AMELIORATIVE EFFECT OF *MORINGA OLEIFERA* LEAVES ON VANADIUM-INDUCED NEUROTOXICITY IN MICE

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ABSTRACT

Moringa oleifera (MO) is a shrub belonging to the family Moringaceae and various reports exist on the medicinal usefulness of its crude extract, including effect on some neurodegenerative diseases. Vanadium (V), a transition metal emitted into the atmosphere during fossil burning and gas flaring, is implicated in various neurodegenerative conditions. However, the ameliorative effect of a pure compound from MO has not been documented. This study was therefore designed to assess the neurotherapeutic properties of a pure compound isolated from MO leaves against vanadium-induced neurotoxicity in mice.

A bioassay-guided fractionation was employed to separate the fractions of the methanol extract of MO leaves. Ferric reducing antioxidant potential assay was used to assess the fraction with the highest anti-oxidant potential, while preparative HPLC was employed to isolate the compound. Nuclear magnetic resonance was employed to elucidate the structure of the pure compound obtained, which was named MIMO2. Cell culture assays (Dihydroethidium, Micronucleus, and Comet assay tests) using immortalised mouse hippocampal cell lines (HT22) were used to assess the effect of MIMO2 on vanadium neurotoxicity. Eighty-four 2-week old mice were randomly and equally divided into seven groups, and dosed for 14 days, in the following groups: controls were water and DMSO, vanadium 3mg/kg (V), MIMO2 5mg/kg (M5), MIMO2 10mg/kg (M10), M5+V, and M10+V. Route of administration for all groups was intraperitoneal. Hanging wire and open field neurobehavioural tests were carried out on day 14, while all animals were humanely sacrificed and perfused on day 15. Histological examination on the brain included H&E, Cresyl Violet (for hippocampal neuronal count in cornu Ammonis 1 and 3 regions), immunohistochemistry (for microglia and astrocytes, with sterological count for microglia), Black Gold II histochemistry and triple immunofluorescence with confocal imaging. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

The concurrent administration of MIMO2 and V in HT22 cells resulted in a significant reduction of the immuno-expression of reactive oxygen species (33% reduction) and vanadium-induced DNA damage (52% reduction). Administration of M10 resulted in a significant amelioration of

the neurobehavioural deficits caused by vanadium. In V group, histology showed Purkinje cell degeneration, depletion and focal multiple layering, with cerebral gliosis, neuronal clumping and degeneration. All these neuropathologies were considerably reduced with the administration of M10. Cresyl Violet stain showed significant amelioration of vanadium-induced neuronal loss in the cornu Ammonis 1 region of M10+V ($4.9\pm2.3 \times 10^{-5}$/sqμm) compared to V ($3.9\pm1.9 \times 10^{-5}$/sqμm). For H&E and Cresyl Violet, no appreciable differences were observed in M5 and M5+V compared to controls and V group, respectively. The somatosensory cortex showed microglia and astrocytic hyperplasia and hypertrophy evident in the vanadium group (12.5±1.5%, area covered by microglia), which was significantly ameliorated in M10+V (9.3±2.3%). Black Gold II histochemistry showed severe vanadium-induced pantropic demyelination, particularly in the middle band of the corpus callosum, somatosensory and motor cortices, which were significantly alleviated in M10+V.

A novel antioxidant compound, MIMO2, was isolated in this study from *Moringa oleifera* leaves. The MIMO2 ameliorated vanadium-induced neurotoxicity both *in vitro* and *in vivo* in mice.

Keywords: *Moringa oleifera* leaves, MIMO2, vanadium neurotoxicity, neurotherapeutic activity

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To my husband,

Mr. Manfred U. Igado

To my daughters,

Roni Ojochetomi Oluwafelami Igado

And

Danielle Ojonemi Oluwasemiloore Igado

And

My Baby Sister,

Omolara Olaitan Olubusayo Ifeyemi Apeke Kenny-Adetola (Nee Oladiran)

1987 – 2017.

You were gone too soon, I love you.

CERTIFICATION

I certify that this work was carried out by Dr. Olumayowa O. Igado, in the Department of Veterinary Anatomy, University of Ibadan, under my supervision.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Vanadium (V) is a transition element (atomic number 23), an important mineral widely distributed on earth, and used extensively in the chemical industry (Cui *et al*., 2015). It is also naturally released into the atmosphere through forest fires, volcanic emissions, marine aerosols, and in the formation of continental dust (Englert, 2004). Vanadium is a trace metal in biological systems. It exists in water, rocks and soils in low concentrations, while existing in oil and coal deposits in relatively high concentrations (Zhang *et al*., 2001). Vanadium has also been reported to be a constituent of virtually all coal and petroleum oils (Eckardt, 1971; Mustapha *et al*., 2014). Vanadium also exists in different oxidative states – II to V (Igado *et al*., 2012)

In vivo, vanadium in combination with fluid (water) results in a reaction that causes the generation of hydroxyl radicals, thereby resulting in oxidative stress with time (Evangelou, 2002). In the presence of V(IV)/V(V), H_2O_2 and oxygen, a free radical production may take place by, mainly, the following chain reactions (Evangelou, 2002):

$$VO^{2+} + H_2O \longrightarrow {}_2VO_2^{+} + H^{+} + OH^{\bullet} \quad (1)$$

$$VO^{2+} + O_2{}^{2-} + H_2O \longrightarrow VO_2^{+} + OH^{-} + OH^{\bullet} \quad (2)$$

Generation of highly toxic hydroxyl radicals ($OH^{\bullet}$) may in turn trigger further radical reactions leading to the relative benign superoxide anion radical, which is spontaneously dismutated by superoxide anion dismutase (SOD), to oxygen and H_2O_2, that, if nothing interrupts, may react with vanadium (VO^{2+}) to perpetuate hydroxyl radical generation (Evangelou, 2002).

Vanadium is a neurotoxicant, proven to cross the blood-brain barrier and induce neurochemical alterations (Garcia *et al*., 2005). Neurotoxic effects have been shown to occur when administered by different routes. Routes include intraperitoneal (Igado *et al.*, 2012; Mustapha *et al*., 2014), *per os* or ingestion (Wenning and Kirsch, 1988; Garcia *et al*., 2005). Vanadium toxicity in humans includes central nervous system disturbances, congestion of the brain and spinal cord (WHO, 1988), while in rats it causes neuro-histopathologies which include necrosis of cerebellar Purkinje cells, demyelination, and also biochemical alterations (Igado *et al.*, 2012). Mental disorders like manic depressive syndrome have been associated with higher levels of vanadium in the blood (Conri *et al*., 1986).

Vanadium exists in different oxidation states and toxicity has been reported to increase with the valency of the compound, with the pentavalent compounds having the highest toxicity (Cui *et al*., 2015). Sodium metavanadate (V^{+5}) has been shown to cause histopathologies, oxidative stress resulting in lipid peroxidation in some brain areas, and also morphological changes in neuronal and glial cells (Garcia *et al*., 2004; Garcia *et al*., 2005; Igado *et al.*, 2012; Mustapha *et al*., 2014)

Moringa oleifera Lam (*M. oleifera*) is one of the 14 species of the family Moringaceae native to India, Africa, Arabia, Southeast Asia, South America, and the Pacific and Caribbean Islands (Iqbal and Bhanger, 2006). The leaves are often eaten as vegetables, and are reputed to be a rich source of essential amino acids such as methionine, cysteine, tryptophan and lysine (Makkar and Becker, 1997). The tree is small to medium sized, and various parts of the plant have been used in traditional medicine for the treatment of various diseases like sores, pneumonia, dysentery, as an analgesic etc (Gupta *et al*., 1999). It has also been used as a food and nutritional supplement (Anwar *et al*, 2007). It exhibits little side effects (Oluduro and

Aderiye, 2009; Ajibade *et al*., 2012) and has been given as supplements to malnourished babies in some parts of Africa (Fulgie, 1999). Almost all the parts of this plant have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders (Parrotta, 2001). *M. oleifera* contains many phytochemicals, for example phenols, flavonoids, alkaloids, glycosides, amino acids, carotenoids, vitamins and sterols (Anwar *et al*., 2007). The leaves are also reported to have anti-inflammatory and hypotensive activities (Caceres *et al*., 1992; Faizi *et al*., 1995). It possesses also a protective effect against oxidative damage (Sreelatha and Padma, 2011). In cell culture, the aqueous extract of the leaf was reported to have cytotoxic effect on human pancreatic cancer cells (Berkovich *et al*., 2013), thereby laying claim to having anti-cancer properties. The aqueous extract of the leave has also been reported to have ameliorating effects on some neurobehavioural deficits observed in Alzheimer's disease rat model (Ganguly *et al*., 2005).

1.2 Research Questions

1. Do *Moringa oleifera* leaves alleviate or cure neurotoxicity?
2. Can a pure compound be obtained from *Moringa oleifera*, which can demonstrate appreciable antioxidant activities?
3. Will the pure compound obtained be effective against vanadium-induced neurotoxicity *in vitro* and *in vivo*?

1.3 Aims and Objectives

This study aims to achieve the following:

- To isolate a potent and pure antioxidant from the methanolic extract of *Moringa oleifera* leaves.
- To discover a potent natural product to combat vanadium neurotoxicity.

- Can *Moringa* or its derivative offer an appreciable level of protection against a neurotoxicant *in vitro* and *in vivo*?

1.4 Justification

Natural products from plants remain an important source of new drugs. Natural products or natural product-derived drugs make up about 28% of all new chemical entities launched between 1981 and 2002, 24% of which are semi-synthetic natural product analogues or synthetic compounds based on natural product pharmacophores (Newman *et al*., 2003). Moringa is a plant that has been reputed to be a miracle plant and is freely eaten in the Nigerian environment. This study is expected to isolate and identify an antioxidant from *Moringa oleifera* leaves that may be effective in combating neurotoxicity both *in vitro* and *in vivo* in mice.

CHAPTER 2

LITERATURE REVIEW

2.1 *Moringa oleifera* (MO)

Moringa oleifera Lam is a member of the family *Moringaceae*. It is a multipurpose plant, widely distributed throughout Asia and Africa (Caceres *et al*., 1992; Iqbal and Bhanger, 2006). Every part of the plant has been reputed to be useful for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders (Parrotta, 2001). *M. oleifera* is a good source of protein (Rajanandh and Kavitha, 2010), and it contains many phytochemicals, for example phenols, flavonoids, alkaloids, glycosides, amino acids, carotenoids, vitamins and sterols (Anwar *et al*., 2007). The leaves are rich in amino acids like methionine, cysteine, tryptophan and lysine (Metha *et al*., 2003). Extracts of the seeds, leaves and roots have been studied for potential usefulness in wound healing, anti-tumour, anti-fertility, antipyretic, hypotensive, analgesic, antiepileptic, anti-inflammatory, anti-ulcer, antispasmodic, antifungal, antibacterial, diuretic, hypocholesterolaemic and antioxidant activities, to mention a few (Chumark *et al*., 2008).

The leaves are a good source of antioxidants and a paste from it has also been used as an external application for wound healing (Rajanandh and Kavitha, 2010),

2.1.1 Botany and Nomenclature

The plant is a medium sized tree of the *Moringaceae* family, consists of a single genus *Moringa.* Botanical name is *Moringa oleifera* Lam. The family has parietal placentation, 3-valved fruit, elongated, non-dehiscent berry and winged seeds. Over the years, different numbers of species have been reported by different people, from four, to ten. Notably, two species are

common, namely, *M. oleifera* and *M. cocanensis*. *M. oleifera* is distinguished by tripinnate leaves, 12-18mm long leaflets, yellow or white petioles without red streaks, and a medium sized tree. *M. cocanensis* has bipinnate leaves, 15-30mm long leaflets, petals have red streaks or are reddish at the base, and the tree is large (Ramachandran *et al*., 1980).

M. oleifera flowers are fragrant, bisexual, oblique, stalked, axillary, have many panicles, are densely pubescent, joined at the apex, and about 0.7 – 1.0 cm long (Ramachandran *et al*., 1980). *M. oleifera* tree is fast growing and drought-resistant (Fuglie, 1999).

Every part of the plant is useful. The trees are used as live fences, wind breaks, to reduce the effect of erosion, or as ornamental plants; the seeds can be used in water purification, the leaves are edible and the barks and roots are used for medicinal purposes (Ramachandran *et al*., 1980).

2.1.2 Origin and Distribution of *M. oleifera*

The tree is reportedly indigenous to Northwest India. It is widely distributed in India, Egypt, Philippines, Ceylon, Thailand, Malaysia, Burma, Pakistan, Singapore, West Indies, Cuba, Jamaica and Nigeria (Ramachandran *et al*., 1980).

2.1.3 Vernacular Names

Moringa oleifera Lam (MO) is known by a variety of names, depending on the location. In Burmese, it is known as *dandalonbin*; Sanskrit, *sobhanjana*; Hindi, *shajmah, shajna, segra*; (Ramachandran *et al*., 1980). In English, it is known as the 'Miracle tree', 'Horse-radish tree' or 'Ben-oil tree' (Luqman *et al*., 2012). In Nigeria, it goes by different names depending on the tribe. Some names are *garawa, konamare, rini maka* (Fulani), *zogale, bagaruwa maka* (Hausa), *ikwe oyibo* (Ibo), *ewe igbale, idagbo monoye* (Yoruba) (Fuglie and Sreeja, 2011).

2.1.4 General Uses of *M. oleifera*

All parts of the MO tree are useful and edible and have been consumed for a long time by humans. The trees have been used for live fencing, alley cropping, gum and wood. The seeds have been used for honey and sugar cane juice clarifier and also water purification. Ben-oil, a non-drying oil that resists rancidity is derived from the seed. This oil is used in salads, for the lubrication of fine machines and in the manufacture of cosmetics (Tsaknis *et al*., 1999). The leaves are used for animal forage, as a domestic cleaning agent when crushed, green manure, foliar nutrient and as a biopesticide, while the bark and gum have been used as tannin for tanning hides. All parts of the plant have been used for food and medicinal purposes (Fuglie, 1999; Fahey, 2005). One of the best known uses for MO in the western nations is the use of the powdered seeds to purify drinking water (Olsen, 1987). The seeds are also eaten green, in powdered or roasted form, steeped for tea or used in curries (Gassenschmidt, *et al*., 1995).

In the Philippines, women have been known to eat *Moringa* leaves mixed in chicken or shellfish soups to enhance breast milk production. The leaves are also used to increase the shelf-life of some foods in Southern India (Pari *et al*., 2007).

Moringa oleifera has been advocated for use in the tropics as food with high nutritive value to combat malnutrition in the developing world. It has been reported to contain a good amount of digestible protein, calcium, iron, vitamin A, C and E, carotenoids, amino acids and various polyphenols (Fahey, 2005; Rajanandh and Kavitha, 2010),

2.1.5 Medicinal and Pharmacological uses of *M. oleifera*

The MO plant has been used to treat a number of ailments like cardiovascular diseases due to the fact that the roots, leaves, gum, flowers have been reported to contain nitrile, mustard oil glycosides and thiocarbamate glycosides as their chemical components. These components

are probably responsible for the diuretic, cholesterol lowering, antiulcer, cardiovascular protective and hepatoprotective properties (Ghasi *et al*., 2000; Dangi *et al*., 2002; Metha *et al*., 2003; Anwar *et al*., 2007; Luqman *et al*., 2012). The roots have been used to treat diarrhoea in traditional medicine due to its antispasmodic activity through calcium channel blockade (Caceres *et al*., 1992). It also possesses antimicrobial activity (Luqman *et al*., 2012). Juice from the fresh leaf has also been found to inhibit the growth of some bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Caceres *et al*., 1991; Nikkon *et al*., 2003).

The flowers contain quercetin, and therefore confer hepatoprotection (Bharali *et al*., 2003), the seeds are used as biosorbent for the removal of cadmium from aqueous media, have antipyretic properties, and show antimicrobial activity (Oliveira *et al*., 1999). The ethanolic extract of the leaves was shown to have a lowering effect on hyperglycaemic, streptozocin-induced diabetic rats, and not in hypoglycaemia. This effect was possibly due to the stimulation of the β-cells of the pancreas or the insulin like activity of the extract (Tende *et al*., 2011). This insulin-like activity is of potential value in Type 2 Diabetic Mellitus patients. Leaves of MO have also been reported to regulate thyroid status and possess radioprotective (Rao *et al*., 2001) and antitumor (Murakami *et al*., 1998) activities. Anti-tumour activities have also been reported by Guevara *et al*. (1999), using the seed extracts in an *in vitro* assay, and by Berkovich *et al*. (2013), using the aqueous extract of the leaves on pancreatic cancer cells.

2.1.6 *M. oleifera* Leaves as an Antioxidant

One of the best known functions or uses of MO is its use as an antioxidant. In recent years, the incidences of chronic diseases have increased, probably due to factors like changes in lifestyle and diet, and a higher consumption of processed foods and drinks (Ara *et al*., 2008; Tsao, 2010). Free radicals are known to play a very important role in the pathogenesis of chronic

diseases like cancer, cardiovascular diseases, and rheumatoid arthritis (Ara *et al*., 2008; Tsao, 2010). This is due to the fact that major cellular components like lipids, proteins, carbohydrates and nucleic acids are susceptible to damage by free radicals, which are generated as by-products of normal aerobic metabolism and also metabolic reactions with drugs, toxins and alcohol (Ogbunugafor *et al*., 2012). Antioxidants are powerful metal chelators which employ a number of redox reactions to detoxify free radicals (Middleton *et al.*, 2000).

Polyphenols and flavonoids are natural antioxidants present in MO leaves (Verma *et al*., 2009; Rajanandh and Kavitha, 2010; Ogbunugafor *et al*., 2012; Vinoth *et al*., 2012; Berkovich *et al*., 2013). Vitamin C is also an antioxidant reported to be abundant in MO leaves (Lockett *et al*., 2000; Dillard and German, 2000; Siddhuraju and Becker, 2003; Ogbunugafor *et al*., 2012).

2.1.6.1 Polyphenols and Flavonoids

Production of free radicals exceeded (commonly designated as reactive oxygen species, ROS) was ascertained to play multiple important roles in tissue damage and loss of function in a number of tissues and organs (Zheng and Huang, 2001). Plant and its products are rich sources of phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Craig, 1999). The antioxidants could attenuate this oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radical species. Several epidemiological studies have shown that caretenoids, tocopherols, ascobates and dietary intake of natural phenolics antioxidants correlates with reduced risk of cancers, cardiovascular diseases, neurodegenerative diseases, aging, asthma and inflammation (Aruoma, 1998; Triantaphyllou *et al*., 2001). Therefore, gradually growing attention is being paid to phytochemicals characterized as antioxidant natural products, very good examples of which are polyphenols or flavonoids.

Recent research strongly supports high intake of fruits, vegetables and whole grains, which are rich in polyphenols, as a link to lowered risks of many chronic and degenerative diseases (Tsao, 2010). Furthermore, the role of serum lipoprotein disturbances and abnormal lipid metabolism characterized by hyperlipidaemia as an etiological factor in the development of coronary heart diseases has also been reported (Ara, 2008). Therefore, evaluation of plants for their antioxidant and lipid lowering capacity is a good approach to finding safer and cheaper ways of managing chronic diseases (Ogbunugafor, 2012).

Polyphenols are the largest class of phytochemicals, playing an important role in health. They are naturally occurring antioxidants in plants, and protect against free radicals when consumed (Blokhina *et al*., 2003; Ogbunugafor *et al*., 2012). Phenolic compounds are present in both edible and non-edible plants and have been shown to possess multiple biological effects, including antioxidative property (Rajanandh and Kavitha, 2010).

Flavonoids are water soluble polyphenolic compounds found abundantly in plants as their glycosides. They react directly with superoxide anions and lipid peroxyl radicals thereby inhibiting and breaking the chain of lipid peroxidation (Rajanandh and Kavitha, 2010).

Flavonoids comprise the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants. Major dietary sources of flavonoids include fruits, vegetables, cereals, tea, wine, and fruit juices (Manach *et al*., 2004). More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves (de Groot and Rauen, 1998). Research on flavonoids received an added impetus with the discovery of the French paradox, that is, the low cardiovascular mortality rate observed in Mediterranean populations in association with red wine consumption and a high saturated fat

intake. The flavonoids in red wine are responsible, at least in part, for this effect (Formica and Regelson, 1995).

Structurally, flavonoids consist of two aromatic carbon rings, benzopyran (A and C rings) and benzene (B ring), and may be divided into various subgroups based on the degree of the oxidation of the C ring, the hydroxylation pattern of the ring structure, and the substitution of the 3-position. The main dietary groups of flavonoids are:

i. Flavonols (e.g., kaempferol, quercetin), which are found in onions, leeks, and broccoli

ii. Flavones (e.g., apigenin, luteolin), which are found in parsley and celery

iii. Isoflavones (e.g., daidzein, genistein), which are mainly found in soy and soy products

iv. Flavanones (e.g., hesperetin, naringenin), which are mainly found in citrus fruit and tomatoes

v. Flavanols (e.g., (+)-catechin, (−)-epicatechin, epigallocatechin, epigallocatechin gallate (EGCG)), which are abundant in green tea, red wine, and chocolate

vi. Anthocyanidins (e.g., pelargonidin, cyanidin, malvidin), whose sources include red wine and berry fruits (Williamson and Manach, 2005; Manach *et al*., 2005; Williams and Spencer, 2012).

Once ingested, flavonoids undergo extensive metabolism in the small and large intestines, liver, and in cells, resulting in forms in the body which are very different from those found in foods (Williams and Spencer, 2012). The study of the association between flavonoid intake and the longterm effects on mortality yielded results suggesting that flavonoid intake is inversely correlated with mortality due to coronary heart disease (Hertog *et al*., 1995; Knekt *et al*., 1996).

2.1.6.1.1 Flavonoids

Until about 50 years ago, information on the working mechanisms of flavonoids was scarce. However, it has been widely known for centuries that derivatives of plant origin possess a broad spectrum of biological activity (Robak and Gryglewski, 1996). In 1930, a new substance was isolated from oranges, which is believed to be a member of a new class of vitamins, and was designated as vitamin P. When it became clear that this substance was a flavonoid (rutin), a flurry of research began in an attempt to isolate the various individual flavonoids and to study the mechanism by which flavonoids act (Nijveldt *et al*., 2001).

2.1.6.1.2 Phenolic Acid and its Derivatives

Some naturally occurring phenolic acids and analogues have been reported to display a wide variety of biological functions in addition to their primary antioxidant activity. Epidemiological studies have also indicated that diets rich in fruits and vegetables may result in a healthier population by preventing cancer (Fiuza *et al*., 2004). *Moringa oleifera* is a plant that has been reputed to have a high level of phenolic compounds, and contain anti-inflammatory agents of the glycoside type (Cheenpracha *et al*., 2010).

2.1.7 General Preparation of the *Moringa oleifera* Leave Extract

The *Moringa oleifera* leaves (MOL) are prepared in different ways before being administered to the animal subjects, the most favoured being the common laboratory rodents (rats and mice). The extracts vary from aqueous form (Ganguly *et al*., 2005; Adedapo *et al*., 2009), alcohol extracted form (Ganguly and Guha, 2008; Kirisattayakul *et al*., 2012; Sutalangka *et al*., 2013), to the air-dried and pulverised form (Nkukwana *et al*., 2014). The alcohol extracted form seems to be the most widely used form of the extract, with the reported yield varying from 1.34% (Mohan *et al*., 2005), 10% (Ganguly and Guha, 2008), to 17.49% (Kirisattayakul *et al*.,

2012; Sutalangka *et al*., 2013). Further analysis or fractionations by some authors (Verma *et al*., 2009; Rajanandh and Kavitha, 2010; Ogbunugafor *et al*., 2012; Vinoth *et al*., 2012; Berkovich *et al*., 2013), recorded the presence of flavonoids, phenolic compounds, vitamins and some amino acids. The extracts were normally administered *per os*.

2.1.8 Therapeutic Potential, Toxicity Studies and LD_{50} of MO

Before the advent of orthodox medicine, Africans relied on herbs for health care problems, while also using them as a source of food (Abalaka *et al*., 2009; Awodele *et al*, 2012). Many herbal medicines are believed to have preventive effects on chronic diseases due to their radical scavenging or antioxidative properties (Potterat, 1997). MOL has been shown to have a high level of phenolic contents, which have antioxidative effects. Many phenolic compounds display an antioxidative effect more potent than vitamin E *in vitro* and also inhibit lipid peroxidation by chain-breaking peroxyl-radical scavenging. They also directly scavenge reactive oxygen species like hydroxyl, superoxide and peroxynitrite radicals (Tsao and Akhtar, 2005); and to scavenge free radicals associated with 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) radical, superoxide, and nitric oxide as well as to inhibit lipid peroxidation (Sreelatha and Padma, 2009). Polyphenols constitute the largest class of phytochemicals, while dietary polyphenols have been shown to play important roles in human health (Ogbunugafor *et al*., 2012).

So far, only six studies on the use of MOL, using powdered whole leaf preparations in humans, have been conducted and published in literature (William *et al.*, 1993; Kumari, 2010; Nambiar *et al*., 2010; Ghiridhari *et al*., 2011; Kushwaha *et al*., 2012; Asare *et al*., 2012). These publications demonstrated the anti-hyperglycemic (antidiabetic) and anti-dyslipidemic activities of MOL. No adverse effect was reported in these human studies (Stohs and Hartman, 2015). None of these human studies evaluated the effect on the nervous system.

Several animal studies have been conducted to assess the toxicity of various preparations of MOL and also the ideal dose, all giving varied results and values. Administering the aqueous extract, at doses of 400, 800, 1600 and 2000 mg/kg daily for 21 days (single acute dose at the highest dose) were deemed to be safe in rats, using indices such as blood cell counts and serum enzyme level, although a dose-dependent decline in body weight was observed (Adedapo *et al*., 2009). Ambi *et al*. (2011) gave rats varying amounts of the powdered MOL incorporated into the feed, for 93 consecutive days, where observable lesions were reported in all organs, including the brain which reportedly showed neuronal degeneration and necrosis of glial cells. Concentrations of MOL used in this study were up to 75% of the feed. The reasons given for some of the pathologies observed were speculated to be probably due to the presence of some trace elements in the leaves, even though observed in the least detectable limits e.g. strontium (69 ± 3 ppm), rubidium (12 ± 2 ppm) and zirconium (11 ± 2 ppm). The authors went further to caution against indiscriminate eating of large quantities of MOL in the area in which the study was carried out.

In another study by Asare *et al*., (2012), the aqueous extract of MOL were found to be genotoxic, based on blood analysis at 3000 mg/kg. This dose is higher than what is normally consumed in humans. A further $_{assay}$ by the same authors revealed cytotoxic effects at 20 mg/kg using human peripheral blood mononuclear cells in vitro.

Other authors have reported very high doses of the aqueous extract in mice, and established the LD_{50}. Awodele *et al*. (2012) in an acute study administered 6400 mg/kg orally, and 1500 mg/kg intraperitoneally in an acute study. The same authors in a subchronic study of 60 days, daily administered 250, 500 and 1500 mg/kg of the extract *per os*. The authors claimed no significant toxicity signs were observed, even with evaluation of haematological and biochemical parameters, except the dose-dependent reduced food consumption observed in the

group treated with 1500 mg/kg. LD_{50} was estimated to be 1585 mg/kg. The oral dose appeared to be the safest form of administration of the extract.

The toxicological effects of the prolonged use of the alcoholic extract of MOL has also been documented by Bakre *et al*. (2013) and Oyagbemi *et al*. (2013). According to Bakre *et al*. (2013), the ethanolic extract of the leaves showed a significant dose-dependent decrease in rearing, grooming, head dips and locomotion; although they also reported an increased anxiogenic effect and enhanced learning in mice. The authors concluded that the leaves possessed a CNS depressant and anticonvulsant properties, the action of which was possibly mediated through the enhancement of the central inhibitory mechanism. This probably justifies the use of the leaves to treat epilepsy in traditional medicine. Oyagbemi *et al*. (2013) demonstrated that the chronic administration of the leaves may predispose the subject to hepatic and kidney damage.

2.1.9 Previous Work on *Moringa oleifera* Leaves (MOL) and Neurological and Related Diseases

In spite of the presence of the defence mechanisms in the nervous system, the nervous system still remains susceptible and vulnerable to various dangers and damages, which are up to 600, already identified disorders (Giacoppo *et al*., 2015). With the study of neurodegenerative diseases, there is an increase in the discovery of new natural compounds possessing pharmacological activities. Consequently, a number of studies have shown that plant-derived chemical compounds have potential-health promoting abilities (Giacoppo *et al*., 2015). A wide variety of phytochemicals have been shown to prevent the risk of carcinogenesis and some chronic diseases like neurodegenerative diseases (Calabrese *et al*., 2012; Alrawaiq and Abdullah, 2014; Fuentes *et al*., 2015).

Derivatives of MOL that have been shown to be effective against neurodegeneration include glucosinolates. Glucosinolates and their breakdown products, isothiocyanates have been reported to be present in little amount in Moringaceae plants. (Galuppo *et al*., 2014; Giacoppo *et al*., 2015). In recent years, glucosinolates have attracted a lot of research interest due to their reported protective effect against neurodegeneration (Giacoppo *et al*., 2015). Some types of glucosinolates (R,S-Sulforaphane – SFN) have been reported to offer protection to mesencephalic dopaminergic neurons from cytotoxicity and oxidative stress by removing intracellular quinone products, prevent reactive oxygen species production, DNA fragmentation and membrane breakdown (Han *et al*., 2007). SFN also protected primary cortical neurons against injuries caused by the oxidized products of dopamine (Spencer *et al*., 2002; Vauzour *et al*., 2007).

2.1.9.1 Use of MOL and Derivatives in *in vivo* Studies

The hippocampus plays a vital role in spatial memory, while the dorsal hippocampus provides animals with a spatial map of their environment (Parron *et al*., 2006). This it does by making use of reference and working memory (Liu and Bilkey, 2001). Lesions in this region cause problems relating to goal-directed navigation and also impair the ability to remember precise location (Herbert and Dash, 2004). Mohan *et al*. (2005) reported the nootropic activity of MOL and so, the ability to improve memory in male and female rats. The leaves displayed a facilitatory effect on retention and acquired learning, using the passive shock avoidance test and elevated plus maze. The extract administered at 100 mg/kg significantly reduced the number of mistakes and latency time to reach the shock free zone. With the elevated plus maze, the extract at 50 mg/kg significantly reduced the transfer latency on the second day of testing, while also antagonising the effect of scopolamine.

In a previous study by Sutalangka *et al*. (2013), experimental rats were administered AF64A (a cholinotoxin) via the intracerebroventricular route, to induce dementia, administration of the alcoholic extract of MOL showed a significant reduction in the escape latency time when subjected to Morris water maze. Also, a corresponding increase in neuronal density of the CA1, CA2, CA3 and the dentate gyrus regions were also observed in groups administered the extract as a treatment to AF64A. The extract also significantly attenuated the decreased activities of superoxide dismutase and catalase induced by AF64A, and decreased malondialdehyde level.

Some compounds isolated from MOL have been shown to have protective effects on the components of the CNS. Protease inhibitors (proline and alanine) isolated from MOL was successfully used to alleviate the extent of axonal damage and treat degenerating axons in rats induced with spinal cord injury resulting in paraplegia in the experimental rats. The protease inhibitors were administered intraperitoneally for the first three post-operative days. Recovery of some level of hind limb function was reported to be better in the drug-treated rats after 7 days post-operation. Quantitative analyses of secondary axonal degeneration at sites remote from the direct mechanical insult was reported to have provided solid evidence for the beneficial effects of protease inhibitors. In the rats treated with proline, the amount of degenerating axons was 13% less than that in untreated controls ($P < 0.001$), and a similar effect was observed in the rats treated with alanine at a dose of 500 mg/kg of body weight, the amount being 12% less than in untreated controls. These protease inhibitors however were said to not cross the blood brain barrier (Singh *et al*., 2012).

Kirisattayakul *et al*. (2012; 2013) demonstrated the potential benefit of the hydroalcohol MOL extract in decreasing brain infarct volume, and also its neuroprotective effect against focal cerebral ischemia. Ischemic stroke was induced by occlusion of the middle cerebral artery, and

the animals were fed extract of MOL. Results showed cerebroprotective effect and enhanced superoxide dismutase activity in the hippocampus, and decreased malondialdehyde levels in cerebral cortex, hippocampus and the striatum.

2.1.9.2 Use in Neuronal Cell Culture

MOL has been used in ayurvedic medicine to treat a number of central nervous system (CNS) ailments, ranging from paralysis, nervous debility to nerve disorders (Hannan *et al*., 2014). There has been evidence for nootropic and neuroprotective disorders in cell cultures of neural cells and in animal models (Hannan *et al*., 2014). Using hippocampal neurons, Hannan *et al*., (2014) reported that the addition of MOL ethanolic extract significantly increased the number and length of neurites and their branching, in a dose-dependent manner, with the optimal concentration achieved at 30 μg/ml. In the same experiment, neuronal viability was increased, cellular injury was decreased and the rate of neuronal differentiation was also accelerated. No cytotoxicity was observed. Neurons also exhibited more extended and multiple branching, an increase in the number and length of primary dendrites and also the appearance of more secondary and even tertiary dendrites. MOL was also observed to modulate axonal development and promote synaptogenesis. The reasons for this multiple branching and differentiation observed could be due to the presence of β-carotene, which is abundant in MOL. β-carotene has been reported to be an inducer of neuronal cell differentiation (Lee *et al*., 2013).

The mechanism of action of *Moringa oleifera* leaves is probably due to the high level of polyphenols and other antioxidative compounds it possesses, which confer neuroprotection by scavenging free radicals or activating cellular antioxidant system (Luqman *et al*., 2012). There is an abundance of data on the use of MOL to treat conditions relating to diabetes, hyperlipidemia, hypertension, hypoglycaemia and some other related conditions, but currently very little

information on pure compounds derived from MOL which have been successfully used to treat neurodegeneration, neurological or related conditions. The current economic recession being experienced world-wide, especially in African countries, is likely to make people seek out the use of herbal medicine more, thereby necessitating the need for further research on this plant. Further investigation still needs to be carried out to isolate and determine a compound that is ideal for combating neurodegeneration.

2.2 Vanadium

Heavy metals are chronically persistent in the environment and severely affect human health through the food chain (Verma and Dubey, 2003). Vanadium (V) is one of the heavy metals with a wide distribution in the world. V is mainly mined in South Africa, Russia and China (WHO, 2001). The average concentration of vanadium in soils around the world is estimated to be around 150 mg kg^{-1} (Edwards *et al.,* 1995; WHO, 2001; Panichev *et al.,* 2006). Like many other heavy metals, V is released into the environment through anthropogenic activities, and its concentration in the environment has been gradually increasing in recent years due to an increased demand for V in high-temperature industrial activities (Krachler *et al*., 2003). V has been recognized as a potentially dangerous pollutant in the same class as mercury (Hg), lead (Pb) and arsenic (As) (Naeem *et al.,* 2007).

Vanadium (V) is a transition element (atomic number 23), an important mineral widely distributed on earth, and used extensively in the chemical industry (Cui *et al*., 2015). It is also naturally released into the atmosphere through forest fires, volcanic emissions, marine aerosols, and also in the formation of continental dust (Englert, 2004).

2.2.1 History of Vanadium

Vanadium was discovered by Andrés Manuel del Río, a Spanish-Mexican mineralogist, in 1801. Del Río extracted the element from a sample of Mexican "brown lead" ore, later named vanadinite. He found that its salts exhibit a wide variety of colors, and as a result he named the element *panchromium* (Greek: παγχρώμιο "all colors"). Later, Del Río renamed the element *erythronium* (Greek: ερυθρός "red") as most of its salts turned red upon heating. In 1805, the French chemist Hippolyte Victor Collet-Descotils, backed by del Río's friend Baron Alexander von Humboldt, incorrectly declared that del Río's new element was only an impure sample of chromium. Del Río accepted Collet-Descotils' statement and retracted his claim (Cintas, 2004). In 1831, the Swedish chemist Nils Gabriel Sefström rediscovered the element in a new oxide he found while working with iron ores. Later that same year, Friedrich Wöhler confirmed del Río's earlier work (Sefström, 1831). Sefström chose a name beginning with V, which had not been assigned to any element yet. He called the element *vanadium* after Old Norse *Vanadís* (another name for the Norse Vanr goddess Freyja, whose facets include connections to beauty and fertility), because of the many beautifully colored chemical compounds it produces (Sefström, 1831).

