer retention was found in younger compared to older rats (Raabe and Al-Bayati, 1997). In another study, weight loss, decreased motion, weakness, bleeding of the eyes and red-nose were reported in the vanadium exposed rats (de la Torre *et al.,* 1999). In addition, reduction in blood glucose physiological changes in cellular structure and renal changes (vacuolisation of cells and evidence of necrosis) were recorded following vanadium administration.

In a related study, Avila-Costa *et al.* (2006) investigated the consequences of vanadium inhalation on memory in 48 clusters of differentiation-1 (CD-1) mice. Mice were first trained on the Morris Water Maze and then subjected to either 0.2 M V_2O_5 or deionized water via inhalation for one hour twice a week for four weeks. Vanadium treated mice exhibited significantly longer latencies compared to control mice on this task. Additional analyses also indicated dendritic spine loss in the hippocampus in mice exposed to vanadium. Other important findings were that neurones in the hippocampus showed necrotic cell death. The Morris Water Maze is a hippocampal dependent task, therefore a damage to the hippocampus as a result of vanadium exposure provides an explanation as to why mice show impaired performance on this task. This study supports evidence that vanadium inhalation may be harmful to humans and animals.

In another study, Azami *et al.,* (2011) investigated the effects of oral administration of sodium metavanadate on Morris Water Maze performance. Rats were administered with sodium vanadate every day for 2 weeks (at a dose of 0, 15, 20, or 25 mg/kg/day). One day after vanadium or vehicle treatment, rats were tested on the Morris Water Maze. Rats that were treated with sodium vanadate showed significant deficits in spatial memory as measured by this task.

Garcia *et al.* (2004) and Todorich *et al*. (2011) reported a significant decrease in grooming responses and locomotor activity respectively in rats treated intraperitoneally with sodium metavanadate; also, behavioural alterations were observed in lactating rat pups whose dams were exposed to sodium metavanadate (Soazo and Garcia, 2007, Olopade *et al.,* 2011).

Igado *et al.* (2012), reported several interesting neuropathological findings following intraperitoneal administration of vanadium at doses of 1.25 mg/kg, and 1.50 mg/kg for five days. Damage to myelin tracts and degeneration of the Purkinje cell layer of the cerebellum were observed. Furthermore, vanadium levels were high in the hippocampus, congestion was

observed in the meningeal blood vessels in the group receiving 1.50 mg/kg vanadium, and increases in lipid peroxidation (LPO) were found in the olfactory bulb. The authors reported that the results may account for previous findings of impairments in cognition, coordination, and movement in previous studies. A finding from this study was the implication of the olfactory bulb suggesting that exposure to vanadium may decrease the sense of smell (Igado *et al.,* 2012).

Consistently researchers have found a range of negative physiological and cognitive effects following vanadium toxicity using different dosages and routes of administration (Gomez *et al.,* 1991; Raabe and Al-Bavati, 1997; Haider *et al.,* 1998; Sanchez *et al.,* 1998; Torre *et al.,* 1999;; Avila-Costa *et al.,* 2005; Soazo and Garcia, 2007; Olopade *et al.,* 2011; Igado *et al.,* 2012; Radike *et al.,* 2012).

The toxicities resulting from the exposure to vanadium have resulted in the call for the establishment of an occupational exposure limit (OEL) in regards to vanadium for industrial workers and other occupational settings that expose workers to vanadium (Assem & Levy, 2009). The German MAK Commission, U.S. American Conference of Governmental Industrial Hygenists (ACGIH) and The National Toxicology Program (NTP) are currently working on an OEL for their various countries.

2.2.3 Mechanism of action of vanadium toxicity

Vanadium causes toxicity in living cells by ionic mechanism resulting from oxidative stress (Mahmoud *et al.,* 2011). Many researchers have shown that oxidative stress in living cells is caused by the imbalance between the production of free radicals and the generation of antioxidants to detoxify the reactive intermediates or to repair the resulting damage. Figure 2.2 shows the attack of heavy metals on a cell and the balance between ROS production and the subsequent defence presented by antioxidants.

Oxygen is vital for all living cells whether neuronal or other kinds of cells taking part in tissue formation but it are potentially dangerous in excess. Thus, it is kept under tight check of complex system that regulates and monitors the usage and uptake of this essential element. Oxygen is involved in glucose break down in the mitochondrion through oxidative phosphorylation and generation of energy currency for the cell which is ATP (Harvey *et al.,* 1999). The mitochondrion has its own molecular machinery (Mt DNA) for the synthesis of enzyme and proteins required for oxidative phosphorylation. Any mutation in Mt DNA leads

to impaired ATP generation and perturbed oxidative phosphorylation cascade that may further lock the neuronal function (Guido and John, 2000). Oxidative stress arises due to disturbed equilibrium between pro-oxidant/antioxidant homeostasis that further takes part in generation of ROS and free radicals which are potentially toxic for neuronal cells. The reason for neuronal cell hypersensitivity towards oxidative stress arises due to anatomic and metabolic factors.

In the brain, various types of glial cells are present and these are involved in anatomic support and metabolic requirement. The endothelial cells surrounding these glial cells are less permeable for uptake of various molecules and protective cells viz. macrophages compared to other endothelial cells in the body. In addition, glial cells in brain require more oxygen and glucose consumption to generate continuous ATP pool *in vivo* for normal functioning of brain as it is one of busiest organ to keep all other organs active and under control. That makes them more susceptible towards oxygen over load and consequently free radical generation (Lepoivre *et al.*, 1994). Under physiological condition, 1-2% of O_2 consumed is converted to ROS but in vanadium toxicity this percentage becomes higher due to reduced surveillance of antioxidants and low regenerative capacity of the brain (Lepoivre *et al.*, 1994).

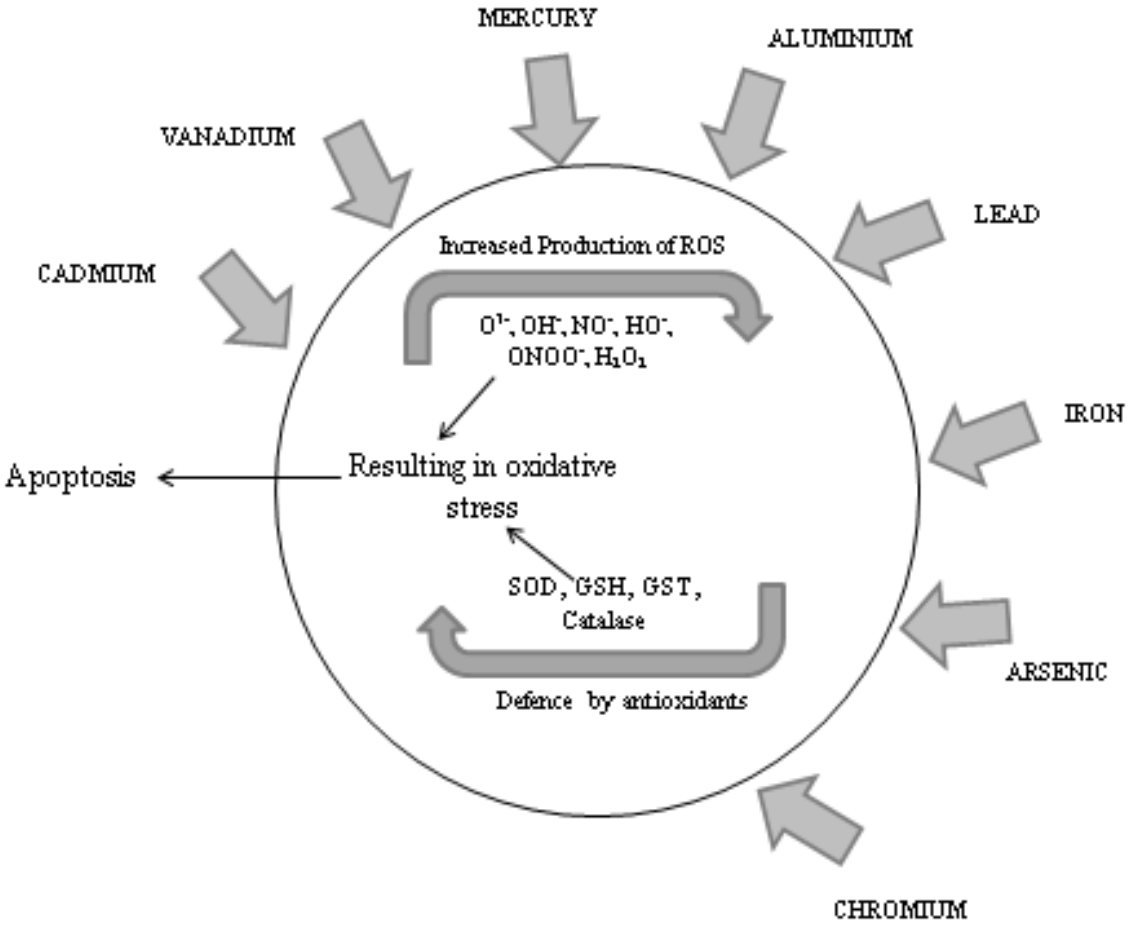


Figure 2.2: The oxidative stress of heavy metals on a cell and the balance between reactive oxygen species production and the subsequent defence presented by antioxidants (Jaishankar *et al.,* 2014).SOD, superoxide dismutase, GSH, Glutathione; Glutathione S transferase.

2.2.4 Vanadium Toxicity, Reactive Oxygen Species Generation and Neuronal Degeneration

Reactive Oxygen Species (ROS) comprise hydrogen peroxide (H_2O_2), superoxide anions and the highly reactive hydroxyl (OH^-) and monoxide radicals ($OH\cdot$, $NO\cdot$). Damaged mitochondria and activated microglia acts as reservoir of ROS. Initially ROS generation was believed to be an outcome of imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS), but recently many chemists and molecular biologists have discovered that regulating ROS may play fundamental role in modulating key cellular functions (Klaus and Heribert, 2004). For example, Haber-Weiss and Fenton reaction initiate ROS generation that activates mitogen activated protein (MAP) kinase cascade, excitotoxic calcium mobilisation and finally apoptotic cell death (Hyman, 2004). Free radicals have been reported for their great contribution to neuronal loss in other conditions such as cerebral ischaemia, seizure disorders, schizophrenia, Parkinson's disease and Alzheimer's disease (Demopoulos *et al.,* 1980; Pryor, 1987; Richardson and Subbarao, 1990; Torbati et al., 1992; Yoshikawa, 1993; Youdim and Lavie, 1994; Cadet, 1998).

Neuronal biochemical composition is mainly susceptible to ROS since it involves pool of unsaturated lipids that are susceptible to peroxidation and oxidative modification. Double bonds of unsaturated fatty acids are target spots for attack by free radicals thus initiating a cascade or chain reaction to damage neighbouring unsaturated fatty acids (Butterfield *et al.,* 2002). Several researchers considered the brain to be highly sensitive to oxidative damage and many studies have demonstrated the ease of peroxidation of brain membranes supporting this notion (Chance *et al.,* 1979; Zaleska and Floyd, 1985; Floyd and Carney, 1992). The brain contains high level of fatty acids, which are more susceptible to peroxidation, consuming an inordinate fraction (20%) of total oxygen for its relatively small weight (2%). In addition, it is not particularly enriched with antioxidant defences. The brain is lower in antioxidant activity in comparison with other tissues, for example, about 10% of that of the liver. Thus, the neural cells are considered to be more susceptible to oxidative damage induced by vanadium compared to other body tissues (Floyd and Carney, 1992).

ROS generation is a pre-requisite for metabolic system to interact with organic molecules *in vivo* as interaction of organic molecules with oxygen is energetically unfavourable. In all forms of ROS generation, molecular oxygen needs to be activated and cellular system have evolved a range of metallo-enzymes that facilitate ROS generation upon interaction of redox

metals (such as vanadium) with O_2 using various catalytic pathways. Since free radicals are toxic to cells, under normal circumstances, cells have efficient regulating system for O_2 and metal ion interaction, leading to free radical and ROS generation (Bush, 2000). In biological systems, animal studies have shown that vanadium compounds induce oxidative stress and lipid peroxidation *in vivo* (Stohs and Bagchi, 1995). Different vanadate-induced effects in biological systems were described to be dependent on the oligomeric species present (Aureliano and Madeira, 1994; Aureliano *et al.*, 2002; Tiago *et al.*, 2004).

Apart from direct ROS generation, there are other *in vivo* pathways that contribute substantively to ROS generation by calcium activation following vanadium toxicity. Calcium is an important signalling molecule, and it is required for many cellular responses and cell-cell communication. Thus, any disturbance in stimulus and regulation of calcium pathway may disrupt the cellular physiology (Angelo *et al.*, 2005).

Antioxidants, such as glutathione, present in the cell protect it from free radicals such as H_2O_2. Under the influence of vanadium, however, the level of the ROS increases and the level of antioxidants decrease. Since glutathione exists both in reduced (GSH) and oxidised (GSSG) state, the reduced form of glutathione gives its reducing equivalents ($H^+ + e^-$) from its thiol groups of cystein in order to make them stable. In the presence of the enzyme glutathione peroxidase, reduced glutathione readily binds with another molecule of glutathione after donating the electron and forms glutathione disulfide (GSSG). The reduced form of glutathione (GSH) accounts for 90% of the total glutathione content and the oxidised form (GSSG) accounts for 10% under normal conditions. Yet under the condition of oxidative stress, the concentration of GSSG exceeds the concentration of GSH.

Oxidative stress is a pathophysiological process in which intracellular balance between endogenous as well as exogenous pro-oxidants and antioxidants is shifted towards pro-oxidants, leaving cells unprotected from free radical attack, which in turn may cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals (Chen *et al.*, 2001).

Recent studies have indicated that vanadium-mediated generation of reactive oxygen species (ROS) may play an important role in vanadium-induced toxicity (Shi and Dalal, 1991; Parfett and Pilon, 1995). For example, reduction of vanadate by glutathione reductase in the presence of NAD(P)H generates vanadium (IV) (Parfett and Pilon, 1995). During the reduction process, molecular oxygen is reduced to O^{2-}, then to H_2O_2 via dismutation. The reaction of

vanadium (IV) with H_2O_2 also generates hydroxyl radical ($OH^{\cdot}$) via a Fenton-like reaction (Shi and Dalal, 1992) as shown in the reactions below;

$Fe^{3+} + \bullet O_2^{-} \rightarrow Fe^{2+} + O_2$ (equation I)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + \bullet OH$ (equation II) *

Combining equations I and II

$\bullet O_2^{-} + H_2O_2 \rightarrow \bullet OH + HO^{-} + O_2$ * Known as Fenton reaction (Roger *et al.,* 1997)

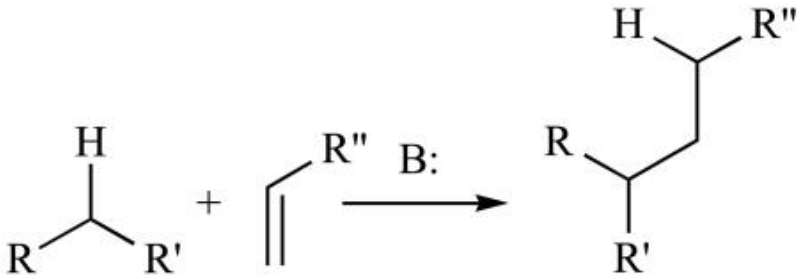
It has been reported that deleterious effects of ROS on human cells may end in oxidative injury, leading to apoptosis (Salganik, 2001).

2.2.5 Mechanism of Reactive Oxygen Species Mediated Cellular Apoptosis

Reactive Oxygen Species (ROS) are extremely reactive with different fundamental molecules in cellular pool and initiating a cascade of reactions and at the same time leading to neuronal cell death. Oxidative over-load in neuronal microenvironment causes oxidation of lipids, proteins and DNA and generates many by-products such as peroxides, alcohols, aldehydes, ketones and cholesterol oxide. Therefore, another biomarker for oxidative stress is lipid peroxidation, since a free radical detaches electron from lipid molecules present inside the cell membrane, which eventually causes lipid peroxidation (Wadhwa *et al.*, 2012; Flora *et al.*, 2012). At very high concentrations, these ROS may cause structural damage to cells, proteins, nucleic acid, membranes and lipids, resulting in a stressed situation at cellular level (Mathew *et al.,* 2011). Most of the ROS are toxic to blood lymphocyte and macrophages, paralysing the *in vivo* defence system (Ferrari, 2000). Cystine, lysine and histidine residues in protein are hot spot for acrolein (oxidatively modified lipid) and NHE (sodium hydrogen exchanger) for modification, and they cross link these amino acid residues via Michael addition (Ian, 2008). Acrolein hampers glutamate and sugar uptake whereas NHE block neuronal ion transporters and activates c-Jun and Mitogen-activated protein (MAP) kinase pathways to invoke cellular apoptosis (Keller *et al.,* 1997). Protein modification leads to loss of function of enzymes regulating oxidative balance in cellular system viz. glutamine synthase and superoxide dismutase. The most significant adverse effect on neuronal health takes place by dysregulation of intracellular calcium signalling pathways initiated by ROS, and in recent years heavy evidences suggest the role of ROS in neuronal cell death (Ermak and Davies, 2002). Other conditions such as ageing and neurodegenerative diseases (such as

Alzheimer's disease, Parkinson disease, Multiple sclerosis, amylotrophic lateral sclerosis) are also associated with ROS generation. For example, Alzheimer's disease is characterised by deposition of amyloid plaques by chelating amyloid-β peptide (Aβ) with transition metal ions. Toxicity of Aβ is attributed to histidine residues at positions 6, 13 and 14 (structural site for transition metal coordination). Binding of these metals produces toxic chemical reaction, altering oxidation state of the metals, producing H_2O_2 and finally gives toxic $OH^{\cdot}$ free radicals (Opazoi *et al.,* 2002). In multiple sclerosis unregulated iron metabolism and ROS generation have been named as a major player in pathogenesis of disease causing demylination in the CNS.

In addition, DNA mutations can add further insult after ROS mediated modifications (Mattson, 2003a) following vanadium toxicity.



Where B is the Base e.g. NaOH, KOH etc.

The ionic mechanism of vanadium toxicity occurs mainly due to its ability to replace other bivalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cation like Na^{+}, which ultimately disturbs the biological metabolism of the cell. The ionic mechanism of vanadium toxicity causes significant changes in various biological processes such as cell adhesion, intra- and inter-cellular signalling, protein folding, maturation, apoptosis, ionic transportation, enzyme regulation, and release of neurotransmitters (Palmer, 2010)

2.2.6 Vanadium Toxicity Treatment

Consequent to the general toxicity observed in the central nervous system as well as other systems, several studies have been aimed at providing a protection to the brain and other body tissues following exposure to toxic levels of vanadium. Inflammatory reactions are most common features of all forms of neuronal disorders induced by metals. Consequently, non-steroidal anti-inflammatory drugs (NSAIDS) have been proposed as downstream therapeutics

to prevent inflammatory infiltration of macrophages. They act as antioxidants to reduce inflammatory cascade induced by oxidative stress (Floyd and Hensley, 2002). CPI-1189, a nitrone related compound is supposed to down-regulate the pro-inflammatory cytokine cascade of genes in primary glial cells. In this regard, nitron and related compounds are under phase III clinical trial for potential commercial applications (Floyd, 1996). In addition, *in vivo* proteins and growth factors such as brain-derived neurotrophic factors, responsible for enhancing memory and cognitive function in oxidative stress can be induced in response to promote growth and survival of deteriorating neurones (Mattson *et al.,* 2003b) following damage by vanadium.

Co-administration of triglyclamol chloride at doses of 50 mg with vanadium tablets has been reported to diminish some intestinal symptoms (Diamond *et al.,* 1963) in animal models. Triglyclamol chloride is a cholinergic-blocking drug suggesting that vanadium may have an influence on the cholinergic system (Diamond *et al.,* 1963). Recently others have proposed the use of antioxidants to counteract the effects of ROS generated following vanadium exposure.

Antioxidants are classified as exogenous (natural or synthetic) or endogenous compounds, both responsible for scavenging ROS or their precursors, inhibiting formation of ROS and binding metal ions, needed for catalysis of ROS generation (Gilgun-Sherki *et al.,* 2001). Natural antioxidant system includes enzymatic and non- enzymatic antioxidants. Enzymatic antioxidants are comprised of limited number of proteins such as catalase, glutathione peroxidase as well as superoxide dismutase (SOD) along with some supporting enzymes. Non-enzymatic antioxidants include direct-acting antioxidants, which are extremely important in defence against oxidative species. Most of them include ascorbic and lipoic acid, polyphenols and carotenoids, derived from dietary sources. The cell itself synthesises a minority of these molecules. Indirectly-acting antioxidants mostly include chelating agents, binding to redox metals to prevent ROS generation (Gilgun-Sherki *et al.,* 2001). They neutralize ROS and other kinds of free radicals produced, and have attracted the attention of many researchers due to their therapeutic potential. The key focus of antioxidant therapy should be to interrupt and modulate the neuronal protein interaction with culprit redox metals instead of therapeutic approaches surrounding down-stream effect of ROS. In certain cases, anti-inflammatory drugs are given as supplement at onset of neurological disorder to pacify immune system as in certain circumstances; immune system is significantly provoked due to oxidative stress. Antioxidant therapy involving enzymes and anti-inflammatory drugs

constitute upstream therapy in ROS generation and prevent down-stream pathologies in advanced neurodegeneration. Oxyradicals have a very short life (1 μs) and usually are inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. In this regard, pathology due to ROS can be checked by at least two mechanisms

i. Inactivation of oxyradicals by dietary antioxidants like vitamin C, vitamin E, β-carotene.
ii. Replacement of esterified membrane phospholipids with polyunsaturated fatty acids (PUFAs) by dietary supplementation with essential fatty acids (Burton and Ingold, 1990)

It has been reported in epidemiological studies that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumour, anti-mutagenic, anti-carcinogenic, antibacterial and antiviral activities (Mitscher *et al,* 1996; Owen *et al.,* 2000; Sala *et al.,* 2002). Though the intake of natural antioxidants has been reported to reduce risk of cancer, cardiovascular diseases, diabetes and other diseases associated with increased production of ROS or failure of antioxidant defence (Halliwell and Gutteridge, 1990; Baynes, 1991; Chang *et al.,* 1993; Ceriello 2000), there uses are novel and it is still a growing field in vanadium toxicity intervention (Olopade and Connor, 2010).

Vitamins C and E, β-carotene and coenzyme Q are the most famous antioxidants of animal diet, Vitamin E is present in vegetable oils and it is a fat-soluble vitamin, absorbed in the gut and carried in the plasma by lipoproteins. Out if 8 natural state isomeric forms of vitamin E, α-tocopherol is the most common and potent isomeric form. Being lipid soluble, vitamin E can effectively prevent lipid peroxidation of plasma membrane (Burton and Ingold, 1989; Burton and Ingold, 1990). The leading study in this regard was by El-Shaari *et al.* (2002), who investigated the role of ascorbic acid protection in lipid peroxidative damage, perturbation of enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and acetylcholinesterase (AChE) activities in discrete regions of rat brain after vanadium exposure. They reported that the increase in lipid peroxidation by vanadium was inhibited by ascorbic acid treatment. Ascorbic acid was also documented to increase the activity of G6PDH and AChE towards normal values but ascorbic acid did not have effect on the oedema, marked vacuolation of white matter and segmental demyelinantion in the hypothalamic region of rat brain in this study. The results confirm that ascorbic acid may be

able to improve enzymatic activity, reduce sensorimotor dysfunctions in vanadium toxicity, but has a limited impact on axonal degeneration.

Olopade *et al.* (2011), explored the possible protective role of vitamin E in nursing dams and suckling pups via lactation following toxic exposure to vanadium. The result indicated a reverse in the trend of behavioural deficit, body and brain weight loss and astrogliosis induced by the metal. This innovative result depicted the significant protective effects of this antioxidant against vanadium, suggesting a supplementation with this antioxidant in nursing dams.

Plants (fruits, vegetables, medicinal herbs) contain a wide variety of free radical scavenging molecules such as phenolic compounds such as phenolic acids, flavonoids, quinons, coumarins, lignans, stilbenes, tannins), nitrogen compounds (including alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites which are rich in antioxidant activity (Cotelle *et al.,* 1996; Zheng and Wang, 2001; Cai *et al.,* 2003). These natural antioxidants prevent oxidation of proteins, lipid peroxidations and prevent generation of ROS, thus acting as up-stream therapeutic barrier to ROS. Therefore, increasing attention has been given to plants in a bid to discover potentially safer, more effective and cheaper antioxidants (Mundhe, *et al.,* 2011; Rajamurugan, *et al.,* 2013). Medicinal plants play a key role in human and animal health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant material (WHO, 1993). Some natural and synthetic products have been used in neuroprotection to combat the ill effects of oxidative stress. For example *Ginkgo biloba* (EGb 761), is a famous Chinese herb has been known for its excellent antioxidant properties that restricts brain damage and neurodegeneration (Yang *et al.,* 2001). It has been shown that EGb 761 improves cognitive activities and neuronal function in Alzheimer's disease but in severe Alzheimer's disease neuroprotective role of EGb 761 seems to be reduced.

Several herbal products had been reported to mitigate oxidative stress due to ROS and ameliorate the effects of heavy metals such as lead and arsenic (Dailiah and Padmalatha, 2012; Ola-Davies *et al.,* 2013). Igado *et al.* (2012), studied the neuroprotective effect of kolaviron from *Garcinia kola* on vanadium toxicity in rats. In the study, vanadium was administered via i.p route either alone at doses of 1.25 mg/kg, and 1.50 mg/kg for five days or following oral doses of *Garcinia kola* (first five days on *Garcinia kola*, and second five days on vanadium). This result showed a promising ability of antioxidants from plant sources

to mitigate vanadium induced neurotoxicity. It further suggests that vanadium toxicity may be ameliorated upon dietary intake or supplementary intake of natural antioxidants that have been known to play a vital role in neuroprotection in variety of neurological disorders (Peter *et al.,* 2004).

Disruption of homeostatic metal metabolic pathways by ROS leads to increased intracellular calcium levels that cause neuronal cell death due to dysregulated microtubule assembly and axonal transport. *In vitro* studies have shown the capabilities of natural oxidants such as Taxol in preventing neuronal cell death by preventing microtubule disaggregation (Burke *et al.,* 1994). This gives a promising therapeutic target to prevent calcium-smediated neurotoxicity in vanadium toxicity.

2.2.7 Challenges in Designing Therapeutics following Vanadium Intoxication

The major challenge in antioxidant therapy to protect the brain and cognitive functions due to vanadium-induced neuronal death is movement across blood-brain barrier (BBB). Chemical opening due to osmotic differential pressure at BBB tight junction or pathological openings due to trauma or pathogenic causes are the means to molecular motion across BBB. Coenzyme Q_{10} (ubiquinone), GSH and oxidised form of vitamin C have shown only a little substantive ability to cross BBB in human and rodent models (Gilgun-Sherki *et al.,* 2001) Thus, antioxidant for neuroprotection must accompany afore-mentioned structure analogue in order to cross BBB and circulate in brain neuronal circuitry.

Information about permeability of antioxidant candidates at the BBB is important to decrease the attrition rate in discovery/development, as poor pharmacokinetics has been recognised as one of the leading causes of failure (Kennedy, 1997; van de Waterbeemd and Gifford, 2003).

Figure 2.3. shows a schematic diagram of the brain and its barrier systems. The BBB and the blood-CSF barrier (B-CSF-B) differ in location, size, morphology, and function. The BBB is an endothelial barrier, where tight junctions between the endothelial cells seal off the vascular lumen from the abluminal side. In contrast, the B-CSF-B is formed by the plexus epithelial cells, which are connected by tight junctions, whereas the capillaries are fenestrated. The available experimental tools to study transport include *in vivo* techniques, *in vitro* models, and computational approaches (Bickel, 2005).

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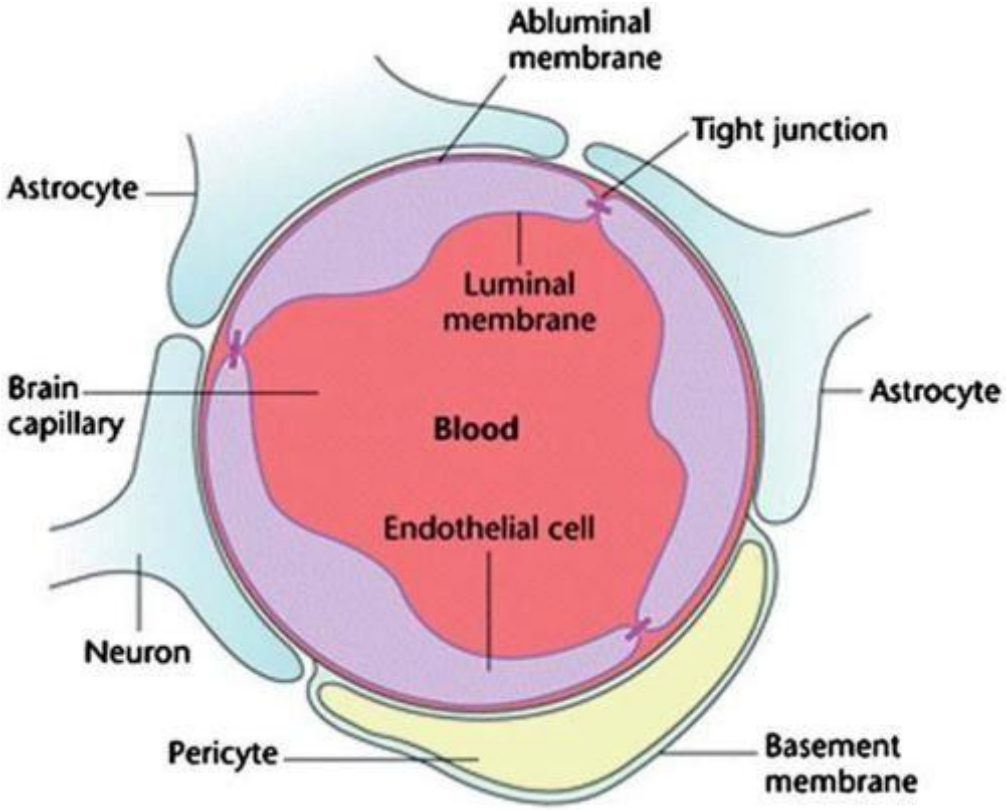


Figure 2.3: Schematic diagram of the blood-brain barrier (Bickel, 2005)

2.2.7.1 Cellular properties of the Blood-Brain Barrier

The BBB consists of a monolayer of brain micro-vascular endothelial cells (BMVEC) joined together by much tighter junctions than peripheral vessels, and this forms a cellular membrane which is known as the main physical barrier of BBB (Abbott, 2005; Cardoso *et. al.,* 2010). The main characteristics of this cellular membrane are; uniform thickness, no fenestrae, low pinocytotic activity, continuous basement membrane, and negative surface charge. In addition to the BMVECs, the neurovascular unit consists of the capillary basement membrane, pericytes, astrocytes and microglia. The BMVECs are surrounded by a basement membrane, which is composed of structural proteins (collagen and elastin), specialised proteins (fibronectin and laminin) and proteoglycans. This structural specificity gives the basement membrane a cell establishment role. Pericytes are cellular constituents of microvessels including capillaries and post-capillary venules that covered about 22-32% of the capillaries and share the same basement membrane. Pericytes are responsible for a wide variety of structural and non-structural tasks in BBB. In summary, they synthesise some structural and signalling proteins, and they are involved in the BMVEC proliferation, migration and differentiation (Cardoso *et al.,* 2010). Fine lamellae closely opposed to the outer surface of the capillary endothelium and respective basement membrane are formed by astrocytes end feet. Like pericytes, astrocytes are involved in various functional and structural properties of neurovascular unit. Microglias are immunocompetent cells of the brain that continuously survey local micro-environment with highly motile extensions and change the phenotype in response to the homeostatic disturbance of the CNS (Prinz and Mildner, 2011). The interactions of brain micro vascular endothelial cells with basement membrane, neighbouring glial cells (microglia and astrocytes), neurones and perivascular pericytes lead to specific brain micro vascular biology. Presence of matrix adhesion receptors and signalling proteins form an extensive and complex matrix which is essential for maintenance of the BBB (Cardoso *et al.,* 2010). The neurovascular unit and BBB cellular components are illustrated above (Figure 2.3).

2.2.7.2 Molecular properties of the blood-brain barrier

The BMVEC assembly is regulated by molecular constituents of tight junctions, adherence junctions and signalling pathways. Tight junctions are highly dynamic structures which are responsible for the barrier properties of BBB (Lanevskij *et al.* 2009). Apical region of the endothelial cells sealed together by tight junctions and paracellular permeability of BMVECs are limited by them. Structurally, tight junctions are formed by interaction of integral

transmembrane proteins with neighbouring plasma membrane. Among these proteins junction adhesion molecules, claudins and occludins (inter membrane), which bind to the cytoplasmic proteins (e.g. zonula occludens, cinguline) are well studied; their role in tight junctions and BBB have been evaluated (Mensch *et al,* 2010). Beyond the main role in physical restriction of BBB, other functions such as control of gene expression, cell proliferation and differentiation have been suggested for tight junctions. Below the tight junctions, actin filaments (including cadherins and catenins) are linked together forming a belt of adherence junctions. In addition to the contribution in the barrier function some other events such as adhesion of BMVEC to each other, the contact inhibition during vascular growth, the initiation of cell polarity and the regulation of paracellular permeability have been suggested for adherence junctions (Mehdipour and Hamidi (2009). A dynamic interaction between tight junctions and adherence junctions through signalling pathways regulate the permeability of BBB. These signalling routes mainly involve protein kinases, members of mitogen-activated protein kinases, endothelial nitric oxide synthase and G-proteins. Dynamic interactions between these pathways control the opening and closing of the paracellular route for fluids, proteins and cells to move across the endothelial cells through two main types of signal transduction procedures (for example, signals from cell interior to tight junctions to guide their assembly and regulate their permeability, signals transmitted from tight junctions to cell interior to modulate gene expression, proliferation and differentiation).

In addition to the proteins with enzymatic activities, there are other specific proteins (drug efflux transporters, multi-drug resistance proteins, organic anion transporting polypeptides) working as BBB transporters which are responsible for rapid efflux of xenobiotics from the CNS (Losscher and Potschka, 2005) and delivery of the essential nutrients and transmitters to the brain. The combined effect of the special cellular and molecular properties of CNS result in the specific barrier functions of BBB, which is important for preventing CNS from harmful xenobiotics. In view of these properties, drug delivery to the CNS is among the most challenging drug development areas. In order to develop successful drug candidates for CNS induced disorders caused by vanadium drug uptake mechanisms are very important.

2.2.7.3 Active transport

Hydrophilic and lipophilic substances which cannot penetrate the brain through passive diffusion can be delivered via active transport. Some compounds are however, substrates for transporters and at the same time they are delivered by passive diffusion. Drug transporters

are integral membrane proteins which are able to carry the drug usually against the concentration gradient into and out of the cell. The overall exposure of xenobiotics to brain through these transporters depends on their location and expression level according to the normal and pathophysiologic conditions. Two types of drug transporters according to their driving forces (ATP-dependent and ATP-independent) are known. Active transporters broadly categorised as primary (ATP-dependent), secondary or tertiary (ATP-independent) (Mruk *et al.*, 2010).

There are two types of transporters:

i. Carrier-mediated transporters, which express on both the luminal and abluminal membranes and operates in both blood to brain and brain to blood directions.
ii. Active efflux transporters which mediate extruding drugs and other compounds from brain (Alam *et al.*, 2010).

Although the main role of the drug transporters is carrying the drugs and other xenobiotics into and out of the brain but they are responsible for other cell processes such as inflammation, differentiation of immune cells, cell detoxification, lipid trafficking, hormone secretion and development of stem cells (Mruk *et al.*, 2010).

2.2.7.4 Influx transporters

Essential hydrophilic nutrients (for example, glucose, amino acids, fatty acids, organic and inorganic ions) reach the brain through influx transporters and receptors. According to the structural similarity of the target drug to the biologic molecules, it can be delivered to the brain using appropriate transporter. Solute carrier family encodes most of the influx transporters, which include facilitated, ion-coupled and ion exchange transporters that do not need ATP (Eyal *et al.*, 2009). These transporters are responsible for uptake of a broad range of substrates, including glucose, amino acids, nucleosides, fatty acids, minerals and vitamins (Alam *et al.*, 2010).

2.2.7.5 Efflux transporters

Efflux occurs in BBB through both passive and active routes in order to detoxify the brain and prevent from drugs and xenobiotics exposures. There are several kinds of efflux transporters such as ATP-binding cassette transporters (ABC), organic anion transport systems and amino acid transport systems (Ueno, 2009). The ABC transporters are primary

active systems which are responsible for different efflux activities including P-glycoprotein (P-gp), multi-drug resistance proteins (MRPs), and breast cancer-related protein (BCRP). P-gp (the most studied ABC transporter), located in luminal side of BBB, immediately pump most of the drugs and xenobiotics back to the blood and decrease the net penetration to the brain. A broad range of drugs, generally including unconjugated and cationic substances are substrates for P-gp, and where some of them are able to inhibit P-gp and lead to increased permeability of co-administered drugs. This fact can be used as a drug delivery strategy to the brain. Along with P-gp, MRPs and BCRP are responsible for main part of drug efflux in BBB, and their effects are dependent on their localisation and expression level in normal and pathologic conditions. Over-expression of these transporters is considered as one of the major reasons of pharmacoresistance of brain diseases and their inhibition, by-passing and regulating methods are important for CNS drug development (Loscher and Potschka, 2005).

2.2.7.6 Metabolism in the Blood-Brain Barrier (Enzymatic barrier)

Existing enzymes in BBB can be regarded as second barrier after negative surface charge. These enzymes involve in disposition of drugs and xenobiotics before entering the endothelial cells of capillaries. Alkaline phosphatases, acid phosphatase, 5'-nucleotidase, adenosine tri-phosphatase and nucleoside di-phosphatase are among well-studied enzymes, distributed within BBB (Ueno, 2009).

2.3 THE PLANT *Grewia carpinifolia* JUSS

2.3.1. Taxonomy of *Grewia carpinifolia*

Domain: Eukaryota

Kingdom: Plantae

Phylum: Angiospermae (flowering plants)

Subphylum: Euphyllophytina

Class: Eudicota

Subclass: Rosidae

Order: Malvales

Family: Malvaceae (formerly in Tiliaceae)

Subfamily: Grewioideae

Tribe: Grewieae

Genus: *Grewia*

Species: *carpinifolia* (From the Latin *carpinus-folium* meaning "hornbeam-leaved")

Botanical name: *Grewia carpinifolia* (Heywood *et al.*, 2007; *The Plant List, 2015)*
Common names: Yoruba (Nigeria): *itakun okere* (Soladoye *et al.*, 2005),
Tamil (India): *Panripputukkan*

2.3.2. Plant description

Leaf is generally alternate, often palmately lobed or compound and palmately veined (Watson and Dalwitz, 1995) (Figure 2.4). The margin may be entire, but when dentate, a vein ends at the tip of each tooth (malvoid teeth). Stipules are present.

The stems contain mucous canals and often also mucous cavities. Hairs are common, and are most typically stellate. Secretory cavities present with mucilage canals within the tissues, and stellate (star-shaped) hairs present on both the vegetative parts and stipules (Wafaa, 2009). The bark is often very fibrous and tough because of the stratified phloem (Heywood, 1979).

The flowers are commonly borne in definite axillary inflorescences, which are often reduced to a single flower, but may also be cauliflorous, oppositifolious, or terminal. They often bear supernumerary bracts. They can be unisexual or bisexual, and are generally actinomorphic, often associated with conspicuous bracts, forming an epicalyx. They generally have five valvate sepals, most frequently basally connate, with five imbricate petals (Wafaa, 2009). The stamens are five to numerous, and connate at least at their bases, but often forming a tube around the pistils. The pistils are composed of two to many connate carpels. The ovary is superior, with axial placentation, with capitate or lobed stigma (Maas and Westra, 2005). The flowers have nectaries made of many tightly packed glandular hairs, usually positioned on the sepals. Androecium varies from 5 to many fertile stamens, branched, adnate to petals with coherent filaments forming monadelphous staminal tube (Cronquist, 1981). Spore tetrads are tetrahedral or decussate with polysiphonous pollen grains, which are shed as single grains. Pollen apertures differ in number and type, from 3 to even 100, colporate or foraminate with either rugate or spinulose exine (Hinsley, 2006). Gynaecium is superior, syncarpous, synovarious to synstyleovarious from one to many carpelled and ovary from one to many locules. Locules are sometimes, secondary divided by false septa, resulting in one-ovule segments (Perveen *et al*., 2004). Styles apical, free or partially joined ended with dry papillate or non-papillate stigmas. Polar nuclei fuse prior to fertilisation and three antipodal cells are formed. Synergids are pear-shaped or hooked with nuclear endosperm formation and asterad embryogeny (Thorne, 2001).

2.3.3. Geographical distribution

The *Grewia* consists of about 280-300 species well represented in tropical Africa, Asia and Australia (Cronquist, 1981; Chung *et al.,* 2003). In West Africa, 17 species occur; sixteen of these are recorded for Nigeria (Hutchinson and Dalziel, 1954). Nigeria is a major centre of diversity for *Grewia* in West Africa (Czarnecka *et al*., 2006). Six species are known to occur in lowland rainforest, eight in dry woodland savannah while two species occupy both ecological zones (Hutchinson and Dalziel, 1954). The lowland rainforest species are distinguished by presence of panicle inflorescence, while the savannah species are distinguished by the presence of cymose inflorescence (Burret, 1926; Hutchinson and Dalziel, 1954; Bayer and Kutbitzki, 2003; Cowie et al., 2011). Although considerable works have been done on the species of the genus, data on Nigerian species are sparse.

Nigerian lowland rainforests and Guinea Savannah provide suitable habitats for the sixteen species in the genus *Grewia.* Six species, *G. malacocarpa, G. barombiensis, G. brunnea, G. coriacea, G. hookerana* and *G. oligoneura* are confined to the high rainforest of the Southern part of Nigeria, while eight species (*G. barteri, G. bicolor, G. cissoides, G. flavescens, G. lasiodiscus, G. venusta, G. tenax* and *G. villosa*) are found in the Guinea Savannah in the Northern part of Nigeria. Only two species (*G. carpinifolia and G. mollis*) occupy both ecological zones.

Figure 2.4: Picture of *Grewia carpinifolia* leaf

2.3.4 Medicinal Uses of the Genus *Grewia*

The different parts of different species of genus *Grewia* are used as folk medicine in the different part of globes (Goyal., 2012). Diverse bioactivity studies on different species of genus *Grewia* have been reported. The roots of *G. abutilifolia* are applied to abscesses . The fruit of *G. asiatica* (commonly referred to as phalsa in Asia) is used as astringent, while the leaf are used in pustular eruptions (Simal., 1980), and root bark as a remedy for rheumatism. Ethanol extract (50%) of *G. asiatica* showed hypotensive activity, while the aqueous extract of stem bark is reported to be antidiabetic (Simal., 1980). Its seed extract and seed oil exhibited antifertility activity (Asolkar *et al*., 1992). Fruit extract of *G. asiatica* showed radioprotective effect in Swiss mice against lethal dose of γ-irradiation (Ahaskar *et al.*, 2007). The fruit is astringent and stomachic. It is reported that unripe phalsa fruit is anti-inflammatory, antidiarrhoeal, haematinic and reduces fever (Morton, 1997; Nair *et al.*, 2005).

G. asiatica contains anthocyanin type cyanidin 3- glucoside, vitamin C, minerals and dietary fibers (Yadav, 1999; Nair *et al.*, 2005). The antioxidant properties of vitamin C are well known and anthocyanin has recently emerged as a powerful antioxidant.
Grewia bicolor is a part of Sudanese traditional medicine, and it is used in the treatment of skin lesions and sometimes also as a tranquiliser (Jaspers *et al.*, 1986). The three alkaloids: Harman, 6-methoxyharman, and 6-hydroxyharman, isolated from the methanol extract of this plant, have antibacterial properties ((Jaspers *et al.*, 1986)

Chloroform extract of the aerial parts of *G. bilamellata* exhibited antimalarial activity against the D6 and W2 clones of *Plasmodium falciparum* (Ma *et al.*, 2006). *G. carpinifolia* is antiparasitic (Goyal *et al.*, 2011) Ethanol extract of stem bark of *G. elastica* showed CNS depressant activity (Bhakuni *et al.*, 1987). Various parts of *G. hirsuta* are used as analgesic , and the ethanol extract of its stem bark exhibits antiviral and diuretic activities (Dhawan, 2006). The plant *G. microcos* is used for treating indigestion, eczema and itch, small pox, typhoid fever, dysentery and syphilitic ulceration of the mouth. *Grewia mollis* is known to be a strong fire resistant (Heinz and Helene, 1978). Various parts of the plant are used in food and medicine. In Nigeria, the stem bark powder or mucilage is used as a thickener in local cakes made from beans or corn flour, commonly called “Kosai” and “Punkasau” in Hausa (Nigeria), respectively. The dried stem bark is ground and the powder mixed with beans or corn flour, thereby enhancing the texture used as a soup or sauce vegetable. The mucilage of the bark or leaf is applied to wound to aid healing. The Yorubas in Nigeria use it medicinally

at times of child-birth to induce uterine contraction (Dalziel, 1977). Some findings demonstrated that the mucilage obtained from the stem bark can serve as a good binder in paracetamol formulations (Martins *et al.,* 2008; Muazu *et al.,* 2008). Phytochemical studies of *G. mollis* indicate the presence of tannins, saponins, flavonoids, glycosides, phenols, steroids and the absence of alkaloids in the leaf and stem bark (Onwuliri and Umezurumba, 2003). Crude methanol extract of *G. mollis* also exhibits antimicrobial activity (Youssef *et al.,* 2012). The roots of *G. sclerophylla* are prescribed in cough and irritable conditions of intestine and bladder. Its decoction is used as an emollient enema, while alcoholic extract of aerial parts demonstrate anticancer activity (Dhawan *et al.,* 2005) and that *of G. serrulata* shows anti-inflammatory activity (Muazu *et al.,* 2008). The stem extract of *G. villosa* was found to be anticarcinogenic culture (Hussein and Kingstar, 1981). An extract of *Grewia villosa* also contains harman alkaloids. Harman alkaloids belong to the class of β-carbolines and bind strongly to receptors in the brain and affect the CNS (Pfau and Skog, 2004). In Sudan, the ethanol extract of the aerial parts was found to exhibit CNS depressant activity (Dhar, 1988).

2.4 Current Study

There exists a plethora of studies on the toxicity of vanadium exposure with evidences indicating that antioxidants may mitigate some of these toxic effects (El-Shaari *et al.,* 2002; Olopade *et al.,* 2011). Several of the *Grewia species* have been demonstrated to possess antioxidant activities. To date, no study (either on human or animals) has examined the improvement of the behavioural and neurocognitive effects using plant extract, localised in Nigeria, a country known for oil exploration. Hence, the purpose of the current study was to investigate whether *Grewia carpinifolia* (with a high biodiversity in Nigeria) could protect the brain following acute vanadium toxicity.

CHAPTER THREE

3.0 STUDY 1

Phytochemical, acute and sub-chronic toxicity studies of the leaf and stem of *Grewia carpinifolia*

3.1 INTRODUCTION

Plants have been used in the management of human and animal health over the years (Adebiyi and Abatan, 2013). In developing countries, the use of plant extracts in traditional medicine is the mainstay of health care delivery or a complement to it. Whereas effects of some of these plants used in folk medicine have been investigated and documented in literature, the safety profile of *Grewia carpinifolia* leaf and stem remains invalidated despite their widespread uses. Plant chemical constituents may be therapeutically active or inactive, thus, there is a need phytochemical screenings to detect diverse groups of naturally occurring phytochemicals (Masih and Singh, 2012). The phytochemical research approach is considered effective in discovering bioactive profile of plants of therapeutic importance. During the present study, the major chemical groups contained in the ethanol extract of the leaf and stem of *Grewia carpinifolia* were examined. Furthermore to justify the folklore uses, its safety and potential toxic effects are carried out following acute and sub chronic administration. Acute toxicity of the plant extract was determined by calculating LD_{50}, which is, the dose that will kill 50% of animals of a particular species.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material and Authentication

Fresh leaf and stem of *Grewia carpinifolia* were collected from the Botanical Garden of the University of Ibadan. The plant was identified and authenticated at the Forestry Research Institute of Nigeria, Ibadan, where herbarium specimen (voucher number FHI. 109693) was deposited. A total of 18125 g of the plant was collected. They were cleaned to remove adhering dirt and the leaf (about 6125 g) were separated from the stem (about 11 085 g).

3.2.2 Extract Preparation

The leaf and stem of *Grewia carpinifolia* were extracted according to the method of Panovska *et al*. (2005). The leaf and stem were air-dried for six weeks and ground into coarse powder using an electric blender (Blender/Miller III, model MS-223, Taiwan, China). Extraction was carried out by cold maceration of the coarse powder with 100% v/v ethanol

for 72 h, with constant shaking using the GFL shaker (no. 3017GBh, Germany). Each batch of harvested solvent were stored in glass containers and refrigerated at 4 ^{o}C.

3.2.3. Separation of the extract

The resultant mixture was filtered using Whatman filter paper (No.1) and the filtrate was concentrated to dryness *in vacuo* at 40°C, using rotary evaporator to give a yield of 20% w/w of the extract. The ethanol remaining in the extract was finally removed by placing small volumes in porcelain dishes in the oven set at low temperature of 4 ^{o}C. The extract obtained for the leaf and stem was semi-solid greenish-brown paste, with varied colour intensities. Aliquot portions of the extract were weighed and dissolved in warm distilled water for use in this study.

3.2.4 Experimental Animals

Male and female Wistar rats were purchased and housed at the Animal House, Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine. The animals were housed under standard conditions of temperature, (25 ± 2°C) and light, (approximately 12/12 h light-dark cycle) and given access to standard diet (Animalcare® Feeds Ltd., Ogere, Nigeria) and fresh water *ad libitum*. The cages were cleaned of waste once daily. All the animals were acclimatised to laboratory conditions for two weeks before the commencement of the experiment. All experiments performed on the laboratory animals in this study followed the OECD approved Standard Operation Procedures (SOPs) and were approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/025) (appendix 1).

3.2.5 Drugs and Chemicals

Ethanol and all other chemicals used were of analytical grade, and were prepared in all glass distilled water.

3.3 Preliminary Phytochemical Analysis

Standard methods were used to detect the presence and quantity of phytoconstituents present in the ethanol extract of *G. carpinifolia* leaf and stem (Kokate, 1998; Khandelwal, 2005).

3.3.1 Qualitative Phytochemical Screening

Dry solid samples of the ethanol extracts of the stem and leaf were screened for the presence of phytochemicals according to the methods of Trease and Evans (1989) as follows:

3.3.1.1 Tannins

Each of the extract (about 0.5 g) was boiled seperately with 10 ml of water for 15 minutes, filtered and made up to 100 ml with distilled water. 10 ml of distilled water and 1 drop of $FeCl_3$ was added to 2 ml of the filtrate. Blue or green colouration indicated the presence of tannins.

3.3.1.2. Phlobotanins

Exactly 5 ml of the filtrate from the test on tannins was added to 3 drops of 40 % formaldehyde and 6 drops of dilute hydrochloric acid. The temperature of the mixture was raised to boiling point, cooled and the bulky precipitate formed was washed with hot distilled water, ethanol and warm 5% potassium hydroxide. The presence of brown coloured residue indicated the presence of phlobotanins.

3.3.1.3. Flavonoids

Each of the extract (0.5 g) was added to some magnesium ribbons and 5 ml of concentrated hydrochloric acid. The red colouration indicated the presence of flavonoids.

3.3.1.4 Saponins

The extract (0.5 g) was added to 5 ml of hot water in a test tube and shaken vigorously. Persistent frothing indicated the presence of saponins.

3.3.1.5. Alkaloids

The extract (0.5 g) was added to 5 ml of distilled water. Hydrochloric acid (1 ml) was added to acidify the mixture and it was filtered. The filtrate (1 ml) was treated with 2 drops of Mayer's reagent. A precipitate was taken as an evidence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent. A turbidity or precipitate further confirmed the presence of alkaloids.

3.3.1.6. Free anthraquinones

Exactly 0.5 g of extract was dissolved in distilled water and extracted by 5 mins with heat. It was filtered hot, cooled and extracted with 10 ml carbon tetrachloride (CCl_4). The CCl_4 layer

was washed with 5 ml dilute ammonium hydroxide. A pink colouration in the ammonia layer indicated the presence of free anthraquinones.

3.3.1.7 Combined anthraquinones

Iron chloride ($FeCl_3$) was added and mixed with 0.5 g of extract and 5 ml HCl. The mixture was warmed in a water bath for 10 min. It was filtered hot, cooled and treated as for free anthraquinones. The presence of colour indicated the presence of combined anthraquinones.

3.3.1.8. Cardiac Glycosides

Exactly 0.5 g of the extract was dissolved in 2 ml of chloroform and 2 ml concentrated H_2SO_4 was carefully added to form a layer. Reddish-brown colouration at the interface indicated the presence of steroidal cardiac glycosides.

3.3.2. Quantitative Phytochemical Screening

3.3.2.1 Determination of total phenolics

Using modified Folin-Ciocalteu method (Wolfe *et al*., 2003). Total phenol contents in the extracts were determined. Aliquots (50 μL) of 12.5, 25, 50, 100, 200, and 400 μg/mL methanol gallic acid solutions were mixed with 100 μL Folin–Ciocalteu reagent (diluted ten-fold) and 100 μL (75 g/L) sodium carbonate. The mixture was incubated at 25°C for 30 min; the quantitative phenolic estimation was performed at 765 nm. The calibration curve was constructed by plotting the value of absorbance versus concentration. A similar procedure was adopted for the tested samples as described above. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract using the following equation, based on the calibration curve: $y = 0.0003x + 0.0716$, $R^2 = 0.9365$, where x was the absorbance and y was the tannic acid equivalent (mg/g).

3.3.2.2 Determination of total flavonoids

Estimation of the total flavonoids in the plant extracts was carried out using the method of Ordonez *et al* (2006). A volume (0.5 ml) of 2% aluminium chloride ($AlCl_3$) ethanol solution was added to 0.5 ml of sample. After one hour at room temperature (25 °C), the absorbance was measured at 420 nm. Extract samples were evaluated at a final concentration of 1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation,

based on the calibration curve: y = 0.0255x, R^2 = 0.9812, where x was the absorbance and y was the quercetin equivalent (mg/g).

3.3.2.3 Determination of anthraquinone

Determination of anthraquinone was based on the procedure reported by Sun *et al.* (1998). A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total anthraquinone content was expressed as catechin equivalents (mg/g) using the following equation, based on the calibration curve: y = 0.5825x, R^2 = 0.9277, where x was the absorbance and y is the catechin equivalent (mg/g).

3.4 Pilot Toxicological Studies

3.4.1 Acute Toxicity Study

The acute toxicity study was done according to the method described by Miller and Tainter, (1944) and Ghosh (1984). The estimation of the dose range of the LD_{50} was initially determined as a pilot study using the 'staircase method' involving a small number of animals (3 each dose) and increasing the doses of the extract in two phases.

Animals were randomly divided into four groups (A-D) with three rats in each group. Group A was administered with 50 mg/kg ethanol extract of *G.carpinifolia* group B, 100 mg/kg, group C, 1000 mg/kg and group D, received distilled water (10 ml/kg) only, and served as control. In the second phase the animals were further administered with 2000 mg/kg ethanol extract of *G. carpinifolia*; 4000 mg/kg, and 6000 mg/kg. These doses were given orally in a single oral dose.

Animals were observed at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h for any toxic symptoms. Parameters observed include; mortality of animals, motor activity, tremors, convulsions, posture, spasticity, opisthotonicity, ataxia, righting reflex, sensations, pilo-erection, ptosis, lacrimation, exophthalmos, salivation, diarrhoea, writhing, skin colour and respiratory rate. The monitoring of the parameters commenced immediately after oral administration the drug. After 24 hours, the number of dead rats was counted in each group and percentage of mortality calculated.

3.4.2 Sub-chronic Toxicity Study

Sub-chronic toxicity study was carried out in accordance with WHO (1992) and OECD 407 (1995) guidelines. Thirty male rats were divided into five groups of six rats each. Group A, received normal saline and served as the controls while rats in groups B, C, D and E were given 100, 200, 400 and 800 mg/kg bw, respectively daily for 28 days. All the rats had free access to food and water throughout the duration of the experiment, and were observed daily for general symptoms of toxicity and mortality.

3.4.2.1 Feed and water intake

Pre-weighed food was provided in standard stainless steel hoppers. After 24 h, rats were briefly removed from their cages and weighed, and the amount of food remaining, including any on the bottom of the cages or any that had spilled onto plastic sheets placed under each cage, was recorded. Intake was calculated as the weight (in grams) of food provided less that recovered (Vento *et al.*, 2008).

3.4.2.2 Body weight change

Rats in all the groups were weighed (Kitchen Scale, Model FKS, India) twice every week during the period of treatment and on the last day of study. Doses of the extract administered were adjusted accordingly.

3.4.2.3 Behavioural Tests

Following the administration of the last dose of the extract on day 28, behavioural tests were carried out on rats in each group.

3.4.2.4 The Open-Field Test

The open-field apparatus was constructed with white plywood and measured 72 x 72 cm with 36 cm walls. One of the walls was covered with Plexiglas so rats could be visible in the apparatus. Black lines were drawn on the floor with a marker and were visible through the uncovered opening. The lines divided the floor into sixteen 18 x 18 cm squares. A central square (18 cm x 18 cm) was drawn in the middle of the open-field (Brown *et al.*, 1999). The central square was used because some rats' species have high locomotor activity and crossed the lines of the test chamber many times during a test session. The central square also had sufficient space surrounding it to give meaning to the central location as being distinct from the outer locations (Carrey *et al.*, 2000).

The open-field maze was cleaned between each rat using 70 % ethyl alcohol to remove any odour biase by previous animal. Behaviour was scored with Hindsight for MS-DOS (version 1.5), and each trial was recorded for later analysis, using a video camcorder (Hitachi, VM-7500LA), positioned above the apparatus. Measures of line crosses were obtained with an automated camera-based computer tracking system (Limelight, Actimetrics) on an IBM PC computer with the camera fixed at about 0.7 m above the apparatus.

Procedure

Rats were carried to the test room in their cages and were handled by the base of their tails at all times. Rats were placed into the centre or one of the four corners of the open-field and allowed to explore the apparatus for 5 minutes. After the 5 minute test, mice were returned to their cages and the open-field was cleaned with 70 % ethyl alcohol and permitted to dry between tests. To assess the process of habituation to the novelty of the arena, rats were exposed to the apparatus for 5 minutes on two consecutive days.

Behaviours scored

The behaviours scored (Brown *et al*, 1999) included:

1. Line crossing: Frequency with which the mice crossed one of the grid lines with all four paws.
2. Centre square entries: Frequency with which the mice crossed one of the red lines with all four paws into the central square.
3. Centre square duration: Duration of time the mice spent in the central square.
4. Rearing: Frequency with which the mice stood on their hind legs in the maze.
5. Stretch-attend postures: Frequency with which the animal demonstrated forward elongation of the head and shoulders, followed by retraction to the original position.
6. Grooming: Duration of time the animal spent licking or scratching itself while stationary.
7. Freezing: Duration with which the mouse was completely stationary.
8. Urination: number of puddles or streaks of urine.
9. Defaecation: number of faecal pellets produced.

Factor Analysis

Several dependent variables measured in the open-field correlated significantly with one another, such as: line crosses and rearing, as well as line crosses and central square activity

(Walsh & Cummins, 1976). Factor analysis of open-field behaviour generally yields three factors, but the names of these factors vary. Jahkel *et al.* (2000) identified three factors: Activity (53.5% of the variance) (line crosses, time active); Exploration (15.5% of the variance) (centre squares crossed, time in centre); and Irritation (9.5% of the variance) (time passive).

Ramos *et al.* (1997) also identified three factors: Anxiety or Approach/Avoidance (36.6% of variance) (locomotion in centre squares); locomotor activity (30.3% of variance) (total lines crossed, lines crosses in outer squares) and defaecation scores (18.3% of variance). Crusio & Schwegler (1987) also found three factors: Locomotion (rearing, lines crossed); Grooming (grooming frequency and duration); and defaecation (sniffing, defaecates). These studies did not always measure the same behaviours and the different factors identified indicated that the definitive study of the open-field test has yet to be conducted.

Repeated Exposure

Repeated exposure to the open-field apparatus result in time dependent changes in behaviour (Choleris *et al.,* 2001). At first, when the apparatus was novel to the animals, more fear-related behaviours (such as stretch attends and activities in the corners and walls of the open field) were displayed. However, with repeated trials, more exploration and locomotor activity (such as rearing and line crosses as well as more central square activity) were observed. There were, however, strain differences in behaviour after repeated testing in the open field. With repeated exposure, some strains showed increased activity, while others showed habituation and decreased activity levels, and others showed no change (Bolivar *et al*. 2000).

Figure 3.1: A picture of the open-field test box showing a mouse (Tatem *et al.* 2014)

3.4.2.5 Fore-limb Support (Hanging Wire) Test

Each mouse's forepaws were placed on a horizontally suspended wire (1 mm in diameter), placed 47 cm above a soft foam landing area (Fig. 3.2). Both ends of the hanging wire were tightly secured. Each of the experimental mice was placed with its forepaws on the wire. Rats were timed from the moment they were placed on the wire until they dropped from the wire. This reflected muscular strength in rats (Soazo and Garcia, 2007). The hanging wire was cleaned between each rat using 70 % ethyl alcohol. Each rat was tested on thrice on this test.

3.4.2.6 Negative Geotaxis

Each rat was placed in the middle of a slab, 30^{o} inclined to the surface plane, in a head-down position, and latency to turn 180^{o} to a head up position was measured. This test reflected vestibular function, and motor development and activity (Carter *et al.*, 2001).

Figure 3.2.: A picture of a mouse on the fore-limb support test

Figure 3.3.: A rat on the negative geotaxis test

3.5 Blood Collection

Blood collection was via puncture of the retro-orbital plexus of anaesthetized rats using haematocrit tubes. About 5 ml of blood was collected. Two separate blood samples were collected from each animal; one into heparinised bottle for haematology, and the other into plain bottles without anticoagulant for collection of serum samples for biochemistry tests.

3.5.1 HAEMATOLOGY

3.5.1.1 Erythrocyte Count

Erythrocyte diluting pipette marked 101 above the bulb was filled with blood sample to exactly 0.5 mark, the tip of the pipette was inserted into the erythrocyte diluting fluid (Grower's solution). Through a steady suction, the pipette was filled with the fluid to 101 mark above the bulb, rotating gently while filling. The pipette was then gently rotated for 3 minutes by a simple wrist movement, with the pipette held horizontally between the thumb and the middle finger. The haemocytometer was filled with the diluted blood in the pipette and then allowed a few minutes for the cells to settle. The erythrocytes were then counted under high power microscope lens (x10), counting all the erythrocytes in the five of the 25 small squares in the central area of the counting chamber of the haemocytometer. The erythrocyte was determined from the sum of all cells in the five small squares multiplied by 10,000, and this gave the total erythrocytes per cubic millimetre.

3.5.1.2 Estimation of Haemoglobin

Cyanmethaemoglobin method which measures total haemoglobin (Hb) including carboxyhaemoglobin was used. Exactly 4 ml of Drabkin's diluent solution (4 ml) was placed in a tube, into which 0.02 ml of the blood sample measured with a pipette was added, rinsing the pipette three times. The mixture was allowed to stand for 10 minutes. After thorough mixing, the mixture was read in a colorimeter at 540 nm wavelength. The Hb value of the sample was calculated, thus:

$$\text{Haemoglobin (Hb)} = \frac{\text{Photometer reading of unknown x g\% Hb of standard x Dilution factor}}{\text{Photometer reading of standard}}$$

$$\text{Dilution factor} = \frac{\text{Volume of whole blood used + Volume of diluents}}{\text{Volume of whole blood used}}$$

3.5.1.3 Determination of Packed Cell Volume

The heparinised capillary tubes were filled with blood samples to about two-third of the tube and the vacant end of each tube was sealed with plasticine. The tubes were then placed in the heamatocrit-centrifuge (Perkin Elmer, USA) for 5-6 min at 3000 rpm. Thereafter, the capillary tubes were then removed and read in the graphic reader.

3.5.1.4 Total Leucocyte Count

The leucocyte diluting pipette, marked 11 above the bulb was filled with blood sample to the mark 0.5 and the pipette was then filled with leucocyte diluting fluid to the mark 11 above the bulb. The mixture was gently rotated for three minutes until well mixed. 2-3 drops from the pipette were discarded before filling the counting chamber of the haemocytometer. The leucocytes were allowed one minute to settle and the number of the leucocytes in the four large corner squares of the counting chamber was counted under low power microscope lens (x40). The sum of the cells in the four corner squares multiplied by 50 gave the total leucocytes per cubic millimetre.

3.5.1.5 Leucocytes Differential Count

The count was estimated using the method of Osim *et al* (2004). A dry micropipette was used to suck in blood from the blood sample bottle. A small drop of blood was applied to one end of a slide and quickly placed on the bench holding it in position and the end of the second slide was then placed in the drop and held there until the blood had spread across it.

It was then drawn slowly over the whole length of the first slide, being held at an angle of 45°. After the blood had spread, it was dried before staining with Leishman's stain. The film which was washed off in a gentle stream of water, dried with filter paper and examined under low and high power microscope (x10) and the different types of cells were respectively counted.

To eliminate inefficiencies associated with mechanical desktop tally counters counting software (EasyCell Counter) was used (Dogsa, 2006). Each leucocyte was assigned to one of the following categories; lymphocytes, monocytes, segmented neutrophils, band neutrophils, eosinophils, basophils, and other cells. For each category, the percentage was calculated by dividing the number of cells in each category of leucocytes by the total number of leucocytes counted. The slides were counted three times for precision. The absolute neutrophil count

(ANC) was calculated as the total leucocyte count (WBC) multiplied by the percentages of segmented neutrophils (Ns) and band neutrophils, (Nb) as follows:

$$ANC = WBC \times (N_s + N_b)$$

3.5.1.6 Platelet Count Determination

The platelet count was determined using the method of Brecher and Cronkite (1950). Whole blood was diluted with a 1% ammonium oxalate solution. The isotonic balance of the diluent was such that all erythrocytes were lysed while the leucocytes, platelets, and reticulocytes remained intact (Brecher and Cronkite, 1950). The standard dilution for platelet counts was 1:100. This dilution was prepared using the leucocyte/platelet unopette system (Rutherford *et al.,* 1996). The dilution was mixed thoroughly and incubated to allow for the lysis of the erythrocytes. Following the incubation period, the dilution was mounted on a haemacytometer. The cells were allowed to settle and counted in a specific area of the haemacytometer chamber under the microscope. The number of platelets was calculated per µL (x 10_9/L) of blood.

3.5.2 BIOCHEMICAL ASSAYS

The other blood collected into plain sample bottles without anticoagulant was used for serum analysis. This portion was allowed to clot and centrifuged at 3500 rpm for 10 minutes. The serum was separated, stored at -4 °C and used for evaluation of biochemical parameters, including activities of alanine transaminase (ALT), aspartate transaminase (AST) levels and alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) levels, total protein, albumin, bilirubin and serum urea concentrations using commercial kits (Randox Laboratories, UK). Globulin levels were calculated as the difference between the total protein and albumen.

3.5.2.1 Alanine aminotransferase activity

The activity of alanine aminotransferase (ALT) was determined following the principle described by Reitman and Frankel (1957).

Principle

Alanine aminotransferase (ALT) was measured by monitoring the concentration of pyruvate hydrazine formed with 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957). The enzyme ALT catalysed the transfer of amino group from L-alanine to α- oxoglutarate to form L- glutamate and oxaloacetate.

α- oxoglutarate + L- alanine $\xrightarrow{\text{ALT}}$ L- glutamate + Pyruvate

The oxaloacetate formed was unstable and quantitatively decarboxylated to pyruvate which then formed a complex with 2, 4- dinitrophenylhydrazine (DNPH) to produce an intensely coloured hydrazine on the addition of NaOH. This coloured complex absorbed radiation at 530-550 nm.

Procedure

0.1 ml of test serum was dispensed into a test tube, 0.5 ml of solution R1 (containing phosphate buffer 100 mmol/l, pH 7.4, L-alanine and α-oxoglutarate) was added to the same test tube. The mixture was then incubated for 30 minutes at 37 ^{o}C. Following incubation, 0.5 ml of 2 mmol/l, 2, 4-dinitrophenylhydrazine (solution R2) was added and allowed to stand for 20 minutes at room temperature (25 ^{o}C). Sodium hydroxide (0.5 ml) was added and the final mixture was read in a spectrophotometer (Spectrolab 2250, India) at 546 nm wavelength. Reagent blank was prepared as described above by replacing sample with 0.1 ml of distilled water according to the description by ALT kit, Randox Chemicals, Netherlands. The absorbance of the test samples were read against the reagent blank after 5 minutes.

Reagents	**Concentration**
Phosphate buffer	100 mmol/L, pH 7.4
L-alanine	100 mmol/L
α-oxoglutarate	2 mmol/L
2,4-dinitrophenylhydrazine	2 mmol/L
sodium hydroxide	0.4 mmol/L

Table 3.1: Determination of alanine aminotransferase activity in serum

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

(Schmidt *et al.*1963).

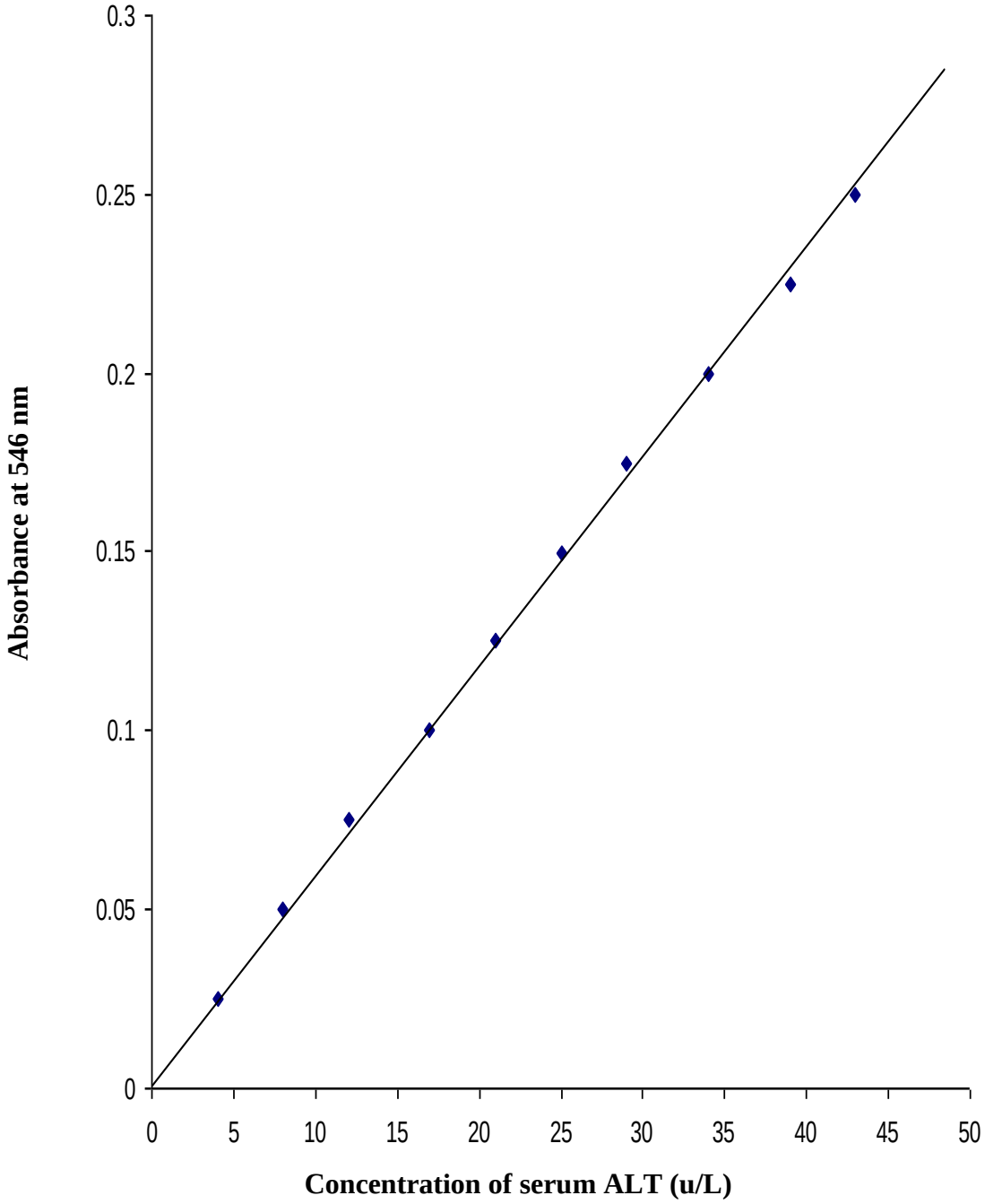


Figure 3.4: Alanine aminotransferase (ALT) standard curve

3.5.2.2 Aspartate Aminotransferase Activity

Aspartate Aminotransferase (AST) activity was determined following the principle described by Reitman and Frankel (1957).

Principle

Aspartate aminotransferase (AST) was measured by monitoring the concentration of oxaloacetate formed with 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957).

α- oxoglutarate + L- alanine $\xrightarrow{\text{AST}}$ L- glutamate + oxaloacetate

Procedure

Test serum (0.1 ml) was dispensed into a test tube and 0.5 ml of solution R1 (containing phosphate buffer 100 mmol/l, pH 7.4, L-aspartate and α-oxoglutarate) was added to the same test tube. The mixture was then incubated for 30 minutes at 37 ^{o}C. Following incubation, 0.5 ml of 2 mmol/l 2, 4-dinitrophenylhydrazine (solution R2) was added and allowed to stand for 20 minutes at room temperature. A total of 5 ml of sodium hydroxide was added and the final mixture was read in a spectrophotometer (Spectrolab 2250, India) at 546 nm wavelength. Reagent blank was prepared as described above by replacing sample with 0.1 ml of distilled water according to description by AST kit, Randox Chemicals, Netherlands. The absorbance of each test sample was read against the reagent blank after 5 minutes.

Table 3.2: Calibration of the aspartate aminotransferase standard curve

Absorbance	U/l	Absorbance	U/l
0.02	7	0.10	36
0.03	10	0.11	41
0.04	13	0.12	47
0.05	16	0.13	52
0.06	19	0.14	59
0.07	23	0.15	67
0.08	27	0.16	76
0.09	31	0.17	89

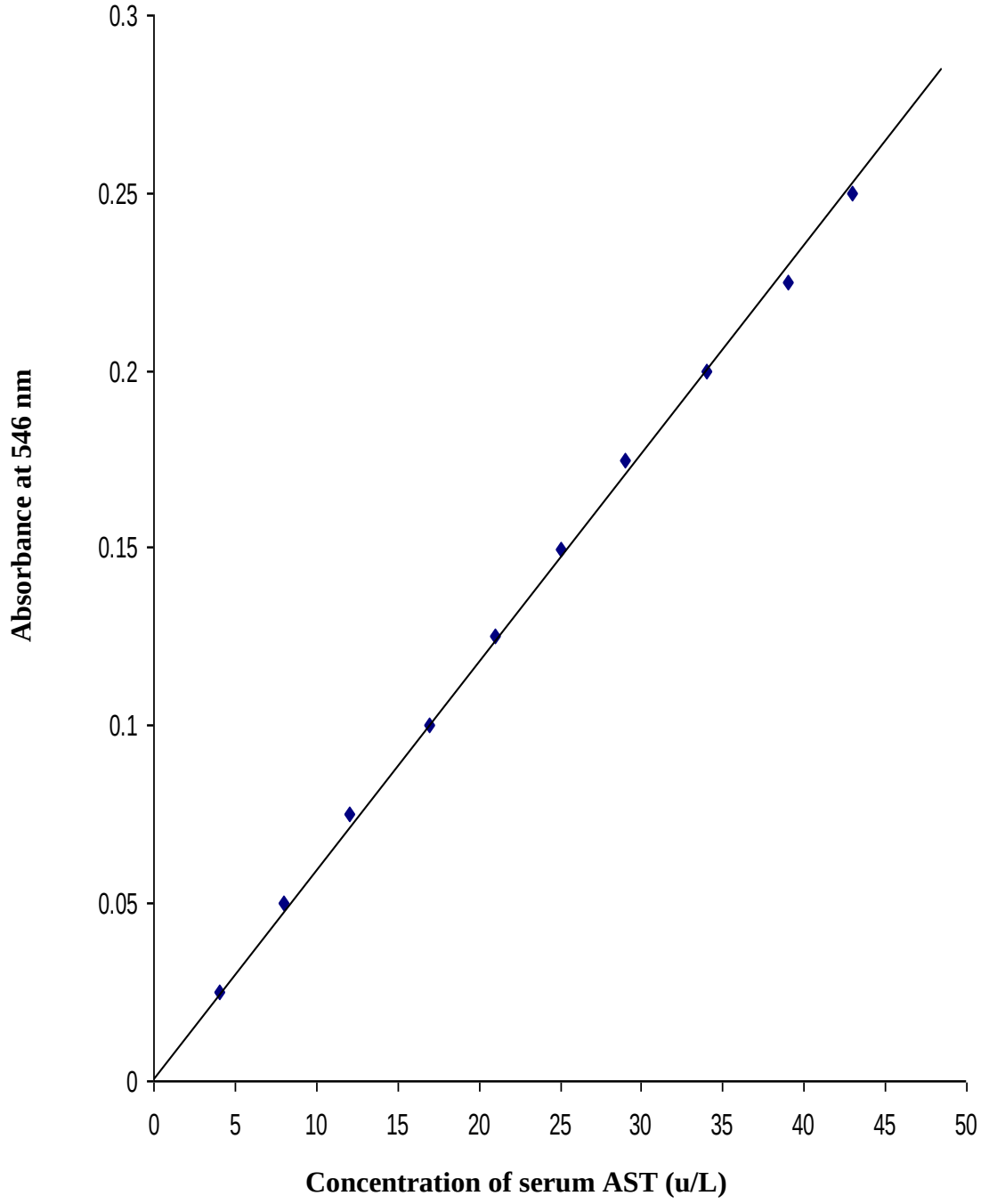


Figure 3.5: Aspartate Aminotransferase standard curve

3.5.2.3 Alkaline Phosphatase Activity

Test serum (0.02 ml) was added to 1 ml p-nitrophenylphosphate (10 mmol/l) in the same test tube. The initial absorbance of the mixture was read in a spectrophotometer (Spectrolab 2250, India) at 405 nm wavelength. The absorbance was read again after 1, 2 and 3 minutes. The alkaline phosphatase (ALP) activity was calculated as follows;

$$\text{ALP (U/L)} = 2760 \times \Delta A \text{ 405 nm/min}$$

3.5.2.4 Gamma Glutamyl Transferase Activity

Test serum (0.1 ml) was added to 1 ml L-γ-glutamyl-3-carboxy-4-nitroanilide (2.9 mmol/l) in the same test tube. The initial absorbance of the mixture was read in a spectrophotometer (Spectrolab 2250, India) at 405 nm wavelength. The absorbance was read again after 1, 2 and 3 minutes. The gamma glutamyl transferase (GGT) activity was calculated as follows;

$$\text{GGT (U/L)} = 1158 \times \Delta A \text{ 405 nm/min}$$

3.5.2.5 Total Protein

Test serum (0.2 ml) was added to 1 ml of Biuret reagent in a test tube. The mixture was incubated for 30 minutes at room temperature (25 °C). The absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) were read against the reagent blank in a spectrophotometer (Spectrolab 2250, India) at 546 nm wavelength. The total protein concentration was calculated as;

$$\text{Total protein (g/dl)} = A_{sample}/A_{standard} \times \text{Standard concentration}$$

3.5.2.6 Albumin

Test serum (0.01 ml) was added to 3 ml of bromocresol green in a test tube. The mixture was incubated for 5 minutes at room temperature. The absorbance of the sample (A_{sample}) and that of the standard ($A_{standard}$) were read against the reagent blank in a spectrophotometer (Spectrolab 2250, India) at 578 nm wavelength. Two minutes later the absorbances A2 of the standard and test sample were also read. The albumin concentration was calculated as:

$$\text{Serum albumin (g/dl)} = A_{sample}/A_{standard} \times \text{Standard concentration}$$

3.5.2.7 Urea

A total of 10 µl of test serum was dispensed into a test tube and 100 µl of solution R1 (containing sodium nitroprusside and urease) was added to the same test tube. The mixture was then incubated for 10 minutes at 37 ^{o}C. Following incubation, 2.5 ml of 120 mmol/l phenol (solution R2) and 2.5 ml of 27 mmol/l sodium hypochlorite (solution R3) was added. The mixture was incubated for another 15 minutes at 37 ^{o}C. The final colour reaction of the solution was read in a spectrophotometer (Spectrolab 2250, India) at 546 nm wavelength. The absorbance of the test samples were read against the standard. The urea concentration was calculated as;

$$\text{Urea concentrartion (mg/dl)} = A_{sample}/A_{standard} \text{ x Standard concentration}$$

3.5.3 Relative organ weight ratio

Different organs, namely the brain, heart, spleen, liver and kidneys were removed, weighed and observed macroscopically. The relative organ body weight ratio (ROW) of each rat was calculated as follows:

ROW = Absolute organ weight (g)/Body weight of rats on sacrifice day (g).

3.5.4 Histological analysis

The brain, heart, spleen, liver and kidneys were removed carefully dissected out, and fixed in 10% formalin and processed routinely for paraffin embedding. Sections (5 µ thickness) was obtained with rotary microtome and processed for Haematoxylin and Eosin Stalin (H / E). Sections were observed with light microscope and photomicrograph taken (Chowdhury *et al.,* 1986).

3.6 STATISTICAL ANALYSIS

Results were analysed using one-way analysis of variance (ANOVA). The comparisons between control and treated groups were made using unpaired Student's t-test (Snedecor and Cochran, 1980) while comparisons within groups were done using the paired Student's t-test.

3.7. RESULTS

3.7.1. PHYTOCHEMICAL STUDIES

Phytochemical screening of the ethanol extract of *Grewia carpinifolia* leaf and stem revealed the presence of alkaloid and reducing sugar (Table 3.3). Tanins, saponins, flavonoids, terpenoids, cardiac glycosides, alkaloids and anthraquinones were present in both the leaf and stem of the plant. Phlobatinins were present only in the leaf of the plant while coumarins were not found in both the leaf and stem of *Grewia carpinifolia.*

Following quantitative analysis it was observed that the leaf had significantly ($\alpha \leq 0.05$) higher phenolics, cardiac glycosides and anthraquinones contents when compared to the stem. However, flavonoids and alkaloids were significantly ($\alpha \leq 0.05$) higher in the stem. No statistically significant ($\alpha \geq 0.05$) difference was obtained between the content of the saponins for the leaf and stem of *Grewia carpinifolia* (Table 3.4).

Table 3.3: Qualitative phytochemical analyses of ethanol extract of *Grewia carpinifolia* leaf and stem

Phytochemical	Leaf	Stem
Tannins	+	+
Phlobatinins	+	-
Saponins	+	+
Flavonoids	+	+
Terpenoids	+	+
Cardiac glycosides	+	+
Coumarin	-	-
Alkaloids	+	+
Anthraquinones	+	+

+ Present, - Absent

Table 3.4: Polyphenol contents of the ethanol extract of *Grewia carpinifolia* leaf and stem

Phenolics	Leaf	Stem
Total polyphenol [a]	19.08 ± 1.21[*]	14.85 ± 1.09
Total flavonoids [b]	9.00 ± 0.13	13.22 ± 1.53[*]
Alkaloids[c]	5.02±1.21	17.33± 2.02[*]
saponins[c]	15.67± 0.12	15.68± 1.40
anthraquinone[c]	165.04± 21.20*	14.87 ± 1.74
Cardiac glycoside[c]	890.21± 100.60	229.33± 45.07[*]

[a] Expressed as mg of gallic acid equivalent (GAE)/g of dry plant material. [b] expressed as mg quercetin/g of dry plant material. [*] indicates that this value is significantly ($\alpha<0.05$) different from the other, [c]expressed as mg of dry plant material

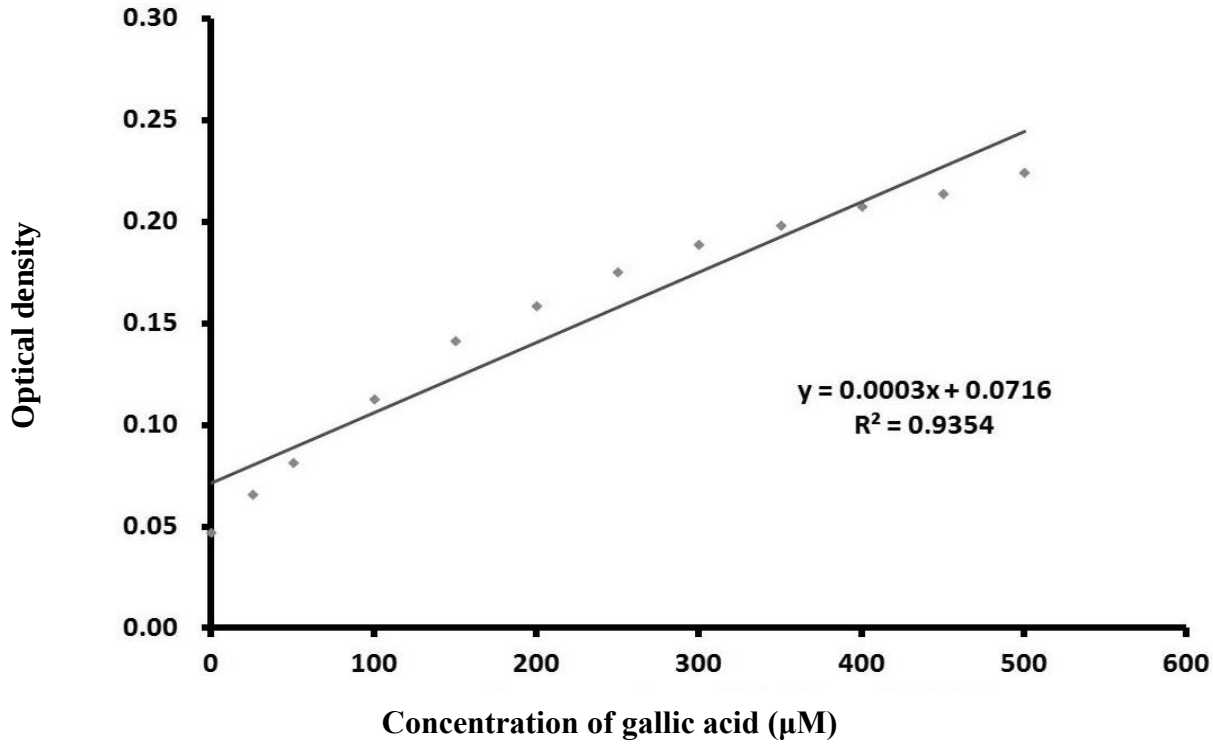


Figure 3.6: Standard curve for Total Phenolic Content

3.7.2 ACUTE TOXICITY STUDY

3.7.2.1 Clinical signs

There was a decrease in locomotion as well as reduction in feed intake in the experimental animals at the dosage of 4000 mg/kg for the leaf and stem of the plant *Grewia carpinifolia.*

3.7.2.2 Mortality

Two mortalities were recorded at 6000 mg/kg for the leaf, one at 6000 mg/kg for the stem and all the animals died at 8000 mg/kg following administration of *Grewia carpinifolia* stem (Tables 3.5 and 3.6).

3.7.2.3 Conversion of percentage mortalities to probits and calculation of LD_{50}

The percentage of animals that died at each dose levels was transformed to probit (Table 3.9). The percentage dead for 0 and 100 were corrected (Tables 3.7 and 3.8.) before the determination of probits as:

Corrected % formula for 0 and 100% mortality (Ghosh, 1984):

For 0% dead: 100(0.25/n)

For 100% dead: 100(n-0.25/n)

where n is number of animals in each group

The probit values were plotted against log-doses and then the dose corresponding to probit 5; that is, 50%, was found out (Figures 3.7 and 3.8). In the present case the Log LD_{50} was 3.75 and 3.77 for the leaf and stem, respectively.

Thus LD_{50} for the leaf and stem were 5623.41 mg/kg and 5879.11 mg/kg respectively.

3.7.2.4 Calculation of the Standard Error of Mean of LD_{50}

The standard error of mean (SEM) of LD_{50} were calculated using the formula described by Ghosh (1984).

$$\text{SEM of } LD_{50} = \frac{\text{Log } LD_{84} - \text{Log } LD_{16}}{\sqrt{2n}} \quad \text{..... Formula 1}$$

where n is number of animals in each group

The probits of 84 and 16 from Table 3.9 were 5.99 and 4.01 (approximately 6 and 4), respectively. The log-LD values for the probits 6 and 4 were obtained from the line on the graph in Figures 3.7. and 3.8. In the present study, for the leaf of *Grewia carpinifolia* it was

4.0 and 3.43 and their antilog were 10000 and 2691.53, for the stem of *Grewia carpinifolia* it was 3.95 and 3.62 their antilog were 7943.28 and 4168.69. Using these values in formula 1 the SEM of LD_{50} of the leaf and stem were 342.20 and 189.11. Therefore, LD_{50} of *Grewia carpinifolia* leaf when given orally was 5623.41 ± 342.40 mg/kg with 95% confidence interval of 5281.01–5965.81 and LD50 of *Grewia carpinifolia* stem when given orally was 5879.11 ± 189.11 mg/kg with 95% confidence interval of 5690–6068.22.

Table 3.5: Percentage mortality after administration of varying (graded) doses of ethanol extract of *Grewia carpinifolia* leaf in rats

Doses (mg/kg)	No of animals	No of Death	Percentage mortality (%)
Control	3	0	0
50	3	0	0
100	3	0	0
1000	3	0	0
2000	3	0	0
4000	3	0	0
6000	3	2	67

Table 3.6: Percentage mortality after administration of varying (graded) doses of ethanol extract of *Grewia carpinifolia* stem in rats

Doses (mg/kg)	No of animals	No of Death	Percentage mortality (%)
Control	3	0	0
50	3	0	0
100	3	0	0
1000	3	0	0
2000	3	0	0
4000	3	0	0
6000	3	1	33.33
8000	3	3	100

Table 3.7**:** Calculation of LD_{50} of the ethanol extract of *Grewia carpinifolia* leaf in rats

Dosage (mg/kg)	Log dose	Dead/Total	Dead (%)	Corrected (%)	Probit
Control		0/3	0		
50	1.7	0/3	0	8.33	3.6
100	2	0/3	0	8.33	3.6
1000	3	0/3	0	8.33	3.6
2000	3.3	0/3	0	8.33	3.6
4000	3.6	0/3	0	8.33	3.6
6000	3.8	2/3	66.67	67	5.44

Table 3.8: Calculation of LD_{50} of the ethanol extract of *Grewia carpinifolia* stem in rats

Dosage (mg/kg)	Log dose	Dead/Total	Dead (%)	Corrected (%)	Probit
Control		0/3	0		
50	1.7	0/3	0	8.33	3.6
100	2	0/3	0	8.33	3.6
1000	3	0/3	0	8.33	3.6
2000	3.3	0/3	0	8.33	3.6
4000	3.6	0/3	0	8.33	3.6
6000	3.8	1/3	33.3	33.3	4.6
8000	3.9	3/3	100	91.67	6.3

Table 3.9: Transformation of percentage mortalities to probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

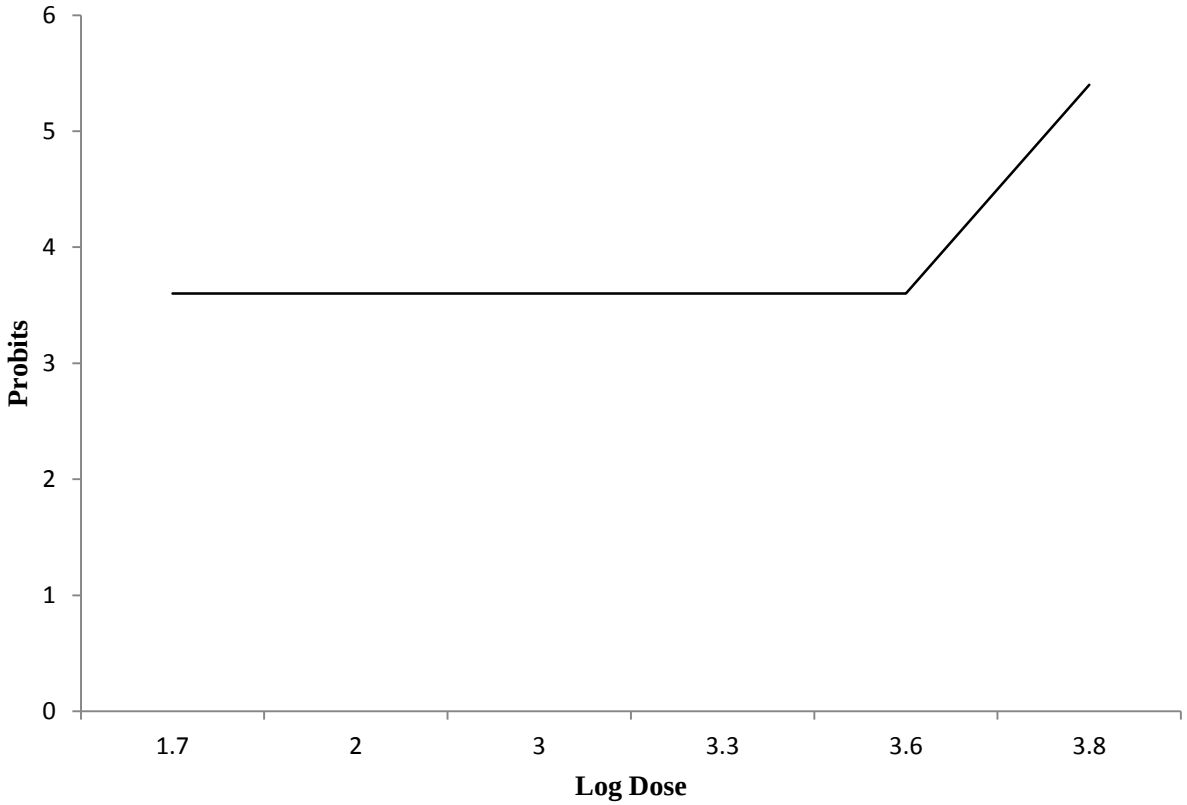


Figure 3.7: Plot of log-doses versus probits (from Table 3.7) for calculation of LD_{50} of *Grewia carpinifolia* leaf administered orally

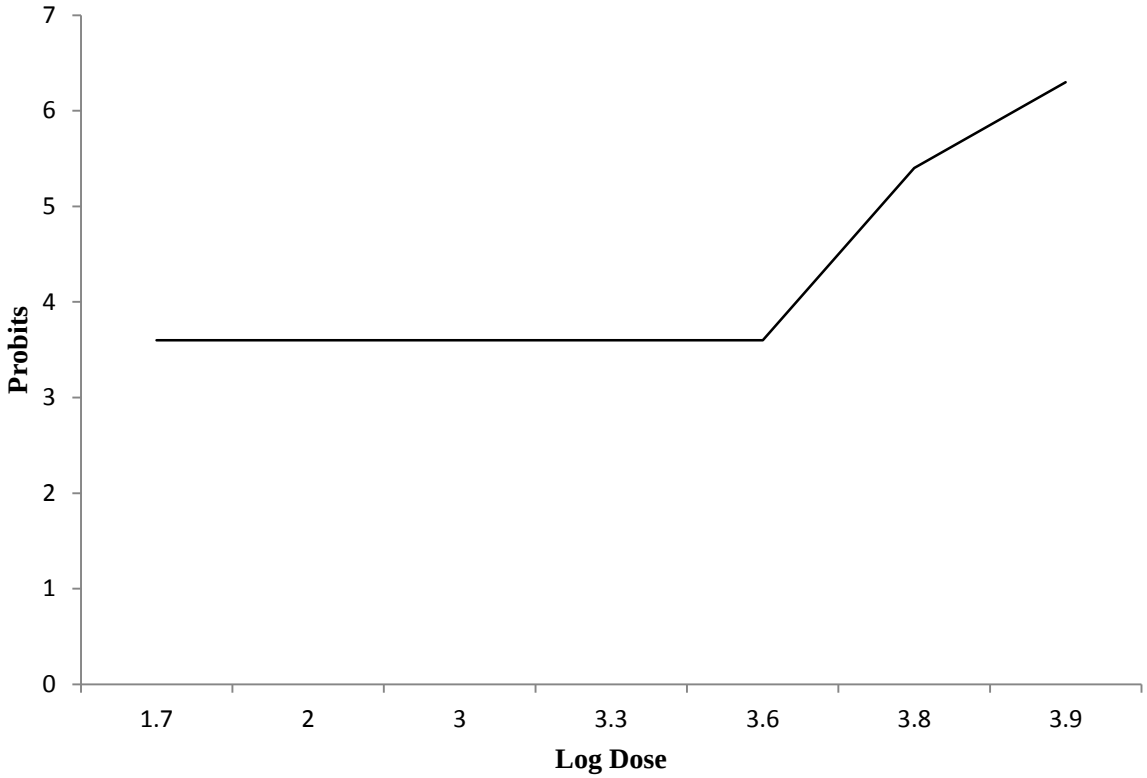


Figure 3.8: Plot of log-doses versus probits (from Table 3.8) for calculation of LD_{50} of *Grewia carpinifolia* stem administered orally

3.7.3 SUB-CHRONIC TOXICITY STUDIES

3.7.3.1 Effect of ethanol extract of *Grewia carpinifolia* leaf and stem on average daily feed and water intake of rats treated for 28 days

The effect of *G. carpinifolia* leaf and stem ethanol extract on average daily feed and water intake of albino rats treated for 28 days is presented in Tables 3.10 and 3.11, respectively. There was no significant difference in the amount of feed consumed by the control and the extract treated groups following administration of *G. carpinifolia* leaf and stem. However, a significant ($\alpha \leq 0.05$) reduction in water intake was observed in the 800 mg/kg dosage.

Table 3.10: Effect of ethanol extract of *Grewia carpinifolia* leaf on average daily feed and water intake

Groups	Feed consumption (g)	Water intake (ml)
control	78.89 ± 2.02	180.10 ± 3.79 [a]
100 mg/kg	75.18 ± 1.11	163.70 ± 4.91[a]
200 mg/kg	57.47 ± 1.97	161.20 ± 8.90 [a]
400 mg/kg	62.47 ± 2.12	164.30 ± 10.52 [a]
800 mg/kg	56.00 ± 3.66	139.50 ± 8.68 [b]

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within columns are significantly ($\alpha \leq 0.05$) different

Table 3.11: Effect of ethanol extract of *Grewia carpinifolia* stem on average daily feed and water intake

Groups	Feed consumption (g)	Water intake (ml)
control	80.02 ±11.25	192.10 ± 7.49 [a]
100 mg/kg	76.18 ± 11.11	173.76 ± 9.801[a]
200 mg/kg	74.37 ± 9.17	170.61 ± 8.90 [a]
400 mg/kg	67.82 ± 12.01	174.73 ± 12.34 [a]
800 mg/kg	60.56 ± 6.75	169.50 ± 10.27 [b]

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within columns are significantly ($\alpha \leq 0.05$) different

3.7.3.2 Effect of ethanol extract of *Grewia carpinifolia* leaf and stem on body weight of rats treated for 28 days

There was an increase in body weight of the animals across test groups throughout the experiment; the difference in values was insignificant ($\alpha \geq 0.05$) when compared to that of the control (Tables 3.12 and 3.13.).

Table 3.12: Effect of graded doses of ethanol extract of *Grewia carpinifolia* leaf on body weights of rats

		Dose of *G. carpinifolia* leaf extract			
WEIGHT (g)	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Week 1	170.40 ± 15.10	187.8 ± 12.88	207.00 ± 10.91	196.40 ± 19.79	193.40 ± 13.42
Week 2	196.80 ± 20.29	216.00 ± 10.65	221.30 ± 14.77	192.90 ± 12.28	198.00 ± 6.04
Week 3	208.40 ± 20.97	212.00 ± 12.43	221.30 ± 14.01	200.00 ± 13.42	205.80 ± 11.54
Week 4	215.76 ± 18.54	217.43 ± 12.54	223.65 ± 15.78	205.36 ± 11.65	209.34 ± 11.87

n=5; Mean ± S.E.M (Standard Error of Mean)

Table 3.13: Effect of graded doses of ethanol extract of *Grewia carpinifolia* stem on body weights of rats

		Dose of *G. carpinifolia* stem extract			
WEIGHT (g)	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Week 1	162.40 ± 11.50	164.81 ± 12.88	164.56 ± 10.91	166.49 ± 19.79	169.40 ± 13.42
Week 2	166.70 ± 10.09	166.00 ± 8.72	168.13 ± 17.74	162.90 ± 12.28	168.00 ± 10.64
Week 3	160.40 ± 17.04	162.00 ± 11.13	167.92 ± 12.15	167.54 ± 10.24	165.80 ± 8.01
Week 4	162.76 ± 13.40	164.37 ± 11.08	165.65 ± 18.08	165.92 ± 9.05	169.51 ± 9.27

n=5; Mean ± S.E.M (Standard Error of Mean)

3.7.3 BEHAVIOURAL TESTS

3.7.4.1 Open-field Test

There was no significant ($\alpha \geq 0.05$) difference in the values obtained in the open-field test. The number of groomings, rearing, centre square duration and other parameters in the extract treated groups were similar to those of the controls (Tables 3.14 and 3.15)

3.7.4.2 Hanging wire Test

There was a significant ($\alpha \leq 0.05$) increase in time spent on the hanging wire within all the test groups when compared with the control. Following oral administration of extract of *G. carpinifolia* leaf, the increase was in the order of 800 mg/kg > control > 400 mg/kg > 200 mg/kg > 100 mg/kg (Figure 3.9.). A dose-dependent increase in latent time was observed in the stem (Figure 3.10.).

3.7.4.2 Negative Geotaxis Test

There was no significant ($\alpha \geq 0.05$) difference in the time it took the rats in the control and extract treated group to turn round in the head-up position in the negative geotaxis tests. The latent time was similar across all the groups (Figures 3.11 and 3.12).

Table 3.14: Mean values for behaviour in the open-field test on day 28 following administration of ethanol extract of *Grewia carpinifolia* leaf ethanol extract

	Dose of *G. carpinifolia* leaf extract				
	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Rearings	17.65± 4.32	16.56 ± 3.73	16.98 ± 3.23	17.64 ± 3.42	17.56 ± 4.45
Groomings	13.52± 3.98	9.56 ± 2.34	12.61 ± 2.43	11.09 ± 2.21	12.08 ± 2.61
Number of new squares	25.40 ± 4.99	18.80 ± 5.56	13.00 ± 6.43	17.60 ± 5.38	13.75 ± 2.66
Freezing (sec)	17.64 ± 1.20	17.54 ± 3.03	17.07± 3.41	17.98 ± 2.96	17.85 ± 2.35
Centre square duration (secs)	32.42 ± 4.53	30.23 ± 4.72	34.01 ± 2.13	30.54 ± 1.65	36.32 ± 3.03
Faecal bolus	3.00 ± 1.14	5.00 ± 1.45	4.67 ± 2.91	4.00 ± 1.27	3.75 ± 1.38
Urine	0.60 ± 0.40	2.80 ± 1.16	1.33 ± 0.67	2.20 ± 1.02	1.50 ± 0.65

n=5; Mean ± S.E.M (Standard Error of Mean)

Table 3.15: Mean values for behaviour in the open-field test on day 28 following administration of *Grewia carpinifolia* stem ethanol extract

		Dose of *G. carpinifolia* stem extract			
	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Rearings	16.55 ± 4.07	16.59 ± 4.71	15.19 ± 3.13	17.31 ± 3.35	16.19 ± 3.58
Groomings	11.64 ± 3.04	14.91 ± 3.42	16.42 ± 4.11	13.44 ± 4.27	12.31 ± 3.74
Number of new squares	20.94 ± 3.92	22.80 ± 5.26	23.91 ± 7.11	16.60 ± 5.32	17.75 ± 3.62
Freezing (secs)	17.08 ±4.32	17.00 ± 3.59	18.03 ± 2.86	17.06±5.41	18.06 ± 3.2
Centre square Duration (secs)	23.96 ± 8.65	28.87 ± 5.45	32.81 ± 8.72	21.07 ± 6.06	26.93± 9.02
Faecal bolus	3.00 ± 1.14	5.00 ± 1.45	4.67 ± 2.91	4.00 ± 1.27	3.75 ± 1.38
Urine	0.60 ± 0.40^{a}	2.80 ± 1.16^{b}	1.33 ± 0.67^{b}	2.20 ± 1.02^{b}	1.50 ± 0.65^{b}

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within rows are significantly ($\alpha \leq 0.05$) different

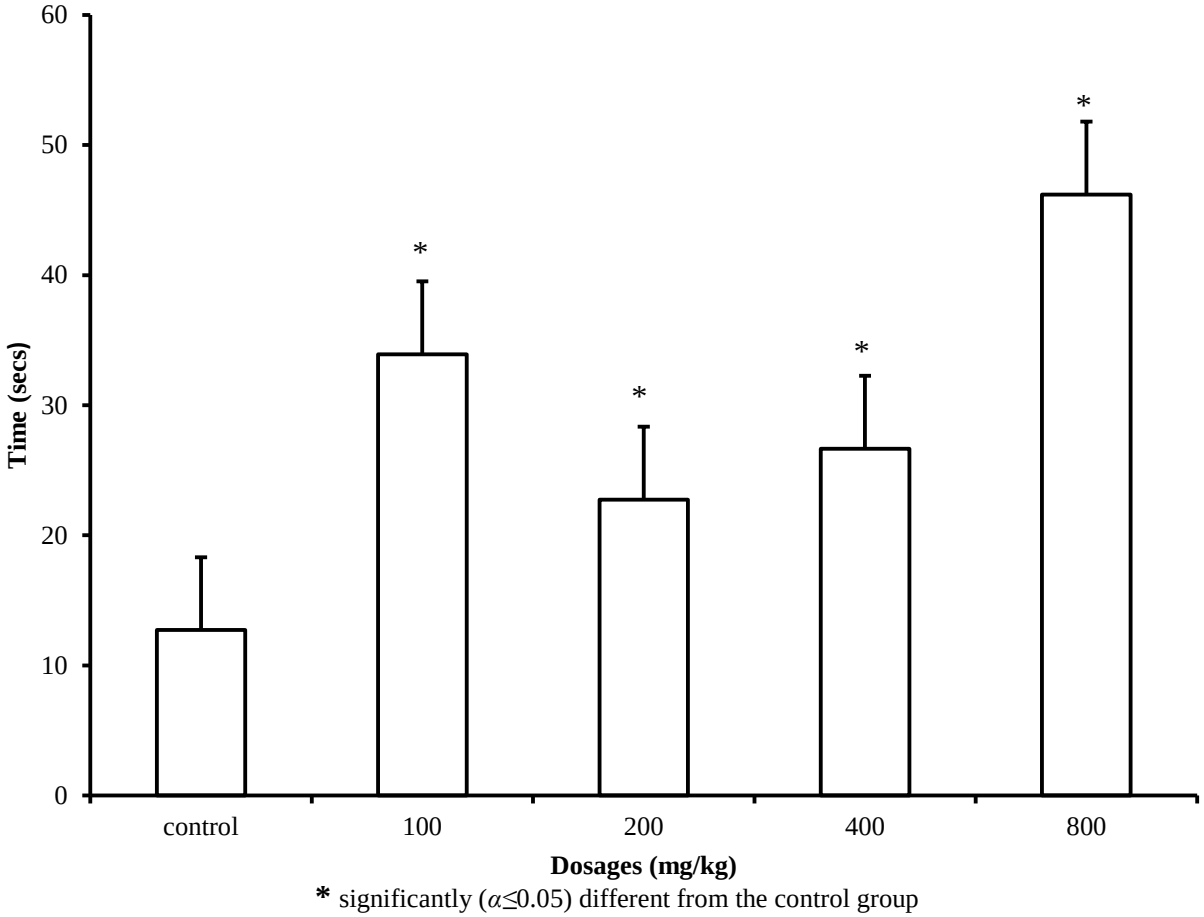


Figure 3.9: Latency on the hanging wire test following an oral administration of extract of *Grewia carpinifolia* leaf for 28 days

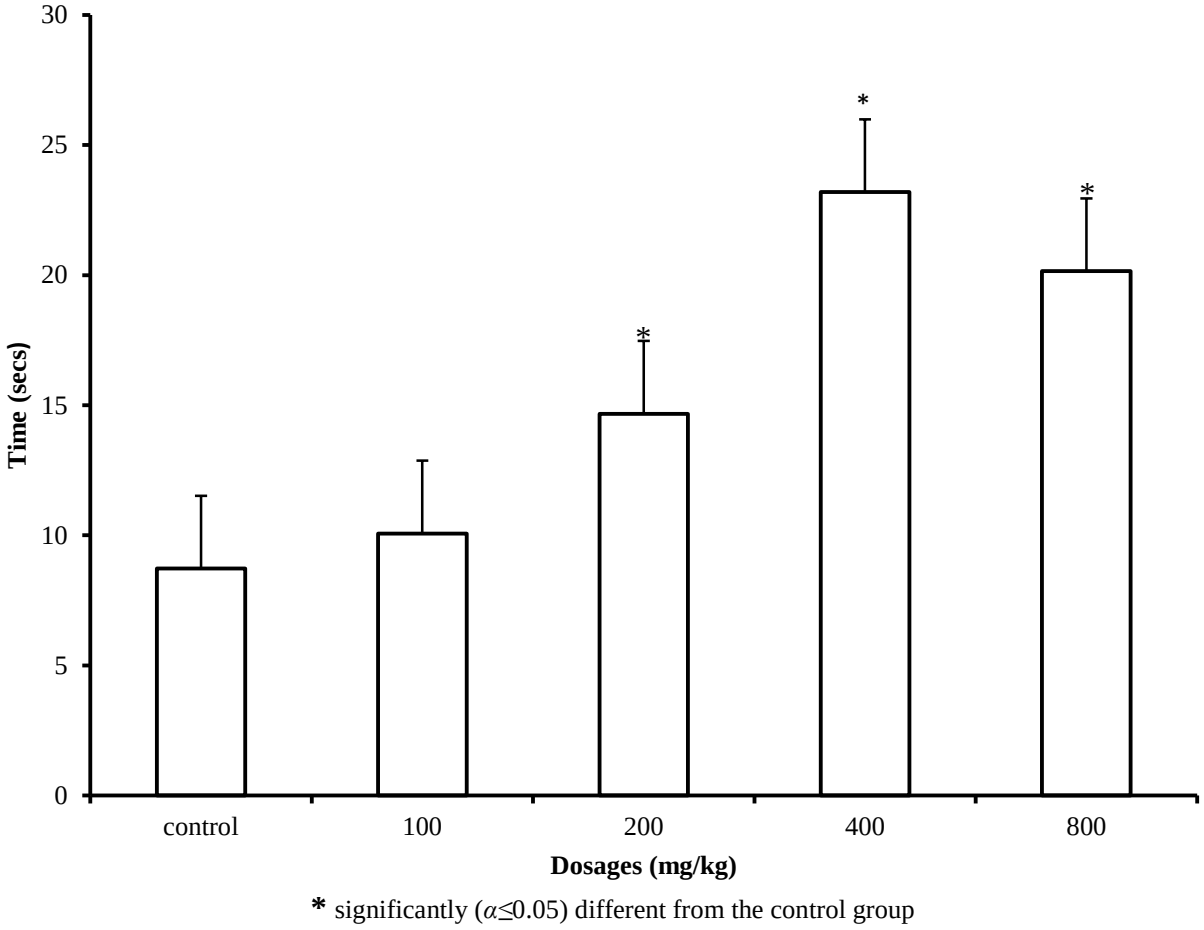


Figure 3.10: Latency on the hanging wire test following an oral administration of extract of *Grewia carpinifolia* stem for 28 days

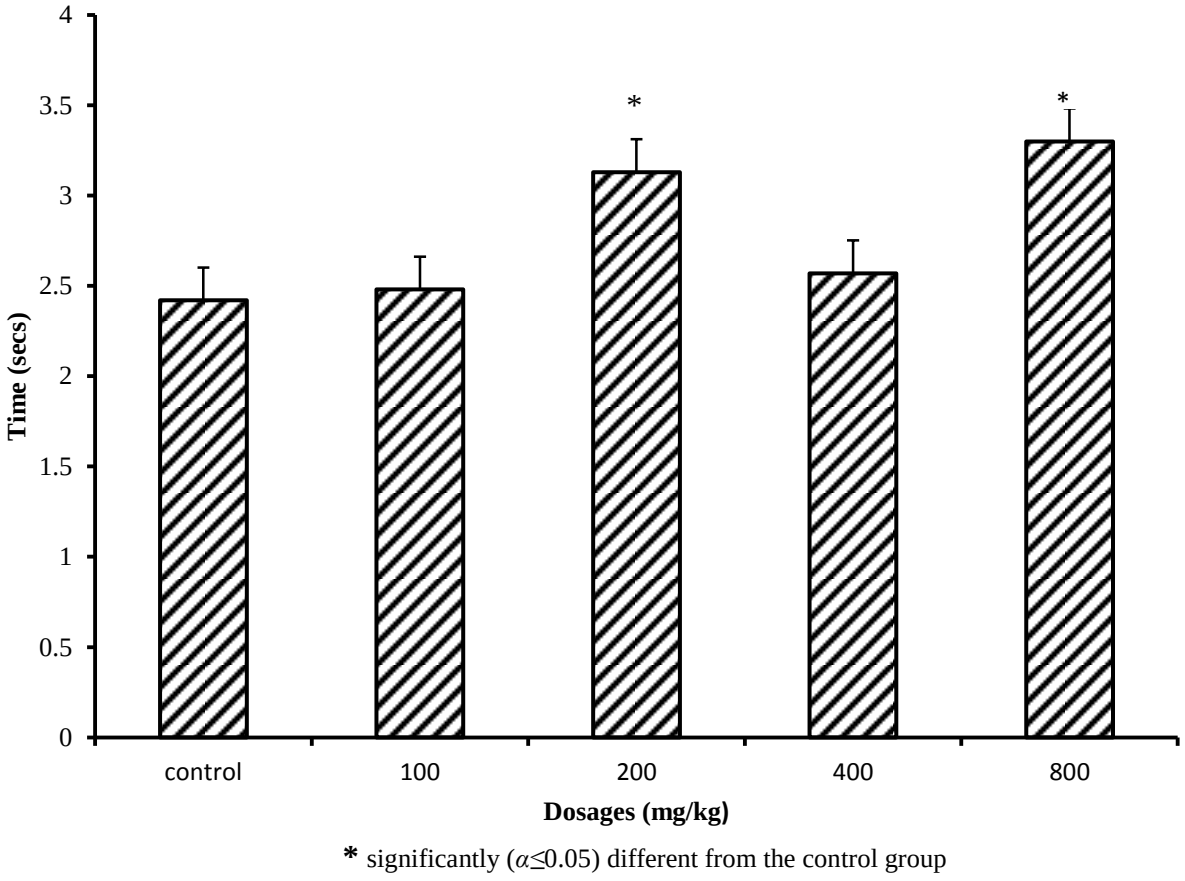
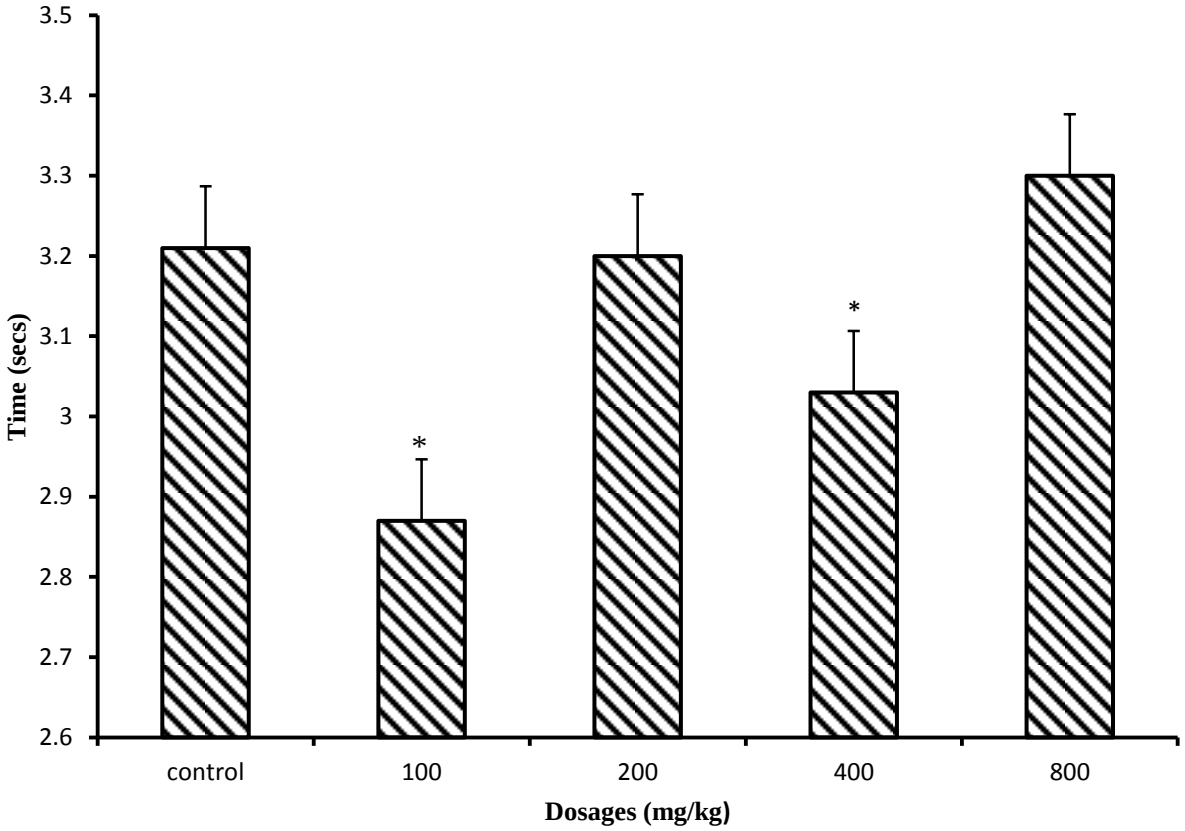


Figure 3.11: Latent time on negative geotaxis test following an oral administration ethanol extract of *Grewia carpinifolia* leaf for 28 days



***** significantly ($\alpha \leq 0.05$) different from the control group

Figure 3.12: Latent time on negative geotaxis test following an oral administration of ethanol extract of *Grewia carpinifolia* stem for 28 days

3.7.5 HAEMATOLOGY

3.7.5.1 Effect of *Grewia carpinifolia* leaf and stem on blood parameters of rats treated for 28 days

A significant ($\alpha \leq 0.05$) decrease in the count of neutrophils was observed following an oral administration of 100 mg/kg of the extract of the leaf when compared with the controls. All other haematological parameters were not significantly ($\alpha \geq 0.05$) different from the control (Tables 3.16. and 3.17.).

Table 3.16: Effect of *Grewia carpinifolia* leaf on blood parameters of rats treated for 28 days

		Dose of *G. carpinifolia* leaf extract			
	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
PCV (%)	44.20 ± 0.58	45.60 ± 1.60	42.00 ± 0.58	44.80 ± 1.91	42.50 ± 1.20
Hb (g/dl)	14.00 ± 0.26	14.24 ± 0.39	13.60 ± 0.17	14.40 ± 0.59	13.88 ± 0.43
RBC ($x10^{12}$/L)	7.96 ± 0.43	8.18 ± 0.63	8.03 ± 0.81	8.14 ± 0.46	8.01 ± 0.29
WBC (10^3 μL^{-1})	9.78 ± 0.87	15.14 ± 2.25	14.43 ± 2.98	11.82 ± 2.78	13.38 ± 1.00
Neutrophils	4.30 ± 0.07	3.84 ± 0.45	8.18 ± 0.81	4.07 ± 0.60	5.75 ± 0.34
(10^3 μL^{-1})	(44.00 ± 4.66)[a]	(25.40 ± 2.54)[b]	(50.67 ± 3.48)[a]	(34.40 ± 1.17)[a]	(43.00 ± 2.86)[a]
Lymphocytes	4.81 ± 0.11	9.69 ± 0.06	5.63 ± 0.27	6.80 ± 0.23	6.22 ± 0.17
(10^3 μL^{-1})	(49.20 ± 4.57)[a]	(64.00 ± 3.11)[a]	39.00 ± 3.00[b]	(57.60 ± 1.29)[a]	(46.50 ± 2.99)[b]
Monocytes	0.63 ± 0.02	1.36 ± 0.02	1.25 ± 0.01	0.95 ± 0.02	1.03 ± 0.06
(10^3 μL^{-1})	(6.40 ± 1.63)	(9.00 ± 0.45)	(8.67±0.67)	(8.00 ± 0.32)	(7.75 ± 0.25)
Eosinophils	0.39±0.12	0.24 ± 0.02	0.24 ± 0.09	0.00 ± 0.00	0.37 ± 0.05
(10^3 μL^{-1})	0.40 ± 0.10	1.60 ± 1.17	(1.67 ± 0.88)		(2.75 ± 0.25)
Platelets ($x10^3$)	585.80 ± 43.72	899.00 ± 143.80	521.30 ± 18.56	684.20 ± 83.15	618.00 ± 145.00
MCV (%)	56.00 ± 0.55	58.00 ± 0.84	57.33 ± 0.88	58.20 ± 1.63	57.50 ± 1.71
MCH (%)	18.00 ± 0.32	18.80 ± 0.20	18.67 ± 0.33	18.80 ± 0.49	19.00 ± 0.71
MCHC (%)	31.60 ± 0.40	32.00±0.55	32.33 ± 0.33	32.20 ± 0.20	32.75 ± 0.25

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within rows are significantly ($\alpha \leq 0.05$) different; values in parenthesis indicate relative differential count; PCV: Packed cell volume; Hb: haemoglobin; WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concenrtration

Table 3.17: Effect of *Grewia carpinifolia* stem on blood parameters of rats treated for 28 days

		Dose of *G. carpinifolia* stem extract			
	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
PCV (%)	38.24.20 ± 1.83	35.03±2.74	36.00±1.62	34.21±1.631	32.86±2.23
Hb (g/dl)	13.02 ± 0.26	13.24±0.21	12.66±0.17	13.09±1.09	13.88±0.43
RBC ($x10^{12}$/L)	7.96 ± 0.43	8.18 ± 0.63	8.03 ± 0.81	8.14 ± 0.46	8.01 ± 0.29
WBC (10^3 μL^{-1})	9.23 ± 1.34	12.94 ± 1.25	12.01 ± 2.28	9.82 ± 1.08	11.38 ± 1.37
Neutrophils	3.80 ± 0.07	3.74 ± 0.45	7.10 ± 0.81	6.84 ± 0.60	5.75 ± 0.34
(10^3 μL^{-1})	$(41.34 \pm 4.66)^a$	$(26.40 \pm 2.54)^b$	$(43.37 \pm 5.02^a$	$(44.40 \pm 1.17)^a$	$(42.84 \pm 2.86)^a$
Lymphocytes	4.80 ± 0.11	8.66 ± 0.06	5.61 ± 0.27	6.96 ± 0.23	6.22 ± 0.17
(10^3 μL^{-1})	$(49.20 \pm 4.57)^a$	$(64.00 \pm 3.11)^a$	$(35.67 \pm 3.03)^b$	$(54.60 \pm 1.29)^a$	$(49.50 \pm 2.99)^b$
Monocytes	0.63 ± 0.02	1.54 ± 0.02	0.30 ± 0.01	0.92 ± 0.02	1.03 ± 0.06
(10^3 μL^{-1})	(6.40 ± 1.63)	(9.00 ± 0.45)	(5.67 ± 0.67)	(8.15 ± 0.32)	(7.75 ± 0.25)
Eosinophils	0.39 ± 0.12	0.24 ± 0.02	0.24 ± 0.09	0.50 ± 0.08	0.37 ± 0.05
(10^3 μL^{-1})	(1.40 ± 0.40)	(1.62 ± 1.31)	(1.72 ± 0.58)	(1.50 ± 0.13)	(2.75 ± 0.25)
Platelets ($x10^3$)	798.25 ± 76.06	854.81 ± 984.80	521.30 ± 18.56	695.20 ± 83.15	721.00 ± 153.07
MCV (%)	56.70 ± 0.85	58.00 ± 0.84	57.33 ± 0.88	58.20 ± 1.63	59.25 ± 1.71
MCH (%)	17.54 ± 0.32	18.80 ± 0.20	18.67 ± 0.33	18.80 ± 0.49	17.92 ± 0.71
MCHC (%)	31.60 ± 0.40	32.00 ± 0.55	32.33 ± 0.33	32.20 ± 0.20	32.75 ± 0.25

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within rows are significantly ($\alpha \leq 0.05$) different; values in parenthesis indicate relative differential count; PCV: Packed cell volume; Hb: haemoglobin; WBC: white blood cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: mean corpuscular Hb concenrtration

3.7.6 SERUM CHEMISTRY

3.7.6.1 Effect of *Grewia carpinifolia* leaf and stem on serum chemistry of rats

No statistical difference was observed in the serum chemistry values measured within and across the groups. An exception was the significant ($\alpha \leq 0.05$) increase in the activities of ALP obtained in rats administered 800 mg/kg of the extract of the leaf when compared with the control group (Table 3.18.). The administration of the extract of the stem at 800 mg/kg also resulted in a significant increase in activities of ALT, AST and ALP (Table 3.19).

Table 3.18: Effect of *Grewia carpinifolia* leaf on serum chemistry of Wistar rats treated for 28 days

	Dose of *G. carpinifolia* stem extract				
	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
ALT (U/L)	64.40 ± 3.49	48.00 ± 5.48	49.67 ± 1.45	55.75 ± 3.12	53.67 ± 8.67
ALP (U/L)	44.85 ± 9.78 [a]	52.45 ± 24.82 [a]	63.72 ± 20.49 [a]	62.45 ± 17.51[a]	101.40 ± 4.56[b]
GGT (U/L)	10.89 ± 4.51	9.25 ± 5.06	14.46 ± 13.34	2.60 ± 0.56	5.00 ± 2.79
AST (U/L)	68.20 ± 23.1	55.00 ± 23.20	61.30 ± 29.8	98.20 ± 28.8	111.00 ± 35.00
Urea (mg/dl)	33.78 ± 6.56	24.32 ± 13.74	41.48 ± 15.41	24.76 ± 1.07	34.94 ± 8.16
TP (g/dL)	5.48 ± 0.86	4.96 ± 0.65	5.92 ± 0.16	5.89 ± 0.23	5.03 ± 0.25
ALB (g/dL)	2.81 ± 0.43	2.54 ± 0.25	2.82 ± 0.14	2.89 ± 0.66	2.43 ± 0.43
GLB (g/dL)	2.67 ± 1.24	2.43 ± 0.68	3.10 ± 0.05	3.00 ± 0.81	2.60 ± 0.27

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within rows are significantly (α<0.05) different; AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; ALT = Alanine aminotransferase; GGT- Gamma Glutamyl Transferase TP = Total Protein; ALB = Albumin; GLB = globulin;

Table 3.19: Effect of *Grewia carpinifolia* stem on serum chemistry of Wistar rats treated for 28 days

		Dose of *G. carpinifolia* stem extract			
	Control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
ALT (U/L)	54.40 ± 4.19	56.00 ± 1.33	54.50 ±0.63	62.15 ± 3.12	63.02 ± 7.83
ALP (U/L)	41.39 ± 2.61 [a]	52.45 ± 24.82 [a]	63.72 ± 20.49 [a]	62.45 ± 17.51[a]	91.16 ± 9.56[b]
GGT (U/L)	9.19 ± 1.32	9.25 ± 5.06	12.31 ± 3.40	12.05 ± 1.16	15.06 ± 3.29
AST (U/L)	55.00± 1.41 [a]	58.02 ± 7.30 [a]	61.30 ± 29.8 [a]	59.75 ± 2.40 [a]	85.25 ±1.80 [b]
TP (g/dL)	5.42 ± 0.69	4.31 ± 0.23	4.08 ± 0.11	5.90 ± 1.04	5.00 ± 0.25
ALB (g/dL)	2.75 ± 0.15	1.88 ± 0.23	1.98 ± 0.19	2.89 ± 0.31	2.62 ± 0.30
GLB(g/dL)	2.67 ± 1.24	2.43 ± 0.68	3.10 ± 0.05	3.01 ± 0.42	2.38 ± 0.16

n=5; Mean ± S.E.M (Standard Error of Mean); Means with different superscripts within rows are significantly ($\alpha \leq 0.05$) different; AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; ALT = Alanine aminotransferase; GGT- Gamma Glutamyl Transferase TP = Total Protein; ALB = Albumin; GLB = globulin;

3.7.7 Absolute Organ Weight

There was no significant ($\alpha \geq 0.05$) difference between the absolute organ weight of the test groups and that of the control group following a daily single oral administration of the extract of the leaf and stem for 28 days (Tables 3.20 and 3.21).

Table 3.20: Effect of *Grewia carpinifolia* leaf on absolute organ weights (g) of Wistar rats treated for 28 days

Groups	Brain	Heart	Left Kidney	Spleen	Right Kidney	Liver
Control	1.64 ± 0.08	0.77 ± 0.07	0.59 ± 0.05	0.76 ± 0.15	0.59 ± 0.09	8.10±0.92
100 mg/kg	1.68 ± 0.08	0.81 ± 0.04	0.61 ± 0.05	0.79 ± 0.11	0.64 ± 0.04	8.23±0.59
200 mg/kg	1.78 ± 0.02	1.01 ± 0.30	0.63 ± 0.06	0.80 ± 0.09	0.67 ± 0.09	8.23±1.63
400 mg/kg	1.61 ± 0.05	0.76 ± 0.02	0.60 ± 0.04	0.90 ± 0.01	0.67 ± 0.04	8.15±0.46
800 mg/kg	1.66 ± 0.02	0.73 ± 0.02	0.55 ± 0.61	0.67 ± 0.03	0.5 8± 0.03	7.59±0.36

n=5; Mean ± S.E.M (Standard Error of Mean)

Table 3.21: Effect of *Grewia carpinifolia* stem on absolute organ weights (g) of Wistar rats treated for 28 days

Groups	Brain	Heart	Left Kidney	Spleen	Right Kidney	Liver
control	1.54 ± 0.07	0.76 ± 0.07	0.56 ± 0.05	0.73 ± 0.15	0.62 ± 0.11	8.23±1.28
100 mg/kg	1.61 ± 0.06	0.76 ± 0.12	0.61 ± 0.03	0.74 ± 0.11	0.63 ± 0.09	8.59±1.59
200 mg/kg	1.57 ± 0.04	0.81 ± 0.30	0.53 ± 0.03	0.78 ± 0.09	0.61 ± 0.12	8.17±1.34
400 mg/kg	1.64 ± 0.05	0.77 ± 0.12	0.56 ± 0.04	0.90 ± 0.12	0.64 ± 0.10	8.78±0.96
800 mg/kg	1.54 ± 0.05	0.73 ± 0.05	0.60 ± 0.02	0.71 ± 0.11	0.59 ± 0.11	8.59±0.65

n=5; Mean ± S.E.M (Standard Error of Mean)

3.7.8 Relative Organ Weight

There was no significant ($\alpha \geq 0.05$) difference between the relative organ weight of the test groups and that of the control group following a daily single oral administration of the extract of the leaf and stem for 28 days (Tables 3.22 and 3.23).