The isolation of vanadium metal proved difficult. In 1831, Berzelius reported the production of the metal, but Henry Enfield Roscoe showed that Berzelius had in fact produced the nitride, vanadium nitride (VN). Roscoe eventually produced the metal in 1867 by reduction of vanadium (II) chloride, VCl_2, with hydrogen (Roscoe, 1869). In 1927, pure vanadium was produced by reducing vanadium pentoxide with calcium (Marden and Rich, 1927). The first large-scale industrial use of vanadium in steels was found in the chassis of the Ford Model T, inspired by French race cars. Vanadium steel allowed for reduced weight while simultaneously

increasing tensile strength (Betz, 2003). German Chemist Martin Henze discovered vanadium in the blood cells (or coelomic cells) of Ascidiacea (sea squirts) in 1911 (Henz, 1911; Michibata *et al.*, 2002).

2.2.2 Characteristics of Vanadium

Vanadium is a medium hard, ductile, steel-blue metal. Some sources describe vanadium as "soft", perhaps because it is ductile, malleable and not brittle (Voort, 1984; Cardarelli, 2008). Vanadium is harder than most metals and steels. It has good resistance to corrosion and it is stable against alkalis, sulfuric and hydrochloric acids (Holleman *et al.*, 1985). It is oxidized in air at about 933 K (660 °C, 1220 °F), although an oxide layer forms even at room temperature (Holleman *et al.*, 1985).

2.2.3 Isotopes of Vanadium

Naturally occurring vanadium is composed of one stable isotope ^{51}V and one radioactive isotope ^{50}V. The latter has a half-life of 1.5×10^{17} years and a natural abundance 0.25%. ^{51}V has a nuclear spin of 7/2 which is useful for NMR spectroscopy (Georges *et al*., 2003). Twenty four artificial radioisotopes have been characterized, ranging in mass number from 40 to 65. The most stable of these isotopes are ^{49}V with a half-life of 330 days, and ^{48}V with a half-life of 16.0 days. The remaining radioactive isotopes have half-lives shorter than an hour, most of which are below 10 seconds. At least 4 isotopes have metastable excited states. Electron capture is the main decay mode for isotopes lighter than the ^{51}V. For the heavier ones, the most common mode is beta decay. The electron capture reactions lead to the formation of element 22 (titanium) isotopes, while for beta decay, it leads to element 24 (chromium) isotopes (Georges *et al*., 2003).

The chemistry of vanadium is noteworthy for the accessibility of the four adjacent oxidation states 2-5. In aqueous solution, vanadium forms metal aquo complexes; the colours are

lilac $[V(H_2O)_6]^{2+}$, green $[V(H_2O)_6]^{3+}$, blue $[VO(H_2O)_5]^{2+}$, yellow VO^{3-}. Vanadium (II) compounds are reducing agents, and vanadium (V) compounds are oxidizing agents. Vanadium (IV) compounds often exist as vanadyl derivatives which contain the VO^{2+} center (Holleman *et al*., 1985).

Vanadium exists in aqueous solution as tetravalent (IV) vanadyl (VO^{2+}) and pentavalent (V) vanadate (HVO_4^-, VO_3^- and/or $H_2VO_4^-$) (Djordjevic *et al.,* 1995; Kresja *et al.,* 1997; Morinville *et al.,* 1998). A number of monomeric and polymeric tetravalent [V(IV)] and pentavalent [V(V)] vanadium species can be present in aqueous solutions, their composition depending upon pH and vanadium concentrations (Evangelou, 2002).

In the presence of oxidizing agents, the vanadium ion is present as the hydrated monomer of vanadate (HVO_4^{2-} or $H_2VO_4^-$) at micromolar concentrations near neutral pH, whereas in the presence of extracellular or intracellular reducing agents (e.g. glutathione), the anion is reduced to the cation vanadyl (VO_2^+), within a few minutes (Djordjevic and Wampler, 1985; Tassiopoulos *et al.,* 1999, 2000). Bioreduction of vanadium (V) to vanadium (IV) by glutathione (Sabbioni *et al.,* 1993) or by microsomes through hydroxyl radicals generation (Shi and Dalal, 1992) as well as by NADH-depended flavoenzymes (Shi and Dalal, 1991) seems to regulate various cellular actions of vanadium compounds such as its cytotoxic and morphological effects on various cells. Vanadate also begins to polymerize at concentrations greater than 0.1 mM at neutral pH (Gordon, 1991). The monomer and polymers V(V) species up to the hexamer, undergo rapid polymerization and depolymerization with pH changes, whereas the decamer is formed rapidly when a V(V) solution is acidified but only slowly depolymerized when the solution is brought back to neutral pH. There is evidence indicating that V(V) at different states of polymerization may also have different toxicity (Wei *et al.,* 1982). Vanadium may also exist

at low oxidation states as trivalent [V(III)] and divalent [V(II)] ions (Meier *et al.,* 1995). Vanadium existing at trivalent state [V(III)] as in two newly synthesized complexes with the sulfhydryl-containing pseudopeptide *N*-(2-mercaptopropionyl) glycine and the amino acid cysteine, is slowly oxidized to V(IV) in aqueous solutions (Tassiopoulos *et al.*, 1999; 2000; Liasko *et al.,* 2000). At physiological pH vanadium is found in the pentavalent oxidation state, as vanadate anion, which is the most stable form among its various oxidation states (Erdman *et al.*, 1984). The oxidation state of vanadium *in vitro* and *in vivo* is related to a variety of its biological effects among which is the participation in free radical reactions (Evangelou, 2002).

The oxidation state of vanadium seems also to determine some differences in biological effects of vanadium compounds. Vanadate (V) and oxovanadium (IV) complexes are, for example, both inhibitors of protein phosphotyrosines (PTPs) in many cells, acting however, with different mechanisms. Vanadate appears to act as a phosphate analogue mimicking the transition state (Bevan *et al.,* 1995a, b; Huyer *et al.*, 1997; Zhang *et al*., 1997). Thus vanadate forms a weak and reversible bond with the thiol group of the PTPs, acting as a competitive inhibitor. Peroxovanadium complexes and aqueous peroxovanadates are however irreversible and more potent PTPs inhibitors since the critical cysteine residue in their catalytic domain is oxidized by peroxovanadium complexes (Huyer *et al.,* 1997; Morinville *et al.,* 1998). The oxidation state of vanadium may also play a role in the activation of intracellular signal transduction pathways regulating the activation of cytosolic protein tyrosine kinases (Elberg *et al.*, 1994; Kresja *et al*., 1997).

2.2.4 Occurrence of Vanadium

Metallic vanadium is not found in nature, but vanadium compounds occur naturally in about 65 different minerals. Economically significant examples include patronite (VS_4), vanadinite ($Pb_5(VO_4)_3Cl$), and carnotite ($K_2(UO_2)_2(VO_4)_2 \cdot 3H_2O$) (Magyar, 2011). Much of the world's vanadium production is sourced from vanadium-bearing magnetite found in ultramafic gabbro bodies. Vanadium is mined mostly in South Africa, north-western China, and eastern Russia. In 2010, these three countries mined more than 98% of the 56,000 tonnes of produced vanadium (Magyar, 2011).

Vanadium is also present in bauxite and in fossil fuel deposits such as crude oil, coal, oil shale and tar sands. In crude oil, concentrations up to 1200 ppm have been reported. When such oil products are burned, the traces of vanadium may initiate corrosion in motors and boilers (Pearson and Green, 1993).

An estimated 110,000 tonnes of vanadium per year are released into the atmosphere by burning fossil fuels (Anke, 2004). Vanadium has also been detected spectroscopically in light from the Sun and some other stars (Cowley *et al*., 1978).

2.2.5 Industrial Applications and uses of Vanadium

Vanadium is used as an alloy with other metals. Approximately 85% of vanadium produced is used as ferrovanadium or as a steel additive (Moskalyk and Alfantazi, 2003). The considerable increase of strength in steel containing small amounts of vanadium was discovered in the beginning of the 20th century. Vanadium forms stable nitrides and carbides, resulting in a significant increase in the strength of the steel (Chandler, 1998). Since the discovery of the increased strength conferred on steel by vanadium, vanadium steel has been used for applications in axles, bicycle frames, crankshafts, gears, and other critical components. There are two groups

of vanadium containing steel alloy groups. Vanadium high carbon steel alloys contain 0.15% to 0.25% vanadium and high-speed tool steels (HSS) have a vanadium content of 1% to 5%. For high-speed tool steels, hardness above HRC 60 can be achieved. HSS steel is used in surgical instruments and tools (Davis, 1995). Some powder metallurgic alloys can contain up to 18% percent vanadium. The high content of vanadium carbides in those alloys increases the wear resistivity significantly. One application for those alloys are tools and knives (Oleg *et al.*, 2009). Vanadium stabilizes the beta form of titanium and increases the strength and temperature stability of titanium and when mixed with aluminium in titanium alloys, it is used in jet engines, high-speed airframes and dental implants. One of the common alloys is Titanium 6AL-4V, a titanium alloy with 6% aluminium and 4% vanadium (Peters and Leyens (2002).

Vanadium is compatible with iron and titanium, therefore vanadium foil is used in cladding titanium to steel (Lositskii *et al.*, 1966). The moderate thermal neutron-capture cross-section and the short half-life of the isotopes produced by neutron capture makes vanadium a suitable material for the inner structure of a fusion reactor (Matsui *et al*., 1996; Chang, 2009). Several vanadium alloys show superconducting behavior. The first A15 phase superconductor was a vanadium compound, V_3Si, which was discovered in 1952 (Hardy and Hulm, 1953). The most common oxide of vanadium, vanadium pentoxide, V_2O_5, is used as a catalyst in manufacturing sulfuric acid by the contact process (Eriksen *et al*., 1995) and as an oxidizer in maleic anhydride production (Abon and Volta, 1997). Vanadium pentoxide is also used in making ceramics (Lide, 2004). Another oxide of vanadium, vanadium dioxide VO_2, is used in the production of glass coatings, which blocks infrared radiation (and not visible light) at a specific temperature (Manning *et al.*, 2002). Vanadium oxide can be used to induce color centers in corundum to create simulated alexandrite jewelry, although alexandrite in nature is a

chrysoberyl (White *et al*., 1962). The possibility to use vanadium redox couples in both half-cells, thereby eliminating the problem of cross contamination by diffusion of ions across the membrane is the advantage of vanadium redox rechargeable batteries (Joerissen *et al.,* 2004). Vanadate can be used for protecting steel against rust and corrosion by electrochemical conversion coating (Guan and Buchheit, 2004). Lithium vanadium oxide has been proposed for use as a high energy density anode for lithium ion batteries, at 745 Wh/L when paired with a lithium cobalt oxide cathode (Kariatsumari, 2008). It has been proposed by some researchers that a small amount, 40 to 270 ppm, of vanadium in Wootz steel and Damascus steel, significantly improves the strength of the material, although it is unclear what the source of the vanadium was (Verhoeven *et al.,* 1998). Lithium vanadium phosphate has been proposed for a new battery as well, and is very commercially applicable because phosphates are inexpensive and vanadium makes the battery very energy dense.

2.2.6 Biological Effects and Role of Vanadium

Vanadium plays a very limited role in human biology (Rehder, 2013). It is more important in marine environments than terrestrial ones (Sigel and Sigel, 1995).

2.2.6.1 Vanadoenzymes

A number of species of marine algae produce vanadium containing vanadium bromoperoxidase as well as the closely related chloroperoxidase (which may use a heme or vanadium cofactor) and iodoperoxidases. The bromoperoxidase produces an estimated 1–2 million tons of bromoform and 56,000 tons of bromomethane annually (Gordon, 1999). Most naturally occurring organobromine compounds, arise by the action of this enzyme (Butler and Carter-Franklin, 2004). They catalyse the following reaction (R-H is hydrocarbon substrate):

$$R\text{-}H + Br^- + H_2O_2 \rightarrow R\text{-}Br + H_2O + OH^-$$

A vanadium nitrogenase is used by some nitrogen-fixing micro-organisms, such as *Azotobacter*. In this role vanadium replaces more common molybdenum or iron, and gives the nitrogenase slightly different properties (Robson *et al*., 1986).

2.2.6.2 Vanadium Accumulation in Tunicates and Ascidians

Vanadium is essential to ascidians and tunicates, where it is stored in the highly acidified vacuoles of certain blood cell types, designated vanadocytes. Vanabins (vanadium binding proteins) have been identified in the cytoplasm of such cells. The concentration of vanadium in these ascidians' blood is up to ten million times higher (MacAra *et al.,* 1979; Smith, 1989) than the concentration of vanadium in surrounding seawater, which normally contains 1 to 2 μg/l (Weiss *et al.,* 1977; Trefry and Simone, 1989). The function of this vanadium concentration system, and these vanadium-containing proteins, is still unknown but the vanadocytes are later deposited just under the outer surface of the tunic where their presence may deter predation (Ruppert *et al*., 2004).

2.2.6.3 Fungi

Several species of macrofungi, namely *Amanita muscaria* and related species, accumulate vanadium (up to 500 mg/kg in dry weight). Vanadium is present in the coordination complex amavadin (Kneifel and Ernst, 1997), in fungal fruitbodies. However, the biological importance of the accumulation process is unknown (Falandysz, *et al.,* 2007). Toxin functions or peroxidase enzyme functions have been suggested.

2.2.6.4 Mammals and Birds

Deficiencies in vanadium result in reduced growth and impaired reproduction in rats and chickens (Schwarz and Milne, 1971). Vanadium is a relatively controversial dietary supplement, used primarily for increasing insulin sensitivity and body building (Yeh *et al.,* 2003). Whether it

works for the latter purpose has not been proven; some evidence suggests that athletes who take it are merely experiencing a placebo effect (Talbott and Hughes, 2007). Vanadyl sulfate may improve glucose control in people with type 2 diabetes (Boden *et al.*, 1996; Halberstam *et al*., 1996; Badmaev, *et al*., 1999; Goldwaser *et al*., 1999; Goldfine *et al*., 2000). Decavanadate and oxovanadates appear to play a role in a variety of biochemical processes, such as those relating to oxidative stress (Aureliano and Crans, 2009).

2.2.7 Vanadium Safety and Toxicity

All vanadium compounds should be considered toxic. Tetravalent $VOSO_4$ has been reported to be over 5 times more toxic than trivalent V_2O_3. (Roschin, 1967). The Occupational Safety and Health Administration (OSHA) has set an exposure limit of 0.05 mg/m^3 for vanadium pentoxide dust and 0.1 mg/m^3 for vanadium pentoxide fumes in workplace air for an 8-hour workday, 40-hour work week (OSHA, 2009). The National Institute for Occupational Safety and Health (NIOSH) has recommended that 35 mg/m^3 of vanadium be considered immediately dangerous to life and health. This is the exposure level of a chemical that is likely to cause permanent health problems or death (OSHA, 2009). Vanadium compounds are poorly absorbed through the gastrointestinal system. Inhalation exposures to vanadium and vanadium compounds result primarily in adverse effects on the respiratory system (Sax, 1984; Ress *et al,* 2003; Wörle-Knirsch *et al*., 2007).

Quantitative data are, however, insufficient to derive a subchronic or chronic inhalation reference dose. Other effects have been reported after oral or inhalation exposures on blood parameters (Ścibior *et al*., 2006; Gonzalez-Villalva *et al*., 2006), on liver (Kobayashi *et al*., 2006), on neurological development in rats (Soazo and Garcia, 2007), and other organs (Barceloux, 1999). The effect of vanadium on reproduction and development showed that

vanadium caused decreased fertility, embryolethality, fetotoxicity and teratogenicity in rats, mice and hamsters (Domingo, 1996). Vanadium also causes low sperm count, decreases sperm motility and increases the incidence of sperm abnormalities (Altamirano-Lozano *et al.,* 1996; Aragón and Altamirano-Lozano, 2001). Vanadium pentoxide was reported to be carcinogenic in male rats and male and female mice by inhalation in an NTP study (Ress *et al*., 2003), although the interpretation of the results has recently been disputed (Duffus, 2007). Vanadium has not been classified with respect to carcinogenicity by the United States Environmental Protection Agency (Opreskos, 1991). Vanadium traces in diesel fuels present a corrosion hazard; it is the main fuel component influencing high temperature corrosion. During combustion, it oxidizes and reacts with sodium and sulfur, yielding vanadate compounds with melting points down to 530 °C, which attack the passivation layer on steel, rendering it susceptible to corrosion. The solid vanadium compounds also cause abrasion of engine components (Totten *et al*., 2003; Woodyard, 2009).

Acute exposure to vanadium has been reported to cause gastrointestinal disturbances, headache, diarrhea, skin and eye irritation, upper respiratory tract inflammation leading to rhinitis, wheezing, nasal hemorrhages, cough, sore throat, chest pain, emphysema and pneumonia (Barceloux, 1999; Shrivastava *et al*., 2007); and cardiovascular diseases (Haider *et al.,* 1998; Soares *et al*., 2008). Pathological evidence shows that vanadium leads to marked necrosis of hepatocytes in the liver, proximal convoluted tubules in the kidney and seminiferous tubules in the testis. Vanadium also caused the depletion of lymphoid cells in the spleen and disruption of the parenchymal integrity of most organs (Olopade *et al.,* 2009). At the subcellular level, there is increased oxidation of DNA bases, formation of nucleoplastic bridges and nuclear buds, blood micronuclei, apoptosis and necrosis in leucocytes of workers in a vanadium

pentoxide factory compared to matched controls (Ehrlich *et al*., 2008; Olopade and Connor, 2010).

Vanadium is widely used in industry; therefore, occupational exposure is common with workers at workplaces and among those living near vanadium related industries such as metallurgical plants (Shrivastava *et al*., 2007; Afeseh-Ngwa *et al*., 2009; Igado and Olopade, 2010). Atmospheric concentrations of vanadium have increased, partly due to emissions from petrochemical plants and increased combustion of fuel derived products (Avila-Costa *et al*., 2006). In the last two decades, restive communities as seen in some parts of the Arabian Gulf and Niger Delta region of Nigeria have witnessed large scale crude oil burning which is a potential source of atmospheric vanadium (Haider *et al*., 1998: Todorich *et al*., 2011). Vanadium has been reported to be the most abundant trace metal in petroleum samples (Amorim *et al*., 2007) and accumulates in the soil, groundwater and vegetation that may be consumed by animals and humans (Pyrzynska and Wierzbicki, 2004).

2.2.8 Vanadium Neurotoxicity

Vanadium is considered as the most abundant metallic ingredient in petroleum. Burning of fossil fuels increases vanadium levels in the atmosphere (Fortoul *et al*., 2002). In humans, exposure to vanadium results in neurobehavioral alteration, such as the reduced functions in emotion, cognition, and motor accuracy (Barth *et al*., 2002; Li *et al*., 2013). In adult rats, vanadium exposure causes neuropathological lesions in hippocampal neurons (Avila-Costa, 2006), as well as oxidative stress and demyelination in the cerebellum (Garcia *et al*., 2004; Cuesta *et al*., 2011; Igado *et al*., 2012). Because vanadium is present in breast milk and easily passes the blood brain barrier, it could cause developmental neurotoxicity in the exposed neonatal animals (Barceloux, 1999; Olopade *et al*., 2011). Lactational exposure of vanadium

delays the development and impairs the locomotor activity in adult rats (Soazo and Garcia, 2007; Olopade *et al*., 2011). These data suggest that vanadium exposure at early life may exert adverse effects on the development and maturation of nervous system (Wang *et al*., 2015).

Vanadium crosses the blood brain barrier and vanadium compounds can have neurologic and neuropathologic consequences through different routes of administration (Edel and Sabbioni, 1989). Lactating rat pups of vanadium exposed dams developed neurological deficits (Edel and Sabbioni, 1989), while Garcia *et al.* (2004; 2005), Olopade *et al.* (2009) and Todorich *et al.* (2011) reported neurobehavioral changes, neuropathologies and increased brain vanadium content after intra-peritoneal administration of sodium metavanadate in rats. Also, Avila-Costa *et al.* (2004; 2005) described neuroinflammatory changes in the brain of rats exposed to vanadium fumes, and reported a peak seven-fold increase in vanadium brain concentration after one week of inhalation which remained fairly constant (0.10- 0.12mg/g dry weight tissue) during eight weeks of exposure. The inhalation route for vanadium induced neurotoxicosis is epidemiologically important because this may actually be the route of entry to the brain during environmental and occupational exposure of vanadium (Olopade and Connor, 2010). Igado *et al.* (2008) reported that vanadium levels in the olfactory bulb of goats in Southwest Nigeria were higher than the other brain regions examined suggestive of increased entry from the inhalation route. This observation can be compared to an observed three-fold increase in brain vanadium concentration in rats after nine days of intra-peritoneal administration of 3mg/kg of sodium metavanadate (Olopade *et al*., 2009).

In cell culture, vanadium easily enters into neuronal cells; metal ions including vanadium can be transported by transferrin and divalent metal ion transporter (DMT 1) in neuronal cultures (Aschner and Aschner, 1991; Erikson *et al*., 2004). Afeseh-Ngwa *et al.* (2009) reported an

upregulation of transferrin and DMT1 over time in dopaminergic neurons in culture after exposure to vanadium and a parallel increase in intracellular levels of vanadium suggesting a direct correlation between transferrin, DMT1 and intracellular vanadium accumulation.

One of the earliest reports on neurobehavioral effect of vanadium was by Done (1979), who stated that vanadium toxicity in humans is manifested as tremor and CNS depression. Behavioral and psychiatric manifestations of toxicity in some vanadium exposed workers have also been reported such as reduced cognitive abilities in humans chronically exposed to vanadium using the Block Design (visuospatial abilities) and Digit Symbol Substitution (attention task) neuropsychological tests (Barth *et al*., 2002). Neurobehavioural deficits have also more recently been reported in Chinese workers exposed to vanadium (Li *et al*., 2013). Higher vanadium levels in the blood are also reportedly associated with some mental disorders such as manic-depressive disorders (Naylor, 1983; Nechay, 1984; Conri *et al*., 1986). Experiments using rats showed that uninterrupted exposure to vanadium sulphate to male rats through drinking water for over 100 days led to reduced outer ambulation, rearing posture and grooming activity in the open field (Olopade and Connor, 2010). Garcia *et al.* (2004) also reported a significant decrease in locomotor activity and grooming responses in rats treated with sodium metavanadate as shown by reduced crosses, rearing and grooming in the open field. Furthermore, Todorich *et al.* (2011) observed a significantly reduced performance on the rotarod test for rats exposed intraperitoneally to sodium metavanadate for 14 days from post natal day one. The importance of lactational exposure in producing behavioral alterations was highlighted in rats by Soazo and Garcia (2007), who reported that treatment of dams with sodium metavanadate for 12 days starting at postnatal day (PND) 10, resulted in deficits in surface righting reflex, negative geotaxis and hind limb support tests. Studies have reported that vanadium has a selective effect

on the adrenergic pathways (Sharma *et al*., 1986), altering norepinephrine, dopamine and 5-hydroxytryptamine brain levels (Witkowska and Brzezinski, 1979). Furthermore, vanadium produces CNS myelin alteration as seen in delay in eye opening of suckling litters whose dams have been treated with sodium metavanadate (Edel and Sabbioni, 1989; Soazo and Garcia, 2007). The impact of vanadium on motor performance raises an interesting possibility about the role of vanadium in motor neuron diseases (Olopade and Connor, 2010).

Vanadium causes disruption of the CSF-brain barrier as evidenced by a disintegration of the histoarchitecture of the ependymal epithelium after eight weeks of vanadium pentoxide inhalation characterised by cilia loss, cell sloughing and ependymal cell layer detachment (Avila-Costa *et al*., 2005). The authors concluded that such damage could allow modification of the permeability of the epithelium and promote access of inflammatory mediators to the underlying neuronal tissue causing injury and neuronal death. After intraperitoneal administration of sodium metavanadate for five consecutive days, Garcia *et al.* (2004) observed decreased myelinated fiber density in the corpus callosum and cerebellum of treated rats. Also, Garcia *et al.* (2005) reported increased demonstration of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH) activity as indicated by increased nitroblue tetrazolium (NBT) staining in the cerebellum and anterior hypothalamus. Many workers have shown through immunohistochemistry increased heat shock protein (hsp70), glial fibrillary acidic protein (GFAP), S-100 activity in brain regions following vanadium exposure (Edel and Sabbioni, 1989; Haider *et al*., 1998). These morphological changes of neurotoxicity indicate vanadium induced stress in the brain and suggest vanadium mediated free-radical generation. Other cytological and immunohistochemical changes resulting from vanadium neurotoxicosis include a time dependent loss of dendritic spines, necrotic-like cell death and a marked alteration of the hippocampus CA1 neuropile

(Avila-Costa *et al*., 2006) and also a decrease in thyrosine hydroxylase (TH) positive neurons in the substantia nigra (Garcia *et al*., 2005). These data clearly demonstrate a direct impact of vanadium on both neurons and glia in the brain. Neurons express transferrin (Tf) receptors so vanadium may gain neuronal access through the Tf mediated uptake described earlier (Afeseh-Ngwa, 2009).

Specific biochemical changes studied in the brain after vanadium exposure reveals processes that support oxidative stress and lipid peroxidation as the major *sequelae* of vanadium administration (Sasi *et al*., 1994). Vanadium is a catalytic metal which induces reactive oxygen species (ROS) in the variety of biological systems (Thompson and McNeill, 1993; Ehrlich *et al*., 2008). The mammalian brain contains large amounts of lipid substances which are susceptible to free radical attack, and in addition contains relatively low levels of free radical-eliminating enzymes (Braughler and Hall, 1989; Floyd, 1990). Though proteins, carbohydrates and nucleic acids are damaged in the brain, lipids in membranes are the primary targets of ROS (Garcia *et al*., 2005); the brain tissue known for its high content of polysaturated fatty acids and aerobic catabolism is thus a most vulnerable target for peroxidative attack (Tayarani *et al*., 1989). Vanadium depresses antioxidative enzyme system in the brain by depleting glutathione and non protein sulfhydryl group (NP-SH). The mechanism of this depletion could be as a result of the inhibition of the enzyme gluthathione reductase which reduces oxidized glutathione to glutathione, and the use of NP-SH in covalent bonding with vanadium (Haider *et al*., 1998).

Demyelination has been reported to be one of the major phenotypes of vanadium induced neurotoxicity. The treatment of dams with sodium metavanadate for 12 days starting at postnatal day (PND) 10, led to delay in eye opening in the suckling litters of these dams after two weeks (Soazo and Garcia, 2004). The same authors also reported that these pups had decreased

myelinated fiber density and anti Myelin Basic Protein staining in the cerebellum and corpus callosum at PND21. In another investigation, sodium metavandate administration for five days resulted in a significant decrease in the levels of total lipids, phospholipids, cholesterol, and cerebrosides, and protein though ganglioside concentration was significantly increased in various brain regions (Sasi *et al.,* 1994). The same authors also reported a selective loss in the brain of polyunsaturated fatty acids (oleic, linoleic, linolenic and arachinodic), and that sphingomyelin was substantially decreased followed by phophatidyl choline and phophatidyl ethanolamine but that phosphatidyl serine and phophatidyl inositol were slightly affected. The authors' speculation was thus that the deterioration of the myelin sheath by vanadium exposure contributed to the preferential lipid loss but lesser loss of protein. These observations corroborate the increased lipid peroxidation seen in the brain of vanadium (sodium metavanadate) exposed rats using the thiobarbituric acid (TBA) reaction test (Garcia *et al*., 2004).

Recently, Todorich *et al* (2011) reported a selective loss in oligodendrocyte population in the corpus callosum of rats after vanadium exposure from PND1 till PND14 indicating that vanadium-induced hypomyelination in neonates could result from destruction of primary oligodendrocyte leading to reduced myelinogenesis or in addition to myelin destruction via oxidative stress. In an attempt to investigate relative sensitivity of glial cells to the cytotoxic effect of vanadium, Todorich *et al* (2011) exposed primary progenitor oligodendrocytes (OPCs), matured oligodendrocytes and astrocytes to increasing concentrations of sodium metavanadate and observed that primary progenitor oligodendrocytes were two -fold more sensitive than mature oligodendrocytes and astrocytes.

The suggestion of oxidative stress as the mechanism underlying vanadium induced tissue pathologies is reproduced in cell culture. Afeseh-Ngwa *et al* (2009) observed a threefold increase

in H_2O_2 production compared to control in N27 mesencephalic neuronal cells after vanadium treatment. Using the dichlorofluoroscein diacetate (DCFH-DA) dye, Olopade *et al* (2009) and Todorich *et al* (2011) reported 200-300% increases in intracellular ROS production in primary oligodendrocyte and astrocytoma (U251) cell culture respectively. The relative sensitivity of some cell types can be assessed through review of the cell culture studies. The LD_{50} (lethal dose) of a dopaminergic cell line was reported to be 40μM when treated with vanadium pentoxide after 12 hours (Afeseh-Ngwa *et al.,* 2009) while in primary oligodendrocyte culture the LD_{50} was 100μM when treated with sodium metavanadate (Haider *et al.,* 1998). In astrocytoma cells, the LD_{50} using sodium metavanadate was attained at about half the dose required to produce the same response in primary rat astrocytes cultures (Olopade *et al.,* 2009) and was lower than the LD_{50} exerted on primary oligodendrocytes (Todorich *et al*., 2011). This latter observation was the basis for the suggestion that vanadium should receive further evaluation as a relatively safe and an effective chemotherapeutic agent against glioblastoma multiforme (GBM) (Olopade *et al.,* 2009).

It is obvious from these reports that differences occur in the effects of different vanadium compounds and even in cell types exposed to vanadium. In fact, a review of vanadium's cellular actions (Shrivastava *et al*., 2007) show varied mechanisms without a clear bias for what actually the dominant biological mechanism is. Wang *et al* (2015) suggested that differences in the genotypic backgrounds of the cell types, such as expression of tumor suppressors or oncogenes and redox states of various cells, may act as biological determinants that allow vanadium compounds to exert cell-specific effects. Research into the exact mechanisms as it affects different cell types, the type of vanadium compound used and the specific biological milieu of the cells will surely remain an ongoing field (Wang *et al*., 2015).

One of the major mechanisms of vanadium induced cytotoxicity in cell culture appears to be apoptosis. Many studies have observed apoptotic changes at varying doses using different vanadium compounds ranging from sodium metavandate with primary oligodendrocyte and U251 astrocytoma cells using Annexin V marker (Haider *et al.,* 1998; Olopade *et al.,* 2009), in N27 mesencephalic cells using vanadium pentoxide with Caspase 9 and Caspase 3 as markers (Afeseh-Ngwa, 2009).

The relationship of vanadium to free radical generation and the sensitivity of primary oligodendrocytes and astrocytoma cells have raised the question of the possibility of a relationship between iron acquisition and management in the neurotoxicogenesis of vanadium due to relatively high concentration of iron in both cell types (Olopade *et al.,* 2009; Todorich *et al*., 2011). Todorich *et al* (2011) also reported a significant increase in apoptotic staining of primary oligodendrocytes exposed to vanadium. Their work showed that the limiting of iron availability to OPCs at the time of vanadium exposure provided partial protection from vanadium induced toxicity while on the other hand, increasing iron availability to OPCs exacerbated cytotoxic effects of vanadium. The work further revealed increased cytopathological effects caused by the interaction of vanadium with ferritin constructs high in iron than constructs with low iron suggesting that the synergistic relationship between vanadium and ferritin on vanadate mediated cytotoxicity is based on the iron content of the latter.

In a similar study, treatment of astrocytoma culture with sodium metavanadate resulted in a down regulation of ferritin expression (Olopade *et al.,* 2009). The effect on ferritin could be blocked when vitamin E was added to the cells with vanadate. This observation indicated that there is disruption of the ferritin molecule by vanadium induced free radicals and the authors proposed that vanadium induced ferritin degradation leading to an iron-mediated free radical

production. The relationship between vanadium and iron in the brain has thus become an issue of importance due to vanadium transport into the brain by the iron-related protein transferrin (EVM, 2002; Erikson *et al*., 2004), and the role of ferritin protein in vanadium induced pathology as previously explained.

2.2.8.1 Antidotes to Vanadium Neurotoxicity

Over the years, using both *in vitro* and *in vivo* studies, a number of antidotes have come up to combat vanadium neurotoxicity, for example, selenium (Haider *et al.,* 1998), tiron and desferroxamine (Aschner *et al.,* 2010), vitamin E (Olopade *et al.,* 2011), *Garcinia kola* (bitter kola) and kolaviron – a flavonoid from bitter kola (Igado *et al.,* 2012), and erythropoietin (Mustapha *et al*., 2014). The fact however to note is that some metal chelators do not pass through the blood brain barrier (Olopade and Connor, 2010). This makes it imperative to obtain an antidote that is non-toxic, but possesses both chelative and antioxidative properties.

2.3 The Brain

2.3.1 Brief History

The word *brain* is derived from the Anglo-Saxon word, *Braegen*, which may have a common root with the Greek word *Bregma* (the upper part of the head). The first mention of the brain as an organ occurs in the papyri of ancient Egypt. The ancient Egyptians (3,000 – 2,500 BC) did not consider the brain to be of importance. They promoted the cardiocentric concept in which the heart was the seat of the soul. Among the ancient Greeks, Plato promoted the cephalocentric theory and coined the term “encephalon”. According to Plato, the brain gyri and sulci were analogous to ridges and furrows of a plowed field for planting of a divine seed to produce consciousness. In contrast, Aristotle promoted the Egyptian cardiocentric theory. He considered the heart to be the centre of the body. The brain was analogous to clouds of steam

where the blood pumped by the heart is cooled. Gyri and sulci were believed to be ripples on the clouds. For centuries, arguments persisted as to the function of the brain, whether it was the seat of emotions or the abode of the soul. It is only within the last two hundred years that any real conception of the function of the brain began to be obtained (Afifi and Bergman, 2005).

2.3.2 Basic Gross or Surface Anatomy of the Brain

The brain can be divided into four lobes (viewed from the lateral surface) – frontal, parietal, temporal and occipital. It is semisolid in consistency and conforms to the shape of its container. It is protected from the external environment by three barriers:

- The skull (neurocranium)
- The meninges
- The cerebrospinal fluid (CSF) (Afifi and Bergman, 2005)

The bony skull acts as the major barrier against physical trauma to the brain (Afifi and Bergman, 2005).

The brain is housed in the protective neurocranium comprising the frontal, parietal, temporal, ethmoid and sphenoid bones. The brain can be anatomically and functionally divided. Anatomically, it can be divided into three divisions, namely, the forebrain or prosencephalon, the midbrain or mesencephalon, and the hindbrain or the rhombencephalon. The forebrain can be further divided into the telencephalon and diencephalon, and the hindbrain into the metencephalon and the myelencephalon. The mesencephalon remains undivided (Thomson and Hahn, 2012).

Functionally, the brain can be divided into the forebrain, brainstem, and the cerebellum. The brainstem includes the midbrain, pons and medulla oblongata. The thalamus is sometimes included in brains subdivisions (Thomson and Hahn, 2012).

Anatomically, dorsally and laterally, the brain is dominated by two large, ovoid cerebral hemispheres that are elongated in the longitudinal direction. Caudal to the hemispheres, on the midline, and extending for a variable distance laterally, depending on the species, is the cerebellum. The longitudinal fissure separates the hemispheres from each other while the transverse fissure separates the hemispheres from the cerebellum (Thomson and Hahn, 2012).

Ventrally, the rostral aspect of the brain of domestic animals is dominated by the paired olfactory bulbs, which are small in humans and miniscule or totally absent in cetaceans. Caudally, the olfactory bulbs are connected via the olfactory tract to the more laterally placed piriform lobes. On the midline is the diencephalon with the optic chiasm, hypophysis (pituitary gland), and mammilary bodies. Caudal to this is the midbrain with the crus cerebri (cerebral peduncles), which convey efferent information from the forebrain to the brainstem. Caudal to the midbrain is the pons, and then the medulla oblongata with the trapezoid body and the pyramids extending to the spinal cord junction (Thomson and Hahn, 2012).

The brainstem forms a stalk connecting between the spinal cord and the cerebral hemispheres. It has a similar cross-sectional arrangement to that of the tubular spinal cord except that in the rostral medulla oblongata, the dorsal aspect of the tube has been opened up and the roof plate expanded. The fluid-filled ventricular system is enlarged in this region. The white matter is superficial and the grey matter is located deeper within the brainstem (Thomson and Hahn, 2012).

2.3.3 The Cerebrum

The cerebral cortex is derived from the dorsal component (or pallium) of the embryonic telencephalon, which is itself the anterior-most subdivision of the fore brain (Kiecker and Lumsden, 2005). It is the layer of gray matter capping the white matter core of the cerebral

hemispheres. In humans, its thickness varies from 1.5 to 4.5 mm, with an average thickness of 2.5 mm. It is thickest in the primary motor area and thinnest in the primary visual cortex. The cortex is irregularly convoluted, forming gyri separated by sulci or fissures. No gender based differences have been reported in humans, although neuronal density is higher in the male with a reciprocal increase in neuropil and neuronal processes in the female (Afifi and Bergman, 2005).