Table 3.22: Effect of extract of *Grewia carpinifolia* leaf on relative organ weights (%) of rats treated for 28 days

Groups	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Brain ($x10^{-3}$)	7.60 ± 0.04	7.72 ± 0.26	7.96 ± 0.88	7.84 ± 0.89	7.93 ± 0.87
Heart ($x10^{-3}$)	3.57 ± 0.07	3.73 ± 0.34	4.52 ± 0.98	3.70 ± 0.82	3.49 ± 0.71
Left Kidney ($x10^{-3}$)	2.73 ± 0.45	2.81 ± 0.39	2.82 ± 0.69	2.92 ± 0.23	2.63 ± 0.73
Spleen ($x10^{-3}$)	3.52 ± 0.04	3.63 ± 0.21	3.58 ± 0.70	4.38 ± 0.55	3.20 ± 0.64
Right Kidney ($x10^{-3}$)	2.73 ± 0.45	2.94 ± 0.33	3.00 ± 0.75	3.26 ± 0.56	2.77±0.61
Liver ($x10^{-3}$)	37.51 ± 0.41	37.84 ± 0.77	36.80 ± 0.55	39.69 ± 0.64	36.26 ± 0.82

n=5; Mean ± S.E.M (Standard Error of Mean)

Table 3.23: Effect of extract of *Grewia carpinifolia* stem on relative organ weights (%) of rats treated for 28 days

Groups	control	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Brain ($x10^{-3}$)	6.76 ± 0.12	6.79 ± 0.94	7.06 ± 0.98	6.94 ± 0.89	7.93 ± 0.87
Heart ($x10^{-3}$)	3.75 ± 0.07	3.73 ± 0.34	3.89 ± 1.01	3.70 ± 0.82	3.94 ± 0.97
Left Kidney ($x10^{-3}$)	2.47 ± 0.45	2.81 ± 0.39	2.82 ± 0.67	2.92 ± 0.23	2.83 ± 0.93
Spleen ($x10^{-3}$)	3.59 ± 0.04	3.63 ± 0.21	3.58 ± 0.70	4.38 ± 0.55	3.26 ± 0.74
Right Kidney ($x10^{-3}$)	2.77 ± 0.57	2.94 ±0.33	3.03 ± 0.96	3.26 ± 0.56	3.75 ± 0.68
Liver ($x10^{-3}$)	39.75 ± 0.40	37.84 ± 0.77	39.98 ± 0.75	39.93 ± 2.41	39.26 ± 3.82

n=5; Mean ± S.E.M (Standard Error of Mean)

3.7.9 HISTOPATHOLOGY

The results of the histopathology of rats treated with ethanol extract of *G.carpinifolia* leaf on the brain, heart, liver, kidney and spleen are shown in Plates I - X. The liver section of rat in the control group as well as groups administered with 100, 200 and 400 mg/kg showed no visible pathological lesion. Conversely, congestion of the central nucleus and sinusoids were observed in the liver of rats treated with 800 mg/kg of *G.carpinifolia* leaf. The heart of the rat administered with 800 mg/kg of extract of *Grewia carpinifolia* leaf showed coronary congestion while no visible lesion was observed in the heart at other tested doses. No visible lesion was observed in the kidney and brain sections of experimental rats administered with ethanol extract of *G.carpinifolia* leaf and stem.

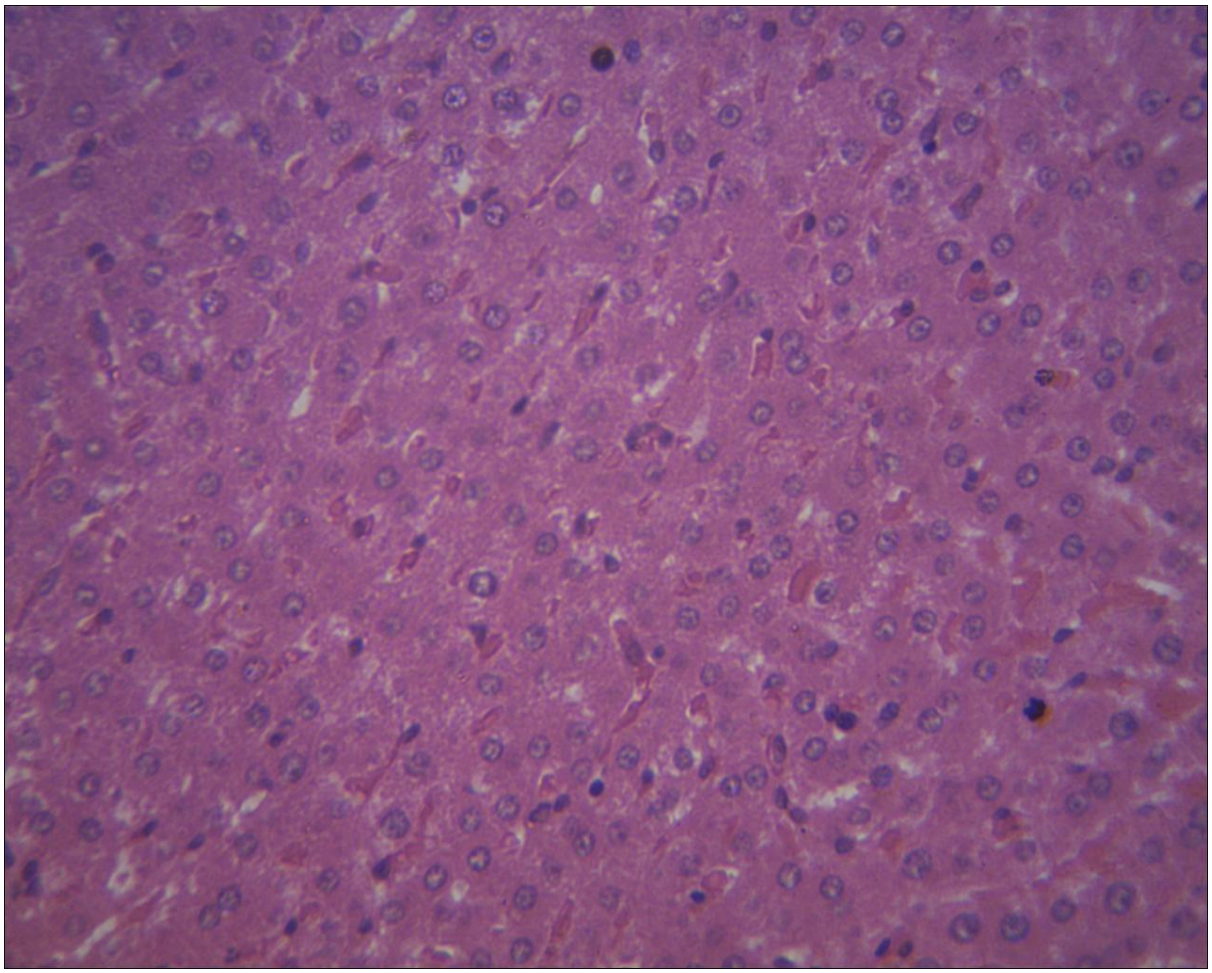


Plate I: The liver section of rat in the control group (group A) showing no visible lesion (mag x 100).

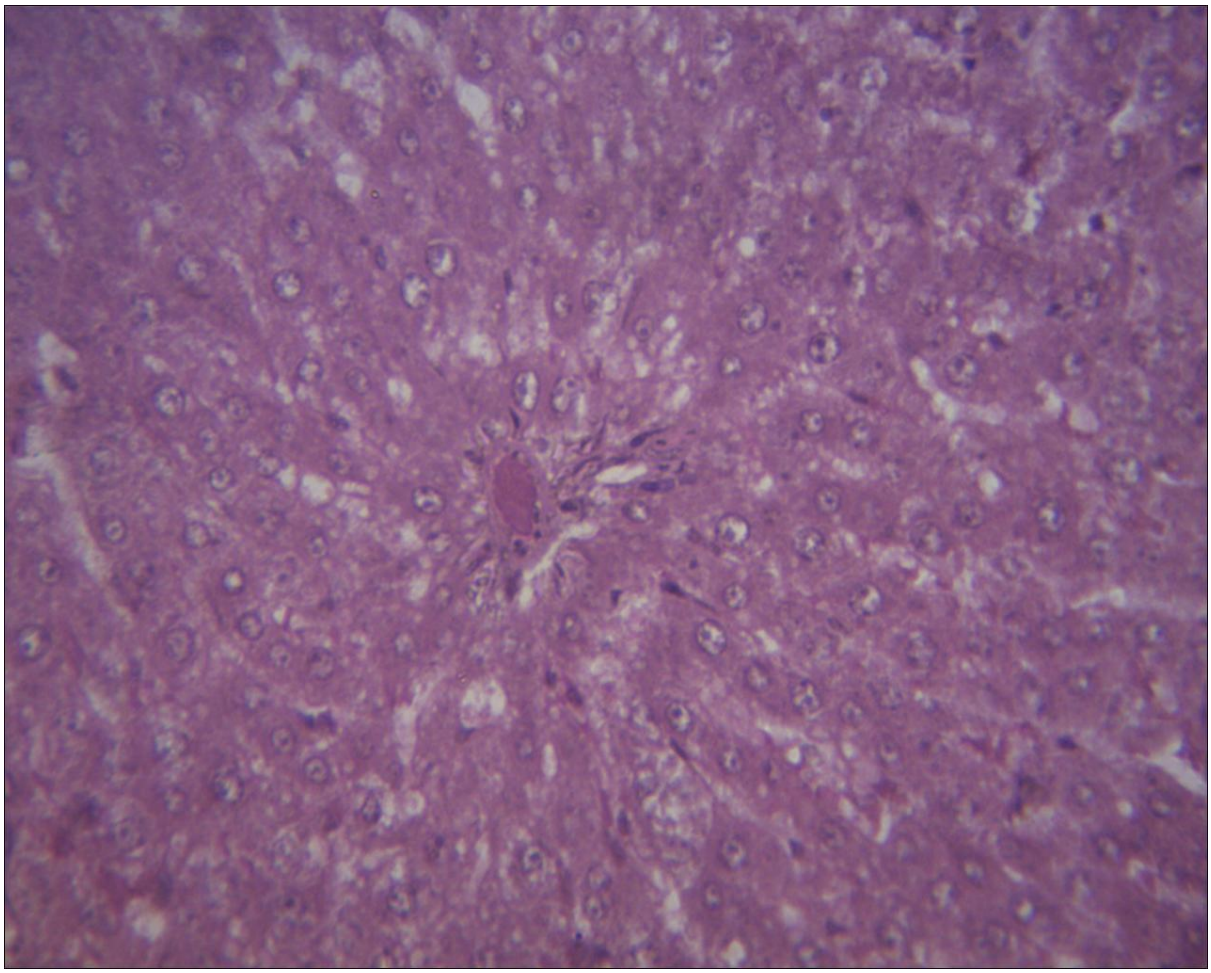


Plate II: The liver section of rat administered with 100 mg/kg (group B) of *Grewia carpinifolia* leaf for 28 days showing no visible lesion (mag x 100).

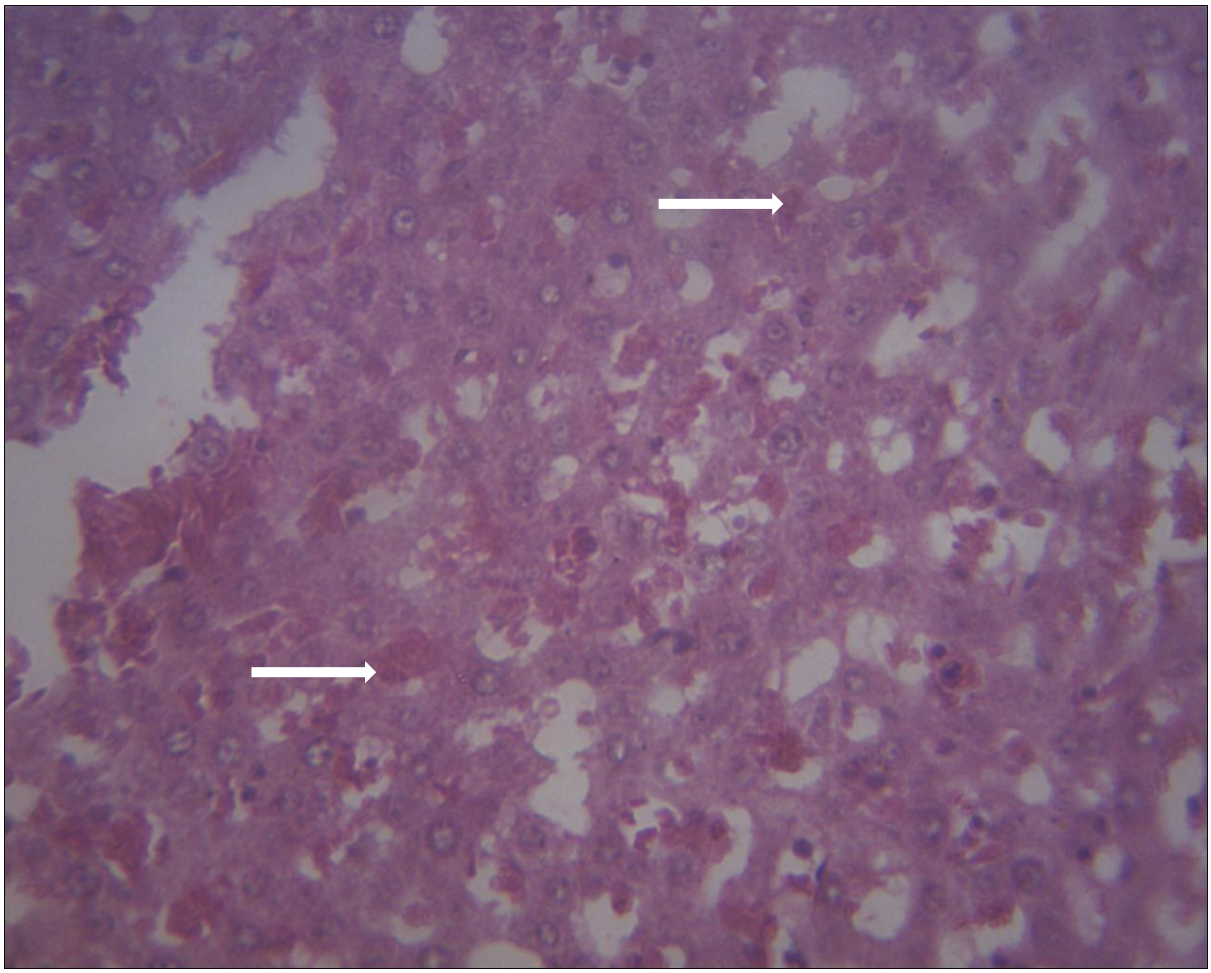


Plate III: Liver of rats treated with 800 mg/kg showing central nucleus and sinusoidal congestion (mag x 100).

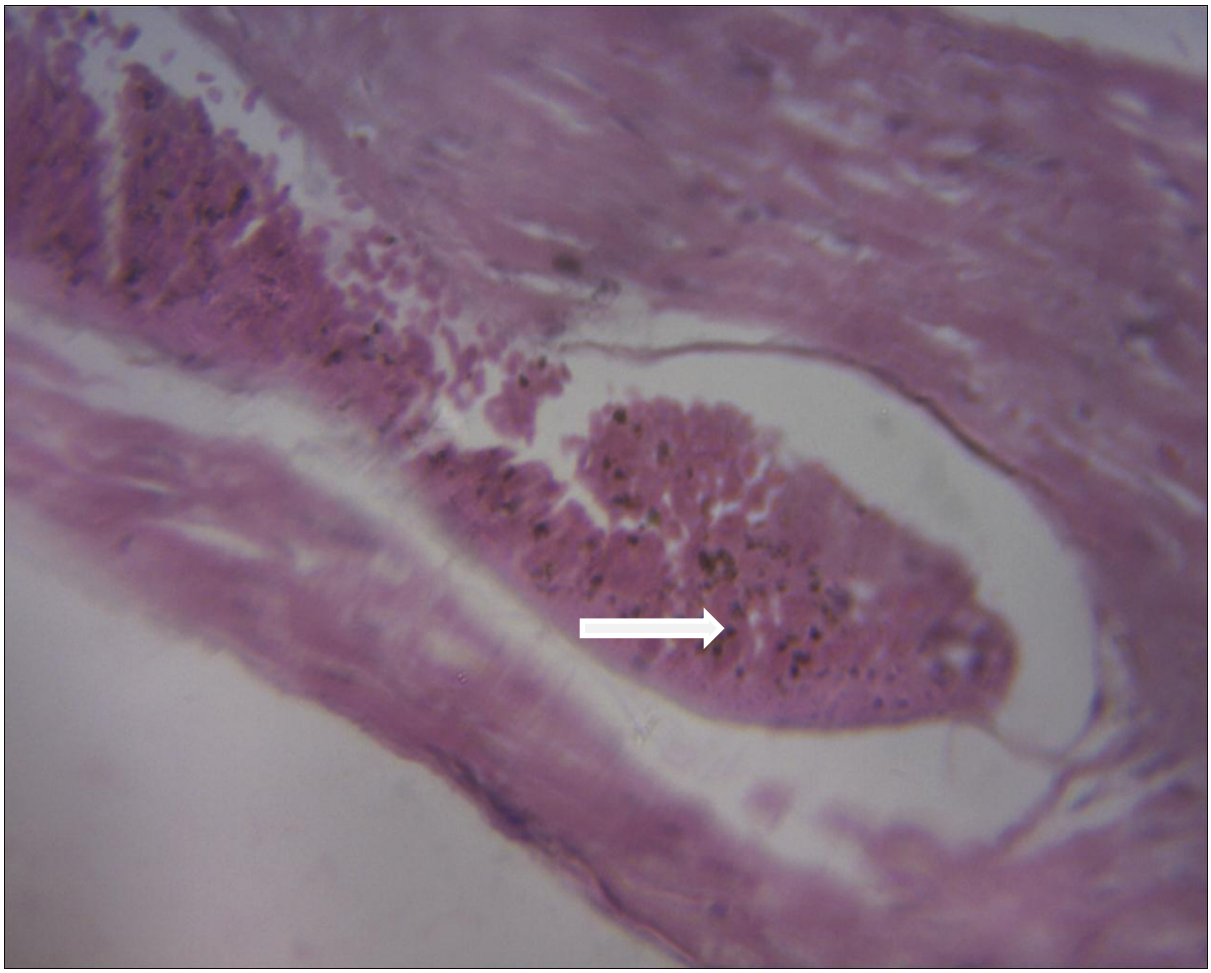


Plate IV: Heart group E administered with 800 mg/kg of *Grewia carpinifolia* leaf showing coronary congestion (mag x 100).

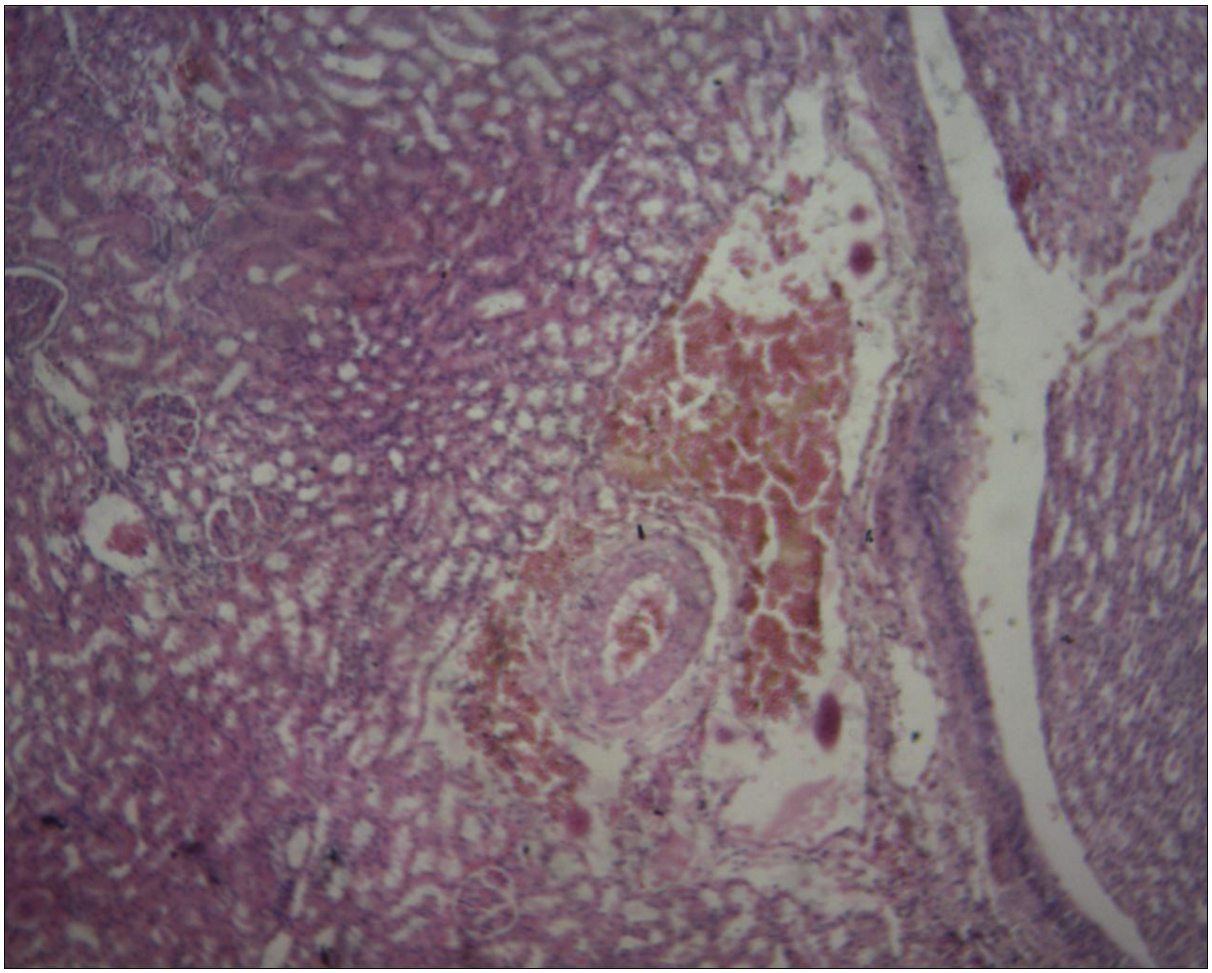


Plate V: The heart section of rat administered with 100 mg/kg (group B) of *Grewia carpinifolia* leaf for 28 days showing no visible lesion (mag x 100).

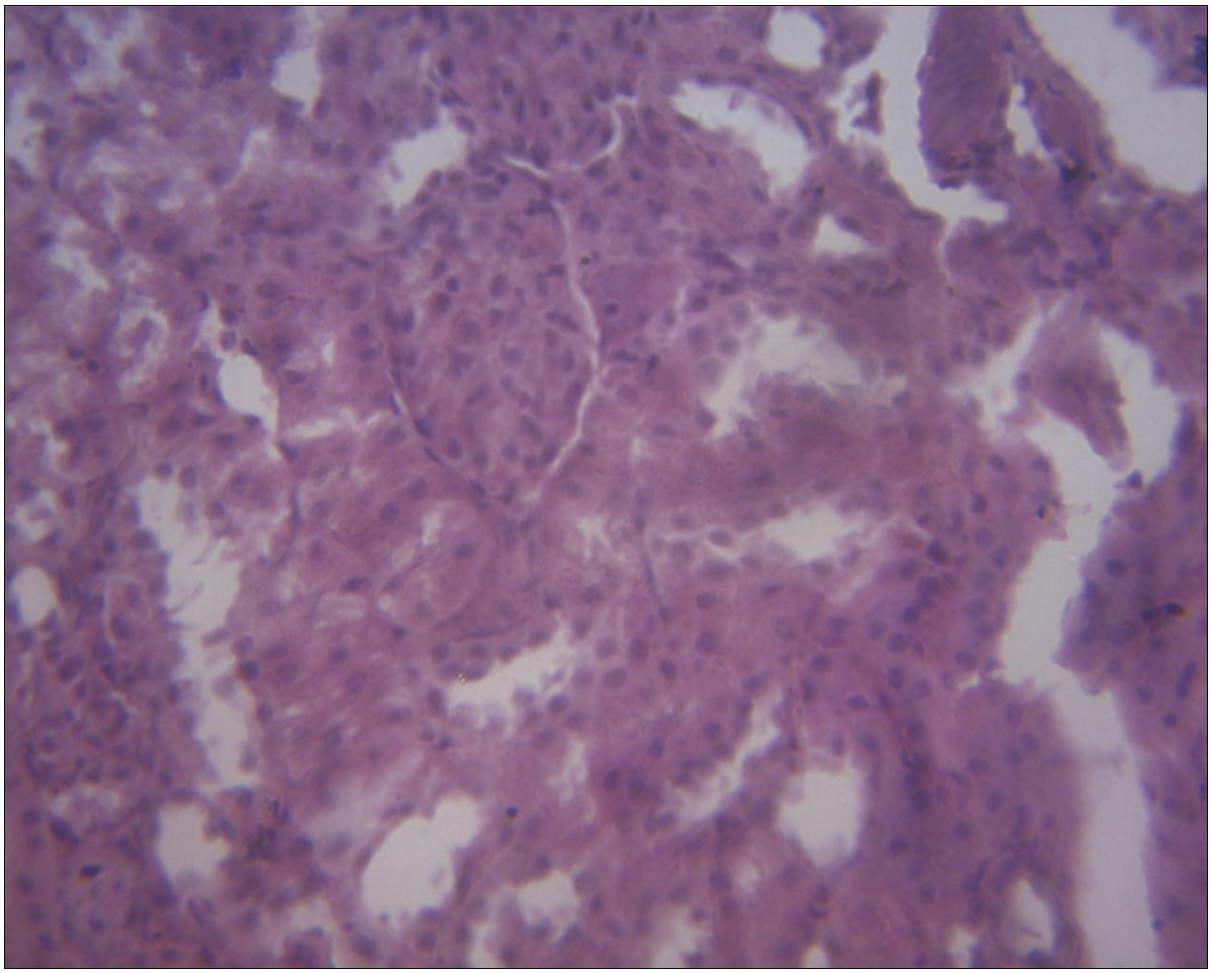


Plate VI: The kidney section of rat administered with 200 mg/kg (group C) of *Grewia carpinifolia* leaf for 28 days showing no visible lesion (mag x 100).

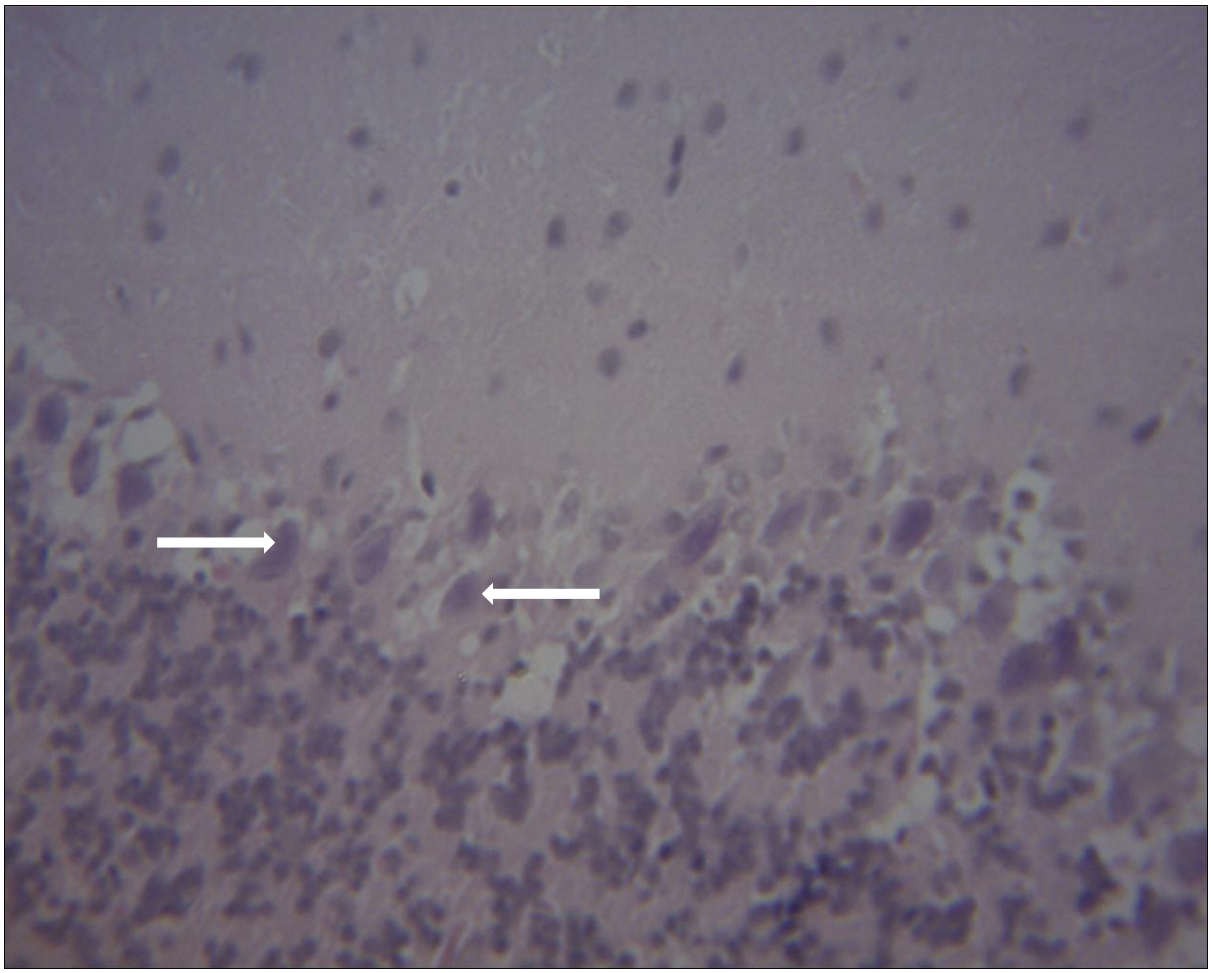


Plate VII: The brain section of rat of control group showing the Purkinje cells of the cerebellum with no visible lesion (mag x 100).

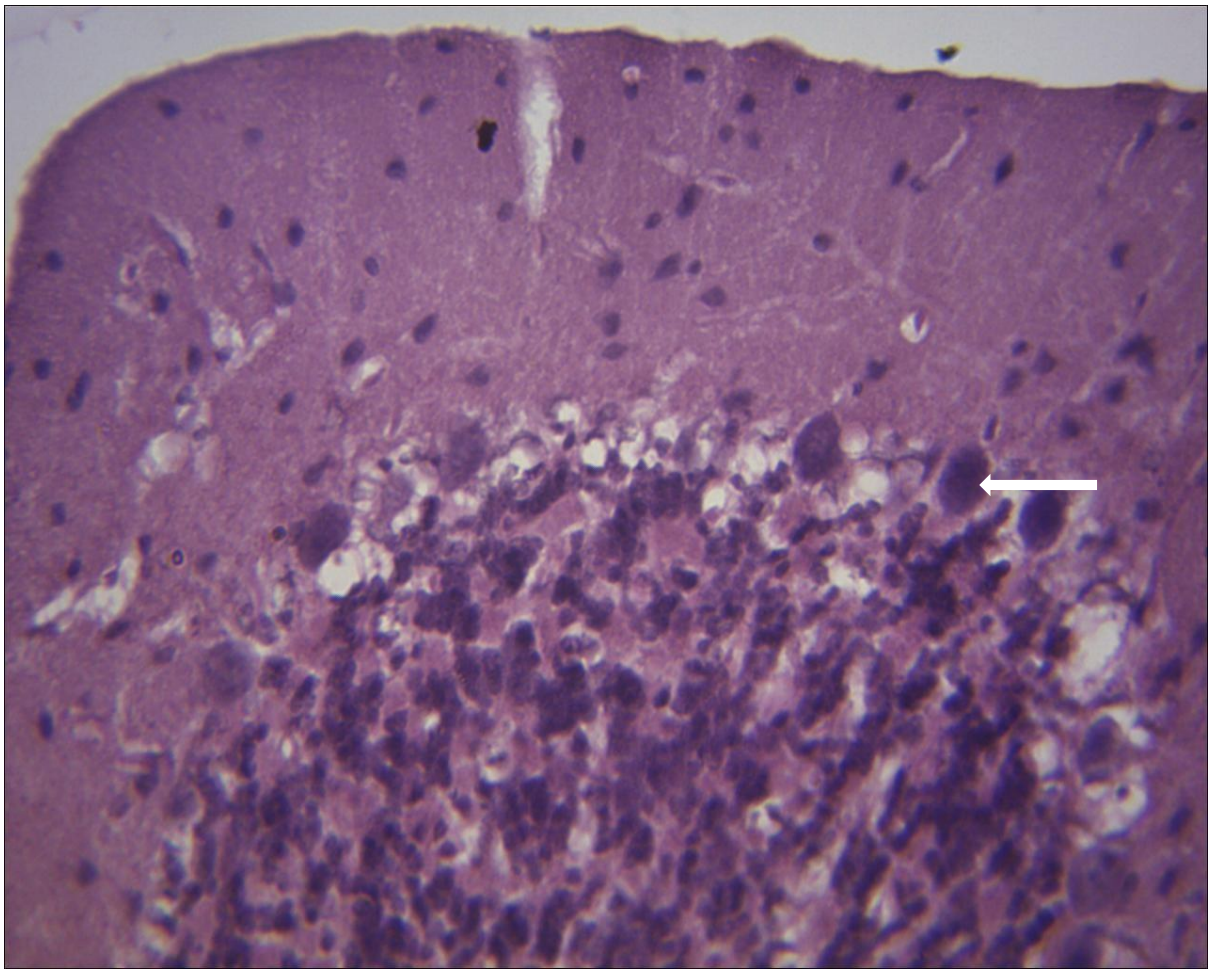


Plate VIII: The brain section of rat administered with 100 mg/kg (Group B) showing the Purkinje cells of the cerebellum with no visible lesion (mag x 100).

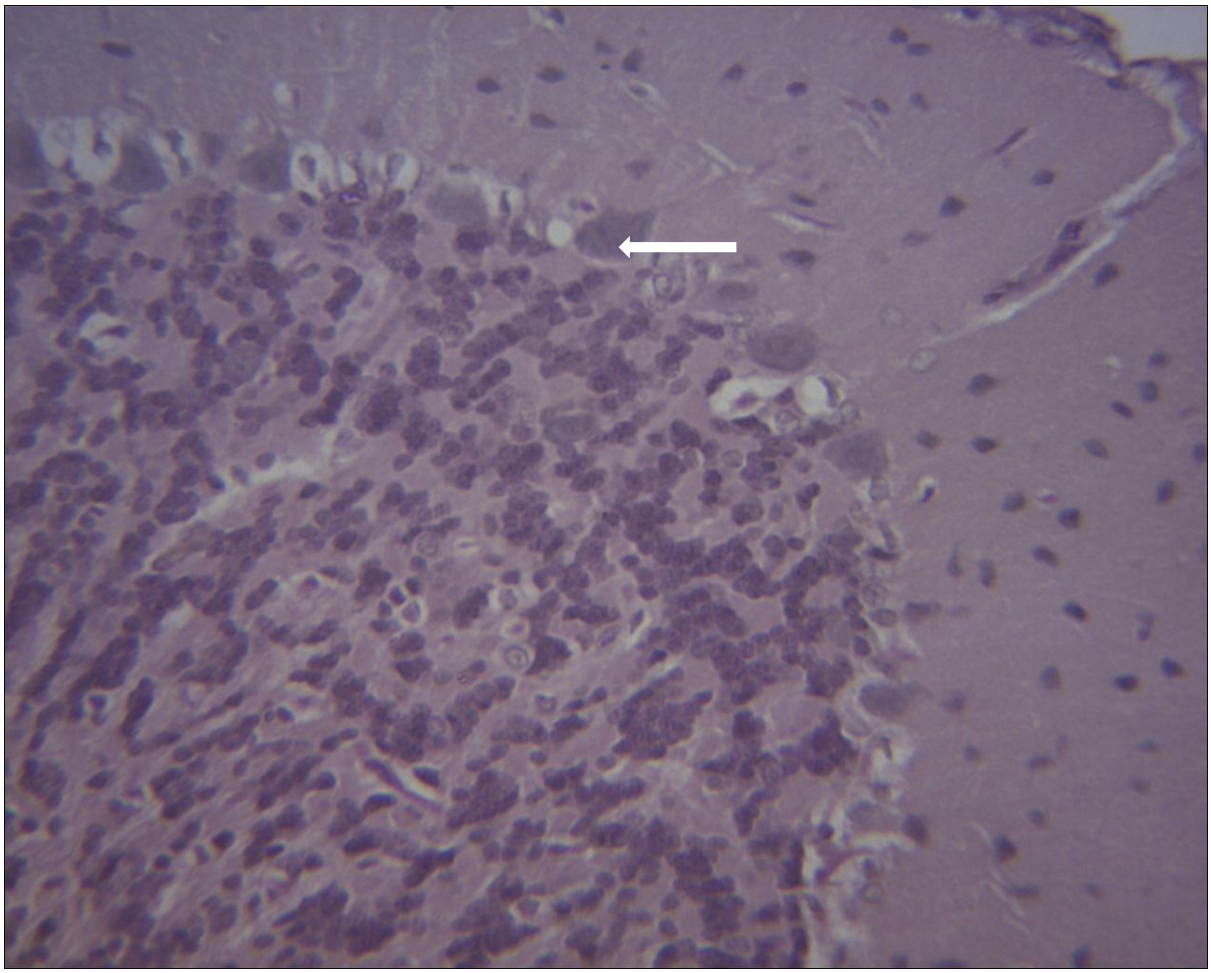


Plate IX: The brain section of rat administered with 200 mg/kg (Group C) showing the Purkinje cells of the cerebellum with no visible lesion (mag x 100).

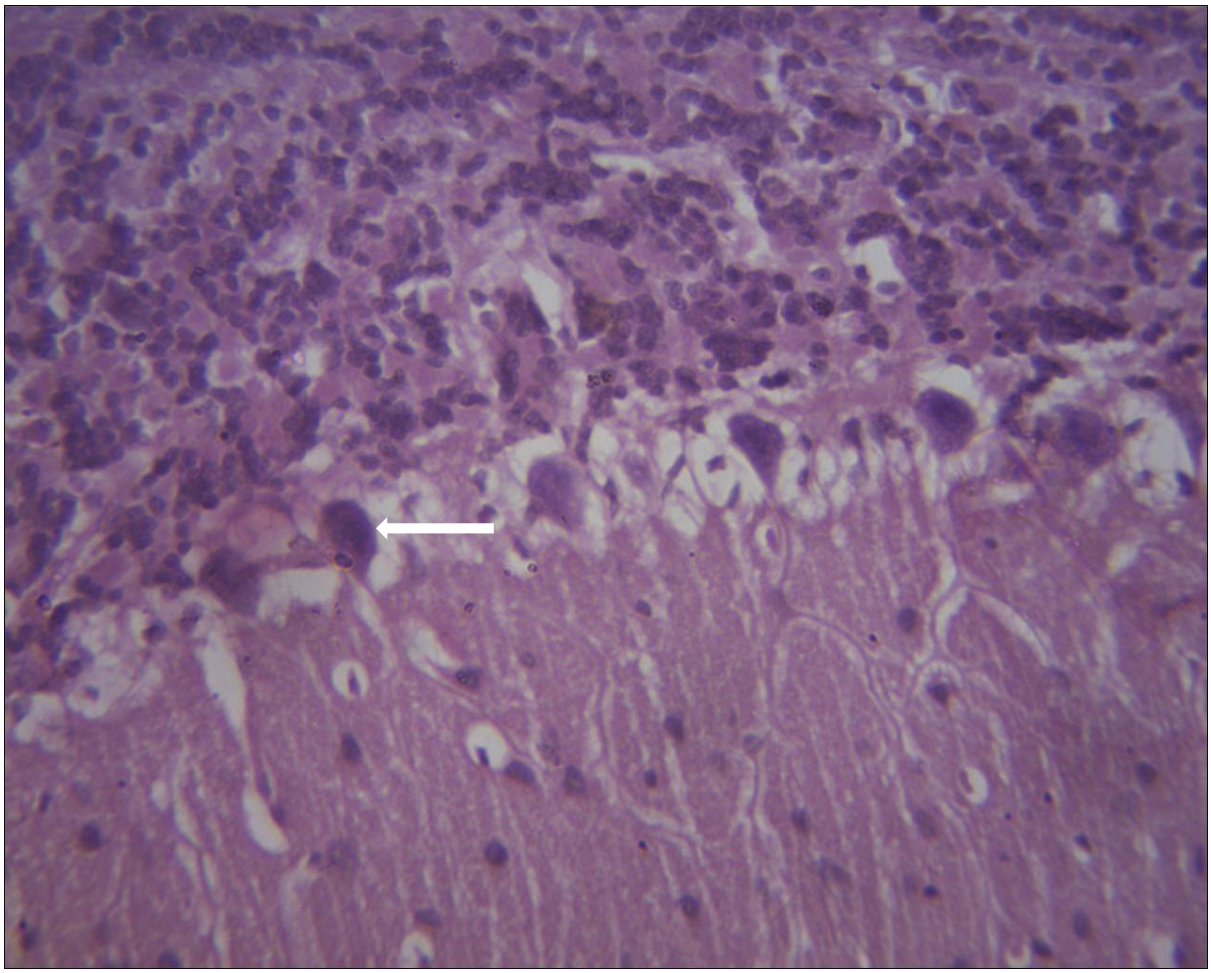


Plate X: The brain section of rat administered with 400 mg/kg (Group D) showing the Purkinje cells of the cerebellum with no visible lesion (mag x 100).

3.8 DISCUSSION

The uses of herbs require good understanding of the toxicity dosage, phytochemical constituents' purity, suitable extraction solvent and adverse effects (Murray, 1998). This can be used to explain their toxicity and probably their folklore in the treatment of numerous human and animal diseases (Farnsworth, 1966; DMP, 2004; CAPES, 2006).

3.8.1 Phytochemical Studies

A knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutics, potent bioactive compounds found in medicinal plant, or the synthesis of new drugs (Sofowora, 1993) but also because such information may be of value in disclosing new sources of economic materials such as tannins, oils and gums. The qualitative phytochemical screening of *G. carpinifolia* of the present study, showed the presence of tannins, saponins, flavonoids, alkaloids, phlobatinins, terpenoids, cardiac glycosides and anthraquinones (Table 3.3).

Alkaloids generally play some metabolic roles and they control development in living system. They are also involved in protective function in animals and are used as medicine, especially the steroidal alkaloids (Raquel, 2007). Tannins are known to inhibit pathogenic fungi (Sodipo *et al.,* 2000). The flavonoids and phenolic compounds in plant have been reported to exert multiple biological effects, including antioxidant, anti inflammatory, antimicrobial and anti carcinogenic abilities (Krings and Berger, 2001; Okwu, 2004; Lalitha and Jayanthi, 2012). The plant extract was also revealed to contain saponins, which are known to inhibit inflammation (Just *et al.,* 1998). Saponins precipitate and coagulate erythrocytes and bind to cholesterol (Okwu, 2004). Steroids have been reported to have antibacterial properties (Raquel, 2007), and they are mportant compounds such as sex hormones (Okwu, 2001). Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Han *et al.,* 2007). Several workers have reported the analgesic (Nobori *et al.,* 1994), antispasmodic and antibacterial (Okwu and Okwu, 2004) properties of alkaloids. Glycosides are known to lower the blood pressure according to many reports (Nyarko and Addy, 1990)

The results obtained in this study, thus suggest that the identified phytochemical compounds may be the bioactive constituents and that *G. carpinifolia* leaf hold promises as source of pharmaceutically important phytochemicals. The result obtained in this study is in

consonance with that obtained by Arun *et al.,* (2013) in their phytochemical investigation of ethanol extract of *Grewia microcos* Linn leaf. Nidhi and Vidya (2013) in their study however reported the absence of anthraquinones, and saponin in extract of *Grewia damine and G. asiatica* leaves. The absence of certain phytochemicals in one species and its presence in another may be safely attributed to the physiological and biosynthetic reactions taking place inside the different plant species and the effect of the environment attributed to this variance (Farhat *et al.,* 2011). According to Simonovska *et al*. (2003), the solvent used in the preparation of plant extracts can qualitatively and/or quantitatively affect the biologically active chemical constituents extracted. Therefore, the results indicate that the form of preparation of the extract is very important for its biological activity and that probably the active principles responsible for the effect of the extract obtained from *Grewia carpinifolia* may be better extracted better with an organic solvent.

The large amounts of polyphenols observed following a quantitative phytochemical screening might indicate that the stem and leaf possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (Anderson *et al*., 2001; Djeridane *et al*., 2006). The results strongly suggest that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

3.8.2. Acute Toxicity Study

Acute toxicity is usually the adverse change(s) occurring immediately or a short time following a single or short period of exposure to a substance or substances, or as adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 h. The adverse effect is any effect that causes functional impairment and/or biochemical lesions, which may affect the performance of the whole animal or that decreases the organ's ability to respond to additional challenges (Barahona and Sanchez-Fortu’n, 1999). Consequently, a substance that enters the organism via the oral route during a limited time and produces any adverse effect with little latency is orally and acutely toxic. It has been claimed that when properly performed and closely observed, an acute toxicity test can give more information about the biologic properties of a chemical compound than any other single test (Walum, 1998).

The acute toxicity study of *G. carpinifolia* leaf and stem showed no mortality or physical changes in skin and fur, eyes and mucus membrane (nasal), respiratory rate, circulatory signs

(heart rate and blood pressure), autonomic effects (salivation, perspiration, piloerection, urinary incontinence and defaecation) and CNS (ptosis, drowsiness, gait, tremors and convulsion) in rats administered doses below 6000 mg/kg b.wt. This observation may indicate that the drug could be considered relatively safe especially when administered orally (Shatoor, 2011). The finding of the present study is in consonance with that of Obidah *et al.,* (2010) that *Grewia mollis* at high concentrations consumption may elicit toxic effects in rats. Ukwuani *et al.,* (2012) also estimated that the LD_{50} of *Grewia crenata* was more than 5000 mg/kg.

Toxic effects are not commonly observed in acute experiments with natural products (Alvarez *et al*., 2004). This may be due to the intrinsic nature of those products, which contain different compounds, usually in low concentrations, as against synthetic drugs whose toxicity is more prone to appear in acute assays (Féres *et al.,* 2006). The popular perception that natural products do not present toxic effects might be explained within this context, since the recognition of product toxicity is only associated to its use, when the effects do manifest immediately after administration. Therefore, multiple dose studies are necessary to assure the safety of natural products. On the other hand, clinical observations of acute assays are valuable tools to define the doses to be tested in the multiple dose experiments, along with pharmacological studies in animals and in humans (Alvarez *et al*., 2004; Hasumura *et al*., 2004). This necessitated the subchronic study.

3.8.3 Sub-chronicToxicity Study

The highest dose used in the sub-acute toxicity study was reduced to 1/5th of the dosage that caused mortality during the acute study. Daily treatment with *Grewia carpinifolia* leaf and stem at the dose of 100, 200, 400 and 800 mg/kg body weight orally for 28 days did not cause mortality or any other signs of toxicity. Ukwuani *et al*. (2012) obtained similar results, although their study involved the use of hydromethanol extract of *Grewia crenata* leaf administered orally to the animals.

3.8.3.1. Feed and water Intake

In the sub-chronic administration of ethanol extract of *G. carpinifolia* leaf and stem, water intake was not significantly affected in the animals administered 400 mg/kg and lower dosages. Water is an essential nutrient due to its physio-chemical properties. Both dehydration and excessive hydration may cause serious health problems (Iversen and Nicolaysen, 2003). Dehydration as little as 2% can result in impaired physiological and

performance responses. Several factors may increase the likelihood of chronic and mild dehydration, including common consumption of alcohol (Sluyter *et al.,* 2000). Hence, in spite of the use of ethanol in the extraction of *G. carpinifolia* leaf and stem, the water intake was not affected in the treated animals, a crucial condition for maintaining the organic equilibrium during the therapy with this plant. The decrease in mean value of water intake at high doses may be due to the bitter taste of the test substance. For instance, the presence of some constituents in the leaf such as tannins (Onwuliri *et al.,* 2006) may cause the observed reduced water intake. Tannins at high dosages have been shown to reduce water intake in animals fed tannin diets, attributed to the astringent property of tannins and induction of internal malaise in mammals (Hotellier and Delaveau, 1975; Toma *et al.,* 2009).

The treated rats did not show any significant alteration in food consumption. This may be responsible for the body weight gain in the study. Loss of appetite is often synonymous with weight loss due to disturbances in carbohydrate, protein or fat metabolisms (Klaassen, 2001). Crude plant extracts may metabolise to a toxic end-product, which could interfere with gastric function and decrease food conversion efficiency (Chokshi, 2007). Interestingly, the food intake was found to be unaltered during the 28-day treatment period when compared to the control group in this study, suggesting that the extract did not metabolise to any toxic product or cause any significant alterations in carbohydrate, protein or fat metabolism in the experimental animals. It also shows that *G. carpinifolia* did not adversely interfere with the nutritional benefits (for example, weight gain, stability of appetite) expected of animals that are continually supplied with food and water *ad libitum* (Aniagu *et al*., 2005). Changes in body weight have been used as an indicator of adverse effect of drugs and chemicals (Mukinda and Syce, 2007).

3.8.3.2 Effect of ethanol extract of *Grewia carpinifolia* leaf and stem on body weight of rats following sub-chronic oral administration

Body weight changes serve as a sensitive indication of the general health status of animals (Hilaly *et al.,* 2004). The results of this study showed that albino rats were able to maintain growth irrespective of dose of the extract of *G. carpinifolia* leaf and stem administered to them. There was no significant ($\alpha \geq 0.05$) difference in body weights of the extract-treated groups when compared with the control group. Although the effect of extract of *G. carpinifolia* leaf on body weight has not been earlier reported, the present observation suggests that *G. carpinifolia* did not interfere with body weight.

3.8.3.3 Effect of ethanol extract of *Grewia carpinifolia* leaf and stem on absolute (g) and relative organ weights of rats following sub-chronic oral administration

The absolute (g) and relative weights (%) of all the isolated organs (liver, kidneys, lungs, spleen and heart) of the treated and the control groups remained normal, indicating that the plant extract was non-toxic in these vital organs. The organ weight is an important indicator of physiological and pathological state in humans and animals. The relative organ weight is fundamental to diagnose whether an organ was exposed to injury or not (Vaghasiya *et al.,* 2010). The heart, liver, kidneys, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicants (Dybing *et al.,* 2002). According to Moore *et al,* (2008), an increase in organ- body weight ratio is an indication of inflammation in these organs, while a reduction in the same parameter can be adduced to cellular constriction. Kluwe (1981) documented that the increase in absolute weight of the kidneys and liver is an indicator of nephrotoxicity and hepatotoxicity, respectively. In this study, no significant differences ($\alpha \geq 0.05$) were found in the absolute and relative organ weights of the extract-treated groups compared to controls. The ethanol leaf extract of *G. carpinifolia* did not induce any toxic effect on the kidneys and the other organs based on this indicator. This is in line with the findings of Ukwuani *et al.* (2012) that extracts of *G. crenata* possess hepatoprotective properties. Sharma and Sisodia (2010) and Asuku *et al.* (2012) had also observed the hepatoprotective efficacy of *Grewia asiatica* fruit and *G. mollis* leaf, respectively against oxidative stress.

3.8.3.4. Effect of ethanol extract of *Grewia carpinifolia* leaf and stem on behaviour following sub-chronic oral administration

The open-field test provides simultaneous measures of locomotion, exploration and anxiety (Walsh and Cummins, 1976). The number of line crosses and the frequency of rearing are often used as measures of locomotor activity, but are also measures of exploration and anxiety. A high frequency of these behaviours indicates increased locomotion and exploration and/or a lower level of anxiety. The number of central square entries and the duration of time spent in the central square are measures of exploratory behaviour and anxiety. A high frequency/duration of these behaviours indicates high exploratory behaviour and low anxiety levels. The *Grewia carpinifolia* spontaneous activities were studied by rearing and the number of line crossed. The rearing (vertical movement) is an index of the locomotor activity (Vogel, 2002), while the increased number of line crossed (horizontal

movement) is an indication of the CNS stimulant properties. Rats treated with extract at varying doses of *G. carpinifolia* leaf and stem showed no significant increase in number of rearings, groomings and time spent in the centre**.** This indicates that the plant extract did not induce anxiety in treated rats.

Defaecation and urination are often used as measures of anxiety, but the validity of defaecation as a measure of anxiety has been questioned (Lister, 1990). Although the effect of leaf extract of members of the *Grewia spp* on behaviour has not been reported to the best of my knowledge, these results taking together indicate that the ethanol extract of *G. carpinifolia* showed no anxiety-like effects, did not affect locomotion or produce CNS. However, the significant increase in the frequency of urine passed in the extract treated groups requires a further investigation. Several investigators have reported the anxiolytic potential of plant extracts (Farhat *et al.*, 2011; Harquin *et al.*, 2012; Harsha and Anilakumar, 2012).

The length of time a rat holds the hanging wire is considered as an indirect measure of grip and muscle strength (Stockdale *et al*., 2017); which was significantly increased in all the tested groups. This finding suggests that the phytochemical constituent of the extract of *Grewia carpinifolia* leaf and stem have roles in their CNS effects as it relates to muscle strength and motor co-ordination.

3.8.3.5. Effect of ethanol extract of *Grewia carpinifolia* leaf on haematology of albino rats treated for 28 days

Assessment of haematological parameters can be used to determine the extent of deleterious effects of extracts on the blood of an animal. It can also be used to explain blood-relating functions of a plant extract or its products (Yakubu *et al.,* 2007). Such analysis is relevant to risk evaluation as changes in the haematological system have higher predictive value for human toxicity, when the data are translated from animal studies (Olson *et al.,* 2000). The haematological parameters (haemoglobin, PCV, MCV, MCH and MCHC) estimated for all the treated groups showed no significant difference from those of the control groups. The MCHC, MCH and MCV relates to individual red blood cells while Hb, RBC and PCV are associated with the total population of red blood cells. Therefore, the absence of significant effect of *G. carpinifolia* leaf extract on RBC, Hb, PCV, MCH, MCHC and MCV could mean that neither the incorporation of Hb into RBCs or the morphology and osmotic fragility of the

RBCs were altered (Adebayo *et al.,* 2005). However, significant ($\alpha \leq 0.05$) differences were obtained in neutrophil and lymphocyte counts in the treated groups when compared with the control group. Ukwuani *et al.* (2012) in their toxicological evaluation of *G. crenata* leaf extracts also observed no significantly ($\alpha \geq 0.05$) difference in the haematological parameters however contrary to the present study, they reported a reduction in platelet count and increases in differential blood count.

3.8.3.6. Effect of *Grewia carpinifolia* leaf ethanol extract on serum chemistry of albino rats treated for 28 days

Blood serum parameters of the common target organs, for example, urea and creatinine level for renal injury and AST, ALT and ALP for hepatic injury were measured. The application of blood serum or plasma enzymes as markers to measure organ damage, cell damage, enzyme induction, activation or inhibition of enzymes become very common in toxicology studies. A variety of blood biochemical measurements could be used to evaluate the severity of tissue damage, possible target organs and measure impaired organ functions. With the combination of these tests, a broad range of information on their physiological and their metabolic functions may be obtained.

Several serum enzymes are used as indicators or markers for hepatocellular injuries such as ALT and AST (Lu and Kacew, 2002). Hepatic "leakage" enzymes are usually cytosolic enzymes. Altered permeability of hepatocellular membrane caused by injury on liver results in the release of soluble cytosolic enzymes into blood (Negishi *et al.*, 1993). They generally escape through baso-lateral side of hepatocytes facing the sinusoids which causes their elevation in blood (Ramaiah and Jaeschke, 2007). These enzymes are released into the blood from cytosol and subcellular organelles of hepatocytes once the liver is injured or damaged. A significant increase in certain hepatic enzymes is commonly associated with the development of liver damage. Alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) are good indicators for hepatobiliary disease. ALP is located mostly within the biliary canaliculi, whereas GGT is mostly in epithelial cells of bile ducts and some periportal hepatocytes. ALP is a group of phosphatase (pH optimum approximately 10) found in almost every tissue in the body. Serum urea level or blood urea nitrogen (BUN) and creatinine test provide information regarding kidney function (Tortora and Derrickson, 2006). Blood nitrogen is part of the urea, resulting from catabolism and deamination of amino acid in the liver and it is mainly excreted through urine by the kidneys. If glomerular filtration rate

(GFR) decreases severely which may be caused by renal failure or obstruction of urinary tract, BUN will rise steeply.

Creatinine, another indicator of kidney function, is a metabolite of creatine phosphate in skeletal muscle. Its level in blood remains constant normally because the rate of excretion through urine is equivalent to the discharge rate from muscle. If kidney damage or failure is found, the rate of excretion will be lower than the discharge rate from muscle. Thus, the serum levels of urea and creatinine will increase accordingly (Tortora and Derrickson, 2006).

The observed increase in the activities of liver enzymes of rats administered 800 mg/kg of the leaf extract in the present study might be due to damage to the membrane of this organ at this dosage.

3.8.3.7. Histopathology

Hypertrophy of organs is first-hand indication of toxicity of chemical or biological substance (Sellers *et al.,* 2007). However, no hypertrophy of organs was observed in this study amongst all the groups studied. In addition, the microscopic examination revealed that none of the organs from the extract treated rats showed any alteration in cell structure or any unfavourable effects, when viewed under the light microscope using multiple magnification powers. However congestion of portal central nucleus and sinusoids were observed in the liver section of rats in group E, administered with 800 mg/kg of the leaf extract, moderate coronary congestion was also observed at 400 and 800 mg/kg. No pathologies were recorded in the histological sections of the kidneys and cerebellum at tested doses. Generally, any damage to the parenchymal liver cells results in elevations of both transaminases in the blood (Olson *et al.,* 2000) Thus, the non-significant increases observed in serum ALT, AST, and alkaline phosphatase activities strongly correlates with the histopathology observations of liver tissue. Equally, there was also no significant increase in urea and creatinine in the sub-acute administration of *G. carpinifolia* extract, when compared to the control group. Any rise in urea and creatinine levels indicates damage to functional nephrons. This finding was further confirmed by histopathological observations of the kidney tissue in this study.

Therefore, the results recorded in this study demonstrate that the *G. carpinifolia* extract did not alter the liver or renal function at doses below 400 mg/kg, and further support the non-toxic nature *G. carpinifolia* extract at these doses.

3.9 CONCLUSION

In the light of the findings, it may be concluded that extract of *G. carpinifolia* leaf and stem have a high safety margin following acute toxicity. *G. carpinifolia* extract was apparently safe at doses below 800 mg/kg body weight, and did not produce any toxic signs or evident symptoms at acute and sub-chronic oral toxicity. *G. carpinifolia* extract did not cause any lethality or produce any remarkable histopathological signs, serum chemical alteration or signs of anxiety after sub-chronic administration. The preliminary phytochemical results suggest promising therapeutic and pharmaceutical activities of *G. carpinifolia* leaf and stem extract.

CHAPTER FOUR

4.0 STUDY TWO

Protective effects of crude extract of *Grewia carpinifolia* on changes im mice following acute vanadium induced toxicity

4.1. INTRODUCTION

Vanadium is a heavy transition metal (Habib and Ibrahim, 2011), which has been recognised to be acutely toxic by most routes of introduction following environmental exposure in large doses. Disposition of vanadium in specific tissues may be involved in the pathogenesis of certain neurological disorders and cardiovascular diseases (Venkataraman and Sudha, 2005). Human activities, such as combustion of fossil fuels (Aragón and Altamirano-Lozano, 2001), inhalation in the vicinity of metallurgical plants and oil exploratory activities as seen in the Niger Delta region of Nigeria, have further led to environment exposure to vanadium. Consequently, this has impacted negatively on the aquatic and terrestrial habitats in this region, which represents about 12% of Nigeria's total surface area with over 28 million inhabitants (Federal Government of Nigeria, 1991).

The toxicity of vanadium compounds is linked to its oxidation state or valency, which increases with the oxidation state (Erdmann *et al.* 1984; Venkataraman and Sudha, 2005). Vanadium within the cells has predominantly an oxidation state of +4 as a result of reactions with intracellular antioxidants (Aureliano and Gândara, 2005; Kordowiak and Holko, 2009). Furthermore, vanadium compounds in the +4 oxidation state are oxidised by atmospheric oxygen to the +5 oxidation state, with accompanying emission of a superoxide anion radical and generation of a hydroxyl radical via a Fenton-like reaction (Cuesta *et al.*, 2011). Following reduction with nicotinamide adenine dinucleotide phosphate (NADPH), the reaction may proceed with generation of hydrogen peroxide (Cuesta *et al.*, 2011).

Vanadium has been shown to induce various toxicities via these effects of reactive oxygen species (ROS) generation (Obianime *et al.*, 2009; Olopade and Connor, 2011). For example, it has been investigated that vanadium compounds induce ROS generation in the brain, thus contributing to degeneration of dopaminergic neuronal cells of the substantia nigra, which in turn may lead to Parkinson's disease (Afeseh Ngwa *et al.*, 2009; Cuesta *et al.*, 2011).

Vanadium could also damage genetic material; induce haemato-toxicity, immune, hepatic, lung, neuro and reproductive toxicities (Shi *et al.*, 1996; Yang *et al.*, 2004; Soares *et al.*,

2008; Hosseini *et al.,* 2012; Altamirano-Lozano *et al,* 2014). Therefore, reduction of +5 forms to +4 forms represents an effective means of reducing the impact of vanadium on a living system (Galli *et al.,* 1991). Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, nuts, seeds, leaf, roots and barks (Narayanaswamy and Balakrishna, 2011; Sharma *et al.,* 2013). Several herbal products have been reported to mitigate oxidative stress due to increased ROS generation and ameliorate the effects of heavy metals (Dailiah and Padmalatha, 2012; Ola-Davies *et al.,* 2013). Several members of the genus *Grewia* have been documented to possess antibacterial, analgesics and antioxidant properties (Ahamed *et al.*, 2007; Goyal, 2012).

Following the knowledge of the phytochemical constituents of *G. carpinifolia* as well as an establishment of a safe dose and lethal dose for the plant in study 1, the aim of the present study was to ascertain the protective role of the plant against the deleterious effects of vanadium in experimental animal model. The protective effects of *Grewia carpinifolia* following haematotoxicity, hepatotoxicity and behavioural deficits induced by vanadium in the mice were investigated. The findings from this work may add to the overall value of the medicinal potential of this plant.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and Authentication

Fresh leaf and stem of *G. carpinifolia* was collected, identified and authenticated as described in study one (Section 3.2.1.)

4.2.2 Extract Preparation

Ethanol extract of the leaf and stem of *G. carpinifolia* was prepared according to the method of Panovska *et al*. (2005) as described in Section 3.2.2 of study 1.

4.2.3. Separation of the extract

The different parts of the plant *G. carpinifolia* were collected and extracted with ethanol as already described in section 3.2.3 of study 1.
The extract was dissolved in distilled water and warmed before administration.

4.2.4 Experimental Animals

Male mice of average weight of 12-15 g were purchased and housed at the Animal House, Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine. The animals were housed under standard conditions of ambient temperature, (25 ± 2°C) and light, (approximately 12/12 h light-dark cycle), fed on standard diet (Animalcare® Feeds Ltd., Ogere, Nigeria) and given accesss to fresh water *ad libitum*. The cages were cleaned of waste daily. All the animals were acclimatized to laboratory conditions for two weeks before the commencement of the experiment. All experiments performed on the laboratory animals in this study followed the OECD approved Standard Operation Procedures (SOPs).

4.2.5 Drugs and Chemicals

Ethanol and the rest of the chemicals used were of analytical grade and were prepared in all glass distilled water.

4.2.6 Experimental Design

The experiment to determine the ameliorative effect of *Grewia carpinifolia* in laboratory mice following acute vanadium intoxication was designed to span 8 days. Sixty juvenile mice were randomly divided into five groups of twelve animals per group.

Group A received distilled water throughout the experimental period and served as control; group B, the standard group received vitamin E (500 mg/kg) every 72 h orally along with a daily dose of sodium metavanadate (Sigma-Aldrich, St. Louis, USA) at 3 mg/kg (Olopade *et al.*, 2011; Mustapha *et al.*, 2014) intraperitoneally (i/p) for 7 days consecutively, group C and group D received *Grewia carpinifolia* leaf extract at a single daily dose of 100 and 200 mg/kg orally respectively along with sodium metavanadate at 3 mg/kg i/p for 7 days consecutively, and group E received only sodium metavanadate at 3 mg/kg i/p for 7 days consecutively. The present study was conducted in line with the European laws on the protection of animals (86/609/EEC). The protocols for this experiment were approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/025) (Appendix 1).

4.2.7. BEHAVIOURAL TESTS

4.2.7.1 The Light/dark Box Test

The test apparatus was constructed according to that described by Hascoet and Bourin (1998) (Figure 4.1). The light/dark box (45 x 27 x 27 cm)was made of plywood and consisted of two chambers connected by an opening (7.5 x 7.5 cm) located at floor level in the centre of the dividing wall. The floor was divided into 9 x 9 cm squares. The small chamber (18 x 27 cm) was painted black and the larger chamber (27 x 27 cm) was painted white. The small compartment was painted black and illuminated by a dim red light (60 W; 4 lx), whereas the large compartment was painted white and brightly illuminated with a 60-W (400 lx) located 40 cm above the centre of both chambers.