2.3.3.1 Divisions of the Cerebrum

The cerebral cortex can be further sub-divided into distinct regions, including the neocortex, a novel acquisition of mammals that has evolved between the phylogenetically older archicortex (comprising entorhinal cortex, retrosplenial cortex, subiculum, and hippocampus) and paleocortex (olfactory piriform cortex). The evolutionary expansion of the neocortex is thought to account for much of the increase in overall brain size and complexity in more advanced species (Krubitzer and Kaas, 2005; O'Leary *et al*., 2007). In humans, more than 80% of the cortex serves an association function specially related to integrative and cognitive activities such as language, calculation, planning and abstract reasoning (Afifi and Bergman, 2005).

On the basis of phylogenetic development and microscopic structure, three cortices are recognised:

- The Neocortex (Isocortex or Homogenetic cortex)
- Allocortex (Paleocortex, Archicortex or Heterogenetic cortex)
- Mesocortex (Periallocortex, Periarchicortex) (Afifi and Bergman, 2005).

The neocortex (also commonly referred to as the cortex) contains an extraordinarily large number of neurons arrayed in a six-layered sheet, with neurons in each layer organized into a complex network of local circuits and subcortical connections. In primates, some of these layers

are subdivided in some cortical areas: for example, in primary visual cortex, layer 4 is subdivided into layers 4a, b, and c. The primate cortex is also characterized by an expansion of the superficial layers of the cortex, layers 2 and 3 (also known as the supragranular layers), which have an important function in the transfer of information between cortical areas. Increased intracortical information processing is likely to have contributed to heightened cognitive ability. In all mammals, the cortex comprises two major groups of neurons: the majority are excitatory glutamatergic projection neurons (70–80%), which exhibit a characteristic pyramidal morphology and extend axons to distant intracortical, subcortical, and subcerebral targets; a minority are inhibitory GABAergic non-pyramidal interneurons (25–30% in primates, 15–20% in rodents), which have short axons and project locally (Hendry *et al*., 1987; Beaulieu, 1993). An appropriate balance between the excitatory and inhibitory circuitry of the cortex is critical for its normal function (Manuel *et al*., 2015).

The allocortex is three layered and phylogenetically older. It is subdivided into paleocortex (rostral insular cortex, piriform cortex and primary olfactory cortex) and archicortex (hippocampal formation) (Afifi and Bergman, 2005).

The mesocortex is found in much of the cingulated gyrus, entorhinal, parahippocampal and orbital cortices and its intermediate in histology between the isocortex and allocortex. The terms periallocortex and periarchicortex are used to refer to this cortex to denote its transitional nature between neocortex and allocortex (Afifi and Bergman, 2005).

The structural organization of the cerebrum and its connection with the brain stem are best appreciated in a frontal (coronal) section of the brain that passes through the ventral part of the midbrain. The rostral end of the midbrain merges with the diencephalon ("between brain"), which has four subdivisions on each side, separated by the third ventricle, which is a slit-like

cavity in the midline (Kiernan, 2005). The subthalamus, which is closest to the midbrain, contains the subthalamic nucleus, which is involved in motor circuitry, and ascending tracts that are about to terminate in the thalamus: the medial lemniscus, spinothalamic tract and fibers from the cerebellum (Kiernan, 2005).

The hypothalamus is medial and rostral to the subthalamus, and has landmarks on the inferior (ventral) surface of the brain. This region controls important autonomic and endocrine functions. Neural and vascular links from the hypothalamus control the pituitary gland (Kiernan, 2005).

The thalamus is the largest part of the diencephalon. It forms much of the wall of the third ventricle and floor of the lateral ventricle. It's many constituent nuclei communicate with the cerebral cortex. Most thalamic nuclei also receive input from subcortical sources and some are stages in pathways for sensory, motor and cognitive activities. At the anterior (rostral) end of each thalamus, the third ventricle becomes continuous with the lateral ventricle, through the interventricular foramen of Monro. The Epithalamus is a poorly understood region of the brain associated with the junction of the cerebral aqueduct and third ventricle. It includes the pineal gland, a much studied but still mysterious probable endocrine organ, which is dorsal to the superior colliculi (Kiernan, 2005).

Only one large body of white matter links brain stem and diencephalon with the cerebral cortex; this is the internal capsule (Figure 2.3.3.1a, b). It consists largely of ascending thalamocortical fibers and fibers descending from the cortex to the brain stem and spinal cord. The posterior limb of the internal capsule includes corticospinal, corticobulbar and corticoreticular fibers with important motor functions (Kiernan, 2005).

The telencephalon ("end-brain") is associated with the lateral ventricle. Its central gray matter, the corpus striatum comprises the large caudate and lentiform nuclei (Figure 2.3.3.1a, b). The external surface of the telencephalon is formed by the cerebral cortex. The central sulcus (fissure of Rolando) and the lateral sulcus (sylvian fissure) demarcate lobes of the cerebral cortex, which are named for the overlying bones of the skull: frontal, parietal and temporal. The smaller occipital lobe forms the posterior pole of the hemisphere, and the insula (or insular lobe) is the cortex of the expanded floor of the lateral sulcus, overlying the lentiform nucleus. Areas of the cortex serve different functions, which have been determined from the effects of lesions, electrical stimulation in the course of surgery, and modern functional imaging techniques (Kiernan, 2005).

The thick layer of white matter separating the cerebral cortex from the corpus striatum and lateral ventricle contains bundles of fibers of three types. Association fibers connect different cortical areas of the same hemisphere. Short association fibers connect within a lobe between adjacent gyri, while long association fibers connect between lobes within the same hemisphere. Commissural fibers connect the left and right cerebral cortices (across the midline between hemispheres); most pass through the corpus callosum, but parts of the temporal lobes are connected by the anterior commissure. Ascending and descending fibers, connecting the cortex with subcortical regions, are known as projection fibers, they connect between the hemispheres and brainstem; the internal capsule is a site of concentration of many such fibers (Kiernan, 2005, Thomson and Hahn, 2012). The main commissural fibers are bundled together as the corpus callosum. The corpus callosum forms an elongated, transverse band of white matter at the base of the longitudinal fissure (Thomson and Hahn, 2012).

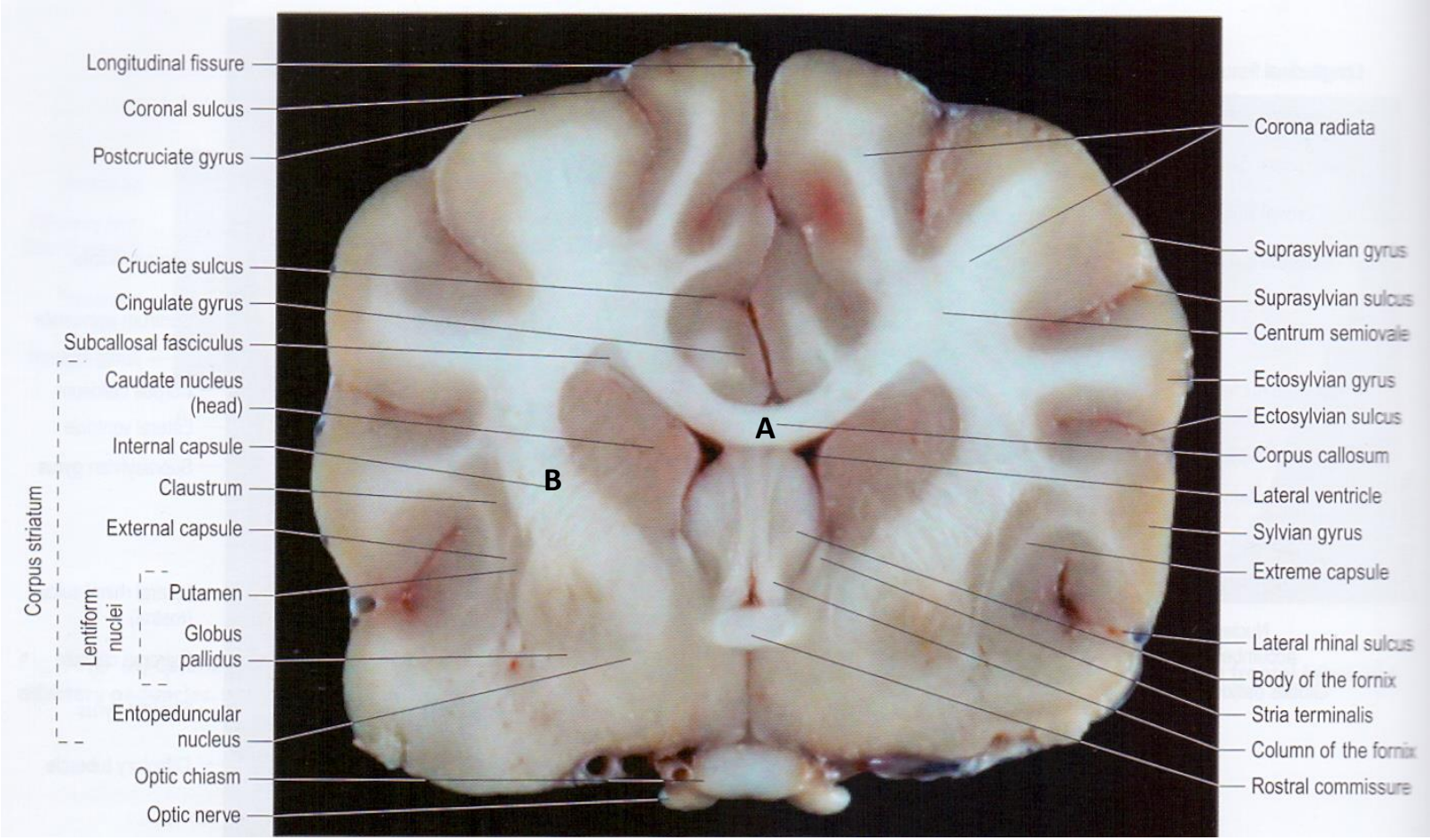


Figure 2.3.2.1a: A coronal section of the canine brain, at the level of the optic chiasma. Note the corpus callosum (A), internal capsule (B), components of the corpus striatum (labelled). Thomson and Hahn, 2012.

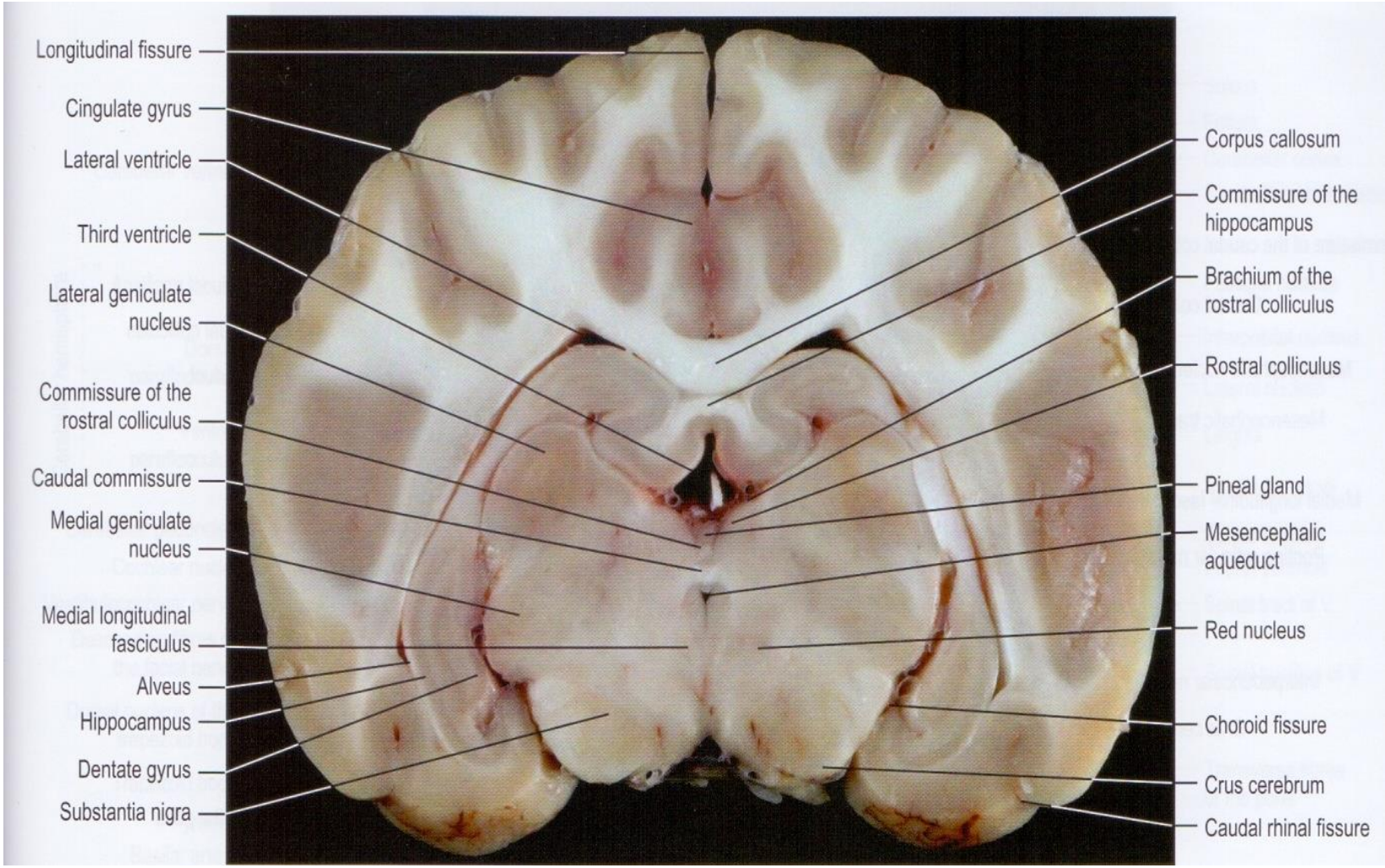


Figure 2.3.2.1b: A coronal section of the canine brain, at the level of the geniculate nuclei. Note the corpus callosum (A), internal capsule (B). Note the increased appearance of the hippocampus compared to Figure 2.3.3.1a (fornix and crus of the fornix). Thomson and Hahn, 2012.

2.3.3.2 Cell Types in the Cerebrum

Attempts to make a comprehensive inventory of types of cortical neurons started with Ramon y Cajal in 1911, and still continue till date (Nicholls *et al*., 2001; Afifi and Bergman, 2005).

The neurons of the cerebral cortex are of two functional categories:

i. Principal (projection) neurons
ii. Interneurons

The principal neurons provide corticocortical and corticosubcortical outputs while interneurons are concerned with local information processing. Recent evidence suggests that the two neuronal types are generated in distinct proliferative zones. Principal neurons are derived from neuroepithelium in the ventricular zone. Interneurons, in contrast, arise from the ganglionic eminence of the ventral telencephalon, which gives rise also to the basal ganglia. The cerebral cortex has its full complement of neurons (10 to 20 billion) by the 18th week (in humans) of intrauterine life (Afifi and Bergman, 2005).

2.3.3.2.1 Principal (Projection) Neurons

Two types of cortical neurons belong to the principal category. They are the pyramidal neurons and the fusiform neurons. The excitatory neurotransmitter in both neuron types is glutamate or aspartate. Principal neurons constitute more than half of all cortical neurons (Afifi and Bergman, 2005).

***Pyramidal Neurons*:** These neurons derive their name from their shape. The apex of the pyramid is directed toward the cortical surface. Each pyramidal neuron has an apical dendrite directed toward the surface of the cortex and several horizontally oriented basal dendrites that arise from the base of the pyramid. Branches of all dendrites contain numerous spines that

increase the size of the synaptic area. A slender axon leaves the base of the pyramidal neuron and projects on other neurons in the same or contralateral hemisphere or else leaves the cortex to project on subcortical regions. The axon gives rise within the cortex to two types of axon collaterals. These are the recurrent axon collaterals (RACs), which project back on neurons in more superficial layers, and the horizontal axon collaterals (HACs), which extend horizontally to synapse on neurons in the vicinity. Pyramidal neurons are found in all cortical layers except layer I. They vary in size; most are between 10 and 50 μm in height. The largest are the giant pyramidal cells of Betz, which measure about 100 μm in height and are found in layer V of the motor cortex (Fix, 2000; Afifi and Bergman, 2005).

***Fusiform, Spindle Neurons*:** These are small neurons with elongated perikarya in which the long axis is oriented perpendicular to the cortical surface. A short dendrite arises from the lower pole of the perikaryon and arborizes in the vicinity. A longer dendrite arises from the upper pole of the perikaryon and extends to more superficial layers. The axon enters the deep white matter. Fusiform neurons are found in the deepest cortical laminae (Afifi and Bergman, 2005).

2.3.3.2.2 Interneurons

Several types of cortical interneurons are recognized on the basis of dendritic architecture. They include the stellate neurons, the horizontal cells of Cajal, and the cells of Martinotti.

Stellate or Granule Neurons

These are small (4 to 8 μm) star-shaped neurons with short, extensively branched, spiny dendrites and short axons. They are most numerous in lamina IV. Stellate cells are the only type of excitatory interneurons in the cortex. The neurotransmitter is glutamate. All other interneurons

exert inhibitory influence by gamma-aminobutyric acid (GABA) (Fix, 2000; Afifi and Bergman, 2005).

Horizontal Cells of Cajal

These are small fusiform neurons with their long axes directed parallel to the cortical surface. A branching dendrite arises from each pole of the perikaryon, and an axon arises from one pole. The dendrites and axon are oriented parallel to the cortical surface. The horizontal cells of Cajal are found only in lamina I and disappear or are rare after the neonatal period (Fix, 2000; Afifi and Bergman, 2005).

Cells of Martinotti

Giovanni Martinotti in 1890 first described cells whose axons ascend toward the surface of the cortex. Martinotti neurons are multipolar with short branching dendrites and an axon that projects to more superficial layers, giving out horizontal axon collaterals en route. The Martinotti neurons are found in deeper cortical laminae (Afifi and Bergman, 2005).

2.3.3.3 Layers of the Cerebrum

The division of the neocortex into layers has been the outcome of extensive cytoarchitectonic (organization based on studies of stained cells) and myeloarchitectonic (organization based on studies of myelinated fiber preparations) studies. Although several such studies are available, the most widely used are the cytoarchitectonic classification of Brodmann and the myeloarchitectonic classification of the Vogts. According to these two classifications, the neocortex is divided into six layers. The six layers of the neocortex are recognizable by about the seventh month of intrauterine life, in humans. The neurons in the six cortical layers develop in waves from the periventricular germinal matrix. Successive waves of migrating neuroblasts become situated progressively farther away from the germinal matrix (inside-out gradient of

cortical histogenesis). Interruption of the normal process of migration or its arrest is associated with cortical gyral malformations such as agyria, pachygyria, micropolygyria, and heterotopia. Many of these are associated with mental retardation, seizures, and other neurologic deficits (Banks, 1974; Kiernan, 2005; Afifi and Bergman, 2005).

Layer I (Molecular, Plexiform)

Layer I consists primarily of a dense network of nerve cell processes among which are scattered sparse interneurons (horizontal cells of Cajal) and neuroglia. The nerve cell processes in this layer comprise projection axons from extracortical sites as well as axons and dendrites of neurons in other cortical areas. This layer of the cortex is primarily a synaptic area (Afifi and Bergman, 2005).

Layer II (External Granular)

Layer II consists of a dense packing of small and medium-sized pyramidal neurons and interneurons intermingled with axons from other cortical layers of the same and opposite hemispheres (association and commissural fibers), as well as axons and dendrites passing through this layer from deeper layers. The dendrites of pyramidal neurons in this layer project to layer I, while their axons project to deeper layers. This layer of the cortex contributes to the complexity of intracortical circuitry (Fix, 2000; Afifi and Bergman, 2005).

Layer III (External Pyramidal)

Layer III consists of pyramidal neurons that increase in size in deeper parts of the layer. The dendrites of neurons in this layer extend to layer I, while the axons project to other layers within the same and contralateral hemisphere (association and commissural fibers) or leave the hemisphere as projection fibers to more distant extracortical sites. This layer receives primarily axons of neurons in other cortical areas (association and commissural fibers), as well as axons of

neurons in extracortical regions such as the thalamus. This layer contains the distinctive stripes of Kaes-Bekhterev, a thin band of myelinated fibers located between the external granular layer and the external pyramidal layer of the cerebral cortex (Fix, 2000; Afifi and Bergman, 2005; Kiernan, 2005).

Layer IV (Internal Granular)

Layer IV consists of pyramidal cells and densely packed small stellate cells with processes that terminate within the same layer, either on axons of other stellate cells or on axons of cortical or subcortical origin passing through this layer. The cell packing density in layer IV is the greatest of all cortical layers. Few of the larger stellate cells in this layer project their axons to deeper cortical layers. Layer IV is especially well developed in primary sensory cortical areas. In the primary visual (striate) cortex, this layer is traversed by a dense band of horizontally oriented thalamocortical nerve fibers known as the external band of Baillarger or the stripe of Gennari. The band of Baillarger was described by the nineteenth-century French neurologist and psychiatrist Jean-Gabriel-Francois Baillarger. The stripe of Gennari was first described in 1782 by Francesco Gennari, an eighteenth-century Italian medical student, and independently by Vic d'Azyr in 1786. Because of the presence of this stripe, the primary visual cortex is known as the striate cortex. The internal granular layer is the major recipient of thalamocortical fibers from modality-specific sensory relay nuclei (visual radiation, auditory radiation, and primary sensory radiation) (Fix, 2000; Afifi and Bergman, 2005; Kiernan, 2005).

Layer V (Internal Pyramidal)

Layer V consists of large and medium-sized pyramidal cells, stellate cells, and cells of Martinotti. The cell packing density in this layer is the lowest of all cortical layers. The largest pyramidal cells in the cerebral cortex (cells of Betz) are found in this layer (hence the name

ganglionic layer). Dendrites of neurons in this layer project to the more superficial layers. Axons project on neurons in other cortical areas but mainly to subcortical sites (projection fibers) except the thalamus, which receives fibers from layer VI. This layer receives axons and dendrites arising in other cortical sites or in subcortical sites. It is also traversed by a dense band of horizontally oriented fibers; this is the internal band of Baillarger. Fibers originating in thalamic sensory nuclei contribute heavily to the formation of the lines of Baillarger, especially the outer one in lamina IV. The lines of Baillarger are thus prominent in primary cortical sensory areas (Fix, 2000; Afifi and Bergman, 2005).

Layer VI (Multiform)

Layer VI consists of cells of varying shapes and sizes, including fusiform cells and the cells of Martinotti, which are prominent in this layer. Dendrites of smaller cells arborize locally or in adjacent layers, while those of large neurons reach the molecular layer. Axons of neurons in this layer project to other cortical laminae or to subcortical regions.

Layers I, V, and VI are present in all types of cortex (neocortex, paleocortex, and archicortex). Layers II, III, and IV, however, are present only in neocortex and thus are considered of more recent phylogenetic development. In general, layers I to IV are considered receptive. The somata of the majority of cells that establish intracortical connections (ipsilaterally and contralaterally) lie in layers II and III. Layers V and VI are efferent. Neurons in lamina V give rise to corticofugal fibers that target subcortical areas (brain stem and spinal cord). Neurons in lamina VI give rise to corticofugal fibers to the thalamus (Afifi and Bergman, 2005; Kiernan, 2005).

In contrast to the horizontal anatomic lamination, the vertical lamination described by Mountcastle seems to be the more functionally appropriate. The studies of Lorente de Nó,

Mountcastle, Szentágothai, and others have shown that the functional unit of cortical activity is a column of neurons oriented vertically to the surface of the cortex. Each such column or module is 300 to 500 μm in diameter, with its height the thickness of the cortex, and contains 4000 neurons, 2000 of which are pyramidal neurons. All neurons in a column are activated selectively by the same peripheral stimulus. There are approximately 3 million such modules in the human neocortex. Each module sends pyramidal cell axons to other modules within the same hemisphere or to modules in the other hemisphere. Of interest is the fact that activation of a module tends to inhibit neuronal activity in adjacent modules. The columnar organization of the neocortex is established in fetal life, but the synaptic connections increase in number in the postnatal period in response to stimulation from the external environment. Lack of external stimuli during a critical period of cortical maturation, usually in the first year of life, will adversely affect normal cortical development (Fix, 2000; Afifi and Bergman, 2005).

2.3.4 The Structure of the Cerebellum

The mammalian cerebellum is located in the posterior cranial fossa and is critical for motor coordination and non-motor functions including cognitive and emotional processes. The anatomical structure of cerebellum is distinct with a three-layered cortex (Marzban *et al.*, 2015).

Microscopic anatomy of the cerebellum was described in detail at the end of the 19th century by Ramon y Cajal and has attracted the attention of many researchers over the last century, and yet many questions remain unanswered. The cerebellum plays an important role in motor coordination (Ito, 1984; Glickstein, 1993; Schmahmann, 1997; Glickstein *et al*., 2009). Increasing evidence shows that the cerebellum also plays a significant role in cognitive functions such as attention, language, emotional behaviour, sleep, and even non-somatic visceral responses (Leiner *et al*., 1991; Wiser *et al*., 1998; Schmahmann and Caplan, 2006).

The mammalian cerebellum is characterized by a midline vermis flanked by hemispheres on each side (Figure 2.3.4a&b). Folds and fissures divide the cerebellum into lobes, lobules, and folia. Mammalian and avian cerebellum is conventionally divided into 3 lobes that are further subdivided into 10 lobules (I–X) (Larsell, 1970; Sotelo and Wassef, 1991; Voogd and Glickstein, 1998; Glickstein *et al*., 2009). The cerebellum contains relatively few cell types that are aggregated in the cerebellar gray matter including the cerebellar cortex and cerebellar nuclei. The cerebellar cortex is formed by three layers, whose neuronal components include stellate and basket cells in the molecular layer; Purkinje and candelabrum cells in the Purkinje layer; and granule cells, Golgi cells, unipolar brush cells, and Lugaro cells in the granular layer. Neurons of the cerebellar nuclei are located close to the roof of the fourth ventricle deep within the cerebellar white matter. The cerebellar nuclei along with some vestibular nuclei constitute part of the output of the cerebellum (Ito, 1984; De Zeeuw and Berrebi, 1995; Voogd *et al*., 1996).

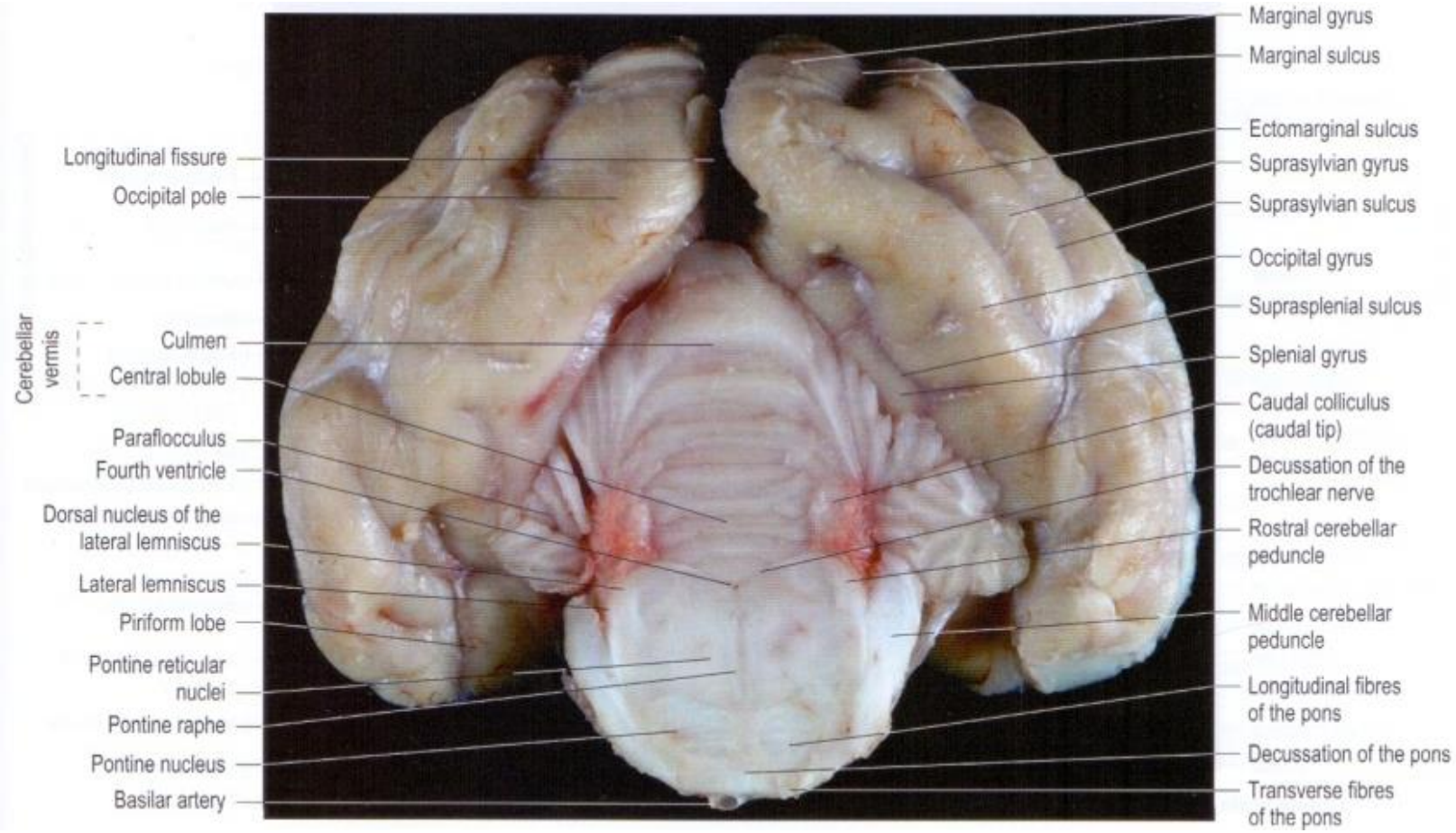


Figure 2.3.4a: Canine brain, transverse slice at the level of the pons. Note the cerebellar vermis, peduncles and nuclei (all labelled). Thomson and Hahn, 2012.

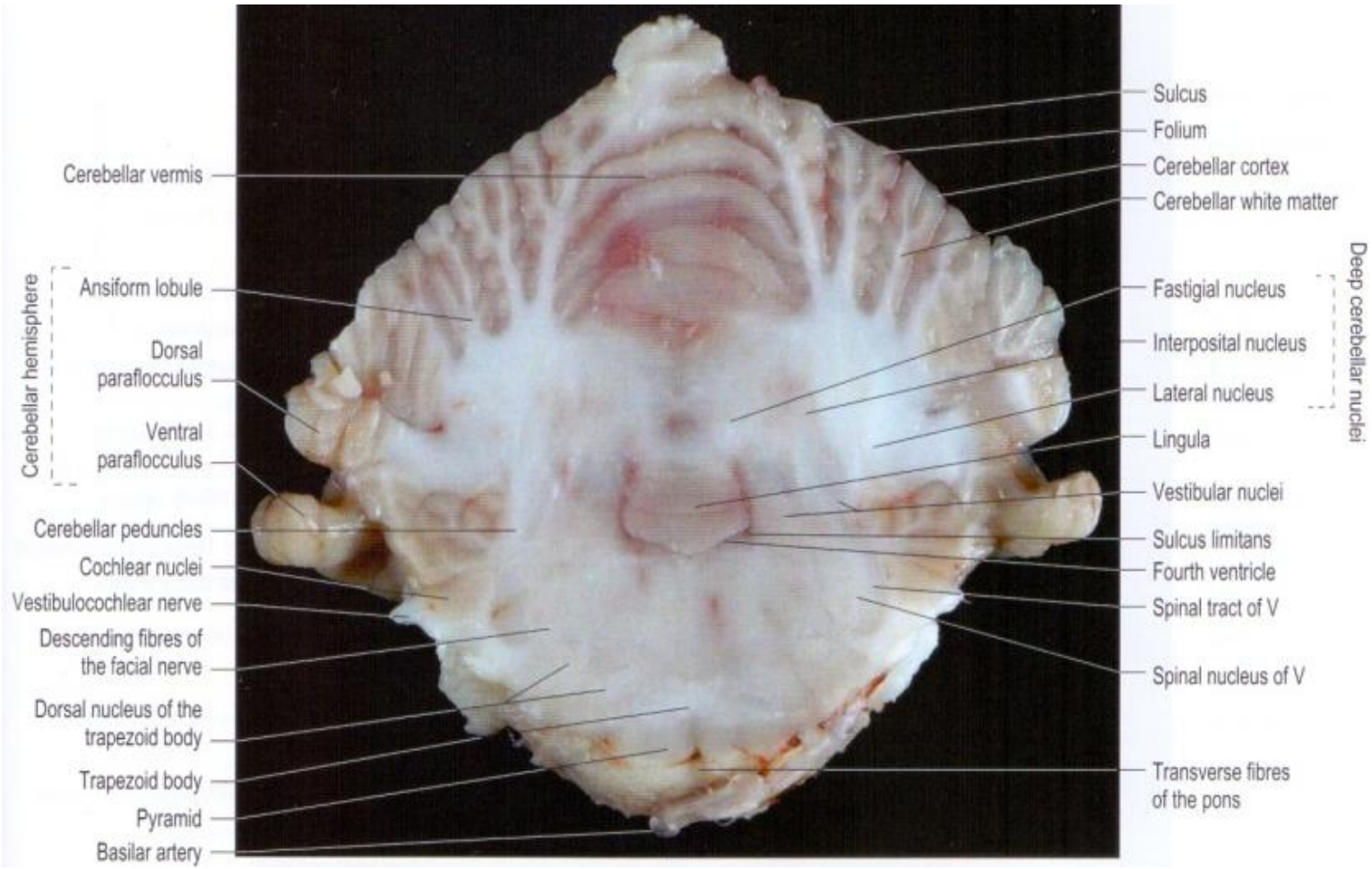


Figure 2.3.4b: Canine brain, transverse slice at the level of the cerebellar peduncles. Note the cerebellar vermis, the folia, white matter, peduncles and nuclei (all labelled). Thomson and Hahn, 2012.

Cerebellar neurons can be classified into inhibitory gamma- butyric acid (GABAergic) and excitatory glutamatergic neurons (Hoshino, 2006; Carletti and Rossi, 2008). Purkinje cells, which are GABAergic, are the principal neurons of the cerebellar cortex with an elaborate dendritic arborization that extends into the molecular layer. Purkinje cells, the sole output of cerebellar cortex, project to the cerebellar nuclei neurons (Leto and Rossi, 2012; Mordel *et al*., 2013; Steuber and Jaeger, 2013). Candelabrum cells (interneurons) are also GABAergic; they are uniformly distributed throughout the entire cerebellar cortex. The small somata are roughly pyriform and vertically located between the Purkinje cell somata (Lainé and Axelrad, 1994; Ambrosi *et al*., 2007; Carletti and Rossi, 2008). Other cerebellar cortex GABAergic interneurons include basket and stellate cells in the molecular layer; and Golgi and Lugaro cells located in the granular layer (Leto *et al*., 2008; Leto and Rossi, 2012; Castejón, 2013).

The cerebellar nuclei are comprised of four major subdivisions:

i. The medial (fastigial), which is subdivided further into caudomedial, middle, and dorsolateral (rostrolateral) nuclei
ii. Anterior interposed nuclei
iii. Posterior interposed nuclei
iv. Lateral (dentate) nuclei (De Zeeuw and Berrebi, 1995; Manto *et al*., 2013).

The medial nuclei generally communicate with the vermis, the interposed nuclei with the paravermis, and the lateral nuclei with the hemispheres (Voogd and Glickstein, 1998). Cerebellar nuclei are composed of several neuronal types: excitatory glutamatergic neurons, which project to different parts of the brain, inhibitory GABAergic neurons that terminate in the inferior olive (Ruigrok, 1997), and inhibitory GABAergic and glycinergic interneurons (Uusisaari *et al*., 2007; Uusisaari and Knöpfel, 2012).

The cerebellum receives two major and one minor types of afferent input. Mossy fibers constitute the majority of afferent fibers in the adult cerebellum. Arising from multiple sources in the central nervous system, they project to the Purkinje cells through granule cells/parallel fibers (Valle *et al*., 2001; Voogd *et al*., 2003; Voogd, 2011). Climbing fibers are exclusively derived from the inferior olivary complex; they synapse on the dendrites of Purkinje cells (Campbell and Armstrong, 1983). A third set of afferents called neuromodulatory cerebellar afferents terminate in all three layers of the cerebellar cortex (Jaarsma *et al*., 1997; Schweighofer *et al*., 2004; Manto *et al*., 2013). All afferents to the cerebellum also send a direct branch to the cerebellar nuclei; these nuclei neurons also receive the Purkinje cell input that are essential in monitoring the whole cerebellar output (Marzban *et al*., 2010; Hashimoto and Hibi, 2012).