The model is based on the observations that although nocturnal rodents such as mice will naturally tend to explore a novel environment, open fields appear to have aversive properties, which inhibit exploratory behaviour. Here, the safe area is the small dark compartment (one third) and the aversive area is the large illuminated compartment. Locomotion and time spent in each zone, latency of the first crossing from one compartment to the other, and shuttle crossings between both compartments were recorded. The number of leaning out (or peeking-out) from the dark chamber by the mice as described by Lapin (1999) was also recorded. Each mouse was allowed to explore the apparatus for 5 minutes (Hascoet and Bourin, 1998). The box was cleaned between each rat using 70 % ethyl alcohol.

Figure 4.1.: The Light/Dark Transition Test Box

4.2.7.2 Open-field test

Locomotor and exploratory activities were measured by an open-field task box (Coulbourn Instruments L.L.C., PA, USA), as described in chapter 3, section 3.2.8.2.3.1 of study 1.

4.2.7.3 Fore-limb Support (Hanging Wire) Test

This test is based on the latency of a mouse to fall off a metal wire upon exhaustion based on the method described by Van Putten *et al.* (2012). A 2 mm thick metallic wire secured to two vertical stands was used. The wire was tightly attached to the frame to avoid vibration or unwanted displacement of the wire during the measurements. Each mouse was placed on the wire with its fore-limbs and monitored for a maximum period of 120 seconds. The period of time it took the animal to stay on the wire before falling was taken and recorded (Brooks and Dunnett, 2009; Carlson *et al.*, 2010). The animals that did not fall off the hanging wire during the test period of 120 seconds were given a maximum score (Kulkarni, 1999). Two trials were performed for each mouse. The second trial was done after all the animals had successfully completed the first trial; hence, all mice were well rested before the second trial.

4.2.7.4 Negative geotaxis Test

Each animal was placed in the middle of a board, 30^0 inclined to the surface plane, in a head-down position and the latency to turn and orient its position; to be facing up the slope, is recorded (Bouet *et al.*, 2004).

4.2.8 HAEMATOLOGY AND SERUM CHEMISTRY

Blood collection was via puncture of the retro-orbital plexus of mice anaesthetized with ketamine (100 mg/kg bw) and using haematocrit tubes. About 3 ml of blood was collected. Two separate blood samples were collected for each animal; one into heparinised bottle for haematology and the other into plain bottles without anticoagulant for collection of serum chemistry tests.

4.2.8.1 Haematology

Packed cell volume (PCV), Hb concentration, red blood cell (RBC) count, platelets, total and white blood cell differential (that is lymphocytes, neutrophils, monocytes, eosinophils) were done following methods described in study 1 section 3.2.8.2.4.1. Mean corpuscular volume (MCV), MCH and MCHC were calculated from the values obtained from the count, respectively.

4.2.8.2 Serum chemistry

Following the centrifugation, the serum were separated, the samples were stored at -4°C and used for evaluation of biochemical parameters which include, ALT, AST and phosphatase ALP activities, total protein using assay kits obtained from Randox Laboratories, UK as described in study 1.

4.2.8.2.1 Creatinine

Creatinine levels were estimated using the creatinine Randox kit (Randox Laboratories, UK).

Principle

In alkaline solution, creatinine reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration. The protocol was done as summarized in Table 4.2 below.

4.2.8.2.2 Urea estimation

The estimation was carried out using the Urea Randox kit (Randox Laboratories, UK).

Principle

Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction.

$$\text{Urea} + H_20 \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenol (blue compound)}$$

Exactly 10 µl of test serum was dispensed into a test tube, 100 µl of solution R1 (containing sodium nitroprusside and urease) was added to the same test tube. The mixture was then incubated for 10 minutes at 37 ^{o}C. Following incubation, 2.5 ml of 120 mmol/l phenol (solution R2) and 2.5 ml of 27 mmol/l sodium hypochlorite (solution R3) were added. The mixture was incubated for another 15 minutes at 37 ^{o}C. The final colour reaction of the solution was read in a spectrophotometer (Spectrolab 2250, India) at 546 nm wavelength. The absorbance of the test samples were read against the standard. The protocol is summarised in Table 4.2 below.

Table 4.1: Creatinine assay protocol

Contents	Initial concentration of Solutions
CAL. Standard	
RIa. Picric Acid	35mmol/l
RIb. Sodium Hydroxide	0.32 mol/l

Procedure	
Wavelength	492(490-510nm)
Cuvette	1 cm light path
Temperature	25/30/37^{o}C
Measurement	against air

Pipette in cuvette:

Working reagent	Standard	Sample
Standard Solution	1.0 ml	1.0 ml
Sample	0.1 ml	-
	-	0.1 ml

After mixing, the absorbance A_1 of the standard and sample was read after 30 seconds. Exactly 2 minutes later, the absorbance A_2 of standard and sample was again read.

Calculation

$A_2 - A_1 = \Delta A$ sample or ΔA standard

$$\frac{\Delta A \text{Sample}}{\Delta A \text{ standard}} \times \text{standard conc (mmol/l)} = \mu\text{mol/l}$$

Table 4.2: Urea assay protocol

Contents	Initial concentration of Solution
$R_{1.}$ Ethylenediaminetetraacetic acid	116 mmol/l
Sodium nitroprusside	6 mmol/l
Urease	1g/l
R_2 Phenol (diluted)	120 mmol/l
R_3 Sodium hypochlorite (diluted)	27 mmol/l
Sodium hydroxide	0.14 N
CAL Standard	

Procedure

Wavelength:	546 nm (530 – 570 nm)
Cuvette	1 cm light path
Temperature:	37^{o}C
Measurement	Against regent blank

Pipette into test tubes:			
Sample	Blank	Standard	Sample
Standard	-----	----	10 µL
Distilled Water	----	10 µL	----
Reagent 1	10 µL	----	----
	100 µL	100 µL	100 µL

Mixed and incubated at 37^{o}C for 10 minutes			
Reagent 2	2.50 ml	2.50 ml	2.50 ml
Reagent 3	2.50 ml	2.50 ml	2.50 ml

The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) was read against the blank.

Calculation

The urea concentration was calculated as;

Urea concentrartion (mg/dl) = $A_{sample}/A_{standard}$ x Standard concentration

4.2.9 Body Weight

Rats in all the groups were weighed daily throughout the experiment.

4.2.9.1 Relative brain weight

Rats were anaesthetized with ketamine (10 mg/kg) intraperitoneally following which the frontal, parietal and temporal bones were gently removed to expose the brain, which was carefully removed, weighed and observed macroscopically (Villers and Ris, 2013). The relative brain weight (RBW) of each rat was calculated as follows:

$$\text{RBW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}}$$

4.2.10 STATISTICAL ANALYSIS

Results were analysed using the statistical package GraphPad prism version 5.01 (San Diego, USA). Data were subjected to one-way analysis of variance (ANOVA) and subsequently to the Tukey multi comparison post-hoc test to perform multiple comparisons in order to assess statistical significance of differences between all possible pairs of groups. Repeated measures on the same animals were analysed using the Wilcoxon matched-pair, signed-rank test (Siegel, 1957). Differences were regarded as statistically significant when $\alpha < 0.05$.

4.3 RESULTS

4.3.1 Mortality and clinical signs

During the study period, all the animals were observed daily for clinical signs and mortality patterns before dosing, immediately after dosing and up to 4 hours after dosing. Animals given 100 mg/kg of the leaf and those administered with vanadium only were observed to develop diarrhoea from the fourth to the 7th day of the experiment. Reduction in feed consumption and activity were observed in the entire test groups. In addition, body scratching, restlessness, shivering and lethargy were observed in the vanadium only group.

4.3.2. BEHAVIOURAL TESTS

4.3.2.1 Light/Dark Box Transition Test

The mean values for observations of the light/dark box test in experimental animals are presented in Figures 4.2 to 4.5**.** There was a significant reduction in the number of leaning out

in the mice administered with sodium metavanadate alone, while the extract significantly increased this to level comparable with the control group. The animals concurrently treated with the extract and vanadium spent more time in the light zone and this was significant when compared with the vanadium only group. In general locomotion was also significantly increased in the extract-treated group, when compared to the sodium metavanadate only group.

4.3.2.2 Open-field Test

The mean values for observations of the open-field test in experimental animals are presented (Tables 4.4 and 4.5). A significant ($\alpha < 0.05$) decrease was observed in the number of line crossing in the group administered with 100 mg/kg of the leaf extract (group C) and the vanadium group (E) when compared with the control and other test groups. There was also a significant ($\alpha < 0.05$) increase in the number of rearing in the group administered with only vanadium (E); however, there was no difference in these parameters in the other test groups when compared with the control. Time spent at the centre of the Open-field after seven days of co-administration of ethanol extract of *G. carpinifolia* leaf at 200 mg/kg, p.o. with vanadium (group D) was not significantly different from those of the control and standard group administered with α-tocopherol. Conversely, a significant increase in time spent at the centre of the box was recorded in the sodium metavanadate only group (E), when compared with the other groups.

4.3.2.3 Hanging wire test

The groups simultaneously administered with the leaf extract of *Grewia carpinifolia* and sodium metavanadate (C and D) spent more time on the hanging wire before falling off although this was of no significant difference ($\alpha > 0.05$) when compared to the control group but was of significance when compared with the sodium metavanadate only group; however, sodium metavanadate only at 3 mg/kg resulted in a significant ($\alpha \leq 0.05$) decrease in the time spent on the hanging wire test (Figure 4.6). Following the administration of the stem extract there was a significant reduction in time spent on the hanging wire in the α-tocopherol, 100 mg/kg of the stem extract, and the sodium metavanadate only group when compared with the control group (Figure 4.7).

4.3.2.3 Negative geotaxis Test

The mean latent time on the negative geotaxis test of the vanadium only group (E) was significantly ($\alpha \leq 0.05$) higher than those of the leaf extract at tested doses and the standard drug (Figure 4.8). The mean latent time on the negative geotaxis of the vanadium only group (E) was statistically ($\alpha \leq 0.05$) higher than those of the other groups; however the stem extracts had values similar to the standard, α-tocopherol group (Figure 4.9).

4.3.3 Body weight

An increase in daily body weight was observed in all the groups from day 1 to 5. There was a decrease in body weight in groups B-E on day 6, after which daily weight gain resumed in groups B-D on days 7 and 8; however, the daily body weight continued to decrease in group E after day 6 (Figures 4.10 and 4.11).

4.3.4 Relative brain weight

There was no significant ($\alpha \geq 0.05$) difference in the relative brain weight of the animal across all the groups (Table 2).

4.3.5 Haematological parameters

At the end of the study, the groups administered with 100 mg/kg of *G. carpinifolia* leaf concurrently with sodium pentavanadate and those administered with sodium pentavanadate only had significantly ($\alpha \leq 0.05$) lower PCV and Hb concentrations when compared to the other groups. A significant ($\alpha \leq 0.05$) reduction in WBC and neutrophil counts was also observed in the sodium pentavanadate only group. The lymphocyte count in the group administered with 100 mg/kg of *Grewia carpinifolia* leaf concurrently with sodium pentavanadate was significantly ($\alpha \leq 0.05$) higher than those of other groups (Table 4.6). The values of the haematological parameters of group administered with α-tocopherol simultaneously with sodium pentavanadate and those administered with 100 mg/kg of *Grewia carpinifolia* leaf concurrently with sodium pentavanadate were not statistically different from those the control group.

At the end of the study using *Grewia carpinifolia* stem, the results were also similar to those of the leaf extract.

4.3.6 Serum chemistry

Significant ($\alpha \leq 0.05$) reductions in total serum protein and cholesterol levels were observed following concomitant administration of 100 mg/kg of *G. carpinifolia* leaf with sodium pentavanadate and those administered with sodium pentavanadate only. There was a significant ($\alpha \leq 0.05$) increase in the serum levels of ALT and AST activities in the sodium pentavanadate only. Furthermore, a significant ($\alpha \leq 0.05$) decrease in potassium levels was observed in groups C, D and E (Table 4.8).

Following the administration of *G. carpinifolia* stem, potassium levels in the extract plus sodium metavanadate groups were not different from the control group, but potassium level in the sodium pentavanadate only (group E) was significantly increased (Table 4.9.).

Table 4.4: The mean values for observations in the open-field test of mice following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium

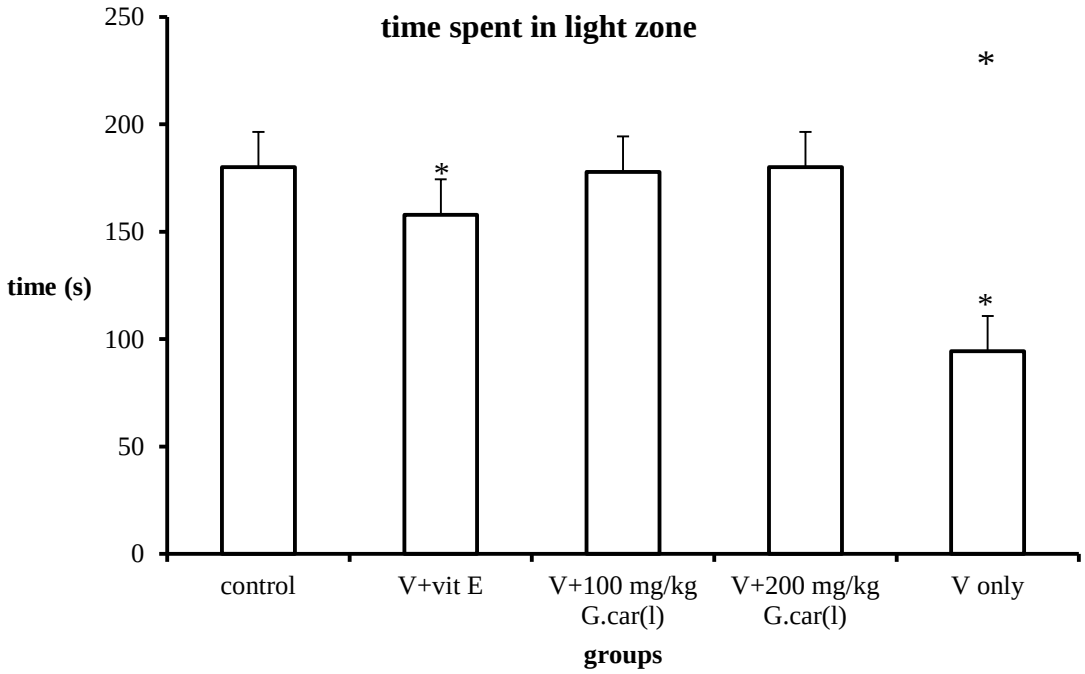
Observation	GROUP A (Control)	GROUP B (V + vit E)	GROUP C (V + *G carpinifolia* (l) at 100mg/kg)	GROUP D (V + *G carpinifolia* (l)at 200mg/kg	GROUP E (V only)
Line crossing	55.64 ± 27.29	59.00 ± 2.53	24.00 ± 16.15*	59.00 ± 5.93	18.60 ± 4.67*
Rearing	17.67 ± 13.50	13.00 ± 1.41	3.25 ± 1.89*	10.00 ± 1.82	73.25 ± 7.23*
Grooming (seconds)	21.33 ± 11.85	16.00 ± 11.31	12.00 ± 4.69	21.65 ± 4.72	14.40 ± 7.33
Stretched attend posture (seconds)	34.36 ± 4.09	38.60 ± 5.02	36.08 ± 7.32	35.21 ± 2.58	62.09 ± 10.36*
Time spent at the centre (seconds)	2.54 ± 0.03	2.86 ± 0.83	3.81 ± 0.51	3.00 ± 0.12	22.64 ± 10.40*
Freezing (seconds)	110.08 ± 9.06	103.00 ± 7.43	98.21 ± 7.65	112.61 ± 14.56	135.73 ± 13.48

Note: Mean ± S.D (Standard Deviation) number of animals in the group (12), * statistically different from the control at $\alpha < 0.05$; Vit E= α-tocopherol; V= sodium metavanadate; *G. carpinifolia* (l) = *Grewia carpinifolia* leaf; *significantly ($\alpha \leq 0.05$) different from the control and extract treated group

Table 4.5: The mean values for observations in the open-field test of mice following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium

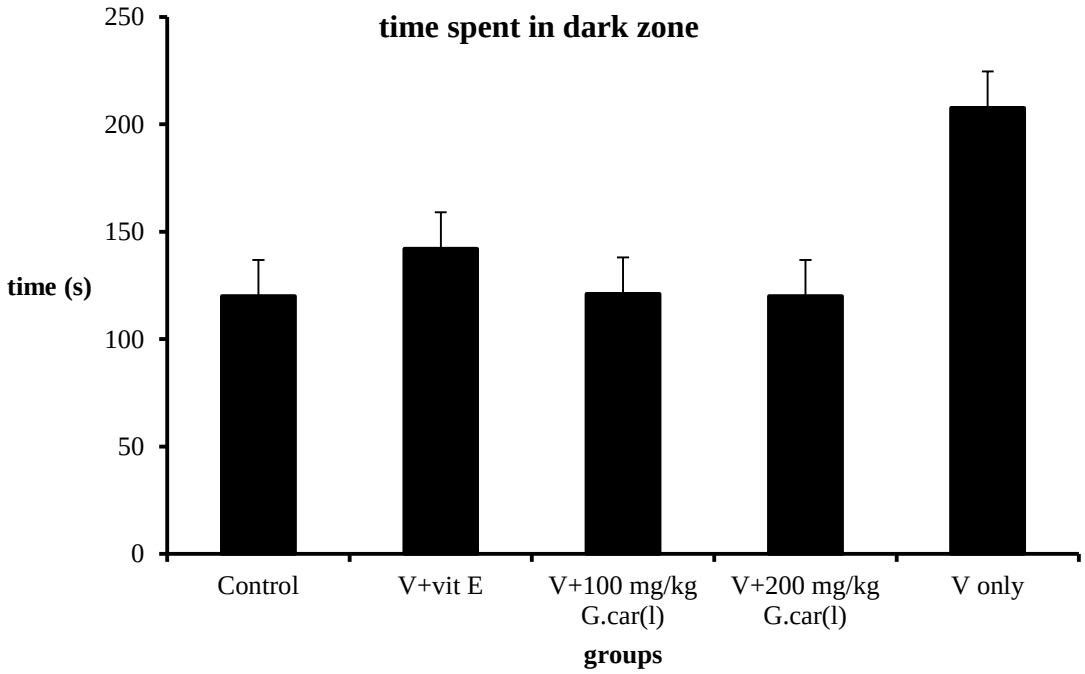
Observation	GROUP A (Control)	GROUP B (V + vit E)	GROUP C (V + *G carpinifolia*(s) at 100mg/kg)	GROUP D (V + *G carpinifolia*(s) at 200mg/kg	GROUP E (V only)
Line crossing	64.03 ± 23.97[a]	49.00 ± 2.53 [a]	28.41 ± 6.81[b]	29.12 ± 3.38[b]	14.05 ± 6.42[c]
Rearing	19.04 ± 8.54 [a]	16.73 ± 3.81 [a]	7.53 ± 2.37 [a]	11.82 ± 2.23 [a]	62.09 ± 7.23[b]
Grooming (seconds)	27.49 ± 13.00	18.31 ± 5.67	17.41 ± 5.53	21.32 ± 6.18	12.01 ± 6.33
Stretched attend posture (seconds)	36.21 ± 3.54 [a]	34.01 ± 4.91 [a]	23.64 ± 7.32 [a]	35.21 ± 2.58 [a]	56.91 ± 8.67[b]
Time spent at the centre (seconds)	5.25 ± 0.27 [a]	4.91 ± 0.94 [a]	4.32 ± 0.72 [a]	5.20 ± 0.24 [a]	18.05 ± 7.61[b]
Freezing (seconds)	90.13 ± 12.53[a]	83.82 ± 9.21 [a]	88.11 ± 5.61 [a]	92.73 ± 11.07[a]	175.04 ± 26.12[b]

Note: Mean ± S.D (Standard Deviation) number of animals in the group (12); means with different superscripts within rows are significantly different at $\alpha<0.05$, Vit E= α-tocopherol; V= sodium metavanadate; *G. carpinifolia* (s) = *Grewia carpinifolia* stem; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group



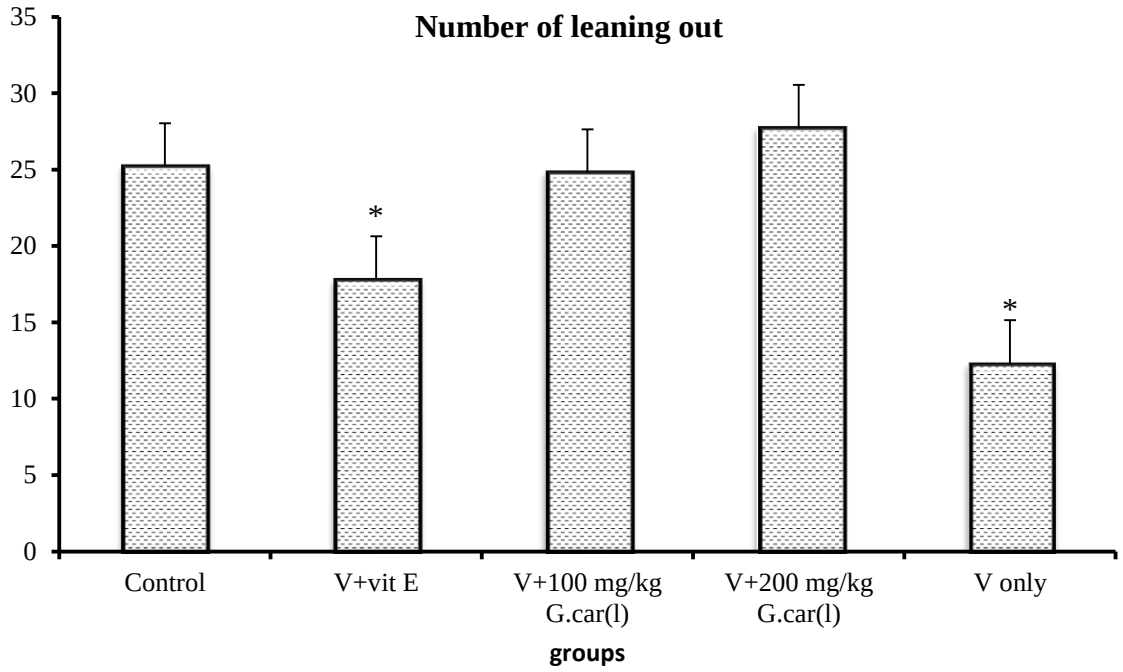
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.2: Time spent by mice in the light zone in the light/dark box test following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



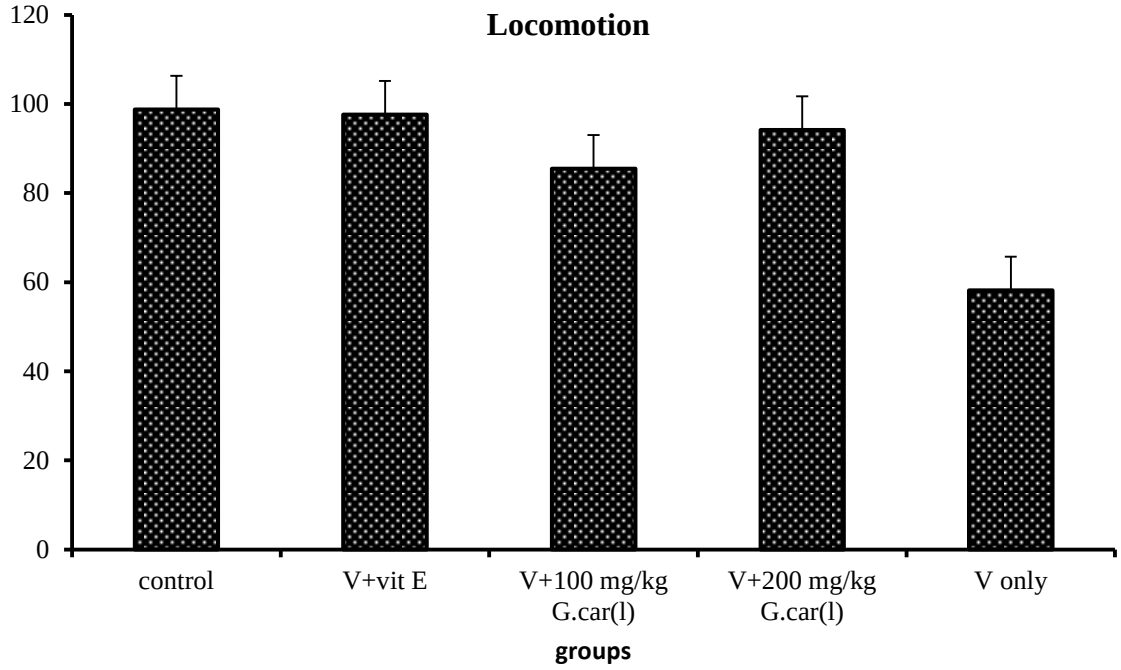
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.3: Time spent by mice in the dark zone in the light/dark box test following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



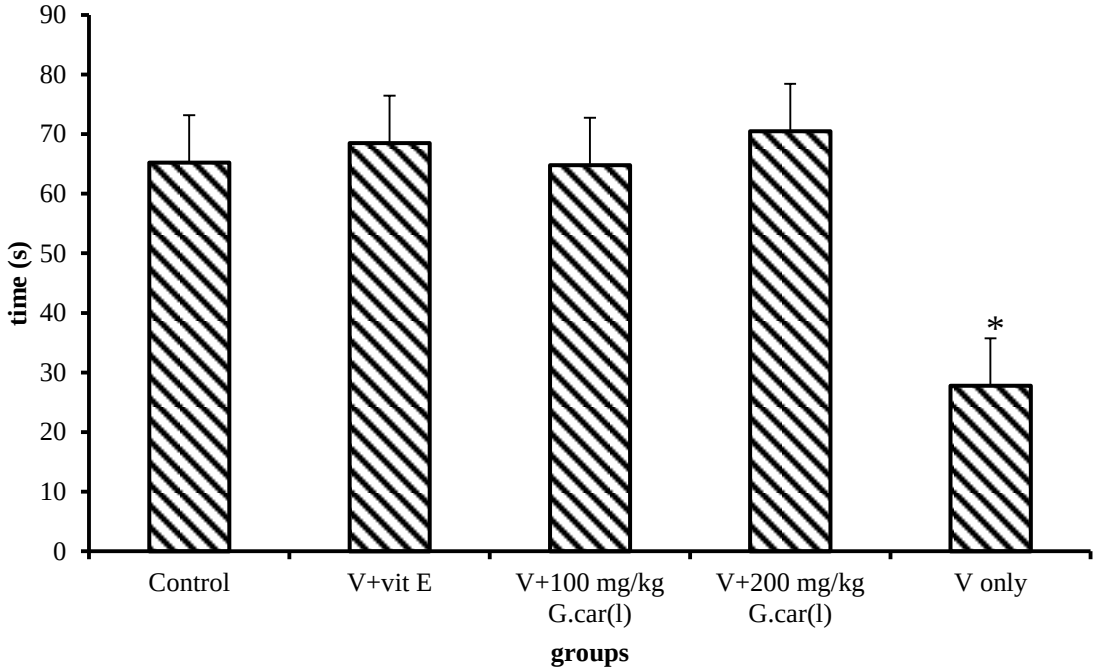
Vit E= α-tocopherol; V= sodium metavanadate; *G. ca r*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.4: The number of leaning out by mice in the light/dark box test following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



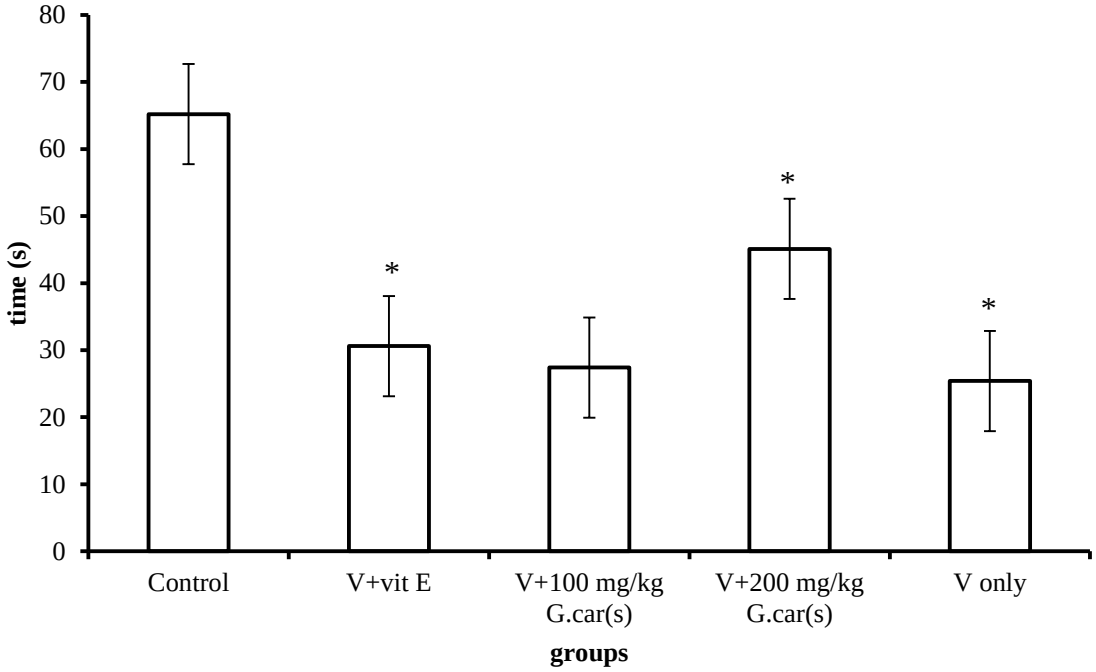
Vit E= α-tocopherol; V= sodium metavanadate; *G. ca r*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.5: The number of line crossings in the light/dark box test following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



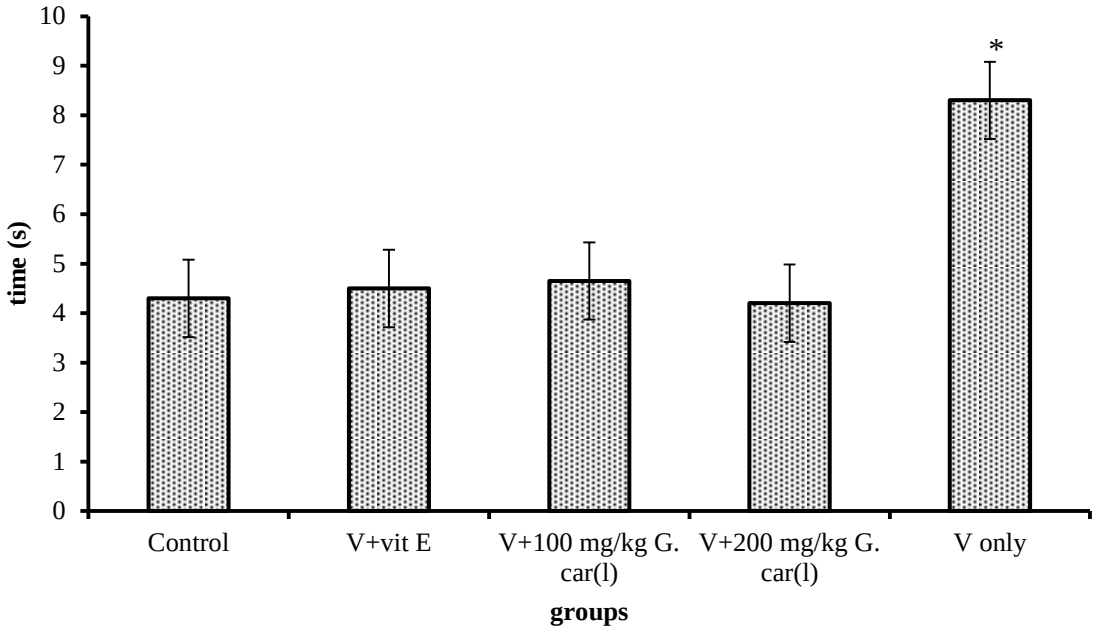
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.6: Time spent on the hanging wire by mice following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



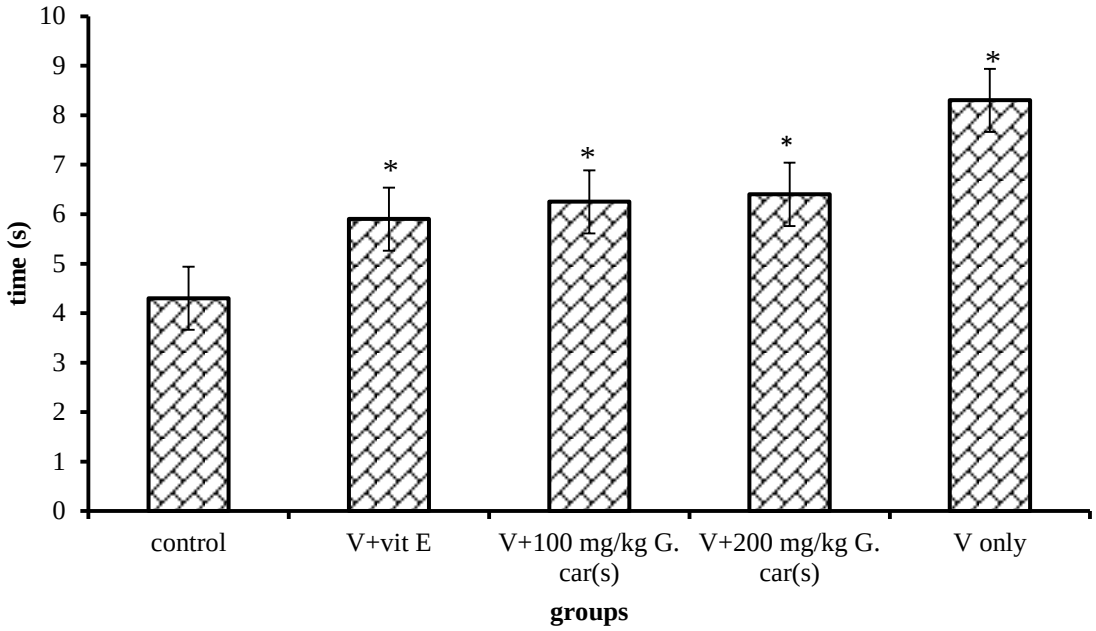
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.7: Time spent on the hanging wire by mice following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium



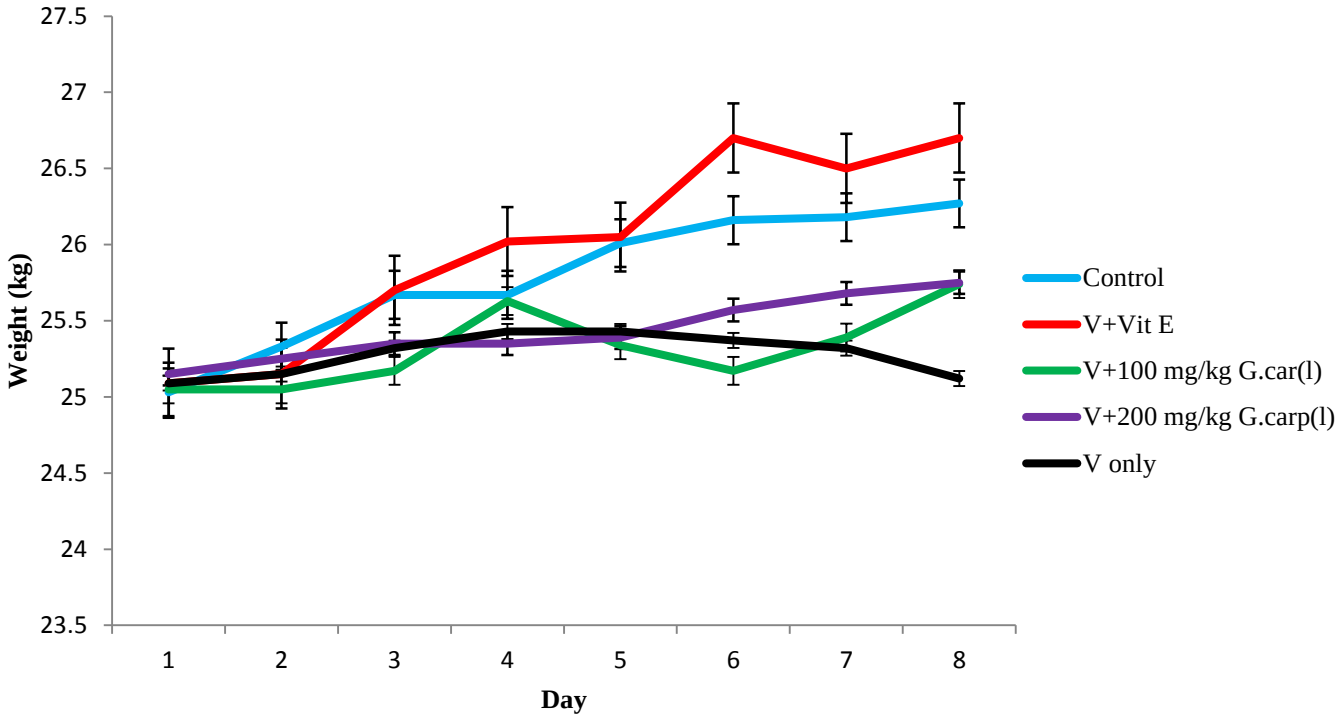
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.8: Mean Values for latency of mice in the negative geotaxis test following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



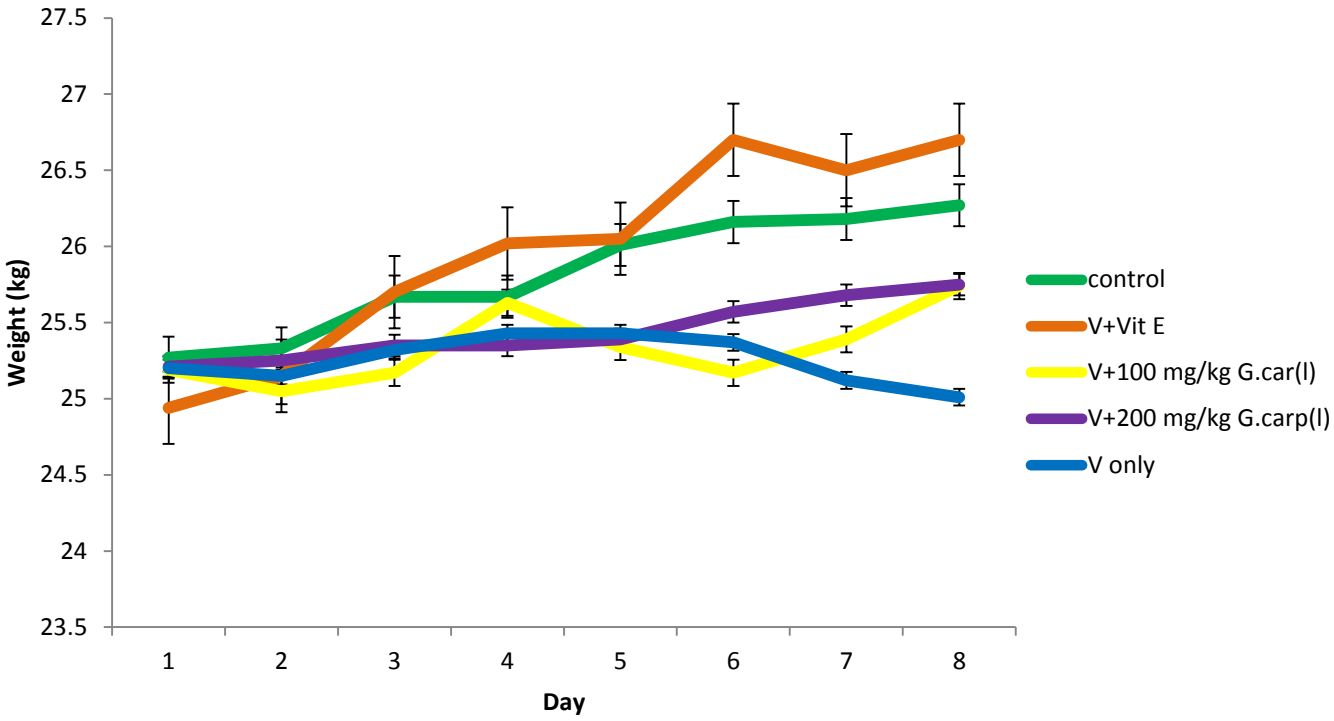
Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem; * significantly ($\alpha \leq 0.05$) different from the control and extract treated group; n=12

Figure 4.8: Mean Values for latency of mice in the negative geotaxis test following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium



Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem; *statistically different from the control at α<0.05

Figure 4.10: Daily fluctuations in body weight following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium



Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem; *statistically different from the control at α<0.05

Figure 4.11: Daily fluctuations in body weight following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium

Table 4.6: Mean haematological parameters of experimental mice following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium

Parameter	GROUP A (Control)	GROUP B (V + vit E)	GROUP C (V + *G carpinifolia*(l) at 100 mg/kg)	GROUP D (V + *G carpinifolia*(l) at 200 mg/kg	GROUP E (V only)
PCV (%)	44.50±6.36	41.50±9.19	33.75±3.20*	40.50±3.54	32.40±4.10*
Hb (g/dL)	15.05±1.63	13.80±2.97	11.13±1.08*	13.38±1.27	11.21±3.61*
RBC ($X10^{12}$/L)	11.50±1.95	9.85±1.17	8.07±2.32	8.96±1.36	11.16±3.21
MCV (fl)	39.00±1.41	42.00±4.24	43.75±9.00	39.50±2.12	35.00±11.42
MCH (pg)	13.50±0.50	14.00±1.41	14.25±2.87	13.50±0.71	12.20±4.15
MCHC (g/dL)	33.00±0.00	33.00±0.00	33.00±0.00	33.00±0.00	33.00±0.00
Platelets ($X10^{9}$/L)	13.00±1.41	12.00±2.83	10.00±0.00	10.00±0.00	10.80±1.10*
WBC ($X10^{9}$/L)	28.10±8.06	18.40±3.39	21.20±4.36	18.40±11.31	12.80±4.40*
Lymphocytes (%)	67.00±4.24 (18.83)	67.00±7.07 (12.33)	74.75±3.69* (15.85)	70.50±10.61 (12.97)	70.60±7.50 (9.04)
Neutrophils (%)	32.50±4.95 (9.13)	32.00±7.07 (5.88)	28.00±3.23 (5.09)	30.00±11.31 (5.15)	28.20±7.22* (3.61)
Monocytes (%)	1.00±0.02 (0.281)	1.00±0.01 (0.18)	1.25±0.50 (0.27)	1.50±0.71 (0.30)	1.20±0.45 (0.15)
Eosinophils (%)	0.00	0.00	0.00	0.00	0.00

Values are expressed as mean ± SEM (n= 5 mice/ group); *statistically different from the control at $\alpha \leq 0.05$; PCV: Packed cell volume; Hb: haemoglobin; WBC: white blood cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concenrtration, absolute values of differential wbc are indicated in parenthesis; Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf

Table 4.7: Mean haematological parameters of experimental mice following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium

Parameter	GROUP A (Control)	GROUP B (V + vit E)	GROUP C (V + *G carpinifolia*(l) at 100mg/kg)	GROUP D (V + *G carpinifolia*(l) at 200mg/kg	GROUP E (V only)
PCV (%)	42.15±5.23	40.51±7.32	39.51±3.16*	40.02±4.14	30.91±5.11*
Hb (gm/dL)	14.56±1.56	13.99±1.47	13.50±1.18*	12.93±2.27	12.38±1.61*
RBC ($X10^{12}$/L)	11.50±1.95	9.85±1.17	8.07±2.32	8.96±1.36	11.16±3.21
MCV (fl)	39.00±1.41	42.00±4.24	43.75±9.00	39.50±2.12	35.00±11.42
MCH (pg)	13.50±0.71	13.45±1.34	13.52±2.32	13.50±0.71	13.20±1.05
MCHC (g/dL)	33.00±0.00	33.00±0.00	33.00±0.00	33.00±0.00	33.00±0.00
Platelets ($X10^{9}$/L)	12.05±3.41	12.34±1.93	11.54±0.00	11.01±0.00	11.76±2.51
WBC ($X10^{9}$/L)	28.10±8.06	18.40±3.39	21.20±4.36	18.40±11.31	12.80±4.40*
Lymphocytes (%)	67.00±4.24 (18.83)	67.00±7.07 (12.33)	74.75±3.69* (15.85)	70.50±10.61 (12.97)	70.60±7.50 (9.04)
Neutrophils (%)	32.50±4.95 (9.13)	32.00±7.07 (5.88)	28.00±3.23 (5.09)	30.00±11.31 (5.15)	28.20±7.22* (3.61)
Monocytes (%)	1.00±0.02 (0.281)	1.00±0.01 (0.18)	1.25±0.50 (0.27)	1.50±0.71 (0.30)	1.20±0.45 (0.15)
Eosinophils (%)	0.00	0.00	0.00	0.00	0.00

Values are expressed as mean ± SEM (n= 5 mice/ group); *statistically different from the control at $\alpha \leq 0.05$; PCV: Packed cell volume; Hb: haemoglobin; WBC: white blood cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concenrtration, absolute values of differential wbc are indicated in parenthesis; Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem

Table 4.8: Mean serum chemical parameters of experimental mice following concurrent administration of ethanol extract of *Grewia carpinifolia* leaf and vanadium

Parameter	GROUP A (control)	GROUP B (V + vit E)	GROUP C (V + *G car*(l) 100 mg/kg)	GROUP D (V + *G car*(l) at 200 mg/kg	GROUP E (V only)
Total Protein (g/dL)	4.50 ± 0.42	3.85 ± 0.92	3.18 ± 0.21*	4.35 ± 0.21	3.46 ± 0.43*
Albumin (g/dL)	1.35 ± 0.25	1.25 ± 0.15	1.13 ± 0.75	1.20 ± 0.100	1.36 ± 0.15
Globulin (g/dL)	3.15 ± 0.71	2.60 ± 0.71	2.05 ± 0.58*	2.15 ± 0.71*	2.18 ± 0.16
Creatinine (mg/dL)	2.25 ± 0.21	1.13 ± 0.14	1.50 ± 0.58	1.25 ± 0.21	1.65 ± 0.63
BUN (mg/dL)	29.97 ± 1.95	26.3 ± 0.91	26.71 ± 2.07	22.7±1.81	29.8 ± 2.64
Sodium (mmol/L)	59.00 ± 7.07	36.00 ± 11.31	31.00 ± 1.155*	34.50 ± 0.71*	34.20 ± 8.79*
Potassium (mmol/L)	47.00 ± 7.07	32.00 ± 11.31	23.25 ± 1.50*	39.00 ± 1.41	26.00 ± 5.10*
ALT (IU/L)	43.00 ± 4.24	32.00 ± 14.14	47.50 ± 1.91	45.00 ± 5.82	75.40 ± 9.07*
AST (IU/L)	49.50 ± 6.36	39.00 ± 18.40	41.50 ± 2.52	455.00 ± 7.07	81.20 ± 10.06*
ALP	34.25 ± 4.90	42.32 ± 7.21	50.07 ± 11.90	38.25 ± 3.14	78.25 ± 6.01*
Cholesterol (mg/dL)	30.00 ± 2.83	26.00 ± 5.66	22.00 ± 1.63*	25.50 ± 0.71	21.40 ± 4.10*
Triglyceride (mg/dL)	40.00 ± 7.07	26.00 ± 2.83	24.50 ± 3.00	25.00 ± 4.24	21.80 ± 8.90

Values are expressed as mean ± SEM (n= 5 mice/ group); *significantly ($\alpha \leq 0.05$) different from the control; BUN- blood urea nitrogen; ALT- alanine aminotransferase; AST- aspartate aminotransferase, Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(l) = *Grewia carpinifolia* leaf.

Table 4.9: Mean serum chemical parameters of experimental mice following concurrent administration of ethanol extract of *Grewia carpinifolia* stem and vanadium

Parameter	**GROUP A (control)**	**GROUP B (V + vit E)**	**GROUP C (V + *G car*(s) 100 mg/kg)**	**GROUP D (V + *G car*(s) at 200 mg/kg**	**GROUP E (V only)**
Total Protein (g/dL)	4.50 ± 0.42	3.85 ± 0.92	3.18 ± 0.21**	4.35 ± 0.21	3.46 ± 0.43**
Albumin (g/dL)	1.35 ± 0.25	1.25 ± 0.15	1.13 ± 0.75	1.20 ± 0.10	1.36 ± 0.15
Globulin (g/dL)	3.15 ± 0.71	2.60 ± 0.71	2.05 ± 0.58**	2.15 ± 0.71**	2.18 ± 0.16
Creatinine (mg/dL)	2.25 ± 0.21	1.13 ± 0.14	1.50 ± 0.58	1.25 ± 0.21	1.65 ± 0.63
BUN (mg/dL)	29.97 ± 1.95	26.3 ± 0.91	26.7 ± 2.07	22.7 ± 1.81	29.8 ± 2.64
Sodium (mmol/L)	59.00 ± 7.07	36.00 ± 11.31	31.00 ±1.155**	34.50 ± 0.71**	34.20 ± 8.79**
Potassium (mmol/L)	47.00 ± 7.07	32.00 ± 11.31	23.25 ± 1.50**	39.00 ± 1.41	26.00 ± 5.10**
ALT (IU/L)	43.00 ± 4.24	32.00 ± 14.14	47.50 ± 1.91	45.00 ± 5.82	75.40 ± 9.07**
AST (IU/L)	49.50 ± 6.36	39.00 ± 18.40	41.50 ± 2.52	455.00 ±7.07	81.20 ± 10.06**
ALP	30.00 ± 2.83	26.00 ± 5.66	22.00 ± 1.63**	25.50 ± 0.71	21.40 ± 4.10**
Cholesterol (mg/dL)	40.00 ± 7.07	26.00 ± 2.83	24.50 ± 3.00	25.00 ± 4.24	21.80 ± 8.90

Values are expressed as mean ± SEM (n= 5 mice/ group); **significantly ($\alpha \leq 0.05$) different from the control; BUN- blood urea nitrogen; ALT- alanine aminotransferase; AST- aspartate aminotransferase; Vit E= α-tocopherol; V= sodium metavanadate; *G. car*(s) = *Grewia carpinifolia* stem.

Table 4.10: Mean values of relative brain weight following concurrent administration of *Grewia carpinifolia* leaf and stem after vanadium induced acute toxicity

Groups	Relative Brain Weight (x 10^{-2}) (leaf extract)	Relative Brain Weight (x 10^{-2}) (stem extract)
control	5.34 ± 0.17	5.04 ± 0.73
V + vit E	5.02 ± 0.21	5.22 ± 0.19
V + *G car* at 100mg/kg	5.20 ± 0.22	5.18 ± 0.37
V + *G. car* at 200mg/kg	5.45 ± 0.10	5.51 ± 0.11
V only	5.14 ± 0.23	5.01 ± 0.76

Values are expressed as mean ± SEM (n= 12 mice/ group); *G. car* = *Grewia carpinifolia*; Vit E= α-tocopherol; V= sodium metavanadate;

4.4 DISCUSSION

Vanadium (V) is a pro-oxidant and indirectly induces the production of ROS leading to oxidative damage (Dhar *et al.,* 1973) and the possible role of V in physiological, biochemical disorders and behavioural changes have been studied (Olopade *et al.,* 2011). It crosses the blood brain barrier and possibly alters biochemistry of brain of treated animals. The CNS myelin could be a preferential target of V- mediated lipid peroxidation in rats and mice since the brain has a high metabolic activity as well as a high concentration of myelin (Sasi *et al.,* 1994). This occurrence may consequently increase the susceptibility of the nervous tissue to peroxidative damage (Garcia *et al,* 2004; Saxena *et al.,* 2013) by V which causes behavioural alterations. Therefore, this study 2 attempted to investigate the effect of ethanol extract of *Grewia carpinifolia* leaf and stem against V toxicity.

Behavioural studies can be used in risk assessment following neurotoxicity as it represent the net output of the sensory, motor and cognitive functions occurring in the nervous system (Evangelista and Duffard, 1996).

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors which are novel environment and light. Thus, the test may be useful in predicting anxiolytic/anxiogenic-like activity in mice. Transitions have been reported to be an index of activity-exploration because of habituation over time, and the time spent in each compartment to be a reflection of aversion (Bourin and Hascoet, 2003). In the present study the increased time spent in the dark zone as well as reduction in locomotion and number of leaning out in the light/dark box test suggested the anxiety-inducing property of vanadium. On the contrary, the simultaneous administration of extract of *Grewia carpinifolia* significantly increased locomotion and time spent in the light zone which might be indicative of its anxiolytic properties (Imaizumi *et al.,* 1994a, b).

The open-field test is one of the most commonly used tests in animal behavioural studies (Seibenhener and Wooten, 2015) to evaluate the emotional state of an animal. The Open-field model examines anxiety related behaviour characterised by the normal aversion of the animal to an open area. Thus, animals removed from their acclimatised cage and placed in environment express anxiety and fear, by showing alteration in all or some parameters (Harquin *et al.,* 2012). Grooming behaviour is a displacement response and is expected to be

displayed in a novel environment (Espejo, 1997). Therefore it is expected to decrease with repeated exposure to the testing apparatus.

In the present study, number of rearing and grooming was significantly increased after vanadium exposure in the Open-field test which may indicate an increase in anxiety in the vanadium-only-treated group, when compared to the control animals as well as the extract-treated groups. Locomotion was also reduced in the vanadium treated group as indicated by an increase in time spent at the centre of the Open-field maze as well as a reduction in the number of new square crossing. Mice administered with leaf and stem extract at 200 mg/kg each conversely had values comparable with the control and standard groups, and this finding may be linked to the ability of the extract at this dosage to reduce or eliminate anxiety-like behaviours in vanadium-exposed mice.

Stretched attend Posture is an essential component of risk-assessment defensive behaviour in rodents (Grewal *et al.,* 1997), which indicate that the animal is hesitant to move from its present location to a new position (Blanchard *et al.,* 2001) and thus a high frequency of these postures indicates a higher level of anxiety. The mice exposed to vanadium in this study, unlike the control and extract-treated groups, recorded a significant increase in stretched-attend posture. This increase in stretched-attend posture suggests anxiety of the mice, indicated to result in low motivation to explore novelty and makes an animal acquire the stretch-attend posture. This is in concordance with results obtained by Domingo (1996) and Soazo and Garcia (2007) following vanadium intoxication. Furthermore, this hesitance by the vanadium treated group to move may be attributed to vanadium-induced muscular weakness (Olopade *et al.,* 2011). In general, observations in the Open-field test were similar in the *G. carpinifolia* extract treated and control groups in this study. Although Dhar *et al.* (1973) and Jaspers *et al.* (1986) documented that the genus *Grewia* possess some anxiolytic activity, the mechanism by which the extract of *G. carpinifolia* reduced anxiety is still not certain.

In this study, muscular strength was found to be significantly decreased after vanadium treatment as observed in the hanging wire test. This finding highlights the fact that heavy metals have been implicated in muscle and joint pain (Alloway, 2013), and further supports that vanadium intoxication results in muscular weakness (Olopade *et al.,* 2011). The present study may indicate that high levels of vanadium may interfere with motor functions, leading to decreased motor activities and grip strength in mice. However, the findings of this study also showed that concurrent administration of *G. carpinifolia* extract to vanadium exposed

animals proved beneficial as the animals performed better on the hanging wire test, evidenced by an increase in the suspension time. The hanging wire test may be used to assess global sub-acute muscle function and coordination over time in mice (Klein *et al.,* 2012) and the ability of the mice to produce sustained tension in the limb musculature. Thus, *G. carpinifolia* extract may exert ameliorative effect on muscular weakness, caused by vanadium intoxication by improved muscle function and coordination in mice.

The negative geotaxis is a reflex test which reflects vestibular function, motor development and activity (Thiessen and Lindzey, 1967). Vanadium has been reported to induce vestibular damage (Mustapha *et al.,* 2014), and generally, mice with vestibular dysfunction become disoriented and unable to generally explore a novel setting. This result could explain the increased time in the negative geotaxis obtained in mice given vanadium only in this study. Nevertheless, the similarities in the lower latent time of the extract treated group at 200 mg/kg and those of the control and standard group further supports the neuroprotective activity of the plant extract at this dosage. The result of the negative geotaxis further substantiates the findings in the Open-field test.

In this study, the daily body weights of mice exposed to vanadium decreased continuously after the sixth day following vanadium administration. The observed insignificant decrease (when compared with other test and control groups) in daily body weight observed in the present study is in consonance with findings by Garcia *et al*, (2004; 2005) in acute vanadium toxicity in rats. Vanadium toxicity has been previously reported to induce anorexia (Zaporowska and Wasilewski, 1989; Merriti and Brown, 1995) leading to reduction in daily body weights which becomes significant following its chronic exposure to rats (Sánchez *et al.,*.1991; Todorich *et al.,*2011). The insignificant difference in relative brain weight in this study is similar to that observed by Gracia *et al.* (2005) and Olopade *et al.* (2011) but contrary to that of Altamirano *et al.* (1993), who described a significant decrease in brain weight following vanadium toxicity. The variance in the results may be ascribed to the fact that six-week old mice were used in this study, while in the studies conducted by Altamirano *et al.* (1993), mice were exposed at post- natal day one. It seems that vanadium has more effect on brain weight, when exposed to neonates undergoing a high degree of cellular proliferation.

It was also observed in the present study that vanadium administration resulted in diarrhoea. Although the plant extract at 200 mg/kg was found to reduce vanadium-induced diarrhoeal

episodes, the mechanism of its anti-diarrhoeal activity is still uncertain. This result is in agreement with studies by Fatum *et al,* (2002) and NIEHS (2008) who reported that diarrhoea in rats following acute sodium metavanadate administration.The literature search however, revealed a paucity of information on the mechanism of diarrhoea induction by vanadium. In the present study, normochromic normocytic anaemia was recorded, with a decrease in Hb and PCV in the group administered with sodium metavanadate only. The anaemia could be a consequence of haemolysis, as other researchers have proposed (Zaporowska and Slotwinska 1996; Yang *et al.,* 2003) or might be a kind of suicidal erythrocyte death identified as eryptosis, specifically described for vanadium toxicity (Föller *et al*., 2008) as a result of oxidative stress (Bracci *et al.,* 2002; Barvitenko *et al.,* 2005). An impairment of erythropoiesis could also be assumed. The results are in consonance with earlier observations of anaemia, changes in Hb and reduction in haematocrit concentrations as previously reported (Markku *et al*., 1981; Zaporowska and Wasilewski, 1992), but in contrast with other reports of Dai and McNeill (1994) and Fawcet *et al,* (1997) who observed no changes in haematological indices following acute vanadium intoxication. The variance in the reports may be due to difference in duration and routes of the vanadium exposure as well as difference in vanadium compounds used. In the present study, the plant extract at 200 mg/kg given concomitantly with sodium matavanadate was able to restore PCV and Hb concentrations to levels comparable with the control and standard groups. Sodium metavanadate vanadium also significantly decreased total WBC and neutrophil counts. Earlier reports have indicated that vanadium induces myelosuppression and this property has been employed in cancer therapy (Crawford *et al.,* 2004). Seven-day simultaneous treatment with *G. carpinifolia* significantly reversed vanadium-induced neutropenia and changes in total WBC count. Though the exact mechanism of action of *G. carpinifolia* is unknown, this finding indicates that the extract is a potential agent to be evaluated clinically, and may be useful in improving antitumour treatment-induced myelosuppression (Manju *et al.,* 2011). The presently used agents like granulocyte-macrophage colony stimulating factor (GM-CSF) or sargramostim® and granulocyte colony stimulating factor (G-CSF) or filgrastim® though effective, have a major limitation due to their exorbitant cost. *G. carpinifolia* being an indigenous plant could be a more economical alternative immunostimulant to reduce side-effects of anticancer drugs.

The result of the present study is similar to that of Aglal (2012), who reported that *G. tenax* also decreased white blood cells in mice. Sodium metavanadate resulted in a decrease in

cholesterol levels. The finding in this study is in line with that of Cooper (2007), who showed that vanadium pentoxide inhibits cholesterol biosynthesis and lowers plasma cholesterol levels, being a potent inhibitor of many enzymes. A number of soluble enzymes of blood serum have been considered as indicators of the hepatic dysfunction and damage.

The increase in the activities of these enzymes in plasma is indicative for liver damage and thus causes alteration in liver function (Adedapo *et al*, 2004). The present study, the increased activities of ALT and AST in serum obviously indicate that liver is susceptible to vanadium induced toxicity. These increases could be attributed to the hepatic damage resulting in increased release of functional enzymes from biomembranes or their increased synthesis (Chang, 2009). This elevation of serum liver enzymes is similar to that reported by Sidhu *et al*., (2004), Adedara *et al.,* (2011) Elshaari *et al.*, (2011) and Hoda *et al.,* (2012). The ability of the extract to lower the levels of these enzymes to values comparable with the control group and that of α-tocopherol (the standard antioxidant), may suggest that the plant extracts did not have adverse effect on the liver. This may also be due to the fact that the extracts offer protection and maintain functional integrity of hepatic cells. The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by vanadium (Murugaian *et al*, 2008). The extract could be said to protect against vanadium induced hepatotoxicity in this regard. The liver is also an important site for the synthesis of many serum proteins (Ahsan *et al*, 2009).

The reduction in serum total protein observed in the sodium metavanadate group may also be associated with decrease in the number of hepatocytes which consequently results in decreased hepatic capacity to synthesize protein. Simultaneous treatment by α- tocopherol and ethanol extracts of *Grewia carpinifolia* leaf and stem significantly increased serum total protein indicating the hepatoprotective activity of the extracts most probably through hepatic cell regeneration (Olorunnisola *et a.l,* 2011).

The significant decrease in potassium levels observed in groups C and E may be due to diarrhoea (Zychlinski and Byczkowski, 1990) as reported by Huang and Kuo, 2007 that hypokaelemia may result from excess renal or gastro-intestinal loss.

It is evident from our study that sodium metavanadate result in negative alteration in hematological and serum biochemical parameters of experimental mice. This study has also

demonstrated that administration of α-tocopherol (vitamin E) and *Grewia carpinifolia* concomitantly with vanadium ameliorates this vanadium induced damage, however the *G. carpinifolia* extract could be more effective at 200mg/kg. The results of this study are in congruence with previous report of Haider and El-Fakhri (1991) and Fatum *et al.* (2002) who showed that α-tocopherol (vitamin E) protects the rats against vanadium-initiated damage.

4.5. CONCLUSION

This study has shown that *G. carpinifolia* extract administered in combination with vanadium ameliorates vanadium induced acute toxicity and behavioural impairment.

From the result in study 1, *Grewia carpinifolia* extract contains numerous phytochemical constituents, including tannins, phlobatinins, saponin, flavonoids, terpenoids, cardiac glycosides, coumarin, alkaloids and anthraquinone. Many of these compounds have proven their potential as antioxidants in various oxidative stress models as scavengers of free radicals as reported in prior studies (Dhar *et al.*, 1973; Ajayi *et al.*, 2011; Bhumi and Savithramma, 2014).

The observed beneficial effects of *G. carpinifolia* extract in vanadium-induced acute toxicty may thus be attributed to these diversified chemical components. Therefore, the plant should be given more emphasis as a candidate in developing a modern drug to minimise vanadium induced toxicity. Although *G. carpinifolia* reduced the toxicity of vanadium, much remains to be studied regarding its mechanism of actions. The leaf extract at 200 mg/kg showed the best protective activity from the present study. Further study is aimed at isolating and characterising the bioactive principle(s) of the extract with the aim of determining its exact mechanism of action.

CHAPTER FIVE

5.0. STUDY THREE

Antioxidant activity and isolation of pure compounds from the ethanol extract of *Grewia carpinifolia* leaf and stem.

5.1. INTRODUCTION

Reactive oxygen species (ROS) include free radicals are reactive molecules involved in many physiological processes and have been implicated in acute vanadium toxicity and several other diseases such as cancer, ageing, arthritis and liver injury.

Antioxidants are substances that when present at low concentrations, compared with those of the oxidizable substrate significantly delay or inhibit oxidation of that substrate (Halliwell and Gutteridge, 1999). Endogenous antioxidants are made within the system of a living organisms while exogenousones are derived from sources outside the living systems such as from diet (Jaouad and Torsten, 2010). Endogenous antioxidants repair ROS damage internally by initiating cell regeneration; exogenous antioxidants repair some free radical damage-externally by stimulating cell regeneration (Wolfe *et al.,* 2003). The growing need to complement the activities of endogenous antioxidants has led to the increased supplementation by exogenous sources. Currently, there is a heightened interest and extensive studies on the exogenous antioxidants from natural sources because they are cheaper, readily accessible and are believed to have lesser side-effects when compared with their synthetic counterparts (Tadhani *et al.,* 2007).

Grewia carpinifolia belongs to family Tiliaceae. From Study 2 (chapter 4) *Grewia carpinifolia* given simultaneously with sodium metavanadate corrected behavioural impairment, changes in haematology and serum chemistry to values comparable with the control and α-tocopherol groups. However, its mechanism of action still remains unknown. The present study was therefore designed to evaluate the ROS scavenging activity of ethanol extract of the leaf and stem of *Grewia carpinifolia* using various *in vitro* model systems. Ascorbic acid was used as the reference in this current study. 1 ,1-diphenyl-2-picryl hydroxyl (DPPH) quenching assay, 2, 2'-azinobis-3-ethylbenzothiozoline-6-sulphonic acid (ABTS) cation decolorization test, ferric reducing antioxidant power (FRAP) Assay systems were selected for the present experiment. The present study was also aimed at isolating pure compounds from the ethanol extract of *Grewia carpinifolia* leaf and stem.

5.2. MATERIALS AND METHODS

5.2.1 Plant material

The leaf of *G. carpinifolia* were collected in September, 2013 (during the rainy season) from the Botanical Garden of the University of Ibadan, Ibadan, Nigeria and identified at the Forestry Research Institute of Nigeria where a herbarium specimen was deposited (voucher number FHI 109693). Plant materials were washed with distilled water and dried at room temperature 25 °C). The dried leaf was ground to a fine powder.

5.2.2 Preparation of extract

The leaf and stem of *G. carpinifolia* were air dried at room temperature to constant weights. The dried plant material was ground separately to powder. Exactly 1 kg of each ground plant material was soaked separately in ethanol for 48 h on an orbital shaker. Extracts were filtered using a Buckner funnel and Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40 ºC using a rotary evaporator.

5.2.3 Chemicals

2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid, potassium ferricyanide; catechin, ascorbic acid, catechin, tannic acid, quercetin, Folin-Ciocalteus's phenol reagent, sodium carbonate and $FeCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the chemicals used including the solvents, were of analytical grade.

This present study was carried out at the State Key laboratory for Phytochemistry and Plant resources, Kunming Institute of Botany, Kunming, Chinese Academy of Sciences, China.

5.2.4. *IN VITRO* ANTIOXIDANT ASSAYS

5.2.4.1 2, 2-azinobis-3-ethyl- benzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

The original ABTS assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the ABTS radical cation (ABTS•+), in the presence or absence of antioxidants. This has been criticised on the basis that the faster

reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. A more appropriate format for the assay is a decoluorisation technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of ABTS•+ used in this study involved the direct production of the blue/green ABTS•+ chromophore through the reaction between ABTS and potassium persulphate as described by Re *et al* (1999). Addition of antioxidants to the pre-formed radical cation reduces ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus the extent of decolorization as percentage inhibition of the ABTS•+ radical cation was determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions. This method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, food and plant extracts.

The (ABTS•+) was produced by the reaction between 5 ml of ABTS stock solution and 5 ml of 2.45 mM potassium persulphate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Although, the oxidation of the ABTS commenced immediately, the absorbance was not maximal and stable until more than 6 h had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature.

Before use, the potassium persulphate ($K_2S_2O_8$) solution was diluted with water to get an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated at 30°C. The plant extract at various concentrations were diluted with dimethyl sulphoxide (DMSO) to obtain the sample solution. Exactly 5 µL of sample solution was homogenized with 195 µL ABTS•+ solution, the mixture was incubated at room temperature for 6 minutes and its absorbance was recorded at 734 nm using micro-plate spectrophotometer (Spectra Max 190 micro-plate spectrophotometer, Molecular Devices®, California, USA). Blanks were run in each assay. The standard curve for this assay is presented in Table 5.1. As for the antiradical activity, ABTS scavenging ability was expressed as IC_{50} (µg/ml). The inhibition percentage of ABTS radical was calculated using the following formula:

ABTS scavenging activity (%) = $(A_0 – A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control, and

A_1 is the absorbance of the sample.

5.2.4.2. 1, 1-diphenyl-2-picryl hydroxyl (DPPH) Radical Scavenging Activity

This test was conducted as described by Blois (1958). The DPPH was dissolved in methanol to a 0.025 g/L. The plant extract at various concentrations was diluted with DMSO to obtain sample solution. Exactly 5 µL of the sample solution in a 96-well micro-titre plate following which 195 µL DPPH working solution was added to each well. After a 20 min of reaction at room temperature, the absorbance of the solution was measured at 515 nm using using micro-plate spectrophotometer (Spectra Max 190 micro-plate spectrophotometer, Molecular Devices®, California, USA). The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

5.2.4.3 Ferric Reducing Antioxidant Power Assay

The ability to reduce ferric ions was measured using the method described by Benzie and Strain (1996). The Ferric Reducing Antioxidant Power (FRAP) reagent was produced just before use by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ (tripyridyl triazine) solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Samples at different concentrations (100, 200, 300, 400 and 500 µg/ml) was then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity, based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. The optical density was read at 593 nm using micro-plate spectrophotometer (Spectra Max 190 micro-plate spectrophotometer, Molecular Devices®, California, USA).

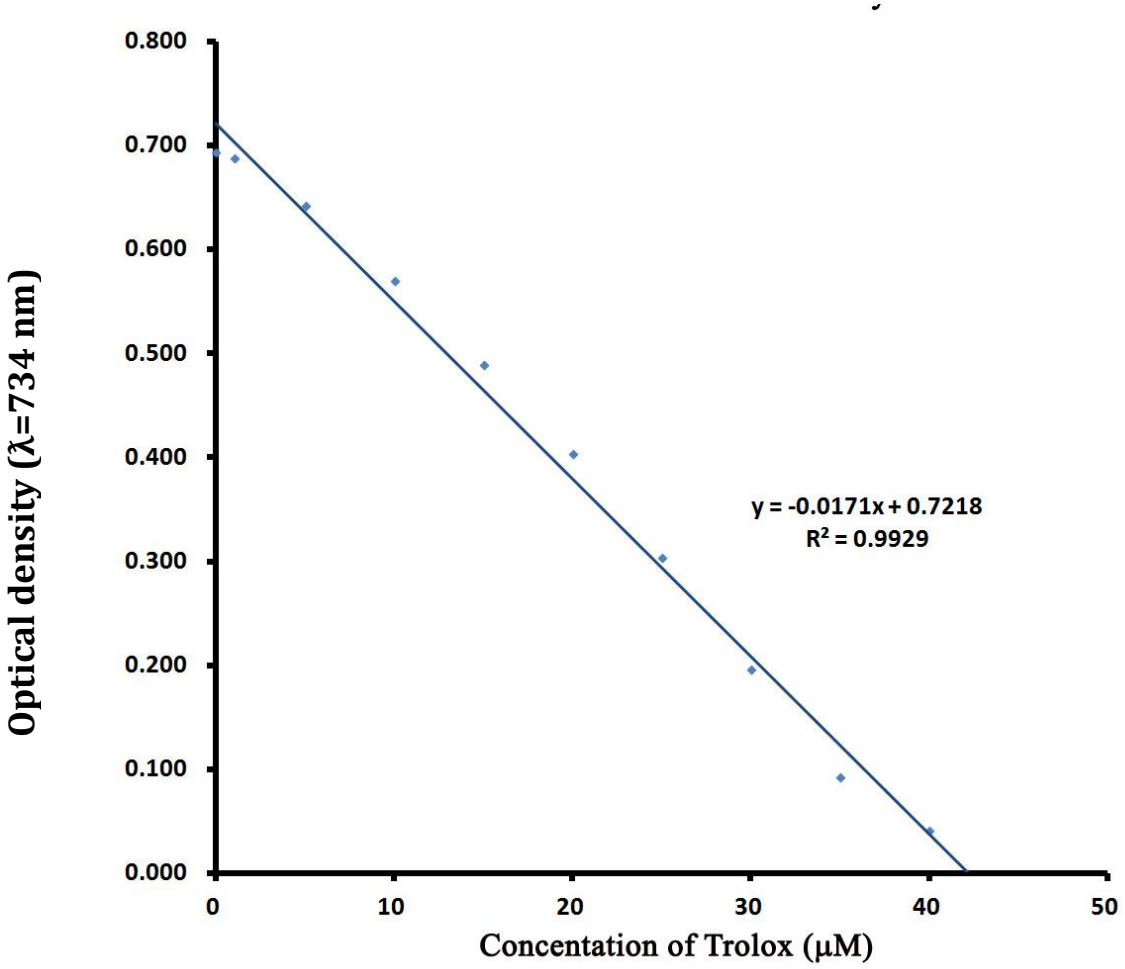


Figure 5.1: Standard Curve for 2, 2-azinobis-3-ethyl- benzothiazoline-6-sulphonic acid) Assay

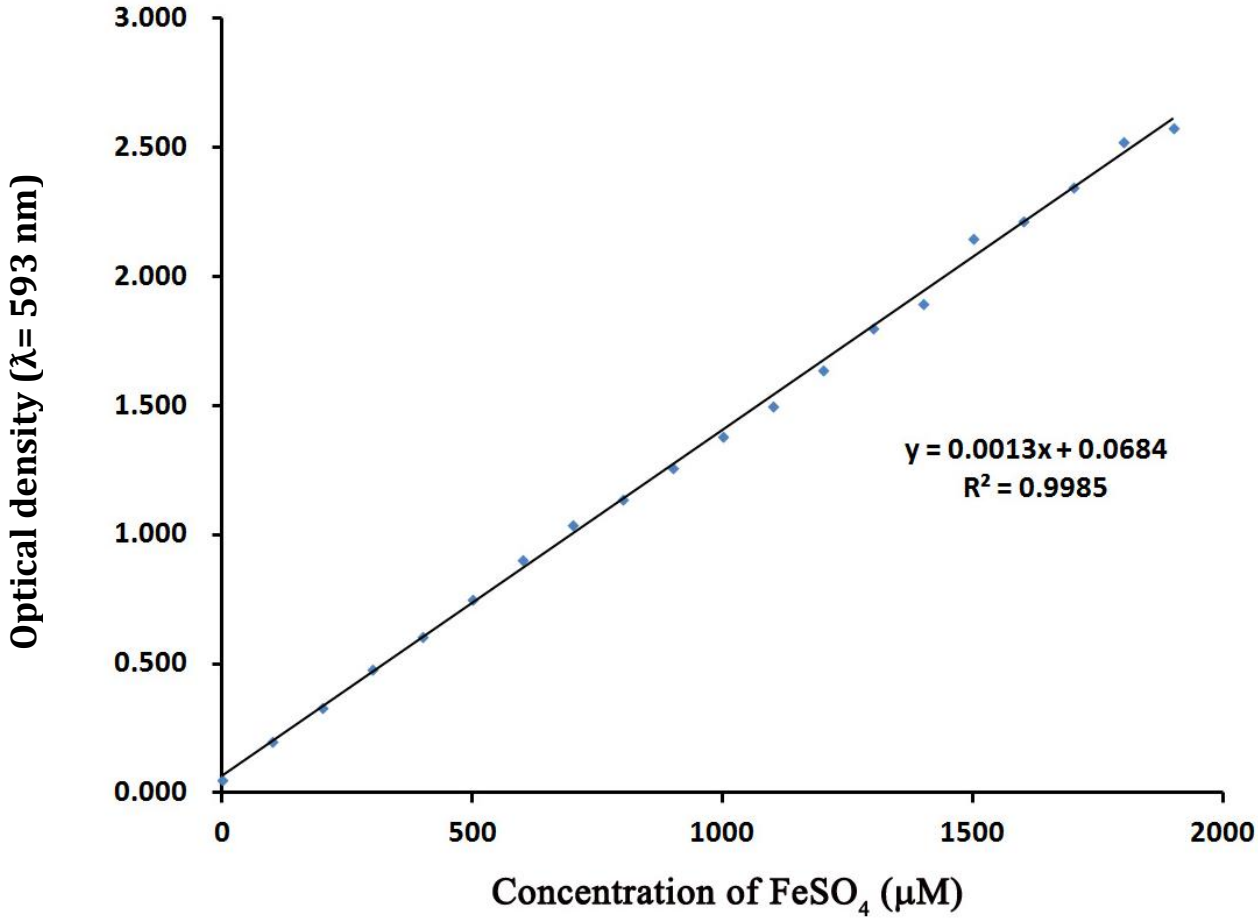


Figure 5.2: Standard Curve for Ferric Reducing Antioxidant Power Assay

5.2.4.4 Thiobarbituric acid-reactive substances assay

The lipid peroxidation ability of the leaf and stem of *Grewia carpinifolia* extract was determined by measuring the formation of thiobarbituric acid-reactive substances (TBARS) according to the method of Varshney and Kale (1990).

Principle

Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of fatty acid membranes and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA) to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm using micro-plate spectrophotometer (Molecular Devices®, California, USA). This reaction is illustrated in Figure 5.3. The pink chromophore is readily extractable into organic solvents such as butanol.

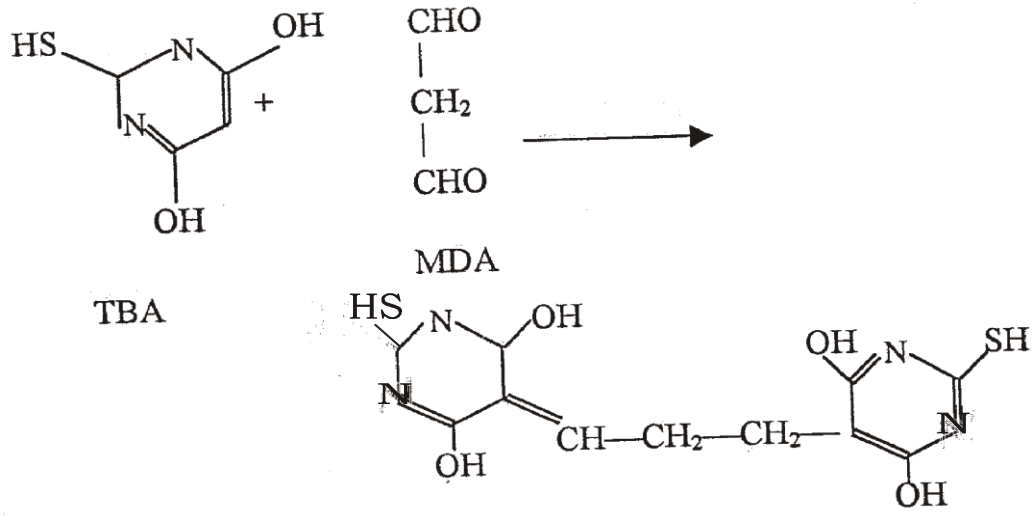


Figure 5.3: Reaction of 2-thiobarbituric acid with malondialdehyde

Reagents

1. 30% Trichloroacetic acid

A total of 6 g of TCA (CCl_3COOH) (BDH Chemicals, England) was dissolved in distilled water and made up to 20 ml.

2. 0.75% Thiobarbituric acid

This was prepared by dissolving 0.15 g of TBA in 0.1 M HCl (May & Baker Ltd.) and made up to 20 ml.

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

Exactly 1.15 g of KCl and 2.36 g of Tris base (Lab Tech Chemicals, Toronto, Canada) were dissolved separately in distilled water and made up to 100ml. The pH was then adjusted to 7.4.

Procedure

A mixture of 1 ml plant extract, 4 ml 99.5% ethanol, 4.1 ml 2.5% linoleic acid in 99.5% ethanol, 8.0 ml 0.02 M phosphate buffer (pH 7.0) and 3.9 ml distilled water was placed in an oven at 40°C in the dark for 1 h. exactly. 2 ml of 20% trichloroacetic acid and 2ml of 0.67% of thiobarbituric acid were added to 1 ml of sample solution. The mixture was placed in boiling water bath for 10 min. It was centrifuged after cooling at 3000 rpm for 20 minutes. The absorbance of the supernatant was measured at 552 nm and recorded again after the maximum was reached. Antioxidant activity was described by percent inhibition as (Varshney and Kale (1990).

% inhibition = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

All analyses were done in triplicate (Kikuzaki and Nakatani, 1993).

5.2.5. ISOLATION OF PURE COMPOUNDS

5.2.5.1 Purification of the extract

The column chromatography was used for the purification of the extract of *G. carpinifolia* in this study. The glass column was packed with silica gel (column chromatography grade) using n-hexane under positive pressure. A piece of cotton wool was introduced into the bottom of a clean dry column, which was clamped vertically using a retort stand. The slurry (silica gel of mesh size 100-200 nm + hexane) was carefully poured into the column to avoid air bubbles and cracking, which could affect the resolution. The column was tapped gently during the introduction of the slurry of silica gel to ensure uniform packing. De-aeration was done using a vacuum pump and the slurry was then allowed to settle. A total of 378 g and 425 g of ethanol extract of *Grewia carpinifolia* leaf and stem were dissolved and pre-adsorbed with silica gel in chloroform. Hexane was used to wash the packed column prior to analysis. This pre-adsorbed sample was allowed to dry before loading unto the column. The ratio of the silica gel (Merck®) to that of the extract was 30:1. The loaded sample was then covered with pure sand and a small piece of cotton wool. The top of the column was usually filled with enough solvent/mixture of solvent to avoid cracking. The elution started with 100% non-

polar hexane solvent in which the polarity of the solvent(s) was increased gradually with time. Exactly 10 ml of each of the fraction was collected.

The total fractions were collected and analysed using thin layer chromatography (TLC) technique to determine the level of purity of each fraction/component.

Table 5.1: Solvent combination used for elution and purification of the extract

n-hexane	Ethyl acetate	Methanol
100	0	0
90	10	0
80	20	0
70	30	0
60	40	0
50	50	0
40	60	0
30	70	0
20	80	0
10	90	0
0	100	0
0	90	10
0	80	20
0	70	30
0	60	40
0	50	50
0	40	60
0	30	70
0	20	80
0	10	90
0	0	100

Figure 5.4: Purification of the ethanol extract of *Grewia carpinifolia* using the column chromatography

5.2.5.2 Separation of column fractions by thin layer chromatography

A line was drawn with pencil parallel to and 2 cm from the bottom of the aluminium-coated thin layer chromatography (TLC) plate. About 0.2 ml of each fraction was spotted on this line, called the origin, at least 1cm from each other. The fraction was spotted on the TLC plates using a capillary tube filled with the fraction to ascertain the level of its purity. The spotted plate was allowed to dry and then developed in different solvent systems depending on the degree of polarity of each constituent. Once the plate had been developed for the predetermined distance, it was removed from the tank and the position of the solvent front was marked. Visualisation of the position was further enhanced by placing in iodine tank for colour development.

To locate the compounds in the sample, the TLC plate was examined under (354 nm) ultraviolet light and any spots visualised with this procedure were ringed using a pencil. With this procedure, fractions with similar retention or retarding factor (Rf) were pooled together. All the TLC analyses were carried out at room temperature in the laboratory. Some of the solvents used for the thin layer chromatography are listed below

Hexane/ethyl acetate (1:1) (3:1) (1:3) (1:2) (2:1) (4:1) (1:4) (3:2)

Hexane/methanol (1:1) (3:1) (1:3) (1:4)

Hexane/ethyl acetate/ methanol (1:1:1) (3:1:1) (4:1:1) (2:1:4)

$$R_f = \frac{\text{distance moved by solute}}{\text{distance moved by solvent from the origin to solvent front}}$$

Fractions having the same R_f were pooled/mixed together and dried in rotary evaporator for further purification using the open column chromatography.

5.2.5.3 Open Column Chromatography

The open column chromatography is a simple and cost effective method used in chemistry to purify individual chemical compounds from a mixture of compounds, using their individual polarity and retention time. The column was packed with silica gel (20-50 nm) as the dry stationary phase and the dried fraction was loaded unto the column. Solvent systems (Table 5.2) with varying polarities were used for elution of the fraction in the column. The eluent were collected into labelled test tubes as shown in Figure 5.4. Each test tube was tested on TLC plate to monitor the elution of each compound. Test tubes with the same compound

were pooled and dried. The pooled sub-fractions were further purified with high performance liquid chromatography (HPLC).

Table 5.2: Solvent combination used for elution and purification of the extract in the open column chromatography

Solvent combination	Ratio used
Petroleum ether/acetone	20:1
Chloroform/methanol	30:1
Methanol/water	1:1

Figure 5.5: A picture showing the collection of sub-fractions of ethanol extract of *Grewia carpinifolia* using an open column chromatography with an automated fraction collector

5.2.5.4 High Performance Liquid Chromatography

The HPLC hardware was Dionex-Thermo Fisher Ultimate 3000 w/auto-sampler (HPLC #2), P-680 pump, TCC-100 Column Compartment, ultraviolet/visible wavelength detector (UV/VIS); Shodex R01 Refractive Index detector. The analytical column was Bio-Rad Aminex HPX-87H (order#125-0140) (ion exclusion); guard column was a Bio-Rad Micro-Guard Cation-H cartridge (order #1250129); the column operated at 50 °C. The column types were specific to the analytes. The 87H column was a hydrogen-form column used for analysis of solution with carboxylic acids, volatile fatty acids, short-chain fatty acids, alcohols, ketones, and many neutral herbal products.

The mobile phase (also known as working buffer, buffer, or solvent) had a flow rate of 2 ml/min with a constant flow rate (isocratic), the running time was determined by the elution time of the analytes being quantified. The mobile phase was tetra-oxosulphate (vi) acid (5 mM) for the 87H column (Fisher #AC124645001). Double deionised water (DD water) was used in preparing the stock solution of the buffer. The buffer was then vacumm filtered through a 0.2 µm x 47 mm nylon filter (Pall Corporation, order #66602) to degas it. The analytes were dissolved in acetone and placed within the rack of the autosampler and chromatograms were generated by the HPLC software. Analytes with single peaks were pure and further subjected to the area under the curve of each peak was used to generate a concentration value. The software was used to establish base-lines for each peak, identify the peak and the drop lines for adjacent peaks. However, the software was only used as a starting point; the chromatogram for each sample was further reviewed manually and corrected to ensure the proper placement of the lines defining the peak's area boundary.

The pure analytes were further elucidated for their chemical structure and for any further information on their chemistry using nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS).

Figure 5.6.: A picture of the High Performance Liquid Chromatography (HPLC) equipment

5.2.6. IDENTIFICATION OF ISOLATED PURE COMPOUNDS

5.2.6.1 Nuclear Magnetic Resonance (NMR) spectroscopy

The structures of the isolated pure compounds were elucidated using NMR spectroscopy which is based on the pulse variant. The groups of pulses may be purely radiofrequency (rf) or include magnetic gradient pulses. The pulse irradiation technique employed was FT NMR at ambient temperature. The acquisition was carried out many times, incrementing the delay (evolution time- t1) between the two pulse groups. The evolution time is labelled t1 and the acquisition time, t2.

Two dimension NMR (2D-NMR) techniques were used as it saves time especially and highlights the connectivity between different types of nuclei (for example, proton and carbon). Proton (^{1}H) and carbon-13 (^{13}C) operating at 200 MHz for proton and 50 MHz for carbon nuclei NMR spectra were recorded on Bruker Avance® software. The positions of proton and carbon resonance in the NMR spectrum were measured relative to the resonance position of tetramethylsilane (TSM) as internal standard. Deuterated solvents were used to measure NMR spectra. The chemical shifts were indicated in parts per million with the solvent shift as reference and coupling constants J in Hertz (Hz). The pure fractions were dissolved in these solvents, after which it was poured inside the NMR tube and the spectra of the compound obtained. The spectra obtained were interpreted to elucidate the structure of the compound(s).

5.2.6.2 Mass Spectrometry (MS)

Mass Spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio (m/z) of charged particles (Sparkman, 2000). It is used for determining masses of particles and the elemental composition of a sample or molecule as well as elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionising compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge rations (Sparkman, 2000; Downard, 2007). The samples were loaded onto the MS instrument and underwent vapourisation. The components of the sample were ionised by impacting them with an electron beam which resulted in the formation of charged particles (ions). The ions were then separated according to their mass-to-charge ratio in an

analyser by electromagnetic fields. The ions were detected quantitatively; the ions signals generated were then processed into mass spectra.

The MS instrument consists of three modules which are an ion source, and can convert gas phase sample molecules into ions (or, in the case of electrospray ionisation, move ions that exist in solution into the gas phase); a mass analyser, which sorts the ions by their masses by the application of electromagnetic fields and a detector, which measures the value of an indicator quantity, and thus provides data for calculating the abundance of each ion present. The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule and determining the structure of a compound by observing its fragmentation. Other uses include the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry.

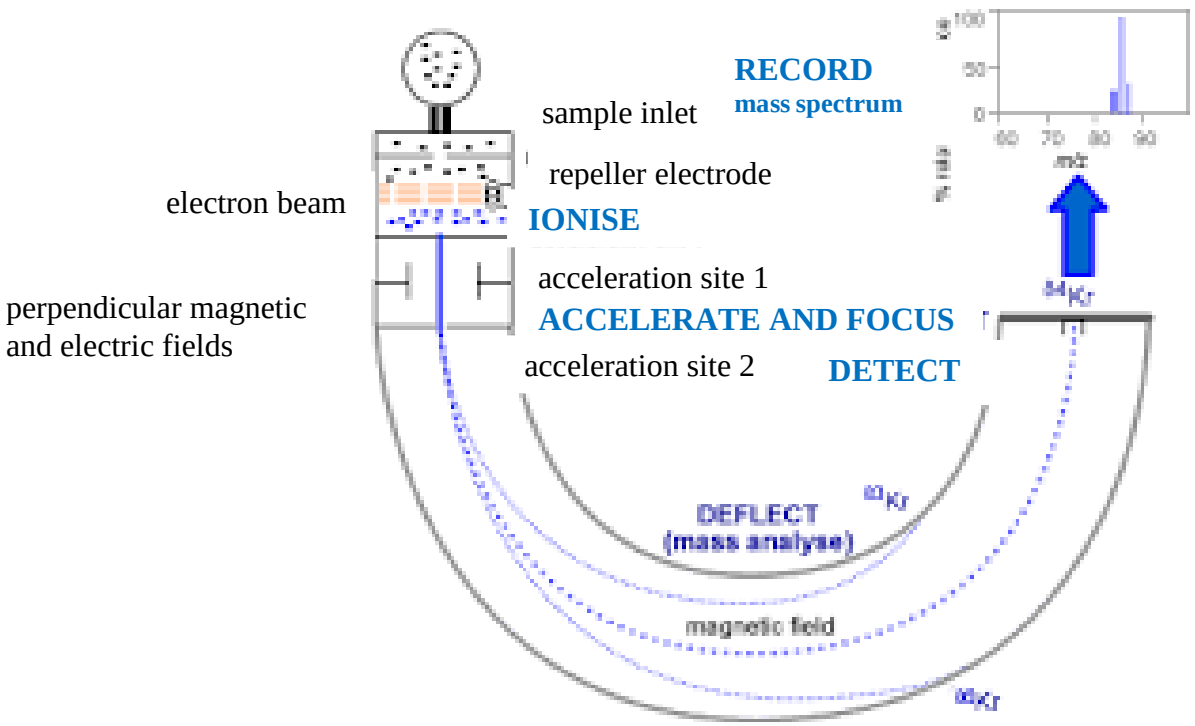


Figure 5.7: A schematic diagram of the Mass Spectrometer (MS) (www.bestchoice.net.nz)

5.2.7 STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard error of mean (SEM) of three replicate. Where applicable, the data were subjected to one-way analysis of variance (ANOVA) and the differences between samples were determined by Duncan's Multiple Range test using Graph pad Prism version 5.0 for Windows, Graph Pad Software. P Values ≤ 0.05 were regarded as significant.

5.3. RESULTS

5.3.1 *In vitro* antioxidant activity of crude extract

The quality of antioxidants in the ethanol extracts of the leaf and stem of *Grewia carpinifolia* was determined by the absorbance and scavenging percentage.
In Figure 5.8., *G. carpinifolia* extract was found to be effective in scavenging the ABTS radical. The percentage inhibition of this radical was concentration-dependent. At 0.5 mg/ml, the inhibition of the leaf and stem extract were 58.15% and 70.25%, and that of ascorbic acid was 80.02%. The IC_{50} of ascorbic acid was 0.31 mg/ml while that of the extract of the leaf and stem were 0.32 and 1.98 mg/ml respectively.

The DDPH method revealed that the scavenging of the free radicals was 28.56, 43.60, 56.85, 58.90 and 72.60% at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml respectively for the ethanol extract of *G. carpinifolia* leaf. The percentage inhibition of the DPPH radical by the extract of *G. carpinifolia* stem was 11.85, 19.11, 22.81, 35.07 and 62.12% at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml, respectively (Figure 5.9.).

Antioxidant activities of the ethanol extracts of the plants as determined by the FRAP assay are depicted in Figure 5.10. In the FRAP assay method, the absorbance of *G. carpinifolia* was found to be maximum 0.105, 0.154, 0.227, 0.678, 2.765 and that of the stem was 0.100, 0.143, 0.215, 0.350, 2.586 at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml, respectively. The standard; ascorbic acid had an absorbance of 4.00 at the maximum dosage of 1 mg/ml (Figure 3)

The percentage inhibition using the TBARS assay ranged from 23.05 to 84.56% for the leaf extract, while that of the stem was between 20.4 and 75.15% at the minimum and maximum concentration respectively. The values were higher than those of ascorbic acid at each of the tested dose.

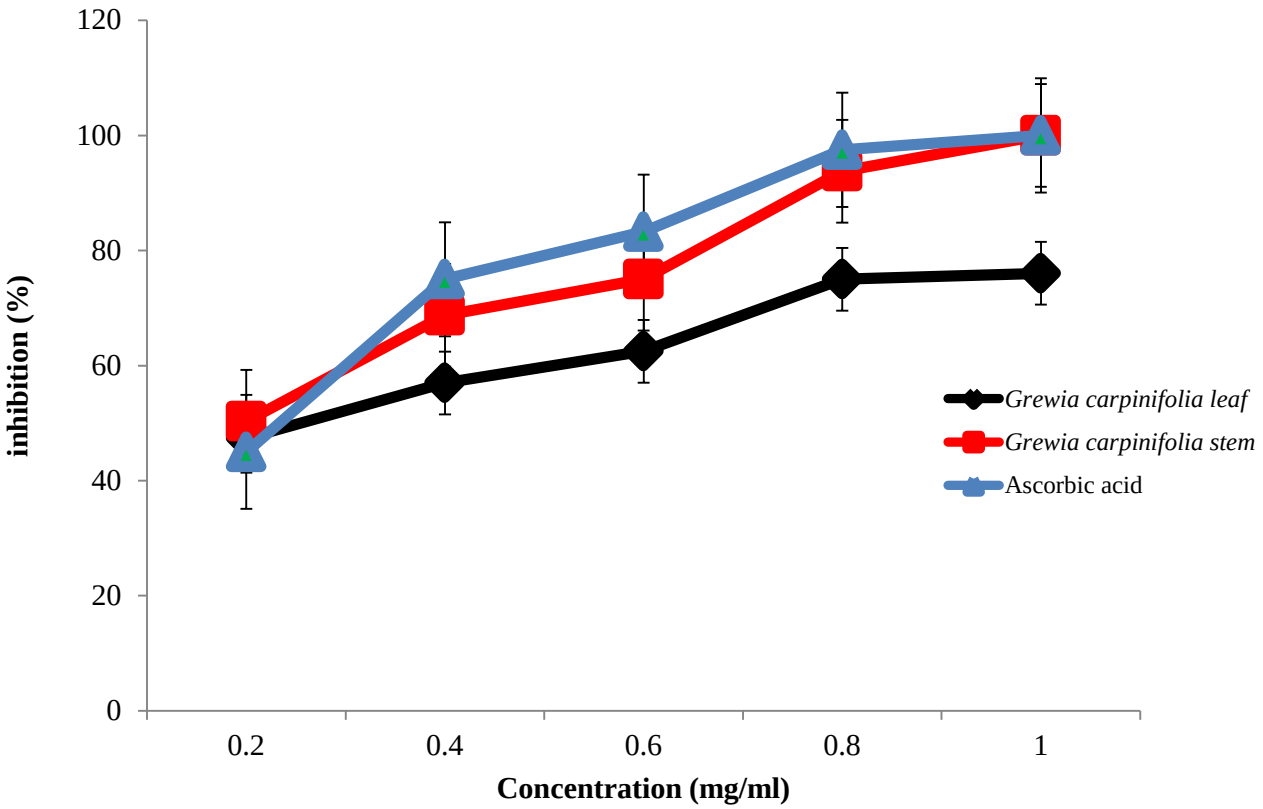


Figure 5.8: ABTS radical-scavenging activity of *Grewia carpinifolia* leaf and stem

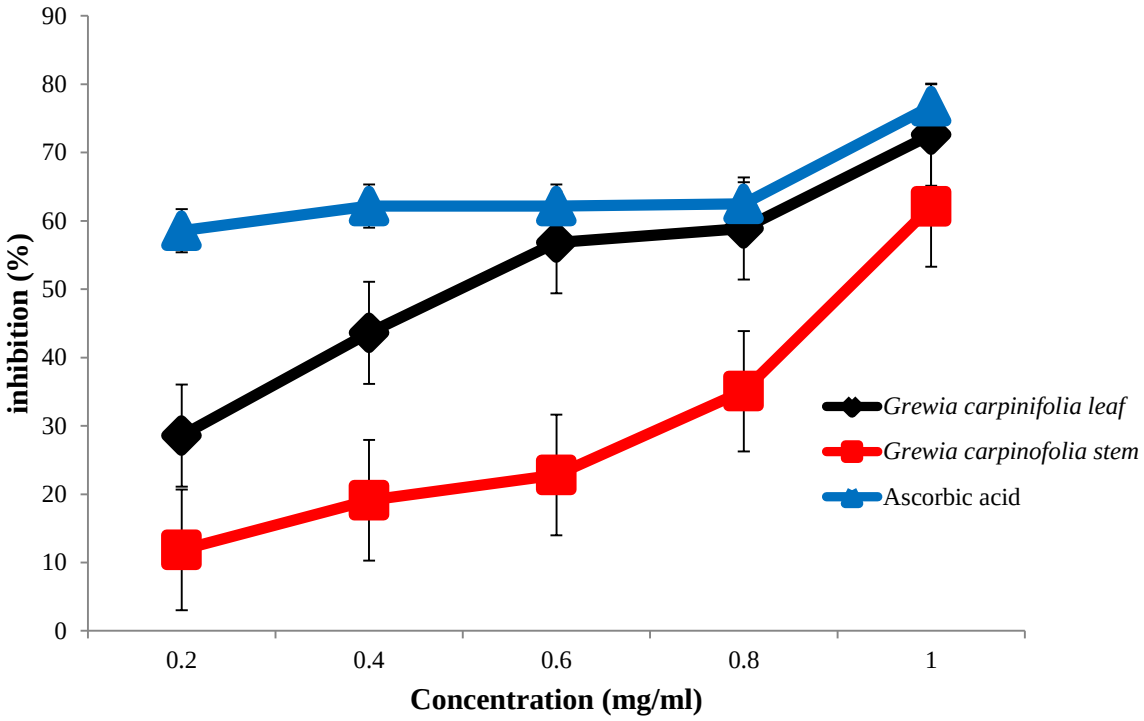


Figure 5.9: DPPH radical-scavenging ability of *Grewia carpinifolia* leaf and stem

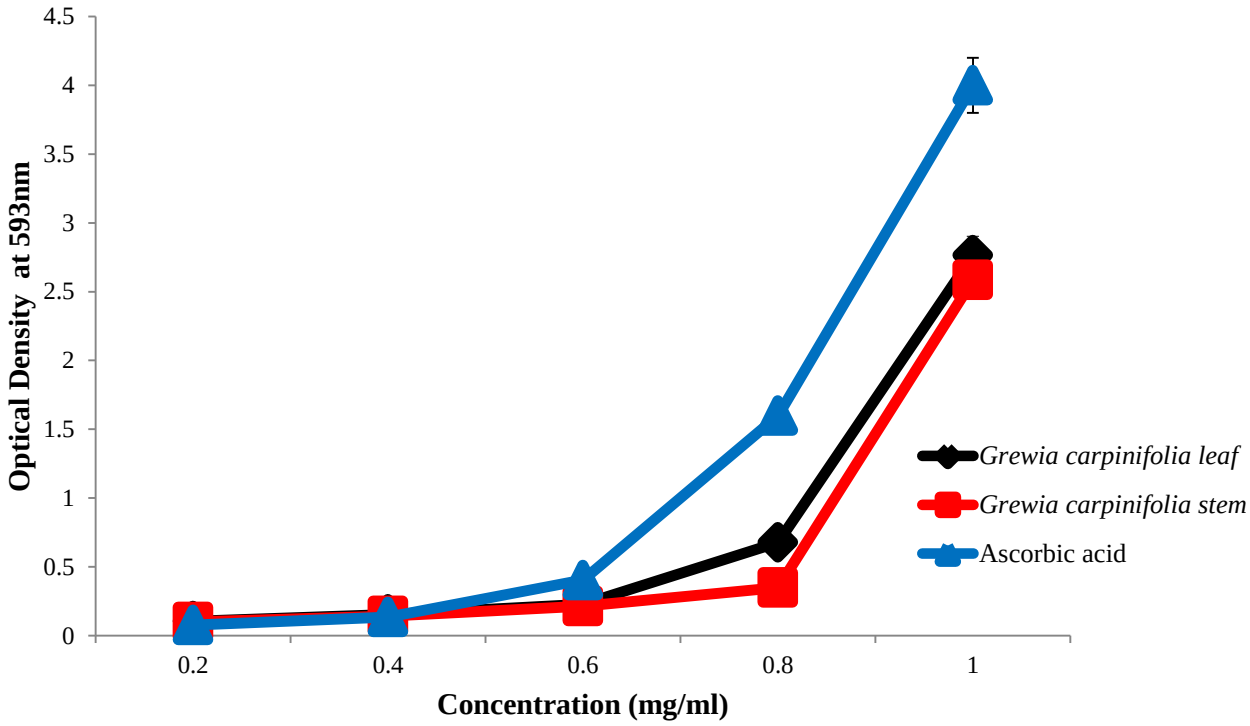


Figure 5.10: Ferric reducing antioxidant power assay of *Grewia carpinifolia* leaf and stem

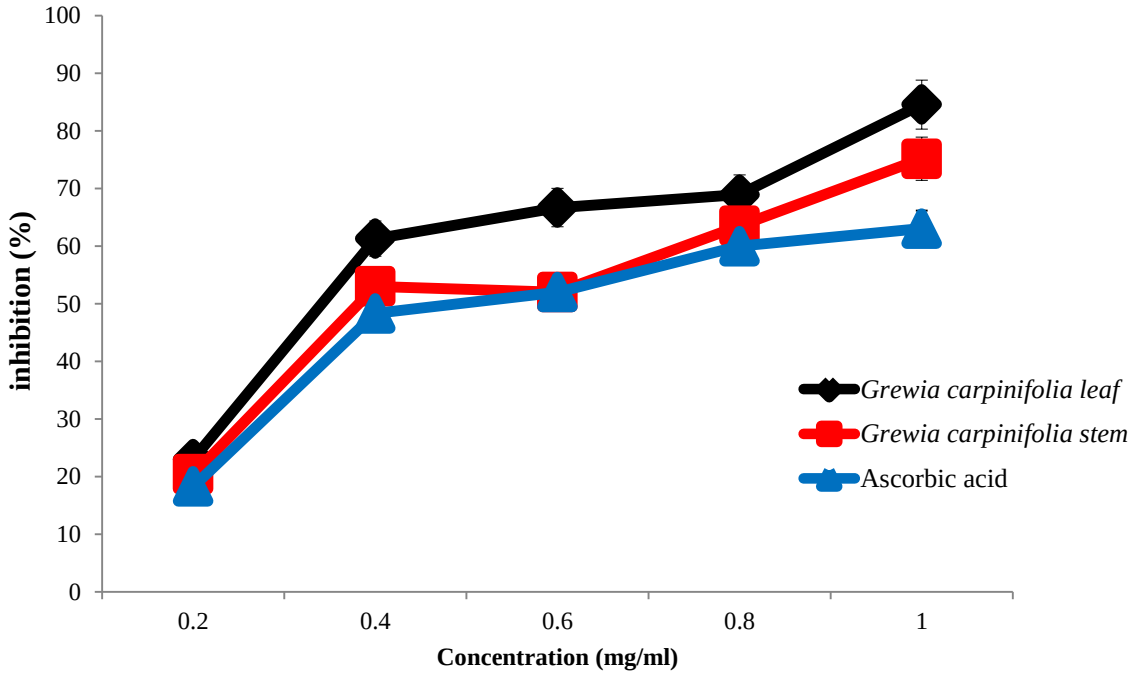


Figure 5.11: Thiobarbituric acid-reactive substances) assay of *Grewia carpinifolia* leaf and stem

5.3.2. Isolation and structure determination of pure compounds from *Grewia carpinifolia.*

As part of the present study in continuous search for potential antioxidant agents, ethanol crude extract of *Grewia carpinifolia* leaf which showed stronger *in vivo* antioxidant property was selected for further investigation.

The ethanol extract *Grewia carpinifolia* leaf (391.1 g) was subjected to liquid-liquid partitioning between hexanes (3 × 100 mL) and aqueous MeOH (MeOH:H_2O, 9:1, 500 ml). The aqueous layer was then diluted to 50% MeOH (v/v) and extracted with ethyl acetate (720 ml). The ethyl acetate fraction displayed the highest activity DPPH radical scavenging (IC_{50} = 0.52 mg/ml) and, hence, selected to isolate its active components. This fraction was passed through a reverse phase SPE-C18 column and eluted with a gradient elution of 60% MeOH:H_2O to 100% MeOH. Seventeen fractions were collected and only 12 were active at IC_{50} = 0.68 µg/ml. These fractions were further purified by column chromatography on a C18 reversed phase HPLC column using a gradient elution of 40% MeOH : H2O to 100% MeOH. This yielded several inactive fractions as well as six pure and active fractions (Tel 1-5). One was a new compound (Tel 5), from this Genus and the others were identified as previously known compounds.

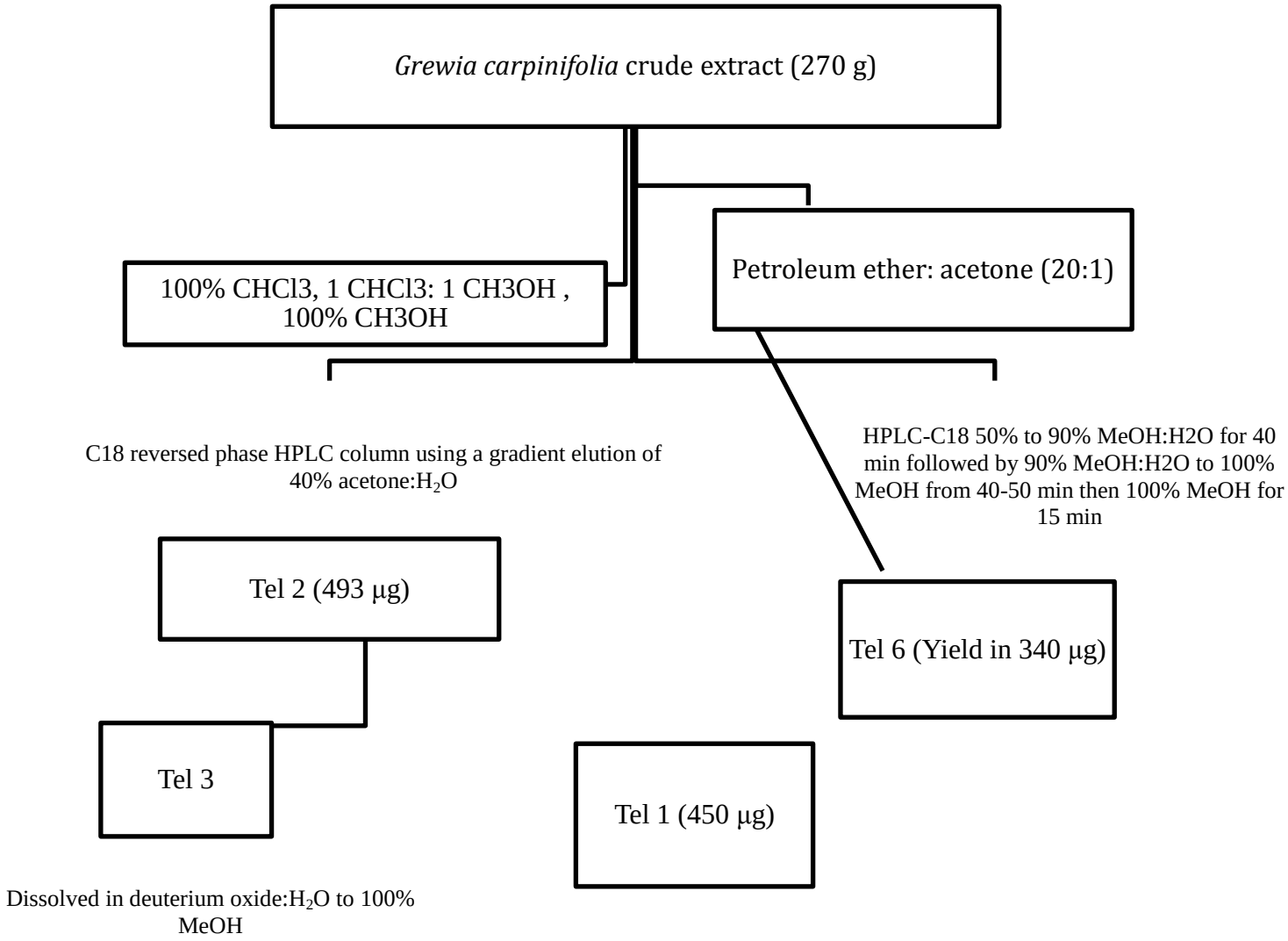


Figure 5.12: Fractionation of ethanol extract of *Grewia carpinifolia* leaves

From TLC, ^{1}H NMR, ^{13}C NMR and mass spectra data compounds identified and isolated from the extracts were β-sitosterol, stigmasterol, spinasterol and dibytyl phthalate. Their structures and spectra characteristics are depicted in Tables 5.3-5.7.

5.3.2.1 Structure Elucidation of Tel 1

Tel 1 was isolated as a pearl white crystalline solid. The IR spectrum of 1 showed a broad band at 3649 cm^{-1}, and a sharp band at 1542 cm^{-1} indicating the presence of hydroxyl and carbonyl groups. The ^{1}H and ^{13}C NMR spectra (Table 5.3) indicated that this compound belonged to the class of triterpenoids. The ^{1}H spectrum of compound 1 in chloroform (Fig. 5.14) indicated that the compound was pure with three oxygenated protons and six methyl groups. A highly shielded peak at δH 0.74 (d, J = 4.4 Hz, H-19) suggested the presence of a cyclopropyl ring. The ^{13}C spectrum of compound 1 contained 30 signals: six methyls, eight methylenes, five methines, three oxygenated carbons, and eight quaternary carbon peaks.

The ^{1}H and ^{13}C NMR signals in chloroform showed typical signals for the cyclopropane methylene protons with peaks at δC/δH 22.0 (C-19)/0.45 (d, J = 4.4 Hz, Hβ-19) and 0.81 (d, J = 4.8 Hz, Hα-19); two quaternary carbons at δC 29.1 (C-9) and δC 29.2 (C-10). Three oxygenated methine peaks were observed at δC/δH 73.5 (C-1)/3.55 (br, Hβ-1); δC/δH 71.4 (C-3)/4.52 (dd, J = 10.4 Hz, 6.8 Hz, Hα-3), δC/δH 70.6 (C-7)/3.56 (br, Hβ-7). The only difference between Tel 1 and Tel 2 was the position of the double bond in the alkyl side chain. The position of the double bond in Tel 2 was confirmed from the stereochemistry correlations of H-19 (δH 0.45, d, J = 4.4 Hz) to Hβ-1 (δH 3.55, m); H-19 (δH 0.81, d, J = 4.5 Hz) to Hβ-8 (δH 1.29, s) and Hβ-11 (δH 1.39, m) as well as H-5 ((δH 2.76, dd, J = 4.0 Hz, 14.0 Hz) to Hα-3 (δH 4.52, dd, J = 10.4 Hz, 6.8 Hz) and H-7 (δH 3.56, m) confirmed the chair conformation of both the rings, A and B, indicating a trans fusion of the two rings and a β-orientation of the cyclopropane ring.

The H-3 signal was observed as a doublet of doublets (dd) due to the axial-equatorial (J = 6.8 Hz) and diaxial (J = 10.4 Hz) interactions, suggesting an α-orientation of H-3. Correlations of Hβ-8 (δH 1.29, m) to Hβ-11 (δH 1.39, m), Hα-15 (δH 1.41, m), Hβ-18 (δH 1.61, s) and H-19 (δH 0.45, d) as well as correlations of Hα-7 (δH 3.56, m) to Hα-30 (δH 1.68, s), suggested a trans fusion C/D rings of this triterpenoid (3.6). , the final flat structure of compound 1 was assigned as shown in Figure 5.15. This compound was identified to be β spinasterol.

Table 5.3: Spectra characteristics of Compound 1

^{1}H NMR	δ 0.74(3H 18XCH_3), δ 0.81(3H, 29XCH_3), δ 0.81 (3H 19XCH_3), δ 0.84 (3H 26XCH_3), δ 0.86 (3H, 26XCH_3), δ 1.06 (3H, 21XCH_3),), δ 3.67 to 3.66(1H, 3-CH), δ 5.33 (1H, 23XCH), δ 5.32 (7XCH_3)
^{13}C NMR	δ142.30, δ139.33, δ 130.04, δ121.51, δ71.66, δ57.71, δ57.61, δ56.87, δ56.76, δ51.18. δ51.16, δ46.66, δ43.30, δ43.04, δ40.60, δ40.50, δ38.19, δ37.26, δ36.89, δ34.64, δ32.73, δ32.66, δ32.48, δ30.25, δ30.10, δ29.95, δ29.83, δ29.79, δ29.64, δ29.48, δ29.33, δ28.92, δ26.68, δ26.05, δ24.93, δ24.90, δ23.69 ppm.
Mass spectra	Molecular weight 451

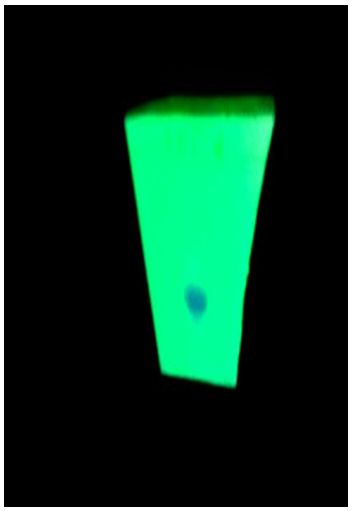


Figure 5.13: Thin Layer Chromatography (TLC) spotting of Tel 1 as seen under the ultraviolet light (356 nm)

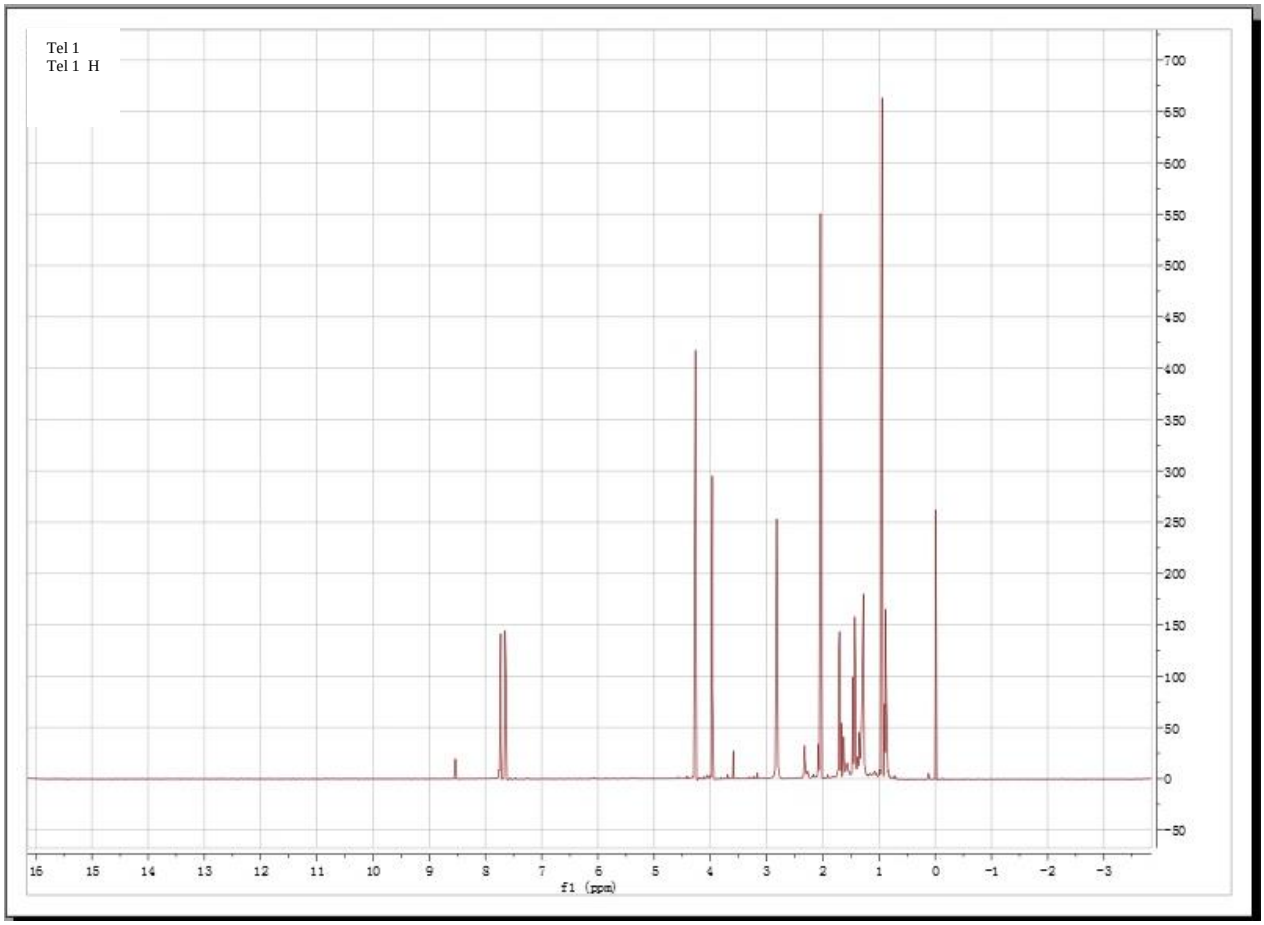


Figure 5.14: The ^{1}H spectrum of Tel 1 in chloroform

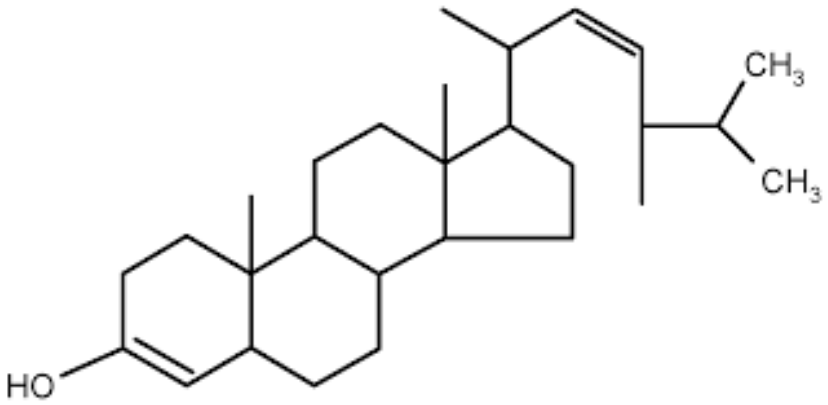


Figure 5.1: Flat structure of Tel 1

5.3.2.2 Structure Elucidation of Tel 2

Tel 2 was also isolated as a white crystalline solid. Mass spectrometry gave a molecular ion peak at m/z 413, confirming the molecular composition of $C_{29}H_{50}O$.

The ^{1}H and ^{13}C NMR spectra indicated that this compound also belonged to the class of triterpenoids. The ^{1}H spectrum of Tel 2 in acetone indicated that the compound has three oxygenated protons and six methyl groups (Table 5.4.). A highly shielded doublet at δH 0.67 (d, J = 4.5 Hz, H-19) suggested the presence of a cyclopropyl ring. The ^{13}C spectrum of Tel 2 contained 31 signals: six methyls, nine methylenes, five methines, three oxygenated carbons, and eight quaternary carbon peaks.

The ^{1}H and ^{13}C NMR signals in acetone showed typical signals for the cyclopropane methylene protons with peaks at δC /δH 36.3 (C-22)/0.59 (d, J = 4.5 Hz, Hβ-19) and 1.43 (d, J = 4.8 Hz, Hα-19); two quaternary carbons at δC 141 (C-5) and δC 122 (C-6). Three oxygenated methine peaks were observed at δC /δH 73.0 (C-1)/4.02 (br, Hβ-1); δC /δH 71.0 (C-3)/4.14 (br, Hα-3), δC /δH 70.0 (C-7)/5.63 (dd, J = 12.4 Hz, 4.5 Hz, Hβ-7) (Figure 5.17). Spectrometry correlations confirmed that compound 2 had a similar cycloartane-type skeleton as that of Tel 1. The fusion of the A/B rings was confirmed by the correlation of H-22 (δH 1.02, s) to C-5 (δC 141.0), C-5 (δC 122), C-17 (δC 56); H-5 to C-4 (δC 55.8), C-13, (δ46) and C-29 (δC 28.27). After connecting the fragments together from spectrometry correlations, the flat structure of Tel 2 was established as shown in Figure 5.18 as that of β-sitosterol.

Table 5.4: Spectra characteristics of Tel 2

^{1}H NMR	δ 0.67 to 1.005 (18 H,6xCH_3)
	δ 1.02 to 1.27 (22 H,11XCH_2)
	δ 1.43 to 2.27 (m, 8H, methane protons)
	δ 5.3 (m, 1H, vinylic proton)
^{13}C NMR	δ141(C-5), δ122 (C-6), δ56 (C-17), δ50 (C-13), δ46(C-13), δ41(C-4), δ 36(C-22), δ37(C-5), δ34(C-1), δ36(C-8), δ34(C-23), δ36.1(C-10), δ31.9(C-16), δ33.9(C-7), δ29.2(C-25), δ28.95(C-27), δ28.27(C-29), δ31.6(-26), δ77.3(C-28)
Mass spectra	Molecular weight 413

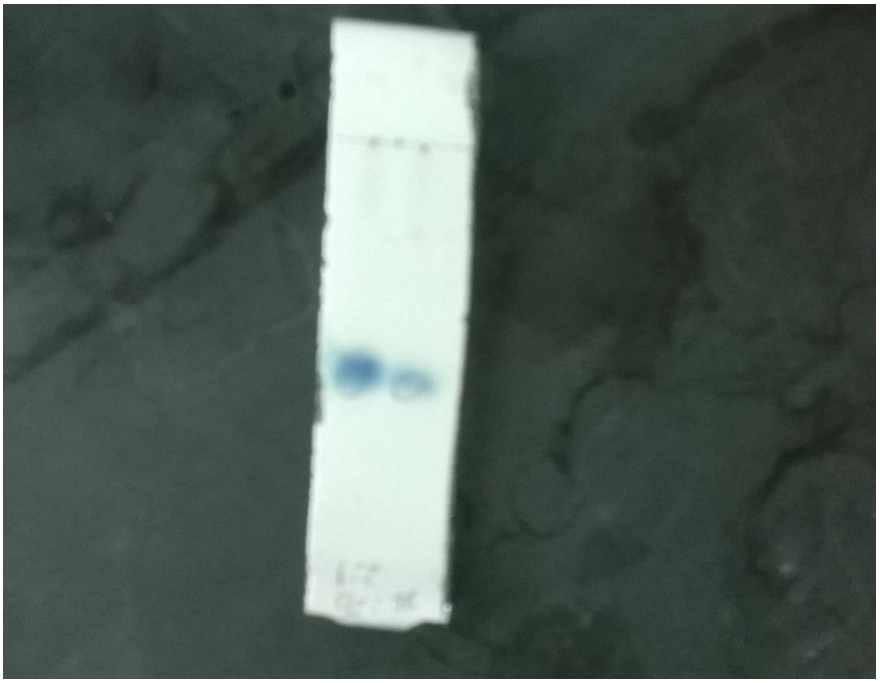


Figure 5.16: Thin Layer Chromatography (TLC) spotting of Tel 2

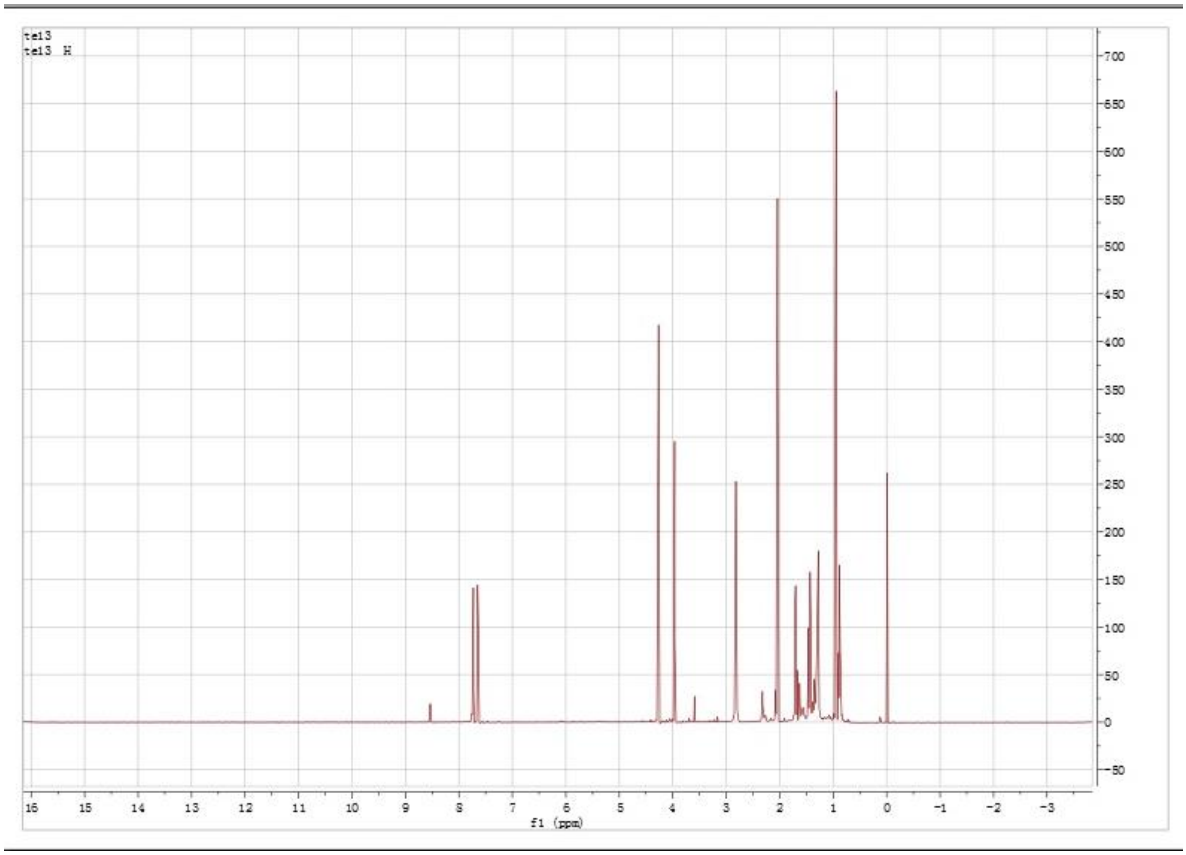


Figure 5.17: The ^{1}H spectrum of Tel 2 in chloroform

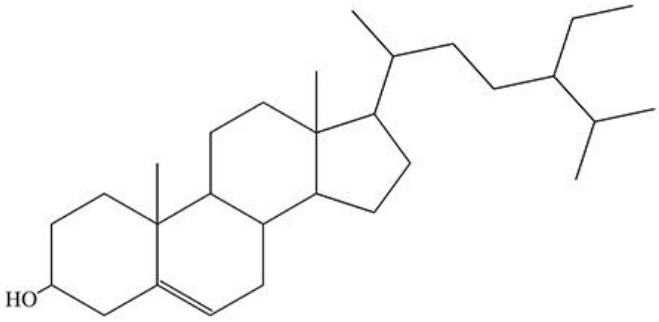


Figure 5.18: Chemical structure of Tel 2 (β- sitosterol)

5.3.2.3 Structure Elucidation of Tel 3

Tel 3 was also isolated as a white crystalline solid. Mass spectrometry gave a molecular ion peak at m/z 1526.

The ^{1}H and ^{13}C NMR spectra indicated that this compound also belonged to the class of aromatic carboxylic acids. From ^{1}H and ^{13}C NMR data spectrum there was a strong peak of ~1750-1700 cm^{-1}, indicating the carboxylic group. There was also a C=O peak at 1711 cm^{-1}. The ^{1}H spectrum of Tel 3 in acetone indicated that the compound has seven carbon atoms, Spectrometry correlations confirmed that compound 3 has a similar structure with that of benzoic acid except that the carbonyl oxygen was deprotonated. The O–H and N–H stretching peaks were broadened by hydrogen bonding, thus the broad 3000 cm^{-1} peak was due to the O–H portion of the COOH functional group. This broad peak typically appeared in the 3200- 2500 cm^{-1} range (Figure 5.20).