The fundamental architecture of the cerebellum is organized into four transverse zones based on gene expression and afferent fiber termination

i. The anterior zone (AZ: corresponding approximately to lobules I–V in mice)

ii. The central zone (CZ: lobules VI–VII), which can be further subdivided into anterior (CZa) and posterior (CZp) components (Marzban *et al*., 2008; Sawada *et al*., 2008)

iii. The posterior zone (PZ: lobules VIII–IX)

iv. The nodular zone (NZ: lobules IX–X; Ji and Hawkes, 1994; Marzban *et al*., 2003, 2004, 2012; Sugihara and Quy, 2007; Marzban and Hawkes, 2011; Bailey *et al*., 2014).

The boundaries of these zones do not align absolutely with the lobe and lobule divisions, but provide a more functionally relevant way of dividing the cerebellum (Marzban *et al*., 2011, 2014). The cerebellum contains the most elaborately patterned circuit of all the central nervous system structures, which may be essential for organizing the large number of functional and topographic zonal circuits (Reeber *et al*., 2013; White and Sillitoe, 2013). Studies on gene

expression patterns in cerebellar nuclei neurons have also revealed molecular heterogeneity that may mirror the molecular complexity of the cerebellar cortex (Chung *et al*., 2009).

The principal cerebellar cytostructure is set during early development and precedes the process of neurogenesis and axono- genesis during which cerebellar circuits and functions are established (Marzban *et al*., 2015).

2.3.5 The Hippocampus

Together with the olfactory bulbs and the piriform lobe, the hippocampus forms the rhinencephalon of the brain (Thomson and Hahn, 2012). The hippocampus appears as a C-shaped structure in coronal sections; it bulges into the inferior horn of the lateral ventricle, and is closely associated with the adjacent dentate gyrus (Afifi and Bergman, 2005) (Figure 2.3.2.1b).

In the late 1500s, Arantius, the anatomist, exposed a convoluted structure in the floor of the temporal horn of the lateral ventricle. He called it the hippocampus due to its resemblance to a sea horse. Two centuries later, anatomists likened the structure to a ram's horn or the horn of the ancient Egyptian deity Ammon, giving it the name Ammon's horn or cornu Ammonis (Afifi and Bergman, 2005).

The hippocampus can be divided into fields designated as cornu Ammonis 1, 2, 3 and 4 (CA1 to 4) (Figure 2.3.5). CA1 is the largest hippocampal field in humans, and is located in the superior division at the interface between the hippocampus and the subiculum. CA2 and CA3 are in the inferior division within the hippocampus. CA4 constitutes the transition zone between the hippocampus and the dentate gyrus. CA2 and CA3 have been referred to as resistant sectors because they are less sensitive to anoxia (Afifi and Bergman, 2005).

The hippocampus is reported to be of special veterinary importance because gross sections can be taken and with proper techniques of tissue preparation and staining, Negri bodies,

intracytoplasmic inclusion bodies that are considered pathognomonic for rabies can be demonstrated (Derakhshan, 1975). However, Bingham and van der Merwe (2002) debunked the fact that the Negri bodies were peculiar to the hippocampus. They conducted an experiment which demonstrated that the reason for the old recommendations is that the hippocampus has the highest frequency of large inclusion bodies, as the reliability of the histological tests used previously depended on inclusion body size.

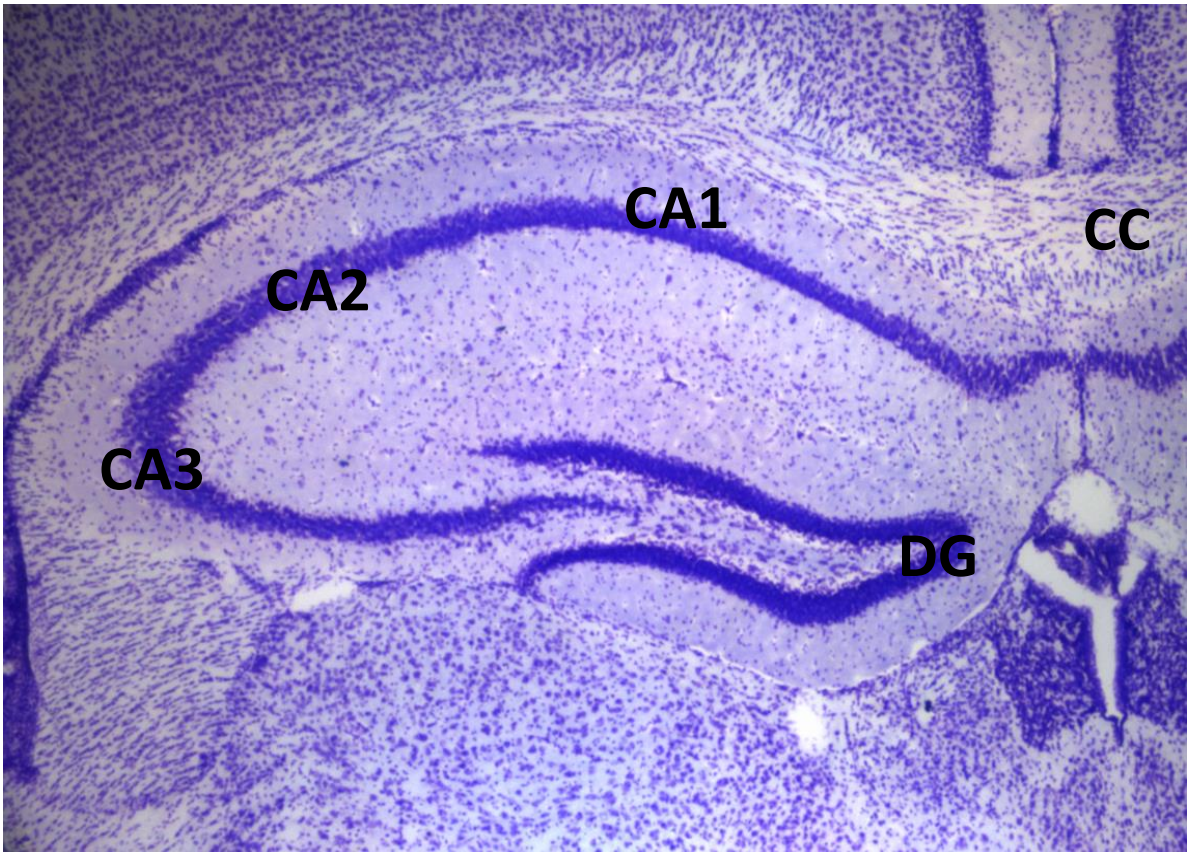


Figure 2.3.5: Coronal section of mouse brain (Cresyl Violet stain) showing the CA1, CA2, CA3 and dentate gyrus (DG) regions of the hippocampus and the corpus callosum (CC). Section prepared by Igado, O.O., labelings adapted from Afifi and Bergman (2005).

2.3.6 Cellular Diversity in the Nervous System

The human brain is estimated to contain 100 billion neurons and several times as many supporting cells. Also, the nervous system has the greater range of distinct cell types than any other organ system. The cellular diversity of any nervous system (any animal) underlies the capacity of the system to form increasingly complicated networks to mediate increasingly sophisticated behaviours (Fix, 2000; Purves *et al*., 2004).

For most of the twentieth century, neuroscientists relied on the same set of techniques developed by Cajal and Golgi to describe and categorise the diversity of cell types in the nervous system. From the late 1970s onward, new technologies have made it possible by the advances in cell and molecular biology provided investigators with many additional tools to discern the properties of neurons. More recently, genetic and neuroanatomical methods have been combined to visualise the expression of fluorescent or other tracer molecules under the control of regulatory sequences of neural genes. This approach shows individual cells in fixed or living tissue in remarkable detail, it allows nerve cells to be identified by both their transcriptional state and their structure. In addition, ways of determining the molecular identity and morphology of nerve cells can be combined with measurements of their physiological activity, and so illuminate structure-function relationships (Purves *et al*., 2004).

2.3.7 Neurons

As one measure of brain complexity, it is generally stated that there are approximately 100 billion neurons in the human brain and even greater numbers of glial cells. Neurons are characterized by wide variations in size as well as shape (especially when special stains are used to reveal their cytoplasmic processes). Neurons may be broadly classified as ‘‘small neurons’’ or ‘‘large neurons,’’ but anatomic subtypes of each of these categories exist. Neurons may also be

classified according to the neurotransmitters that they release (e.g., cholinergic, glutamatergic, GABAergic). Most neurons have multiple dendrites arising from their cell bodies. However, with rare exceptions, each neuron has only a single axon (even though this axon may branch at points distal to its cell body). Axons are specialized for transport, for the conduction of waves of depolarization, and for synaptic transmission. The Nissl substance, which stains quite prominently in large-sized neurons but is usually not apparent in small-sized neurons at the light microscopic level, represents the rough endoplasmic reticulum (RER). The RER is primarily confined to the neuronal soma but may penetrate slightly into the axonal hillock. The axon contains large numbers of neurofilaments and microtubules. These structural elements are important for maintaining cell integrity as well as for axonal transport. Chemicals that affect axonal transport may result in axonal swelling and degeneration that is visible at the light microscopic level (Garman, 2011).

A variety of immunohistochemical markers exist for neurons. Some of these markers include synaptophysin, NeuN, neurofilament protein, neuron-specific enolase (NSE) (which is not entirely specific for neurons), and microtubule-associated protein 2 (MAP2) (Garman, 2011).

2.3.8 Neuroglial Cells

Neuroglial cells, also known as glial cells or simply glia, are quite different from nerve cells. They are more numerous than neurons in the brain, outnumbering them by a ratio of about 3:1 (Purves *et al*., 2004). The major distinction is the fact that glia do not participate directly with in synaptic interactions and electrical signalling, although their supportive functions help define synaptic contacts and maintain the signalling abilities of neurons. Although glial cells also have complex processes extending from their cell bodies, these are generally less prominent than

neuronal branches, and do not serve the same purposes as axons and dendrites (Purves *et al*., 2004).

The term *glia*, is from the Greek word meaning "glue" (Nicholls *et al*., 2001; Purves *et al*., 2004; Afifi and Bergman, 2005). It reflects the 19th century presumption that these cells held the nervous system together in some way. Well established glial roles include:

- Maintaining the iconic milieu of nerve cells
- Modulating the rate of nerve signal propagation
- Modulating synaptic action by controlling the uptake of neurotransmitters at or near the synaptic cleft
- Providing a scaffold for some aspects of neural development
- Aiding in (or sometimes impeding) recovery from neural injury (Purves *et al*., 2004).

2.3.8.1 Types of Neuroglial Cells

Three types of glial cells occur in the mature central nervous system (CNS) – astrocytes, oligodendrocytes and microglial cells (Nicholls *et al*., 2001; Purves *et al*., 2004) (Figure 2.3.8.1).

2.3.8.1.1 Astrocytes

These are restricted to the brain and spinal cord. They have elaborate local processes that give these cells a star-like appearance (hence the prefix 'astro'). The major function of astrocytes is to maintain, in a variety of ways, an appropriate chemical environment for neuronal signalling (Fix, 2000; Purves *et al*., 2004).

Astrocytes have multiple roles within the CNS, including maintenance of the integrity of the blood-brain barrier, uptake and recycling of glutamate and GABA, maintenance of the extracellular ionic milieu (via uptake of K^+ ions released during neuronal activity), and neuronal metabolic support. Radial astrocytes are specialized astrocytes that provide pathways for neuron

migration during brain development. Within the cerebellum, some radial glia transform into the ‘‘Bergmann astrocytes’’ (or ‘‘Bergmann glia’’), the cell bodies of which reside within the Purkinje neuron layer. Proliferation of Bergmann astrocytes, referred to as ‘‘Bergmann gliosis’’, may be seen as a result of chemical toxicities that produce a loss of Purkinje neurons. Corpora amylacea, common within the brains of aging mammals (especially humans but rare in the CNS of rodents), represent glucose polymers (‘‘polyglucosan bodies’’) that reside within the cytoplasm of astrocytes. Corpora amylacea are most frequently present within perivascular and subpial locations, thus corresponding to the location of astrocytic cytoplasmic processes. Astrocyte-like neurons have a variety of neurotransmitter receptors within their cell membranes, and astrocytes are also involved in information processing. Stimulation of astrocytes by neurotransmitters induces cell signaling (via gap junctions and involving elevations in intracellular calcium) to other astrocytes over relatively long distances (Agulhon *et al*. 2008).

The important roles of astrocytes in supporting neuron function is underscored by the large numbers of these cells present in the brain. Astrocytes are the predominant glial cell type and comprise approximately half of the volume of the adult mammalian brain (Agulhon *et al*., 2008). In most brain areas (and depending on the species), there is an approximately 1 to 1 ratio between the numbers of astrocytes and the number of neurons, although the ratio is higher in some brain regions and is also higher in brains from those species possessing greater cognitive abilities (Sofroniew and Vinters, 2010; Sidoryk-Wegrzynowicz *et al*., 2011). To fulfill their various vital roles, astrocytes have cytoplasmic extensions that touch on the surfaces of all major regions of the neuron’s anatomy (i.e., cell bodies, axons, dendrites, and synapses) and also extend to the pial surface of the brain to form the glia limitans (glial limiting membrane). The

glia limitans seals the surface of the brain and also dips into the brain tissue along the perivascular (Virchow-Robin) spaces.

Astrocyte foot processes also surround brain capillaries and, during development, induce endothelial cells to form tight junctions. The term ‘‘astrocyte’’ refers to the multiple radially arranged cytoplasmic processes that can be appreciated only with special stains. However, while astrocytes have long cytoplasmic extensions that reach from neurons to the pial surface and/or to capillaries, these processes are not seen within H&E-stained sections. In fact, nonreactive astrocytes are characterized within Haematoxylin and Eosin stained sections by ‘‘naked nuclei’’ and little observable cytoplasm. Astrocytes are often broadly classified into fibrous and protoplasmic types, with the former being found within white matter regions and the latter residing within the gray matter. This is an oversimplification, however, with newer evidence indicating that astrocyte populations are heterogeneous from one brain region to another (Yeh *et al*., 2009; Hewett, 2009).

In gray matter regions of the CNS, astrocytic cell nuclei are often found to be in close proximity to neurons but may be found anywhere within the neuropil. Astrocyte nuclei typically have pale, finely granular chromatin patterns and relatively small or indistinct nucleoli. One of the many roles of the astrocyte is to remove and detoxify ammonia; and in states of hyperammonemia, ‘‘Alzheimer type II astrocytes’’ with swollen, ‘‘water clear’’ nuclei may be seen in sections from immersion-fixed (but not perfusion-fixed) brains (Norenberg *et al*., 2007)

2.3.8.1.2 Oligodendrocytes

Oligodendrocytes are responsible for the formation and maintenance of the myelin sheaths of the CNS. Although Schwann cells serve this role in the peripheral nervous system, oligodendrocytes will be found to extend out from the brain for some distance into the proximal

segments of the cranial nerves (as well as along the entire optic nerve). Within these cranial nerves, sharp demarcations will be seen between the zones of central and peripheral myelination. Oligodendrocytes, in contrast with Schwann cells, ensheath multiple axons, whereas a single Schwann cell forms the myelin sheath for only one axonal internode. Within tracts of white matter, oligodendrocytes are typically arranged in linear rows between the nerve fibers (Bradl and Lassmann, 2010).

Oligodendrocytes are also restricted to the CNS, lay down a laminated, lipid-rich wrapping called myelin around some, but not all axons. Myelin has very important effects on the speed of the transmission of electrical signals. The Schwann cells elaborate myelin in the peripheral nervous system (Purves *et al*., 2004).

2.3.8.1.3 Microglial Cells

Microglial cells are derived primarily from haematopoietic precursor cells, although some are derived directly from neural precursor cells. They share many properties with macrophages found in other tissues, and are primarily scavenger cells that remove cellular debris from sites of injury or normal cell turnover. Microglia cells also secrete signalling molecules (especially a wide range of cytokines) that can modulate local inflammation and influence cell survival or death. In the case of brain damage, the number of microglia at the site of injury increase dramatically. Some of these cells proliferate from microglia resident in the brain, while others come from macrophages that migrate to the injured area and enter the brain via local disruptions in the cerebral vasculature (Purves *et al*., 2004).

Microglia comprise the reticuloendothelial system of the CNS and constitute 5-20% of the brain's glial cell population. As with neurons and the macroglia, microglia are functionally heterogeneous. In lesions characterized by neuronal degeneration, individual microglia will

typically be seen in close proximity to the degenerating neurons. Greater insults to the CNS may result in denser infiltrates of microglia, some of which will assume a histiocytic cell morphology or even form granuloma-like inflammatory patterns. Under appropriate conditions, microglia may transform into macrophages and, in this state, are sometimes referred to as ‘‘gitter cells.’’. In most ‘‘neurotoxic’’ lesions, neuronal degeneration will be apparent by the time microglia aggregate at the scene. However, this is not always the case (Garman, 2011).

2.3.8.2 Markers for Neuroglia Cells

Cell markers are valuable tools for examining the functions of cells in normal conditions as well as during repair and disease processes. An ideal marker is specific to a given cell type in normal conditions and/or during conditions involving injury or disease. Markers can be expressed on more that one cell type (Redwine and Evans, 2002).

Antibodies to cell-type-specific markers identify a cell at the molecular level and are considered more reliable than identification of cell type by morphology. Therefore, if a marker for a cell type is not available, it is possible that the existence of a particular cell type may be overlooked. An example of this in the CNS was the characterization of an oligodendrocyte progenitor cell type in the adult rodent and human CNS (French-Constant and Raff, 1986; Levine and Card, 1987). Adult oligodendrocyte progenitors are scattered throughout the parenchyma of the brain and spinal cord, but were completely overlooked prior to the availability of in vivo markers specific for these cells. A more accurate understanding of CNS cell types and their distinct functions have been postulated to lead to a better understanding of disease pathology and development of novel therapeutic agents for CNS diseases (Redwine and Evans, 2002).

i. *Adult Oligodendrocyte Progenitors*: This cell type may play a role in

inflammatory and repair processes in the CNS. The function of adult oligodendrocyte progenitors in normal conditions is not well understood. They proliferate to a small extent (Levine *et al.,* 1993) and thus may replace mature oligodendrocytes as needed. These cells are abundant in the CNS (Redwine and Evans, 2002). In normal conditions, adult oligodendrocyte progenitors can be identified by antibodies against NG2 (Dawson *et al.,* 2000), PDGFdR (Hermanson *et al.,* 1992; Nishiyama *et al.,* 1996), and 04 (Reynolds and Hardy, 1997). The anti-NG2 antibody labels adult oligodendrocyte progenitors in normal adult rodent brain and spinal cord (Dawson *et al.,* 2000). NG2 is the most reliable marker for this cell type in rat and mouse since it has been found to specifically label adult oligodendrocyte progenitors in normal and reactive or inflammatory conditions (Redwine and Armstrong, 1998).

ii. *Mature Oligodendrocytes*: Oligodendrocyte precursors originate from germinal regions located in subven- tricular zones in the brain (Levison and Goldman, 1993) and germinal zones in the spinal cord (Pringle and Richardson, 1993).

Oligodendrocytes begin myelinating axons postnatally by extending modified processes that wrap around nearby axons and eventually form a myelin sheath. A single process from an oligodendrocyte forms a single segment of myelin around an axon that consists of layers, or lamellae. The myelin sheath is a modified membrane containing lipids, glycolipids, gangliosides, and myelin-specific proteins. Myelin sheaths and oligodendrocytes are often identified by immunolabeling myelin-specific proteins. Antibodies to myelin proteins include proteolipid protein – PLP (Sobel *et al*., 1994; Greer *et al*., 1996), myelin basic protein – MBP (Shine *et al.,* 1992), 2^{r},3^{r}-cyclic nucleotide-3^{r}-phosphodiesterase – CNP (Reiser *et al.,* 1994), myelin oligodendrocyte glycoprotein – MOG (Birling *et al.,* 1993; Gardinier and Matthieu, 1993), myelin-associated glyco- protein – MAG (Trapp *et al.,* 1989) and myelin-associated

oligodendrocytic basic protein – MOBP (Holz *et al.,* 1996). The most common antigens used to identify oligodendrocytes are PLP, MBP, and CNP. PLP and MBP are the most abundant myelin proteins (50% and 30%, respectively), and antibodies to these proteins label oligodendrocyte cell bodies, processes and the compact myelin sheath. CNP is less abundant than PLP or MBP, and it is not found in the compact myelin sheath. Antibodies against CNP are useful for identifying oligodendrocytes in vivo since they label oligodendrocyte cell bodies and processes, as well as the outer myelin membrane, but not inner compact myelin lamellae. Anti-MOG antibody labels oligodendrocytes and myelin sheaths (Birling *et al.,* 1993), anti-MAG antibodies label the periaxonal myelin membrane closely associated with the axon (Trapp *et al.,* 1989), and anti-MOBP antibody primarily labels the myelin sheath (Holz and Schuab, 1997). A monoclonal antibody named Rip (Friedman *et al.,* 1989) has also been used in vivo to identify rat oligodendrocytes (Reynolds and Hardy, 1997). The Rip antibody stains the oligodendrocyte cell body, processes, and myelin and the staining pattern resembles that by antibodies to CNP. Another antibody that recognizes oligodendrocytes is monoclonal antibody CC-1 (Smith *et al.,* 1993). An additional oligodendrocyte marker less often used is transferrin (Conner and Fine, 1986). Transferrin is primarily expressed by oligodendrocytes, but can be detected in ependymal cells in some periventricular regions and in the choroid plexus of the rat CNS (Benkovic and Connor, 1993).

iii. *Astrocytes*: Astrocytes are involved in local inflammatory responses in many disease models. Reactive astrocytes often seen within and near regions of inflammation and tissue damage have shorter, thicker processes and a larger cell body and nucleus than the resting astrocyte. Astrocytes proliferate in response to viral infection, demyelination, and injury (Norton, 1999). The most well characterized specific marker for the astrocyte cell type is

GFAP (Redwine and Evans, 2002). Antibodies to GFAP recognize astrocytes throughout the brain and spinal cord and label astrocytes with either radial or stellate morphology. S100, a calcium-binding protein, is also expressed primarily in astrocytes (Haan *et al.,* 1982); however, S100 immunoreactivity can be detected in some neuronal populations in rat brain (Yamashita *et al.,* 1999). S100 is a secreted protein that can increase neurite outgrowth and is considered a neurotropic cytokine (Eddleston *et al.,* 1993).

iv. *Microglia*: Microglia share many functions and antigenic markers with peripheral macrophages and are considered to be the resident macrophages of the CNS (Redwine and Evans, 2002). Commonly used markers for microglia (and macrophages) are Mac-1 (complement receptor type 3, CR3), F4/80, and lectin binding. Mac-1 binds complement fragment C3bi and intercellular adhesion molecule-1 (ICAM-1), and is used as a receptor for some strains of gram-negative bacteria (Horwitz, 1992). Mac-1 consists of a d-chain, CD11b, and a þ-chain, CD18. Anti-Mac-1 and anti-CD11b antibodies are available for tissues of mouse (Ho and Springer, 1982) and humans (Beller *et al.,* 1982). In the rat, a commonly used monoclonal antibody that binds Mac-1 is MRC OX-42 (Robinson *et al.,* 1986). Another microglia/macrophage marker, F4/80, is a cell surface glycoprotein expressed by microglia and macrophages in mouse and rat tissues (Starkey *et al.,* 1987). F4/80 has recently been implicated in cell-to-cell signaling pathways (Uarschkau and Hiderlen, 1999). Several lectins that bind to microglia (and macrophages) in mouse, rat, and human tissues have been used to identify these cells (Acarin *et al.,* 1994; Schumacher *et al.,* 1994). Monoclonal antibody ED1, which binds a cytoplasmic antigen in rat microglia and macrophages, has been used as a microglial marker (Dijkstra *et al.,* 1985). In humans, CD68 and Ham-56 are also used as microglial markers (Andjelkovic *et al.,* 1998). Another reliable marker more recently used is

Iba1 (Azeez *et al*., 2016).

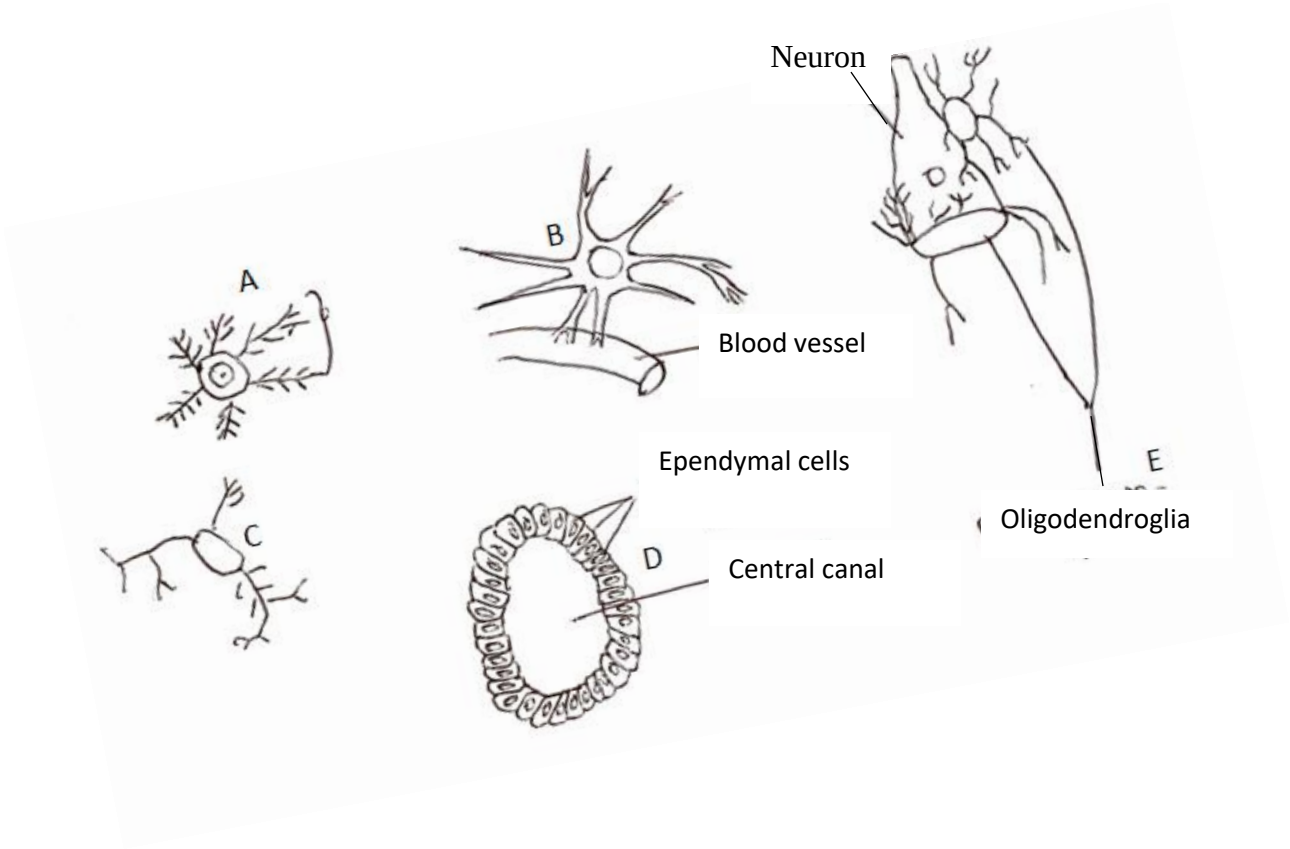


Figure 2.3.8.1: Schematic diagram showing the different types of neuroglia. A = protoplasmic astrocytes; B = fibrous astrocytes; C = microglia; D = ependymal cells lining central canal of spinal cord; E = oligodendroglia (Adapted from Afifi and Bergman, 2005)

2.3.9 The Blood Brain Barrier (BBB)

In order to function effectively, the brain requires a substantial amount of energy to maintain electrochemical gradients across neuronal membranes. Although constituting only 2% of the weight of the body, the brain utilises 20% of its blood supply. The blood is delivered through a uniquely complex network of blood vessels that extends more than 650 km and spans a surface area of about 20 m^2. The mean distance between capillaries is 40 μm, which permits near-instantaneous solute equilibration throughout the brain interstitial space for small molecules. However, unlike nearly all other organs of the body where there is a free exchange between blood and interstitial fluid, the capillaries in the brain have evolved to constrain the movement of molecules and cells between blood and brain. This important characteristic provides a natural defence against toxic or infective agents circulating in the blood, and is conferred by cell adhesion molecules allowing endothelial cells to form tight junctions (Palmer, 2010).

This blood brain barrier (BBB), while fulfilling a protective role, severely limits the movement of medicines and potential medicines into the brain. As a result, most molecules have to take a transcellular route into the brain, so the overwhelming majority of small molecules, proteins and peptides do not cross the BBB. It also limits the entry of red blood cells and leukocytes into the brain (Palmer, 2010).

The blood brain barrier (together with the blood-CSF barrier) prevents some molecules that may be present in the blood from diffusing into the CNS parenchyma. This protective mechanism aims to limit the exposure of the CNS tissues to pathogens and molecules that could have a deleterious effect. Because of these barriers, the composition of the CSF and the

extracellular fluid in the CNS is different to that in the rest of the body. Structurally, the BBB has three components:

a. Tight junctions between the capillary endothelia

b. A thick basement membrane

c. A layer of astrocyte foot processes that surround the capillaries (Thomson and Hahn, 2012).

The concept of the BBB was anticipated by the Nineteenth Century French physiologist Claude Bernard in his phrase: “La fixité du milieu intérieur est la condition d'une vie libre et indépendante” (“The constancy of the internal environment is the condition for a free and independent life”). Direct evidence for the existence of the BBB came, at the dawn of the Twentieth Century, from the German scientist and Nobel Laureate Paul Ehrlich, with his observation that when he injected colored dyes, including trypan blue, into the blood stream they leaked out of capillaries in most regions of the body to stain the surrounding tissue, but the brain and spinal cord remained unstained (Ehrlich, 1885; 1904). Ehrlich wrongly concluded that the differential colouring of the body was due to the CNS having a lower affinity for the dyes than the tissues of the rest of the body. It was his student, Edwin Goldmann, who completed the experiment a number of years later by injecting trypan blue into the subarachnoid space of rats. He observed that the brain and spinal cord did stain, but the rest of the body's organs did not. This falsified the hypothesis that the differential staining was due to a lower affinity for the dyes in CNS tissues, and inevitably led to the conclusion that the dye was unable to cross the specialized walls of brain capillaries. Goldman's experiment thus provided convincing evidence for the existence of the BBB (Palmer, 2010).

Lewandowsky, another German scientist, came to the same conclusion in 1900 on the basis of his observation that small doses of potassium ferrocyanide caused convulsions when injected into the subarachnoid space of a dog's spinal cord, yet larger doses injected systemically were well tolerated; this contrasted with the action of strychnine which caused lethal cramps when administered both centrally and peripherally (Lewandowsky, 1900). He therefore concluded that 'the walls of cerebral capillaries hinder the transit of certain compounds and not for others.' However, Lewandowsky ideas encountered much skepticism, especially from Ehrlich.

Further support for the existence of the BBB came from the work of the Russian neurophysiologist Lina Stern in 1921. She was puzzled why certain medicines and serums injected into the blood stream did not enter the CNS. Thus, for example, intravenous injections of anti- tetanus serum fail to check tetanus once the poison gets into the CNS. Stern concluded that there must be a barrier developed to protect the CNS from toxins and germs. She called this block the "hematoencephalic barrier." This led her to experiments where she directly injected compounds into the brains of dogs and observed profound effects that confirmed the existence of the BBB. During World War II, she gave brain injections to shock victims given up for dead with success that was so dramatic that this approach became a standard measure of care in many Soviet hospitals (Vein, 2008).

Stern's work was confirmed and extended by two German researchers, Friedman and Elkeles, who, in 1934, concluded that: 'It can be stated that toxins differ among themselves in the facility with which they pass the blood–brain barrier' (Friedman and Elkeles, 1934). Despite mounting evidence, the concept of the BBB remained controversial for many years. Even as late as 1962, questions about it as 'fact or fantasy' were being asked (Tschirgi, 1962). But the doubts

subsided with the emergence of electron microscopic data demonstrating the presence of tight junctions in brain capillaries (Brightman and Reese, 1969). On the basis of this and comparative studies of anatomy and physiology, it was accepted that the BBB is formed in most vertebrates by tight junctions between the endothelial cells that form brain capillaries (Abbott, 1992).

The impermeable nature of the BBB means that it acts as a functional interface between the circulatory system(and thus the immune system) and the brain. It maintains homeostasis through its regulation of trans-BBB movement of chemicals and cells. Disruption of the BBB is, thus, a critical event in the development and progression of several brain disorders. In somecases, increased BBB permeability is a consequence of the pathology, such as with stroke and traumatic brain injury, but chronic neurodegenerative diseases, such as Alzheimer's dementia and Parkinson's disease, also. In all cases, it seems that neurodegenerative change is exacerbated by the linked process of BBB disruption and neuro-inflammatory change. In other cases, BBB disruption may be a precipitating event, such as with multiple sclerosis, infectious meningitis and meningoencephalitis, cerebral malaria, dementia associated with AIDS, epilepsy, obesity and, possibly, neuro-developmental diseases such as schizophrenia and autism (Palmer, 2010).

During inflammation or an immune-mediated pathologic process the blood-brain barrier breaks down and allows access of cells and other substances into the brain. The increased permeability of the blood-brain barrier during inflammation depends on several factors: opening of tight junctions, gaps across endothelial cells, increase in receptor-mediated transcytosis, and increase in pinocytosis (Afifi and Bergman, 2005).

Other barrier systems in the brain include the blood-cerebrospinal fluid (CSF) barrier and the blood-nerve barrier (Afifi and Bergman, 2005).

In the blood-CSF barrier, tight junctions that join choroidal epithelial cells constitute the barrier at this site. The surface area of the blood-CSF barrier is only 0.02 % of the surface area of the blood-brain barrier. The ependymal cells lining the ventricles are not joined together by tight junctions and thus do not constitute a barrier between the CSF and brain. A third barrier, the blood-nerve barrier, comprises the perineurium and capillaries of the endoneurium. Walls of capillaries are nonfenestrated, and endothelial cells have tight junctions. This barrier is most effective in dorsal root ganglia and autonomic ganglia (Fix, 2000; Afifi and Bergman, 2005).

The choroid plexus is one of the sites for production of CSF. It is composed of villi extending from the ventricular wall into the CSF. It is distributed in the body, trigone, and inferior horn of the lateral ventricle, foramen of Monro, roof of the third ventricle, and posterior part of the roof of the fourth ventricle. Each villus is composed of an extensive network of fenestrated capillaries embedded in connective tissue stroma. Villi are lined by a single layer of choroidal cuboidal epithelium in continuity with the ependymal cell lining of the ventricular wall. The apical surfaces of the choroidal epithelium in contact with CSF are specialized into microvilli that increase their ventricular surface. Choroidal epithelial cells are attached to each other by tight junctions that constitute an effective barrier to the free passage of substances from the blood vessels in the core of the villus into the CSF (blood-CSF barrier). Hydrostatic pressure within the fenestrated capillaries of choroid plexus forces water, solutes, and proteins out into the connective tissue core of the villus. Macromolecular substances, however, are prevented from free passage to the CSF by the tight junctions between the lining choroidal epithelial cells (Fix, 2000; Afifi and Bergman, 2005).

Studies on the mechanisms of the barrier system have shown that the anatomic substrates of the barrier (endothelial lining, basement membrane, glial processes, tight junctions) cannot

account for all the observed phenomena of the barrier system. It is thus conceivable that other factors are operative in the barrier system. These factors include the following:

- Blood flow: This factor is operative in the entry to the brain of substances of high lipid solubility. The rate of blood flow to a brain region will determine the amount of entry of such substances.
- Metabolic requirement: The rate of entry of some substances into the brain seems to be dependent on the metabolic requirement of that region of the brain for the particular substance. Cholesterol, for example, is accumulated in the brain during myelin formation and decreases when myelination is completed.

The brain barrier system is more permeable in newborn infants than in adults. As the brain matures with age, the barrier system becomes less permeable. The brain of the newborn, for example, is permeable to bilirubin. A rise in bilirubin levels in the blood of a newborn is detrimental to brain function. In contrast, an excessive rise in serum bilirubin in the adult does not affect the brain (Fix, 2000; Afifi and Bergman, 2005).