Table 5.5: Spectra characteristics of Tel 3

^{1}H NMR	δ0.89 (12H, 7XCH_3) δ2.05(2H, H2′), δ2.03 (2H, H3′), δ4.28 (1H), δ7.75 (2H, H 3 & H 4), δ8.55 (2H, H2 & H 5),
^{13}C NMR	167.91 (ester carbonyl carbon), 133.37 (s, C-1& C-6), 131.93 (s, C-7'), 129.57 (d, C-6') ; 66.63(d, C-5'), 65.83 (d, C-2 & C-5), 31.28 (d, C-3 & C-4), 30.25 (d, C -3'), 30.10 (t, C1' and C1"), 31.69 - δ 19.80. (t, C2', C4', C2" and C3" as a methylene envelop), 13.93 (q, methyl groups attached to C7' and C4").
Mass spectrometer	Molecular weight 1526

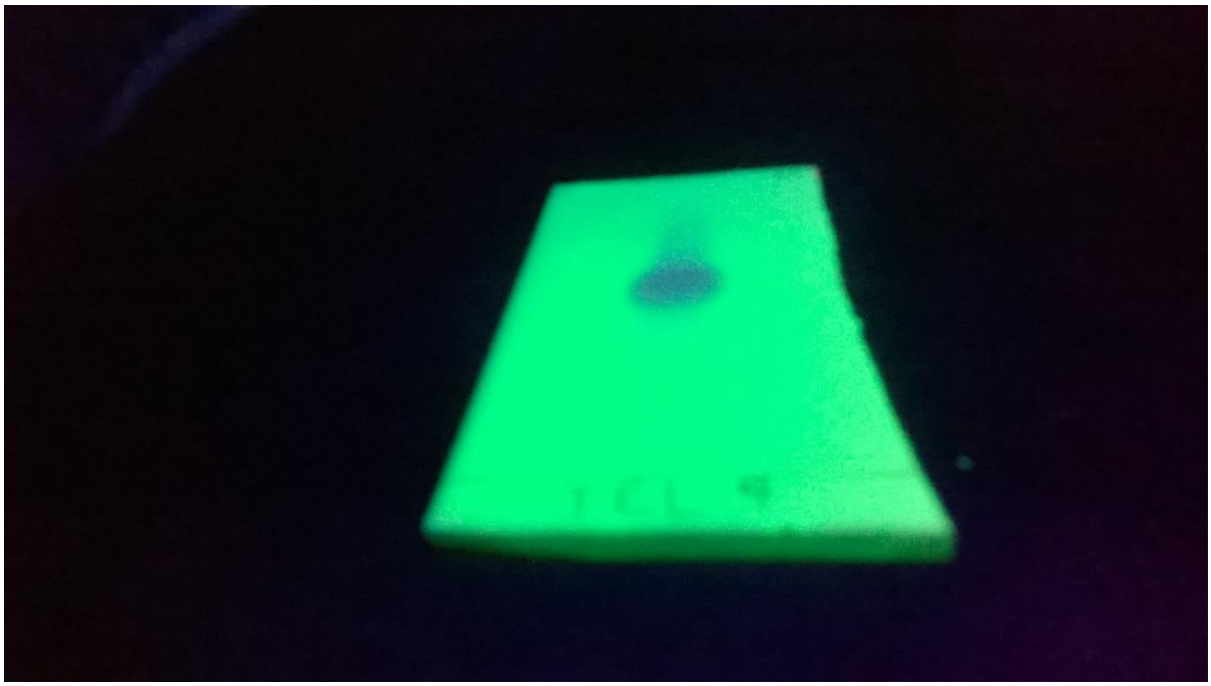


Figure 5.19: Thin layer chromatography spotting of Tel 3 as seen under the ultraviolet light (356 nm)

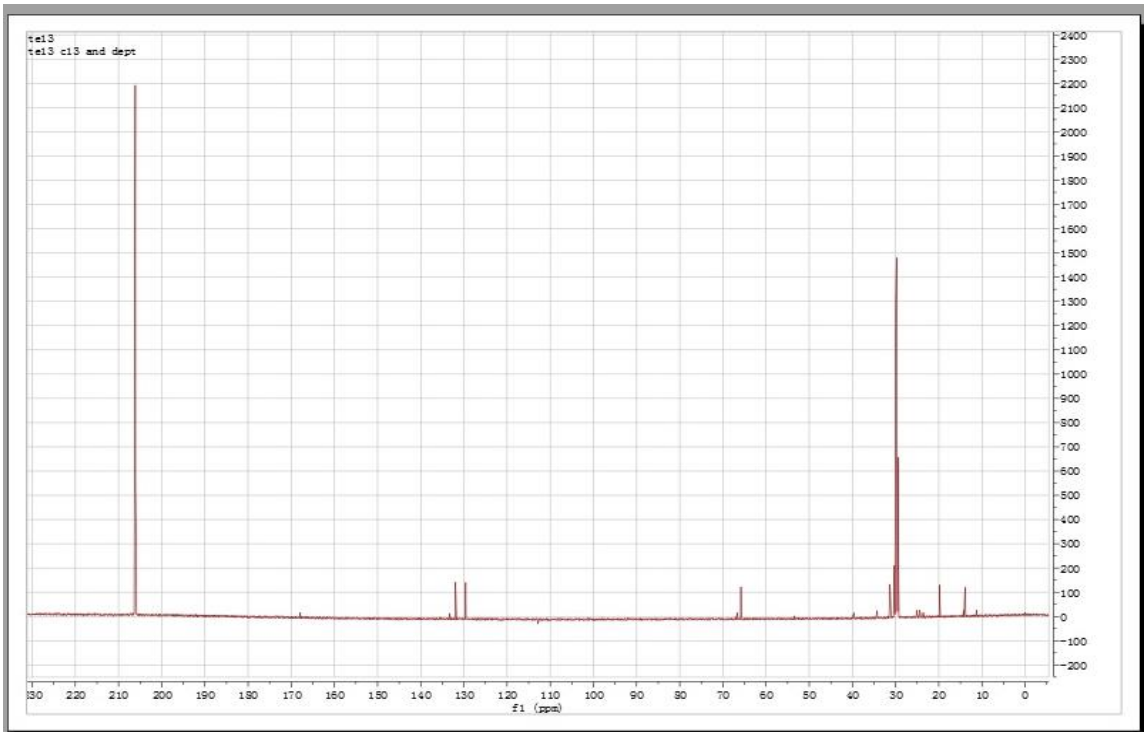


Figure 5.20: ^{13}C and Distortionless enhancement by polarization transfer nuclear magnetic resonance spectra of Tel 3

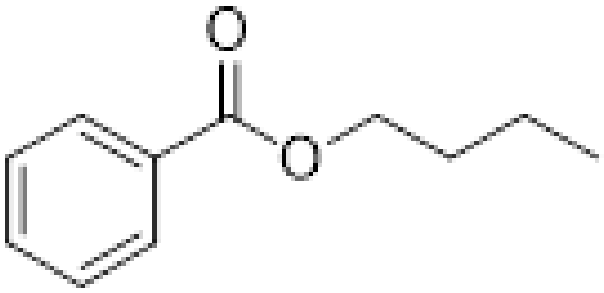


Figure 5.21: Chemical Structure of Tel 3 and 4 (Benzoic Acid butyl ester)

5.3.2.4 Structure Elucidation of Tel 5

Tel 5 was also isolated as a white crystalline solid. Mass spectrometry gave a molecular ion weight peak at m/z 278.343. Its three highest peaks were at m/z 205, 204 and 149.

The ^{1}H and ^{13}C NMR spectra indicated that this compound also belonged to the class of phthalate. From the heteronuclear single quantum corelation, (Table 5.6.) spectrum, it marked that downfield shift of aromatic protons at δ 7.72 and δ 7.53 coincided with aromatic carbon atoms (C2 & C5, δ129.87) and (C3& C4, δ127.81). The relationship of olifinic protons H5' & H6' (δ 5.32 and δ 5.30) is noticed with C5' and C6' (δ122.77 & δ 144.00). The coexistence of H1' & H1" at δ 4.30 and 4.10 is in agreement with C1' and C1" at δ 62.00. The methylene protons H 2' and H 4' at δ 2.04 and δ 2.31, respectively established the correlation with C2' and C4' respectively. The methyl protons at δ 0.89 ascertain correlation with methyl groups, attached to C7' and C 4" (δ 31.10, δ18.80 and δ13.70). The absence of homonulear coupling at δ132 and δ 31.10 for (C1& C6) and C7' indicated, totally substituted carbon atoms. The correlation of C1' and C1" at δ 62 showed connectivity with H1' and H1" at δ 4.30 and δ 4.10 protons respectively.

The correlation of H6' at δ5.3 showed connectivity with C4' at δ27.96 of the methylene envelopes. The correlation of H4' at δ2.31 showed connectivity with C2' at δ 29.24 of methylene envelopes. Correlation experiments (HMBC) confirmed the position of double bonds and hydroxy function.

Table 5.6: Spectra characteristics of Tel 5

^{1}H NMR	δ0.89 (12H, 7XCH_3) δ2.05(2H, H2′), δ2.03 (2H, H3′), δ4.28 (1H), δ7.75 (2H, H 3 & H 4), δ8.55 (2H, H2 & H 5),
^{13}C NMR	167.91 (ester carbonyl carbon), 133.37 (s, C-1& C-6), 131.93 (s, C-7'), 129.57 (d, C-6') ; 66.63(d, C-5'), 65.83 (d, C-2 & C-5), 31.28 (d, C-3 & C-4), 30.25 (d, C -3'), 30.10 (t, C1' and C1"), 31.69 - δ 19.80. (t, C2', C4', C2" and C3" as a methylene envelop), 13.93 (q, methyl groups attached to C7’ and C4").
Mass spectrometer	Molecular weight 1526

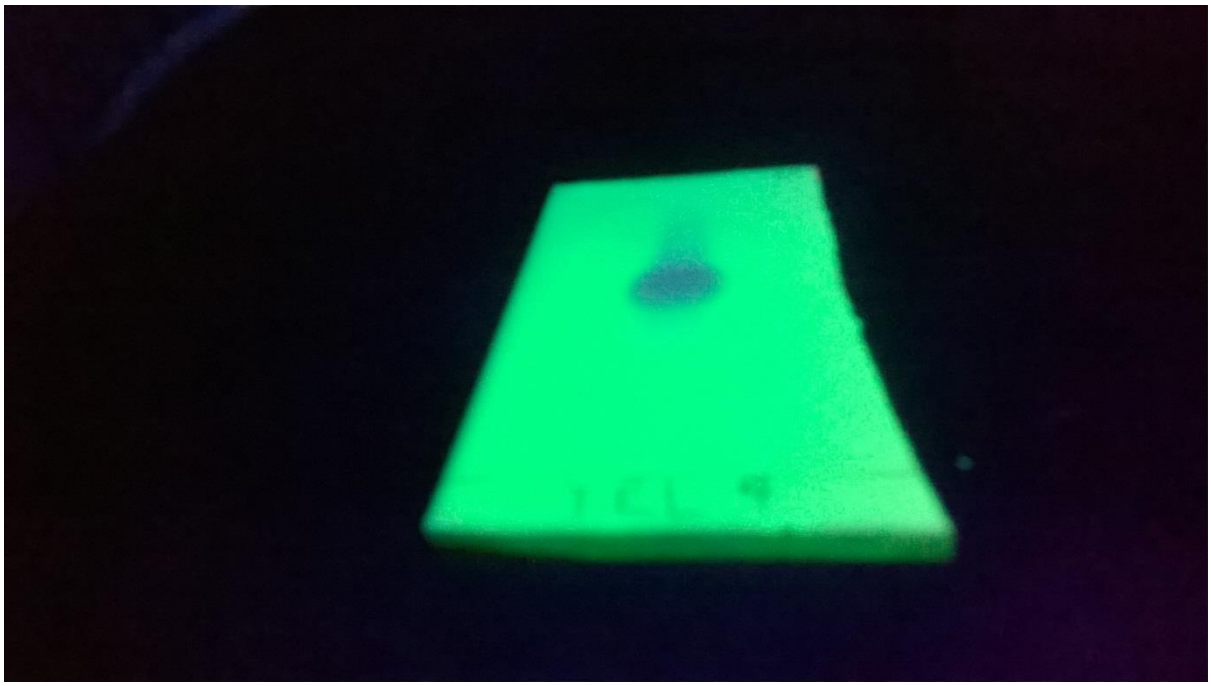


Figure 5.22: Thin layer chromatography spotting of Tel 5 as seen under the ultraviolet light (356 nm)

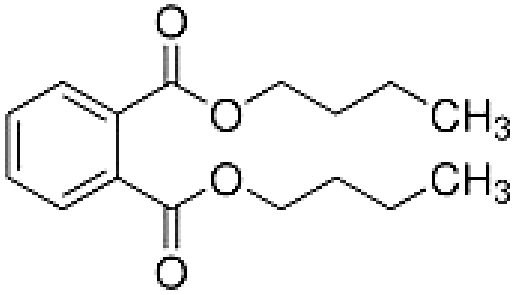


Figure 5.23: Chemical structure of Tel 5 (dibutyl phthalate)

5.3.2.5 Structure Elucidation of Tel 6

Tel 6 was isolated as a white powder. The mass spectral data of the compound gave a molecular formula $C_{29}H_{48}O$, which was supported by the ^{13}C NMR spectral data. ^{1}H NMR spectra of Tel 6 showed the presence of two methyl sinlgets at δ 0.71, and 1.03; three methyl doublets that appeared at δ 0.80, 0.82, and 0.91; and a methyl triplet at δ 0.83. Tel 6 also showed protons at δ 4.98, 5.14, and 5.31, suggesting the presence of three protons corresponding to that of a tri substituted and a di substituted olefinic bond. Liebermann-Burchard reaction indicated Tel 6 is having a sterol skeleton. The proton corresponding to the H-3 of a sterol moiety appeared as a triplet of doublet of doublets at δ 3.51. The ^{1}H and ^{13}C NMR values for all the protons and carbons are given in Table 5.7. The spectral data showed the presence of sterol skeleton with a hydroxyl group at C-3 position and two double bonds at C-5/C-6 and C-20/C-21, with six methyl groups. Thus, the structure of Tel 6 was assigned as the known compound, stigmasterol (Figure 5.24).

Table 5.7: Spectra characteristics of Tel 6

^{1}H NMR	δ5.33 (1H,6XH), 5.32(1H, 23XH) 3.67(1H,22XH), 3.67(1H, 3XH), 2.86(1H, 20XH), 1.8-2.0 (5H, m) ppm. Other peaks are observed at δ 0.740.89(9H), 0.96-1.07(5H), 1.211.48 (4H), 0.69-0.73 (m, 3H), 1.8-2.00 (5H), 1.07-1.13 (m, 3H), 1.32, 1.57 (9H) ppm.
^{13}C NMR	142.3 (C-5), 139.3 (C-22), 130.04, 121.5(C-6), 71.66 (C-3), 57.71(C-14), 57.61(C-17), 52.12 (C-9), 51.18 (C-9), 51.16 (C-20), 46.66(C-12), 43.30 (C-13), 43.04 (C-4), 41.35 (C-12), 40.61 (C-1), 40.50 (C-10), 38.19 (C-8), 35.59(C-20), 37.26 (C-22), 36.89 (C-7), 34.64 (C-8), 32.73 (C-25), 32.66 (C-16), 32.58 (C-2), 32.48 (C-15), 30.25 (C-28), 30.10 (C-11,26), 29.95 (C-27), 29.79 (C-19), 26.05 (C-21), 24.90(C-18, 29).

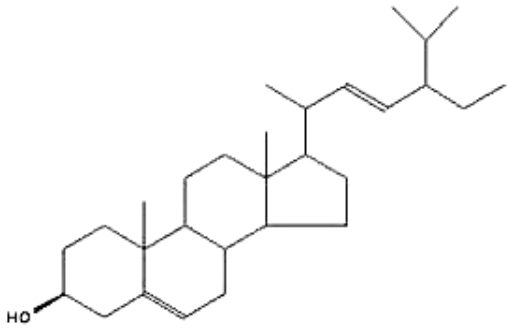


Figure 5.24: Chemical structure of Tel 6 (stigmasterol)

5.3.3. *IN VITRO* ANTIOXIDANT ACTIVITY OF PURE COMPOUNDS

The antioxidant capabilities of the various pure compounds from the ethanol extracts of *Grewia carpinifolia* leaf were determined by the absorbance and scavenging percentage. In Figure 5.22., spinasterol, stigmasterol, sitosterol as well as dibutyl phthalate were found to be effective in scavenging the ABTS radical. The percentage inhibition of this radical was, however, not concentration-dependent. At 0.5 μg/ml, the inhibition of the ABTS radical by spinasterol, sitosterol, benzoic acid butyl ester, dibutyl phthalate, stigmasterol was 65.05%, 47.25%, 25.83%, 22.16% and 58.12%, respectively.

The percentage inhibition using the TBARS assay by the pure compounds ranged from 45.32 to 100% (stigmasterol had the highest percentage inhibition, whereas benzoic acid butyl ester had the lowest), while that of ascorbic acid was 62.05% at the dose of 1 μg/ml.

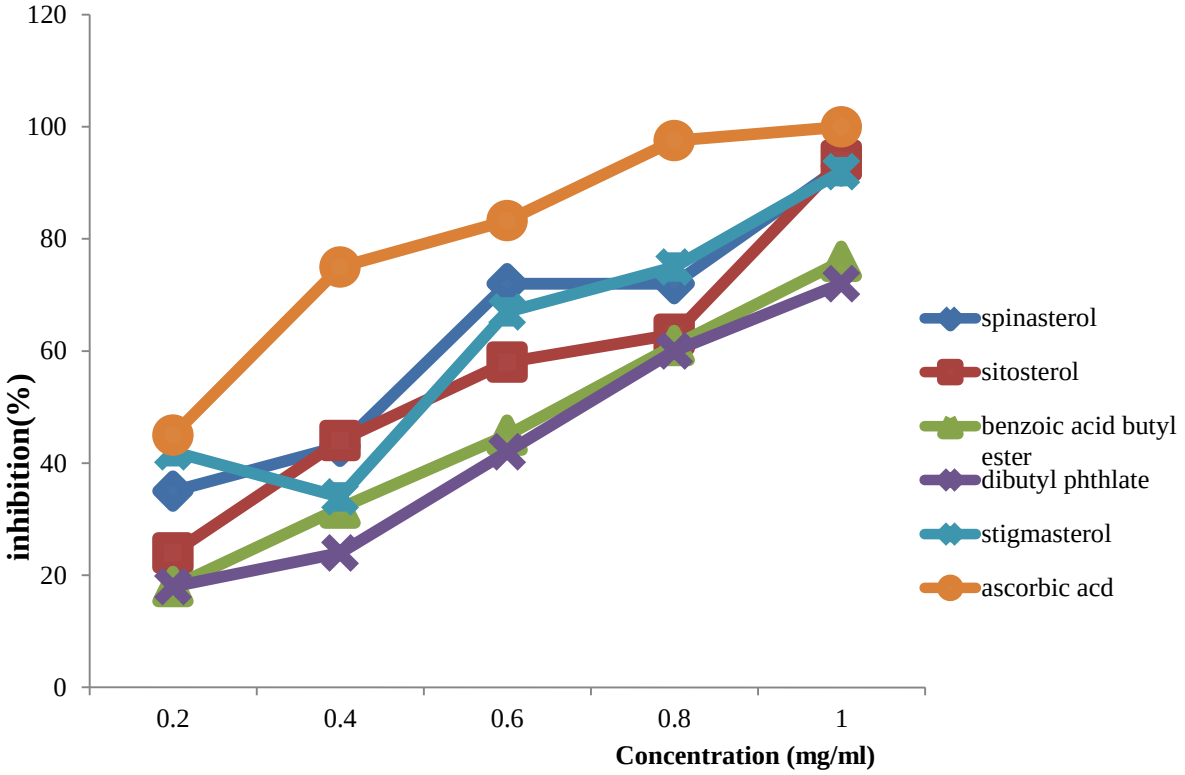


Figure 5.25.: 2, 2'-azinobis-3-ethylbenzothiozoline-6-sulphonic acid radical scavenging ability of isolated pure compounds of *Grewia carpinifolia* leaf

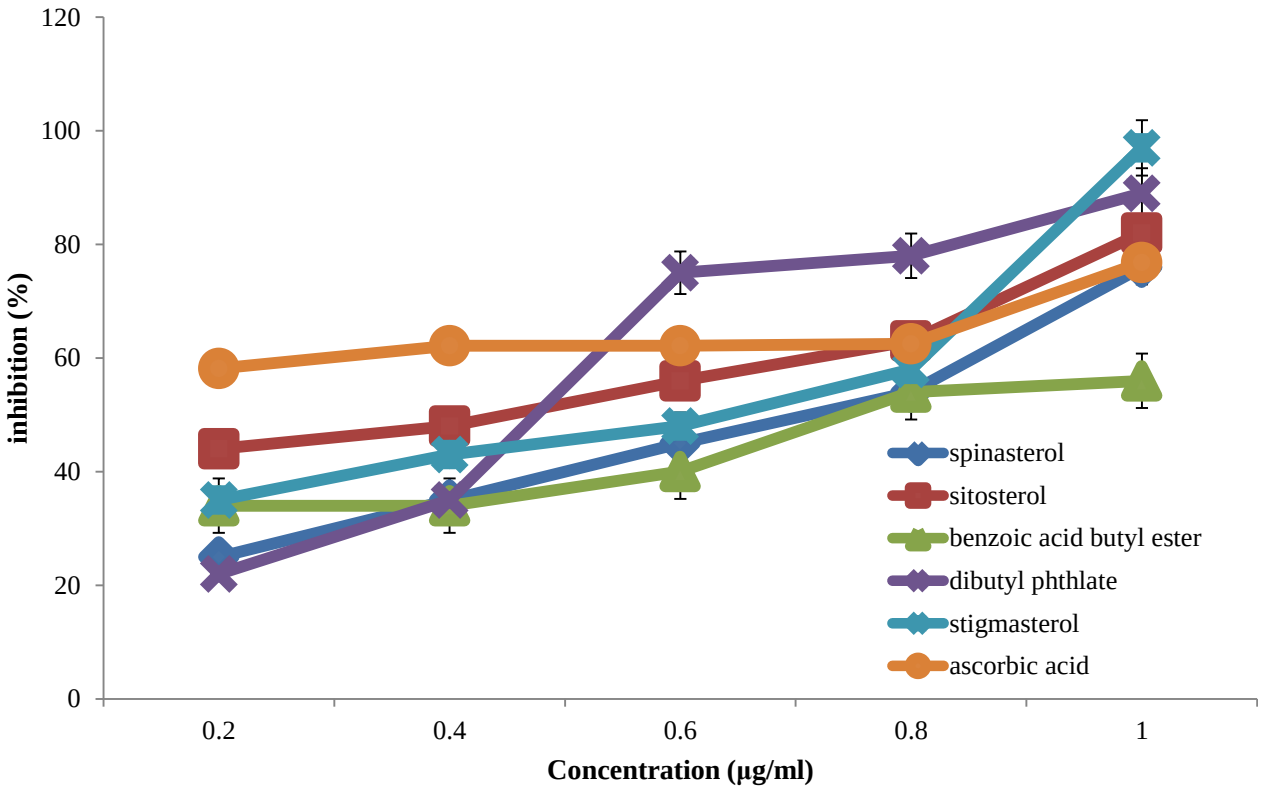


Figure 5.26: Thiobarbituric acid-reactive substances assay of isolated pure compounds of *Grewia carpinifolia* leaf

5.4. DISCUSSION

Plants contain different groups of phenolic compounds, including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids. All the phenolic classes have received extensive attention because of their physiological functions, including free radical scavenging, anti-mutagenic, anti-carcinogenic and anti-inflammatory effects (Manthey, 2000; Bandoniene and Murkovic, 2002).

5.4.1 *In vitro* antioxidant assay

The $ABTS.^{+}$ scavenging assay employs a specific absorbance (734 nm) at a wavelength remote from the visible region has been employed by several authors as an index that reflects the antioxidant activity of test samples (Wu *et al.,* 2006). The scavenging of the ABTS + radical by the stem extracts was found to be as high as 100%; same as the standard at the highest concentration of 1 mg/ml. This shows that *Grewia carpinifolia* extract presents a good ability to scavenge the ABTS radical.

The DPPH radical is a stable free radical which dissolves in methanol and shows characteristic absorption at 517 nm, when an antioxidant scavenges free radicals by donation of hydrogen ion, the DPPH picks up the electron which results in a decrease in absorption consequently the purple solution become lighter in colour (Villaño *et al.,* 2007), the resulting discoloration is stechiometrically dependent on the number of electrons gained (Silva *et al.,* 2005). From the present study, it can be postulated that *Grewia carpinifolia,* has DPPH scavenging activity by reducing the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principles. The scavenging of the DPPH radical by the extracts was found to be much lower than that of $ABTS.^{+}$. Stereoselectivity of the radicals or the solubility of the extract in different testing systems are some factors that have been reported to affect the capacity of extracts to react and quench different radicals (Yu *et al.,* 2002).

Wang *et al*. (1998) found that some compounds which have ABTS + scavenging activity did not show DPPH scavenging activity. This is not the case in this study. This finding further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be potent therapeutic agents for treating radical-related pathological damages.

The FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ) (Benzie and Strain, 1996). Generally, the reducing properties are linked with the presence of compounds which exert their action by breaking the free radical chain through the donation of a hydrogen atom (Duh *et al.,* 1999). In the present study as shown in Figure 5.10., the absorbance of *G. carpinifolia* clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration as standard antioxidant. Hence, the plant should be able to donate electrons to free radicals stable in the actual biological and food system.

The extracts of *G. carpinifolia* leaf and stem showed a considerable antioxidant effect in all the assays. The leaf extract showed a higher antioxidant activity in all the assays with the exception of the ABTS assay where the stem extract had a higher activity at all the tested concentration.

In the TBARS assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation. The high percentage inhibition recorded in this assay might indicate the ability of the extract to inhibit linoleic acid peroxidation.

Previous reports on the antioxidant activity of *G. carpinifolia* are very rare in the literature and, thus, this made the comparison of the present results with that of previous studies very difficult. However, our result in the present study is similar to several other documentations on the antioxidant activities of other *Grewia species* such as *G. asiatica* which is native to Asia and has many pharmacological activities including antioxidant properties (Zia-Ul-Haq *et al.,* 2013). Recently, Anwar *et al*. (2015) also demonstrated that *G. optiva* possesses high antioxidant activity and contains several secondary metabolites such as flavonioids, alkaloids, terpenoids, tannins and saponins. The antihyperglycaemic effect of *G. asiatica* fruit extract has also been linked to its improvement of the pancreatic β-cells as well as its antioxidant effects (Khattab *et al.,* 2015). *G. tiliaefolia* has similarly been reported to have the ability to scavenge free radicals in both *in vivo* and *in vitro* models (Selvam *et al,* 2010).

The present study corroborated the findings in study 1 (chapter 3) that the ethanol extract of *G. carpinifolia* contains substantial amount of phenolics and may be inferred that these phenolics are responsible for its marked antioxidant activity as assayed through various *in vitro* models. It has been shown that there is close relationship between total phenolic

contents and antioxidative activity of the fruits, plants and vegetables (Albayrak *et al*., 2010). The antioxidant activity of phenolics is mainly due to their redox properties, which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may have also a metal chelating potential (Pietta, 2000). The considerable high level of these phenolic compounds in the ethanol extracts of the leaf and stem of *Grewia carpinifolia* might be responsible for the pharmacological activities as well as the folklore use of the plant.

5.4.1 Isolation of pure compounds and their *in vitro* antioxidant assay

All the isolated compounds have been reported to have various pharmacological activities including antibacterial, antihelminthic, anticancer, antiparasitic, antimalarial and antioxidant (Manoharan *et al.,* 2010; Tamiselvan *et al.,* 2011, Aggarwal *et al,* 2011, Ashwini *et al* 2012). Stigmasterol, spinasterol and beta-sitosterol are unsaturated phytosterol (*Gallina et al.,* 2007*;* Kandati *et al.,* 2012), which have been reported to be present in oils of soybean, calabar bean, and rape seed, and in a number of medicinal herbs, including the Chinese herbs *Ophiopogon japonicus* and American Ginseng (Han *et al.,* 2008). Being steroids, they are precursors of anabolic steroid boldenone; boldenone undecylenate is commonly used in veterinary medicine to induce growth in cattle (Ros *et al.,* 2007; Draisci *et al.,* 2007). Interestingly the triterpenoid, δ-sitosterol has been documented to elicit some central nervous system activities (Aguirre-Hernández *et al.,* 2007) and also boost the immune system.

The isolated compound (Tel 5) is a derived condensed product of dibutyl phthalate. It was isolated for the first time from this plant source. Dibutyl phthalate was found to possess antimicrobial as well as potent larvicidal activity; it has also been shown to have anticancer properties. Benzoic acid and its esters which were isolated compounds Tel 3 on the other hand have been documented to occur naturally in plants, where they serve as an intermediate in the biosynthesis of many secondary metabolites (Liu *et al*., 2013).

It has been reported that reactive oxygen species (ROS) such as superoxide anion and hydroxyl radical participate in the process of inflammation following vanadium toxicity in various tissues, including the brain (Trenam *et al.,* 1999). Therefore, compounds that have scavenging activities toward these radicals and/or suppressive activities on lipid peroxidation may, thus, be expected to have therapeutic potentials for vanadium-induced brain damage. Remarkably in this present study, pure compounds isolated from the *Grewia carpinifolia* extract showed better antioxidant capabilities in the various *in vitro* models when compared

to the crude extract. Formation of lipid peroxides, their degradation, and the roles of hydroperoxides in cellular metabolism have recently attracted interest (Hodges *et al.*, 1999).

The most accurate approach to measure lipid peroxidation is to directly quantify their primary hydroperoxide products. However, these are extremely difficult to measure as a result of their lability and required lengthy procedures. Consequently, the determination of MDA, a secondary end-product of oxidative lipid degradation, has become the system of choice for estimating lipid peroxidation. The TBARS assay is often the method of choice because of its simplicity over other available methods such as gas chromatography or high-performance liquid chromatography (Janero 1990). In the TBARS assay, the pure compounds had better results when compared to the standard, ascorbic acid (a water soluble vitamin). The result of the assay revealed that the compounds might be able to terminate chain radical reactions via electron transfer, with the phytosterols having the highest antioxidant activity. Although the results are promising, further studies are needed to evaluate the effectiveness of these pure compounds to prevent oxidative damage to brain cell and tissues based on *in vivo* studies.

Conclusion

The results from the current study further suggest that *Grewia carpinifolia* is an important source of therapeutic agents. The pure compounds isolated from *G. carpinifolia* could contribute to the medicinal and folklore uses of the crude extract of the plant. The antioxidant properties of these compounds might play a role in preventing diseases and toxicities in which free radicals have been implicated.

CHAPTER SIX

6.0 STUDY FOUR

Brain uptake index and neuro-protective activity of pure compounds of *Grewia carpinifolia* following acute vanadium-induced toxicity

6.1 INTRODUCTION

Environmental exposures to vanadium (V) produce several adverse health effects in animals. Vanadium continues to be widely used in various industrial applications, including steel-making; arc welding; temperature-resistant alloy production; and glass, pigment and paint manufacturing (McNeilly *et al.*, 2004; Bunting, 2006). Vanadium is a preferred metal for the production of special steels and temperature-resistant alloys because it is one of the lightest high-strength metals. More than 90% of industrial vanadium is used in steel making. The dominant market driver for vanadium over the past three years has been an increased world-wide demand for higher strength steel, most notably in China (Bunting, 2006). This increased demand for vanadium is not expected to decline as the world-wide demand for high quality steel continues. Welding and the associated exposure of workers to welding fumes have increased along with steel production. The use of vanadium with non-ferrous metals is also of particular importance in the atomic energy industry, aircraft construction and space technology (Hazardous Substance Database, 2006). Particularly, vanadium compounds are often released into the environment in large quantities, mainly by burning fossil fuels.Vanadium is usually found to be the most abundant trace metal in petroleum samples and can be found in concentrations reaching 1500 mg kg^{-1}, depending on the source of the crude oil (Amorim *et al.*, 2007). Vanadium accumulates in the soil, groundwater, and plants that may be consumed by animals and humans (Pyrzynska and Weirzbicki, 2004).

Earlier studies have shown that vanadium exposure may cause CNS depression, tremor, behavioural deficits, neurasthesia and other severe motor deficits, including vegetative symptoms (Done, 1979; WHO, 2000). Another study also showed that inhaled V_2O_5 can damage the nigrostriatal dopaminergic system in rodent models (Avila-Costa *et al.*, 2004); V-induced neurotoxicity has been closely linked to the persuasion of oxidative stress that leads to ROS generation and lipid peroxidation. In addition, oxidative stress induced by vanadium exposure leads to the release of cytochrome C into the cytosol; cytosolic cytochrome C activates the caspase cascade; caspase-3 mediates proteolytic cleavage of protein kinase C delta (PKCδ); proteolytically activated PKCδ mediates DNA fragmentation and apoptosis (Afeseh Ngwa *et al.*, 2009). These evidences imply that apoptosis possibly plays an important

role in acute and chronic intoxication with V. V generally accumulates in the brain (Gracia *et al.,* 2005). However, living cells possess diverse mechanisms to maintain heavy metal concentrations at levels that do not exceed cellular requirements. For example, chelation by either glutathione (GSH) or phytochelatins (PCs) has been described in some plants and algae (Korbecki *et al.,* 2012). GSH is found in all organisms participating in multiple metabolic processes, for example, intracellular redox state regulation, transport of GSH-conjugated amino acids and other molecules.

Despite these widespread uses of vanadium and the health effects of the metal, in particular the CNS effects, compounds to mitigate these effects are not well characterised. Although many chelating agents and antioxidants have been found to reduce V toxicity in some other systems of the body, most of them were ineffective in the brain because of their inability to get to the target organ (brain) via the blood-brain-barrier. Because of the intrinsic limitations and variability in the efficacy of previous heavy metal chelating agents, V detoxication is eagerly awaiting development of novel generation of therapeutic agents with various modes of actions, especially from medicinal plants (because of their perceived reduced side effects in comparism to synthetic drugs).

Study 3 has shows that pure compounds isolated from *G. carpinifolia* possess, to an extent, antioxidant activity. Since antioxidant and chelating agents have been proposed for the treatment of vanadium poisoning, this study focused on the possibility that the administration of the pure compounds (β- spinasterol, sitosterol, dibutyl phthalate, benzoic acid butyl ester and β- stigmasterol) isolated from the plant *G. carpinifolia* may have a beneficial effect against brain injury, induced by vanadium.

6.2 MATERIALS AND METHODS

6.2.1. Plant Material

Pure compounds isolated from *Grewia carpinifolia* as described in study 3 were used for the present study

6.2.2. BRAIN UPTAKE INDEX OF ISOLATED PURE COMPOUNDS

6.2.2.1 Experimental Design

The experiment to determine the rate of transport of β-spinasterol, sitosterol, dibutyl phthalate, benzoic acid butyl ester and β-stigmasterol across the blood-brain barrier (BBB) in

laboratory rat was via the brain uptake index determination. Eighteen adult rats weighing between 115-135 g from the Experimental Animal Holding Unit, Kunming Institute of Botany, Chinese Academy of Sciences were used.

6.2.3 NEURO-PROTECTIVE ACTIVITY

6.2.3.1 Experimental Animals

Seventy two weaned (juvenile) mice of about four weeks old were purchased and housed at the Animal House, Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan and randomly divided into six groups. The animals were housed under standard conditions of temperature, (25 ± 2°C) and light, (approximately 12/12 h light-dark cycle), and given access to standard diet (Animalcare® Feeds Ltd., Ogere, Nigeria) and fresh water *ad libitum*. The cages were cleaned of waste daily. All the animals were acclimatized to laboratory conditions for two weeks before the commencement of the experiment. All experiments performed on the laboratory animals in this study followed the OECD approved Standard Operation Procedures (SOPs) and were approved by the Animal Care and Use Research Ethics Committee, University of Ibadan (UI-ACUREC/App/2016/025) (appendix 1).

6.2.3.1.2 Experimental Design

The experiment to determine the neuro-protective activity of pure compounds of *Grewia carpinifolia* in laboratory mice following acute vanadium intoxication was designed to span eight days. Seventy two juvenile mice were randomly divided into six groups of 12 animals per group.

Group A received distilled water throughout the experimental period and served as control; group B, the standard group received vitamin E (500 mg/kg) every 72 hrs orally along with a daily dose of sodium metavanadate (Sigma-Aldrich, St. Louis, USA) at 3 mg/kg (Olopade *et al.*, 2011; Mustapha *et al.*, 2014) intraperitoneally (i/p) for 7 days consecutively, groups C, D and E received a single oral dose of 100 μg β- spinasterol, sitosterol and β- stigmasterol, respectively, along with sodium metavanadate at 3 mg/kg i/p for 7 days consecutively and group F received only sodium metavanadate at 3 mg/kg i/p for 7 days consecutively.

6.2.4 Chemicals

Sodium metavanadate (NaO_3V) as purchased from Sigma (St. Louis, MO); Myelin basic protein (MBP) antibody as purchased from Molecular Probes (Eugene, OR).. Nitric acid was purchased from Fisher Scientific (Pittsburgh, PA). Ethanol and the rest of the chemicals used were of analytical grade and were prepared in all glass distilled water.

The tritiated water was obtained from New England Nuclear (Boston, MA, USA). The ^{113m}In generator was purchased from New England Nuclear Radiopharmaceuticals Division (North Billerica, MA, USA.). Each 1.0 cc of indium eluted from the generator was chelated by the addition of 10 pl of sterile disodium edetate (150 mg $m1^{-I}$) solution (Endrate, Abbott Laboratories, North Chicago, IL., U.S.A.). This indium-EDTA chelate solution was then titrated with 0.5 M NaOH to neutrality (about 0.1 ml) and adjusted to pH 7.55 by addition of 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) buffer.

6.2.5 Determination of the Brain Uptake Index of Isolated Pure Compounds

The brain uptake index (BUI) was determined as described by Oldendorf (1981). β-spinasterol, sitosterol, dibutyl phthalate, benzoic acid butyl ester and β- stigmasterol were radiolabeled and administered as a rapid bolus injection with a reference substance (^{3}H-water) that was freely diffusible across the BBB into the common carotid artery of aneasthetized rat (0.2 ml in less than 0.5 s) in about 0.2 ml of buffered Ringer's solution. A second reference substance (^{113m} In) was also injected, which does not penetrate the BBB, this helped to correct for the fraction of the bolus remaining in the vascular lumen at the time of brain sampling (about 2%). After about 1-2 s passage time through the brain capillaries, the brain uptake was measured as the single pass extraction by brain tissue sampling after decapitation, which was performed within 5-15 s. The short time interval excluded systemic recirculation and also minimised potential wash-out (Oldendorf, 1981). The assumptions were that there was no transport of drug from brain to blood and that there is no metabolism during the time of the experiment.

The BUI (as percentage) was calculated using the equation below (Cornford *et al.*, 1983):

$BUI = 100[(E_{test} - E_{refv})]E_{refp}$

Where $E_{test} = (C_{brain}/C\ i_{njectate})$ test

$E_{refV} = (C_{brain}/C\ i_{njectate})\ ^{113m}$ In

$E_{refP} = (C_{brain}/C\ i_{njectate})\ ^3$H

C_{brain} is the concentration of the compound in the brain devoid of blood

$C_{injectate}$ is the concentration of the ompound in the injected buffer.

E_{test} is the brain extraction of the unknown test substance

E_{refV} is the apparent extraction of a vascular marker (non-penetrable reference)

E_{refP} is the extraction of the penetrable reference

6.2.6 NEURO-PROTECTIVE ACTIVITY

6.2.6.1 Behavioural tests

6.2.6.1.1 Morris-Water Maze

Materials

The maze was a circular washtub of 100 cm in diameter and 40 cm high. The tank was divided into four quadrants with wire stretched across the top. A height adjustable circular platform (6 cm diameter) was placed in one quadrant. It was filled with water to about 1 cm above the platform (mice were able to stand comfortably on the platform) as described by Morris (1981) (Figure 6.1).

Background

The Morris-Water Maze task is a behavioural task that has been developed to study learning and memory in animals (Sutherland and Ruby, 1989). This task uses a round pool of water in which a platform is submerged beneath the surface. When placed in the maze the animal's task is to find the hidden platform. The "learning" of the animal can be observed by the time it takes to find the platform over a number of trials. The non-cued version Morris water maze test was used in the present study in that the platform was not visible; the animal must learn the platform's spatial position in the surrounding environment in order to escape.

Animals were tried four times a day to condition them to the maze before a single trial on the last day of the study.

Method

This behavioural task consists of an acquisition phase and a probe trial. The protocol was similar to that described by Morris (1984) with minor modifications (Wang *et al.*, 2010). In the acquisition phase, mice were subjected to daily sessions of four trials per day for 3 days to

find the submerged platform that was located in the centre of the SE quadrant of the pool and remained at the same position throughout the whole experiment. At the start of the experiment animals were placed on the platform for about 20 seconds. This allowed the animals to observe the cues in the room and their relationship to the platform. After the 20 seconds, the animals were gently placed in the water in the NW quadrants with its head facing the wall. Timing was started simultaneously to record the time it took the animal to find the platform once it was placed in the water. If on any trial an animal did not find the platform in 120 seconds, such animal was removed from the water and placed on the platform. Once an animal found the platform, it was allowed to sit for 10 seconds and then removed from the maze. The mice were started in the same position (or quadrant of the pool) at the beginning of each trial. The learning curve for each animal was constructed by constructing the trial number on the X-axis while the Y-axis represented the time to platform (TTP). After completion of daily training, the animals were returned to their home cages for rest. 24 h later, each mouse was subjected to the retention test (probe trial) in which the platform was removed. The mice were allowed to swim freely for 120 s. The number of crossings over the position at which the platform had been located and the swimming time in the quadrant of the former platform position was recorded as measures for spatial memory.

Figure 6.1: The water maze for testing learning and memory in mice

6.2.6.1.2 Open-field Test

Locomotor and exploratory activities were measured by an open-field task box (Coulbourn Instruments L.L.C., PA, USA) as described in chapter 3, section 3.2.8.2.3.1 of study 1

6.2.6.1.3 Fore-limb Support (Hanging Wire) Test

This test was performed as described by Van Putten *et al.,* (2012) in chapter 4 of the present study section 4.2.8.3.

Animals were sacrificed on the eight day at the end of the behavioural test.

Tissue homogenisation

Freshly harvested brains (placed on Petri dishes) were individually weighed. The entire organ was placed in a 10ml plastic homogenization tube and appropriate volume of phosphate buffer saline (PBS) was added as the homogenization buffer. The tissue was with a rotor-stator homogenizer (RW16 Basic S1 Overhead stirrer, IKA® Inc., USA) with the rotor turning at 1,500 rpm for about 10-30 sec resting for 5 sec within an interval for a total of 120 s. The tissues were homogenised by going up and down the tube with the stator twice only so as to keep the state of homogenisation of each organ uniform from sample to sample. The homogenising tip was cleaned with distilled water, washed thrice with 70% ethanol and rinsed finally with distilled water between samples. The homogenate was centrifuged for 10 min at 4,000 rpm to yield a pellet that was discarded and a supernatant that was used in the assays (Ohkawa *et al.,* 1979). The homogenates were later poured into test tube and placed in a centrifuge and set at 3 000 rpm for 7 minutes. The supernatants were used for the *in vivo* antioxidant assay

6.2.6.1.4 *IN VIVO* ANTIOXIDANT ASSAY

6.2.6.1.4.1 Hydrogen Peroxide Colorimetric Assay

The hydrogen peroxide concentration in brain tissue was determined using the colorimetric hydrogen peroxide (H_2O_2) assay kit (#CS0270 Sigma)

Principle

The kit employed a colour reagent that contained a dye, xylenol orange, in an acidic solution with sorbitol and ammonium iron sulphate that reacted together to produce a purple colour proportional to the concentration of H_2O_2 in the sample. The exact mechanism of the colour

reaction is not known, but probably involves coordinated iron reacting with H_2O_2 and the dye molecule.

Reagents

Hydrogen peroxide standard (# H 2163) – 0.5 ml solution in water, 100,000 ng/ml, contained stabiliser, light sensitive.

Hydrogen peroxide colour reagent, (# H 2288) – contained xylenol orange dye in an acidic solution with sorbitol and ammonium iron sulphate

Sample Diluent, 50 mM phosphate, pH 6.0

Deionised or distilled water.

Preparation

1. The standard was equilibrated to room temperature.
2. Serial standard dilutions were prepared as indicated in Table 6.1.

Procedure

1. A standard curve was plotted for the assay (Figure 6.1.)
2. 50 µl of sample diluent was added into the blank (zero standard) wells.
3. 50 µl of standard was also added into separate wells.
4. 50 µl of sample was added into wells.
5. 100 µl of hydrogen peroxide colour reagent was added to all the wells.
6. The mixture was incubated for 30 minutes at room temperature.
7. The optical density (OD) of each well was read using a multiwell plate reader (ELx 800, Biotek® USA) set to 550 nm.

All readings were done in duplicate.

Results

Mean absorbance = $(A_0 – A_1)$

Where A_0 is the absorbance of the sample, and

A_1 is the absorbance of the blank (zero standards).

A standard curve was constructed by plotting the mean absorbance against concentration.

Table 6.1: Serial standard dilutions of the colorimetric hydrogen peroxide assay

Tube #	Sample diluent µL	Standard from tube #: -µL	Final standard concentration (ng/ml)
0	Standard vial 100,000 ng/mL		
1	966 µL	34 µL (0)	3,400
2	500 µL	500 µL	1,700
3	500 µL	500 µL	850
4	500 µL	500 µL	425
5	500 µL	500 µL	212
6	500 µL	500 µL	106

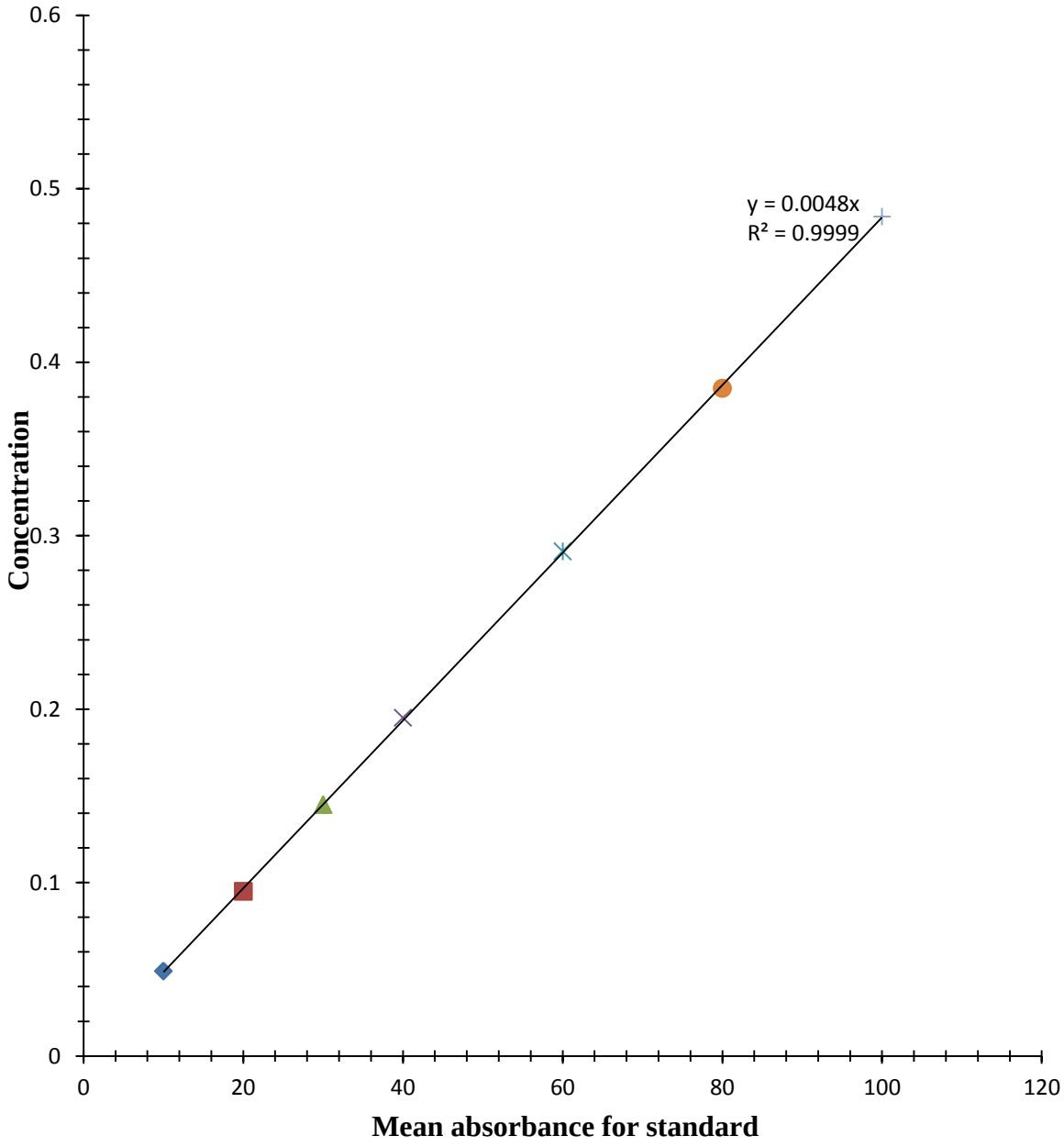


Figure 6.2: Standard curve for the estimation of hydrogen peroxide assay

6.2.6.1.4.2 Determination of Catalase activity

Catalase activity was determined according to the method of Sinha (1972).

Principle

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

Reagents

1. 5% $K_2Cr_2O_7$ (dichromate solution) 2.5 g of $K_2Cr_2O_7$ (Hopkins & Williams, England) was dissolved in 40 ml of distilled water.
2. 0.2 M H_2O_2

Exactly 2.3 ml of H_2O_2 (Surechem Products Ltd.) was mixed with distilled water in a 100ml volumetric flask and the solution made up to the mark with same.

3. Dichromate/acetic acid

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

4. Phosphate buffer (0.01 M, pH 7.4)

Exactly 0.496 g of K_2HPO_4 $12H_2O$ (Oxford Laboratory Unit, India) and 0.973 g KH_2PO_4 $2H_2O$ (Sigma Chemical Company, Oxford) dissolved in 100 ml of distilled water. The pH adjusted to 7.4.

Procedure

Determination of catalase activity of samples

Exactly 0.2 ml of sample was mixed with 0.8 ml distilled H_2O to give 1 in 5 dilution of the sample. The assay mixture contained 2 ml of H_2O_2 solution (400 μmoles) and 2.5 ml of phosphate buffer in a 10 ml flat bottom flask. 1 ml of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown

into 1 ml dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

Calculation

H_2O_2 consumed = 800 µmoles-H_2O_2 remaining

CAT activity = $\frac{H_20_2 \text{ remaining}}{\text{mg protein}}$

One unit of CAT activity represents the amount of enzyme required to decompose 1 µmol of H_2O_2/minute.

6.2.6.1.4.3 Determination of superoxide dismutase activity

The activity profile of superoxide dismutase (SOD) in the homogenates was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of adrenaline at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O^-_2) radical generated by the xanthine oxidase reaction caused the oxidation of adrenaline to adrenochrome and the yield of adrenochrome produced per O^-_2 introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of adrenaline. These results led to the proposal that autoxidation of adrenaline proceeds by at least two distinct pathways, only one of which is a free radical chain reaction, involving superoxide (O^-_2) radical and hence inhibitable by SOD.

Reagents

1. 0.05 M Potassium phosphate buffer (pH 7.8).

6.97 g of K_2HPO_4 (Oxford Laboratory Unit, India) and 1.36 g KH_2PO_4 (Sigma Chemical Company, Oxford) were dissolved in 900 ml of distilled water and the volume made up to 1 litre. The pH was adjusted to 7.8.

2. 0.05M Carbonate buffer (pH 10.2)

Exactly 14.3 g of Na_2CO_3 $10H_2O$ (BDH Ltd; Poole, England) and 4.2 g of $NaHO_3$ (May & Baker Ltd, England) were dissolved in 900 ml of distilled water and then made up to 1 litre. The pH was adjusted to 10.2.

3. 0.3 mM Adrenaline (Tokyo Chemicals Industry Co. Ltd, Tokyo Japan)

0.0137 g of adrenaline was dissolved in 200 ml distilled water and then made up to 250 ml. This solution was freshly prepared.

Briefly, 50 mg of adrenaline was dissolved in 100 ml distilled water and acidified with 0.5ml concentrated hydrochloric acid. This preparation prevents oxidation of adrenaline and is stable for four weeks. 30 µL of erythrocyte extract was added to 2.5 ml 0.05 M carbonate buffer (pH 10.2), followed by the addition of 300 µL of 0.3 mM adrenaline. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation

Increase in absorbance per minute = $A_3 - A_0$

Where, A_0 = absorbance after seconds

A_3 = absorbance 150 seconds

$$\% \text{ inhibition} = \frac{\text{increase in absorbance for substrate}}{\text{increase in absorbance of blank}} \times 100$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute.

6.2.6.1.4.4 Assessment of Lipid Peroxidation

Lipid peroxidation was determined by measuring the formation of TBARS according to the method of Varshney and Kale (1990).

Principle

Under acidic condition, MDA produced from the peroxidation of fatty acid membranes react with the chromogenic reagent, 2-thiobarbituric acid (TBA) to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm using spectrophotometer (ELx 800, Biotek®, USA). This reaction is illustrated in Figure 6.3. The pink chromophore is readily extractable into organic solvents such as butanol.

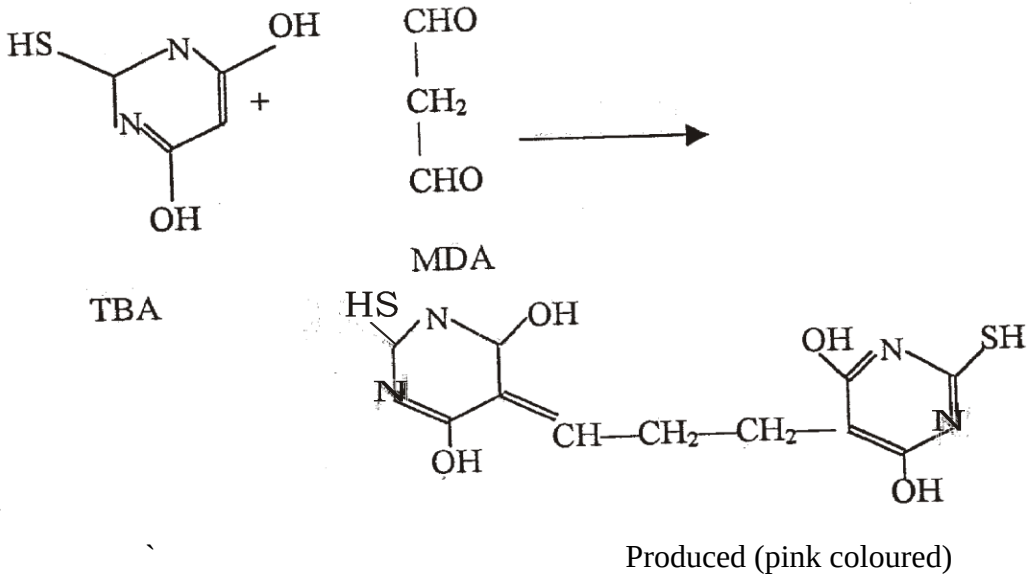


Figure 6.3: Reaction of 2-thiobarbituric acid with malondialdehyde

Reagents

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

6 g of TCA (CCl_3COOH) (BDH Chemicals, England) was dissolved in distilled water and made up to 20 ml.

2. 0.75% Thiobarbituric acid (TBA)

This was prepared by dissolving 0.15 g of TBA in 0.1M HCl (May & Baker Ltd.) and made up to 20 ml.

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

1.15 g of KCl and 2.36 g of Tris base (Lab Tech Chemicals) were dissolved separately in distilled water and made up to 100 ml. The pH was then adjusted to 7.4.

Procedure

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

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

6.2.6.1.4.5 Estimation of reduced glutathione level

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

Principle

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is, therefore, based upon the development of a relatively stable (yellow) colour, when 5′, 5′ – dithiobis-(2-nitrobenzoic acid, DTNB) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412 nm. This reaction is illustrated in Figure 6.4. Reduced glutathione is proportional to the absorbance at 412 nm.

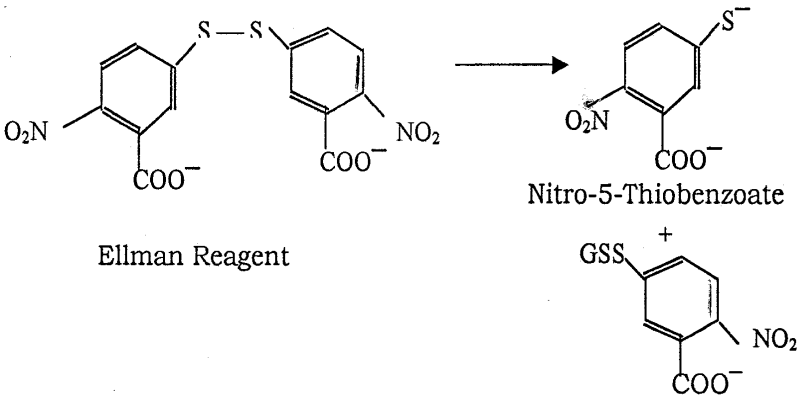


Figure 6.4.: **Reaction of reduced GSH with Ellman's reagent**.

Reagents

GSH working standard

1. 40 mg GSH (Sigma-Aldrich Chemical, USA) was dissolved in 0.1 M phosphate buffer, pH 7.4, and made up to 100 ml with the same.

2. 0.1 M Phosphate buffer (pH 7.4)

a) First 0.1 M K_2HPO_4 $12H_2O$ (Oxford Laboratory Unit, India) was prepared by dissolving 0.992 g in 200 ml of distilled water.

b) 0.1 M KH_2PO_4. $2H_2O$ (Sigma Chemical Company, Oxford) (MW=156.03) was prepared by dissolving 1.946 g in 200 ml of distilled water.

Finally 0.1 M phosphate buffer was prepared by adding 200 ml of (a) to 100 ml of (b) and the pH adjusted to 7.4 with drops of concentrated HCl or NaOH as the case may be.

3. Ellman's reagent (5′, 5′-Dithiobis- (2-nitrobenzoate) (DTNB) (Sigma-Aldrich Ltd)

   a. This was prepared by dissolving 0.04 g of Ellman's reagent in 0.1M Phosphate buffer and made up to 200 ml.

4. Precipitating Solution

4% of sulphosalicyclic acid ($C_7H_6O_6S.2H_2O$ Mol. Wt. 254.22) (Rem Light Laboratories, PVT LTD, India) was prepared by dissolving 4.8 g of sulphosalicyclic acid in 120 ml of distilled water. This reagent is stable for approximately three weeks at 40 °C.

5. Blank

2 ml of the 0.1 M phosphate buffer was added to 3 ml of diluted precipitating solution (3parts to 2 parts of distilled water).

Calibration of GSH standard curve

Procedure

Serial dilutions of the GSH working standard were prepared as shown in the Table 6.2. To each was added 4.5 ml of Ellman's reagent. GSH was proportional to the absorbance at 412 nm (as well as at 430 nm using colourimeter). The readings were taken before five minutes. This is because the colour is stable for at least 5 minutes after the addition of Ellman's reagent. After 10 minutes of standing, there was frequently a loss of 1 to 2% of the colour. However, an additional delay of 5–15-minutes will result in only a small error. Each sample was prepared in duplicate. A graph of optical density against concentration was plotted.

Estimation of GSH level

Exactly 0.5 ml of sample was placed into the tubes and 0.5 ml of the precipitating solution was mixed with sample. The mixture was centrifuge at 4,000 rpm for 5 minutes. 0.5 ml of the supernatant was put in another test tube; 4.5 ml of Ellman's reagent was added to the supernatant in the tube. The optical density was read at 412 nm against distilled water as blank. GSH was proportional to the absorbance at that wavelength and the estimate was obtained from the GSH standard curve illustrated in Figure 6.5.

Table 6.2: Preparation of glutathione Standard Curve

Stock (ml)	PO_4 buffer (ml)	Ellman's reagent (ml)	Absorbance (412 nm)	Glutathione conc. (µg/ml)
0.02	0.48	4.5	0.04	8
0.05	0.45	4.5	0.101	20
0.10	0.40	4.5	0.194	40
0.20	0.30	4.5	0.380	80
0.30	0.20	4.5	0.572	120
0.40	0.10	4.5	0.749	160

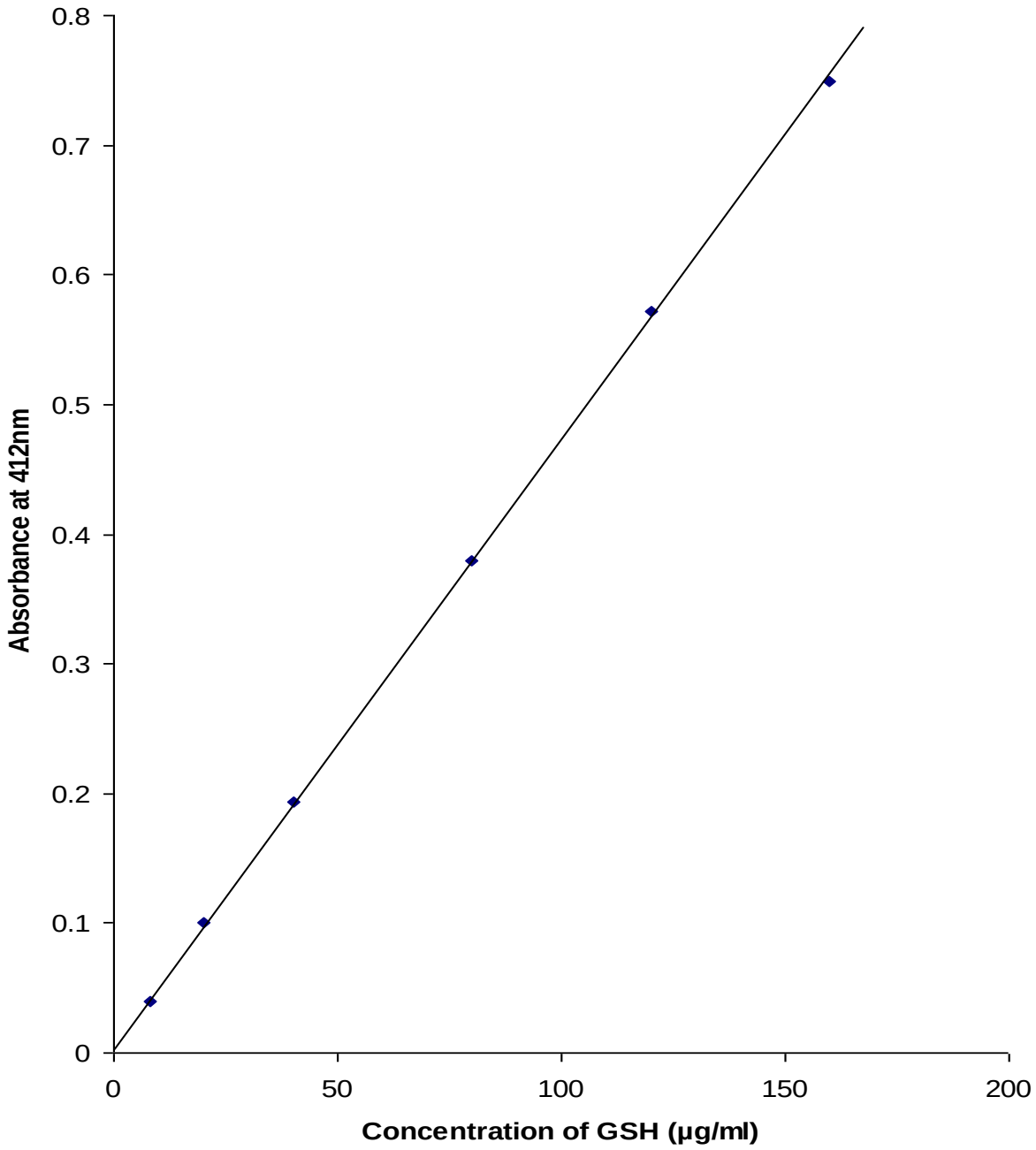


Figure 6.5: Glutathione standard curve

6.2.6.1.4.6 Glutathione Peroxidase

Glutathione peroxidase (GPx) activity was measured according to Buetler *et al.* (1963).

Reagent	Concentration
• NaNO3	0.0325 g in 15 ml of distilled water
• GSH (Sigma-Aldrich Chemical, USA)	0.123 g in 10 ml in phosphate buffer
• H_2O_2 (Surechem Products Ltd.)	28 uL in 100 ml of distilled water
• TCA (Sigma-Aldrich Chemical, USA)	2 g in 20 ml of distilled water
• K_2HPO_4 (Oxford Laboratory Unit, India)	5.23 g in 100 ml of distilled water
• DTNB (Sigma-Aldrich Chemical, USA)	0.04 g in 100 ml of phosphate buffer
• Phosphate buffer	0.992 g of K_2HPO_4 and 1.946 g of KH_2PO_4 in 200 ml of distilled water

Procedure

0.05 ml of phosphate buffer , 0.1 ml of $NaNO_3$, 0.2 ml of GSH, 0.1 ml of H_2O_2, 0.5 ml of sample, 0.6 ml of distilled water were added into a test tube and the mixture incubated at 37 °C for 3 minutes. 0.5 ml of TCA was then added and centrifuge at 3,000 rpm for 5minutes. 2 ml K_2PHO_4 and 1ml DTNB were added to 1ml of supernatant and the absorbance read at 412 nm against blank.

Calculation

Consumed GSH/mg protein = 245.84-remaining GSH

One unit of GPx activity is defined as the amount of enzyme required to utilize 1 nmol of NADPH/minute at 25 °C.

6.2.6.2 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections. It enables the analysis of protein expression in the context of tissue morphology. It exploits the specific binding of an antibody to its target epitope in combination with a chromogenic or fluorescent readout. This is especially useful for assessing the progression and treatment of diseases such as cancer. The IHC protocol optimised for paraffin-embedded samples was performed as described by Crosby *et al.* (2009). The basic steps for the IHC-P protocol were as follows:

A. Reagents Preparation

Xylene, ethanol, anhydrous denatured, histological grade (100% and 95%)

Wash buffer: 2% phosphate buffered saline (PBS) milk. To prepare 2 % PBS milk 5 g of skimmed milk powder was added to 250 ml 1X PBS, pH 7.4.

Antibody dilution: Primary antibody was diluted (1:1000) with 1% PBS milk+1% Triton X

The secondary antibody was diluted (1:200) with 1% PBS milk+1% Triton X

Citrate. 10 mM- Sodium citrate buffer: To prepare 1 L, 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) was added to 1 L dH_2O. The pH of the mixture was adjusted to pH 6.0 with 1 M NaOH.

3% hydrogen peroxide: To prepare 100 ml, 10 ml 30% hydrogen peroxide was added to 90 ml H_2O.