Certain areas of the brain are devoid of a barrier system. These areas, known as circumventricular organs include:

i. The area postrema, a chemoreceptor center in the caudal medulla oblongata

ii. The neurohypophysis

iii. The organ vasculosum of the lamina terminalis (superior and rostral to the optic chiasma), which is sensitive to plasma osmolarity

iv. The median eminence of the hypothalamus

v. The subcommissural organ located ventral to the posterior commissure at the junction of the third ventricle and aqueduct of Sylvius

vi. The subfornical organ (under the fornix), which is sensitive to circulating angiotensin II

vii. The pineal gland (Fix, 2000; Afifi and Bergman, 2005).

In some circumventricular organs, neurons have specialized receptors for specific proteins. These include the area postrema, subfornical organ, and organ vasculosum. Other circumventricular organs have neurons with secretory properties. These include the median eminence, neurohypophysis, subcommissural organ, and pineal gland. All these areas are characterized by rich vascularity. Unlike vessels elsewhere in the brain, the endothelial lining of vessels in these areas is fenestrated (Fix, 2000; Purves *et al*., 2004; Afifi and Bergman, 2005).

2.3.10 Blood Supply to the Brain

Although the CNS accounts for only 2% of the body weight, it has a disproportionately high metabolic rate and receives about 20% of the total cardiac output. The oxygen requirements of the synapses and neuronal cell bodies are greater than those of the axons, thus the grey matter receives more blood flow than the white matter. Additionally, association/integration areas have greater requirements than other areas and so the forebrain is more vascular than other CNS regions (Thomson and Hahn, 2012).

2.3.10.1 Arterial Blood Supply

Arterial blood supply of the brain is based around five pairs of arteries (Figure 2.3.10). Four of these arise from the cerebral arterial circle located on the ventral surface of the forebrain. These arteries include the rostral, middle and caudal cerebral arteries and the rostral cerebellar artery (Dyce *et al*., 2002; Thomson and Hahn, 2012).

The caudal cerebellar arteries originate from the basilar artery, which is continuous with the ventral spinal artery at the foramen magnum. The basilar artery runs longitudinally on the ventral aspect of the brainstem and connects with the caudal aspect of the arterial circle. The

major brain arteries traverse the surface of the brain in the sulci, and send smaller branches to perfuse the deep tissues. While there are anastomoses between the arteries on the surface of the CNS, the penetrating arteries do not communicate with each other and so blockages of individual end-arteries lead to ischaemia of defined parts of the CNS. Although there are some species differences in the arteries of the brain, these are relatively minor. However, there are major differences in how blood gets to the brain; these differences are clinically important (Dyce *et al*., 2002; Thomson and Hahn, 2012).

Generally speaking there are four possible routes by which the blood may arrive at the arterial circle; the internal carotid artery, the basilar artery, the maxillary artery and the vertebral artery. The common domestic animals have various combinations of these routes. The maxillary artery and the vertebral artery may have rete mirabile *(rete* - L = net, *mirabile* – L = marvellous) where the major vessel breaks into many convoluted small branches before re-anastomosing. The rete mirabile may act as a heat exchanger to protect the brain from major temperature changes and act to reduce pulsations in the blood perfusing the CNS. The cat, sheep and ox have one, or more, rete mirabile (Dyce *et al*., 2002; Thomson and Hahn, 2012).

Patterns of arterial supply to the brain

1. General form (dog, human): The internal carotid artery and the basilar artery both carry blood to the cerebral arterial circle. Carotid blood reaches most of the cerebral hemispheres except the caudal portion. Vertebral blood supplies the rest of the brain.

2. Sheep and cat: The proximal two-thirds of the internal carotid artery is absent in the adult and the direction of blood flow is caudad in the basilar artery. The only supply of blood to the cerebral arterial circle originates from the maxillary arteries via a rete mirabile. Maxillary blood supplies all the brain except the caudal part of the medulla.

3. Ox: As in the sheep and cat the proximal two-thirds of the internal carotid is absent in the adult and the direction of blood flow is caudad in the basilar artery. Unlike the sheep there are two anastomosing branches, one from the maxillary artery and one from the vertebral artery, both of which have rete mirabile. Maxillary and vertebral blood supplies the whole of the brain (Dyce *et al*., 2002; Thomson and Hahn, 2012).

Arterial supply to the spinal cord

The spinal cord is supplied segmentally by spinal branches arising from the vertebral artery and aorta, and entering the spinal canal at the intervertebral foramens. Within the spinal canal, each branch divides into a smaller dorsal, and larger ventral branch. The dorsal branches enter the spinal cord with the dorsal root. The ventral branches unite to form the unpaired, median, ventral spinal artery (Getty, 1975; Dyce *et al*., 2002; Thomson and Hahn, 2012).

Venous drainage of the CNS

The neuraxis and meninges are drained by veins and sinuses (see other texts for details of the veins). In sinuses, valves are absent or poorly developed, hence retrograde blood flow can occur. Sinuses in the brain form a dorsal and ventral set. The dorsal set is primarily midline with the dorsal sagittal sinus, sited in the falx cerebri draining caudally to meet the straight sinus at the confluences of the sinuses located at caudal end of the falx cerebri. The straight sinus drains the great cerebral vein from the deeper cerebrum. Drainage from the confluence is laterally into the ventrally directed, paired transverse sinuses, beginning in the dorsal part of the occipital bone (Getty, 1975; Dyce *et al*., 2002; Thomson and Hahn, 2012).

The transverse sinus terminates by dividing into the sigmoid and temporal sinuses in the ventrocaudal aspect of the skull. The ventral set of sinuses begins with the paired cavernous sinus lying subdurally on the floor of the rostral fossa, surrounding the hypophysis. The

cavernous sinus receives venous drainage from the ophthalmic plexus, and may, depending on the species, interconnect across the midline by the intercavernous sinus. The cavernous sinuses drain caudally into the ventral petrosal sinus that connects to the sigmoid sinus. The dorsal petrosal sinus drains the basal vein of the cerebrum into the transverse sinus. The sinuses drain into the maxillary, vertebral, occipital and internal jugular veins (Getty, 1975; Dyce *et al*., 2002; Thomson and Hahn, 2012).

The spinal cord has an internal vertebral venous plexus lying on the floor of the spinal canal, with paired vessels that diverge to drain into intervertebral veins at each intervertebral foramen (Dyce *et al*., 2002; Thomson and Hahn, 2012).

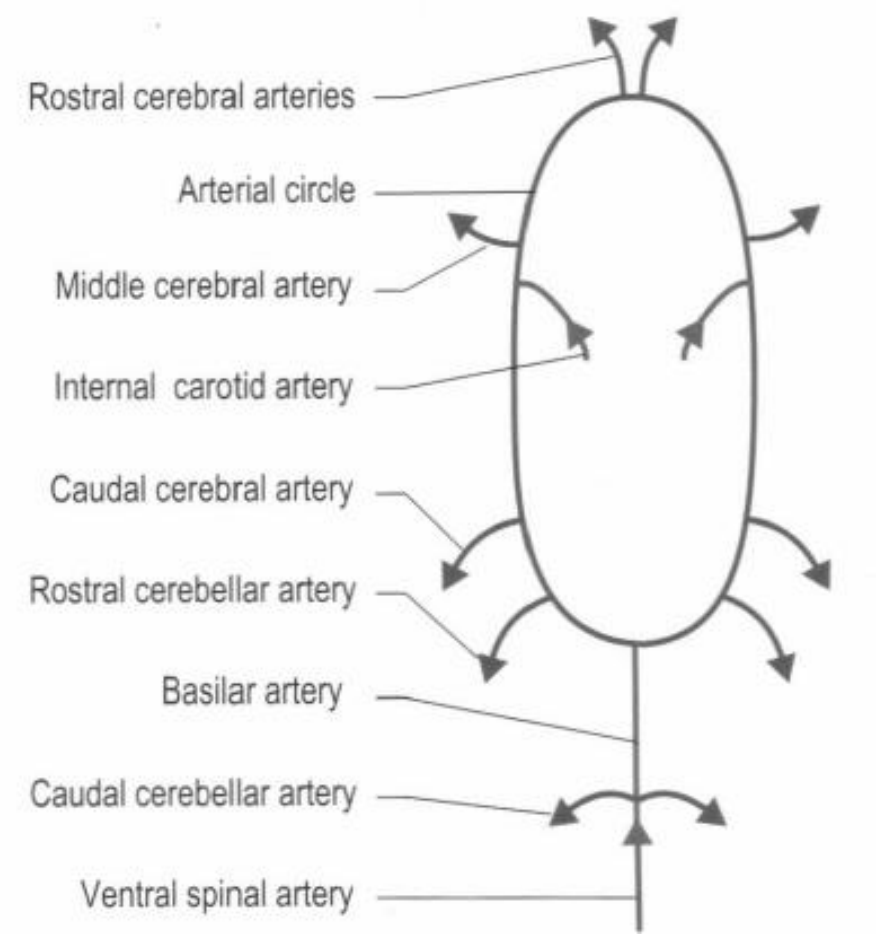


Figure 2.3.10: Schematic of the blood supply to the brain of the dog (Thomson and Hahn, 2012).

CHAPTER 3

MATERIALS AND METHODS

EXPERIMENT 1

EXTRACTION OF METHANOLIC EXTRACT OF *MORINGA OLEIFERA* LEAVES, BIOASSAY-GUIDED FRACTIONATION AND PURIFICATION, STRUCTURE ELUCIDATION AND SYNTHESIS

3.1.1 Preparation of the Methanolic Extract of *Moringa oleifera* Leaves

All leaves used for the study were collected from a single source, in Ibadan, Nigeria and in the rainy period (May-July) to avoid disparity in chemical composition based on differences in soil type, moisture content and other factors. The leaves were identified and authenticated at the Federal Institute of Nigeria (FRIN), with FRIN number FHI No. Moringa Lam. Moringaceae – 109606.

The methanolic extraction was carried out based on a modification of the method described by Rao, *et al.* (2001). The leaves were air-dried by spreading in a dark room, pulverised and weighed. 2,243.6 grams of the pulverised leaves were put into flat-bottom beakers and distilled n-hexane (100%) dispensed into the beakers until the height was at least 2-3 cm above the level of the leaves. The pulverised materials were removed 24 hours later, air-dried and returned into the beakers. Methanol (100%) was dispensed into the beakers until the height was at least 2-3 cm above the level of the leaves. This was left to stand in a dark and dry place for 72 hours, after which the supernatant was decanted and sieved with a Whitman's filter paper (24.0 cm, ashless). The supernatant was evaporated using Buchi® Rotary evaporator, at 40 rpm, in a water bath maintained at $34^{O}C$, to get rid of the excess methanol. The resulting product was

put in an oven at 34^{O}C until a syrup-like consistency was achieved. Weighing the resultant product gave a yield of 138g (about 40 mls).

3.1.2 *Bio-activity Guided Fractionation and in vitro Antioxidant Properties*

The extract was slurried in methanol (MeOH) and filtrated using LiChroprep© RP-18, to remove insoluble parts, and thereafter subjected to a liquid-liquid fractionation according to the chart (Figure 1).

Antioxidant activity of the fractions was assessed using Ferric Reducing Anti-oxidant Potential (FRAP) according to the method previously described by Benzie and Szeto (1999) and Schmid *et al.*, (2008). TEMPOL was used as a standard.

Five series of 2 ml cuvettes were prepared in triplicates. Six hundred microliters of the FRAP reagent was pipetted into each cuvette. Ferrous sulphate (10mM) was pipetted into each series in increasing concentration (7 µl, 14 µl, 21 µl, 28 µl, 35 µl) and also water in decreasing volume (193 µl, 186 µl, 179 µl, 172 µl, 165 µl). These were allowed to stand for 30 minutes and the intensity (absorbance) was read with a spectrophotometer at 593 nm. A line graph was plotted with the values obtained to derive the standard. For the samples to be tested, 200 µl of the samples was pipetted into cuvettes in triplicates, allowed to stand for 30 minutes, read with a spectrophotometer and the average intensity obtained. The µM ferric equivalence/mg extract was calculated using the following formula:

y = (standard) x

Where ‘y’ is the absorbance, ‘x’ is the µM ferric equivalence and the ‘standard’ is the value derived from the graph.

3.1.3 Preparative High Performance Liquid Chromatography (HPLC)

All procedures were based on previous reports by Bringmann *et al.* (2005), Ghosh *et al*. (2011) and Glaser *et al*. (2014). The fraction with the highest antioxidant value (butanol fraction) (Figure 2) was subjected to a gravity column, using silica gel in chloroform and methanol (4.8:0.2). The resultant fractions were pooled together into 4 places based on elution on TLC plates. These four fractions were further subjected to preparative HPLC, using a semi-preparative column 125mm x 10mm, 5μm pore, Nucleodur Spinx RP. Solvents used were water and methanol (MeOH); (A) H2O, (B) MeOH, gradient: 10% B (0 min), 10% (1 min), 100% (15 min), 100% (25 min), 10% (30 min); flow rate 3.3mL/min. Water and methanol were finally used as solvents based on the polarity of the extract after experimenting with different solvents using Thin Layer Chromatography (TLC).

In TLC procedure, using capillary tube, a spot of the extract was placed on a glass slide previously covered with silica gel. This slide was then placed in a glass chamber having a known concentration of solvent in it, to allow the spot of extract to elute. Separation of the extract was then viewed under a UV light (256 nm).

Further fractions obtained are highlighted in Figure 3. Nuclear Magnetic Resonance (NMR) and Liquid Chromatography/Mass Spectrophotometer (LC/MS) were employed to identify the compounds.

3.1.4 *Nuclear Magnetic Resonance (NMR) Data*

The structures were identified with NMR and LC/MS. Samples were loaded into the NMR and LC/MS machines and data obtained were analysed according to Bevinakatti and Banerji (1992) and Glaser *et al*. (2014).

3.1.5 *Synthesis of the p-Hydroxyphenyl Acetic Acid Butyl Ester*

To synthesize MIMO2, based on the structure, the root chemicals were defined and the following procedure carried out:

Two grams (12 mmol) of 4-methoxyphenyl acetic acid, 500 mg (4 mmol) DMAP (4-Dimethylaminopyridine) and 2.30 g (12 mmol) EDC-HCl were dissolved in 20 mL *n*-butanol and stirred for 24 h at room temperature. The solution was washed three times with water and the organic phase dried over Na_2SO_4 and evaporated. Purification of the resulting white syrup by column chromatography (ethyl acetate: petrol ether, 60:40, *v/v*) yielded 2.42 g (10.9 mmol, yield 91%) of butyl *p*-methoxyphenyl acetate (MIMO1) (Figure 4). ^{1}H NMR ($CDCl_3$) δ (ppm) *J* (Hz): 0.81 (t;*7.4*;3H); 1.36 (m;2H); 1.60 (m;2H); 3.55 (s;2H); 3.79 (s;3H;-OC*H*$_3$); 4.08 (t;*6.7*;2H); 6.65 and 7.20 (ABq;4H;aromatic).

O-Demethylation process: To obtain MIMO2 (which was the compound in MO), MIMO1 was demethylated. According to Node *et al.* (1979) 4.50 g (33.8 mmol) $AlCl_3$ were dissolved in 2 mL of ethanethiol. 1.60 g (7.2 mmol) of MIMO1 were added and stirred for 2 hours. To enable better stirring, a small portion of $CHCl_3$ was added. The resulting solution was poured into water, acidified with dilute HCl and extracted with $CHCl_3$. The organic phase was then washed three times with brine, Na_2SO_4 solution and H_2O, respectively. The chloroform phase was dried over $MgSO_4$ and the solvent was evaporated, yielding 1.41 g (6.7 mmol, 93%) of butyl *p*-hydroxyphenyl acetate (MIMO2) as viscous yellowish syrup soluble in DMSO, but insoluble in H_2O. NMR data were in accordance with the literature (Bevinakatti and Banerji 1992). MIMO2 was characterized to be a phenolic compound.

FRAP was performed to determine the antioxidative power of MIMO2 relative to some other fractions. The antioxidative power of MIMO2 relative to TEMPOL, the methanolic extract

of *M. oleifera* leaves (MO), butanol fraction (BF) and MIMO1 was assessed using FRAP, as described previously. All values were expressed as nmol Fe^{2+} equivalence of the extract or substance.

3.1.6 *Antioxidative Potential of MIMO2 using FRAP*

The antioxidative potential of MIMO2 relative to TEMPOL, the methanolic extract of *M. oleifera* leaves (MO), butanol fraction (BF) and MIMO1 was assessed using FRAP, as previously described.

EXPERIMENT 2

CELL CULTURE EXPERIMENTS USING HT22 CELLS (IMMORTALIZED MICE HIPPOCAMPAL CELLS)

3.2.1 *Chemicals and Reagents*

Cell culture media and reagents were obtained from PAA Laboratories GmbH (Pasching, Austria) and Invitrogen Life Technologies (Darmstadt, Germany).

HT22 cells (immortalized mouse hippocampal cells) were grown and expanded in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine serum, 1% L-Glutamine, 0.35% Pen-Strep and 1% Glucose, based on a modification of the method of Chen *et al*. (2005). Cells were maintained at 37 $^{\circ}$C temperature and 5% carbon (II) oxide. Passage number of cells used for experiment was from 4 to 12. An average of 100,000 cells was seeded in 6-well plates in 3 mls of media. Twenty four hours after seeding, the cells were treated with vanadium and / or MIMO2 and maintained at 37 $^{\circ}$C temperature, and 5% carbon (II) oxide. Cells were exposed to 100 μM of vanadium according to previous reports by Todorich *et al*., (2011). The dose used for the Comet assay was increased to 200 μM.

3.2.2 *Measurement of Intracellular Cellular Reactive Oxygen Species by Dihydroethidium (DHE) Staining*

Analysis was carried out according to the method described previously by Fazeli *et al*. (2012). The cell permeable fluorogenic dihydroethidium (DHE) was used to evaluate the release of reactive oxygen species (ROS). The cells were seeded on glass coverslips in 6-well cell culture plates (≈100,000 cells/well in 3 ml medium) and treated with the test compounds in varying doses after 24h. In the cases of combinations of MIMO2 or MO with vanadium, either of the two additives were added 20 min before the 30min vanadium treatment. The media were

discarded, cells washed with phosphate buffered saline (PBS), and fresh medium with 5 μM DHE was added and cells were incubated in the dark for 30 mins. Afterwards the cells were washed with PBS and mounted on slides for taking photograph by using an Eclipse 55i fluorescence microscope (Nikon GmbH, Düsseldorf, Germany), with a Fluoro Pro MP 5000 camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany) respectively. The pictures were then analysed using ImageJ free software (http://rsbweb.nih.gov/ij). Two hundred cells from each sample were analysed by using ImageJ software. DHE fluorescent intensity was expressed as value relative to the negative control group (sterile water).

Wells were treated with MIMO2 at concentrations of 1μM, 0.5 μM and 0.25 μM/well, MO was at a concentration of 0.063 mg/well and vanadium at 100 μM.

3.2.3 *Comet Assay (CA)*

Assay was carried out according to the method described previously by Fazeli *et al*. (2012). MIMO2, MO, and vanadium were used to treat HT22 cells at varying doses for 4 hours. Cells were harvested and CA was performed. Briefly, the cells were embedded in 0.5% low-melting-point agarose and loaded on fully frosted microscope slides, coated with a thin layer of high-melting-point agarose. For lysis, slides were immersed in lysing solution (1% Triton X-100, 10% dimethyl sulfoxide, and 89% lysis buffer containing 10 mM Tris, pH 10; 1% Na-sarcosine; 2.5 M NaCl; and 100 mM Na_2EDTA) and incubated at 4°C in the dark for 1 h. DNA unwinding and alkali-labile damage expression was allowed in alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 20 min at 4°C in the dark, followed by electrophoresis at 4°C, for 20 min in a 1 V/cm and 300 mA electrical field. Afterwards, the slides were neutralised for 5 min in 0.4 M Tris (pH 7.5), dehydrated and fixed in ice cold 100 % methanol for 10 min and stained using GelRed™ Nucleic Acid Gel Stain (10,000X in H_2O, Biotium,

Hayward, CA, USA). A fluorescence microscope at 200-fold magnification and a computer-aided image analysis system (Komet 5; Kinetic Imaging, Bromborough, UK) were employed for analysis. DNA damage was quantified using the percentage of DNA in the tail.

3.2.4 *Statistics*

For all experiments, data from at least three experiments were evaluated, and expressed as mean ± standard deviation. Statistical significance between the individual groups was tested using the ANOVA, Bonferroni post test, GraphPad Prism®, Version 5 (GraphPad Software Inc., La Jolla, CA USA). A p value of ≤ 0.05 was considered significant.

EXPERIMENT 3

ANIMAL EXPERIMENTS

In all animal experiments, body weight was recorded daily.

3.3.1 LD_{50} *of MIMO2*

Ethical approval was obtained from the Animal Ethical Committee of the University of Ibadan, ethical code number UI-ACUREC/App/2016/028.

Four-week old male albino mice were used to ascertain the LD_{50} of MIMO2 based on a modification of Lorke's method (1983). Sixteen 4-week old male mice were assigned randomly into 4 groups and administered 100 mg/kg, 75 mg/kg, 50 mg/kg and 25 mg/kg of MIMO2 on a daily dose for eight days. MIMO2 was diluted with sterile dimethyl sulphoxide (DMSO) and was administered intraperitoneally (i/p) in all cases. Mortality was recorded.

Results obtained were used to calculate the LD_{50} based on Lorke's formula:

$$LD50 = \sqrt{(D0 \; x \; D100)}$$

Where D0 = highest dose that gave no mortality (25 mg/kg), and D100 = lowest dose that produced mortality (50 mg/kg).

$$LD50 = \sqrt{(25 \; x \; 50)} = 35.35 \text{ mg} \approx 35 \text{ mg/kg}$$

3.3.2 *Arriving at the Optimal MIMO2 Dose used in Combination with Vanadium 3 mg/kg*

A pilot study was initially conducted to assess the effect of MIMO2 and vanadium on mortality and morbidity. Sixteen 4-week old male mice were randomly divided into 4 groups of 4 mice each, receiving the dose of 35 mg/kg, 25 mg/kg, 15 mg/kg and 10 mg/kg. Combining vanadium 3mg/kg with MIMO2 35 mg/kg for 5 days resulted in mortality of the mice. Vanadium and MIMO2 were administered i/p, for 5 consecutive days. Vanadium was dissolved in sterile water.

3.3.3 *Effect of MIMO2 on Vanadium Induced Neurotoxicity in Developing Mice*

Eighty-four 2-week old mice were randomly divided into seven groups of 12 mice each.

Group I – Negative control (sterile water – H_2O)

Group II – Positive control (DMSO)

Group III – Vanadium 3 mg/kg (V)

Group IV – MIMO2 10 mg/kg (M10)

Group V - MIMO2 10 mg/kg + V 3 mg/kg (M10+V)

Group VI – MIMO2 5 mg/kg (M5)

Group VII - MIMO2 5 mg/kg + V 3 mg/kg (M5+V)

Treatment was for 14 days. All injections were administered i/p. vanadium was administered daily, while MIMO2 was administered every 72 hours (D0, D2, D5, D8, D11, D14). Mice were subjected to neurobehavioural tests on D14, and sacrificed on D15.

3.3.3.1 *Neurobehavioural Tests*

Two neurobehavioural tests were carried out to test for muscular strength and coordination (hanging wire) and to test for anxiety (open field test). At least 12 mice were sampled for each group. The protocol for the neurobehavioural tests was adapted from Mustapha *et al*. (2014) and Azeez *et al*. (2016).

Hanging wire: Mice were suspended by their forelimbs from a wire, at a height of about 60 cm from the ground. The time it took for each mouse to fall off the wire was recorded in seconds. A trial of 3 times was conducted for each mouse and the average time calculated.

Open field test: A white open wooden box, measuring 80 cm by 80 cm, with 16 smaller squares (demarcated with black paint) measuring 20 cm by 20 cm, and a centre square measuring 20 cm by 20 cm. The mouse was placed in the centre square and released. The duration of the

stay in the box was 5 minutes per animal. The number of line crossings, rearing, grooming, number of faecal boluses voided and urine spots were noted. The average values for these parameters were calculated per group. ANY-maze® software, version 4.99m, was used to analyse the open field test.

3.3.3.2 *Body and Relative Brain Weight*

Body weight was recorded daily for all the groups using a digital weighing scale. The brains were weighed immediately and thereafter removed in non-perfused animals to avoid errors due to perfusion. Relative brain weight was calculated in each animal as $\left(\frac{Brain\ Weight}{Body\ weight}\right) x\ 100$, and expressed in percentage.

3.3.3.3 *Sacrifice of Mice*

Five mice per group were sacrificed on D15. All mice were anaesthetized with ketamine (100 mg/kg, i.p.). Mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were post-fixed in PFA for 2 hours and stored in 0.1% Na azide in 0.1 M phosphate buffered saline (PBS) at 4^{o}C until ready for sectioning. Brains were then put in 30% sucrose in PBS for cryoprotection for 24 hours. Serial frozen sections were cut through the brain at a 30 μm thickness with a freezing microtome, collected in adjacent wells of 24-well plates and kept at 4^{o}C until analysed for immunohistochemistry.

3.3.3.4 *Histopathology*

Haematoxylin and Eosin (H and E) stain: Histopathologies in brain regions (cerebrum, cerebellum and the hippocampus) were assessed.

Cresyl violet stain: This stain was used to count hippocampal neuronal cells in the CA1 and CA3 regions. The neuronal count of total percentage of dead cells was calculated as $\left(\frac{Number\ of\ dead\ cells}{Total\ number\ of\ cells}\right) x100.$

Black Gold II histochemistry: one series of sections from each brain was processed free-floating for myelin staining using the Black Gold II histochemical protocol according to a modification of the method previously described by Schmued *et al.* (2008). Briefly, the sections were washed in distilled water for 2 minutes before adding a 0.3% warm Black-Gold (haloaurophosphate complex) (HistoChem, Jefferson, AR) solution, obtained by adding 100mg of Black- Gold to 50mL of 0.9% NaCl and then heating to 60ºC. The sections were then incubated in an oven for 18 minutes at 60ºC. The staining was monitored visually for 12-18 minutes until full impregnation. The sections were mounted on gelatin coated slides, air-dried for 24 hours, and then dehydrated through graded alcohols, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany) as mounting medium.

Black Gold II counterstained with cresyl violet stain was also employed to assess the brain histology.

Immunohistochemistry: Series of adjacent sections were processed for immunoperoxidase to investigate microglia and astrocyte activation, and the eventual occurrence of damage of myelinated axons, assessed by the presence of nonphosphorylated neurofilaments, a sensitive marker of axonal pathology. Sections were first treated in 0.1% H_2O_2 (Mallinckrodt Baker, Deventer, The Netherlands) for 15 minutes to inactivate endogenous peroxidase and then incubated for 1 hour in 5% normal goat or horse serum (Vector Labs, Burlingame, CA) in PBS. The sections were then incubated overnight at 4ºC in PBS containing 1% normal goat or horse serum and the following primary antibodies: rat anti-CD11b (Serotec, Oxford, United Kingdom; diluted 1:500) for microglial cells, rabbit anti-glial fibrillary acidic protein (GFAP) (1: 500; Merck), to visualize astrocytes; mouse monoclonal anti-SMI-32 (1:100; Covance, Emeryville, CA), which recognizes nonphosphorylated neurofilaments; mouse monoclonal anti-myelin basic

protein (MBP), 1:500, Boehringer Mannheim, Germany) for visualising myelination; rabbit monoclonal anti-Iba-1 (1:1,500; Wako) for visualising microglia. After washing, the sections were incubated for 2 hours at room temperature in the appropriate biotinylated secondary antibodies (diluted 1:200; all purchased from Vector Labs). The sections were then reacted in avidin biotin- peroxidase solution (ABC kit, Vectastain, Vector Labs) using 3,3'-diaminobenzidine as chromogen, and then dehydrated in graded alcohols, cleared in xylene, mounted on gelatinized slides, and coverslipped with Entellan.

The brain sections were also processed for triple immunofluorescence to reveal simultaneously astrocytes, microglia and IL-1β expression. These sections were first rinsed in PBS, incubated for 1 hour at room temperature with a blocking solution containing normal donkey serum, and then incubated overnight at room temperature, on a rocker with primary antibodies: anti-GFAP (1:500), CD11b (1:500) and IL-1β antibody (1:200; Santa Cruz Biotechnology). Anti-goat IgG Alexa 548 and anti-rabbit IgG Cy™ 2-conjugated affiniPure (Jackson Immuno Research Laboratories) were used as secondary antibodies. The sections were then counterstained with the fluorescent nuclear marker 4'-6'-diamidino-2-phenylindole (DAPI), mounted on slides with 0.1% paraphenylendiamine in a glycerol-based medium (90% glycerol and 10% PBS) and coverslipped.

Densitometric analysis was employed to analyse the Black Gold intensity of the commissural region of the corpus callosum, using the Olympus microscope Image Pro Plus® software. Microglia count was conducted with the Neurolucida Stereological count, while the microglia percentage area covered was with the Image Pro Plus® software.

3.3.4 *Statistics*

All data where applicable were expressed as mean ± standard deviation. Statistical significance between the individual groups was tested using ANOVA, post test Bonferroni, GraphPad Prism®, Version 5 (GraphPad Software Inc., La Jolla, CA USA). A p value of ≤ 0.05 was considered significant.

CHAPTER 4

RESULTS

EXPERIMENT 1

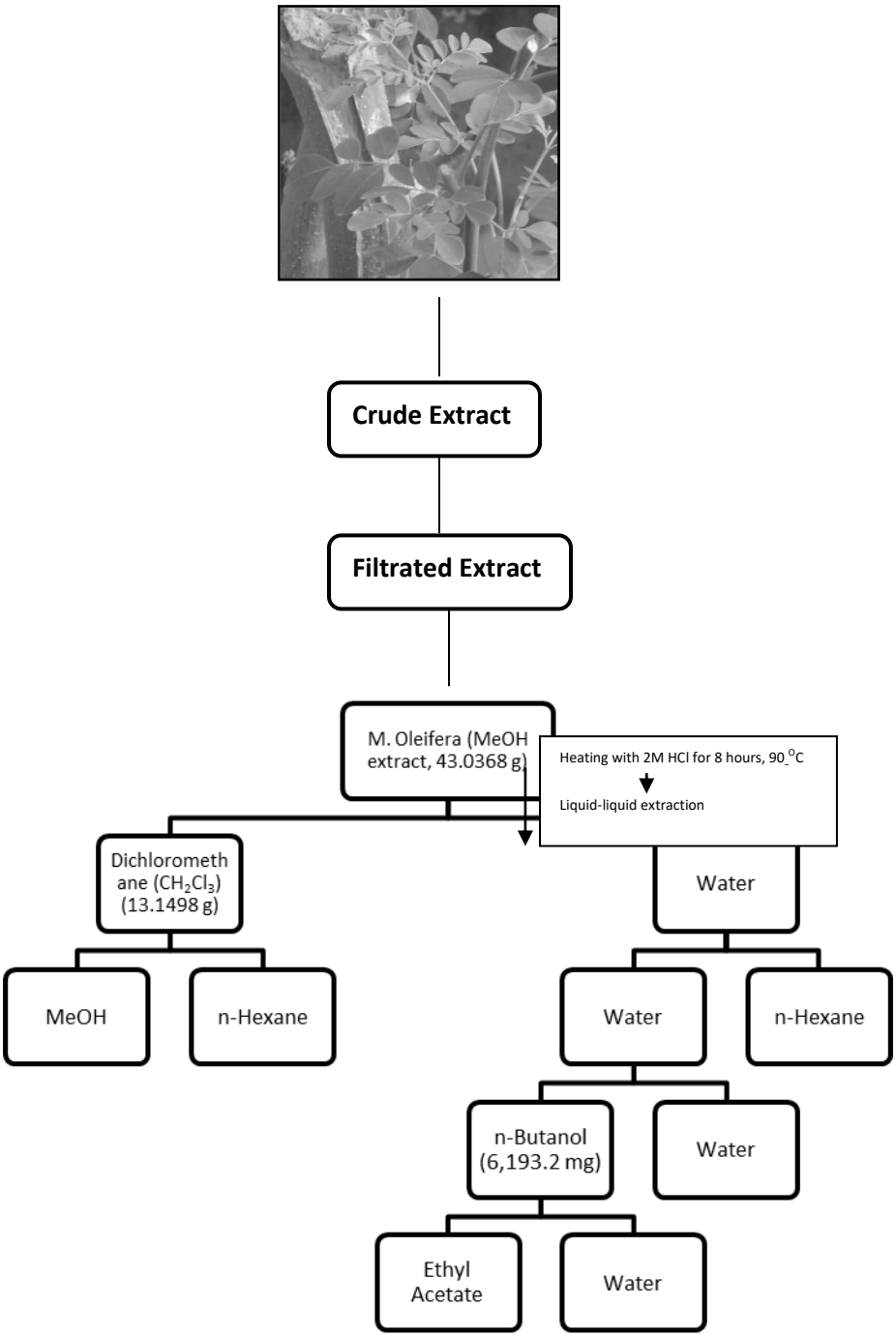


Figure 1: Liquid-liquid fractionation chart of the methanolic extract of *M. oleifera* leaves

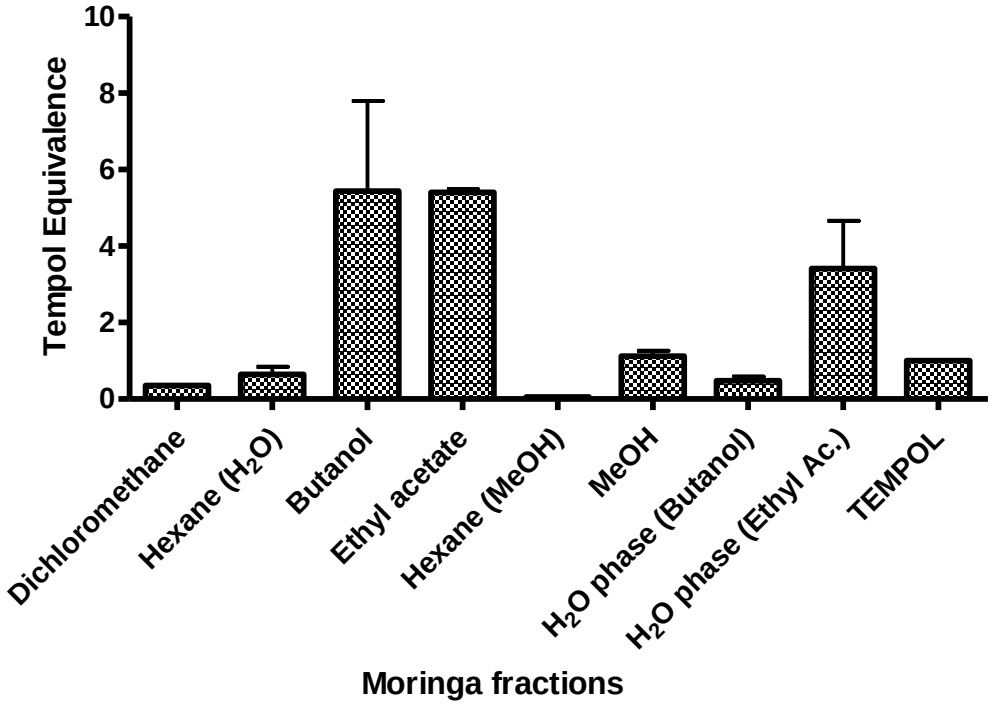


Figure 2: µM TEMPOL Equivalence/ mg extract relative to the fractions obtained after liquid-liquid fractionation. The fractions that gave the best results were butanol, ethyl acetate and water phase (ethyl acetate), the latter two fractions being from the butanol fraction.

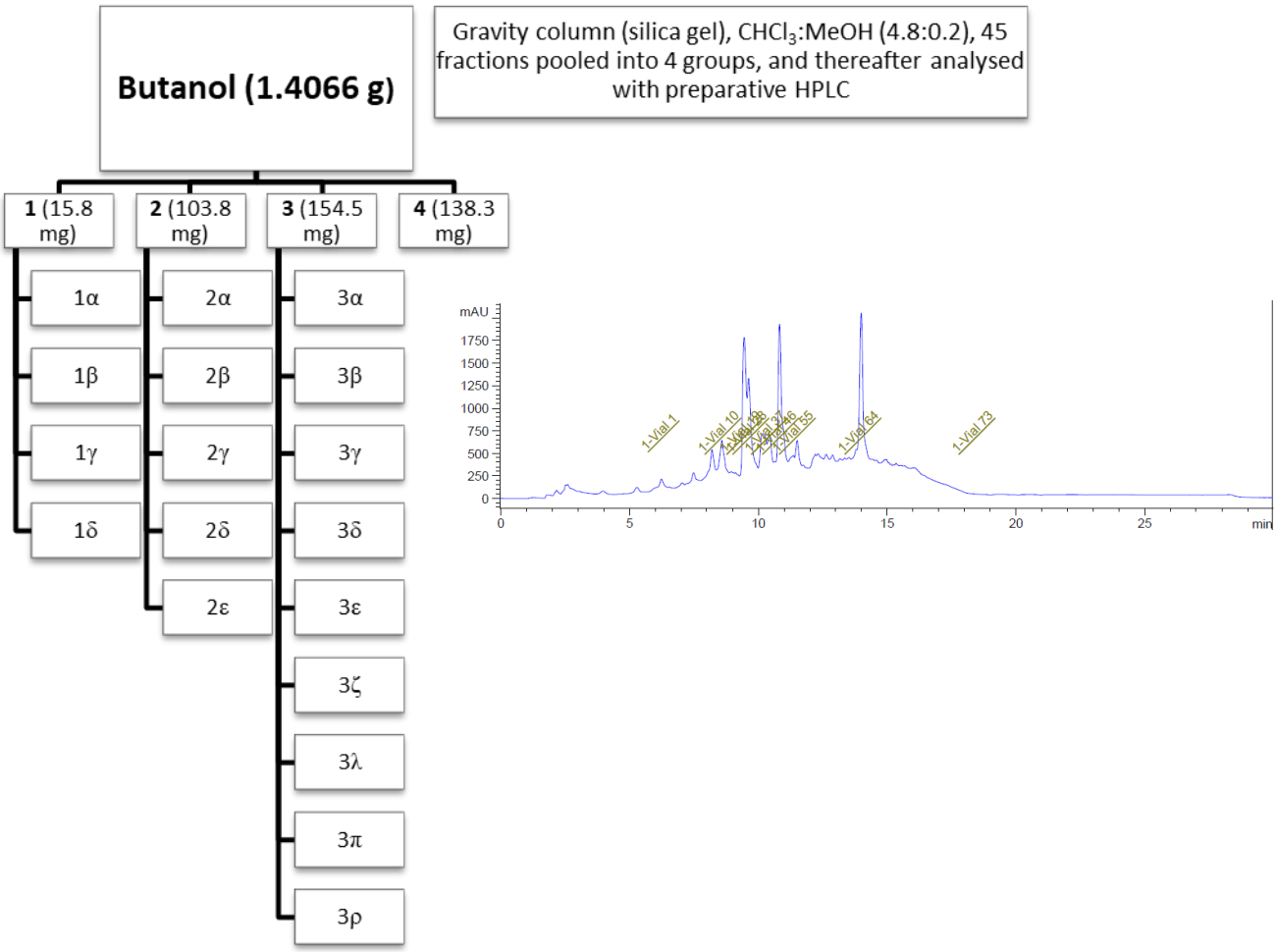


Figure 3: Butanol phase fractionation. 1 = F4; 2 = F5-9; 3 = F10-14; 4 = F15-45 (pooling of fractions based on TLC results, eluted with chloroform:MeOH – 4.8:0.2). 2δ, 2ε and 3γ show identified compounds (MIMO2 - p-Hydroxyphenyl acetic acid butyl ester). 2α, β and γ show high activity relative to other fractions when compared with ascorbic acid, while the others showed low activity, when stained with Phosphomolybdic acid. Graph inset shows the HPLC activity of fraction 2 (2δ, 2ε).

Based on the structure, the isolated compound was named p-Hydroxyphenyl acetic acid butyl ester, with a molecular weight of 208.3g/mol (Figure 5).

NMR data: 6.74 (d; 8.6; 1H), 7.12 (d; 8.6; 1H), 5.33 (si; OH), 6.74 (d; 8.6; 1H), 7.12 (d; 8.6; 1H), 3.53 (si; 2H), 4.1 (ti; 6.7; 2H), 1.57-1.64 (m; 2H), 1.3-1.4 (m; 2H), 0.91 (t; 7.4; 3H).

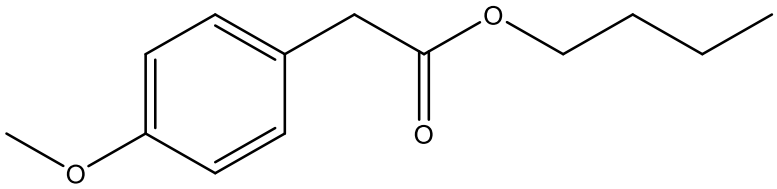


Figure 4: *p*-Methoxyphenyl acetic acid butyl ester (MIMO1)

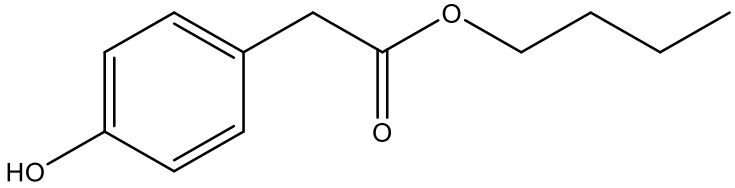


Figure 5: *p*-Hydroxyphenyl acetic acid butyl ester (MIMO2). Molecular Weight: 208.3g/mol.

MIMO2 has a Good Antioxidative Potential using FRAP

The antioxidative potential of MIMO2 relative to TEMPOL, the methanolic extract of *M. oleifera* leaves (MO), butanol fraction (BF) and MIMO1 was assessed using FRAP, as previously described (Figure 6). All values were expressed relative to TEMPOL (µM TEMPOL Equivalence/ mg extract). MIMO1 gave a value of 0.3, BF gave 1.24, MO 4.6, and MIMO2 gave 8.35.

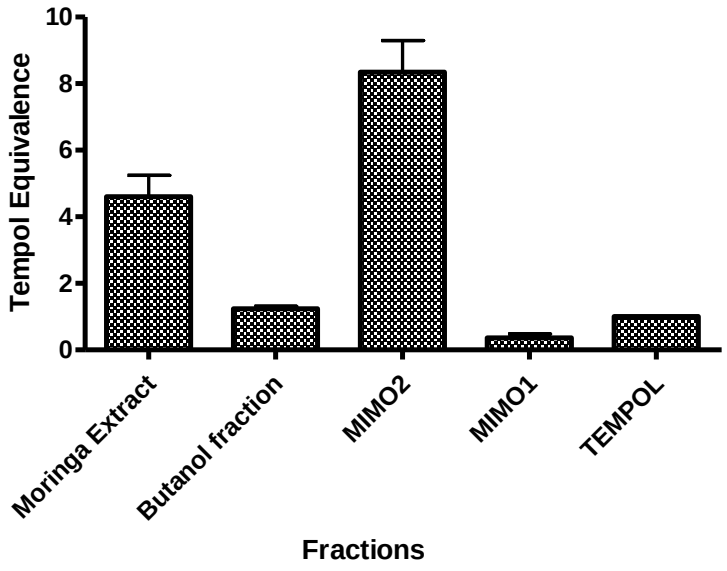


Figure 6: µM TEMPOL Equivalence/mg extract relative to Moringa extract, butanol fraction, MIMO1 and MIMO2**.**

EXPERIMENT 2

CELL CULTURE

4.2.1 *MIMO2 and MO Resulted in Reduced Cellular ROS (Dihydroethidium) caused by Vanadium*

Vanadium treated cells showed increased release of cellular ROS relative to the cells treated with 0.063 mg MO, and MIMO2 1 μM, 0.5 μM and 0.25 μM (Figure 7). Although the combination of MIMO2 and MO at the different concentrations with vanadium gave a remarkable reduction when compared with vanadium alone, no statistically significant difference ($p>0.05$) was observed using Mann Whitney U test (Figures 7 and 8).

4.2.2 *MIMO2 and MO Resulted in an Amelioration of DNA Damage Caused by Vanadium (Comet Assay)*

A significant reduction of DNA-damage was observed after incubation of HT22 cells with MO 0.01 mg/ml and vanadium 200 μM compared to vanadium 200 μM alone (Figure 9). Similar to that, a significant reduction of DNA-damage was observed after incubation of the cells with MIMO2 0.25 μM and vanadium 200 μM compared to vanadium 200 μM. A statistically significant reduction ($p<0.05$) was observed in all groups combined with vanadium 200 μM relative to vanadium 200 μM alone, but the most significant reduction was observed in combination with MIMO2 0.25μM (Figures 9 and 10).

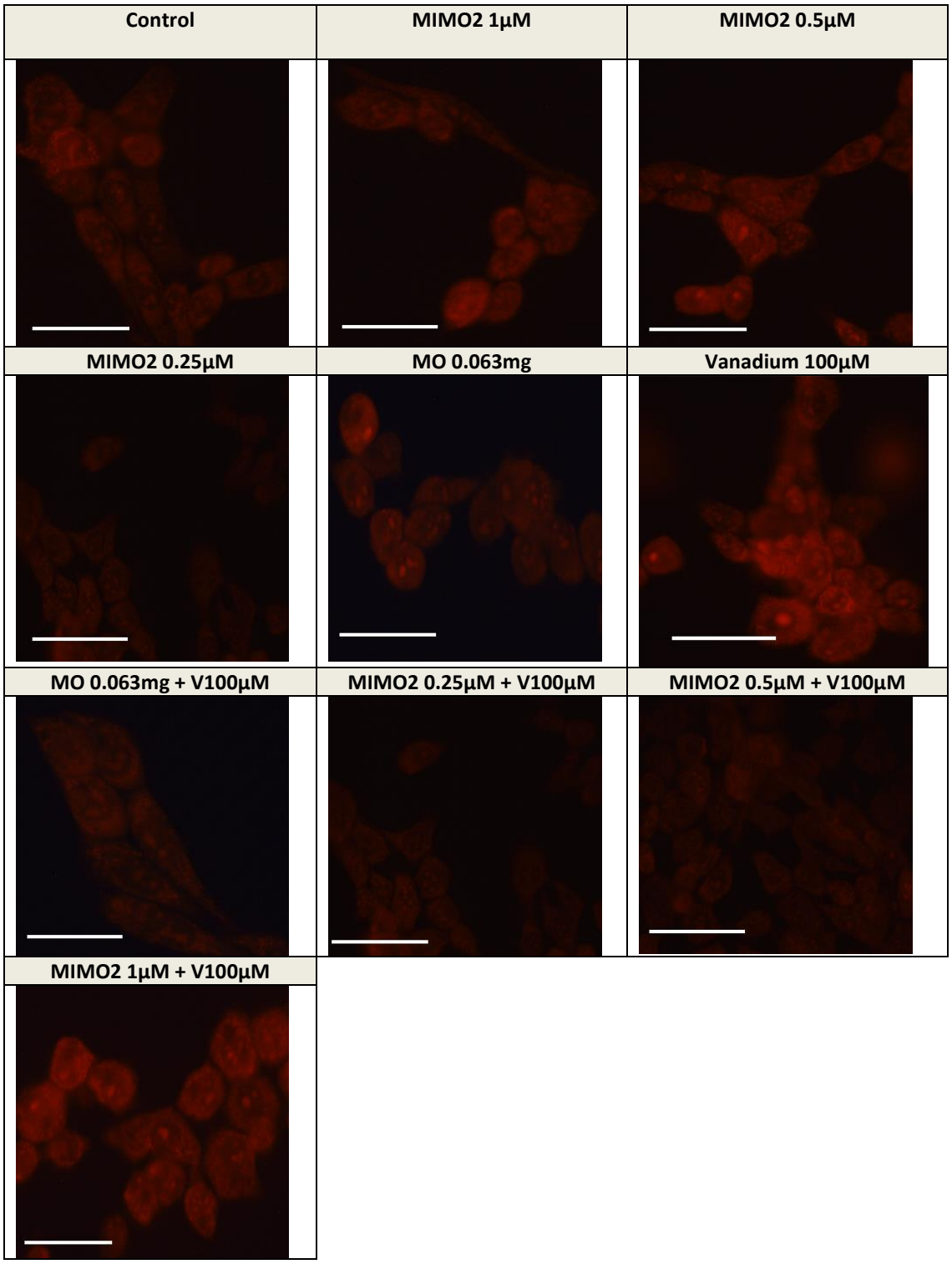


Figure 7: Superoxide level (detected by DHE staining) in HT22 cells following treatment for 30 min with vanadium 100 µM, MIMO2 1 µM, 0.5 µM, 0.25 µM, MO 0.063 mg and a combination of vanadium 100 µM with the different concentrations of additives. Scale bar - 50 µm

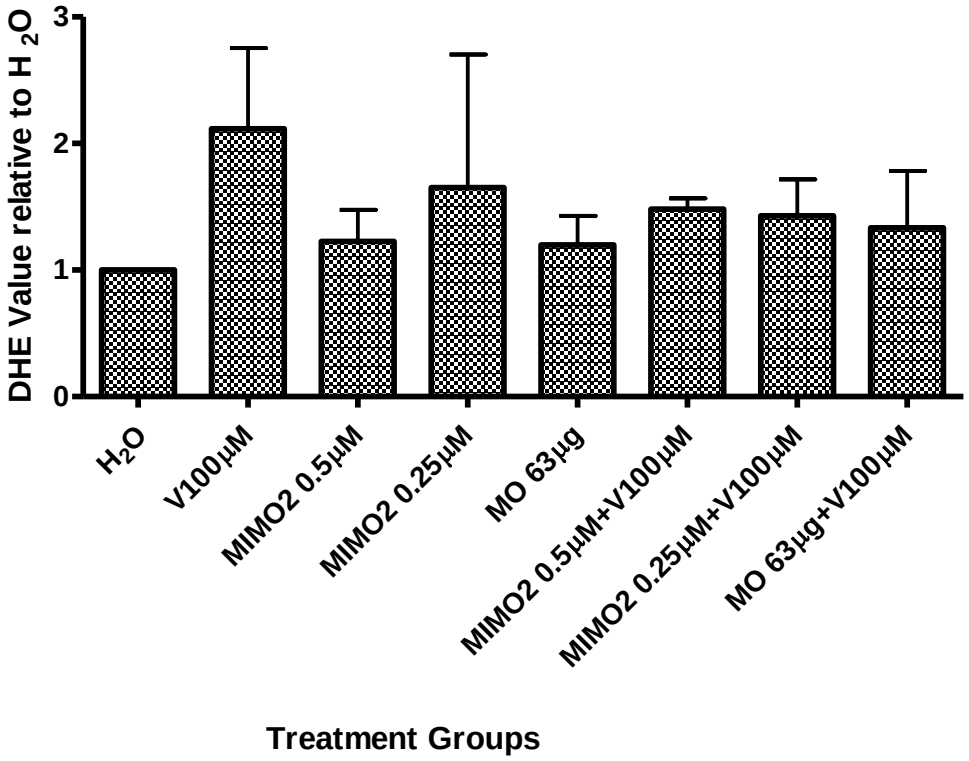


Figure 8: The effect of MIMO2 on vanadium induced superoxide production (detected by DHE staining) in HT22 cells, expressed as the 'fold increase over H_2O. MO 0.063 mg, MIMO2 0.25 μM and 0.5 μM in combination with vanadium showed a reduction in superoxide level. * shows groups that were statistically significantly different from V100 μM ($p<0.05$) using Mann Whitney test, n= 3. Inset: representative of HT22 cells.

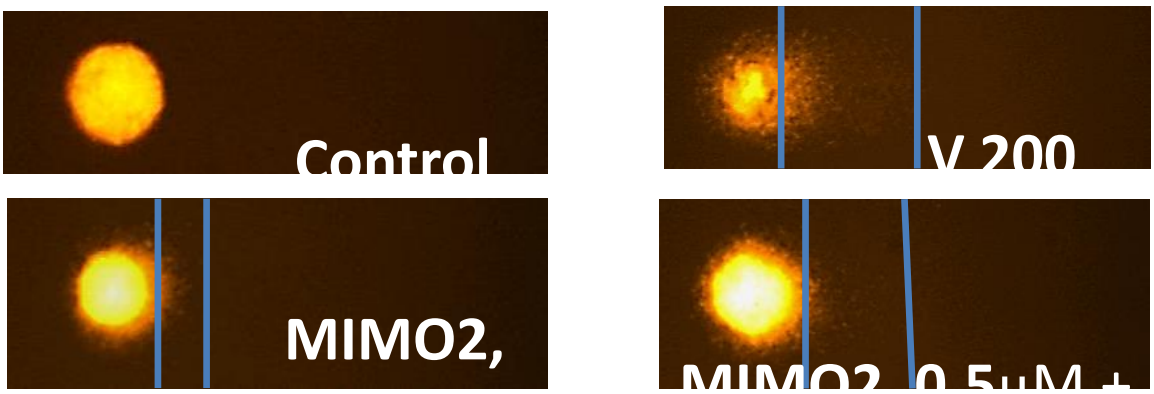


Figure 9: Representative diagrams of Comet assay, showing cell damage induced by vanadium and subsequent protection by MIMO2 in HT22 cells. The blue lines show the extent of DNA in tail. Note the extent of cell damage in V200 μM relative to the other groups, and the ameliorative effect of MIMO2 on the vanadium-induced damage.

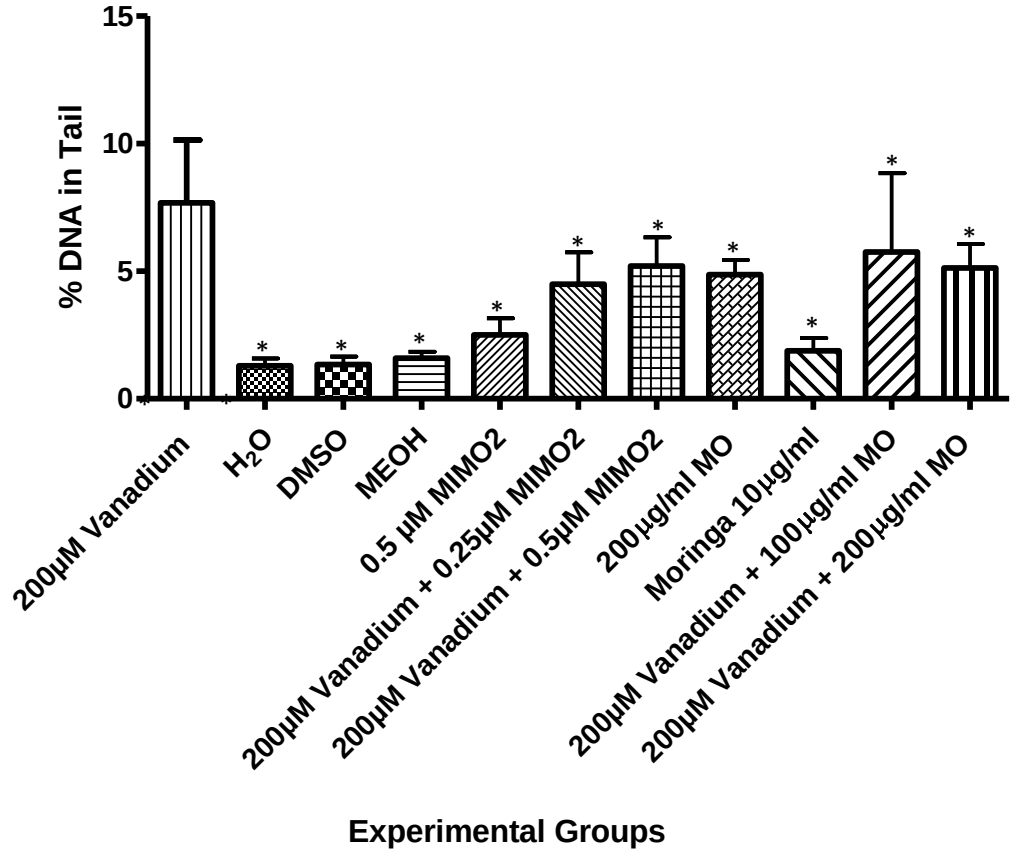


Figure 10: The effect of MIMO2 and MO on vanadium toxicity on DNA using HT22 cells. Graph shows the mean dose response for tail DNA. MIMO2 (0.25 µM and 0.5 µM) and MO (0.01 mg/ml and 0.02mg/ml) gave protection against vanadium neurotoxicity (n = 3 to 4). * indicates groups which show statistically significant differences ($p<0.05$) relative to vanadium 100 µM alone.

EXPERIMENT 3

ANIMAL EXPERIMENTS

4.3.1 *LD_{50} of MIMO2*

Mortalities recorded and days were:

MIMO2 100mg - D2 ($^1/_4$), D3 ($^2/_4$), D5 ($^3/_4$).

MIMO2 75 mg/kg – D1 ($^2/_4$), D3 ($^4/_4$).

MIMO2 50 mg/kg – D3 ($^1/_4$).

MIMO2 25 mg/kg – No mortality recorded (Figure 11)

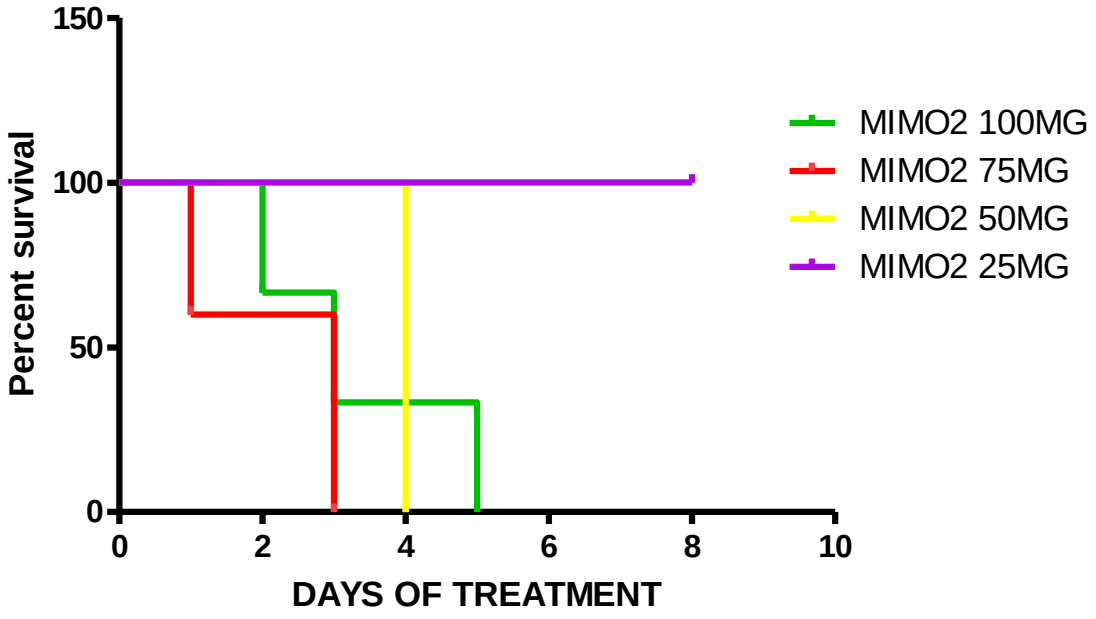


Figure 11: LD_{50} of MIMO2. Kaplan-Meier survival graph, an 8-day experiment showing the mortalities and the days recorded in 4- week old mice. MIMO2 25 mg/kg recorded 100% survival.

4.3.2 *MIMO2 at Different Concentrations used in Combination with Vanadium 3 mg/kg*

Mortality recorded on the different days for the various concentrations were:

MIMO2 35 mg/kg + vanadium 3 mg/kg – D2 ($^{1}/_{5}$), D4 ($^{3}/_{5}$), D5 ($^{5}/_{5}$)

MIMO2 25 mg/kg + vanadium 3 mg/kg – D6 ($^{3}/_{4}$)

MIMO2 15 mg/kg + vanadium 3 mg/kg – No mortality recorded

MIMO2 10 mg/kg + vanadium 3 mg/kg – No mortality recorded (Figure 12)

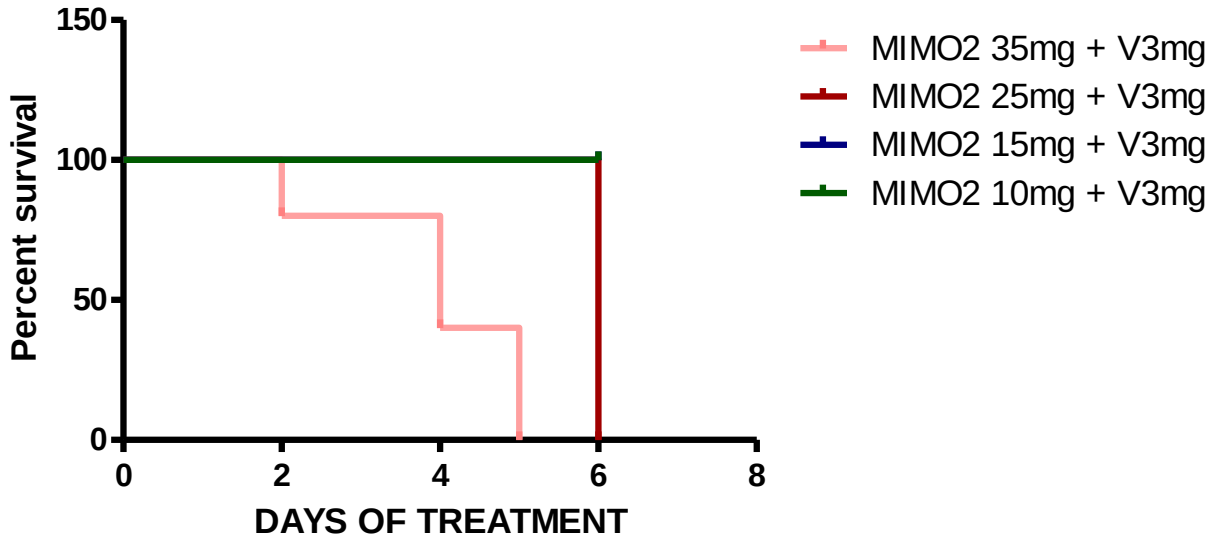


Figure 12: MIMO2 + Vanadium 3 mg/kg. Kaplan-Meier survival graph, a 5-day experiment showing the mortalities and the days recorded in 4-week old mice. MIMO2 15 mg/kg + V 3 mg/kg and MIMO2 10 mg/kg + V 3 mg/kg recorded 100% survival.

4.3.3 *Effect of MIMO2 on Vanadium Induced Neurotoxicity in Developing Mice*

4.3.3.1 *Effect on Body and Relative Brain Weights*

MIMO2 was not administered daily. Since it was not water soluble but fat soluble, it would likely have the tendency to accumulate in body fat, and therefore retained in the body. Weight increase was achieved by comparing increase in body weight relative to D14 (Dx – D1). Comparing the increase in body weight of the different groups on D14, statistical analysis using t-test only showed a statistically significant difference ($p < 0.05$) between the water control group and vanadium 3 mg/kg group, with the vanadium group showing a reduction in body weight relative to control (Figure 13). Relative brain weight was calculated as $\left(\frac{Brain\ weight}{Body\ weight}\right) x\ 100$. The average value was determined for each group.

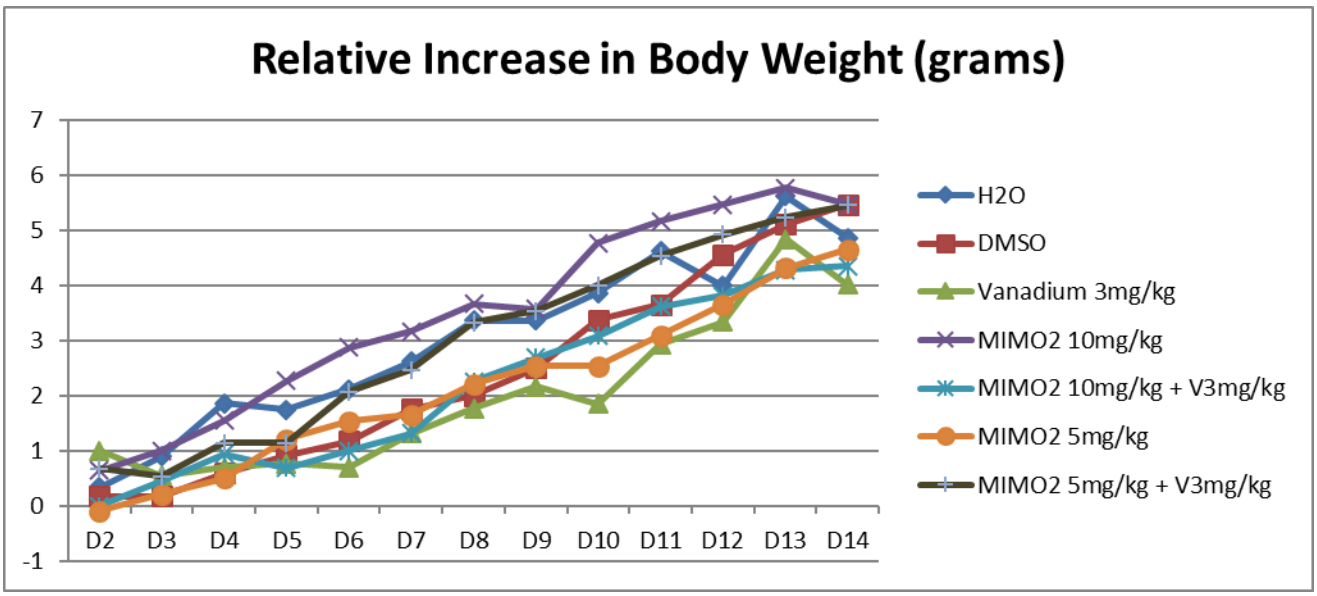


Figure 13: Line graph showing the relative increase in body weight in grams (D1 – D14) of the different groups.

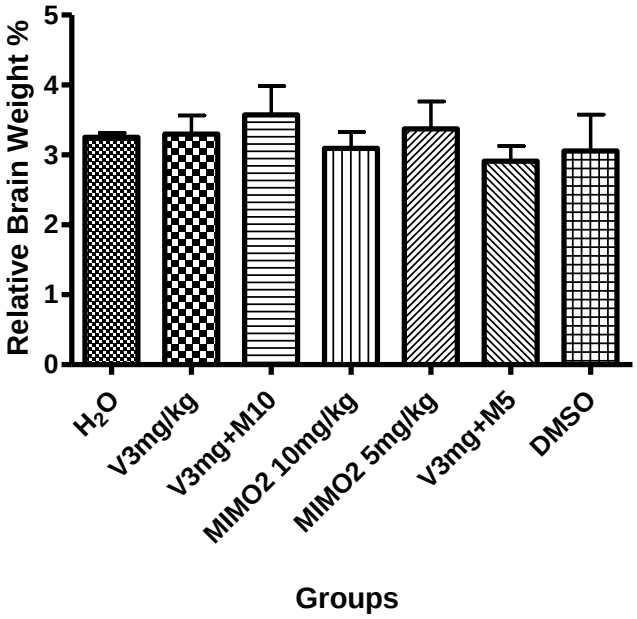


Figure 13b: Mean relative brain weights of the groups, expressed as percentage. No statistically significant difference ($p > 0.05$) was observed among the groups when compared with the control group (H_2O) and V 3mg/kg group.

4.3.3.2 *Neurobehavioural Tests*

Hanging Wire: Statistically significant difference ($p<0.05$) was observed between water and the following groups – V, M10, M5, M5+V, with the water control group having the highest value, followed closely by M10+V group. The V 3 mg/kg group had the lowest value (Figure 14).

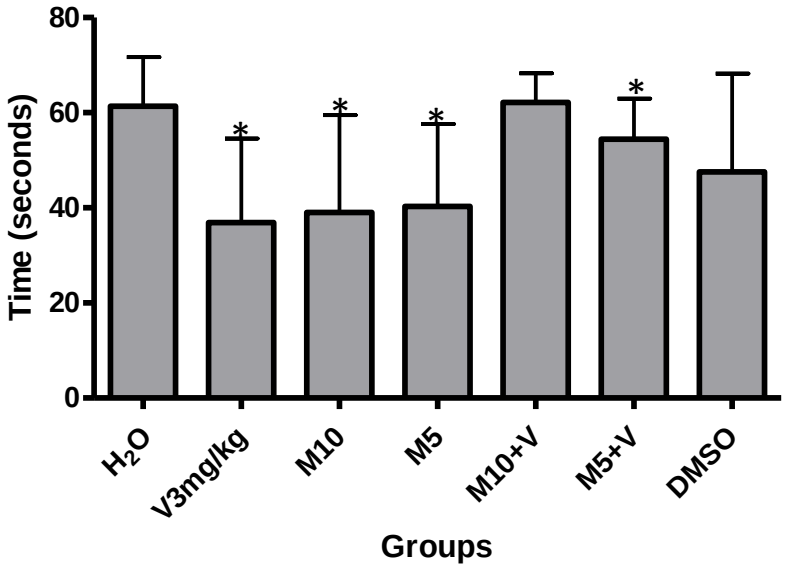


Figure 14: Neurobehavioural Test – Hanging wire. Highest values recorded in water group and M10 + V 3 mg/kg. * indicates statistically significant difference ($p < 0.05$) relative to the control group (water).

Open Field Test: M10 group appeared to be the most active group, having the highest values for rearing and line crossings (Figures15a and b). The vanadium group showed the lowest values for these tests.

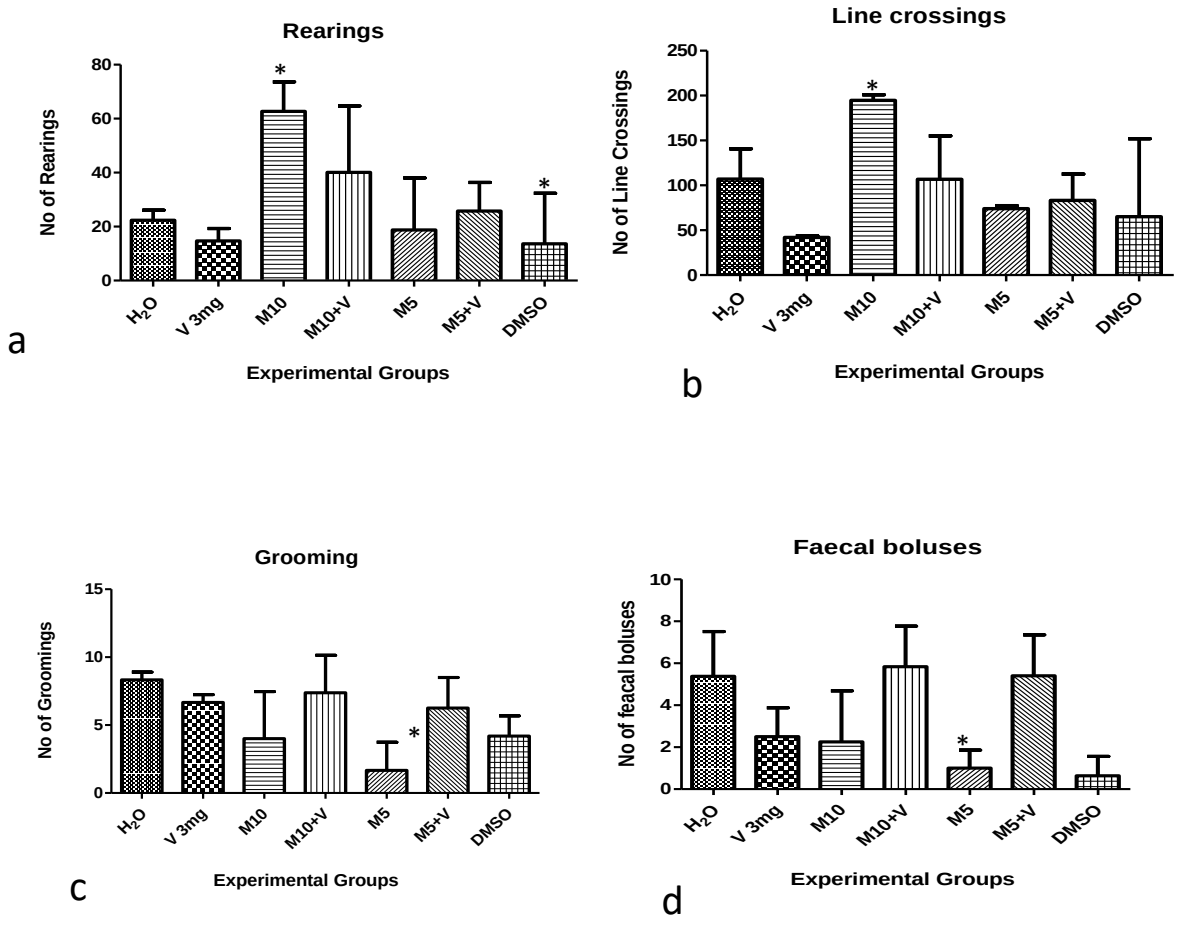


Figure 15: Open Field Test. * indicates statistically significant difference relative to H_2O group

4.3.3.3 *Histopathology*

Haematoxylin and Eosin

Cerebrum: pathologies were observed in the prefrontal cortex of the V and M10+V groups. The neurons appeared pyknotic. This was more pronounced in the V group (Figure 16).