Blocking Solution: H_2O_2 /CH_3OH: 25 ml 30% H_2O_2 was added to 225 ml methanol for 20 minutes.

Substrate: VectorStain® DAB Substrate Kit (#SK-4100)

Fixation

Formalin Solution (10%, buffered neutral): Formaldehyde (37-40%), 100 ml distilled water and 900 ml NaH_2PO_4 were mixed to dissolve.

0.2 M Phosphate Buffer (PB), pH 7.4: 10.9 g Na_2HPO_4 was added to 500 ml distilled water, the solution pH was thereafter adjusted to 7.4.

B. Sample preparation and fixation

Brain samples were fixed in 10% neutral buffered formalin (NBF) for 18-24 hours. After fixation, the tissue blocks were embedded in paraffin in a mould. These were allowed to cool after which thin slices (4-6 μm) were cut on a microtome, and floated in sections in a water bath. Sections were mounted on to charged slides (improved adherence to the slide) and dried overnight.

C. Deparaffinisation/Rehydration

Before the commencement of the staining protocol paraffin wax was removed from the sample and the sample rehydrated to enhance quality staining. Sections were placed in three containers of xylene for 5 minutes each to remove the paraffin wax. Rehydration was done by placing sections in two containers of 100% ethanol for 10 minutes each. Sections were thereafter placed in two containers of 95% ethanol for 10 minutes each. To complete the rehydration process, sections were washed twice in distilled water (dH_2O) for 5 minutes each. Slides were not allowed to dry at any time during this procedure to prevent inconsistent staining.

D. Antigen retrieval

Antigen retrieval was done in order to expose the antigenic sites to allow the antibodies to bind. This was due to the methylene bridges formed during fixation, which cross-linked proteins and therefore masked antigenic sites. The antigen retrieval serves to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind.

Antigen retrieval was done with sodium citrate pH 6.0 as buffer. Following rehydration, slides were placed in a plastic rack dipped the buffer and kept in a microwave and heat at full power for 15 minutes. The container was removed from the microwave and the slides allowed to sit in the warm buffer for 20 minutes until it reached room temperature. The rack was removed and slides rinsed in dH_2O thrice for 5 minutes each.

E. Immunohistochemical staining protocol

To quench endogenous peroxidase activity in the samples, which may lead to high background staining, sections were blocked in methanol/H_2O_2. Following this, slides were rinsed and rocked in PBS thrice for 5 minutes each.

A hydrophobic pen was used to draw a large circle around the sample, taking care not to touch the sample. This created a hydrophobic boundary so that a smaller volume of antibody solution can be used and allowed multiple sections on one slide. To prevent non-specific binding of the antibody to the tissues, each section was further blocked with 2% PBS for 1 hour.

Blocking solution was removed and 100–400 µl primary antibody (mouse monoclonal anti-MBP SMI-94) (Abcam®) was added to each section. This was incubated overnight at 4 °C in a humidified chamber. The overnight incubation allowed ample time for the primary antibody to bind. The primary antibody solution was poured off and sections were rinsed in 1% PBS three times for 5 minutes each.

After removal from PBS, excess liquid was removed by wiping the bottom of the slide with tissue paper and slides were placed back in the humidity chamber. Secondary antibody (donkey anti-mouse IgG H&L (biotin) #ab6819) (Abcam®) was added. Slides were incubated in the secondary antibody for 1 hour at room temperature. The secondary antibody was poured off and sections were washed in PBS buffer three times for 5 minutes each. Slides were placed back in the humidity chamber.

Signal amplification

To achieve a stronger signal/staining, more enzyme or fluorophore were added to the slides by incubating in Avidin-biotin complex (ABC) solution (Vectastain®, CA, USA) for 1 hour as described by Hsu *et al.* (1981). This process utilizes the high affinity of avidin, a protein found in chicken egg white, for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible. In brief, the primary antibody is bound to the protein of interest. A biotinylated secondary antibody is then bound to the primary antibody. In a separate reaction, a complex of avidin and biotinylated enzyme is formed by mixing the two in a ratio that leaf some of the binding sites on avidin unoccupied. This complex is then incubated with the tissue section after the antibody incubations. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody. This allows the attachment of more enzymes to the target than is possible using an enzyme-conjugated secondary or primary antibody only.

The ABC solution was poured off and rinsed in PBS thrice for 5 minutes each.

The coloured product of the myelin basic protein was later developed with a chromogen; 3, 3'- diaminobenzidine (DAB) which has a brown Intense colour. DAB was added to the tissues and incubated on the benchtop for 5-10 minutes. DAB was poured off from slides and immersed in dH_2O.

H. Dehydrating and Mounting Sections

Sections were placed in alcohol as follows for 5 minutes each to dehydrate

100% ethanol

100% ethanol

100% ethanol

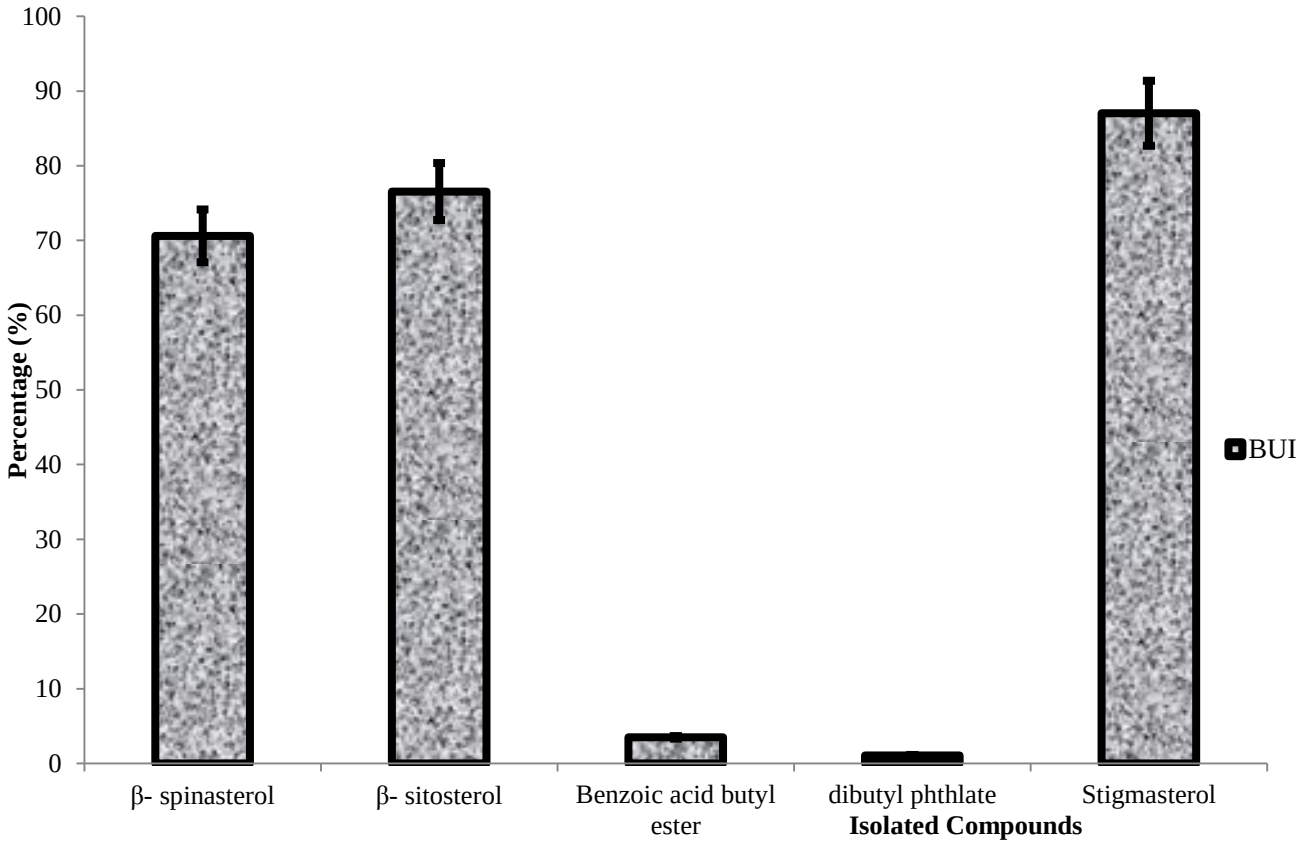
Xylene

Sections were then mounted with cover-slips using mounting medium (Cytoseal) being careful to avoid introducing air bubbles. The mounting medium was allowed to set and slides were viewed on a microscope.

6.3 RESULTS

6.3.1 BRAIN UPTAKE INDEX OF ISOLATED PURE COMPOUNDS

In the barbiturate-anaesthetized adult rat, the brain uptake index of β- spinasterol, sitosterol, benzoic acid butyl ester, dibutyl phthalate and β- stigmasterol were 70.63 ± 10.21%, 76.54 ± 8.36%, 3.5 ± 0.73%, 1.07 ± 0.34%, 87.03 ± 12.05%, respectively on a single capillary passage. Stigmasterol had the highest uptake in the brain, while dibutyl phthalate had the lowest uptake. The principal finding of the present study is that β-spinasterol, sitosterol, and β- stigmasterol are available for transport through the BBB (Figure 6.6.)



BUI- Brain uptake index

Figure 6.6: Brain uptake index of β- spinasterol, sitosterol, benzoic acid butyl ester, dibutyl phthalate and β-stigmasterol isolated from ethanol extract of *Grewia carpinifolia* leaf

6.3.2 BEHAVIOURAL TESTS

6.3.2.1 Morris Water Maze

Acquisition Trails - Two-way repeated-measures ANOVA (day × group) revealed that the group had a significant effect on the escape latency. A significant day effect on the escape latency was also observed, which indicated that mice improved over the course of training trails in all groups, except the group treated with sodium metavanadate only. Subsequent comparisons further suggested that no difference was observed between the control and treated groups on escape latency. The mice injected with sodium metavanadate took a longer time to locate the platform than did mice in the sitosterol and stigmasterol group (Figure 6.7.), but it became significant only on day 2 and 3. β-sitosterol (100 μg) attenuated the spatial learning deficits observed following sodium metavanadate as indicated by a reduction of the escape latency (day 1, P = 116 s; day 3, P = 15 s) in comparison to the standard control group of α-tocopherol (day 1, P = 114 s; day 3, P = 33 s. All the groups treated with the pure compounds showed a trend of increasing the escape latency.

Probe Trail - As seen in Figure 6.8., the swimming time in the target quadrant was significantly ($\alpha \leq 0.05$) lower in the negative control group treated with only sodium meravanadate. Spinasterol slightly increased the swimming time in the target quadrant but without any significant difference. By contrast, β-sitosterol and stigmasterol increased ($\alpha \leq 0.05$) the swimming time in the target quadrant markedly when compared to all other treated groups including the negative control.

6.3.2.2 Open-field Test

In the Open-field test, the frequency of line crossed by animals in the control group and groups treated with the pure compounds was not significantly different. This parameter was however, significantly ($\alpha \leq 0.05$) lower in group administered with sodium metavanadate only (Figure 6.9.). Mean values recorded for the time of stretch attend postures of animals were 36.31± 5.95 for control, 40.09 ± 6.75 for the groups administered with α-tocopherol; 23.64 ± 6.34, 19.16 ± 6.09, 21.70 ± 2.59 for group administered with spinasterol; sitosterol and stigmasterol respectively and 56.91 ± 7.02 in the vanadium only group (Figure 6.10). These values were significantly ($\alpha \leq 0.05$) lower in the pure compound-treated groups in relation to the control groups. The grooming and rearing frequency of experimental animals

were lower in the extract treated group when compared with the control, although this was significant ($\alpha \geq 0.05$) except in the vanadium only group.

6.3.2.3 Hanging Wire Test

Animals treated with sodium metavanadate only had a significant ($\alpha \leq 0.05$) decrease in grip strength and an increased hanging latency time when compared to normal control animals. Co-treatment with pure compounds from *Grewia carpinifolia* (spinasterol; sitosterol and stigmasterol) improved significantly ($\alpha \leq 0.05$) the grip strength and reduced hanging latency, when compared to the standard group (Figure 6.12).

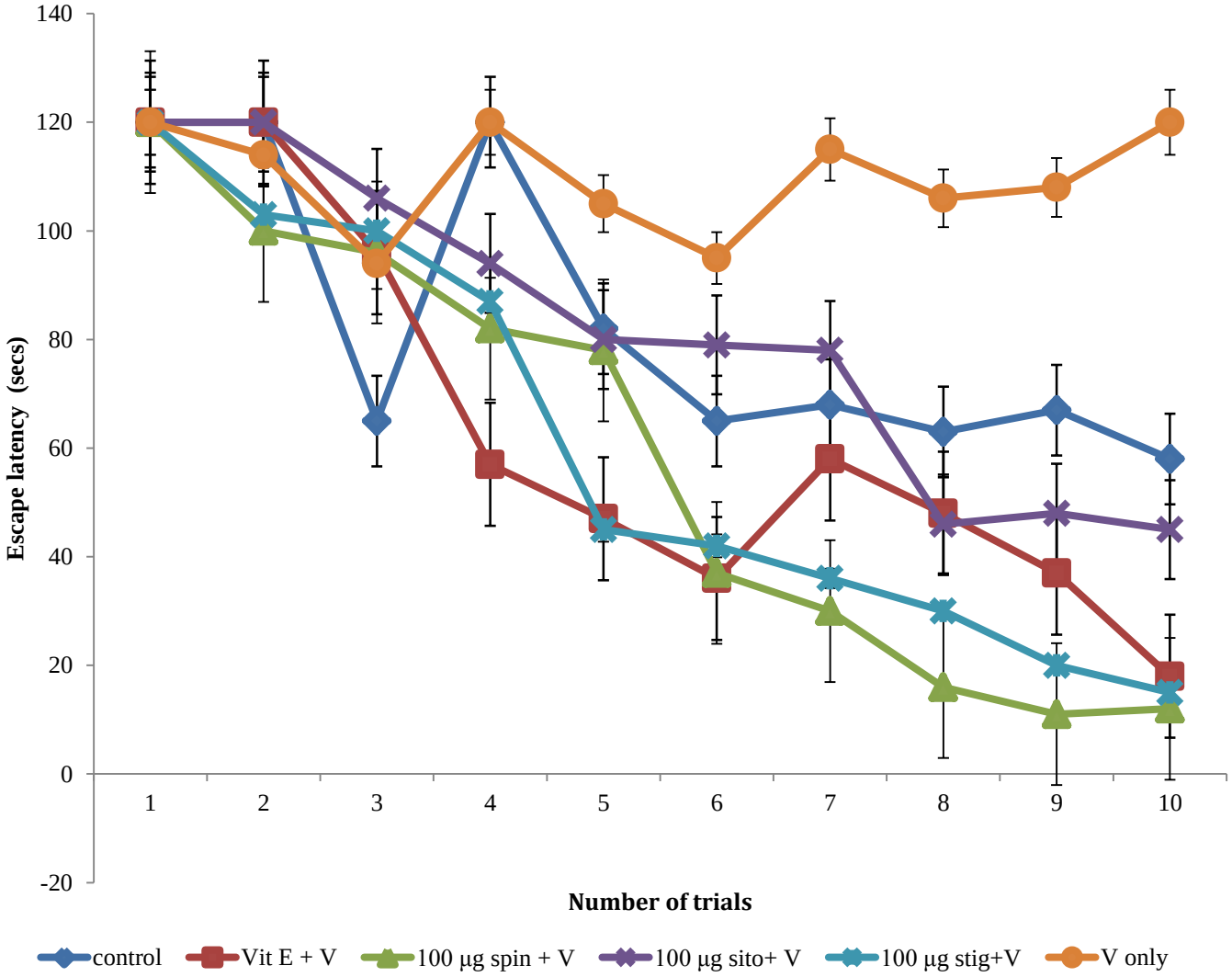
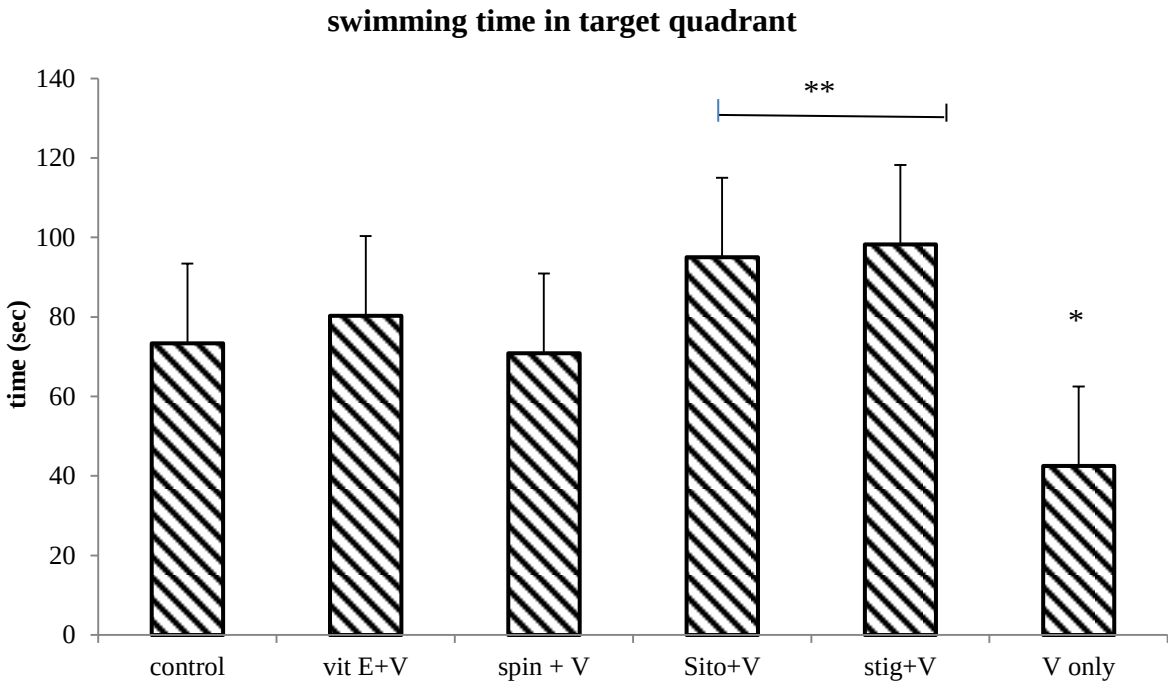
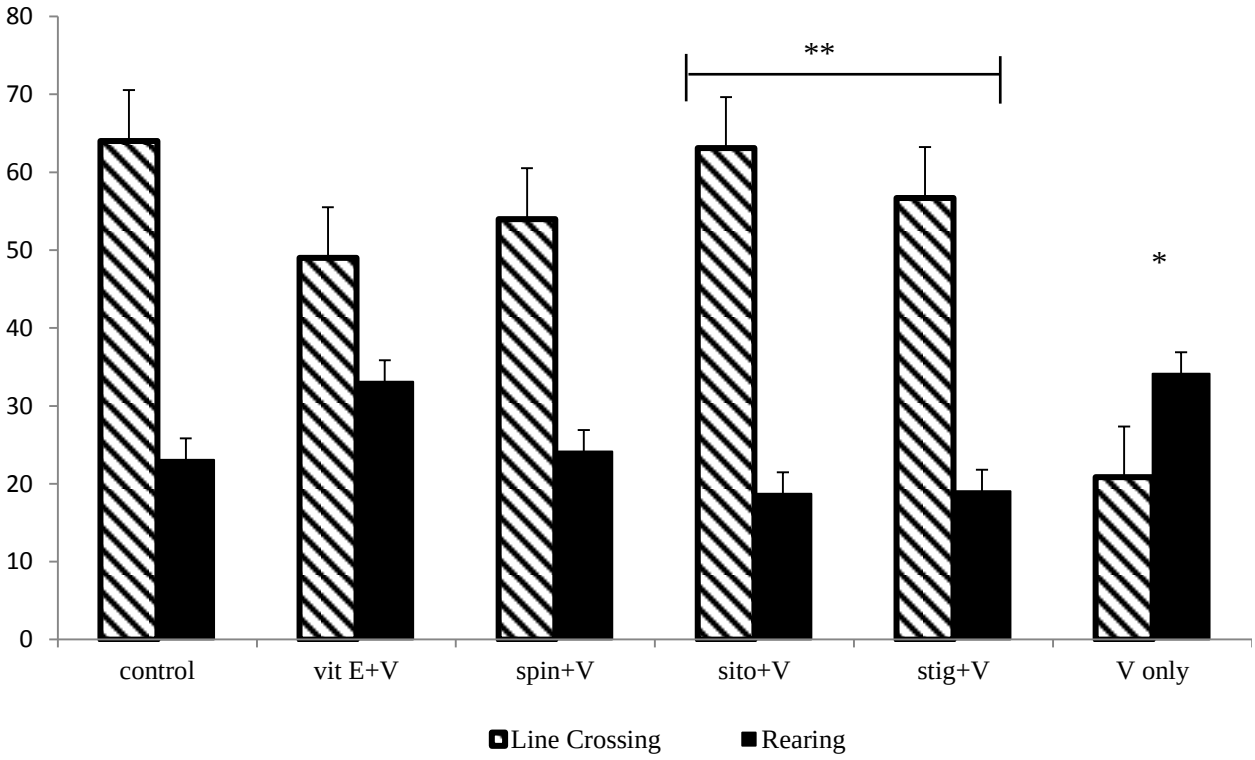


Figure 6.7: Morris-water Maze acquisition trial



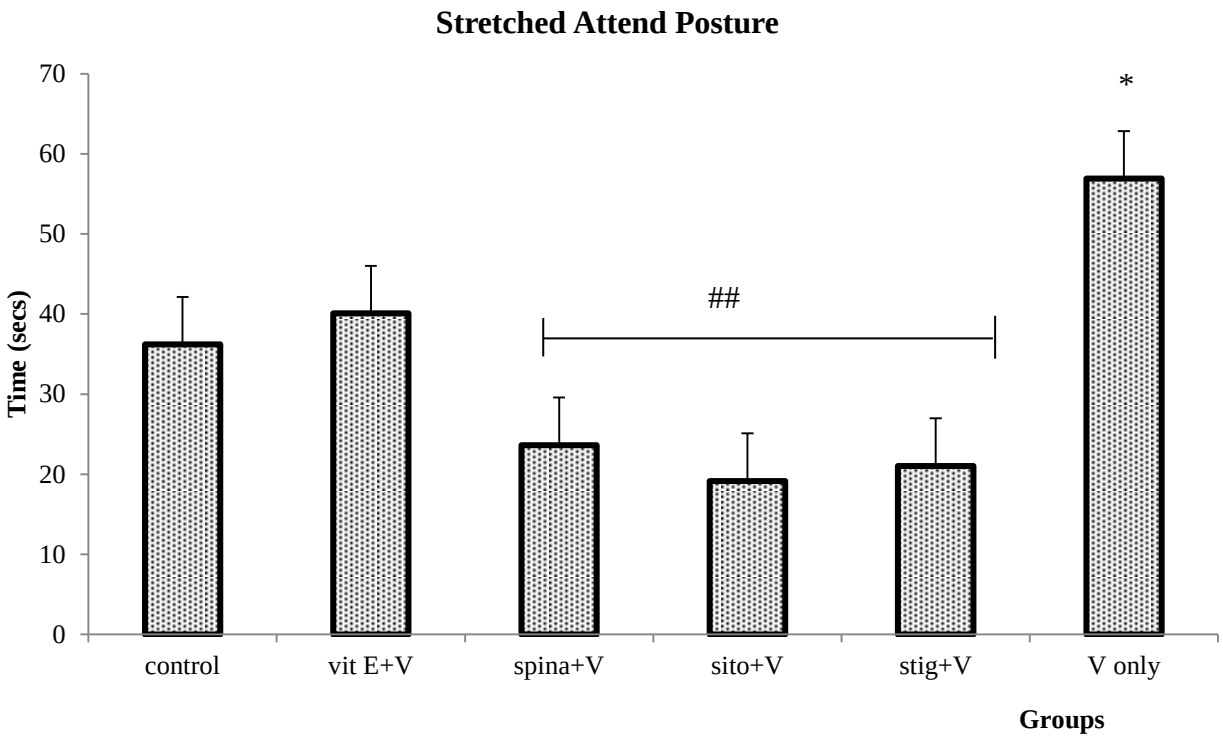
n=12; Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito= sitosterol; stig= stigmasterol *significantly ($\alpha \leq 0.05$) different from the normal control, ** *significantly ($\alpha \leq 0.05$) from the sodium metavanadate only group

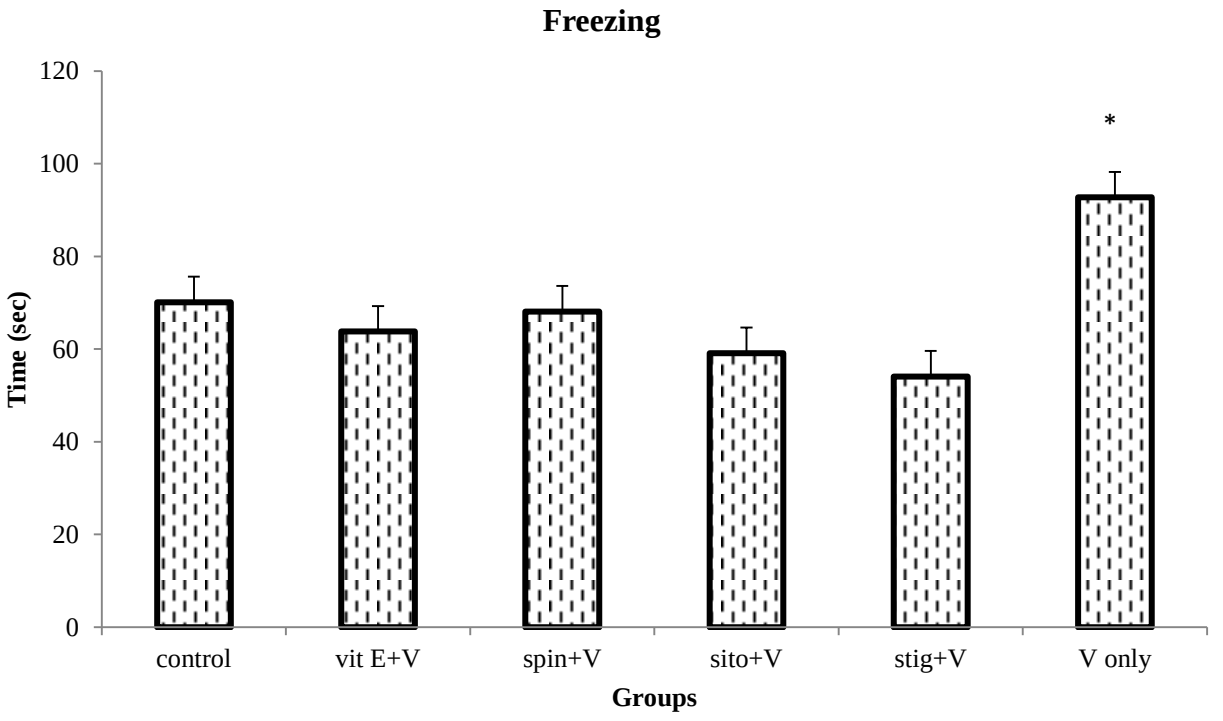
Figure 6.8: Time spent swimming in the target quadrant in the Morris-water maze probe trial following concurrent administration of spinasterol, sitosterol and stigmasterol and vanadium



n=12; Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito= sitosterol stig= stigmasterol *significantly ($\alpha \leq 0.05$) different from the normal control, ** *significantly ($\alpha \leq 0.05$) from the sodium metavanadate only group

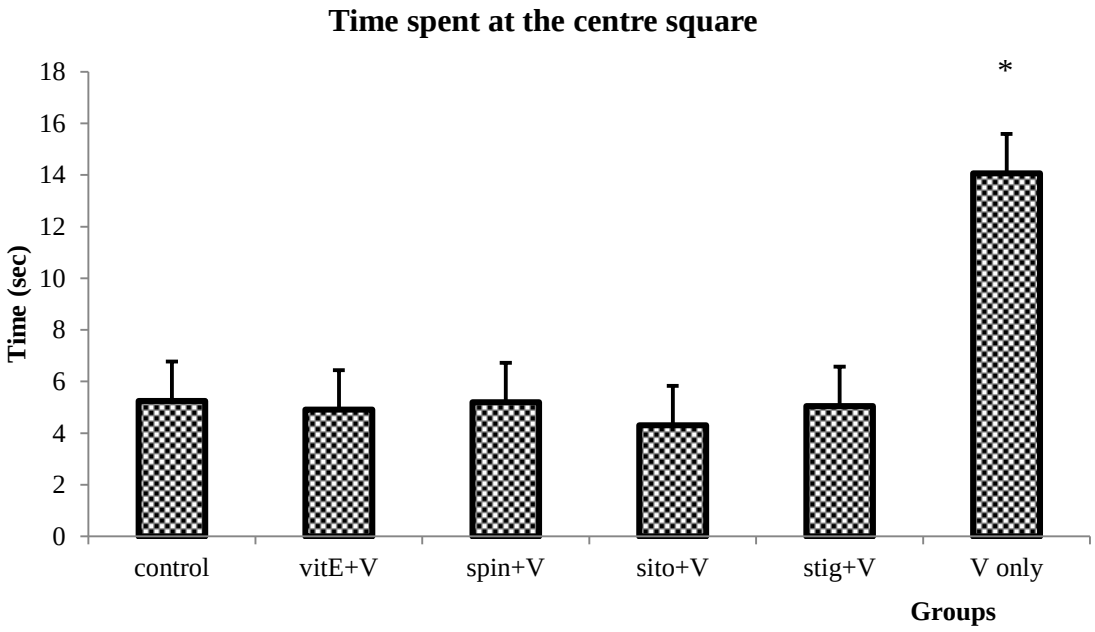
Figure 6.9: Number of line crossings and rearings in the open-field following concurrent administration of spinasterol, sitosterol and stigmasterol and vanadium

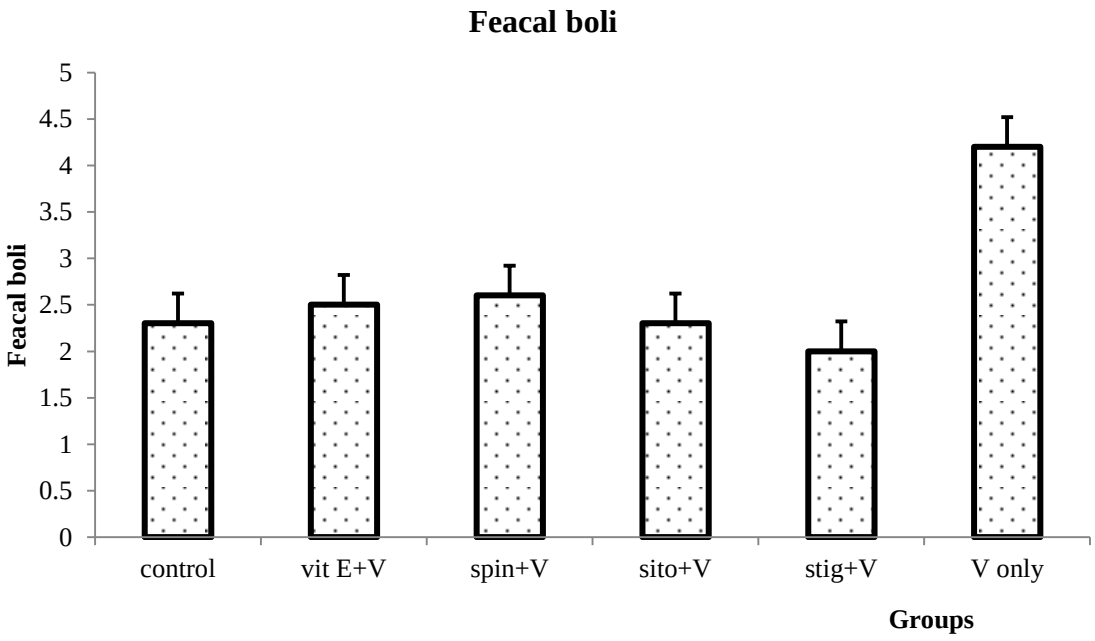




n=12, Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito= sitosterol stig= stigmasterol
*significantly ($\alpha \leq 0.05$) different from the normal control, ## significantly ($\alpha \leq 0.05$) from the sodium metavanadate only group

Figure 6.10: Stretch-attend posture and Freezing in the Open-field test following concurrent administration of spinasterol, sitosterol and stigmasterol and vanadium

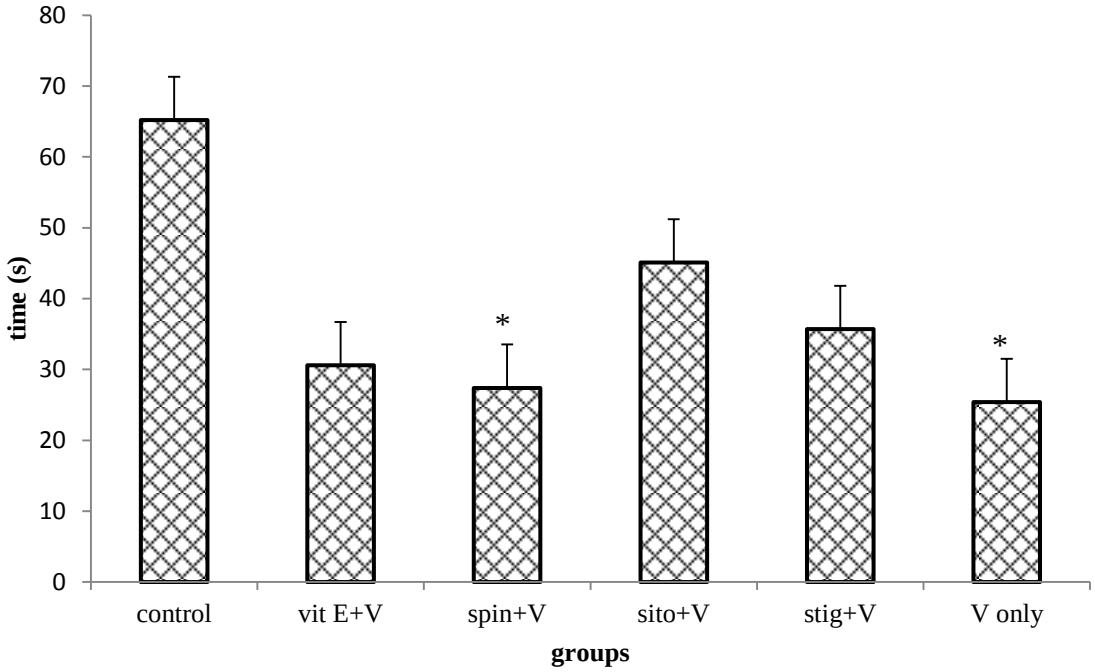




n=12, Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito= sitosterol stig= stigmasterol

*significantly ($\alpha \leq 0.05$) different from the normal control

Figure 6.11: Time spent at the centre square and number of faecal boli in the Open-field test following concurrent administration of spinasterol, sitosterol and stigmasterol and vanadium

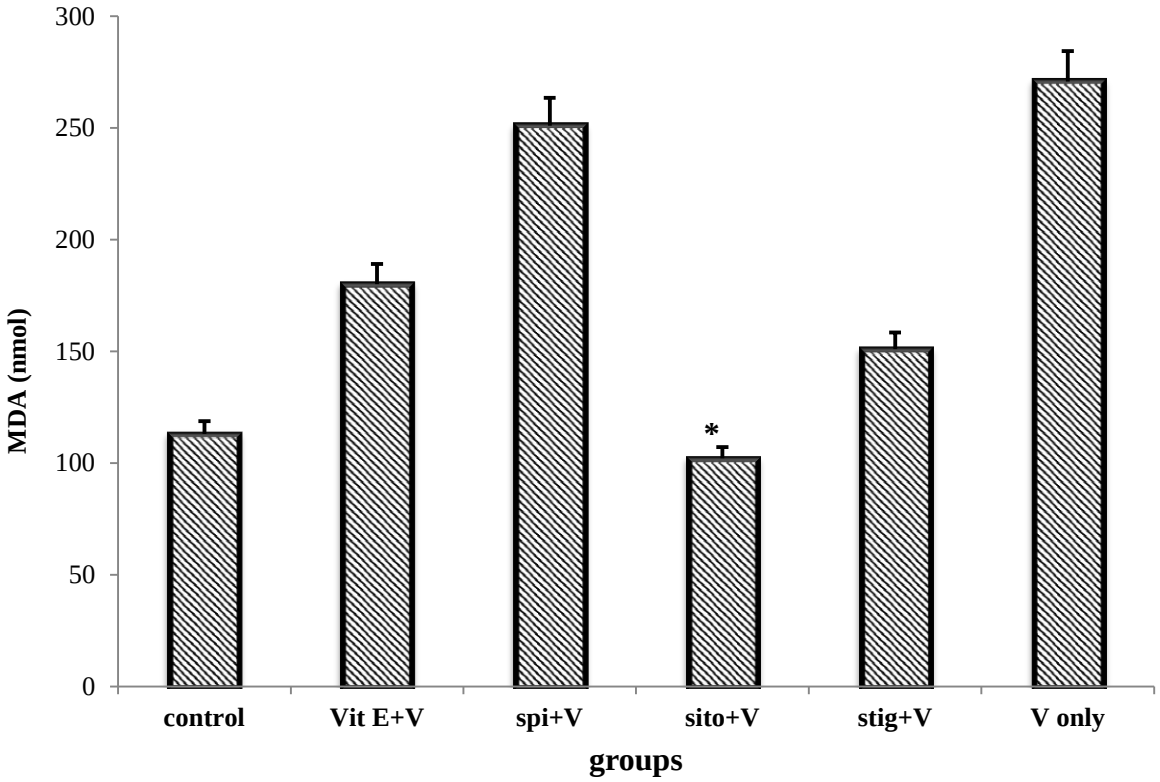


n=12, Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito = sitosterol stig= stigmasterol
*significantly ($\alpha \leq 0.05$) different from the normal control

Figure 6.12: Time spent on the hanging wire following concurrent administration of spinasterol, sitosterol and stigmasterol and vanadium

6.3.3 *IN VIVO* ANTIOXIDANT STUDY

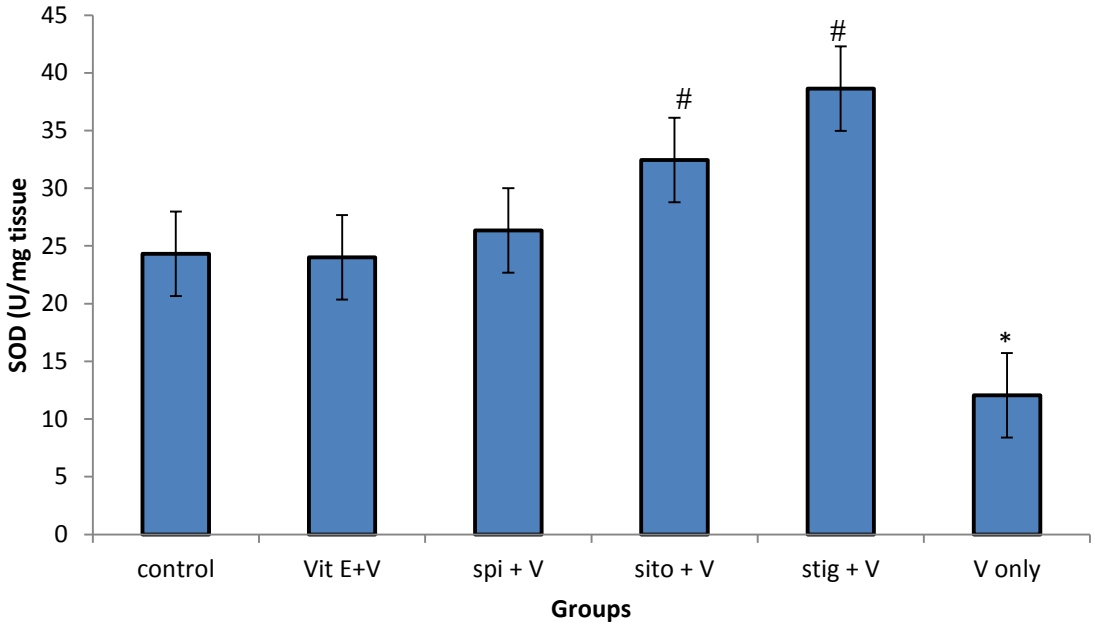
In the present study vanadium toxicity in mice resulted in a significant ($\alpha \leq 0.05$) reduction in the activities of superoxide dismutase (SOD), catalase (CAT), gluthathione peroxidase (GPx), and concentration of glutathione (GSH), and an increase in malondialdehyde (MDA) concentration in the brain (Figs 6.13 - 6.16). These anomalies were prevented upon concurrent treatment with β-sitosterol and stigmasterol; which returned the elevated brain MDA to normal control values and restored the altered activities of these enzymes to values comparable to the normal control group. The activity of catalase in the groups co-administered with spinasterol and sodium metavanadate only had values that were significantly ($\alpha \leq 0.05$) lower than the other groups.



n =5; Vit E = α-tocopherol; V = sodium metavanadate; spin = spinasterol; sito = sitosterol stig= stigmasterol

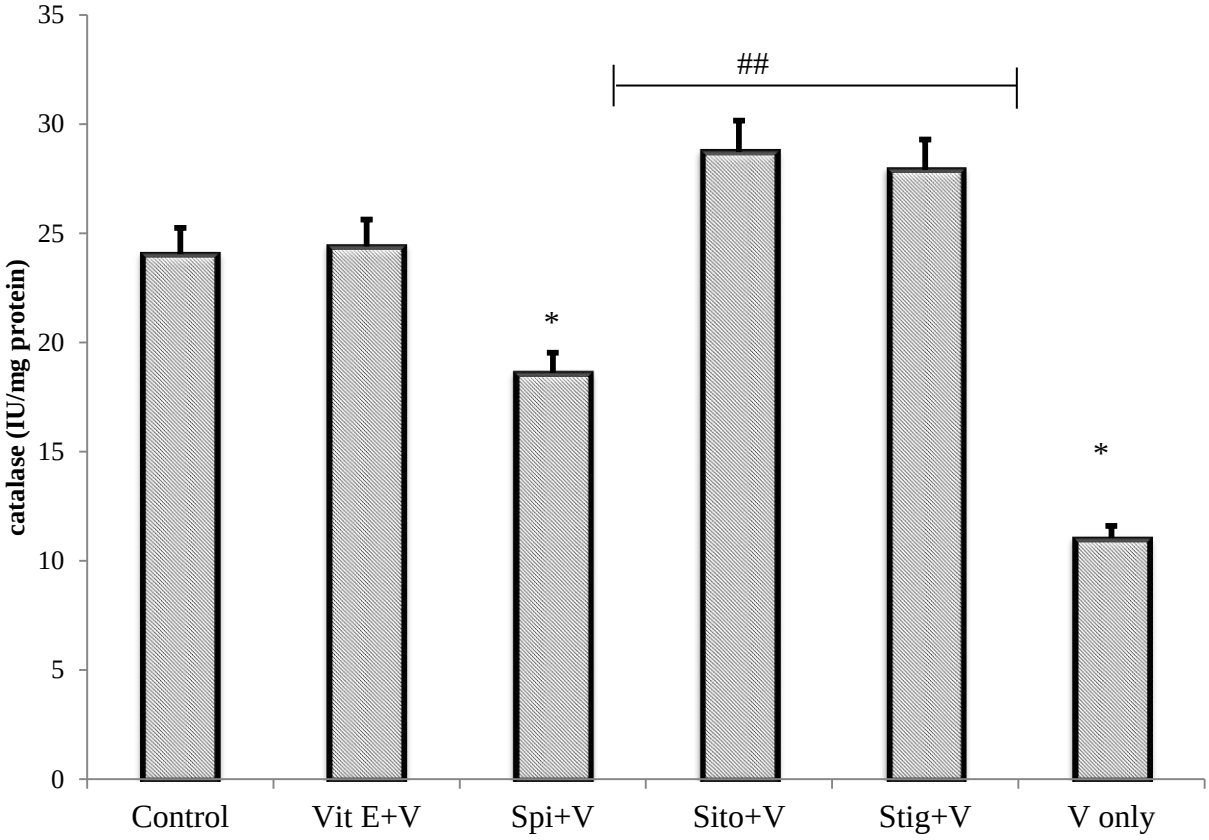
*significantly ($\alpha \leq 0.05$) different from the normal control

Figure 6.13: Effects of α-tocopherol, spinasterol, sitosterol and stigmasterol on vanadium induced changes in brain MDA levels in mice.



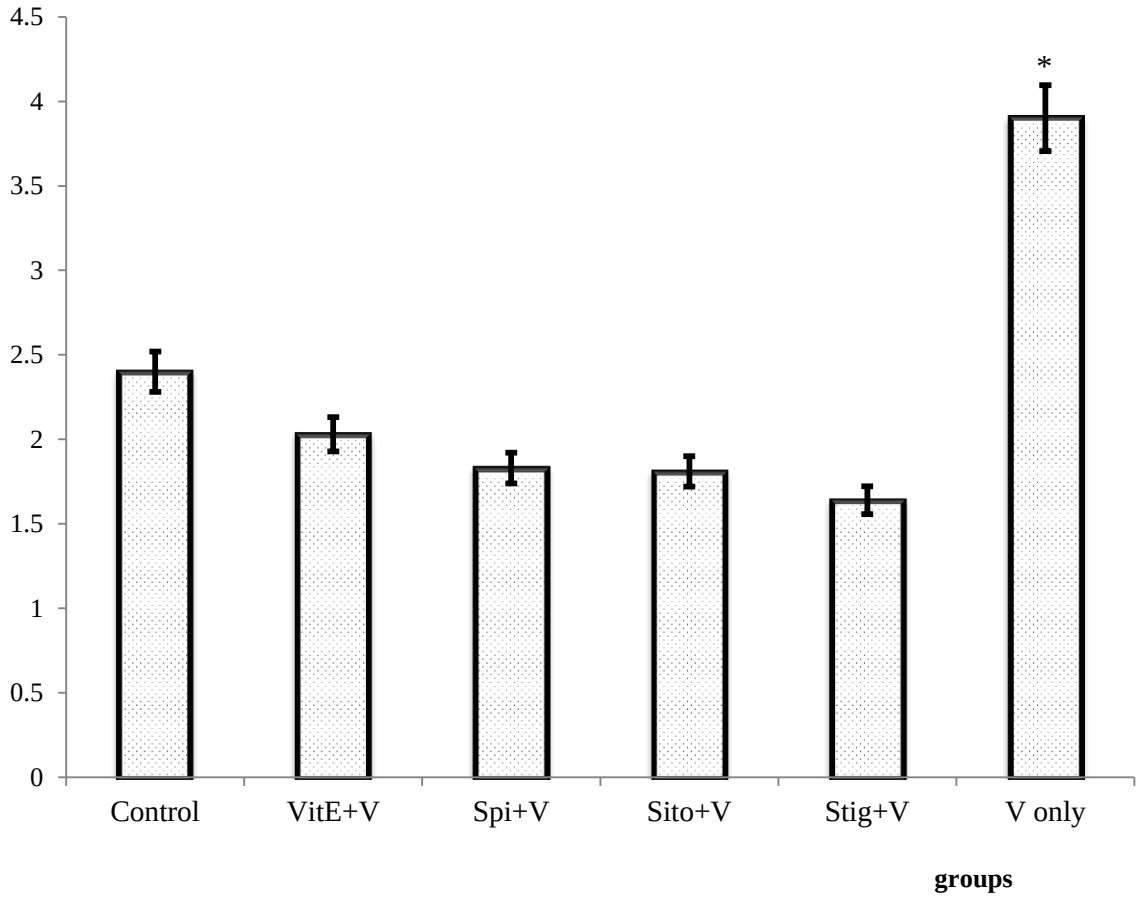
N =5; Vit E = α-tocopherol; V = sodium metavanadate; spin = spinasterol; sito = sitosterol stig = stigmasterol
*significantly ($\alpha \leq 0.05$) different from the normal control, # significantly ($\alpha \leq 0.05$ different from the sodium metavanadate only group

Figure 6.14: Effects of α-tocopherol, spinasterol, sitosterol and stigmasterol on vanadium induced changes in brain superoxide dismutase activities in mice.



n =5; Vit E= α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito = sitosterol stig = stigmasterol; *significantly ($\alpha \leq 0.05$) different from the control, ## significantly ($\alpha \leq 0.05$) different from the sodium metavanadate only group

Figure 6.15: Activities of catalase in brain tissue of mice following co-administration of α-tocopherol, spinasterol, sitosterol and stigmasterol with vanadium



n =5; Vit E = α-tocopherol; V= sodium metavanadate; spin = spinasterol; sito= sitosterol stig= stigmasterol;
*significantly ($\alpha \leq 0.05$) different from the control

Figure 6.16: Hydrogen peroxide (H_2O_2) levels in brain of mice following co-administration of α-tocopherol, spinasterol, sitosterol and stigmasterol with vanadium

6.3.4. IMMUNOHISTOCHEMISTRY

Myelin basic protein (MBP) immunohistochemical staining showed a down regulation of MBP in vanadium exposed groups (acute) as compared with the pure compounds treated groups and control in the cerebellum and hippocampus (Plates 6.1- 6.7). The number of cells expressing the myelin basic protein was thereafter quantified with the ImageJ® software (Figure 6.15). The expression of myelin basic protein (MBP) in the cerebellum was significantly higher in the groups co-administered with β-sitosterol and stigmasterol, when compared with the negative control group of sodium metavanadate only.

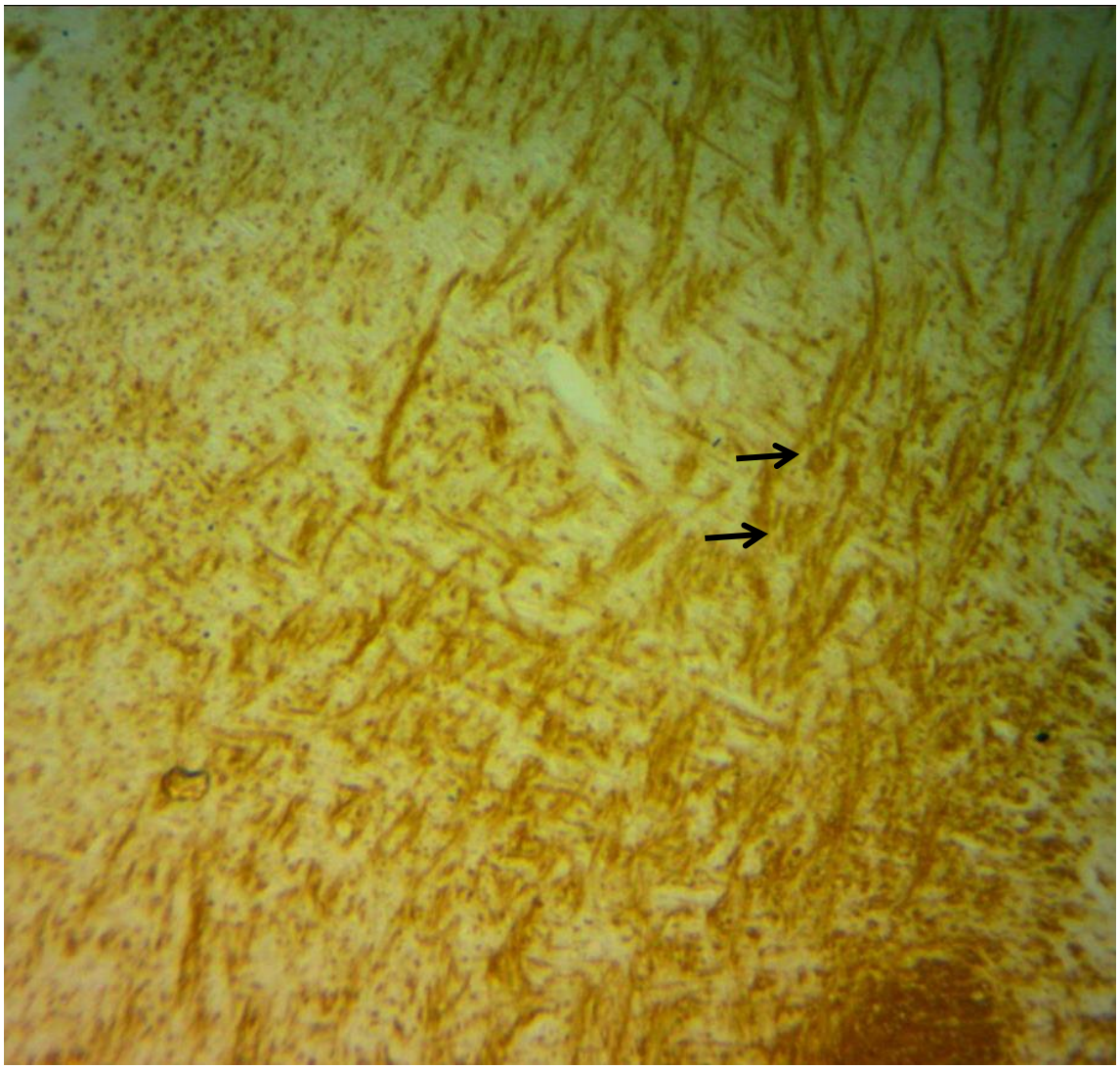


Plate 6.1: of the brainstem of control mice showing MBP-immunolabelled myelin fibres (x100) (arrows) localised at the hippocampal region arranged closely and tidy

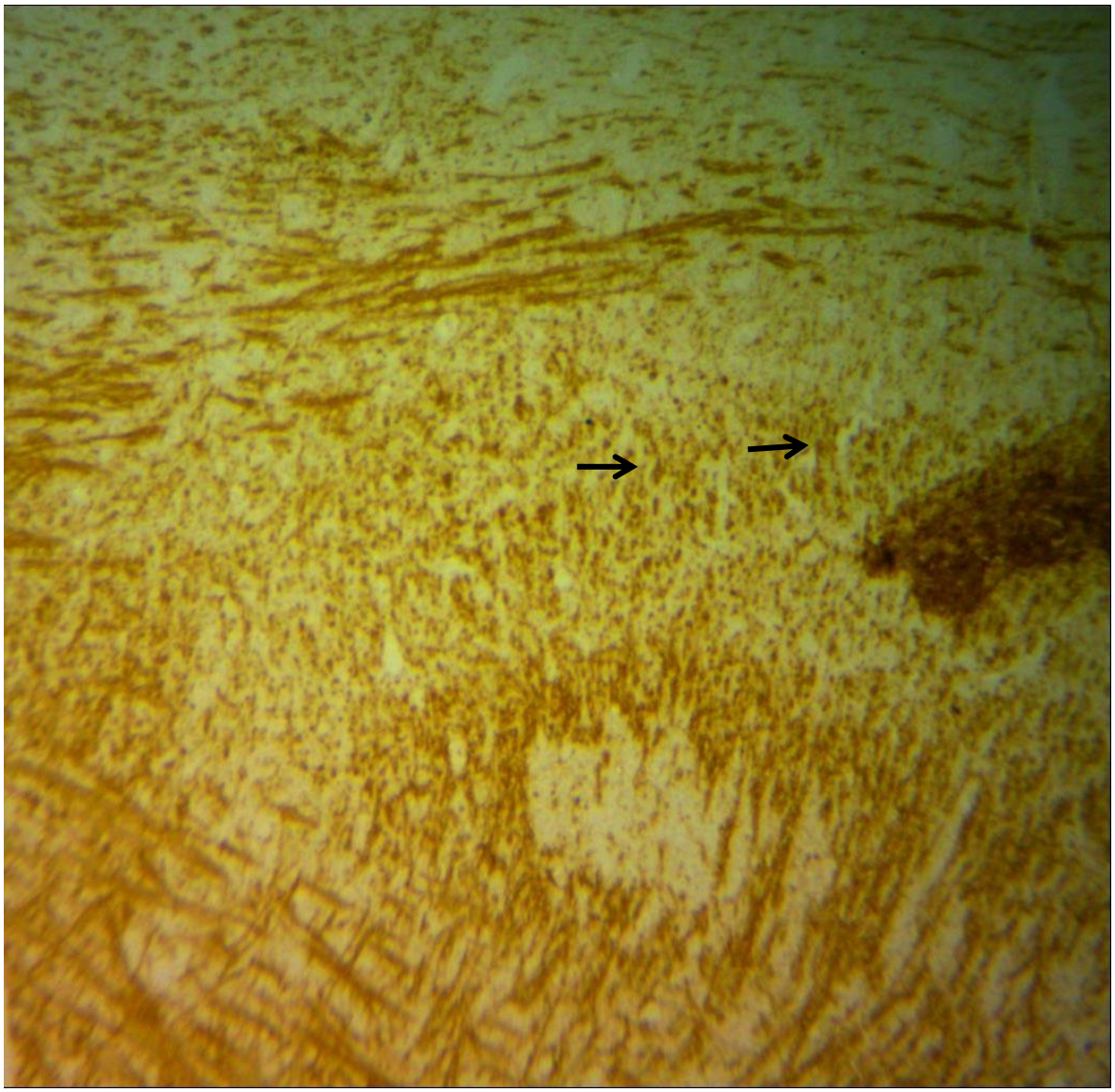


Plate 6.2: Brainstem of mouse co-administered with β-sitosterol and vanadium x100. MBP-immunolabelled myelin fibres (arrows) localized at the hippocampal region arranged orderly and closely

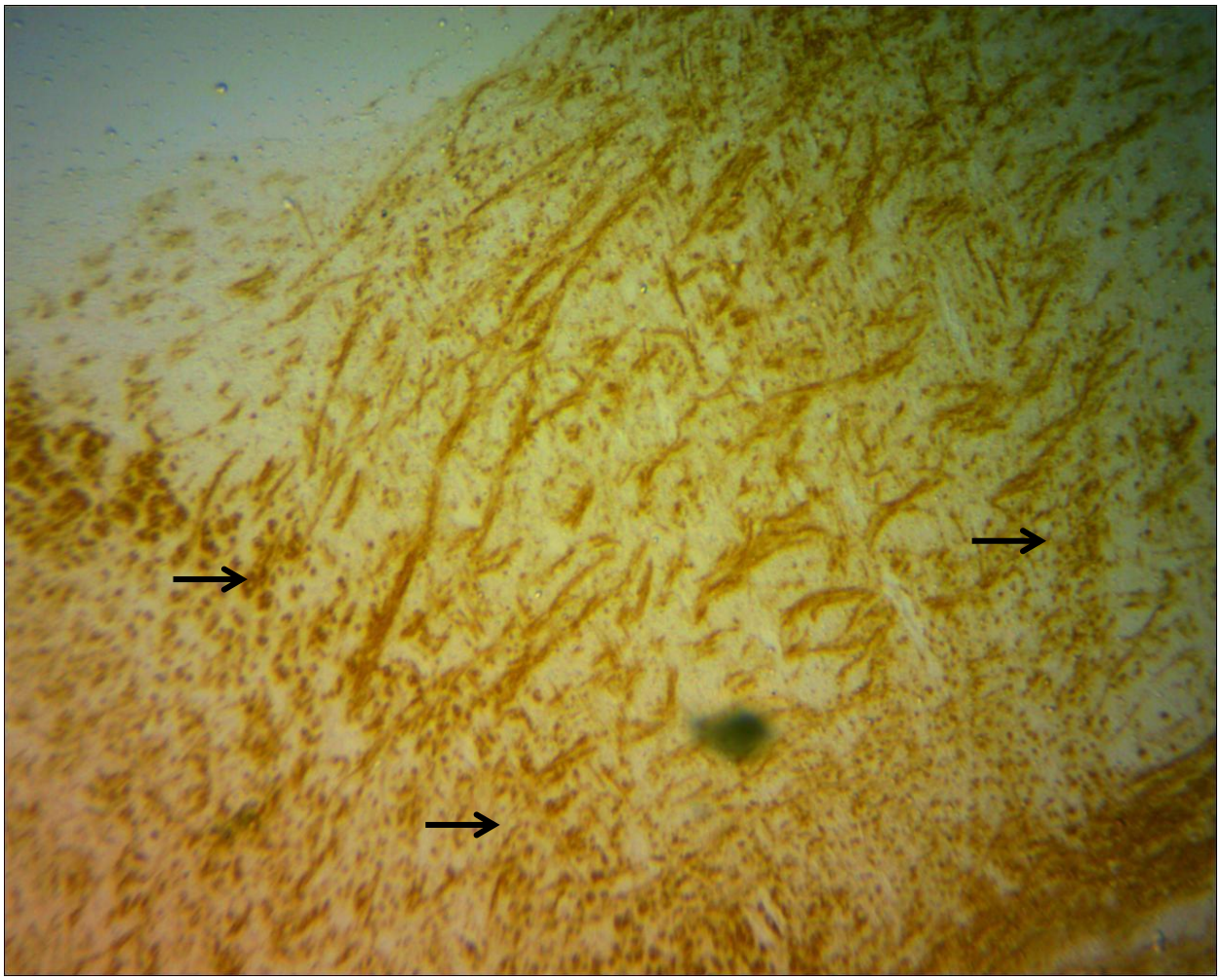


Plate 6.3: Brainstem of mouse co-administered with stigmasterol and vanadium (x100). MBP-immunolabelled myelin fibres (arrows) localized at the hippocampal region were arranged orderly and closely

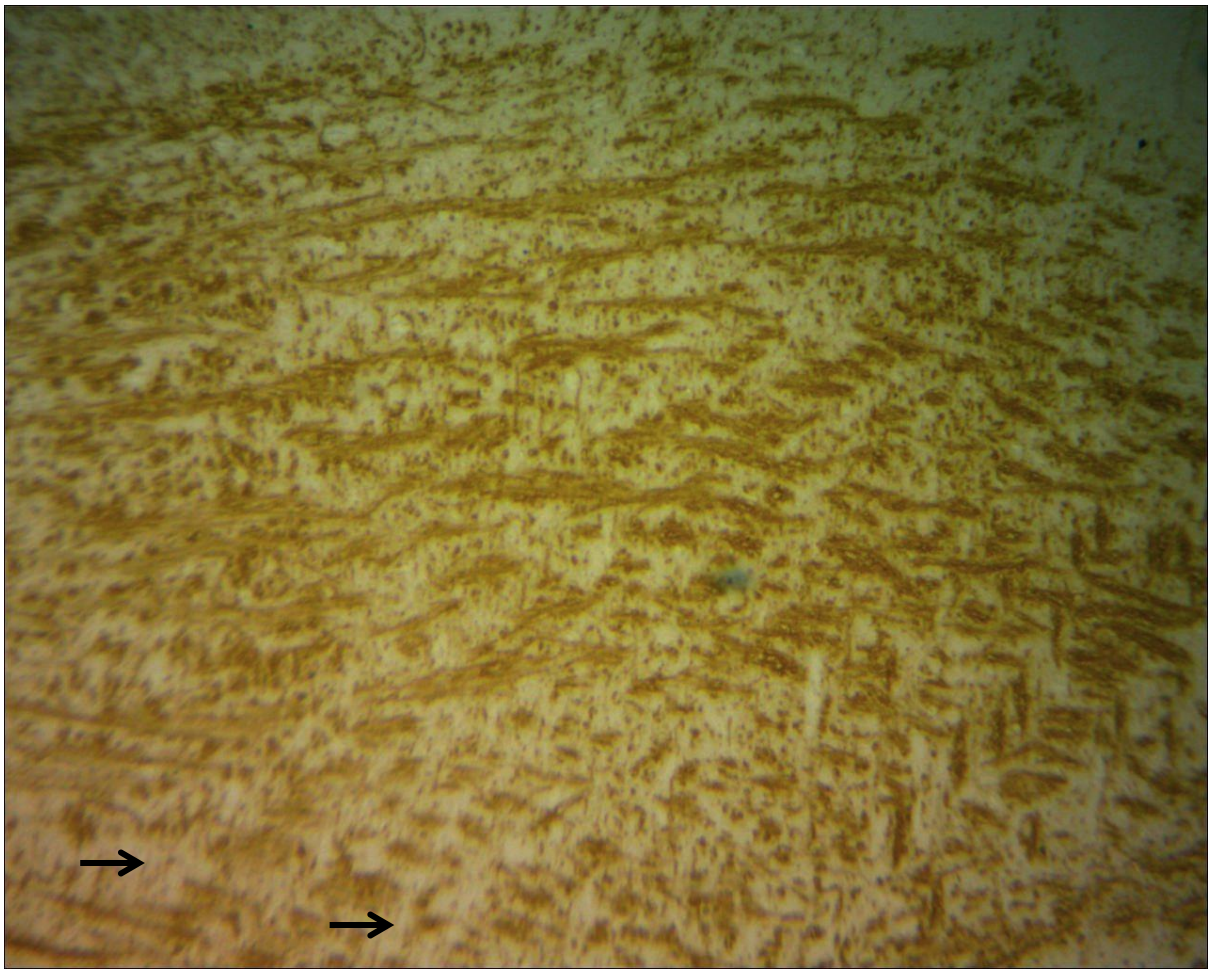


Plate 6.4: Brainstem of Group F administered with sodium metavanadate only (x100). MBP-immunolabelled myelin fibres (arrows) localized at the hippocampal region showing discontinuity in myelin fibres.

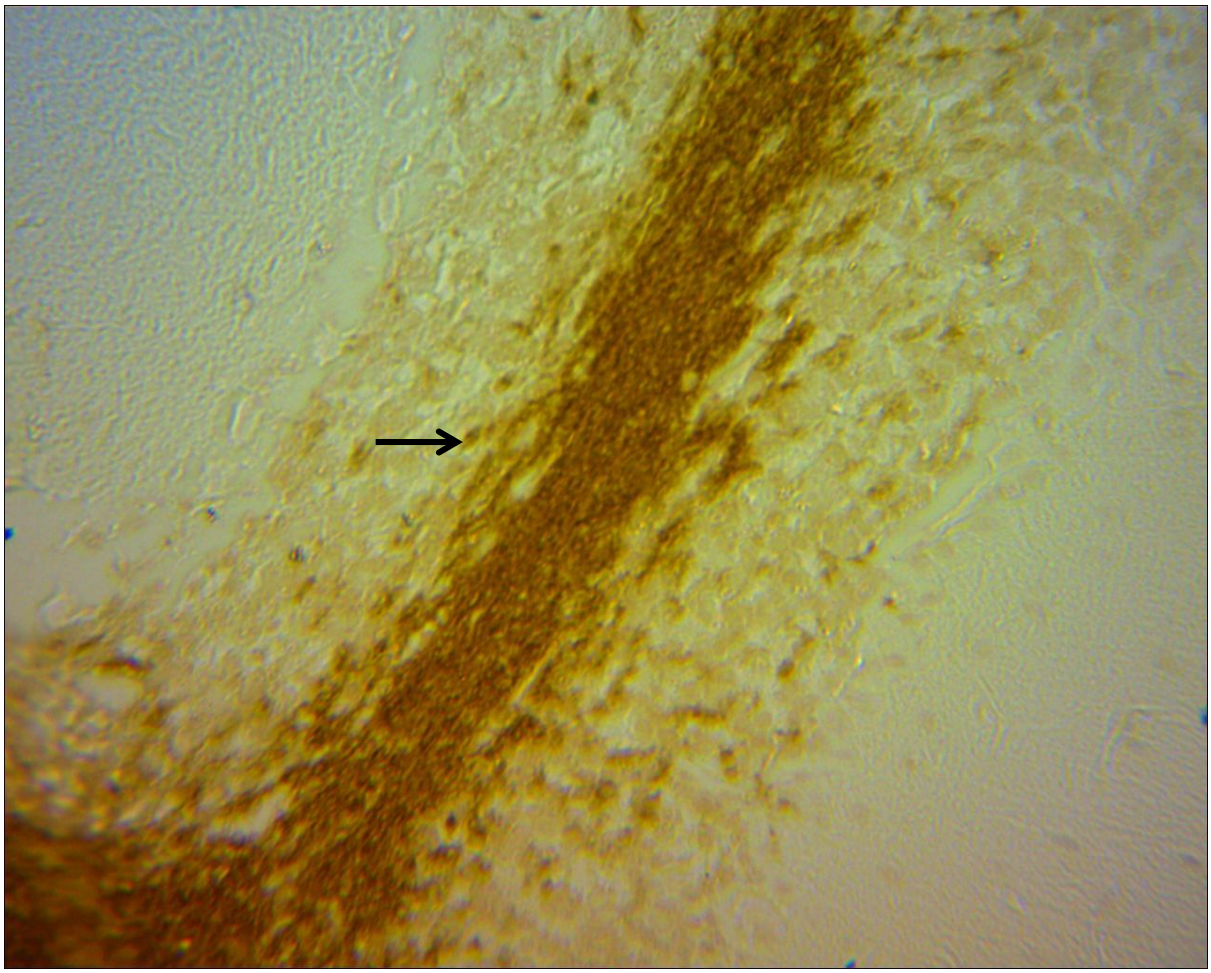


Plate 6.5: Cerebellum of control mice (x100). MBP-immunolabelled myelin fibres (arrows) with no visible lesion

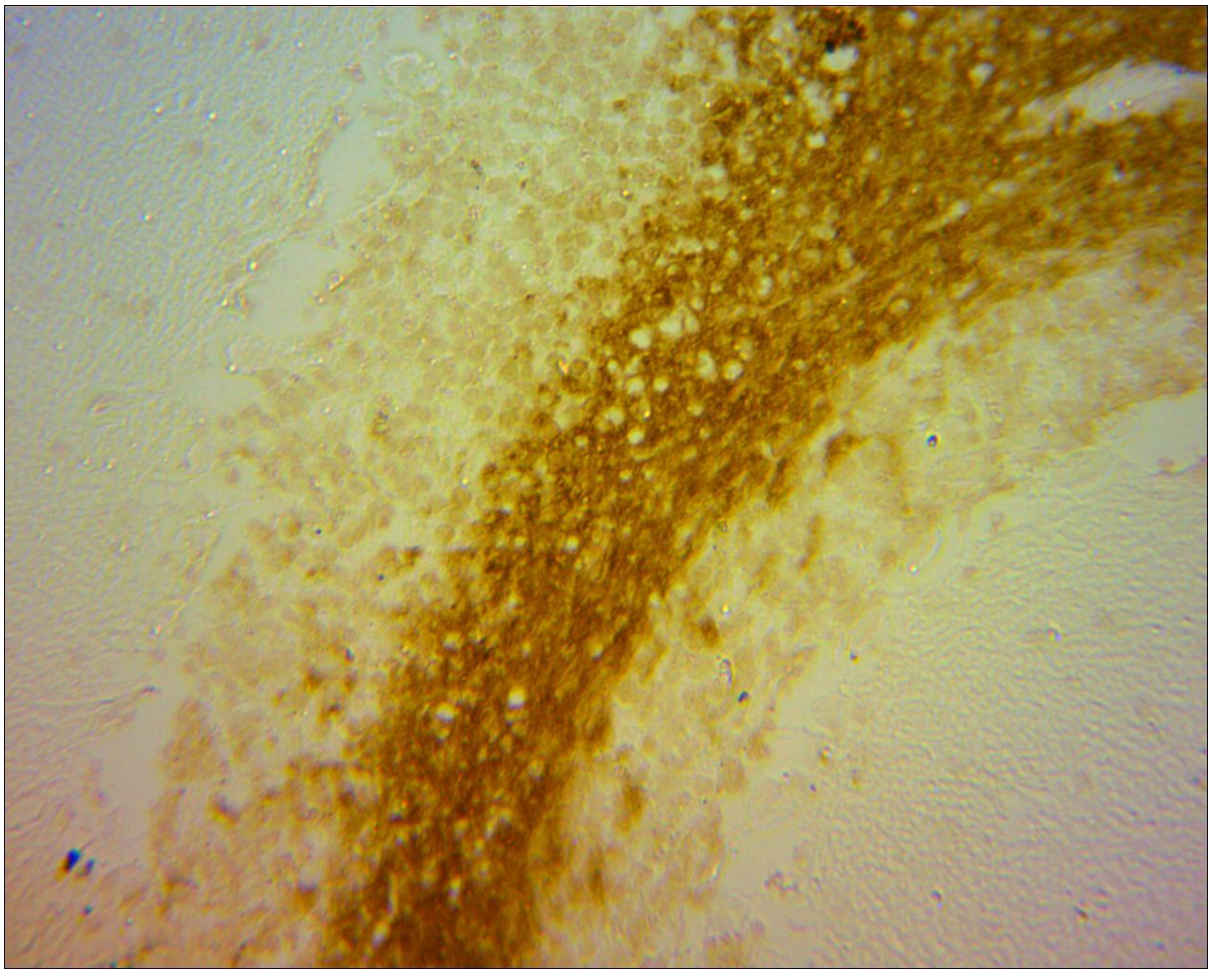


Plate 6.6: Cerebellum (x100) of mouse administered with beta-sitosterol and sodium metavanadate. MBP-immunolabelled myelin fibres (arrows) with neatly arranged myelin

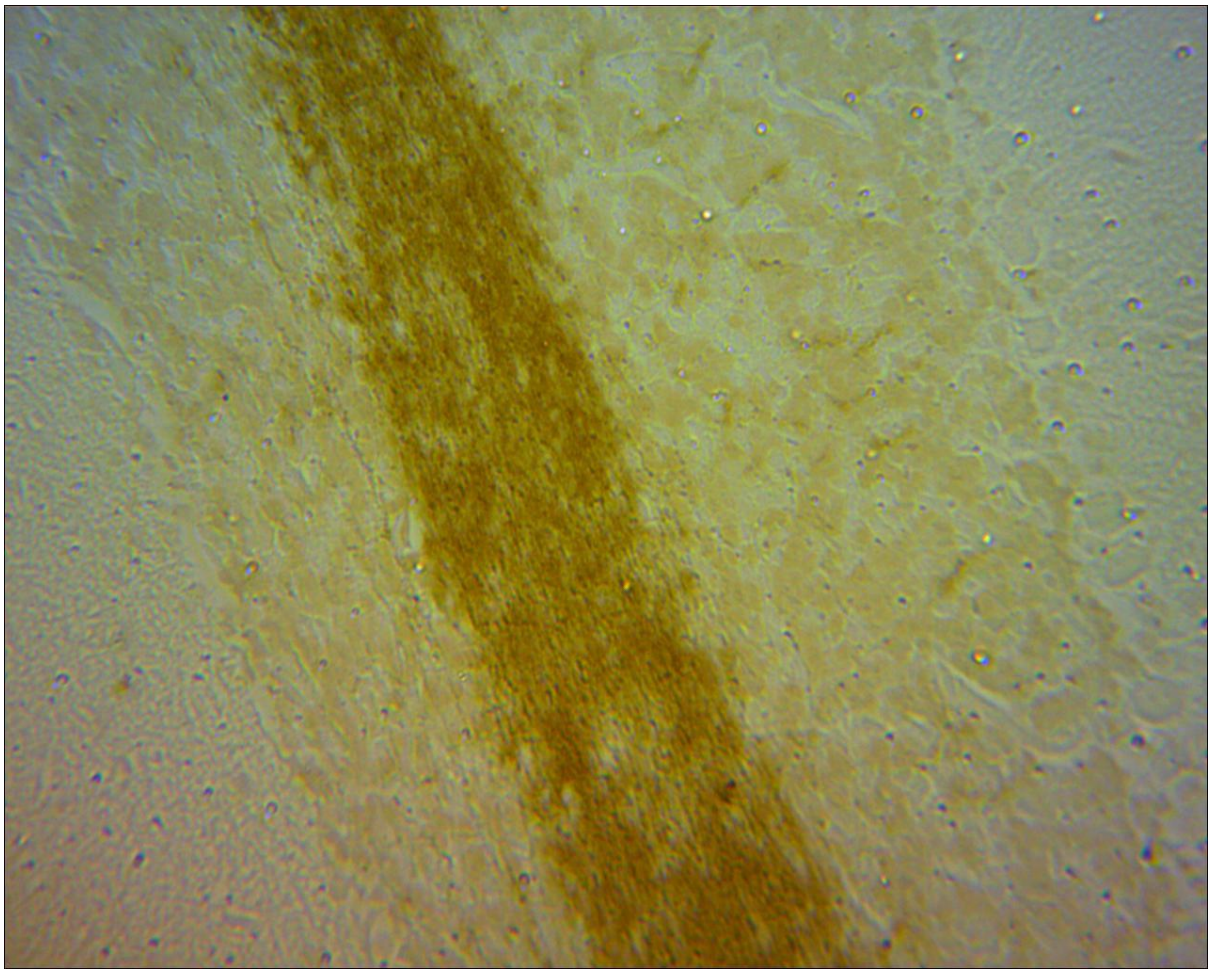


Plate 6.7: Cerebellum of mouse administered with sodium metavanadate only showing poor staining with MBP immunolabelling

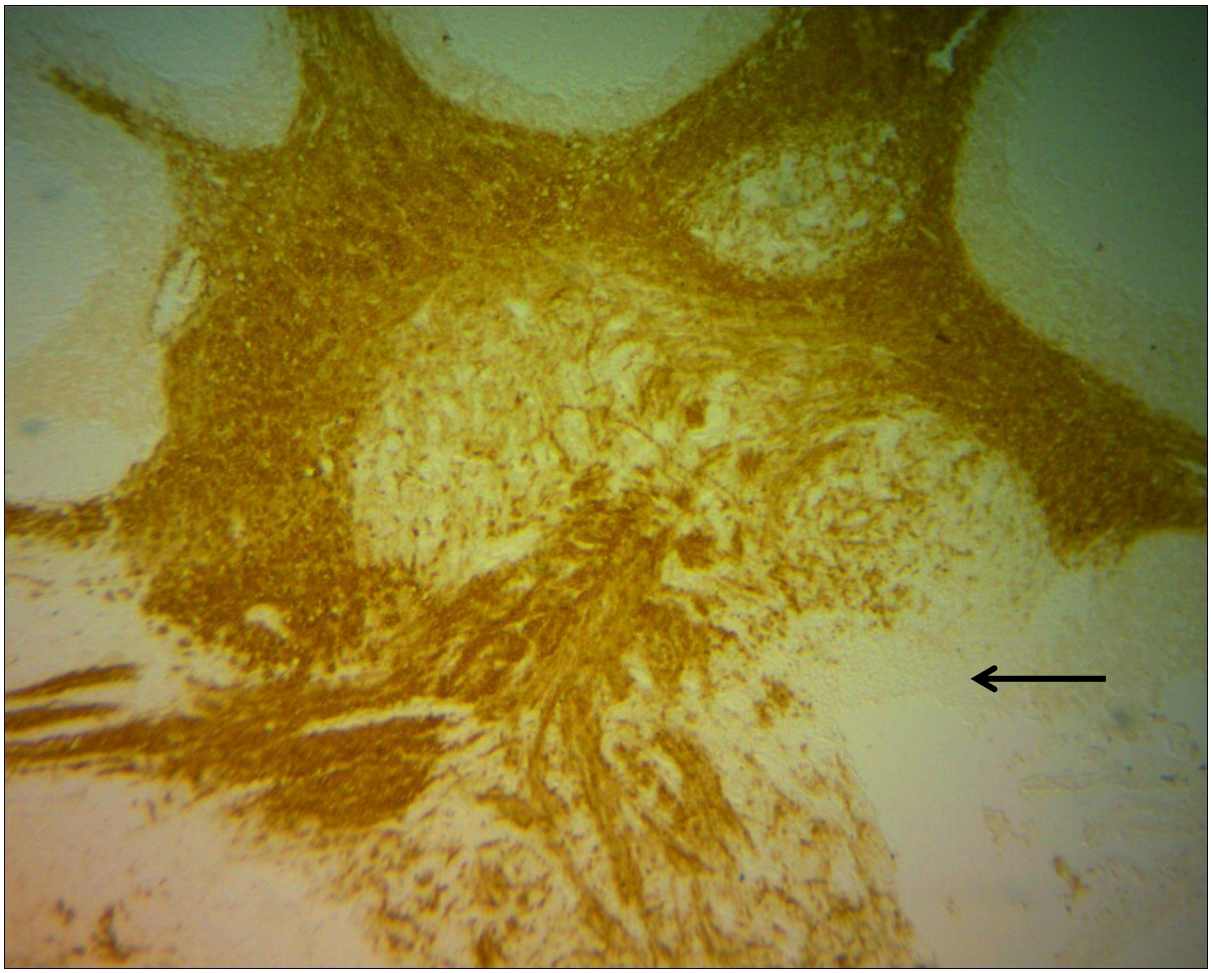


Plate 6.8.: Cerebellum (x 100) of mouse administered with sodium metavanadate only showing discontinuity of myelin fibres with MBP immunolabelling

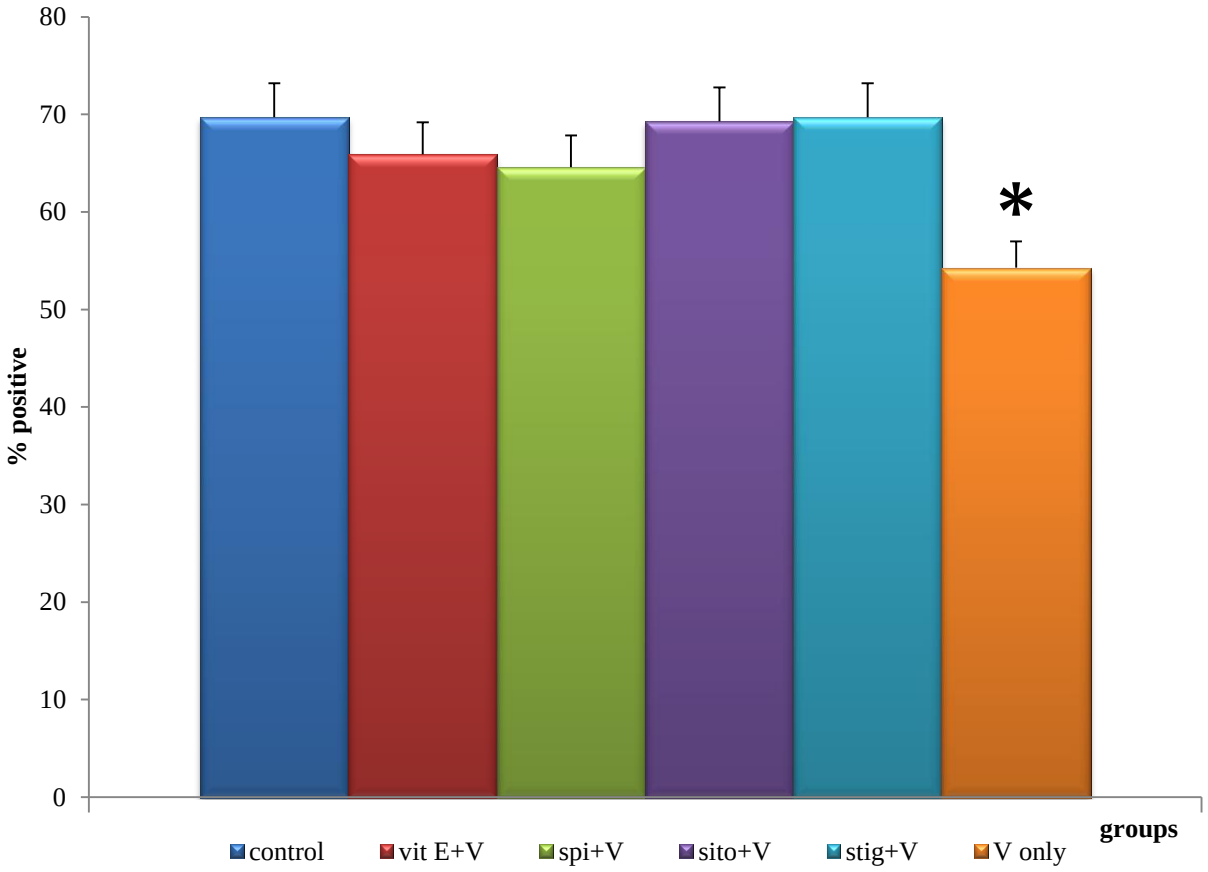


Figure 6.15: Quantification of expression of myelin basic protein in the cerebellum of mice using ImageJ® software

6.4 DISCUSSION

The BBB protects the brain by strictly regulating transport in and out of the brain, thereby maintaining brain homeostasis. The downside of this tightly regulated barrier is that it also limits the transport of therapeutics into the brain. Drugs to treat CNS disorders are often unable to penetrate into the brain to perform their actions. Approximately 98% of the small molecule drugs and nearly 100% of the large molecule pharmaceutics (for example, peptides, proteins and nucleic acids) cannot substantially cross this barrier (Pardridge, 2005). At the same time, treatment is needed for vanadium induced neurotoxictity and other CNS diseases, such as depression, Alzheimer's disease, Parkinson's disease, brain cancer, and cerebrovascular diseases (Gynther *et al.,* 2008), Therefore, there is an upsurge of research targeted at discovery of compounds/drugs that can be delivered across the BBB.

Though the transit time through the brain capillaries is very short (1 s) in estimating BUI, stigmasterol, β-sitosterol and spinasterol were notwithstanding transported into the brain. This may signify that these compounds are readily available for transport through the BBB on a single capillary passage. The data represent the first report *in vivo* demonstration of these compounds across the BBB. The mechanism of crossing the BBB by these compounds still remains unknown. It can be assumed that these compounds have the ability to bind to plasma protein, making them available for transport through the BBB (Pardridge and Mietus, 1979; Pardridge and Landaw, 1984). Another possibility is that their transport into the brain is mediated by a carrier or active transport system. Alternatively, stigmasterol, β-sitosterol and spinasterol may be transported from blood to the brain via lipid mediation.

6.4.1 Behavioural Study

In the present study, intra-peritoneal injection of sodium metavanadate in mice did result in deficits in learning and memory. Vanadium-induced cytotoxicity is associated with oxidative stress, which results increased formation of reactive oxygen species, lipid peroxidation, and modification of proteins by reactive lipid peroxidative products in neurones (Afeseh Ngwa *et al.,* 2009). Deficits produced by lesions of the hippocampus can be detected by hippocampus dependent learning tasks, such as Morris water maze. Morris water maze is generally accepted as an indicator of spatial learning and reference memory, which reflects long-term memory (Morris, 1984; D'Hooge and De Deyn, 2001). Spatial learning is assessed across repeated trials, and reference memory is determined by preference for the platform area when the platform is absent (D'Hooge and De Deyn, 2001). In the present study, after 7 days of

intra-peritoneal injection of sodium metavanadate only, mice showed significant long-term memory impairments. The results are similar to previous findings which demonstrated that water-maze place learning and probe trial performance were impaired after vanadium injection in mice (Folarin *et al.,* 2016). β-sitosterol and stigmasterol significantly shortened the escape latency prolonged by sodium metavanadate injection after 2 days of training. Interestingly, when using the escape latency as an index of learning and memory, these compounds showed stronger improving ability compared to that in the α-tocopherol group. In addition, during the probe trial session, β-sitosterol and stigmasterol also increased the swimming time and distance in the quadrant where the platform was previously placed as did the α-tocopherol treated group. This strongly indicates that β-sitosterol and stigmasterol improved spatial learning and memory.

The open-field maze (OFM) was originally introduced as a measure of emotional behaviour in rats (Hall, 1934; Blanchard and Blanchard, 1989); it has equally proven to be successful with mice (Christmas and Maxwell, 1970). It has attained the status of being one of the most widely used measures of behaviour in animal (Walsh and Cummins, 1979). Rodents have been reported to show distinct aversions to large, brightly lit, open and unknown environments (Choleris *et al.,* 2001). Thus, the test provided an opportunity to analytically assess novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behaviour in rodents (Prut and Belzung, 2003). The number of line crosses and the frequency of rearing are used as measures of locomotor activity, with a high frequency of these behaviours indicating increase in locomotion exploration. In this study, results showed that β-sitosterol and stigmasterol from *Grewia carpinifolia* extract significantly increased the numbers of line crossed and the frequency of rearing decreased in the vanadium only group. In addition in this experiment, β-sitosterol and stigmasterol also increased the number of entries into the central portion of the arena and reduced the number of faecal boli deposit, which are indices of anxiety. This may show a relationship between emotion (anxiety) and locomotion as there may be a possibility that these compounds may induce an increase in alertness, resulting to increase in locomotor activity, which may be indicative of their anxiolytic property. Gadekar *et al.* (2011) had reported that phytosterols have anxiolytic activity. Sedative properties of a drug or plant extract are said to be carried out by Gamma- amino-butyric-acid (GABA), the major inhibitory neurotransmitter in the CNS (Apu *et al.,* 2013), which may be the mechanism through which β-sitosterol and

stigmasterol acts. This may likely lead to a decrease in the rate of firing of major neurones in the brain or may directly activate GABA receptors (Ronok, *et al.,* 2012).

The hanging wire test performed in order to demonstrate motor impairment and coordination in mice (Crestani, *et al,* 2001). Mice as young as four weeks, and as old as approximately 19 months of age have been reliably evaluated with this test (Fougerousse *et al,* 2003). The ability of these compounds to significantly increase latency in the hanging wire test might show their contribution to muscular strength in animal models. These results from the hanging wire (forelimb support) test have conclusively linked the increased locomotor activity and increased swimming speed from the Open-field test and Morris water maze, respectively, indicating the positive role of beta-sitosterol and stigmasterol in motor coordination.

6.4.2. *In vivo* antioxidant study

Plants have been found to have phytoconstituents of relevance in phytomedicine (Dahanukar *et al.,* 2002; Somova *et al.,* 2003). Oxidative stress occurs when pro-oxidant and antioxidant levels become imbalanced in favour of pro-oxidation. Oxidative stress-related disorders can be prevented or managed by antioxidants and the therapeutic effects of many medicinal plants have been hypothesized to result from the free radical scavenging (antioxidant) activity of the constituent antioxidant phytochemicals notably phenols, flavonoids and tannins (Vinson *et al.,* 1995). In view of this, the widely acclaimed beneficial effects of flavonoids on human health are due to their radical scavenging and metal chelation properties in which the hydroxyl functional groups are involved (Kessler *et al.,* 2003). The presence of these antioxidant phytoconstituents in plant extract may be responsible for the inhibition lipid peroxidation the mice. The increase in oxidative stress and the overwhelming of the *in vivo* antioxidant defence system following vanadium-induced toxicity have not been disputed (Sasi *et al.,* 1994; Saxena *et al.,* 2013). Reactive oxygen species generated in the tissue can be efficiently scavenged by the enzymatic antioxidant system and non-enzymatic antioxidants (Seo *et al.,* 2003).

SOD, as an endogenous antioxidant enzyme, plays an important role in the intracellular antioxidant defence in the brain. This antioxidant enzyme is responsible for the dismutation of the O_2^- generated during vanadium metabolism (Ibrahim *et al.,* 2006) to H_2O_2 which are substrates for the peroxisomal catalase and cytosolic GPx. The observed decrease in both

SOD and GPx activities in sodium metavanadate-treated mice strongly suggest an overwhelming superoxide radical generation and H_2O_2 formation following vanadium administration. The antioxidant potential of both beta-sitosterol and stigmasterol *in vivo* is evidenced by the significant inhibition of vanadium effects in the treated mice. The presence of transition metal like Fe^{2+} in the brain could trigger the generation of the highly reactive hydroxyl radicals ($.OH^-$) from the H_2O_2 resulting from vanadium metabolism through Fenton reaction (Zhang *et al.,* 2011). These ROS are all capable of reacting with cellular macromolecules with consequent lipid peroxidation, depletion of sulphydryl-containing peptides and damage to DNA. Reduced level of GSH may promote oxidative stress and membrane lipid peroxidation (Injac *et al.,* 2009).

MDA, an important lipid peroxidation product, can be taken as an indicator for the state of oxidative damage of membranes exposed to oxidative stress. The reported abnormal alteration in MDA content and its relation to memory impairment have been shown in previous studies (Um *et al.,* 2012). The observed decrease in GSH level and corresponding increase in malondialdehyde (MDA) level in the brain of sodium metavadate-treated mice strongly support the hypotheses that increased oxidative stress associated with an impaired antioxidant defence status is one of the mechanisms of vanadium toxicity (Afeseh Ngwa *et al.,* 2009). In the present study, concurrent treatment with stigmasterol and β-sitosterol could protect the brain from vanadium-induced injury by increasing the activity of GSH while decreasing membrane lipid peroxidation. As revealed in the present study, the ability of stigmasterol and β-sitosterol to modulate the endogenous antioxidant enzymes of mice administered with vanadium portrays good signal for neurooprotection.

Catalases are involved in the mechanisms used of protection cells against the damage caused by ROS to cellular components including nucleic acids, lipids and proteins (Imlay, 2002). The present study also showed that co-administration of beta-sitosterol and stigmasterol following vanadium toxicity increased catalase activity finding may be linked to the ability of these compounds in biological system to increase the activity of this antioxidant enzyme so as to combat the increased oxidative stress.

Hydrogen peroxide is reactive oxygen metabolic by- product that serves as a key regulator for a number of oxidative stress-related states (Davies, 1999; Zhang *et al.,* 2001). Functioning through NF kappa-B and other factors, hydroperoxide-mediated pathways have been linked to heavy metal toxicity as well as asthma, inflammatory arthritis, arteriosclerosis, diabetic

vasculopathy, osteoporosis, a number of neurodegenerative diseases and Down's Syndrome (Li and Karin 1999; Uesuge *et al.,* 2000; Emelyanov *et al.* 2001; Kim, *et al.,* 2001; Halliwell, 2001; Mody *et al.,* 2001; Okuda *et al.,* 2001; Peiroet al 2001; Sanji *et al.,* 2001). The significant increase in the level of H_2O_2 in the brain further contributes to its neurotoxicity and confirmed earlier reports of its mechanism of action. It is possible that beta-sitosterol and stigmasterol diminished the levels of H_2O_2 via their reducing properties by donating a hydrogen atom, which breaks the H_2O_2 chain as already established in study three (chapter 5)

Epidemiological studies of human populations, and experiments in animal models of neurodegenerative disorders, have also provided evidence that phytochemicals in fruits, vegetables and plant can protect the nervous system against disease (Liu, 2003; Joseph *et al.,* 2005). The vast majority of studies on these phytochemicals have focused on the fact that many of them possess antioxidant activity. The present findings are in line with previous findings that the neuroprotective effects of various phytochemicals are associated with reduced levels of oxidative stress. For example, resveratrol, quercetin and catechins reduced oxidative stress and protected cultured hippocampal neurones against nitric-oxide-mediated cell death (Larso, 1988). Neuroprotective effects of compounds in natural products, including a-tocopherol, lycopene, resveratrol, *Ginkgo biloba* and ginsenosides have been reported (Ikeda *et al.,* 2003).

6.4.3 Immunohistochemistry

Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction allows detection of highly specific amino acid sequences within a protein, targeting defined regions, domains, or cleavage products; it can also detect specific post-translational modifications (PTM) on a protein. This is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaf a substrate to produce a coloured precipitate at the location of the protein. The myelin basic protein (MBP) staining detects developing and adult myelin, developing oligodendrocytes as well as distinguishes oligodendrocytes from microglia, astrocytes, neurones and other cells in brain sections. Myelination is required if mammalian neural circuits are to function normally. The development process of myelination varies between region and species. In mice, myelination predominantly occurs postnatally (Wiggins 1982; Paus *et al*., 1990). It has been documented that myelin formation in the brain begins at

around postnatal day (PND) 10 in the mice, with the maximal rate of myelin accumulation occurring around PND 20; however, myelin accumulation does continue into adulthood, albeit at a decreasing rate (Doretto *et al*. 2011). The process of myelination has similarities in all species, beginning in the spinal cord and proceeding rostrally. Within the cerebral lobes, myelin development occurs initially in the primary motor and sensory areas of the cerebral cortex and corpus callosum before spreading outward. Toxicants such as vanadium have been documented to be a major leading cause of disruption of myelin (Rodier 1990; Todorich *et al.,* 2011) before, during and after formation (Herring and Konradi 2011).

In the MBP immunohistochemistry, there was increased myelination density in the beta-sitosterol and stigmasterol group compared to vanadium exposed mice which showed areas of myelin pallor and discontinuity of myelin fibres. The present study further points to the fact that vanadium altered myelination after its synthesis since juvenile mice (myelination should have begun at this age) were used in this study; causing hypomyelination. Concurrent administration of beta-sitosterol and stigmasterol however protected the myelin sheaths, and prevented this hypomyelination. This is in consonance with findings that beta-sitosterol aided repair of damaged neurones by neuronal synthesis, and restoration of synaptic activity and ultimately nerve impulse transmission (Russo and Borrelli, 2005).

The general finding that mice with a down-regulation of MBP expression in the hippocampus were also impaired on the learning and memory task emphasize a role for the hippocampus in specific memory such as configural learning. The present results are more compatible with the indication that the hippocampus plays an especially important role in processing and remembering spatial and contextual information (Jarrard, 1993)

6.5 CONCLUSION

It can be concluded that beta-sitosterol and stigmasterol protected vanadium induced neurodegeneration attributed to its antioxidant and behavioural properties. The study also suggests that the protection of activities of antioxidant enzymes by these compounds along with their direct ability to be delivered into the brain may be beneficial. The activity of these compounds can be further explored in amnesia, multiple sclerosis and other degenerative conditions in which impaired neurotransmission plays vital role in their pathogenesis.

CHAPTER SEVEN

7.0 CONCLUSION AND RECOMMENDATION

The leaf and stem of *Grewia carpinifolia* were demonstrated to have a high safety margin with lethal doses of 5623.41 ± 342.40 mg/kg and 5879.11 ± 189.11 mg/kg, respectively, when administered orally. When given at high doses for a prolonged period, however, alterations were observed in biochemical parameters. It is therefore suggested that administration of leaf and stem of *Grewia carpinifolia* at high doses beyond 800 mg/kg should be avoided for extensive periods.

The current study is of paramount relevance to the research on neuroprotection against vanadium toxicity because it investigated the effects of pure compounds from plant extract that can mitigate the effects of acute vanadium in rodent, using *in vivo* and *in vitro* antioxidant studies, different behavioural tasks as well as immunohistochemistry. There is clinical relevance of the present study to human research on vanadium, as the result can be translated to human studies.

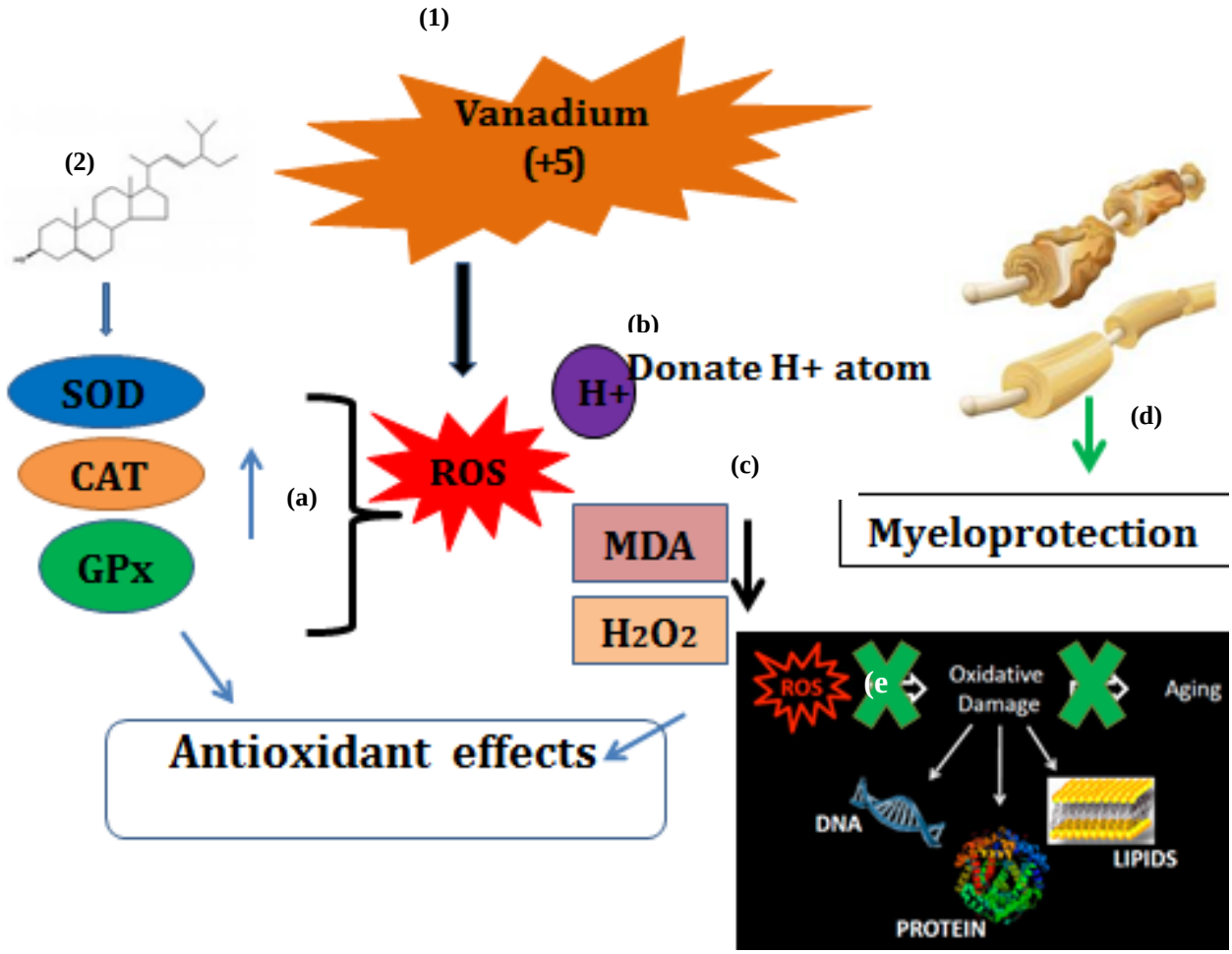
The BBB represents a major obstacle to the delivery of substances to the CNS during vanadium induced toxicity. Although several attempts had been explored in this regard to mitigate the neurotoxicity damage of this heavy metal, the present study represents a major break-through in the research of drug delivery to the brain as it established that beta-sitosterol and stigmasterol successfully crossed from the vascular component to the brain. Furthermore, beta-sitosterol and stigmasterol, which seem to have the greatest *in vivo and in vitro* antioxidant activity, have the potential to donate hydrogen atom and, thus, effectively act as antioxidants.

The results of the present study suggested that β- sitosterol and stigmasterol are memory enhancers and these compounds successfully reversed vanadium induced demyelination.

The present study also reported for the first time the isolation of dibutyl phthalate; a potent anti-carcinogen from *Grewia carpinifolia.* From literature search this is the first time this compound was isolated from any member of the Genus *Grewia.*

The findings of the present study were used to propose a mechanism of action for β-sitosterol and stigmasterol from *Grewia carpinifolia* against vanadium toxicity (Figure 7.1.)

It is, therefore, recommended that beta-sitosterol and stigmasterol be used as dietary supplements in animals and humans following exposure to toxic doses of vanadium. The memory enhancing and myeloprotective abilities of these compounds may be harnessed in other various disease conditions of the CNS such as Alzheimer's disease, multiple sclerosis and epilepsy. These compounds would serve as a potential neuro-pharmaceutical.



(1) Vanadium treatment impairs mitochondrial function with an increase in ROS generation, resulting in oxidative damage caused by lipid peroxidation, reduction in activities of antioxidant enzymes and leading to DNA fragmentation and apoptosis. **(2)** Co-treatment with **β-sitosterol and stigmasterol** increased activities of antioxidant enzymes **(a)** and donates H+ atom to free radical cleaving ROS generated by vanadium induced toxicity **(b)** decreased the level of MDA and H_2O_2 in brain tissues **(c)** crossed the blood brain barrier and prevented damage to myelin sheaths **(d),** which subsequently preserved the integrity of DNA, protein, lipids, and thus prevented premature ageing

Figure 7.1: A proposed model describing the mechanism of action of β-sitosterol and stigmasterol following vanadium-induced acute toxicity

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Appendix 1: Approval of study by the Animal Care and Use Research Ethics Committee (ACUREC), University of Ibadan

UNIVERSITY OF IBADAN
DEPARTMENT OF VETERINARY PATHOLOGY

Head of Department:
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June 8, 2016

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Dept. of Vet. Physio., Biochem.and Pharm.
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Ibadan.

NOTICE OF ETHICAL APPROVAL FOR A RESEARCH PROJECT PROPOSAL

Your letter on the above requesting for Ethical Approval of your proposal titled: "***Protective effects of Grewia carpinifolia on Vanadium-induced neurotoxicity***" *refers*: strictly as outlined in your proposal submitted for assessment.

Please quote **UI-ACUREC/App/2016/025** as reference for this approval.

You are to note that **UI-ACUREC** reserves the right to monitor and conduct compliance visit to your research site without previous notification.

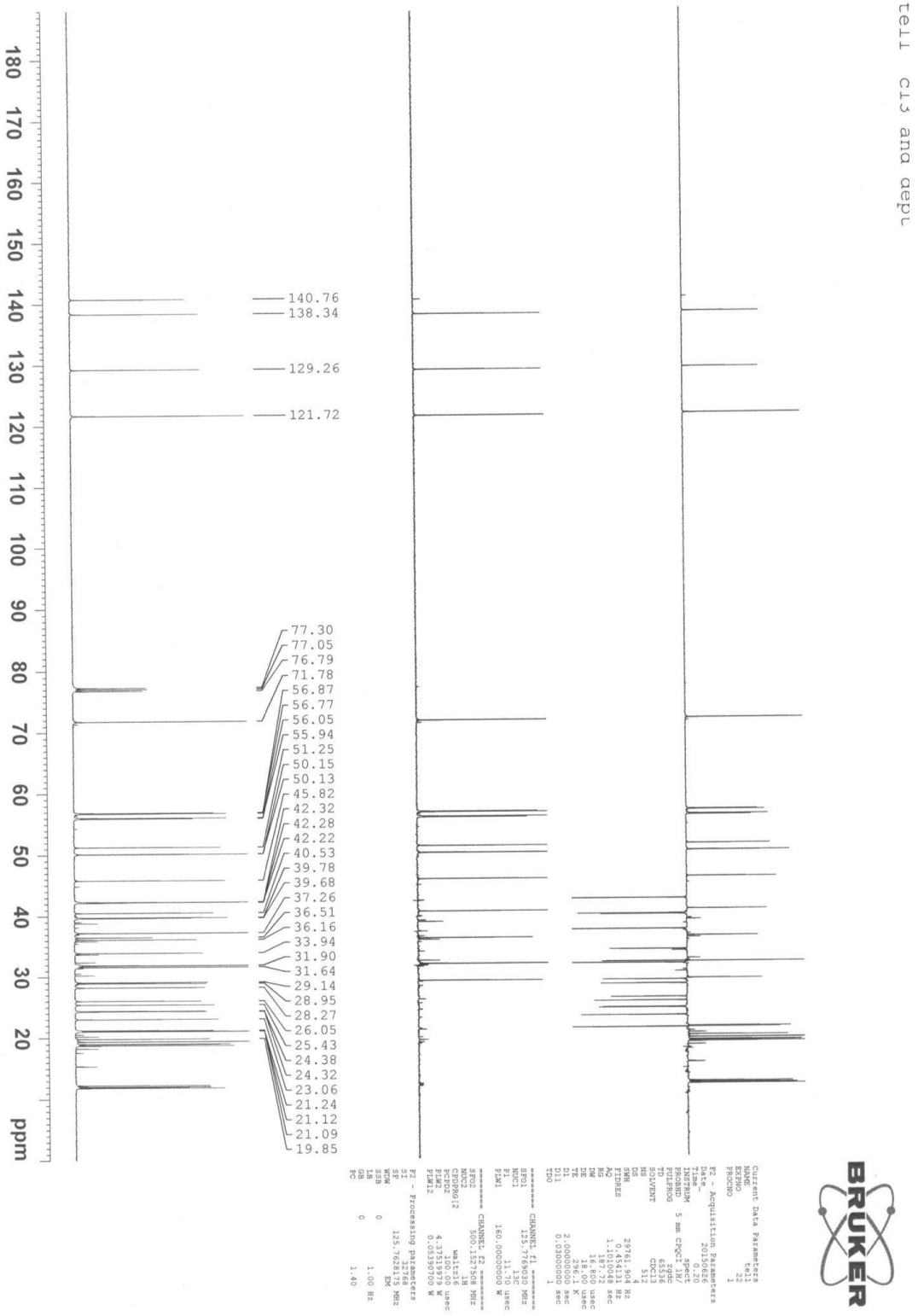
Thank you.

08/06/2016

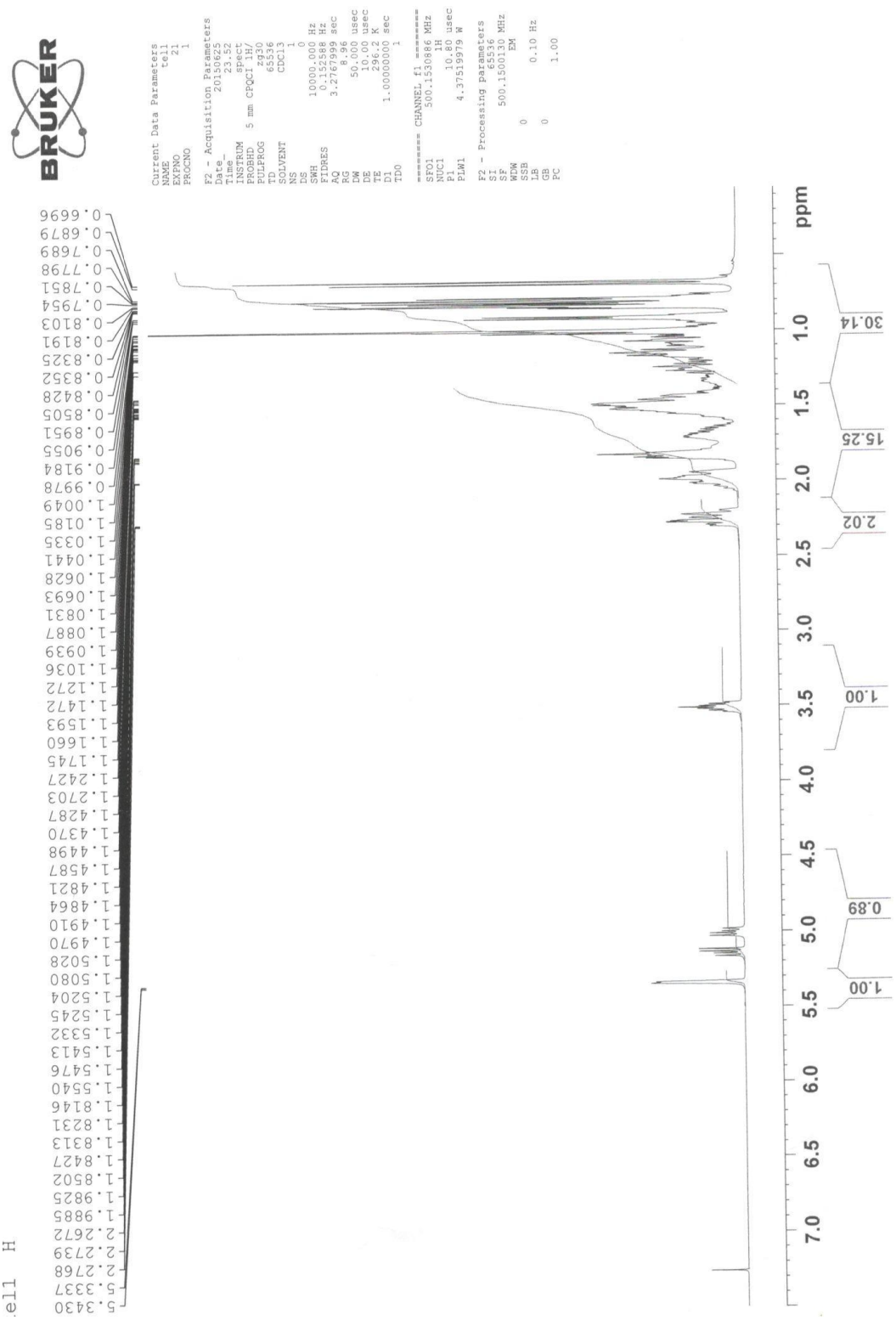
Prof. V.O. Taiwo
Chairperson, UI-ACUREC

Cc: Dean, FVM
Director, Research Management Office

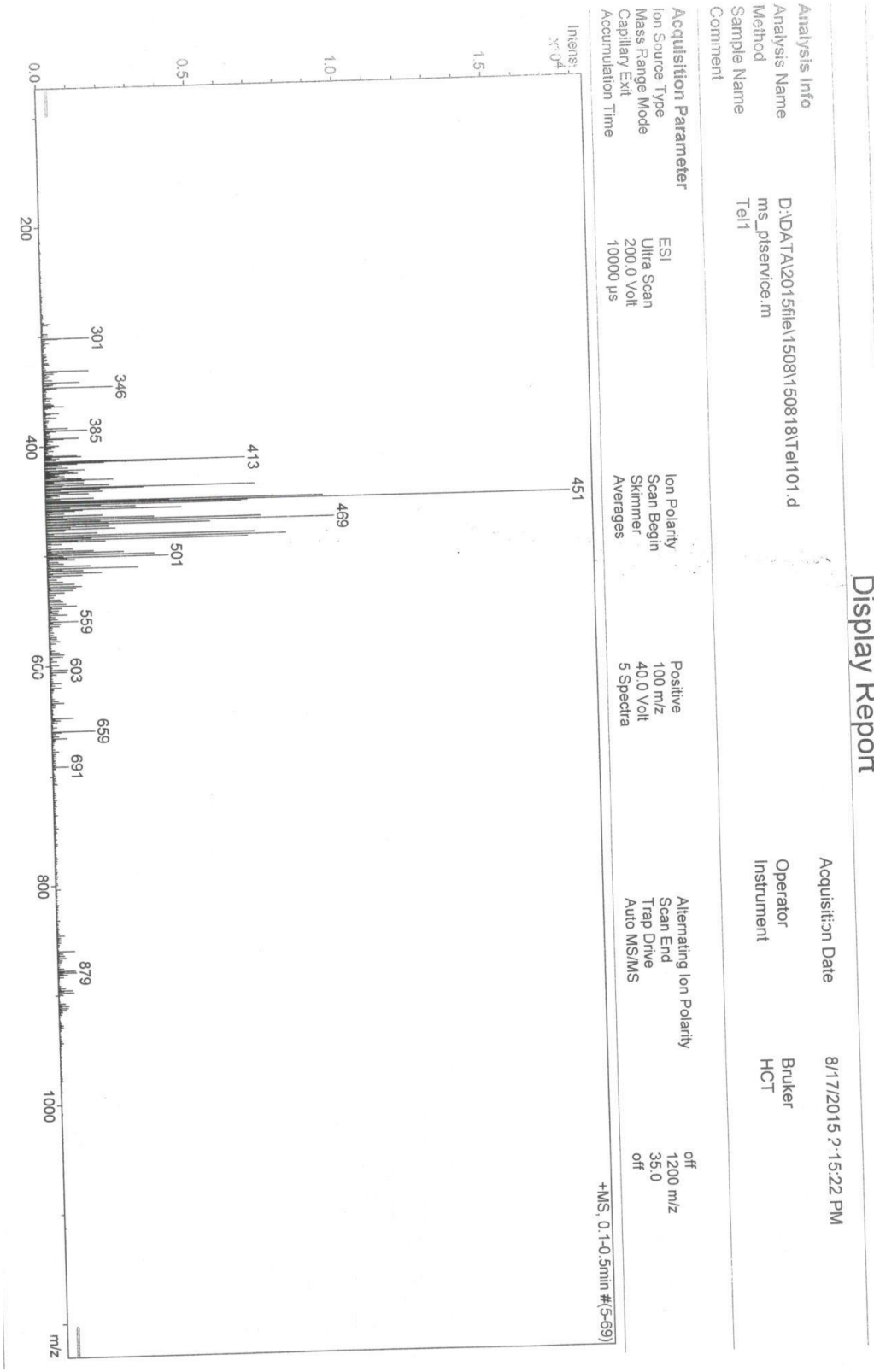
Appendix 2: ^{13}C and DEPT for β-spinasterol (Tel 1)



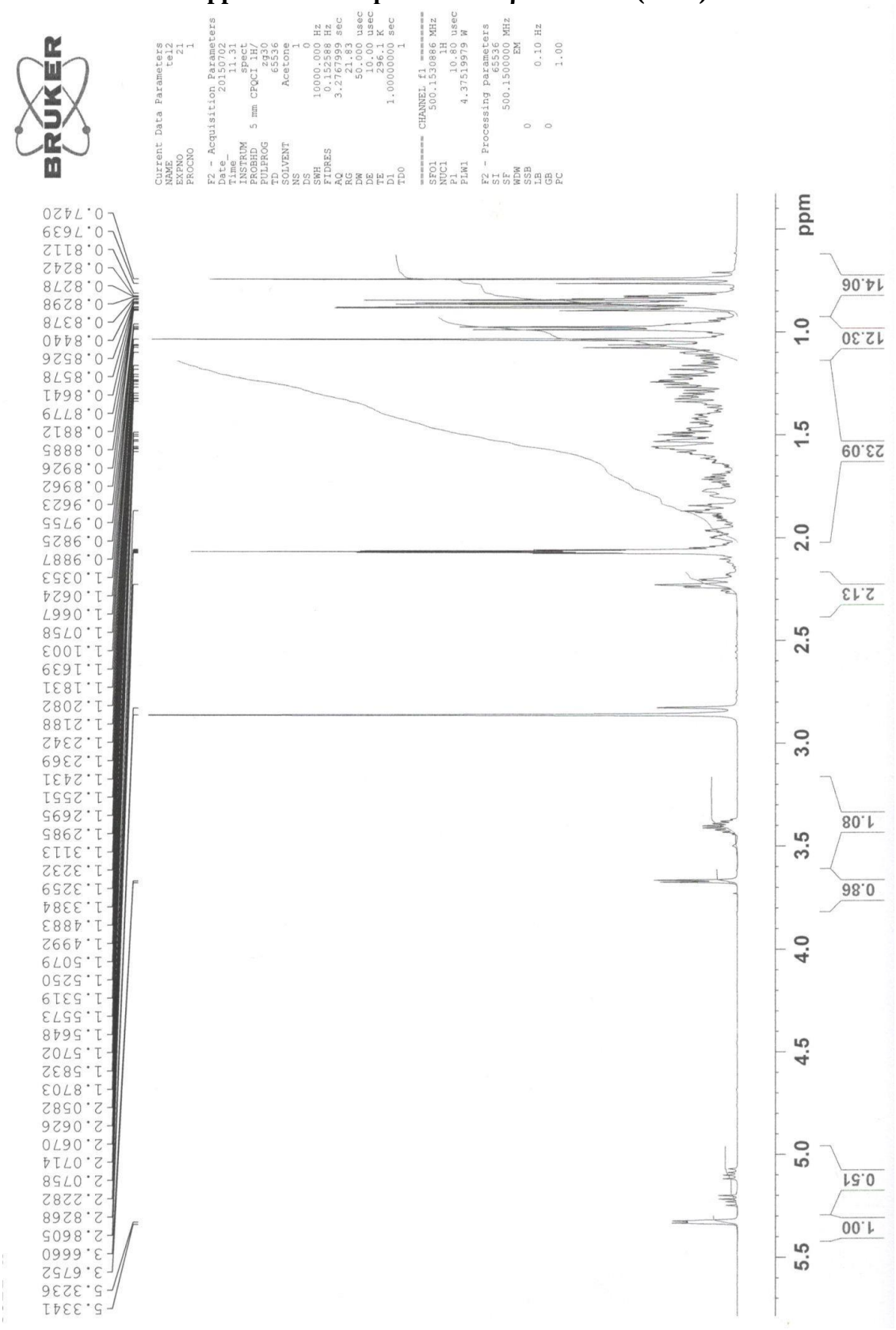
Appendix 3: ^{1}H for β-spinasterol (Tel 1)



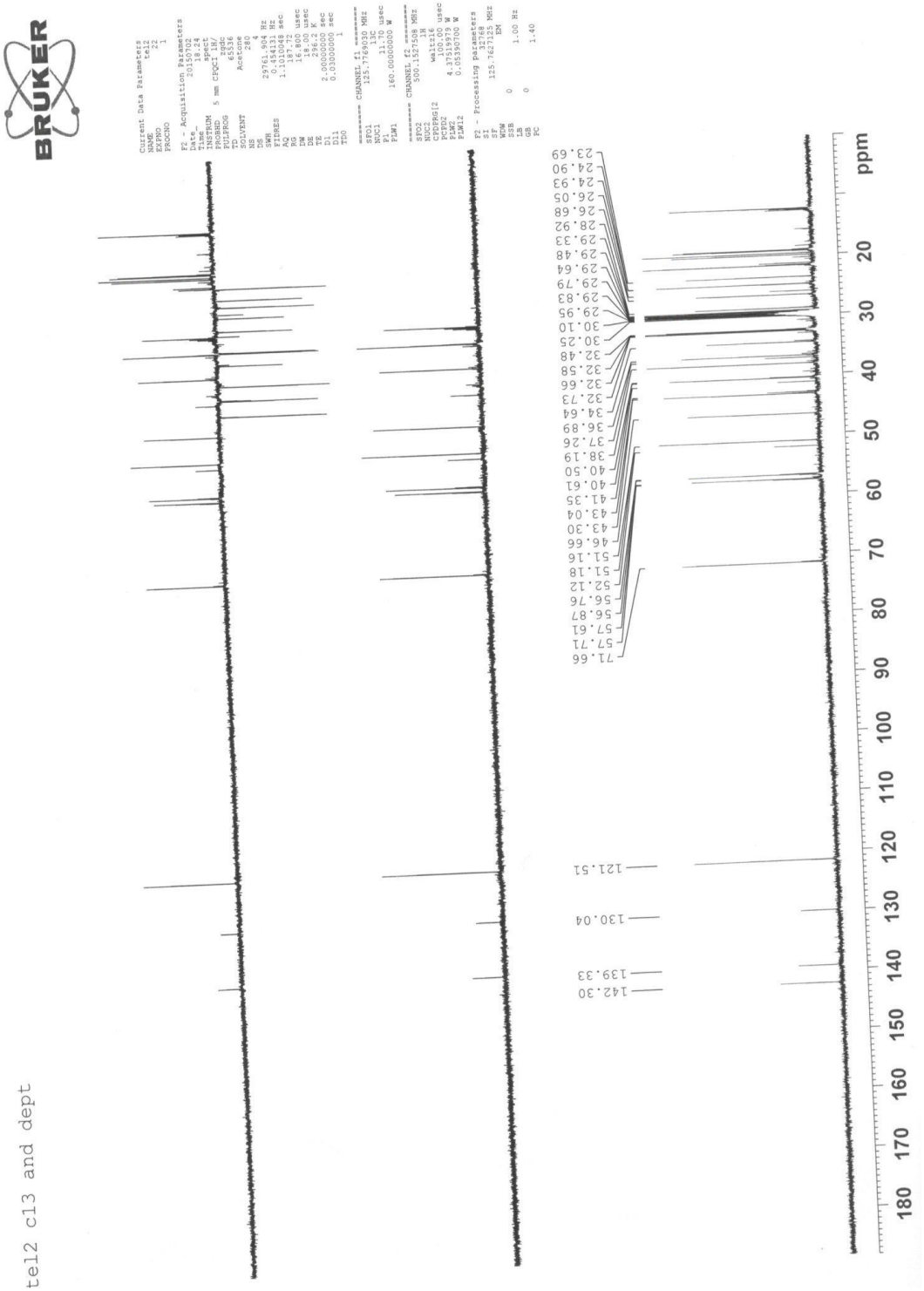
Appendix 4: Mass Spectra (MS) of β –spinasterol (Tel 1)



Appendix 5: ^{1}H spectrum for β-sitosterol (Tel 2)

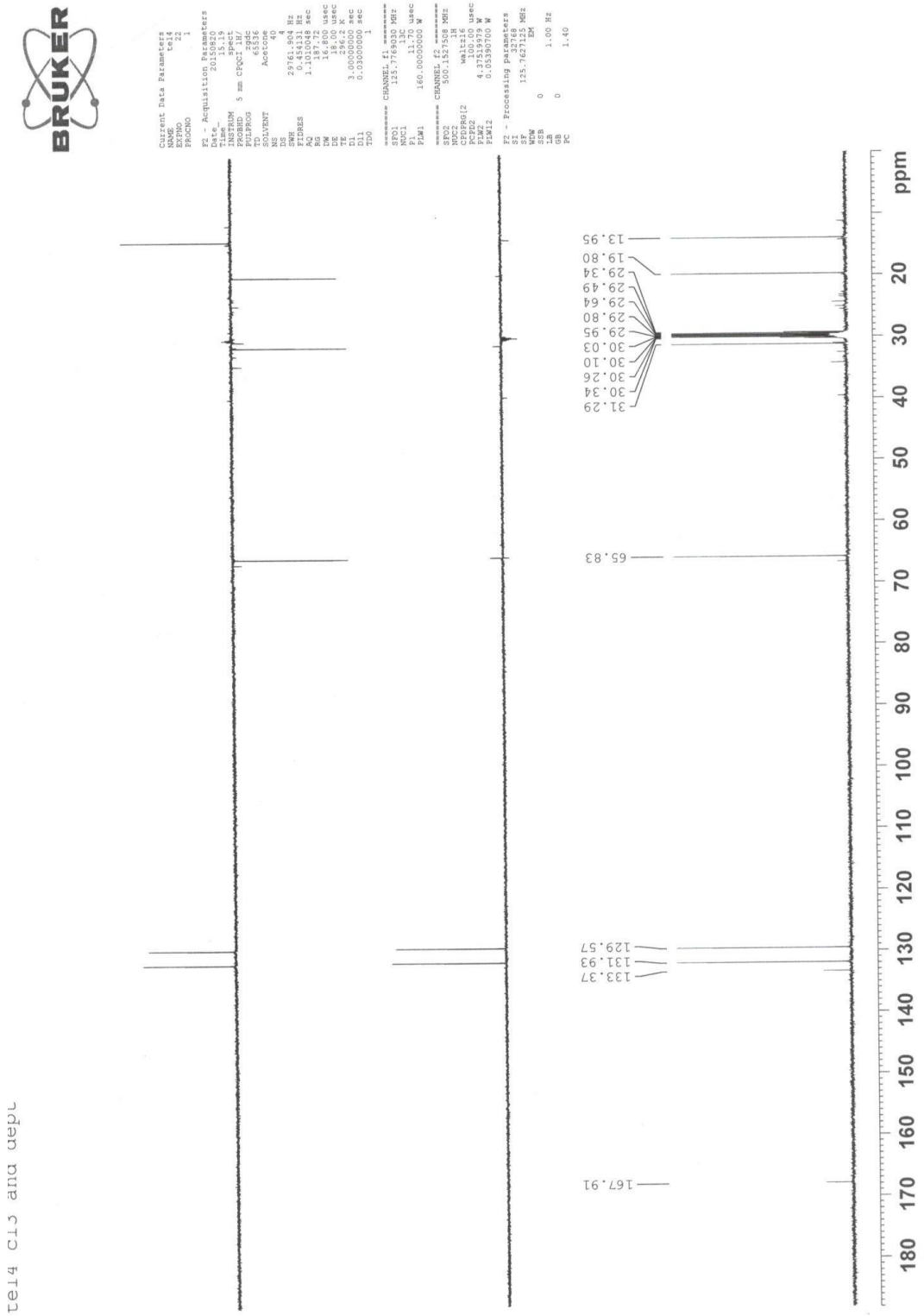


Appendix 6: ^{13}C and DEPT for β-sitosterol (Tel 2)

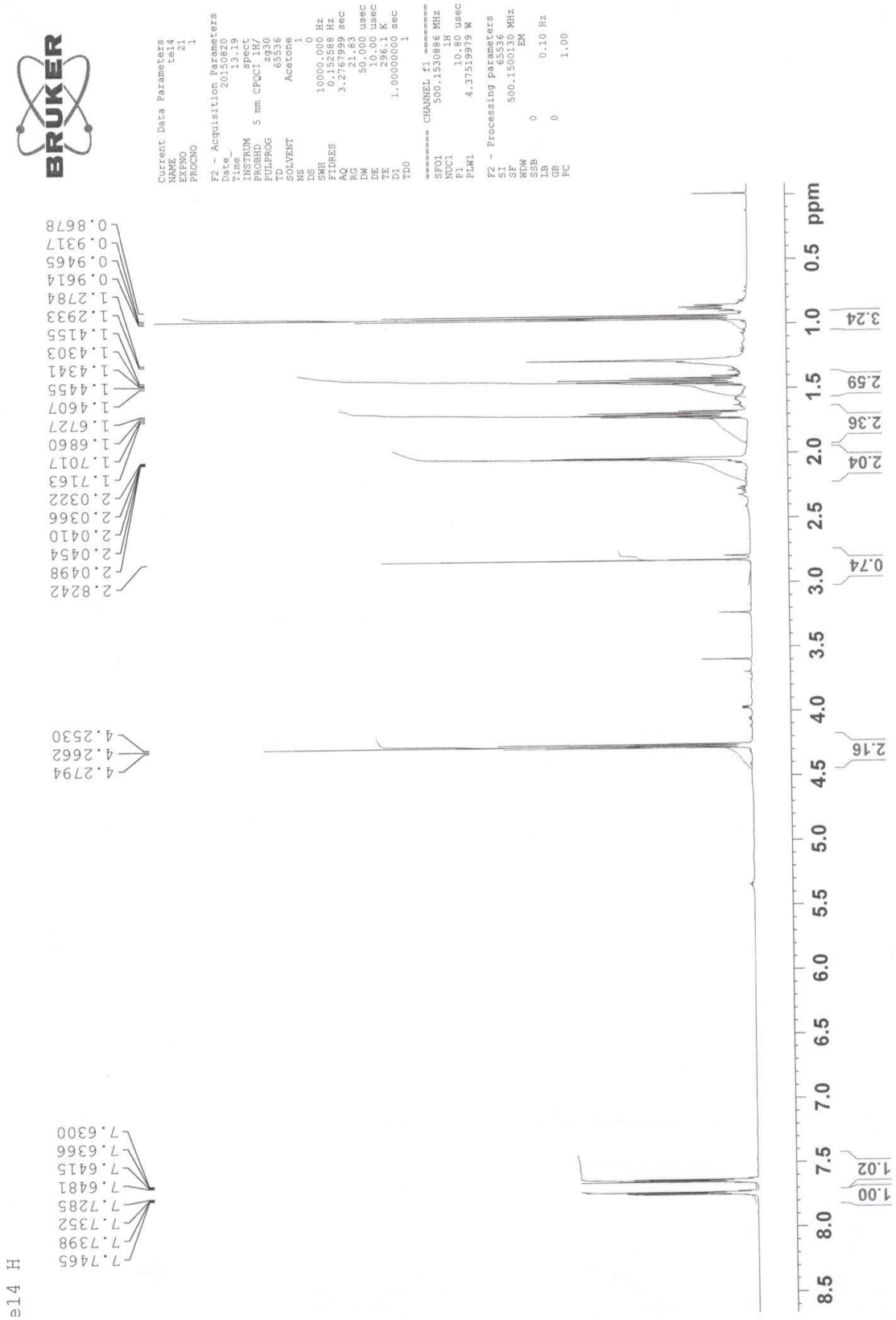


Appendix 7: Mass Spectra (MS) of β-sitosterol (Tel 2)

Appendix 8: ^{13}C and DEPT of benzoic acid butyl ester (Tel 3&4)



Appendix 9: ^{1}H spectrum for benzoic acid butyl ester (Tel 3&4)



Appendix 11: ^{1}H spectrum for fraction Tel 5