Cerebellum: The V group showed degenerated and even loss of Purkinje cells in some regions. Also, the granular layer showed a more intense or increased granulation. The MIMO2 and combination group (M10+V) also showed the same pathologies, although not as intense, implying an amelioration of the effects of vanadium. In the vanadium group, 4 mice out of 5 showed stratification of the Purkinje cell layer in several areas of different folia, while in M10+V, only one mouse showed the stratification in a focal area (Figure 17).

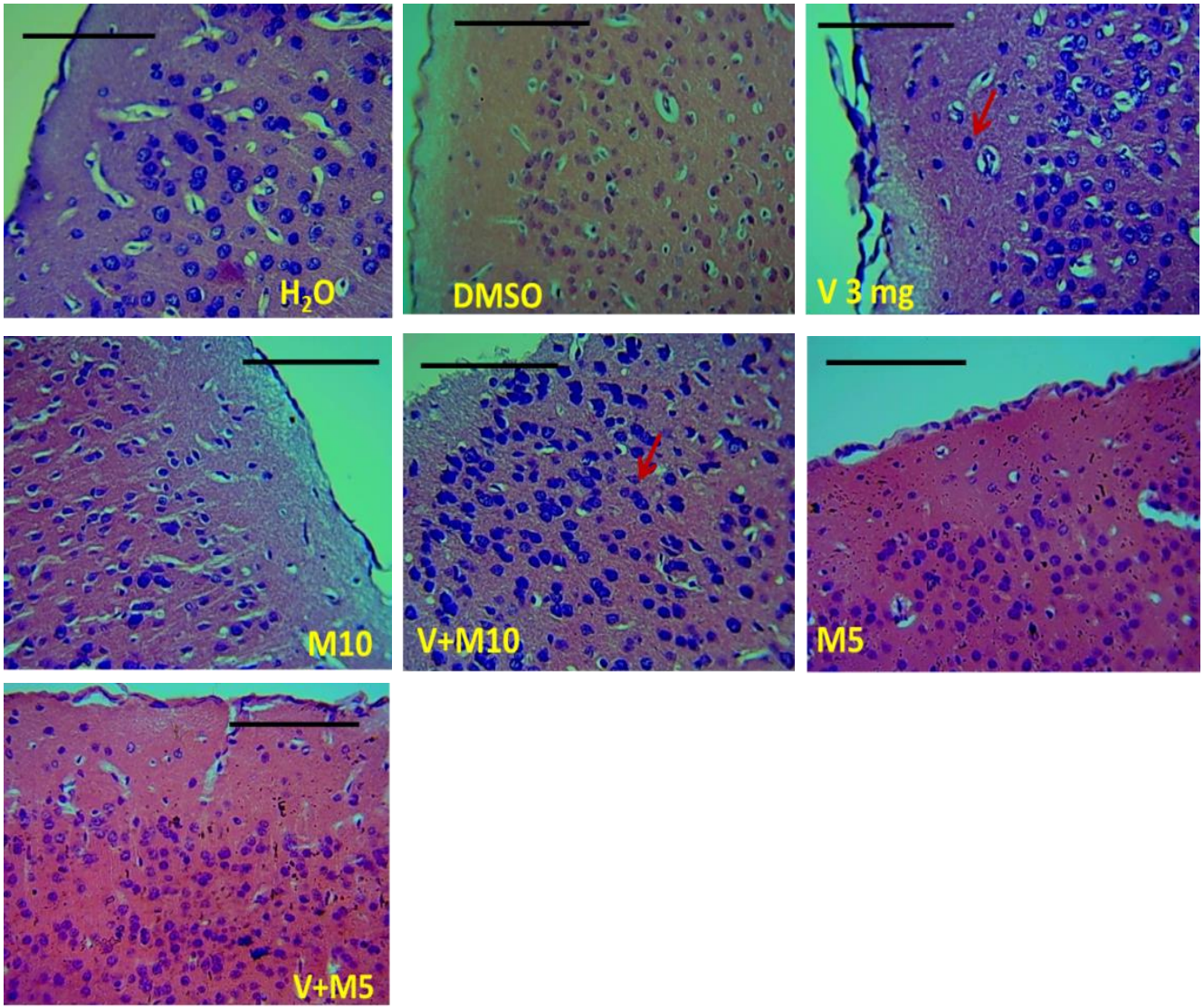


Figure 16: Micrographs of the cerebrum of treated mice. Note the slightly degenerated neurons (red arrows) in the V 3 mg/kg and M10 + V 3mg/kg group. H&E, scale bar = 50 μm.

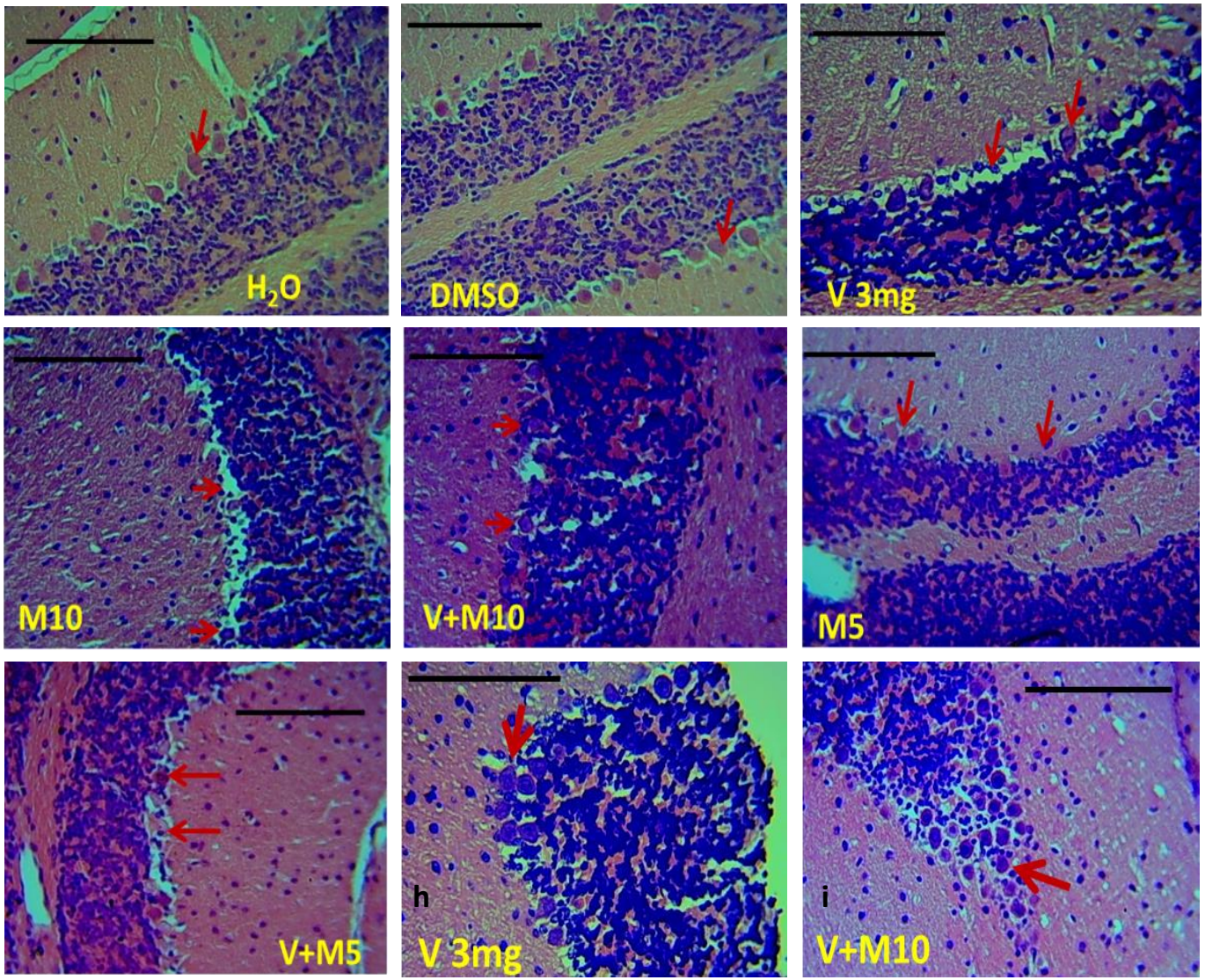


Figure 17: Micrographs of the cerebellum of treated mice. Note the differing appearances of the Purkinje cells (red arrows) in all the groups. Note Purkinje layer stratification in (h) and (i). H&E, scale bar = 50 μm.

4.3.3.4 *Cresyl Violet Stain*

CA1 region: Hippocampal neuronal cells in V 3mg/kg group had a vacuolated appearance. This was ameliorated with the administration of MIMO2 10mg/kg. M10 and M5 groups showed few vacuolated cells (Figure 18). Neuronal cell count of apparently normal cells in all the groups showed all groups to be statistically significantly different ($p < 0.05$) from the water group. M10 gave a slightly higher value, although no statistically significant difference was observed ($p > 0.05$). A neuronal count of total percentage of dead cells showed that the vanadium group had the highest number of dead cells, and was statistically significant ($p < 0.05$) to all the other groups (Figures 19 a, b).

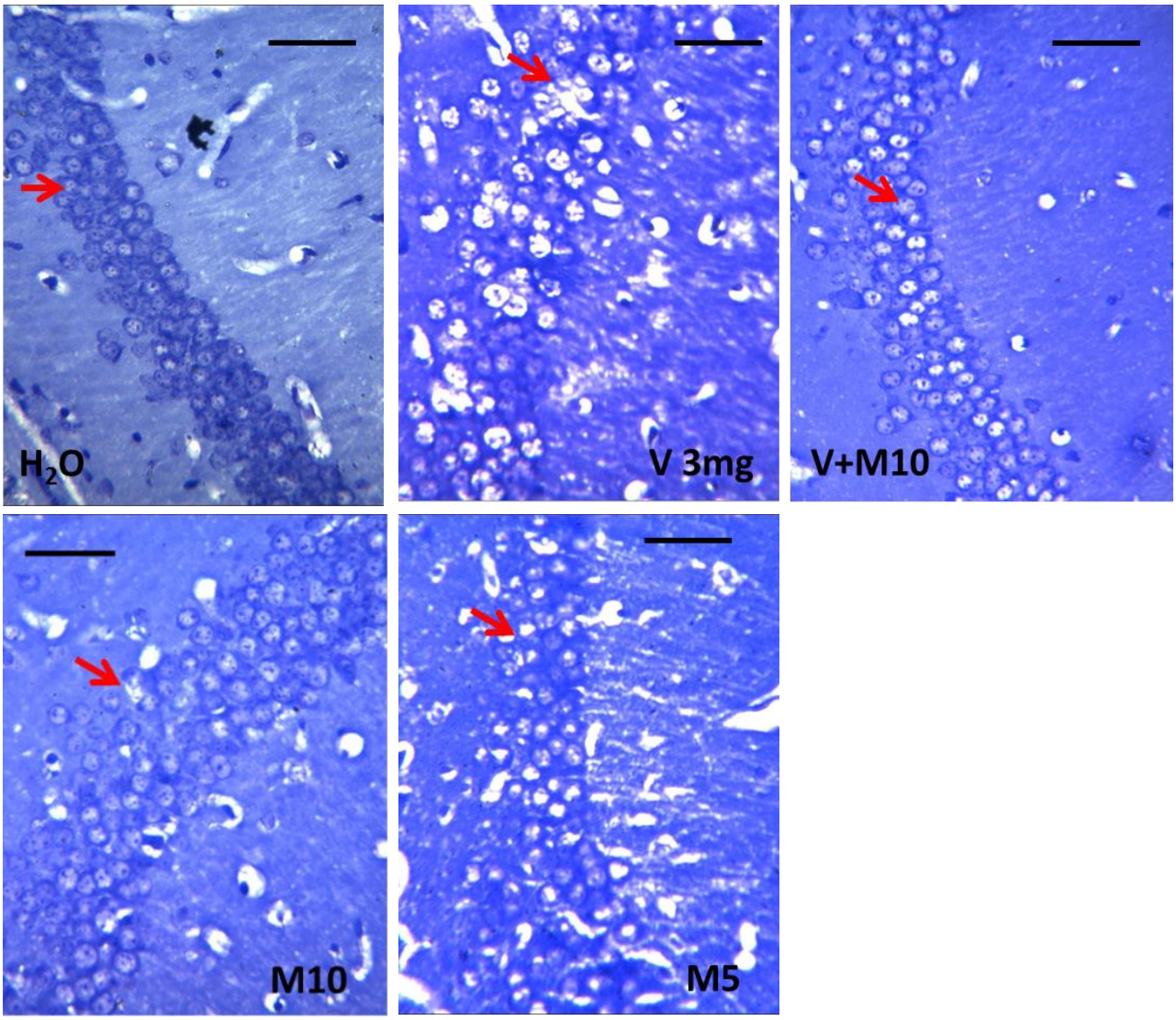


Figure 18: Micrographs of the hippocampus of treated mice, CA1 region, cresyl violet stain. Arrows indicate neuronal cells. Note the vacuolated appearance of the neuronal cells in V 3 mg/kg group. The V 3 mg/kg group had numerous vacuolated cells relative to the other groups. Scale bar = 50 μm.

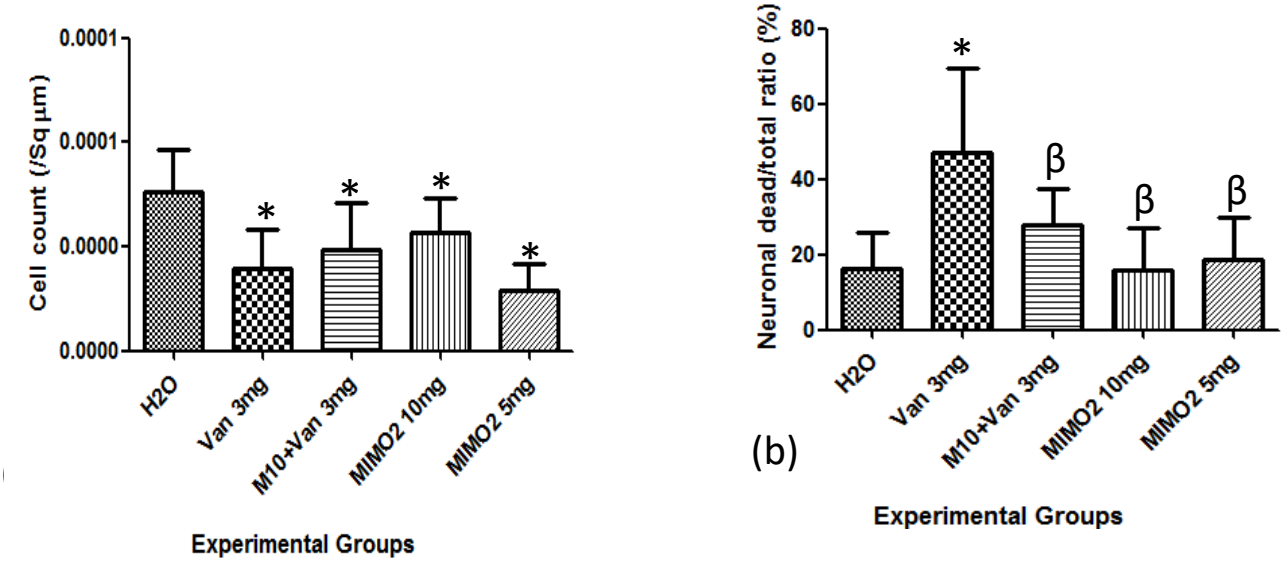


Figure 19: Hippocampal region CA1 neuronal cell count. (a) Cell count of apparently healthy cells. (b) Cell count of relative number of dead cells expressed in percentage. * indicates groups which are statistically significantly different ($p < 0.05$) from water group, while β indicates groups statistically significantly different from ($p < 0.05$) V 3 mg/kg group.

CA3 region: the neuronal cells observed in the V group had a pyknotic appearance (Figure 20). This however was not significantly reflected in the cell count, as no statistical significant difference ($p > 0.05$) was observed amongst the groups, even though the V group had the lowest number of cell count and the highest number of relative dead cell ratio (Figures 21 a & b).

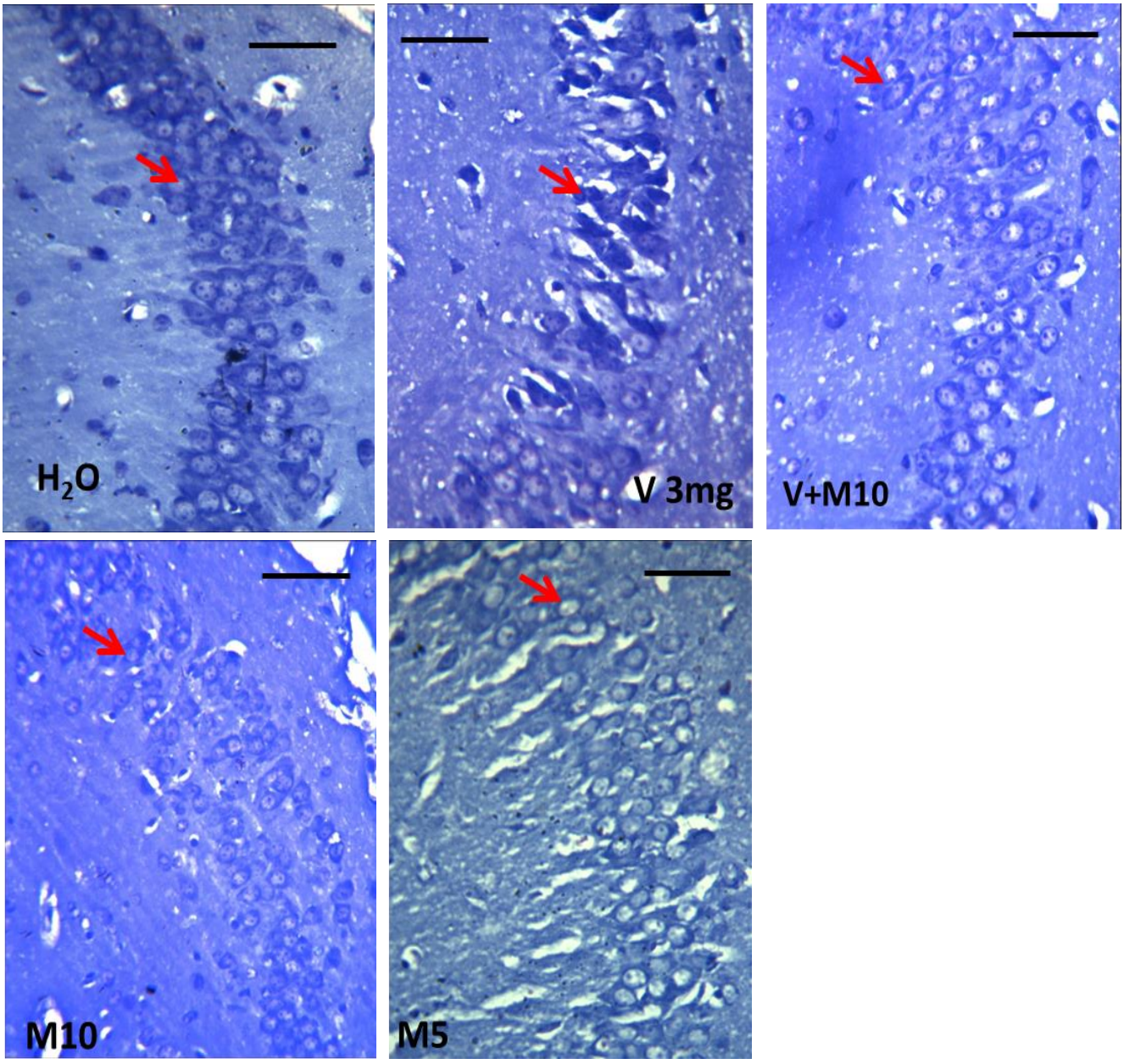


Figure 20: Micrographs of the hippocampus, CA3 region, cresyl violet stain. Arrows indicate neuronal cells. Note the pyknotic appearance of neuronal cells (identified by the loss of the nuclear outline) in vanadium group. Scale bar = 50 μm.

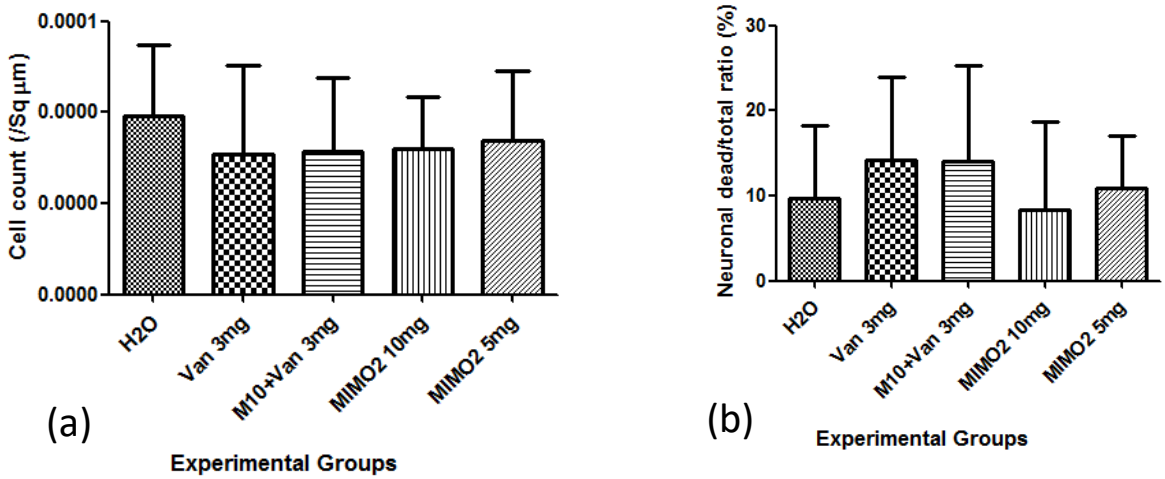


Figure 21: Hippocampal region CA3 neuronal cell count. (a) Cell count of apparently healthy cells. (b) Cell count of relative number of dead cells expressed in percentage. No statistically significant difference ($p > 0.05$) was observed amongst the groups.

4.3.3.5 *Histochemistry (Black Gold II)*

Black gold II histochemistry showed selective demyelination of the corpus callosum, the deeper layers of the motor and somatosensory cortices, the hippocampus, which was more pronounced in the CA1 region, the habenular, the region of the thalamic nucleus, and the rostral commissure (Figures 22b and 23). The internal capsule appeared relatively resistant to the demyelination (Figure 23).

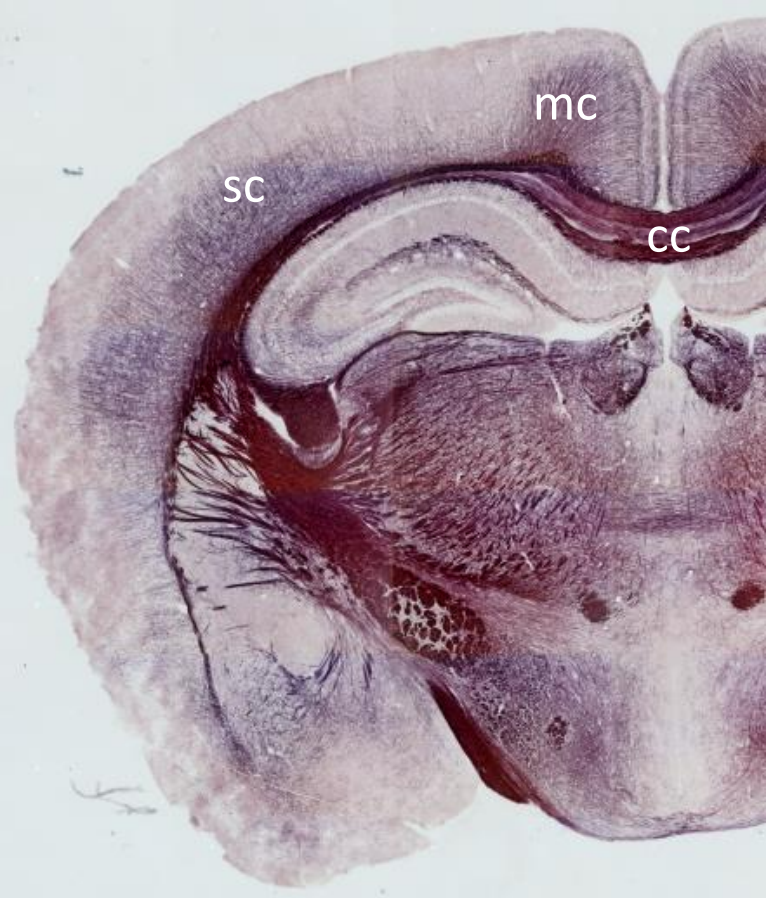


Figure 22a: Micrograph of whole brain stained with Black gold II. The corpus callosum (cc), somatosensory cortex (sc) and motor cortex (mc) are labelled. These were the major regions analysed for densitometry.

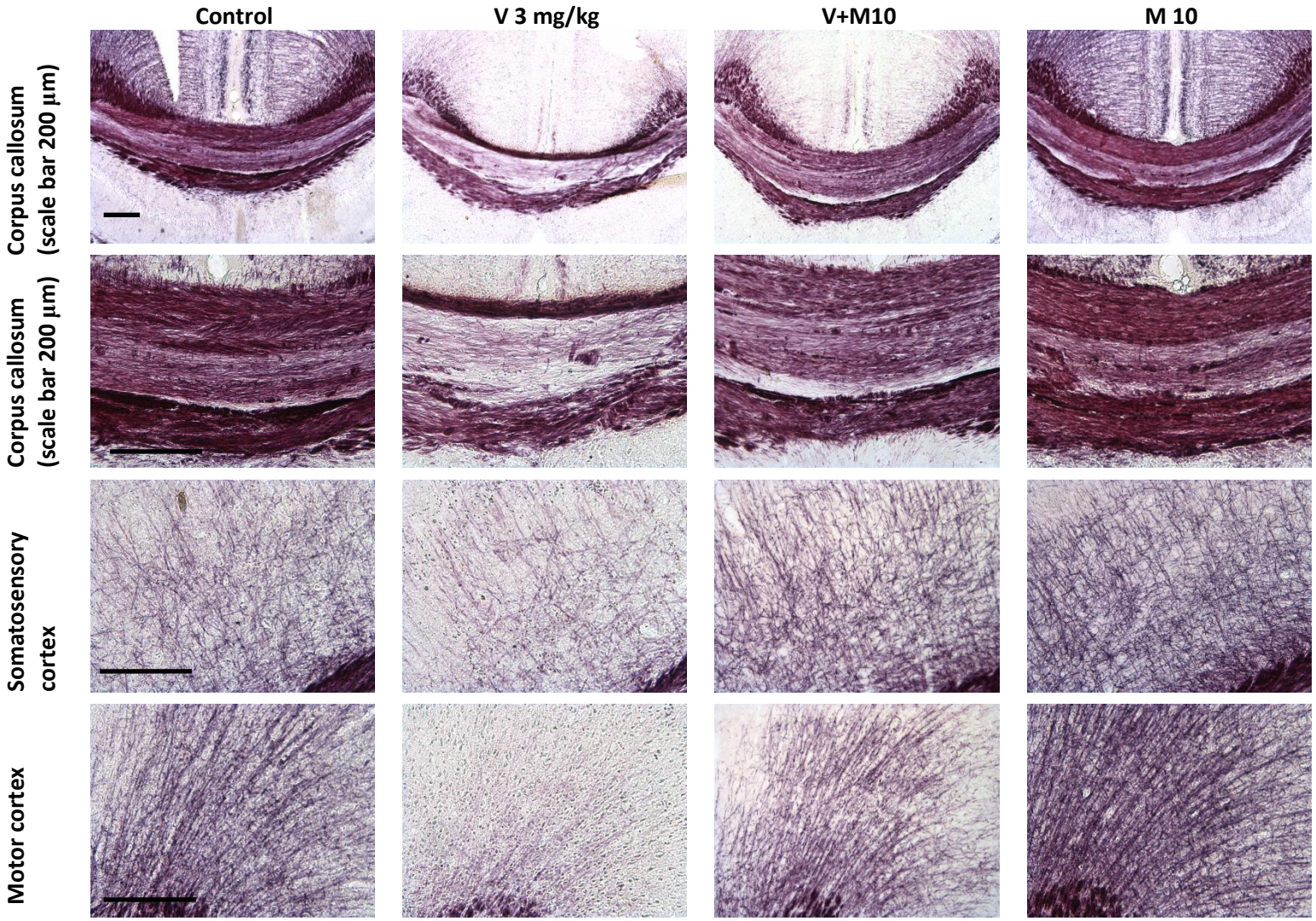


Figure 22b: Black gold II histochemistry. Panels show demyelination in the corpus callosum (different magnifications), deep layers of the somatosensory cortex and the motor cortex in the vanadium administered group. Note that MIMO2 at 10 mg/kg showed a level of myelin recovery. Scale bar 200 µm (note – scale bar is same across the rows).

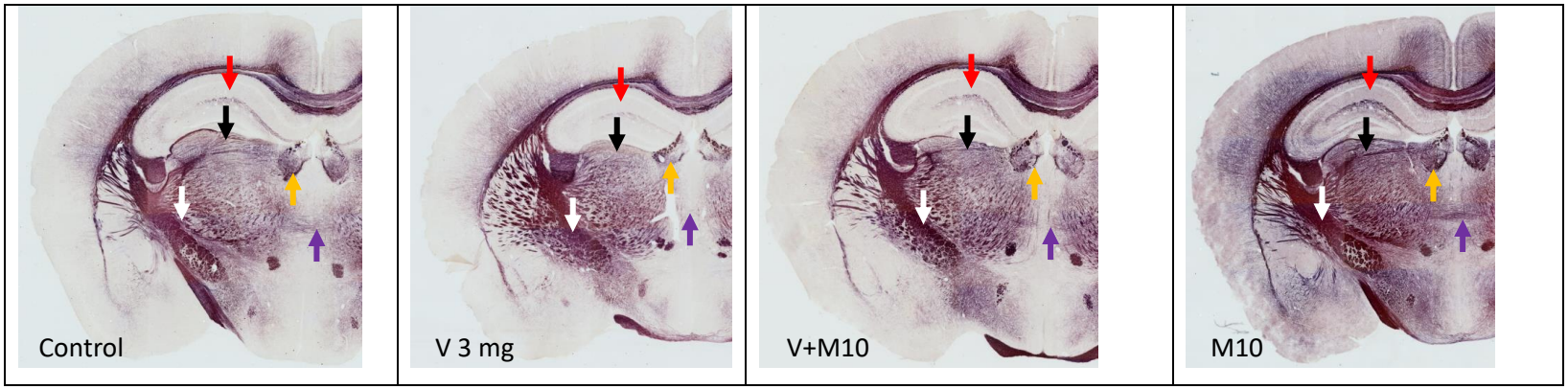
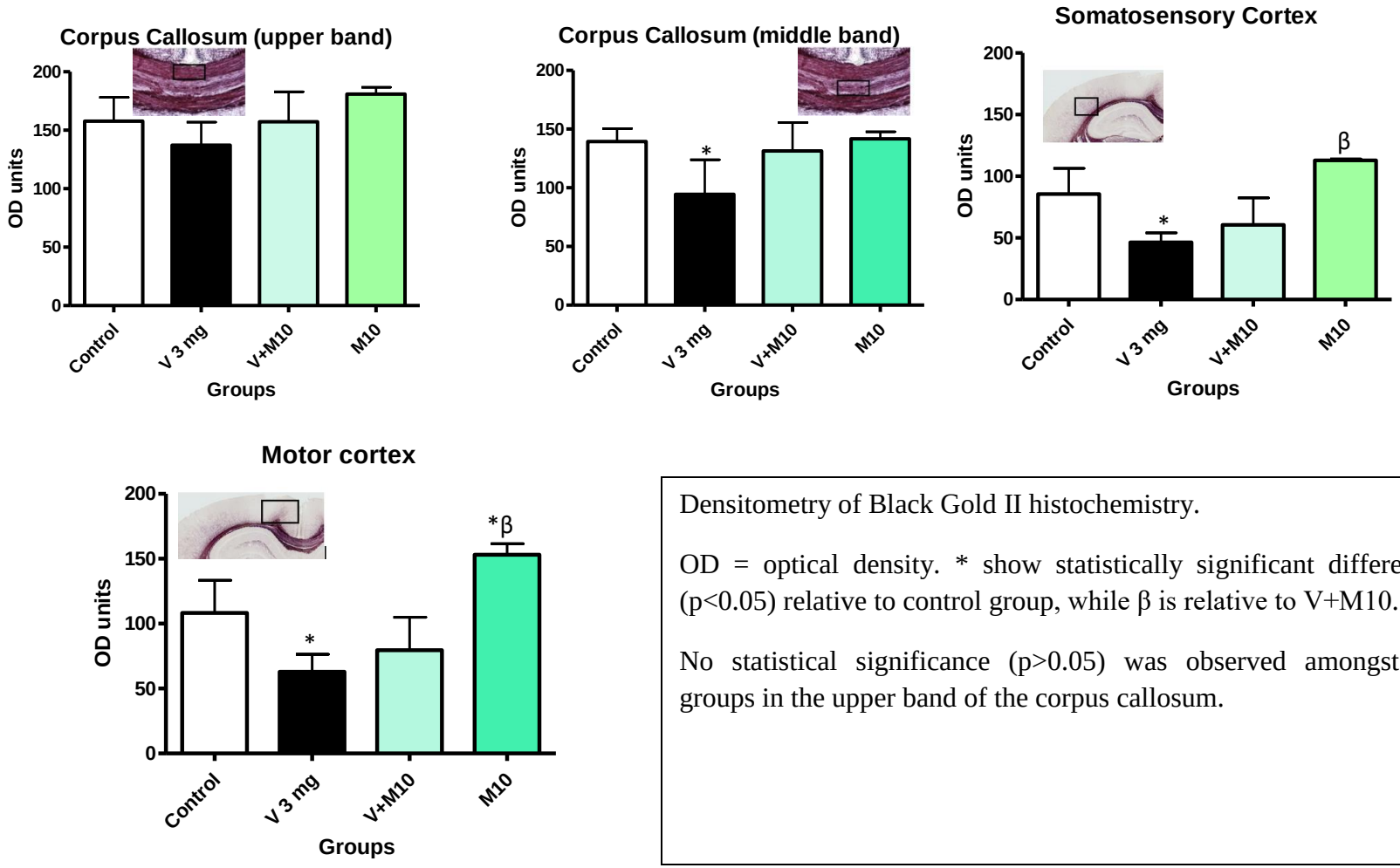


Figure 23: Micrographs of whole brain stained with Black gold II, showing demyelination in the CA1 region (red arrow), the region of the thalamic nucleus (black arrow), the habenular (yellow arrow) and the rostral commissure (purple arrow). The internal capsule (white arrow) appears resistant to the demyelination.

BLACK GOLD DENSITOMETRIC ANALYSIS



Densitometry of Black Gold II histochemistry.

OD = optical density. * show statistically significant differences ($p<0.05$) relative to control group, while β is relative to V+M10.

No statistical significance ($p>0.05$) was observed amongst the groups in the upper band of the corpus callosum.

Figure 23b: Graphs showing the degree of myelin intensity or optical density of the corpus callosum (upper and lower bands), the somatosensory and motor cortices.

4.3.3.6 Iba1 Immunohistology (Microglia)

There was microglial activation in the corpus callosum, somatosensory and motor cortices of the V 3 mg group. The microglia showed bigger soma, and there was also an increase in number. The bigger soma was more obvious in the corpus callosum. There was also microglia activation in the M10+V group, although not as much as the vanadium group. M10 group showed higher number of microglia relative to the control group, although this was not statistically significantly different ($p>0.05$). The use of M10 in combination with vanadium showed an alleviation or reduction in microglia activation (Figures 24 a&b).

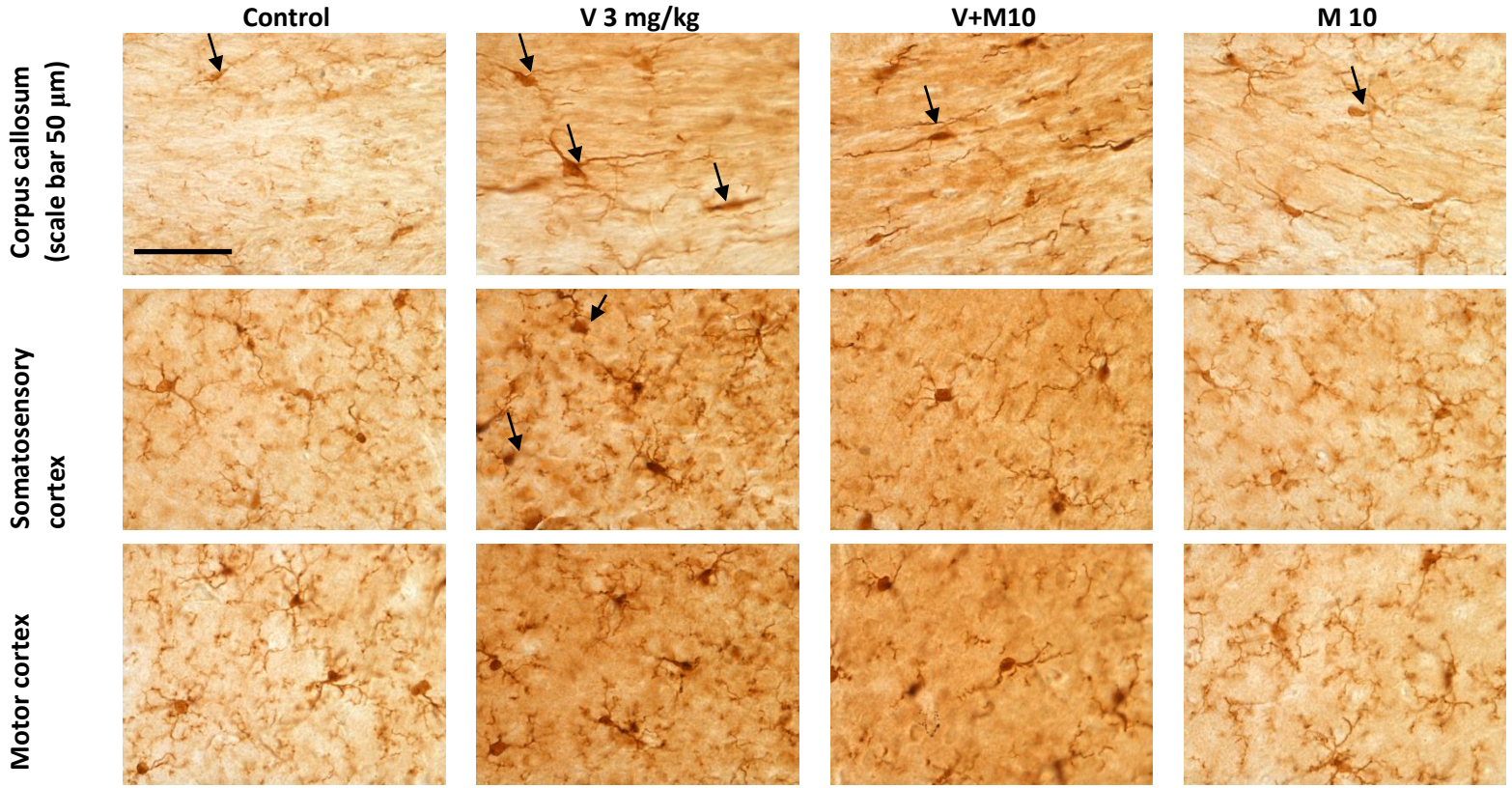


Figure 24: Iba1 stain, showing microglia in the corpus callosum, somatosensory and motor cortices. In the vanadium group, note the increase in the number of the microglia, and the bigger cell body. Scale bar = 50 µm (note – scale bar remains constant for all micrographs).

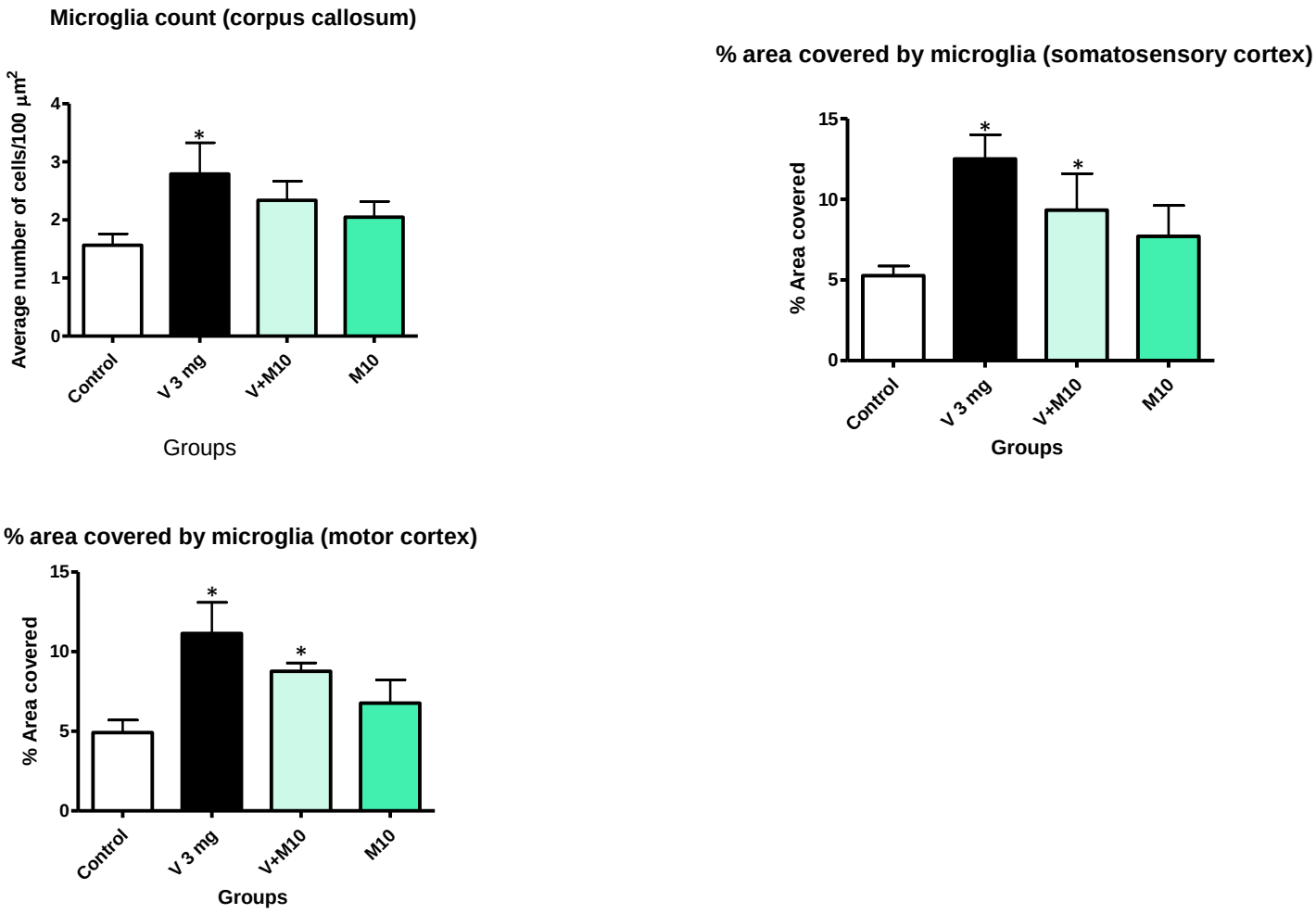


Figure 24b: Graphs showing the microglia count in the corpus callosum, and percentage area covered in the cortices. * Indicates statistical significance relative to control group ($p < 0.05$).

4.3.3.7 Glial Fibrillary Acidic Protein - GFAP (Astrocytes)

There was greater astrocytic activation in the V 3 mg group, which appeared to be alleviated with the co-administration of M10. Astrocytic activation was not observed in the M10 group (Figure 25).

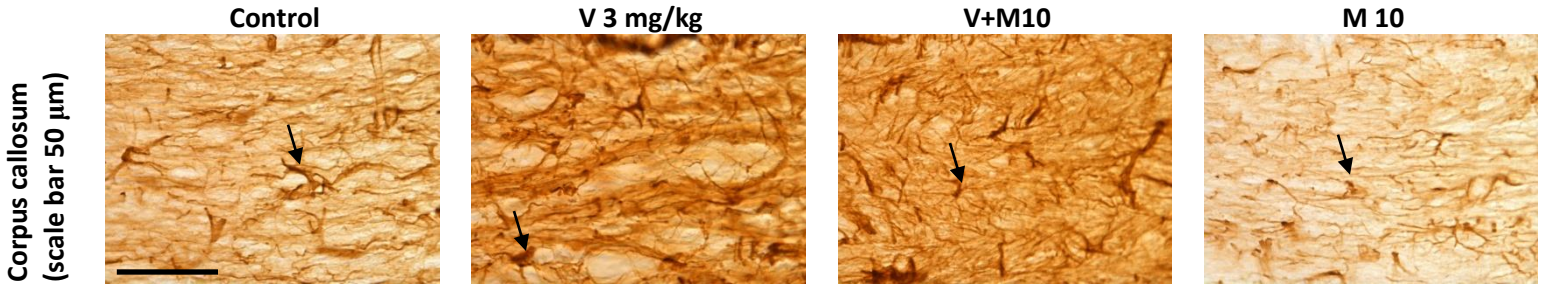


Figure 25: GFAP of the corpus callosum. Note the increased astrocytic activation in the vanadium group, the attenuation observed with the administration of MIMO2. Arrows indicate astrocytes. Scale bar = 50 µm (note – scale bar remains constant for all micrographs).

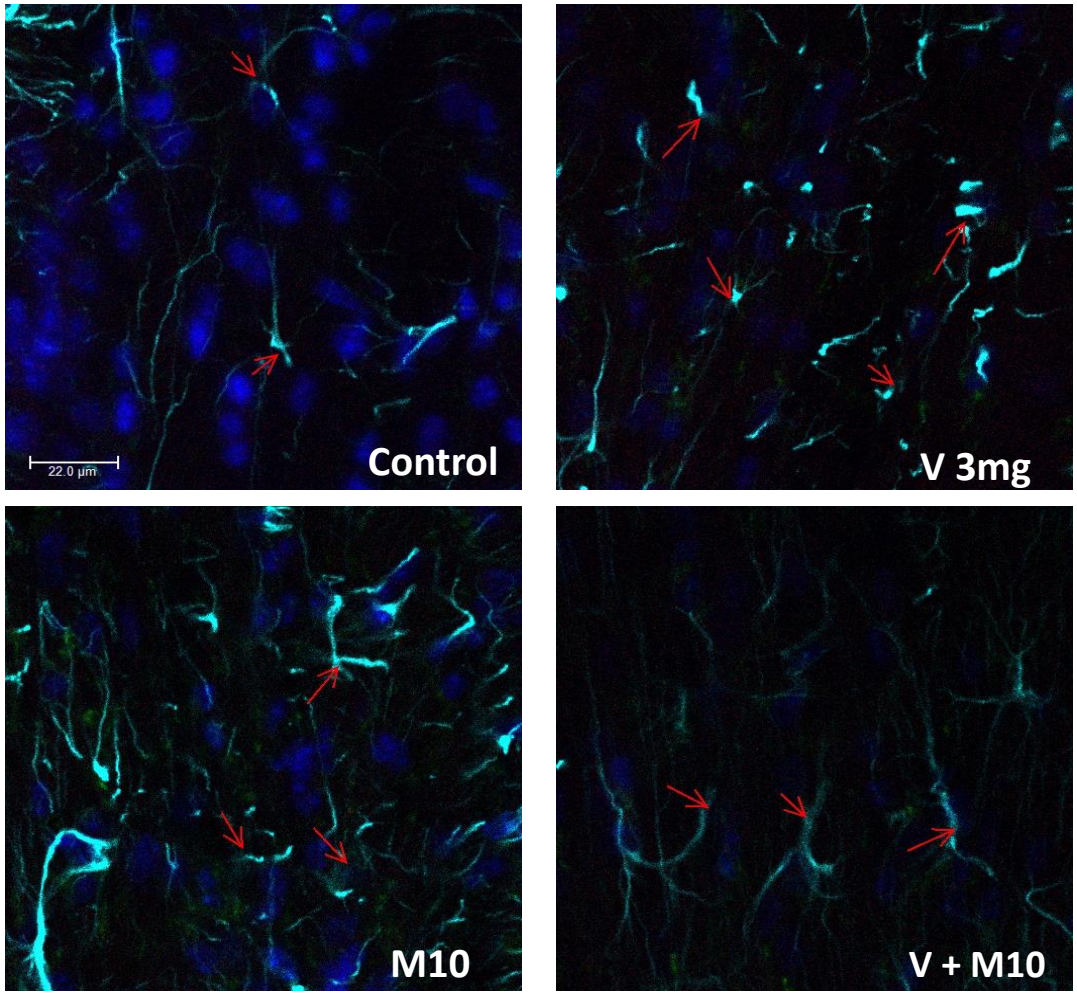


Figure 26: Immunofluorescence/Confocal microscopy, corpus callosum (GFAP/CDIIb/IL-1β/DAPI). Arrows indicate astrocytes. Note the presence of astrocytes in V 3mg group and the amelioration in M10+V group. Scale bar 22 µm (note – scale bar remains constant for all micrographs).

CHAPTER 5

DISCUSSION

Fractionation and Cell Culture

The use of *in vitro* cellular models forms a very important and indispensable aspect of understanding molecular and cellular processes as concerns a particular tissue or organ, or a disease pathogenesis. The hippocampus plays a very important role in memory, and dysfunctions of this structure have been believed to contribute to the pathogenesis of Alzheimer's disease and some other diseases affecting memory (Liu *et al*., 2009).

In this current study, the immortalized mouse hippocampal (HT22) cell line was used to assess the potential protective effect of MIMO2. Using DHE, MIMO2 1 μM (Figure 6) and 5 μM (data not shown) appeared to show higher colour intensity relative to 0.5 μM and 0.25 μM. This is probably due to the fact that at 1 μM and above, MIMO2 was pro-oxidative. This is similar to results obtained from *in vitro* assays in rats using α-tocopherol (Abudu *et al*., 2004) and *Garcinia kola* (Igado *et al*., 2012), when high doses were administered. Even in the presence of the toxicant (vanadium), the toxicity signs observed were still obvious. 0.5 μM and 0.25 μM when given alone and in combination with vanadium 100 μM gave results that were lower than what was previously obtained with higher concentrations of MIMO2. Although MO 63 μg showed results similar to the two lower concentrations of MIMO2 when the DHE intensity was assessed, further tests need to be carried out to determine and ascertain the potency and efficacy of the extract relative to the pure compound.

In the Comet-Assay, DNA-damage in HT22 cells was reduced after incubation with MO 0.01 mg/ml, MIMO2 0.5 μM and MIMO2 0.25 μM (Figure 8) following vanadium incubation suggesting a possible protective effect. The reasons for this ameliorative effect displayed by MIMO2 could not yet be ascertained. It is possible that it had a chelative effect on vanadium, as

seen in kolaviron, a bioflavonoid antioxidant (Farombi *et al*., 2002; Farombi and Nwaokeafor, 2005; Igado *et al*. 2012). Also, due to the fact that the vitality test did not show any significant difference (the lowest vitality value being 88%), it can be deduced that the DNA damage observed was not an indirect effect of cell death, but due to the genotoxicity of vanadium.

Moringa oleifera leaf (MOL) has been extracted in different forms varying from aqueous form (Ganguly *et al*., 2005; Adedapo *et al.,* 2009), alcohol extracted form (Ganguly and Guha, 2008; Kirisattayakul *et al*., 2012; Sutalangka *et al*., 2013), to the air-dried and pulverised form (Nkukwana *et al.*, 2014). The alcohol extracted form seem to be the most widely used form of the extract, with the reported yield varying from 1.34% (Mohan *et al.*, 2005), 10% (Ganguly and Guha, 2008), to 17.49% (Kirisattayakul *et al*., 2012; Sutalangka *et al*., 2013). Further analysis or fractionations by some authors recorded the presence of flavonoids, phenolic compounds, vitamins and some amino acids. There has been evidence for nootropic and neuroprotective disorders in cell cultures of neural cells and in animal models (Hannan *et al*., 2014).

Using hippocampal neurons, Hannan *et al*., (2014) reported that the addition of MOL extract significantly increased the number and length of neurites and their branching, in a dose-dependent manner, with the optimal concentration achieved at 30 μg/ml. In the same experiment, neuronal viability was increased, cellular injury was decreased and the rate of neuronal differentiation was also accelerated. No cytotoxicity was observed. Neurons also exhibited more extended and multiple branching, an increase in the number and length of primary dendrites and also the appearance of more secondary and even tertiary dendrites. MOL was also observed to modulate axonal development and promote synaptogenesis. The reasons for this multiple branching and differentiation observed could be due to the presence of β-carotene, which is

abundant in MOL. β-carotene has been reported to be an inducer of neuronal cell differentiation (Lee *et al*., 2013).

Nigeria is a country that is rich in crude oil, and is one of the highest producers of crude oil worldwide. Increased human activities such as increased exploitation of natural minerals have resulted in an upsurge of environmental pollution in the oil producing countries. The effects of this pollution are more pronounced in the countries of the third world. The increase in mineral exploitation has subsequently resulted into an increase in vanadium being released into the atmosphere (Bascom, 1996; Barceloux, 1999; Igado *et al*., 2008; Olopade *et al*., 2009). Currently, no data appears to be documented on the level of atmospheric vanadium in Nigeria. A previous work by Igado *et al*. (2008) showed that goats from a relatively unindustrialised area in Nigeria still had a relatively high amount of vanadium in the brain (0.34 ± 0.10 ppm). This report on the concentrations of different metals in the brain of goats highlighted the prevalence of environmental pollution and susceptibility of the brain to environmental pollutants in a so-called rural area.

Traditional healers have prescribed different parts of the plant *M. oleifera* for various ailments for hundreds of years, ranging from ear and dental infections, cardiovascular ailments, respiratory diseases and cancer treatment (Luqman *et al*., 2012). Different compounds for different medical purposes have also been isolated from the plant: for example, nitrile glycosides and niaziminins for the treatment of blood pressure (Faizi *et al*., 1995); isolation of glycosides (Sahakitpichan *et al.*, 2011); and the isolation of crypto-chlorogenic acid, isoquercetin and astragalin (Vongsak *et al*., 2013). MIMO2 has a phenol ring, and phenols are natural antioxidants, having the ability to react with superoxide anions and lipid peroxyl radicals, thereby breaking the chain of lipid peroxidation (Rajanandh and Kavitha, 2010). This antioxidant

activity demonstrated by phenolic compounds is believed to be due to their structure – the substitutions on the aromatic ring and the structures on the side chain (Shahidi and Wanasundara, 1992).

Similar to the results we obtained, Sreelatha and Padma (2011) and Sikder *et al*. (2013) had shown a reduction of DNA-damage induced with hydrogen peroxide in KB tumor cells and lymphocytes respectively after treatment with *Moringa* extract. *Moringa* extract alone was shown to induce DNA-damage in alveolar epithelial cells indicating pro-apoptotic and antiproliferative effects that would be of interest in the treatment of cancer (Tiloke *et al*., 2013). The nature of the observed DNA-damage in HT22 cells should be further analysed with additional tests like the micronucleus test.

Vanadium causes oxidative stress in the central nervous system (CNS) through the generation of free radicals and the main areas affected are the hippocampus and the cerebellum. In addition, morphological alterations of neurons and astroglial cells in the CNS of rats, after vanadium exposure has also been reported, resulting into various neurobehavioral deficits (Garcia *et al.*, 2004, Mustapha *et al.*, 2014). A possible way by which this is achieved is probably by vanadium exerting neurotoxic effects in dopaminergic neuronal cells via caspase-3-dependent PKCd cleavage (Afeseh-Ngwa *et al*., 2009). MIMO2 displayed antioxidative properties by reducing the cellular ROS released.

Animal Experiments

MIMO2 was a compound shown to be fat soluble and not water soluble. This property implies that it has the tendency to accumulate in the body, like vitamin E, and can easily cause toxicity in body systems at high concentrations, since it might not be easily excreted. In this case, it was advisable to give MIMO2 at staggered doses. Giving MIMO2 25 mg/kg alone recorded

100% survival in the mice, while using the same dose in combination with V 3 mg/kg resulted in mortality. This shows that the two drugs had a potentiating effect (pro-oxidant) on each other, thereby resulting in increased mortality/morbidity when combined at these doses.

Body weight and relative brain weight did not show any significant difference relative to the control and V 3 mg groups. This is in contrast to what was reported by Mustapha *et al*. (2014) and Azeez *et al.* (2016). This could be attributed to the fact that in the aforementioned experiments, vanadium administration commenced from post natal day 1 (PND1), while in the current experiment, it commenced on PND14. The age of exposure of the pups probably played a very important role in their response to insults.

The present findings show that vanadium administration starting from PND 14 may not necessarily affect the weight gain significantly, but resulted in a decrease in locomotion and exploratory activity. Histopathological analysis revealed demyelination in a region-dependent manner. Activation of astrocytes and microglia was consistent with demyelination observed.

Body Weight and Functional Deficit

The finding in this study of no significant difference in the body weight increase across the groups is similar to reports by Garcia *et al.* (2004, 2005) and Igado *et al*. (2012), who exposed 3 month old rats and 2 month old rats respectively for 5 days. Contrary to the results obtained were experiments by Azeez *et al*., (2016) who reported a significant decrease in body weight with vanadium administration to mice for duration of 90 days, via milk and through the intraperitoneal route. It could be deduced from this result that the effect of vanadium administration on body weight may not only be related to the duration, but also the age at commencement of administration. It is however possible that dosing for a longer period in the current study may result in a statistical significant difference.

Significant decreased muscular strength and locomotion ($p < 0.05$) relative to the control group (H_2O) was observed in V 3 mg/kg, M10 mg/kg, M5 mg/kg and M5 + V 3 mg/kg. There was a significant increase in rearing and line crossing in the M10 mg/kg, implying that the animals in this group were more excitable. Addition of M10 to vanadium showed a significant alleviation of the muscular weakness observed in the vanadium alone group. Administration of vanadium has previously been reported to result in muscular weakness in mice (Mustapha *et al*., 2014; Azeez *et al*., 2016).

Purkinje Layer Stratification and Loss of Purkinje Cells

Stratification of the Purkinje layer is observed in migrating cells in the developing embryo (Yuasa *et al*., 1991). The presence of the stratification in the groups administered vanadium could be due to the fact that the administration of vanadium affected the cell migration. This was less in the group administered vanadium and M10. Pyknosis of the cells observed is consistent with previous reports by Igado *et al*., (2012), where vanadium has been reported to also result in neuronal cell death, or a decrease in neuronal cell population. The pathologies observed in the cerebellum of the vanadium treated group could account for the relative muscle weakness, since the cerebellum is responsible for muscle coordination (Afifi and Bergman, 2005). Previous reports of Purkinje cell stratification have been documented in lead poisonings in mice and in Bicaudal-D protein knockout mice (Jaarsma *et al*., 2014). However, to the best of my knowledge, this current study is the first report of vanadium administration resulting in Purkinje cell stratification.

Hippocampal Neuronal Count

The effect of vanadium on the hippocampal neuronal cells is similar to that observed by Sutalangka *et al*. (2013), when he induced dementia with a cholinotoxin. He reported an

ameliorative effect caused by the oral administration of the hydroalcohol extract of *Moringa oleifera* leaves. The effect of MIMO2 on the cells however, appeared to be more potent compared to what was observed with the administration of the leave extract.

The CA1 region was more susceptible to vanadium-induced damage relative to the CA3. This could be due to the fact that the CA3 region is documented to be the hippocampal region that is least susceptible to insults (Afifi and Bergman, 2005).

Demyelination

Demyelination or hypomyelination has been reported in vanadium exposure, via lactation or direct intraperitoneal injection (Igado *et al*., 2012; Mustapha *et al*., 2014). The lipid content of the brain is the myelin. The high metabolic activity of this myelin increases the vulnerability of nervous tissue to peroxidative damage. Vanadium causes lipid peroxidation in different regions of the brain, resulting in an initiation of oxidative chain reactions. Because myelin possesses a high relative content of phospholipids, it becomes a potential target for membrane oxidative damage caused by vanadium (Garcia *et al.*, 2004; Igado *et al*., 2012).

Demyelination in this study was observed mainly in the midline of the corpus callosum, the hippocampus, and diencephalon. This region-selective demyelination is similar to that reported in cuprizone neurotoxicity (Kipp *et al*., 2009; Zendedel *et al*., 2013). Damage to the corpus callosum has been reported to cause subtle changes in bilateral sensory and motor coordination (Berlucchi, 2012). This demyelination effect of vanadium and the alleviation resulting from the use of M10, is mirrored in the hanging wire results.

The central portion of the corpus callosum commissure has been reported to be vulnerable to toxic challenges probably due to the penetration of toxic agents and arrangement of vascularisation (Berlucchi, 2012). Vanadium crosses the blood brain barrier (Igado *et al*., 2012),

thereby making it easy to cause damage in the brain. The amelioration of demyelination by MIMO2 could probably be due to the fact that it mops up the free radicals produced due to the action of vanadium (being an antioxidant). Probably, it prevents the penetration by and action of vanadium, by a mechanism of action that still needs to be further investigated. However, polyphenolic compounds (like MIMO2) exhibit strong antioxidant and anti-inflammatory activities which may lessen or reduce neurodegeneration (Choi *et al*., 2012). It is also noteworthy, the fact that MIMO2 alone (M10) appeared to potentiate/stimulate the production of myelin. This increased myelin in the corpus callosum, motor and somatosensory cortices could have been responsible for the increased activity observed in the open field and hanging wire tests thereby resulting in improved motor coordination.

Microglia and Astrocyte Activation

Microglia activation and astrogliosis are characteristic features of CNS lesions (Gudi *et al*., 2014).

Microglia are resident immunocompetent cells present in the brain parenchyma; they are key mediators of neuroinflammation (Hanisch and Kettenmann, 2007). Microglia activation has been reported to precede demyelination and persist in chronic myelin lesions (Gudi *et al*., 2014). In this study, although relatively acute compared to the study by Azeez *et al*. (2016), it was observed that there was a significant increase in microglia activation in all the three brain regions sampled, while significant alleviation was observed in the somatosensory and motor cortices. This shows that in the corpus callosum, even with the significant amelioration of demyelination, there was not a corresponding decrease in microglia activation.

Recruitment of microglia is reportedly regulated by astrocytes (Skripuletz *et al*., 2013; Gudi *et al*., 2014). Astrocytes provide trophic support to other cells and also exercise an

inhibitory effect on remyelination (Alizadeh *et al*., 2015). Astrogliosis due to vanadium administration, in different time frames, and in different brain regions have been reported by different authors (Garcia *et al*., 2005; Mustapha *et al*., 2014; Azeez *et al*., 2016). In the current study, immunofluorescence confirmed that astrogliosis was most pronounced in the corpus callosum. This is contrary to what was previously reported by Azeez *et al*. (2016), where astrogliosis was pronounced in both grey and white matter. The reason for this could be due to the shorter period of exposure in the current study.

CONTRIBUTION TO KNOWLEDGE

- Isolation, characterisation and structure elucidation of MIMO2, a pure compound, from *M. oleifera* leaves
- Discovery of MIMO1, a novel compound, previously unreported.
- Demonstration of the protective effect of MIMO2 against vanadium-induced neurotoxicity, *in vitro* (DHE and CA)
- MIMO2 reduced neurobehavioural deficits induced by vanadium
- MIMO2 offered i*n vivo* neuroprotection against neuronal loss, demyelination, astrocytosis and microglia activation
- This work showed evidence that MIMO2 can stimulate/potentiate myelin production

Conclusion

Vanadium administration seemed to show a pantropic damage on the brain, while MIMO2 consistently showed the ability to decrease the toxicity signs exhibited by vanadium due to its antioxidative property. MIMO2 appears to be relatively safe in the animals.

Environmental pollution and its ravages on human and animal system is an ongoing scourge that is on the increase. It is hoped that on the long run, results from this experiments and

subsequent studies regarding MIMO2 as a potential drug, will open up vistas to alleviate the ravages of environmental pollution in the form of vanadium-induced neurotoxicity.

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ADDENDUM

RESULTS TABLES

Table 1: µM TEMPOL Equivalence/ mg extract of the fractions obtained from liquid-liquid fractionation (n = 3).

Liquid Fraction	µM TEMPOL Equivalence/ mg extract (Mean ± SD)
Dichloromethane	0.3575 ± 0.0137
Hexane (H_2O)	0.6481 ± 0.2003
Butanol	5.4407 ± 2.3560
Ethyl acetate	5.4038 ± 0.0846
Hexane (MeOH)	0.0544 ± 0.0028
MeOH	1.1213 ± 0.1435
H_2O phase (Butanol)	0.4763 ± 0.1132
H_2O phase (Ethyl Ac.)	3.4139 ± 1.2479
TEMPOL	1 ± 0.0000

Table 2: µM TEMPOL Equivalence/mg extract of selected fractions and substances (n = 3).

Fraction/Substance	µM TEMPOL Equivalence/ mg extract (Mean ± SD)
Moringa Extract	4.6035 ± 0.6411
Butanol fraction	1.2375 ± 0.0750
MIMO2	8.3496 ± 0.9471
MIMO1	0.3635 ± 0.1139
TEMPOL	1 ± 0.0000

Table 3: DHE intensity values in HT22 cells after vanadium induced superoxide production (n = 4).

Group	DHE intensity relative to H_2O (Mean ± SD)
H_2O	1 ± 0.0000
V100 µM	2.1169 ± 0.6360
MIMO2 0.5µM	1.2270 ± 0.2490
MIMO2 0.25µM	1.6514 ± 1.0524
MO 0.063 mg	1.1980 ± 0.2304
MIMO2 0.5 µM+V100 µM	1.4818 ± 0.0855
MIMO2 0.25 µM+V100 µM	1.4296 ± 0.2873

Table 4: Percentage DNA in tail of HT22 cells after administration of vanadium &/or MIMO2 or MO (n = 3 – 4)

Groups	% DNA in tail (Mean ± SD)
NK H2O	1.290 ± 0.295
NK DMSO	1.344 ± 0.308
NK MEOH	1.589 ± 0.253
200µM Vanadium	7.679 ± 2.462
0.5 µM MIMO2	2.512 ± 0.647
200µM Vanadium + 0.25µM MIMO2	4.499 ± 1.241
200µM Vanadium + 0.5µM MIMO2	5.206 ± 1.126
0.2 mg/ml MO	4.875 ± 0.569
200µM Vanadium + 0.2mg/ml MO	5.136 ± 0.932
Moringa 0.01 mg/ml	1.887 ± 0.499
200µM Vanadium + 0.1mg/ml MO	5.759 ± 3.083

Table 5: Increase in body weight (grams)

	Increase in body weight relative to D1 or D0												
Groups	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
H2O	NA	0.33	0.89	1.86	1.74	2.11	2.61	3.36	3.36	3.86	4.61	3.99	5.61
DMSO	0.16	0.17	0.17	0.58	0.92	1.17	1.75	2.00	2.50	3.37	3.64	4.55	5.10
Vanadium 3mg/kg	NA	1.00	0.54	0.69	0.77	0.69	1.31	1.77	2.15	1.85	2.92	3.34	4.84
MIMO2 5mg/kg	0.30	-0.10	0.20	0.50	1.20	1.53	1.64	2.20	2.53	2.53	3.09	3.64	4.31
MIMO2 5mg/kg + V3mg/kg	0.13	0.67	0.53	1.13	1.13	2.07	2.46	3.32	3.53	3.99	4.53	4.92	5.23
MIMO2 10mg/kg	0.09	0.64	1.00	1.55	2.26	2.86	3.16	3.66	3.56	4.76	5.16	5.46	5.76
MIMO2 10mg/kg + V3mg/kg	0.25	0.00	0.44	0.94	0.69	1.00	1.31	2.25	2.68	3.08	3.61	3.81	4.28

***NA – Not applicable**

Table 6: Relative brain weight (%)

Groups	**Relative brain weight (Mean ± SD)**
H_2O	3.250 ± 0.065
DMSO	3.057 ± 0.519
Van 3mg/kg	3.299 ± 0.267
MIMO2 5mg/kg	3.374 ± 0.390
V3mg+M5	2.910 ± 0.217
V3mg+M10	3.574 ± 0.411
MIMO2 10mg/kg	3.092 ± 0.234

Table 7: Values for neurobehavioural tests (Mean ± SD)

Groups	Hanging wire (60 secs)	Line crossing (5 mins)	Rearings (5 mins)	Grooming (5 mins)	Faecal boluses (5 mins)	Urine spots (5 mins)
H_2O	61.3 ± 10.40	107 ± 33.78	22.33 ± 3.79	8.333 ± 0.58	5.375 ± 2.13	0.25 ± 0.46
DMSO	47.5 ± 20.70	65.2 ± 86.83	13.6 ± 18.80	4.2 ± 1.48	0.6364 ± 0.92	0 ± 0.00
V 3mg	36.9 ± 17.7	42 ± 1.73	14.67 ± 4.62	6.67 ± 0.58	2.5 ± 1.38	0 ± 0.00
MIMO2 5mg	40.3 ± 17.40	74 ± 3.46	18.75 ± 19.31	1.67 ± 2.08	1 ± 0.87	0.11 ± 0.33
MIMO2 5mg+V3mg	54.4 ± 8.54	83.13 ± 29.56	25.75 ± 10.62	6.25 ± 2.25	5.4 ± 1.96	0.5 ± 0.71
MIMO2 10mg	39 ± 20.50	194.7 ± 6.35	62.67 ± 10.97	4 ± 3.46	2.25 ± 2.44	0.125 ± 0.35
MIMO2 10mg+V3mg	62.2 ± 6.10	106.9 ± 48.28	40.13 ± 24.56	7.38 ± 2.77	5.833 ± 1.94	0.17 ± 0.41

Table 8: Hippocampal Neuronal count for CA1 and CA3 regions (Mean ± SD)

Groups	CA1 region		CA3 region	
	Live cells (sq μm) $x10^{-5}$	Dead/Total cells (%)	Live cells (sq μm) $x10^{-5}$	Dead/Total cells (%)
H_2O	7.6 ± 2.03	16.38 ± 9.641	3.93 ± 1.53	9.7 ± 8.55
V 3mg	3.94 ± 1.9	46.94 ± 22.6	3.09 ± 1.95	14.13 ± 9.84
MIMO2 10mg+V3mg	4.87 ± 2.25	27.73 ± 9.566	3.13 ± 1.63	13.98 ± 11.30
MIMO2 10mg	5.66 ± 1.68	16.04 ± 10.83	3.21 ± 1.12	8.42 ± 10.22
MIMO2 5mg	2.9 ± 1.26	18.65 ± 11.03	3.38 ±1.52	10.83 ± 6.21

Table 9: Black gold II densitometric analysis values to evaluate demyelination

Groups	Corpus callosum upper band	Corpus callosum middle band	Somatosensory Cortex	Motor cortex
Control	157.7 ± 20.32	139.3 ± 11.07	85.51 ± 20.94	108.3 ± 25.04
V 3 mg	137.2 ± 19.8	94.46 ± 29.37	46.49 ± 7.539	62.88 ± 13.48
V+M10	157.3 ± 25.65	131.4 ± 24.15	60.56 ± 21.9	79.62 ± 25.3
M10	181 ± 5.81	141.8 ± 5.895	112.9 ± 1.197	153.1 ± 8.55

Table 10: Microglia count values using Iba1 antibody

Groups	Corpus callosum (cells/100 μm^2)	Somatosensory Cortex (% area covered)	Motor cortex (% area covered)
Control	1.56 ± 0.20	5.26 ± 0.60	4.92 ± 0.79
V 3 mg	2.79 ± 0.54	12.51 ± 1.50	11.15 ± 1.94
V+M10	2.34 ± 0.33	9.33 ± 2.27	8.77 ± 0.52
M10	2.05 ± 0.27	7.71 ± 1.91	6.76 ± 1.47

CONFERENCES ATTENDED WITH PAPERS PRESENTED

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PUBLICATIONS FROM THE THESIS

1. **Igado, O.,** Glaser, J., Bankoğlu, E., Ramos-Tirado, M., Wunram, S., Stopper, H., Holzgrabe, U. and Olopade, J. (2016). A pure compound from *Moringa oleifera*: a possible antidote to vanadium neurotoxicity. *Journal of Neurochemistry*, 134 (Supplement 1): 199. *PubMed PMID*: 2848876
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